Endothelial progenitor cell secretome and oligovascular repair in a mouse model of prolonged cerebral hypoperfusion.

SUPPLEMENTARY MATERIAL

METHODS

Cerebral prolonged hypoperfusion model

Briefly, male C57Bl/6 mice (25-30g, 2-3 months old, Charles River Institute) were anesthetized with 4.0% isoflurane and then maintained on 1.5% isoflurane in 70% N_2O and 30% O_2 using a small-animal anesthesia system. Through a midline cervical incision, both common carotid arteries were exposed. A microcoil with an internal diameter of 0.18 mm (Sawane Spring Co.) was applied to bilateral common carotid arteries. Rectal temperature was maintained between 36.5°C and 37.5°C. A total of 24 mice were initially subjected to the BCAS surgery. Since most of the BCAS-operated mice do not exhibit severe neurological dysfunction nor massive infarcts at least within 1 month after the surgery (1-3) we excluded animals that presented signs of paresis and convulsion, remarkable body weight loss (>20% from baseline during the follow-up period) and evident infarcts at the histological evaluation as follows: from 24 mice which were initially subjected to the BCAS surgery, 3 mice were euthanized during or immediately after the surgery after presenting bleeding from the CCA, left hemiparesis, and convulsion, respectively. The other 21 mice were divided into two groups; vehicle-treated (n=11) vs. EPC-CM-treated (n=10) mice. Three mice were excluded for further functional and histological evaluation because they showed remarkable body weight loss during the follow-up period (n=2) or an evident infarction at 28 days after the surgery (n=1).

Therefore, a total of 18 mice (vehicle-treated (n=10) and EPC-CM-treated (n=8) mice) were evaluated for functional outcome and white matter integrity.

Protocol for Conditioned Media Production

Mouse outgrowth Endothelial Cells (OECs) derived from EPC cultures of FVB mice obtained in a previous study (4) and stored in liquid nitrogen were used for the production of EPC-secretome as conditioned media (CM). Briefly, cells were thaw and seeded in fibronectin-coated 75cm^2 plastic flasks and grown in EGM-2MV OR EGM-2 media (consisting in EBM plus supplemental factors such as hEGF, Hydrocortisone, GA, VEGF, hFGF-B, IGF-1 and Ascorbic Acid, from Lonza) plus 10%FBS media. At confluence flasks contained about 4x10^6 cells, growing media was removed and cells were gently washed 3 times with PBS. Finally 12 mL of basal media (EBM) were added for 24 hours when CM was collected, transferred into a sterile tube and centrifuged at 1,500 rpm for 5 minutes to remove any cell debris. Finally the CM was concentrated using 10KDa-membrane centrifuge tubes (Sartorius, V0601) and spun for a total of 22 minutes at 4,000g at 21ºC (about 12x final volume). CM was obtained from 10 flasks at different days. EBM media was also concentrated as vehicle media but only for 3 minutes to obtain similar concentration volumes. Single aliquots of concentrated CM and concentrated basal media (BM, vehicle) were frozen at -80ºC until use.

Y Maze Test

The maze consists of 3 arms (40 cm long, 9.5 cm high, and 4 cm wide, labeled arm-A, -B, or -C) with equal angles between all arms. Experiments were performed in a dimly illumination room between 9:00pm to 11:00pm. Mice were initially placed within one arm and allowed to move in the maze freely. The sequence and number of arm entries were recorded for each mouse over an 8-minute period without reinforces such as food, water, or electrical foot shock. The task was videotaped with a video camera (Everio GZ-MG-77-S). The percentage of triads in which all three arms were represented (ABC, CAB, or BCA but not BAB) was calculated as an

alternation to estimate short-term memory of the last arms entered whereas the number of arm entries serves as an indicator of spontaneous activity.

Western Blot

Corpus callosum protein concentrations were quantified and adjusted to the same concentrations (0.5 μg/μl) by adding PBS, samples were mixed with equal volumes of sample buffer containing 91% SDS (Novex) and 9% 2-mercaptoethanol (Sigma). Subsequently, samples were heated at 95°C for 5 min and each sample (10 μg per lane) was loaded onto 4–20% Tris–glycine gels. After electrophoresis and transferring to nitrocellulose membranes (Novex), the membranes were blocked in Tris buffered saline with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk for 60 min at room temperature. Membranes were then incubated overnight at 4°C with anti-PDGFR-α (1:1000, Santacruz), myelin basic protein (MBP) antibody (1:500, Thermo scientific), anti-CD31 antibody (1:500, BD Pharmingen), or anti-β-actin antibody (1:10000, Sigma Aldrich) followed by incubation with peroxidase-conjugated secondary antibodies and visualization by enhanced chemiluminescence (Amersham).

Separately, EPC-CM were loaded in SDS-PAGE (12%) and transferred into PVDF membranes. Non-specific bindings were blocked with non-fat milk (10% in PBS-Tween), and membranes were incubated overnight with rabbit anti-ANG (NOVUS; 1:500) with non-fat milk (10% in PBS-Tween) at 4ºC. Secondary antibody was diluted 1:10.000 and membranes incubated at room temperature for 1 hour. The substrate reaction was developed with a chemiluminescent reagent and visualized with a luminescent image analyzer (Las-3000, FujiFilm; USA).

Immunohistochemistry

After fixation and cryoprotection, the brain was frozen and 16-μm-thick consecutive coronal sections at 0 to 0.5 mm anterior from the bregma were prepared using a cryostat. Brain sections were incubated at 37°C for 30 min in 1N HCl to detect BrdU labeling. Sections were incubated

overnight with anti-BrdU (1:50; Oxford Biotechnology). Double immunofluorescence staining was performed by simultaneously incubating the sections overnight at 4°C with anti-GST-pi (1:100, MBL) and anti-PDGFR-α (1:100, R&D systems) antibodies. For CD31 staining, the mouse was perfused transcardially with ice-cold saline, and the brain was removed, frozen on dry ice immediately and stored at -80℃ in light-shielded conditions. The brain was cut on a cryostat (20μm) and fixed with 4%PFA (15min), incubated with PBS-0.1%Tween (15min), and blocked with 10% blockase (AbD serotec, 60min) before sections were incubated at 4℃ overnight with anti-CD31 antibody (1:100, BD Pharmingen). Then sections were incubated with secondary antibodies with fluorescence conjugated secondary antibody (1:200; Jackson Immunoresearch Laboratories) at room temperature for 60 min. Subsequently, the slides were covered with Vectashield mounting medium with DAPI (H-1200, Vector Laboratories). Immunostaining was analyzed with a fluorescence microscope (Nikon) interfaced with a digital charge-coupled device camera. The number of the BrdU- and GST-pi- immunoreactive (BrdU+/GST-pi+), and BrdU- and PDGFR-α- immunoreactive (BrdU+/ PDGFR-α+) cells in the corpus callosum was counted by an investigator blinded to the experimental groups**.** Total number of proliferating oligodendrocyte lineage cells (which included both GST-pi+ and PDGFR-α+ / BrdU+) were also analyzed according to treatment.

Protein Profile of EPCs Secretome

Briefly, $2x10^6$ OECs were cultured as described above in 75 cm^2 flasks. After 24 hours they were washed twice and 12 ml of fresh basal media (EBM) were added to obtain the CM 24 hours later. The media were concentrated with 10K-membrane centrifugal filters (Amicon Ultra-0.5ml 10K Ultracel, Millipore, Germany), obtaining 0.4 to 0.5 ml of concentrated CM. The total protein amount in the concentrated CM was determined by Comassie Protein Assay kit (Thermo Scientific, USA). Next, the profile of proteins present in the OECs conditioned

media (n=4) was analyzed using the Proteome Profiler Mouse Angiogenesis Array kit (R&D Systems, USA) which can detect the expression of 53 mouse angiogenesis-related proteins. Briefly, 150ug of total protein of concentrated CM mixed with the biotinylated detection antibodies provided in the kit were incubated with the nitrocellulose membrane containing the capture antibodies (n=4). After an overnight incubation the membranes were washed and the Streptavidin-HRP and chemiluminescent detection reagents applied. The spot-signal was detected with the Luminescent Image Analyzer LAS-3000 (Fujifilm Medical systems, USA) and densities were analyzed with the Searchlight Array Analyst (Axiocor, Canada). Concentrated EBM was used as negative control. Results are expressed as neat chemilluminiscent units (by subtracting the background of the negative control).

Endothelial Cell Proliferation Assay

To test the pro-angiogenic properties of EPCs conditioned media on human cerebral microvascular endothelial cells (hCMEC/D3) we assessed cell proliferation after treatment with EPCs conditioned media, Angiogenin (a potent pro-angiogenic- mitogenic factor identified as aprt of EPCs secretome) and Neomycin (an inhibitor of Angiogenin). Briefly, $1x10^4$ human endothelial cells were seeded in 24/well plates in EGM-2 media (cointaining EBM, ½ of the supplemental factors hEGF, Hydrocortisone, GA, VEGF, hFGF-B, -IGF-1, Ascorbic Acid, Heparin) and 2% FBS) during 3 days. At day 4th wells were gently washed with PBS twice and treatments added in corresponding wells: conditioned media diluted 1/20 in EBM, Angiogenin or Neomycin at the mentioned dilutions. As controls concentrated EBM 1/20 or EBM were used, respectively. Each experiment was run in duplicates and data is expressed as percentage of control condition. The number of total and viable cells was counted with the Muse™ Cell Count and Viability Kit.

Endothelial Cell Viability Assay

Measurement of the reduction of 3-(4,5-dimethylthiazol- 2-yl)2,5-diphenyl-tetrazolium bromide (MTT) to produce a dark blue formazan product was performed to assess the integrity of mitochondrial function as a measure of cell viability in hCMEC/D3 treated with Neomycin at different doses for 24 hours when MTT was diluted basal media and added to cells for 60 minutes. Two wells per condition were run in each experiment and the obtained blue formazan was measured per duplicate to obtain a mean value. Results for treatments are expressed as a percentage of the control (basal media) group absorbance.

Oligodendrocyte Precursor Cell Culture

OPCs were prepared from cerebral cortices of 1-2 day old SD rat pups. Dissociated cortex cells were plated in poly-d-lysine-coated flasks, and cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 20% fetal bovine serum and 1% penicillin/streptomycin. After the cells were confluent, the flasks were shaken for 1 hour on an orbital shaker (220 rpm) at 37°C to remove microglia. The medium was changed and shacked overnight when the medium was collected and plated on non-coated tissue culture dishes for 1 hour at 37°C to eliminate possible contamination by astrocytes and remaining microglia. The non-adherent cells were collected and maintained in Neurobasal (NB) media containing glutamine, 1% penicillin/streptomycin, 10 ng/mL PDGF-AA, 10 ng/mL FGF-2 and 2% B27 supplement onto poly-dl-ornithine-coated plates.

Immunocytochemistry

Cultured EPCs or OPCs were immunostained for Myelin Basic Protein (MBP) or Angiogenin respectively. Briefly, cells were washed with ice-cold PBS (pH 7.4), treated with 4% PFA for 15 min, incubated with 0.1% triton for 10 min and further incubated with 3% BSA for 1 hr. Subsequently, cells were incubated with primary antibody against MBP (1:200, Thermo Scientific) or ANG (1:50) at 4°C overnight. After being washed with PBS, they were incubated with secondary antibodies with fluorescence conjugations for 1 hour at room temperature.

Finally, nuclei were counterstained with DAPI. Image was analyzed with a fluorescence microscope (Nikon) interfaced with a digital charge-coupled device camera and an image analysis system.

REFERENCES

1. Shibata M, Ohtani R, Ihara M et al. White matter lesions and glial activation in a novel mouse model of chronic cerebral hypoperfusion. Stroke. 2004;35:2598-2603.

2. Shibata M, Yamasaki N, Miyakawa T et al. Selective impairment of working memory in a mouse model of chronic cerebral hypoperfusion. Stroke. 2007;38:2826-2832.

3. Reimer MM, McQueen J, Searcy L et al. Rapid disruption of axon-glial integrity in response to mild cerebral hypoperfusion. J Neurosci. 2011;31:18185-18194.

4. Morancho A, Hernández-Guillamon M, Boada C, Barceló V, Giralt D, Ortega L, et al. Cerebral ischaemia and matrix metalloproteinase-9 modulate the angiogenic function of early and late outgrowth endothelial progenitor cells. J Cell Mol Med. 2013;17:1543-1553.

TABLE & FIGURE LEGENDS

Supplementary Table I. List of proteins detected in the EPCs secretome.

Supplementary Figure I. Representation of the analyzed regions of the corpus callosum studied for by western blot and immunohistochemistry studies.

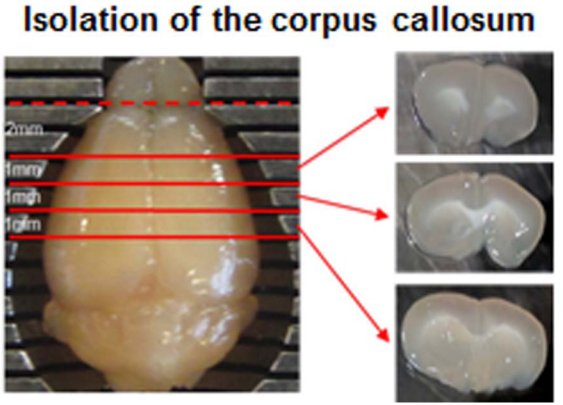
Supplementary Figure II. Chemilluminiscent signal of representative Proteome Profiler™ membranes at different acquisition times including both conditioned media (CM) and vehicle treatments. White circles show membrane positive controls, all other bright signal corresponds to expressed proteins.

Supplementary Figure III. Cell viability of mature human endothelial cells (hCMEC/D3) treated with neomycin. The potential toxic effects of Neomycin (used as an inhibitor for the ANG present in the Conditioned media) were tested by the MTT assay. No significant differences were observed at the tested concentrations (n=4).

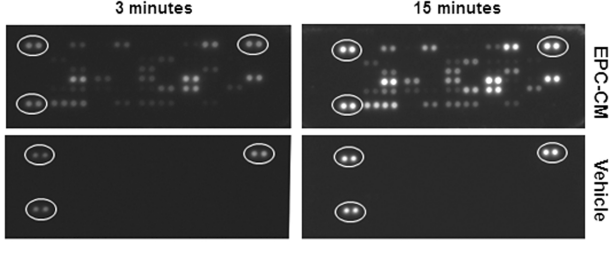
Supplementary Table 1. List of proteins detected in the EPCs secretome.

PROTEIN	Signal Intensity	PROTEIN	Signal Intensity
ADAMTS1	**79.2±40.6**	**IP-10/CXCL10**	**212.5±35.8**
Amphiregulin	n.d	**KC/CXCL1**	**101±46**
Angiogenin	**103.6±44.0**	Leptin	n.d
Angiopoietin-1	n.d	**MCP-1/CCL2**	**110.7±25**
Angiopoietin-3	n.d	MIP-1a/CCL3	6.5±5.8
Cogulation factor III	**37.8±25.5**	**MMP-3**	**203.6±59.1**
CXCL16	**170.5±55.8**	**MMP-8**	**13.2±8**
Cyr61	**44.3±28.2**	**MMP-9**	**7.7±2**
DLL4	n.d	**NOV**	**203.1±43.5**
DPPIV	n.d	**Osteopontin**	**31.7±18.7**
EGF	n.d	**PD-ECGF**	**57.7±38.8**
Endoglin	**21±12.9**	**PDGF-AA**	**26±15.6**
Endostatin/Collagen XVII	**84.8±34.2**	**PDGF-AB/PDGF-BB**	**6.7±5.5**
Endothelin-1	**10.7±7.8**	**Pentraxin-3**	**36.7±29.2**
FGF acidic	**10.5±6.5**	**Platelet factor 4/CXCL4**	**123.2±36.8**
FGF basic	n.d	**PlGF-2**	**197.1±57.6**
FGF-7/KGF	**5±4.5**	Prolactin	n.d
Fractalkine	**58.2±29.7**	**Proliferin**	**74.1±31**
GM-CSF	n.d	**SDF-1/CXCL12**	**159±56.7**
HB-EGF	**2.7±2.84**	**Serpin E1**	**165.3±49.7**
HGF	80.8±26.8	Serpin-E1/PAI-1	n.d
IGFBP-1	n.d	**Thrombospondin-2**	**63.25±24.5**
IGFBP-2	**20.7±12.4**	**TIMP-1**	**66.25±25.6**
IGFBP-3	n.d	**TIMP-4**	**41.25±26**
IL-1 alpha	n.d	VEGF	n.d
IL-1b	**10.2±5.8**	**VEGF-B**	**28.5±18.6**
IL-10	**7.8±5.9**		

Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.

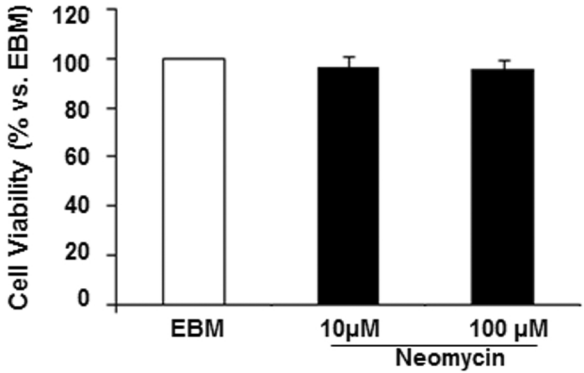


Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☑ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☑ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☑ An overall study timeline is provided.
Inclusion and exclusion criteria	☑ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☑ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☐ Type and methods of randomization have been described. ☐ Methods used for allocation concealment have been reported.
Blinding	☑ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☑ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☐ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☑ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☐ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☑ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☑ Statistical methods used have been reported. ☑ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☑ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☐ Different sex animals have been used. If not, the reason/justification is provided. ☑ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☑ Statements on funding and conflicts of interests have been provided.