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Supplemental Information

miR-195 Has a Potential to Treat Ischemic and Hemorrhagic Stroke through Neurovascular Protection and Neurogenesis

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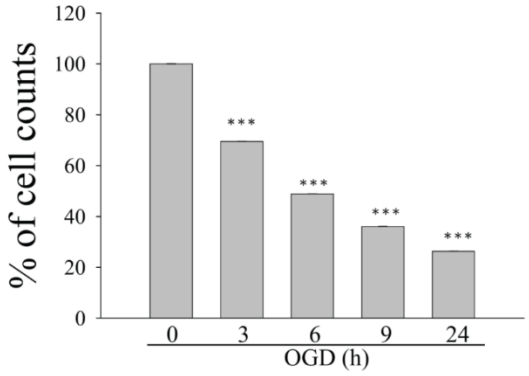
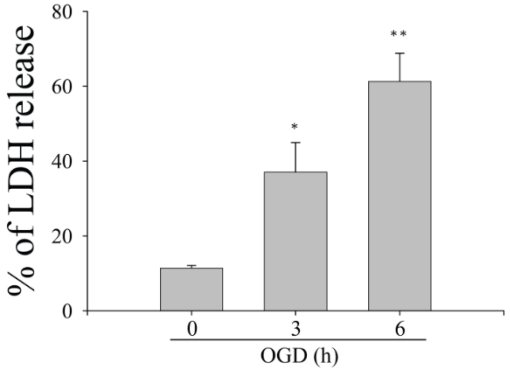
Supplemental Figures

(Suppl Fig1 A)



(Suppl Fig 1B)



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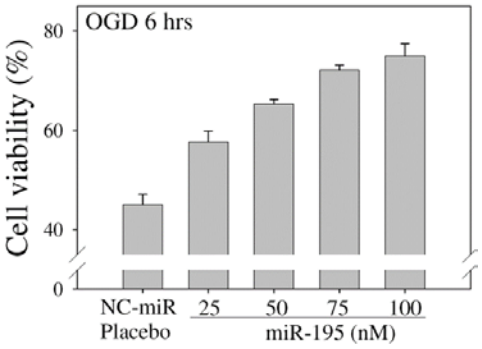
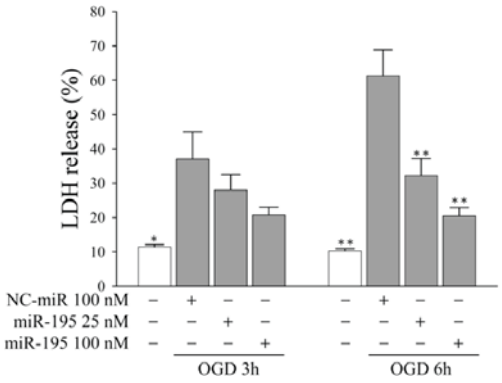
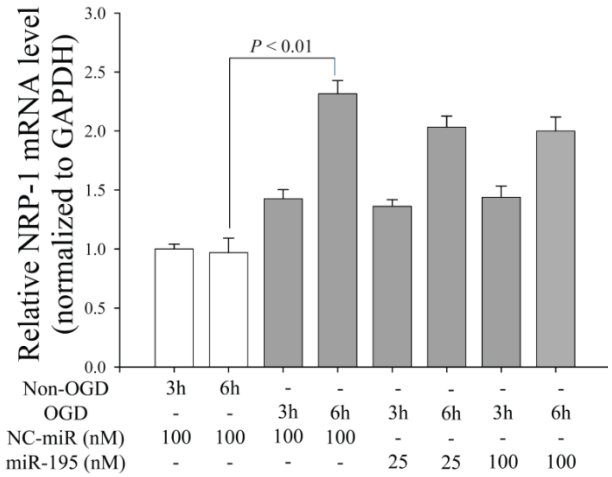
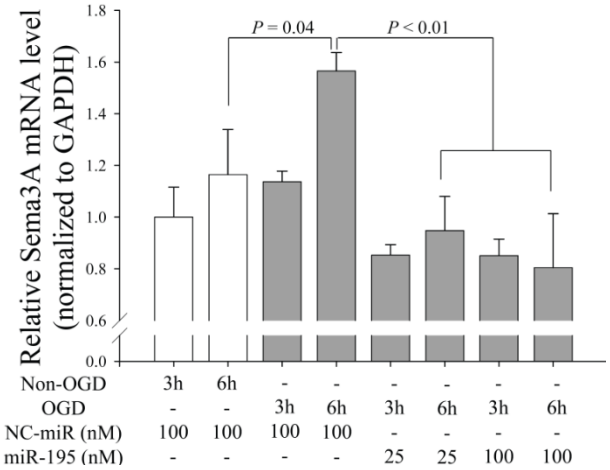


(Suppl Fig 1D)

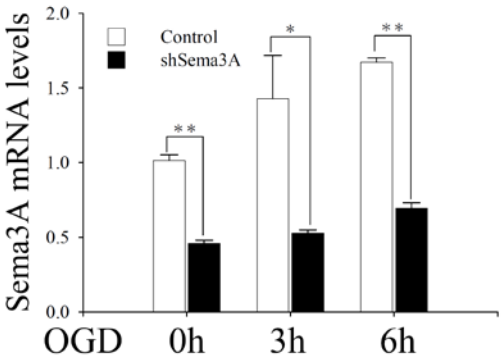


(Suppl Fig 2)

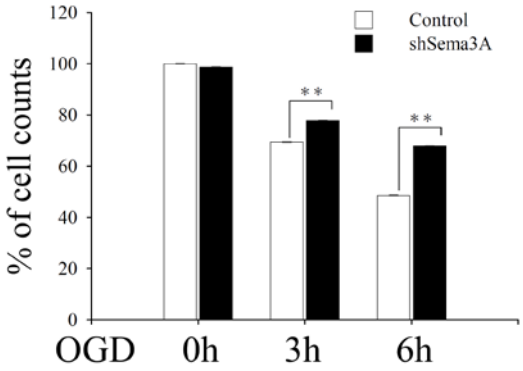

Relative NRP-1 mRNA level (normalized to GAPDH)
P < 0.01
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Non-OGD 3h 6h - - - - - -
OGD - - 3h 6h 3h 6h 3h 6h
NC-miR (nM) 100 100 100 100 - - - -
miR-195 (nM) - - - - 25 25 100 100


Relative Sema3A mRNA level (normalized to GAPDH)
P = 0.04
P < 0.01
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.0
Non-OGD 3h 6h - - - - - -
OGD - - 3h 6h 3h 6h 3h 6h
NC-miR (nM) 100 100 100 100 - - - -
miR-195 (nM) - - - - 25 25 100 100

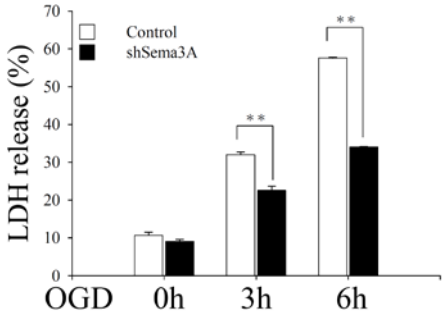
(Suppl Fig 3A)



(Suppl Fig 3B)



(Suppl Fig 3C)



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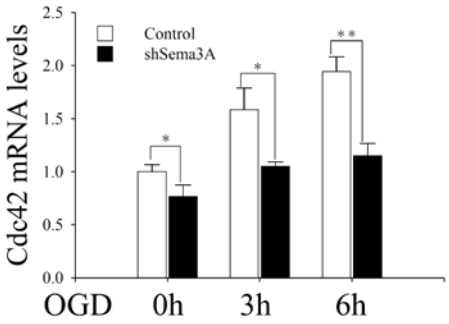
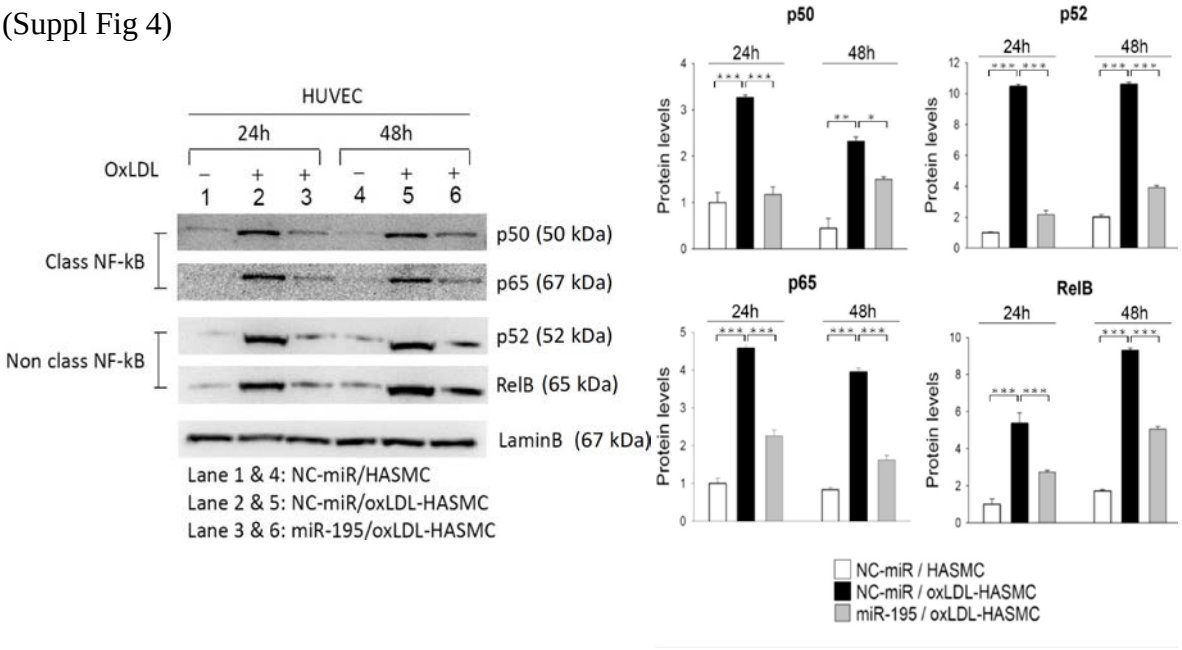
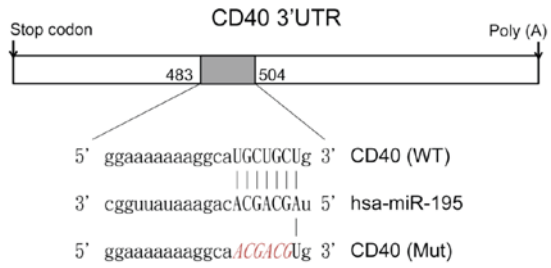


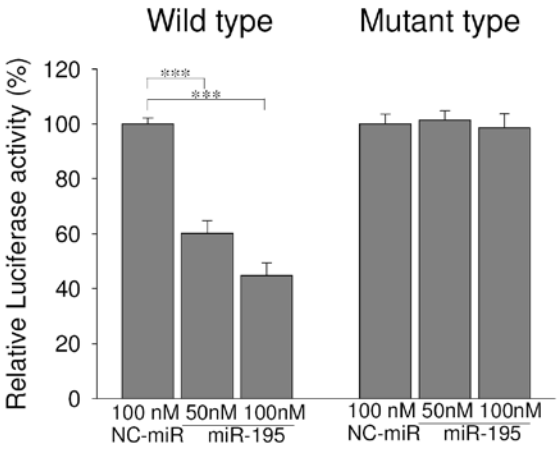
(Suppl Fig 4)


HUVEC
24h
48h
OxLDL
– + + – + +
1 2 3 4 5 6
Class NF-kB
Non class NF-kB
p50 (50 kDa)
p65 (67 kDa)
p52 (52 kDa)
RelB (65 kDa)
LaminB (67 kDa)
Lane 1 & 4: NC-miR/HASMC
Lane 2 & 5: NC-miR/oxLDL-HASMC
Lane 3 & 6: miR-195/oxLDL-HASMC
p50
p52
p65
RelB
24h
48h
Protein levels
NC-miR / HASMC
NC-miR / oxLDL-HASMC
miR-195 / oxLDL-HASMC

(Suppl Fig 5A)



(Suppl Fig 5B)



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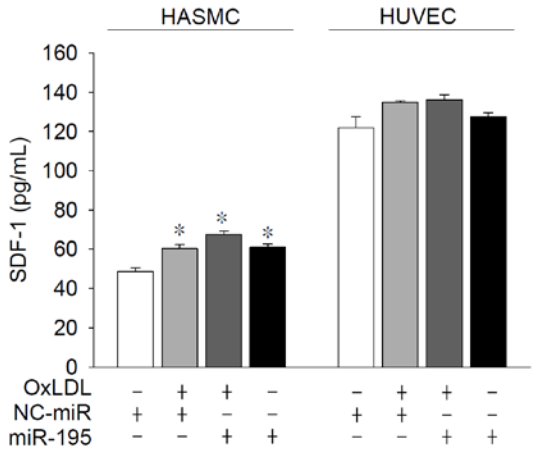

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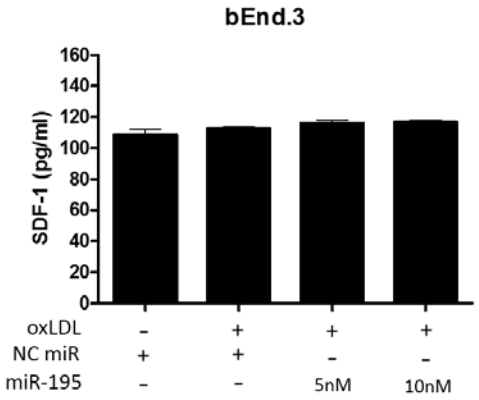
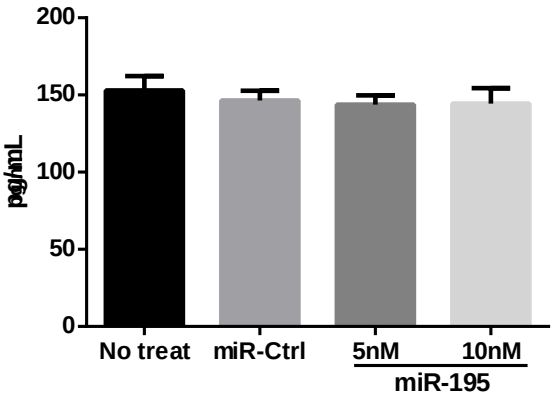


(Suppl Fig 6C, ALT cells)



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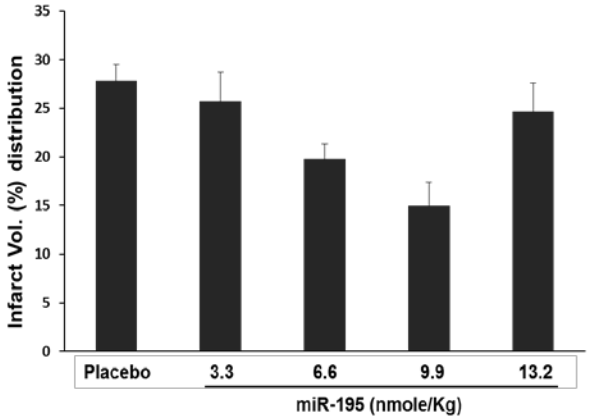

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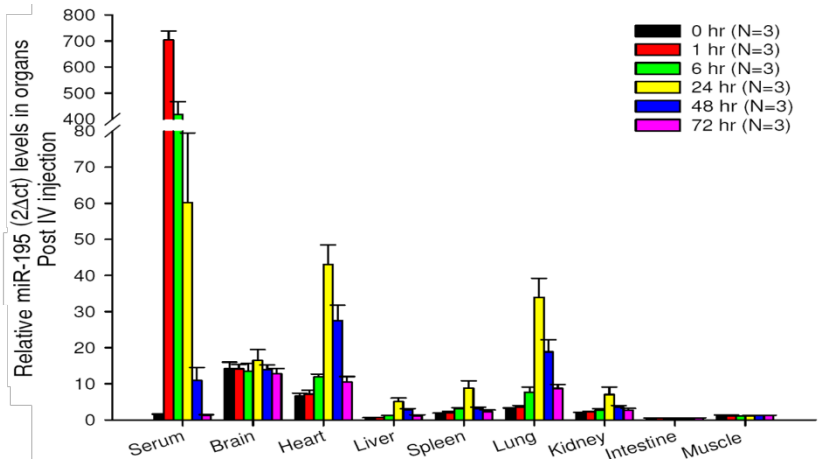

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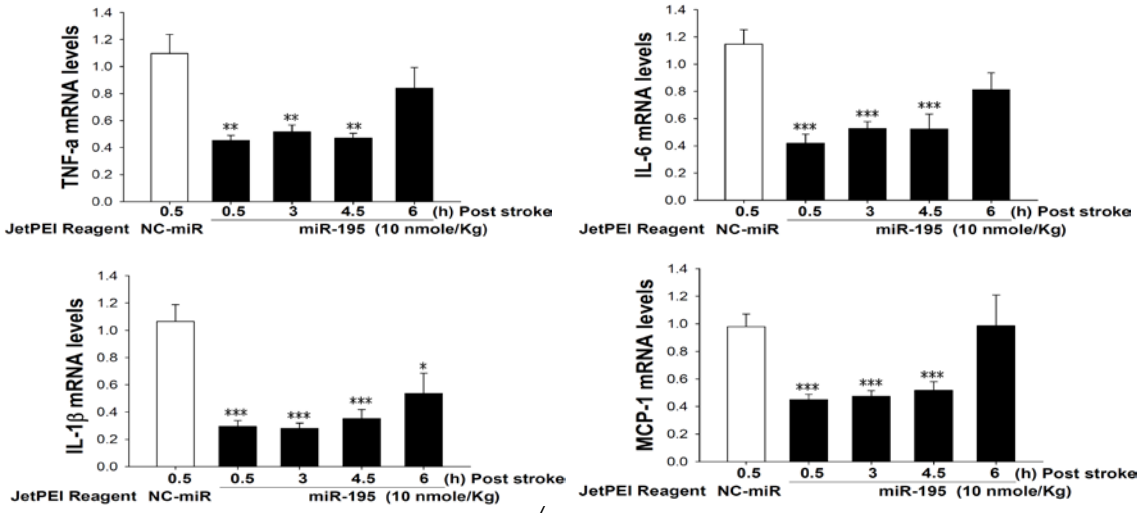

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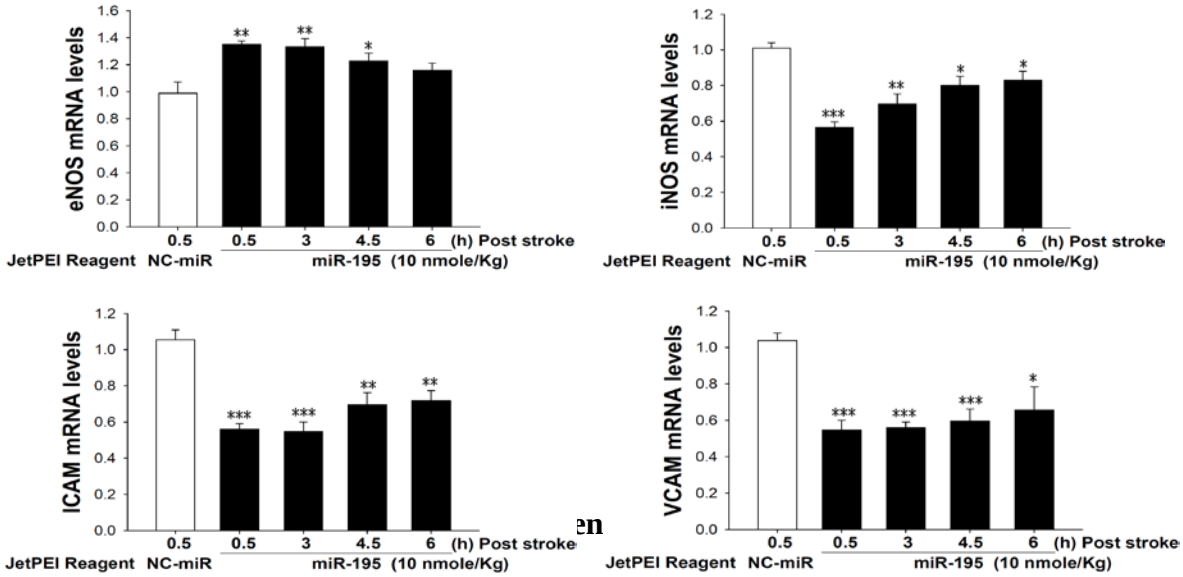
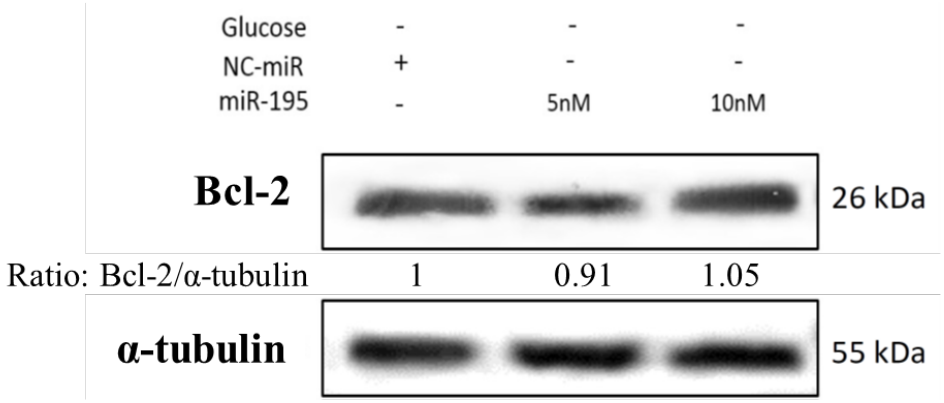


(Suppl Fig 11)



Supplemental Figure Legends

Supplemental. Fig 1. MiR-195 increased survival in OGD-treated SH-SY5Y cells

(A-D) The OGD treatment to SH-SY5Y cells induced a duration-dependent decrease of cell counts and an increase of LDH (A and B), which could be reversed by transfection of miR-195 (C and D). NC-miR: negative control microRNA. Data are means ± SE from three independent experiments performed in triplicates. *P<0.05, **P<0.01 and ***P<0.001 vs. control cells not exposed to OGD.

Supplemental Fig 2. miR-195 knocked down Sema3A mRNA level but had no effect on Nrp-1 mRNA level

Supplemental Fig 3. Knockdown of Sema3A eliminated the OGD effects.

(A) Sema3A level was increased by OGD in a duration-dependent manner and Sema3A-shRNA significantly suppressed OGD-induced Sema3A mRNA level.

(B-D) Sema3-shRNA eliminated the OGD effects on cell viability, LDH release and Cdc42 gene expression. Data are means ± SEM from three independent experiments performed in triplicates. *P<0.05 and **P<0.01 vs. control cells not exposed to OGD. Scrambled shRNA was used as transfection control.

Supplemental Fig 4. miR-195 inhibited nuclear translocation of NF-κB transcription complex

miR-195 influenced the nuclear translocation of NF-κB transcription complex in HUVECs when co-cultured with HASMCs. Nuclear extracts from HUVECs were analyzed by the western blot to detect p50, p65 (also known as RelA), p52 and RelB proteins. Lamin B was used as a nuclear marker. The Western blot data are shown in the left and the quantitative data in the right. Values are presented as mean ± SEM from 3 independent experiments performed in triplicates.

Supplemental Fig 5. miR-195 targeted and suppressed CD40 expression

(A) Sequence pairing revealed a miR-195 target site in the 3' UTR of the CD40 transcript. **(B)** the luciferase reporter assay showed a dose-dependent knockdown of luciferase activity by miR-195 which confirmed the miR-195 binding site in the CD40 3'UTR. miR-195 was transfected by using lipofetamine2000.

Supplemental Fig 6. miR-195 increased SDF-1 secretion

(A) Both miR-195 and oxLDL increased the SDF-1 release in the HASMC culture medium but not in HUVEC culture medium. **(B and C)** miR-195 did not increase SDF-1 release in the culture medium in the mouse brain endothelial cell line

(bEND.3) or in mouse astrocyte cell line (ALT cells). SDF-1 protein levels in culture medium were measured by ELISA. Data are presented as mean ± SEM from at least three independent experiments. **p<0.01, ***p<0.001

Supplemental Fig 7. miR-195 dose-response data

We conducted a dose escalation study to find the optimal dose of miR-195 (carried by jetPEI nanoparticles) in rats of permanent MCAO. The placebo was NC-miR carried by jetPEI nanoparticles. The sample sizes for placebo, 3.3, 6.6, 9.9 and 13.2 miR-195 (nmole/kg) were 3, 3, 9, 10 and 4, respectively. Based on this dose-ranging study, we decided to use 10 nmole/kg for the main animal study.

Supplemental Fig 8. miR-195 biodistribution

miR-195 carried by jetPEI nanoparticles was IV injected (10 nmole/kg) to 18 normal SD rats. miR-195 was detected by real-time PCR. Three rats were sacrificed at the following time points: pre-dose, 1h, 6h, 24h, 48h and 72h.

Supplemental Fig 9. miR-195 had anti-inflammatory effects in stroke animals.

The rats subjected to permanent MCAO were IV injected with miR-195 or NC-miR carried by jetPEI nanoparticles, and the brains were collected at 24hr post-stroke. miR-195 decreased TNFα, IL-1β, IL-6 and MCP-1 mRNA levels in the brains. The

sample size for NC-miR was 6. For miR-195 treatment at the time point of 0.3h, 3h, 4.5h and 6h, the sample size was 10, 10, 10 and 8, respectively. RNA data are presented as mean ± SE from qPCR performed in triplicates. *P< 0.05; **p<0.01; ***p<0.001.

Supplemental Fig 10. miR-195 exerted endothelial protection in stroke animals. The rats subjected to permanent MCAO were IV injected with miR-195 or NC-miR carried by jetPEI nanoparticles, and the brains were collected at 24hr post-stroke. miR-195 improved endothelial functions by altering mRNA levels of iNOS, eNOS, VCAM and ICAM. The sample size for NC-miR was 6. For miR-195 treatment at the time point of 0.3h, 3h, 4.5h and 6h, the sample size was 10, 10, 10 and 8, respectively. RNA data are presented as mean ± SE from qPCR performed in triplicates. *P< 0.05; **p<0.01; ***p<0.001.

Supplemental Fig 11. The effect of miR-195 effect on BCL2 expression in mouse neural cells (N2A)

Supplemental Table1. Negative correlation between miR-195 in the infarct hemisphere and infarct size.

	infarct size %	brain miR-195*	sema3A*
PEI195-D3H-1	3	4.52x	1.07x
PEI195-D3H-2	4.38	3.48x	0.9x
PEI195-D3H-3	5.02	2.97x	1.37x
PEI195-D3H-4	12.2	3.74x	1.16x
PEI195-D3H-5	12.62	2.05x	0.84x
PEI195-D3H-6	16.69	4.08x	0.96x
PEI195-D3H-7	21.49	2.34x	1.76x
PEI195-D3H-8	22.95	1.73x	3.44x
PEI195-D3H-9	24.56	1.85x	1.61x
PEI195-D3H-10	25.42	1.82x	1.54x

Spearman r=-0.78 (p=0.0075) between infract size and fold increase of miR-195.
*the ratio = RNA expression level in the infarct hemisphere/contralateral hemisphere

Supplemental Table 2. The calculate pharmacokinetics parameters.

The injected dose of 10 nmole/kg is equivalent to 6×10^{15} miR-195 copy/kg. Seven rats were used for this pharmacokinetic study. The first blood sample after miR-195 injection was withdrawn at 5 min.

λ_z: the elimination (terminal) rate constant.

$T_{1/2}$: the elimination (terminal) half-life.

T_{max}: the time at which C_{max} is reached.

C_{max}: the observed maximum (peak) plasma miR-195 concentration after dosing.

$AUC_{0\text{-}last}$: the area under the plasma miR-195 concentration-time curve from the time zero to that of the last sample assayed.

$AUC_{0\text{-}\infty}$: the area under the plasma miR-195 concentration-time curve from the time zero to infinity.

CL: the plasma clearance of the miR-195 from the body.

$MRT_{0\text{-}\infty}$: the mean residence time (MRT_0) extrapolated to infinity.

V_β: the volume of distribution at the elimination phase.

V_{ss}: the volume of distribution at steady-state.

Non-compartmental pharmacokinetic parameters are calculated using the WinNonlin™. The apparent plasma terminal elimination half-life ($T_{1/2}$) is calculated according to the following formula:

$$T_{1/2} = \ln(2)/ \lambda_z$$

The observed maximum plasma concentration (C_{max}) and the time of C_{max} (T_{max}) were determined directly from the experimental values. The area under the plasma concentration-time curve of miR-195 from time 0 to the last measurable concentration ($AUC_{0\text{-}last}$) was determined by the linear trapezoidal rule. The area under the plasma concentration-time curve from time 0 to infinity ($AUC_{0\text{-}\infty}$) was determined by $AUC_{0\text{-}last} + C_{last}/\lambda_z$.

Rat	λ_z (1/min)	$T_{1/2}$ (min)	T_{max} (min)	C_{max} (copy/mL)	$AUC_{0\text{-last}}$ (min*copy/mL)	$AUC_{0\text{-}\infty}$ (min*copy/mL)	CL (copy/ (min*copy/mL)/kg)	$MRT_{0\text{-}\infty}$ (min)	V_β (copy/ (copy/mL)/kg)	V_{ss} (copy/ (copy/mL)/kg)
1	0.000776	893.2743	5	3.43694E+13	1.97099E+15	1.97099E+15	2.930183	770.624	3776.192	2258.069
2	0.000435	1593.003	5	2.37986E+13	7.54123E+14	7.54123E+14	7.39451	1033.225	16994.19	7640.189
3	0.000426	1625.835	5	1.88531E+13	9.96471E+14	9.96471E+14	5.572238	1285.021	13070.15	7160.446
4	0.000405	1712.684	5	1.74402E+13	1.05939E+15	1.05939E+15	4.942568	1648.827	12212.49	8149.441
5	0.000647	1071.015	5	3.77593E+13	1.95273E+15	1.95273E+15	3.005403	556.7267	4643.792	1673.188
6	0.000104	6650.806	5	2.51089E+13	1.09389E+15	1.09389E+15	3.319097	6016.793	31847.02	19970.32
7	0.000293	2366.807	5	3.22587E+13	1.15722E+15	1.15722E+15	4.664472	1441.843	15927.22	6725.435

Supplemental Methods and Materials

Cell culture

Human neuroblastoma SH-SY5Y cells, Human THP-1 monocytic leukemia cells, human umbilical venous endothelial cells (HUVEC) were from American Type Culture Collection (ATCC). Mouse neuroblastoma cell line N2A cells and mouse brain endothelial cell line bEnd.3 cells were from Bioresource Collection and Research Center (Taiwan). The rat neural stem cells were isolated from SVZ. The components of culture medium for SH-SY5Y contain a 1:1 mixture of DMEM and Ham's F-12 medium, 10% heat-inactivated FBS, 4mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B at 37^{o}C in a humidified incubator under 5% CO_2 and 95% air. The medium was changed every 3-4 days. Cells were seeded in 96-well culture plates to measure cell viability at a density 1×10^5 cells/ml. N2A cells and bEnd.3 cells were maintained in DMEM supplemented with 10% FBS plus 50 μg/ml gentamicin in a humidified incubator under an atmosphere of 5% CO2/95% air at 37 °C.

Cell co-culture

For HASMC/HUVEC non-contact co-culture, HUVECs were seeded onto the 24-well plate at a density of 3×10^5 cells/cm^2. HASMCs were seeded on the filters of transwell insert (0.4 μm pore membrane; Millipore) at a density of 1×10^5 cells/cm^2. The insert was put on the top of 24-well plate as the co-culture system with or without the presence of oxLDL (40 μg/ml).

Oxygen–glucose deprivation and transfection of miRNA

The oxygen–glucose deprivation (OGD) condition mimics the in vivo ischemic condition. For OGD, SH-SY5Y cells were cultured with glucose-free DMEM (Gibco Inc.) and placed in the hypoxic chamber (5% CO_2, 1% O_2 and 94% N_2) for 3h or 6h. After OGD, SH-SY5Y cells were maintained in glucose-containing DMEM with normoxia (5% CO_2, 95% O_2) for 21h (if OGD duration was 3h) or 18h (if OGD duration was 6h). Control SH-SY5Y cells were cultured in the normoxic condition for 24 hrs. Cellular phenotypes and biomarkers were measured at 24^{th} hour after the initiation of OGD treatment.

Cell viability

The cell viability was determined by cell counts and lactate dehydrogenase (LDH) assay. Cells were seeded at a density of 1.5×10^5 cells per well on collagen coated 12-well plates and maintained at 37°C in a 5% CO2 atmosphere. After OGD and reoxygenation, an aliquots of cells were counted and stained with trypan blue to determine viability. Cell lysis was quantified after 4h of treatment with the test agents by LDH activity using the Cytotoxicity Detection KitPlus (Roche Applied Science, Germany). The plates were centrifuged at 400 g and 4^oC for 4 min and an aliquot of 50 µl was taken to quantify the LDH amount.

Cell cycle Analysis

OGD-induced SH-SY5Y cell death was identified as a sub-G1 peak in the cell cycle. Briefly, 5 x 10^6 harvested cells were washed in ice-cold PBS, fixed in ice-cold 70% ethanol and stored at 4^{o}C. Cells were then washed with PBS, treated with 0.5 mg/mL RNase (Sigma) at 37^{o}C for 15 min and finally stained with propidium iodide (Sigma, 50 μg/mL) in PBS. Cells were analyzed on a Coulter EPICS XL flow cytometer (System II software; Becton-Dickinson, CA, USA) and data were analyzed with Modifit LT 2.0 (Becton-Dickinson, CA, USA). 10,000 cells were counted for each determination. For detailed analysis, the three cell cycle compartments (sub-G1, G0/G1, S, and G2/M phases) were distinguished and the percentage of cells was quantified with CELLQuest software (Becton-Dickinson, CA, USA).

Detection of DNA fragmentation by TUNEL assay

For the TUNEL assay, the ApoAlert DNA fragmentation assay kit (Clontech, CA, USA) was used. The incorporation of fluorescein-dUTP into the fragmented nuclear DNA generates the green fluorescence detected by a standard fluorescein filter set (520 ± 20 nm). All cells stained with propidium iodide exhibited strong red cytoplasmic fluorescence when viewed at > 620 nm.

Luciferase reporter construct and assay

The plasmid construct was created to carry the predictive miR-195 binding site. A DNA segment containing miR-195 binding site in the wild-type 3'UTR was cloned into the Mlu I/Hind III site of the pMIR-REPORT Luciferase vector (Life Technologies). The mutant 3'UTR to destruct the miR-195 binding site was generated by site-directed mutagenesis as described previously (Brons-Poulsen et al., 1998). Plasmid constructs (carrying either wild or mutant type 3'UTR) and miR-195 were co-transfected into the HEK293A cells, and firefly and Renilla luciferase activity were measured at 24h using the Dual-Luciferase Reporter Assay. Each transfection was repeated twice in triplicate.

Target site prediction

Three algorithms were used to predict miR-195 target genes. The algorithms are miRanda algorithm (http://microrna.sanger.ac.uk/targets/v5/), TargetScan (http://targetscan.org/), and PicTar (http://pictar.mdc-berlin.de/). The binding sites of POU2F1 in the promoter region of COMP and Ang1 were predicted by the TFSEARCH promoter program (http:// www.cbrc.jp/research/db/TFSEARCH.html).

Real-time PCR quantification and protein detection

Total RNA was isolated from cells using Trizol reagent (Invitrogen). RNA quality was measured using A260/A280 readings. cDNA was synthesized and real-time PCR were conducted on a 7900 HT Fast Real Time PCR system (Life Technologies). The expression

level was calculated by using the difference of threshold cycle method (ΔCt) with normalization to the reference genes (RNU6B or GAPDH).

For proteins, intra-cellular proteins were detected by the western blot. Cells were homogenized in 100 μl of protein extraction reagent (Thermo Scientific, Waltham, MA, USA) and protease inhibitor (Panomics, Fremont, CA, USA). Protein concentration was determined by the Pierce BCA Protein Assay Kit (Thermo Scientific). Protein of 20 μg was loaded per lane and separated by NuPAGE Novex Bis-Tris 4-12 % mini gel electrophoresis (Invitrogen) in the Novex Xcell-II apparatus for 120 min at 100 V, and then transferred to Immbilon-PVDF transfer membranes (Millipore, Billerica, MA, USA) for immunoblotting. Proteins were visualized by enhanced chemiluminescence according to the manufacturer's instruction. Nonspecific binding was blocked with 5 % nonfat milk for 1 hour at the room temperature.

The antibodies to IKKα, IKKβ, IκBα, p-IκBα and β-actin were purchased from Genetex (Irvine, CA, USA). The antibodies to IκBβ and VCAM-1 were purchased from Abcam (Cambridge, MA, USA). The antibodies to p-IκBs was purchased from Cell Signaling (Danvers, MA, USA). The antibody to E-selectin was purchased from Biovision (Milpitas,CA. USA). The antibodies to ICAM-1, eNOS, CD40 and GAPDH were purchased from BD (Franklin Lakes, NJ, USA). For the NF-κB signaling, the nuclear proteins were extracted by the NucBuster protein extraction kit (Novagen, Madison, WI, USA). The

antibodies to p65, p50, p52, RelB and Lamin B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tie-2 and anti-Ti2-P antibodies were purchased from Millipore (Bedford, MA, USA). Anti-body for POU2F1 was from Cell Signaling Technology (Danvers, MA, USA).

The proteins in the supernatant of culture medium were detected by the enzyme-linked immunosorbent assay (ELISA) kits (BD Bioscience, CA, USA).

Northern blotting assay for miR-195

The northern blot hybridization used the miRNA northern blot assay kit (Signosis, CA, USA). 5 μg total RNA was loaded to each well. RNA was fractionated using 15% denture gel, blotted on membranes, and hybridized with biotin labeled miR-195 and U6 probes.

Monocyte adhesion assay

THP-1 cells were suspended in medium with 5 μM of Calcein AM (C3100MP, Invitrogen). The Calcein AM–loaded THP-1 cells (200 μl at 5 x10^5 cells/ml) were added to the wells containing HUVECs. Non-adherent THP-1 cells were removed and fluorescence of adherent THP-1 cells was detected by a fluorescence microscope and the intensity of fluorescence was measured by a plate reader at 485 nm excitation and 515 nm emission filters.

Transfection of microRNA, siRNA and lentiviral-based short hairpin RNA (shRNA)

The sequences of synthetic miR-195 mimic, miR-195 inhibitor and NC-miR were 5’-UAGCAGCACAGAAAUAUUGGC-3’, 5’-GCCAATATTTCTGTGCTGCTA-3’ and 5’-AGUACUGCUUACGAUACGG-3’ respectively. The Lipofetamine 2000 (Thermo Fisher Scientific) or HiPerFect Transfection Reagent (Qiagen) was used for transfection. The lentivirus clone containing shRNA was obtained from the National RNAi Core Facility (Academia Sinica, Taiwan). shRNA plasmid (1μg /mL) or empty vector (pLKO.1 puro) was transfected to the cells using Lipofectamine 2000 (Invitrogen).

Viral-miR preparation

The DNA fragment encoding pre-miRNA-195 was amplified by PCR from human genomic DNA. The PCR primers were: forward-5'-AAGTGGAGTCTTTGTTGCCCACACCCAGCT-3' and reverse-5'-CCACCCT GCCTGGAGCAGCACAGCCAATAT-3'. The adenovirus expressing miR-195 (Ad-miR-195) and control adenovirus expressing GFP (Ad-GFP) were generated using the RAPAd miRNA Adenoviral Expression System kit (Cell biolabs, CA, USA) according to the manufacturer’s protocols. The inserted fragment was verified by automated sequencing. Ad-miR-195 and Ad-GFP were generated using the RAPAd miRNA Adenoviral Expression System kit (Cell biolabs, CA, USA).

For virus amplification, we infected the adenoviruses into HEK293A cells, and then purified the virus by ViraBind Adenovirus Miniprep kit (Cell biolabs) followed by titration with the QuickTiter Adenovirus Titer Immunoassay kit (Cell biolabs). Viral multiplicity of infection was estimated based on in vitro HEK-293A transduction efficiency: 0.5 ml of undiluted viral stocks was added to 10^6 HEK293A cells cultured in the 12-well plates, and number of GFP-positive cells was counted 48 h after transduction. After transfection for 48 hours, supernatants from virus-containing cell culture were harvested and deep frozen in aliquots.

Rat model for balloon injured carotid artery and stroke

The procedure for balloon injury can be found in our previous publication (Wang et al., 2012). For ischemic stroke model, male SD rats (280 to 350 g) were used for the induction of middle cerebral artery occlusion (MCAO) with previously reported surgical approaches (Candelario-Jalil et al., 2005; Wang et al., 2010). Rats were sedated with isoflurane (Abbott Laboratories Ltd., Queenborough, Kent, UK), anaesthetized by intraperitoneal administration of pentobarbital sodium (40 mg/kg; Sigma – Aldrich). A 3-0 nylon filament with silicon modification at the tip was inserted into a small nick on the right CCA and advanced approximately 22 mm beyond the carotid bifurcation to occlude blood flow to the middle carotid artery (MCA). Thereafter, surgical sutures were ligated along the CCA rostral to the nick to anchor the nylon filament and to seal off the vessel. The skin incision was

closed with surgical suture and topically treated with antibiotic ointment. For the permanent MCAO, the inserted nylon filament was left for 24hr and then the rats were sacrificed by over-dosed isoflurane at 24hr post-stroke because of high mortality and severe morbidity after 24hr MCAO. For transient MCAO, the inserted nylon filament was removed for reperfusion after 2hr occlusion. The rats subjected to transient MCAO were evaluated for the Garcia score and then sacrificed on day5 post-stroke.

For the hemorrhagic stroke, male SD rats (280-320 g) were sedated with isoflurane (Abbott Laboratories Ltd., Queenborough, Kent, UK), anaesthetized by intraperitoneal administration of pentobarbital sodium (40 mg/kg; Sigma–Aldrich), and then placed in a stereotaxic frame in a supine position on a warming pad at 37 °C. intracranial hemorrhage (ICH) was induced by stereotaxic infusion of bacterial collagenase Type IV-S (0.6 U in 1.0 μl sterile saline, Sigma-Aldrich) over a period of 5 minutes (0.2 mm posterior, 3.0 mm right, 6.0 mm depth to bregma at the skull surface). The needle was kept in place for another 10 minutes to prevent backflow. The ICH animals were sacrificed on day3 post-stroke.

miR-195 mimic (10 nmol/kg) or normal control microRNA (NC-miR) was formulated with In vivo-jetPEI® (PolyPlus Transfection Inc. IIIkirch, France) according to the manufacturer's instructions. Formulated microRNA was administrated intravenously (IV)

via tail vein after stroke induction. The treatment was given at 30 min, 3h, 4.5h and 6h for the permanent MCAO group, only 6h for the transient MCAO group, and 4h for the ICH group. The operators were blinded to treatment assignment of miR-195 or NC-miR.

The brains were collected and sliced into 2 mm coronal sections. The ischemic brain slices were stained with 0.1% 2,3,5-TTC. Stained brain slices were scanned with a flatbed scanner. Digital photographs of the brain slices were taken and lesion volume was computed using Image J (version 1.40, NIH, Bethesda, MD, USA). The total lesion volume or lesion percentage of brain (mm^3) was calculated as the sum of lesion area in each section.

Neurological score

Rats subjected to transient MCAO were evaluated for the Garcia score. To exclude potential failure of induced ischemic stroke, only rats with the Garcia score between 6 and 8 at 6h post-MCAO were included for the further studies. This neurological score will test for spontaneous activity, symmetry of movement and outstretch of limbs, circling behavior, climbing ability, body proprioception, and vibrissae touch. Each item was graded from 0-3 with the overall Garcia scores ranging from 0 to 18.

Similarly, to exclude potential failure of induced hemorrhagic stroke, rats were evaluated by the severity of movement impairment, and only impairment reached the moderate or severe

level, the rats were used for further studies. The movement evaluation has 3 grades – (1) “Mild” if a rat walked a straight line, weaker grip of the left forelimb and could stand on their own in a cage, (2) “Moderate” if a rat circled or walked to the left, activity was weakened and almost could not grip the left forelimb, and (3) “Severe” if a rat failed to walk without help, almost could not grip of the left forelimb and no activity.

The graders were blinded to treated and placebo groups. The assessment was performed on day3 and day5 for transient MCAO rats and on day1, 2 and 3 for ICH rats.

Assessment of brain edema

Because the brain edema peaked on day3 post-ICH (National Institute of Neurological and Stroke rt, 1995), the water content in the brain was measured using a common wet/dry method as previously described. On day3 the brain was removed to obtain the wet weight and the cerebellum was used as an internal control. The sample was then dried in an oven at 85°C for 48h to obtain the dry weight. The water content was expressed as a percentage of the wet weight: [(wet weight)-(dry weight)] (wet weight) $^{-1}$ × 100 %.

Evaluation of BBB permeability

Cerebrovascular permeability was assessed by a modified Evans blue extravasation method. On day3 post-ICH, rats were anesthetized and injected with 2% solution of Evans blue in normal saline (4 ml/kg of body weight) into the tail vein. The stain was allowed to circulate

for 2h. After that the rats were perfused with 300 ml normal PBS to wash out any remaining dye in the blood vessels and then the brains were removed and sectioned to 2 mm thickness. The cerebellum was used as an internal control. Evans blue dye was extracted by incubating them in N,N-dimethyl formamide overnight at 55°C and vortexed for 5 minutes. The mixture was subsequently cooled for 30 minutes and centrifuge (1500 g at 4°C) for another 30 minutes. The absorbance of Evans blue in the supernatant was then measured with a spectrophotometer at 610 nm. The results were presented as (g of Evans Blue stain) / (g of brain tissue).

Immunofluorescent staining and immunohistochemistry (IHC) staining

Immunofluorescent staining was used to detect cellular expression of Ang-1 (Sigma-Aldrich), COMP (Biorbyt), SDF-1 (Millipore), Sox2 (Abcam), Nestin (Abcam) and GAP43. IHC was used to stain the markers in brain slices. For brain slices, after 4% paraformaldehyde fixation for 15min, the slices were blocked in blocking buffer for 1 hour, incubated with primary anti-bodies overnight at 4℃, and followed by incubation with the secondary anti-bodies conjugated with anti-rabbit Alexa 488, anti-goat Alexa 647, or anti-mouse Alexa 647 for 1 hour. Nuclei staining was performed with 1ug/ml DAPI (Molecular Probes, Carlsbad, CA) for 5min at room temperature. After extensive washing, the coverslips were dried and mounted on glass slides.

Isolation and culture of neural stem cells

The tissue from SVZ was dissected, kept in 10 ml of ice-cold Hibernate A medium and cut into small pieces. After centrifugation, HBSS containing 0.1% (w/v) trypsin and 0.01% (w/v) DNase 1 was added, followed by rotation at 37°C for 60 min. Fetal bovine serum was used to stop the digestion, followed by centrifugation, and re-suspended in HBSS. The cell and tissue suspension was filtered and washed with the N2 medium. After removing excess myelin and other cell types, cells were seeded on fibronectin-coated culture plates in a plating medium (N2/DF), and maintained at 37°C in a humidified 5% CO_2 atmosphere.

Study approval

The Animal Care and Use Committee of the Kaohsiung Medical University approved the animal experimental protocols, which strictly conforms to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).