Journal of Nuclear Medicine, published on September 8, 2016 as doi:10.2967/jnumed.115.170308

EPO pretreatment of ECFCs enhances functional recovery after transplantation in a rat model of cerebral ischemia through an increase of their homing abilities: a SPECT/CT study.

Short running title: SPECT imaging of ECFCs benefits on MCAO

Philippe Garrigue[1,2,3], Guillaume Hache[1], Youssef Bennis[1], Pauline Brige[4], Jimmy Stalin[1], Lionel Pellegrini[1], Lionel Velly[5], Francesca Orlandi[6], Elena Castaldi[6], Françoise Dignat-George[1,7], Florence Sabatier[1,8], Benjamin Guillet[1,2,3]*.

(1) Aix-Marseille Université, Inserm, UMR_S 1076, Marseille, France.

(2) Aix-Marseille Université, CERIMED, Marseille, France.

(3) APHM, Hôpital Nord, Service Radiopharmacie, Marseille, France.

(4) Aix-Marseille Université, LIIE, Marseille, France.

(5) APHM, Département Anesthésie-Réanimation adulte, Marseille, France.

(6) Advanced Accelerator Applications, Colleretto-Giacosa, Italy.

(7) APHM, Hôpital Conception, Service d'Hématologie, Marseille, France.

(8) APHM, Hôpital Conception, Laboratoire de Culture et Thérapie Cellulaire, Marseille, France.

First author : Philippe Garrigue (philippe.garrigue@univ-amu.fr), Faculté de Pharmacie, 27 Boulevard Jean Moulin, 13005 Marseille, France.

***Corresponding author:** Pr Benjamin Guillet (benjamin.guillet@univ-amu.fr), Faculté de Pharmacie, 27 Boulevard Jean Moulin, 13005 Marseille, France.

Disclosure: The authors have nothing to disclose.

Keywords: Ischemic stroke, brain-blood barrier, apoptosis, cerebral blood flow, ECFC, homing

Word count: 4999

ABSTRACT

Background: endothelial colony-forming cells (ECFCs) are promising candidates for cell therapy of ischemic diseases, as less than 10% of patients suffering from an ischemic stroke are eligible for thrombolysis. We previously reported that erythropoietin priming of ECFCs increased their *in vitro* and *in vivo* angiogenic properties in mice with hind limb ischemia. Objective: the present study aimed to evaluate whether priming of ECFCs with erythropoietin could enhance their homing into the ischemic site after transient middle cerebral artery occlusion (MCAO) followed by reperfusion in rats and potentiate their protective or regenerative effects on brain-blood barrier disruption (BBBd), cerebral apoptosis and cerebral blood flow (CBF) by Single Photon Emission/Computed Tomography (SPECT/CT) imaging. Methods: Rats underwent a 1-hour MCAO followed by reperfusion, and then received either PBS injection (control, n=10), PBS- or erythropoietin-primed ECFCs IV injection ($ECFC_{PBS}$, n=13; $ECFC_{EPO}$, n=10) 1 day after MCAO. ECFCs homing and their effects on BBBd, cerebral apoptosis and CBF were evaluated by SPECT/CT imaging up to 14 days after MCAO. Results were expressed as median±interquartile ipsi-/contralateral ratio of the activity in MCA-vascularized territories in each hemisphere. Histological evaluation of neuronal survival and astrocytal proliferation was performed on day 14. Results: erythropoietin priming increased ECFCs homing to the ischemic hemisphere ($ECFC_{PBS}$: 111.0±16.0%; $ECFC_{EPO}$: 146.5±13.3%). BBBd was significantly reduced (control: 387±153%; $ECFC_{PBS}$: 151±46%, P<0.05; $ECFC_{EPO}$: 112±9%, P<0.001) and negatively correlated with ECFCs homing (*Pearson* r=-0.6930, P=0.0002). Cerebral apoptosis was significantly reduced (control: 161±10%; $ECFC_{PBS}$: 141±9%; P<0.05; $ECFC_{EPO}$:118±5%, P<0.001) and negatively correlated with ECFCs homing (*Pearson* r=-0.7251, P<0.0001). CBF was significantly restored with ECFCs and almost totally with erythropoietin priming (control: 72±2%; $ECFC_{PBS}$: 90±4%, P<0.01; $ECFC_{EPO}$: 99±4%, P<0.001) and positively correlated with ECFCs homing (*Pearson* r=0.7348, P<0.0001). Immunoblocking against the CD146 receptor on ECFCs highlighted its notable role in ECFCs homing with erythropoietin priming ($ECFC_{EPO}$: 147±14%; n=4; $ECFC_{EPO+aCD146}$: 101±12%, n=4, P<0.05). Conclusion: priming with erythropoietin prior

to cell transplantation is an efficient strategy to amplify the migratory and engraftment capacities of ECFCs and their beneficial impact on BBBd, apoptosis and CBF.

INTRODUCTION

Ischemic stroke is a leading cause of morbidity and mortality with a constantly increasing prevalence worldwide (*1*). New approaches including neuroprotective and/or neurorestorative therapies are still under evaluation. Among them, stem cell therapy and more specifically endothelial progenitor cell (EPC) transplantation is considered as one of the most promising strategies. EPCs are endogenous immature cells mobilized from bone marrow that have the ability to home into the site of ischemic injury tissue recovery (*2*). Endothelial cells forming colonies (ECFCs) are of particular interest because they constitute a homogeneous and well-characterized EPC population displaying abilities for *de novo* vessel formation (*3*). We previously reported that ECFCs transplantation in rat after middle cerebral artery occlusion (MCAO) attenuated neurological deficit through a decrease of acute neurodegeneration and an enhancement of tissue regeneration, although the ECFC homing and engraftment into the ischemic tissue appeared relatively poor after intravenous delivery (*4*). Thus, new approaches to enhance the stem cell transplantation effects are desirable for stem cell therapy to the regenerative treatment of stroke: we recently stated that the well-known and pharmaceutical grade erythropoietin pre-treatment (*5*) or co-treatment (*6*) of ECFCs increased their *in vitro* and *in vivo* angiogenic properties. In parallel, new effective tools are needed for evaluation of their benefits and we recently reported SPECT characterization of cerebral blood flow (CBF), blood brain barrier disruption (BBBd) and cerebral apoptosis up to 14 days after MCAO in rat (*7*).

The present study aimed to evaluate whether erythropoietin priming of ECFCs could enhance their homing into the ischemic site after MCAO in rats and potentiate their protective or regenerative effects on BBBd, cerebral apoptosis and CBF.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (Janvier-Labs) weighing 230-280g were used in all experiments. This study was approved by the Institution's Animal Care Committee (CE14, Aix-Marseille Université) and was conducted according to the EU-Directive 2010/63/EU and the recommendations of the Helsinki Declaration. Experiments were performed in an authorized laboratory (C13-055-20) by trained and competent researchers. A blinded experimenter performed neurological evaluations and histological quantifications.

MCAO induction

Rats were intubated and the lungs mechanically ventilated with 3.0vol% sevoflurane in a gas mixture of 30% oxygen and 70% nitrogen. During anesthesia, inspired and expired fraction of sevoflurane, oxygen and carbon dioxide were continuously monitored (Capnomac Ultima, Datex-Ohmeda). A 60-minute MCAO was induced as previously described (*7*).

Cell preparation and priming

Human umbilical cord blood samples from healthy donors were collected in compliance with French legislation and ECFCs were isolated as previously reported (*8*).

Erythropoietin priming consisted in a 24h-incubation of ECFC with human recombinant erythropoietin (10,000 IU/mL, epoietin-alpha, Eprex®, Janssen-Cilag) to a final concentration of 5 IU/mL diluted in Endothelial Basal Growth Medium (EBM-2, Lonza) supplemented with 0.5% fetal bovine serum (FBS). After extensive washing, cells were used in subsequent assays ($ECFC_{EPO}$). Cells maintained in 0.5% FBS/EBM-2 and pretreated with phosphate buffer saline (PBS, Lonza) instead of erythropoietin for 24h, were used as control cells ($ECFC_{PBS}$). In blocking experiments, antibody against CD146 (anti-CD146 Sendo-1 clone, Biocytex, 1µg/mL media) was added after erythropoietin or PBS incubation for 1h ($ECFC_{EPO+aCD146}$ or $ECFC_{PBS+aCD146}$).

Twenty-four hours after MCAO, animals were randomly allocated to PBS-injected control, $ECFC_{PBS}$, or $ECFC_{EPO}$ using the online automated research randomizer (http://www.randomizer.org). For blocking experiments, animals were randomly allocated to $ECFC_{PBS}$, $ECFC_{PBS+aCD146}$, $ECFC_{EPO}$ or $ECFC_{EPO+aCD146}$ group. 0.2mL PBS alone or 4.10^6 ECFCs in 0.2mL PBS were delivered in the tail vein of control and transplanted animals respectively (Fig. 1).

In order to assess a putative toxic effect of CD146-immunoblocking, we evaluated cell survival using the 7AAD-Annexin-V-FITC kit (Beckman-Coulter). ECFCs were plated in 6-well plates and exposed to 0.5% FBS/EBM-2 with anti-CD146 antibody (1µg/ml) for 1h. Samples were read in a FC500 flow cytometer (Beckman-Coulter). Cells that showed low staining for both Annexin-V and 7-AAD were considered pro-survival cells.

Radiotracers and radiolabeling

Commercially available hexamethylpropyleneamine-oxime (HMPAO, Cerestab®) and pentetic acid (DTPA, Pentacis®) were purchased respectively from General-Electrics and IBA. The experimental Annexin-V-128 kit was provided by Advanced Accelerator Applications. Radiolabeling with fresh $^{99m}TcO_4^-$ pertechnetate (physical half-life: 6h; γ-energy: 140keV) was carried out according to the manufacturer's instructions. Radiochemical purities were checked to be ≥95% by thin layer chromatography.

ECFC radiolabeling and labeling with CM-DiI

Cell radioactive labeling was performed as reported by Detante et al. (*9*). Briefly, 40.10^6 ECFCs were trypsinized into single cell suspension, then cells were mixed with 370MBq of ^{99m}Tc-HMPAO for 30min at 37°C, 5%CO_2. Cells were centrifuged and washed twice with PBS and counted. Labeling efficiency was 68±7% of total radioactivity. For the immunoblocking experiment, a co-labeling was realized by incubating cells with 5µL CM-DiI (Vybrant®, Life-Technologies) per mL of suspension for 20min at

37°C, 5%CO_2. ECFCs were washed twice with PBS and resuspended in 3mL of PBS at 37°C, and 4.10^6 labeled ECFCs (25±3MBq) were injected into the tail vein 24h after MCAO.

Assessment of CD146 expression in erythropoietin-primed ECFCs

Thirty micrograms of $ECFC_{PBS}$ and $ECFC_{EPO}$ cell lysates (lysis buffer: Tris 10mM pH8, EDTA 1mM pH8, NaCl 150mM, NP40 1% and proteases inhibitors) were subjected to 4-12% NuPage SDS-polyacrylamide gel electrophoresis (Life) and transferred onto nitrocellulose membrane with the IBLOT transfer device (Life). Membranes were incubated with BSA 4%, with anti-CD146 antibody (clone 7A4, Biocytex), anti-actin (CellSignaling), and a secondary peroxidase-coupled antibody before detection with ECL (Amersham). CD146 membrane expression was also determined on ECFCs by flow cytometry. Cells were labelled with phycoerythrin-conjugated antibodies (anti-CD146 Sendo-1 antibody or isotype-matched IgG as negative control) for 45min at 4°C. After washing, samples were analyzed by flow cytometry on a Beckman Coulter apparatus (FC500). The number of antigenic sites per cell was counted by using a quantitative indirect immunofluorescence assay based on the linear relation between antigen expression and the median fluorescence intensity. Results are expressed as percent of control.

Single photon emission computed tomography data acquisition

According to experimental paradigm (Fig. 1), 20MBq/100µL of ^{99m}Tc-DTPA, 37MBq/150µL of ^{99m}Tc-Annexin-V-128, or 20MBq/150µL of ^{99m}Tc-HMPAO were injected through the tail vein, respectively 2, 3, and 14 days after MCAO. Animals were imaged under a NanoSPECT/CTplus® camera (Bioscan) 30min after ^{99m}Tc-DTPA or ^{99m}Tc-HMPAO injection, and 90min after ^{99m}Tc-Annexin-V-128 injection. Multi-pinhole SPECT parameters were set up as follows: termination condition: 10,000 counts, 256x256 picture with a 1.14 zoom factor and 1.00mm^2 pixel.

Single photon emission computed tomography image analysis

Images were reconstructed and analysis was performed using the 3D-ROI module part of InVivoScope® software v2.0p4 (InviCRO). After co-registration between SPECT and CT, 2 volumes of interest were

drawn according to the MCA-vascularized territories in each hemisphere, CT overlay images providing anatomic guidance. Radioactivity inside each volume of interest was quantified and corrected by the tissue volume (MBq/mm^3). We then calculated the ipsilateral-to-contralateral ratios (i/c, %). Image color scales were normalized for comparing signals between conditions.

Measurement of Neurological Outcome

Neurological deficits were evaluated at 1, 3, 7, and 14 days after MCAO. Neurological function was graded using the modified neurological severity score (*10*) (mNSS, normal score: 0; maximal deficit score: 18). Somatosensory deficit was measured both before and after MCAO with the adhesive-removal somatosensory test. The time necessary to remove a small adhesive-backed paper dot from the limb was recorded during 3 trials per day for 3 days before MCAO and on day 1, 3, 7, and 14. Sensorimotor function was evaluated by the foot-fault test. The rats were allowed to walk on a horizontal 77-bar scale. A computer-assisted device (Locotronic®, Bioseb) was used to record foot faults (falling or slipping paws between bars). Each animal performed the tests 3 times per day for 3 days before MCAO then 1, 3, 7, and 14 days after MCAO.

Immunohistochemistry

Animals were euthanized with a lethal dose of pentobarbital (ClinMidy) 2 days (blocking experiment) or 14 days post-MCAO and perfused by a 4% phosphate-buffered paraformaldehyde solution (Sigma-Aldrich). Brains were harvested, post-fixed for 24h, cryopreserved in 10% and 30% sucrose, snap-frozen and stocked at −80°C. Frozen sections were cut with a sliding microtome (CM1900, Leica). Immunohistochemical labeling used primary anti-GFAP (1:100, Dako) and anti-NeuN (1:1,000, Millipore) monoclonal antibodies, then detected with Bond Polymer Refine Red Detection and Bond Polymer Refine HRP-Detection kits (Novocastra, Leica). Five fields of view were quantified in ipsi- and contralateral hemispheres for each slide under a light and fluorescent microscope (Eclipse-Ni, Nikon) at

2x and 10x using threshold-base labeling detection after saturation and intensity calibration. Results are expressed as NeuN- or GFAP-positive hit i/c ratio.

Statistical Analysis

Values are expressed as median±interquartile unless otherwise indicated. Statistical differences were analyzed using the Kruskal-Wallis analysis of variance with Dunn's post-hoc analysis when comparing 3 groups, Mann-Whitney test when comparing 2 groups. Pearson correlation was assessed between ECFC homing and BBBd, apoptosis, or CBF SPECT/CT quantifications. Statistical analyses were performed with Prism® software (GraphPad Software), $P<0.05$ considered as indicating statistical significance.

RESULTS

Neurological outcome

mNSS scores were significantly lower in both $ECFC_{PBS}$ and $ECFC_{EPO}$-treated groups than control group at day 7 (control: 7.1±3.7, n=10; $ECFC_{PBS}$: 2.7±2.2, n=13, $P<0.001$; $ECFC_{EPO}$: 2.0±2.6, n=10, $P<0.001$) and day 14 (control: 7.7±5.7, n=10; $ECFC_{PBS}$: 2.6±3.0, n=13, $P<0.001$; $ECFC_{EPO}$: 1.4±2.1, n=10, $P<0.001$, Fig. 2A).

Compared to control animals, sensorimotor function was significantly improved in both $ECFC_{PBS}$ and $ECFC_{EPO}$-treated animals at day 7 (control: 25.4±21.0, n=10; $ECFC_{PBS}$: 9.9±13.8, n=13, $P<0.01$; $ECFC_{EPO}$: 5.6±2.6, n=10, $P<0.001$) and at day 14 (control: 23.7±20.1, n=10; $ECFC_{PBS}$: 7.3±3.5, n=13, $P<0.001$; $ECFC_{EPO}$: 4.6±4.2, n=10, $P<0.001$, Fig. 2B).

Adhesive removal test time (Fig. 2C) was significantly shorter in both $ECFC_{PBS}$ and $ECFC_{EPO}$ treated groups than in control group at day 7 (control: 111.6±75s, n=10; $ECFC_{PBS}$: 29.3±29s, n=13, $P<0.001$; $ECFC_{EPO}$: 25.5±43s, n=10) and day 14 (control: 101.4±70s, n=10; $ECFC_{PBS}$: 20.9±34s, n=13, $P<0.001$; $ECFC_{EPO}$: 22.3±24s, n=10, $P<0.001$). At day 3, the adhesive removal test time was significantly shorter in

$ECFC_{EPO}$ group and not in $ECFC_{PBS}$ group, compared to control group (control: 26.8±19s, *n*=10; $ECFC_{PBS}$: 23.3±20s, *n*=13, ns; $ECFC_{EPO}$ 15.6±12s, *n*=10, *P*<0.001).

Erythropoietin-primed ECFCs enhanced neuron survival and decreased astrocyte proliferation

Fourteen days after MCAO, we found a significantly higher NeuN positive-hit i/c ratio in $ECFC_{PBS}$ (65±12%; *n*=13) compared to control (40±5%; *n*=10, *P*<0.05), and even higher in $ECFC_{EPO}$ group (84±10%; *n*=10) compared to control (*P*<0.01) and compared to $ECFC_{PBS}$ (*P*<0.05, Figs. 3A and 3B). Similarly, we found a significantly higher GFAP-positive hit i/c ratio in control animals (3,000±1,171%; *n*=10) compared to $ECFC_{PBS}$ (349.5±365,8%; *n*=13) and $ECFC_{EPO}$ (484±395%; *n*=10, *P*<0.05, Figs. 3A and 3C).

Erythropoietin priming increased ECFC homing to ischemic territory

SPECT/CT imaging 12h after radiolabelled ECFCs injection showed a tight i/c uptake ratio for $ECFC_{PBS}$ (111.0±16.0%, *n*=13) that was significantly increased to 146.5±13.3% with $ECFC_{EPO}$ (*n*=10, *P*<0.0001, Fig. 4A).

Erythropoietin priming induced CD146 overexpression in ECFCs

Western-blotting and flow cytometry experiments showed that erythropoietin priming induced a significant increase of CD146 expression of ECFCs (respectively 154±43% and 108±7% *vs* control, both *P*<0.05, *n*=3, Figs. 4B and C).

CD146-immunoblocking abolished erythropoietin-primed ECFCs homing

While no significant difference in i/c uptake ratio was found between $ECFC_{PBS}$ (119±10%, *n*=3) and $ECFC_{PBS+aCD146}$ (118±6%, *n*=3, Fig. 5A), CD146-immunoblocking induced a significant decrease in transplanted ECFC homing abilities after erythropoietin preconditioning from 147±14% (*n*=4) for $ECFC_{EPO}$ to 101±12% (*n*=4) for $ECFC_{EPO+aCD146}$ (*P*<0.05, Fig. 5B). CD146-immunoblocking did not affect cell survival 12h after incubation (95±4%, Fig. 5C).

Immunofluorescence showed no significant difference in i/c ratio of CM-DiI-positive cells between $ECFC_{PBS}$ (119.0±10.5%, n=3) and $ECFC_{PBS+aCD146}$ (118.0±6.0%, n=3, Fig. 5E) whereas significantly decreased in $EFCF_{EPO+aCD146}$ (101.5±11.5%, n=4) compared to $ECFC_{EPO}$ animals (146.5±13.5%, n=4, P<0.01, Figs. 5D and F).

Effects of ECFC homing potentiation on BBBd, cerebral apoptosis and CBF SPECT imaging

Two days after MCAO, ^{99m}Tc-DTPA i/c ratio was significantly decreased in $ECFC_{PBS}$ (151±46%, n=13, P<0.05) and $ECFC_{EPO}$ (112±9%, n=10, P<0.001) compared to control animals (387±153%, n=10). ^{99m}Tc-DTPA i/c ratio in $ECFC_{EPO}$ rats was significantly lower than $ECFC_{PBS}$ (P<0.05). BBBd was significantly and inversely correlated to the ECFC homing (*Pearson r*=-0.6930, P=0.0002, Fig. 6A).

Three days after MCAO, ^{99m}Tc-Annexin-V-128 i/c ratio was lower in $ECFC_{PBS}$ (141±9%, n=13, P<0.05) and $ECFC_{EPO}$ rats (118±5%, n=10, P<0.001) compared to control (161±10%, n=10). ^{99m}Tc-Annexin-V-128 i/c ratio in $ECFC_{EPO}$ rats was significantly lower than $ECFC_{PBS}$ (P<0.05). Cerebral apoptosis was significantly and inversely correlated to the ECFC homing (*Pearson r*=-0.7251, P<0.0001, Fig. 6B).

Fourteen days after MCAO, ^{99m}Tc-HMPAO i/c ratio was higher in $ECFC_{PBS}$ (90±4% n=13, P<0.01) and $ECFC_{EPO}$ rats (99±4%, n=10, P<0.001) compared to control (72±2% %, n=10). ^{99m}Tc-HMPAO i/c ratio in $ECFC_{EPO}$ rats was significantly higher than $ECFC_{PBS}$ (P<0.05). CBF was significantly correlated to the ECFC homing (*Pearson r*=0.7348, P<0.0001, Fig. 6C).

DISCUSSION

Various cell therapies for stroke, including the use of mesenchymal stem cells (MSCs), cord blood cells, or EPCs, represent a potential innovative strategy with a wide therapeutic time window after stroke and are still under intensive preclinical and clinical evaluations (*11*,*12*). We report here that erythropoietin

priming of ECFCs enhanced cell abilities to home to ischemic tissue and that the number of homed cells correlated with cerebral tissue recovery.

One limitation of our work consists in using immunocompetent rats with no immunosuppressive therapy, known to be neuroprotective (*13–15*). According to the BBBd, we could not exclude the involvement of peripheral cell immunity even if we previously excluded lymphocytic inflammatory reaction (*4*) that could be explained by the low immunogenicity of cord blood cells (*16,17*). Additionally, we previously reported that ECFCs did not proliferate, and only a few cells were visible 14 days after transplantation (*4*).

Early administration of exogenous ECFCs could anticipate the protective effects of endogenous mobilized EPCs after stroke (*18*) since mobilization occurs late from 7 to 28 days after stroke in humans (*19*). Moreover, several strategies aiming to circumvent poor homing efficiency of cell-based therapies for stroke have already been explored including arterial infusion (*20*), C-X-C chemokine receptor type 4 (CXCR4) or vascular endothelial growth factor (VEGF) over-expression or pharmacological co-treatment (*6,21*).

Erythropoietin, mainly known as the regulator of erythropoiesis, has been found to also induce non-hematopoietic effects (*22*) and contributes to the physiologic and pathologic angiogenesis to the body's innate response to tissue injury and especially to ischemic injury (*23*).

Here we reported that erythropoietin priming of ECFCs increased their homing abilities and their benefits in animals up to 14 days after MCAO. Although trends were clearly visible, additional benefits of erythropoietin-primed ECFCs on neurofunctional recovery could not be demonstrated, probably due to the lack of sensibility of clinical evaluation (*24*). However, brain SPECT/CT imaging allowed us to evidence the therapeutic superiority of erythropoietin-primed ECFCs as shown by improved benefits in terms of BBBd, apoptosis activation and CBF compared to unprimed ECFCs.

SPECT/CT is one of the most recommended imaging modalities for cell tracking (*25,26*). We observed that erythropoietin priming of ECFCs increased their homing ability to reach ischemic tissues and

potentiated their benefits on BBBd, brain apoptosis and CBF, as these were all significantly correlated with ECFC homing intensity. Our observations, in line with previous experiments on rodent model of hind limb ischemia (*5*), underlie the major interest of ECFCs homing-enhancing strategies in ischemic tissues after stroke.

Additionally we observed that erythropoietin priming induced an increased ECFC CD146 expression, a transmembrane immunoglobulin involved in ECFCs adhesion to activated endothelium, migration, proliferation and paracrine secretion (*27–29*). We observed that erythropoietin-induced CD146 overexpression mediated the increase in ECFCs' homing, as CD146 immunoblocking fully abrogated erythropoietin homing potentiation.

BBBd, peaking on day 2 post-MCAO as we previously reported (*7*), is an early process involved in stroke physiopathology, consistent with the vasogenic edema reported after ischemic stroke in humans. Here we showed *in vivo* the benefits of ECFCs injection upon BBBd as assessed by ^{99m}Tc-DTPA imaging 2 days post-MCAO. These results are in line with benefits observed from MSCs transplantation on BBB stabilization in rodent models of neuroinflammation (*30*). Additionally, we observed that benefits on BBBd were notably emphasized when cell homing is potentiated using erythropoietin-primed ECFCs. A correlation between BBBd and apoptosis in patients who underwent acute stroke is now proven (*31*) and place the BBBd as a potential innovative target, which is in this case attenuated by transplanted ECFCs.

Clinical follow-up of apoptotic regions has already been correlated with the neurological outcome in patients (*32*). On day 3 post-MCAO, we observed in $ECFC_{PBS}$ and $ECFC_{EPO}$ groups a huge decrease in apoptosis activation, matching previous *ex vivo* reports using bone-marrow-derived EPCs (*33*), CXCR4-primed EPCs (*34*) or $VEGFR2^+$ MSCs transplantation in MCAO rodent models (*35*). Moreover we showed a direct correlation between the amount of transplanted cells homing and apoptosis attenuation.

CBF assessed at 14 days after MCAO by ^{99m}Tc-HMPAO SPECT/CT, one of the most described for experimental stroke imaging (*36*), allows to evaluate definitive tissue injury. We reported here a partial

CBF restoration with the $ECFC_{PBS}$-treated animals, which became nearly total in the erythropoietin-primed ECFCs animals. This is probably obtained through an enhancement of ECFCs angiogenic properties (*5*) and, according correlation between CBF and ECFCs homing, partly through the potentiating of their homing abilities. The ECFC-mediated vascular regeneration mechanisms may imply metalloproteases (*37*), the SDF-1/CXCR4 pathway (*5*), VEGF secretion (*38*) and interleukin IL-8 or IL-1β (*39,40*) but also go through the increased CD146 expression on ECFCs (*29*).

CONCLUSION

Among the various strategies to enhance progenitor cell homing, we showed that erythropoietin could be considered as powerful priming agent for ECFCs based therapy since erythropoietin enhanced their benefits on BBBd, apoptosis and CBF after MCAO through optimization of ECFCs migratory and engraftment capacities to the ischemic tissue, at least partially supported by CD146 expression. Finally, correlations between cell homing and BBBd, apoptosis or CBF validate our hypothesis that increasing the transplanted cells homing abilities may potentiate ischemic tissue recovery.

ACKNOWLEDGEMENTS

The authors thank S. Pons, S. Fernandez, P. Stellmann, M. Skandalovski and S. Vigier for technical assistance, the Obstetrics and Gynecology Department of Conception Hospital (Pr. D'Ercole, C. Gilbertas) and the cord-blood bank of Marseille (Pr. Chabannon). Very special thoughts to Pr Pisano.

REFERENCES

1. Go AS, Mozaffarian D, Roger VL, et al. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation*. 2014;129:e28-e292.

2. Li Y-F, Ren L-N, Guo G, et al. Endothelial progenitor cells in ischemic stroke: an exploration from hypothesis to therapy. *J Hematol Oncol.* 2015;8:33.

3. Medina RJ, O'Neill CL, Sweeney M, et al. Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities. *BMC Med Genomics*. 2010;3:18.

4. Moubarik C, Guillet B, Youssef B, et al. Transplanted late outgrowth endothelial progenitor cells as cell therapy product for stroke. *Stem Cell Rev.* 2011;7:208-220.

5. Bennis Y, Sarlon-Bartoli G, Guillet B, et al. Priming of late endothelial progenitor cells with erythropoietin before transplantation requires the CD131 receptor subunit and enhances their angiogenic potential. *J Thromb Haemost*. 2012;10:1914-1928.

6. Pellegrini L, Bennis Y, Guillet B, et al. Therapeutic benefit of a combined strategy using erythropoietin and endothelial progenitor cells after transient focal cerebral ischemia in rats. *Neurol Res*. 2013;35:937-947.

7. Garrigue P, Giacomino L, Bucci C, et al. Single photon emission computed tomography imaging of cerebral blood flow, blood-brain barrier disruption, and apoptosis time course after focal cerebral ischemia in rats. *Int J Stroke.* 2016;11:117-126.

8. Delorme B, Basire A, Gentile C, et al. Presence of endothelial progenitor cells, distinct from mature endothelial cells, within human CD146+ blood cells. *Thromb Haemost.* 2005;94:1270-1279.

9. Detante O, Moisan A, Dimastromatteo J, et al. Intravenous administration of 99mTc-HMPAO-

labeled human mesenchymal stem cells after stroke: in vivo imaging and biodistribution. *Cell Transplant.* 2009;18:1369-1379.

10. Chen J, Sanberg PR, Li Y, et al. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke*. 2001;32:2682-2688.

11. Lee JS, Hong JM, Moon GJ, et al. A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells*. 2010;28:1099-1106.

12. Hess DC, Sila CA, Furlan AJ, Wechsler LR, Switzer JA, Mays RW. A double-blind placebo-controlled clinical evaluation of MultiStem for the treatment of ischemic stroke. *Int J Stroke.* 2014;9:381-386.

13. Labrande C, Velly L, Canolle B, et al. Neuroprotective effects of tacrolimus (FK506) in a model of ischemic cortical cell cultures: role of glutamate uptake and FK506 binding protein 12 kDa. *Neuroscience*. 2006;137:231-239.

14. Erlandsson A, Lin C-HA, Yu F, Morshead CM. Immunosuppression promotes endogenous neural stem and progenitor cell migration and tissue regeneration after ischemic injury. *Exp Neurol.* 2011;230:48-57.

15. Yuen C-M, Sun C-K, Lin Y-C, et al. Combination of cyclosporine and erythropoietin improves brain infarct size and neurological function in rats after ischemic stroke. *J Transl Med*. 2011;9:141.

16. Vendrame M, Gemma C, Pennypacker KR, et al. Cord blood rescues stroke-induced changes in splenocyte phenotype and function. *Exp Neurol.* 2006;199:191-200.

17. Roura S, Bagó JR, Soler-Botija C, et al. Human umbilical cord blood-derived mesenchymal stem cells promote vascular growth in vivo. *PloS One*. 2012;7:e49447.

18. Mao L, Huang M, Chen S-C, et al. Endogenous endothelial progenitor cells participate in

neovascularization via CXCR4/SDF-1 axis and improve outcome after stroke. *CNS Neurosci Ther.* 2014;20:460-468.

19. Zhou W, Zhu D, Yang G, et al. Circulating endothelial progenitor cells in Chinese patients with acute stroke. *Hypertens Res.* 2009;32:306-310.

20. Chen Y-L, Tsai T-H, Wallace CG, et al. Intra-carotid arterial administration of autologous peripheral blood-derived endothelial progenitor cells improves acute ischemic stroke neurological outcomes in rats. *Int J Cardiol*. 2015;201:668-683.

21. Bo C-J, Chen B, Jia R-P, et al. Effects of ischemic preconditioning in the late phase on homing of endothelial progenitor cells in renal ischemia/reperfusion injury. *Transplant Proc.* 2013;45:511-516.

22. Maiese K, Chong ZZ, Shang YC. Raves and risks for erythropoietin. *Cytokine Growth Factor Rev.* 2008;19:145-155.

23. Velly L, Pellegrini L, Guillet B, Bruder N, Pisano P. Erythropoietin 2nd cerebral protection after acute injuries: a double-edged sword? *Pharmacol Ther.* 2010;128:445-459.

24. Rosell A, Agin V, Rahman M, et al. Distal occlusion of the middle cerebral artery in mice: are we ready to assess long-term functional outcome? *Transl Stroke Res.* 2013;4:297-307.

25. Aicher A, Brenner W, Zuhayra M, et al. Assessment of the tissue distribution of transplanted human endothelial progenitor cells by radioactive labeling. *Circulation.* 2003;107:2134-2139.

26. Gavins FNE, Smith HK. Cell tracking technologies for acute ischemic brain injury. *J Cereb Blood Flow Metab.* 2015;35:1090-1099.

27. Bardin N, Anfosso F, Massé JM, et al. Identification of CD146 as a component of the endothelial junction involved in the control of cell-cell cohesion. *Blood.* 2001;98:3677-3684.

28. Bardin N, Blot-Chabaud M, Despoix N, et al. CD146 and its soluble form regulate monocyte

transendothelial migration. *Arterioscler Thromb Vasc Biol.* 2009;29:746-753.

29. Kebir A, Harhouri K, Guillet B, et al. CD146 short isoform increases the proangiogenic potential of endothelial progenitor cells in vitro and in vivo. *Circ Res.* 2010;107:66-75.

30. Park HJ, Shin JY, Kim HN, Oh SH, Song SK, Lee PH. Mesenchymal stem cells stabilize the blood-brain barrier through regulation of astrocytes. *Stem Cell Res Ther*. 2015;6:187.

31. Lorberboym M, Blankenberg FG, Sadeh M, Lampl Y. In vivo imaging of apoptosis in patients with acute stroke: correlation with blood-brain barrier permeability. *Brain Res.* 2006;1103:13-19.

32. Zgavc T, Ceulemans A-G, Hachimi-Idrissi S, Kooijman R, Sarre S, Michotte Y. The neuroprotective effect of post ischemic brief mild hypothermic treatment correlates with apoptosis, but not with gliosis in endothelin-1 treated rats. *BMC Neurosci*. 2012;13:105.

33. Qiu J, Li W, Feng S, Wang M, He Z. Transplantation of bone marrow-derived endothelial progenitor cells attenuates cerebral ischemia and reperfusion injury by inhibiting neuronal apoptosis, oxidative stress and nuclear factor-κB expression. *Int J Mol Med.* 2013;31:91-98.

34. Chen J, Chen J, Chen S, et al. Transfusion of CXCR4-primed endothelial progenitor cells reduces cerebral ischemic damage and promotes repair in db/db diabetic mice. *PloS One*. 2012;7:e50105.

35. Bao X, Feng M, Wei J, et al. Transplantation of Flk-1+ human bone marrow-derived mesenchymal stem cells promotes angiogenesis and neurogenesis after cerebral ischemia in rats. *Eur J Neurosci.* 2011;34:87-98.

36. Heiss W-D. Radionuclide imaging in ischemic stroke. *J Nucl Med.* 2014;55:1831-1841.

37. Morancho A, Ma F, Barceló V, Giralt D, Montaner J, Rosell A. Impaired vascular remodeling after endothelial progenitor cell transplantation in MMP9-deficient mice suffering cortical cerebral ischemia. *J Cereb Blood Flow Metab.* 2015;35:1547-1551.

38. Urbich C, Aicher A, Heeschen C, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. *J Mol Cell Cardiol.* 2005;39:733-742.

39. He T, Peterson TE, Katusic ZS. Paracrine mitogenic effect of human endothelial progenitor cells: role of interleukin-8. *Am J Physiol Heart Circ Physiol.* 2005;289:H968-H972.

40. Rosell A, Arai K, Lok J, et al. Interleukin-1beta augments angiogenic responses of murine endothelial progenitor cells in vitro. *J Cereb Blood Flow Metab.* 2009;29:933-943.

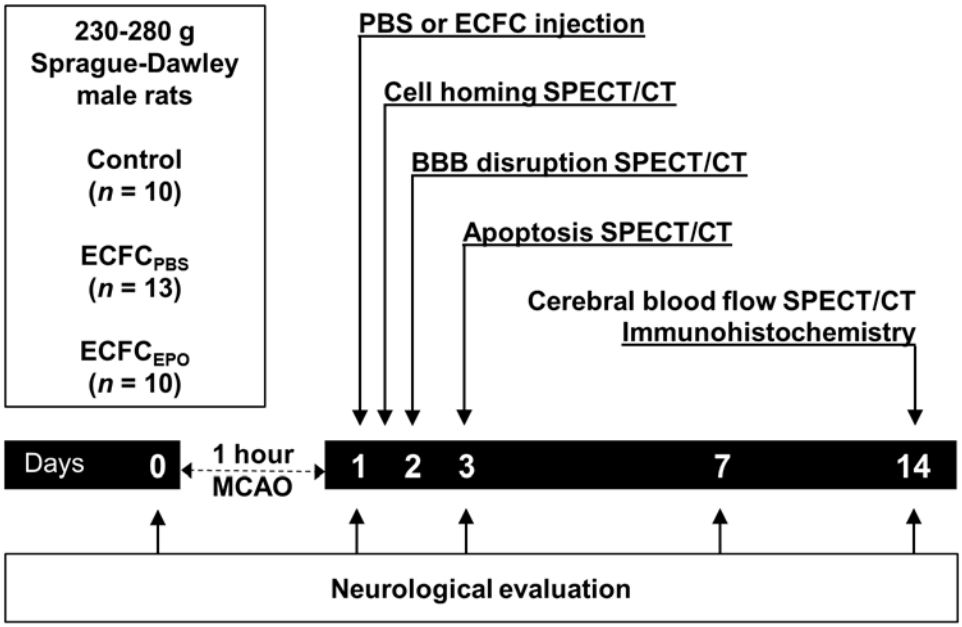

Fig. 1: Experimental Protocol

Rats were submitted to MCAO then treated with PBS (control, *n*=10), PBS-pretreated ECFCs ($ECFC_{PBS}$, *n*=13) or erythropoietin-primed ECFCs ($ECFC_{EPO,}$ *n*=10) 1 day after MCAO. ECFCs homing was assessed by SPECT/CT 12h after injection of radiolabeled cells, BBBd using ^{99m}Tc-DTPA, apoptosis using ^{99m}Tc-Annexin-V-128, and CBF using ^{99m}Tc-HMPAO, respectively 2, 3, and 14 days after MCAO. *Ex vivo* immunohistochemistry evaluation of neuronal survival and astrocytes proliferation was carried out 14 days after MCAO.

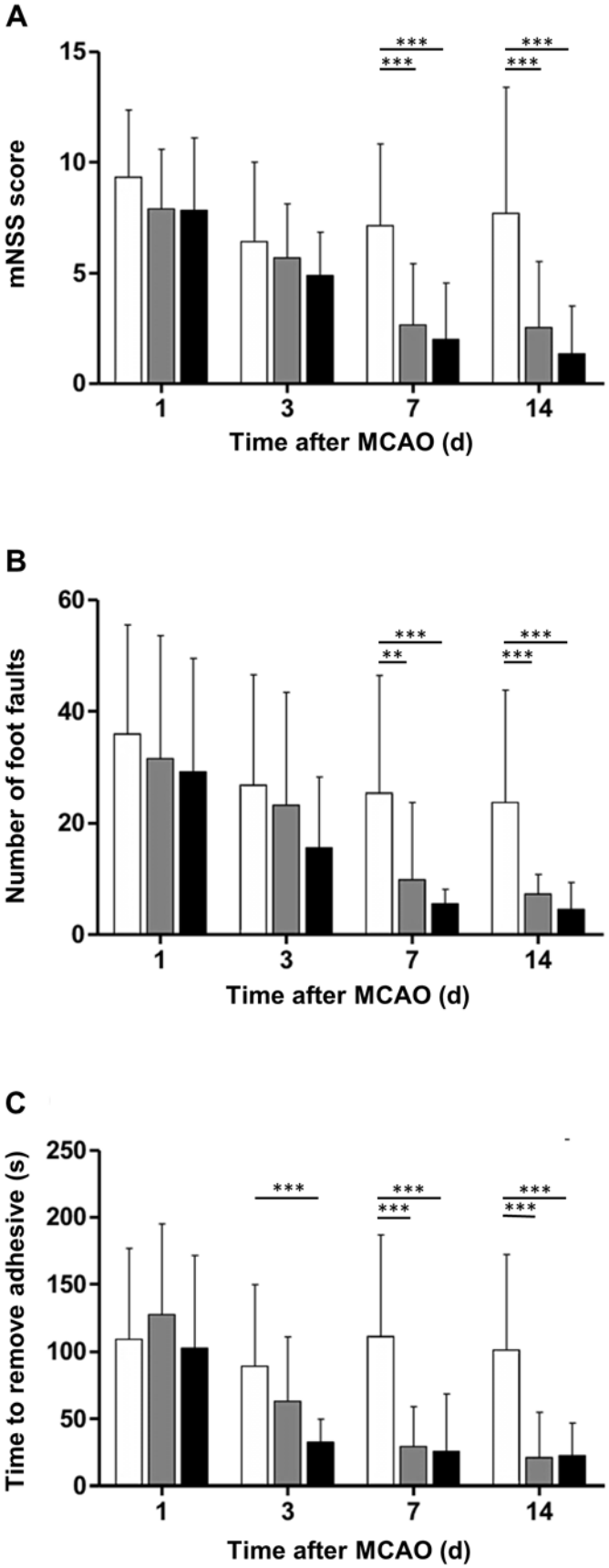

Fig. 2: Effects of ECFCs treatments on neurological evaluations

Evolution of mNSS (A), foot fault number (B) and time to remove adhesive tap in seconds (C) for control (white bars), $ECFC_{PBS}$ (grey bars) and $ECFC_{EPO}$ animals (black bars), up to 14 days after MCAO.

$^{**}P<0.01$, $^{***}P<0.001$

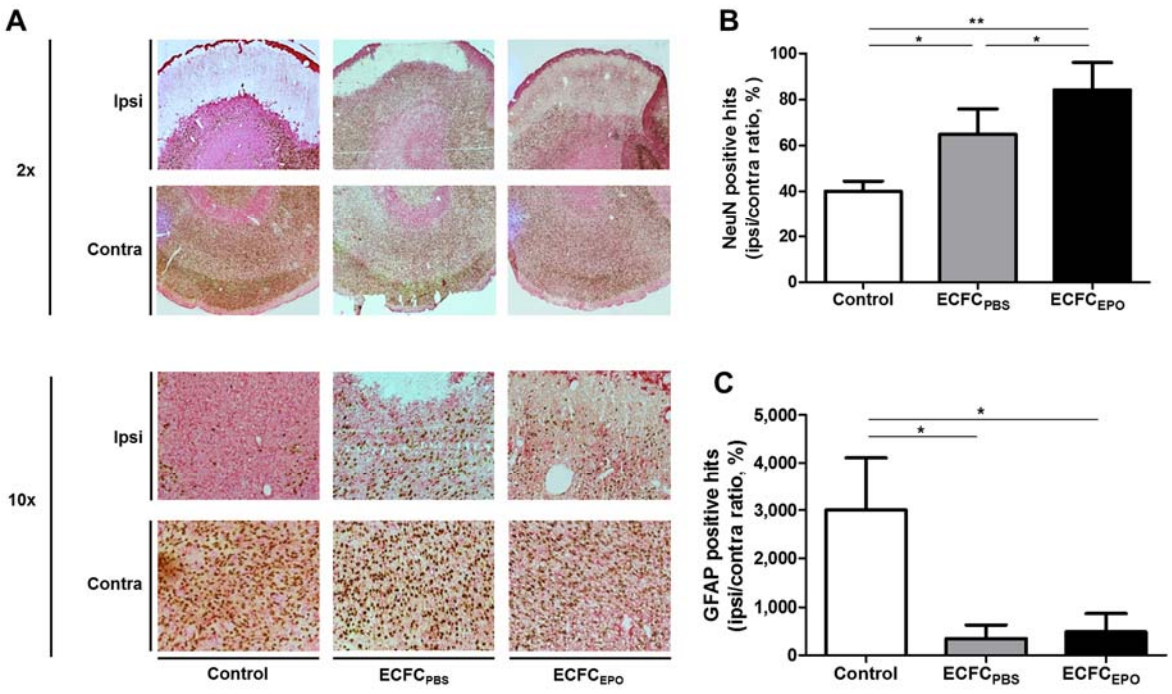

Fig. 3: Effects of ECFCs treatments on neuronal survival and astrocytal proliferation

A: Brain sections of control, ECFC$_{PBS}$ and ECFC$_{EPO}$ rats as examined under a light microscope at 2x and 10x after NeuN/GFAP staining 14 days after MCAO. Quantifications in ipsi- and contralateral hemispheres are expressed as median±interquartile NeuN- (B) or GFAP-positive hits i/c ratio (C).

*P<0.05, **P<0.01

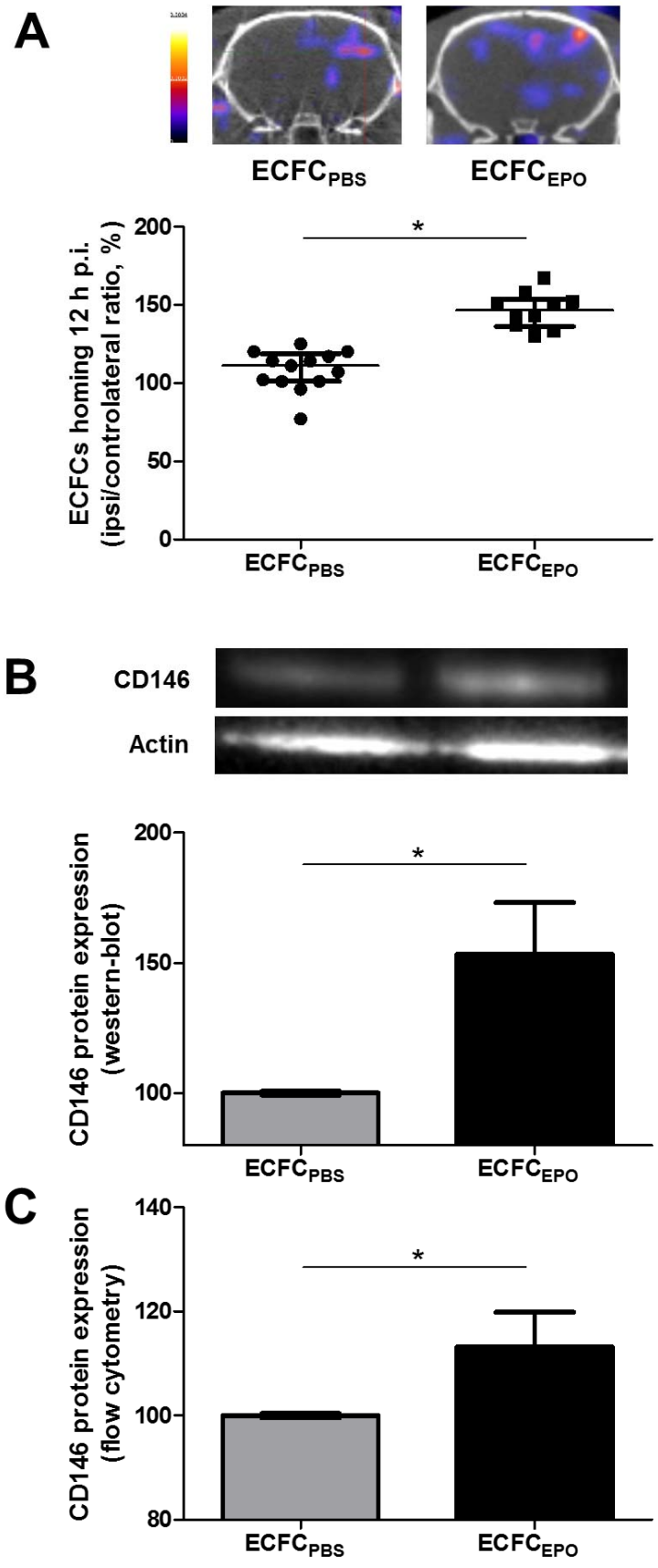

Fig. 4: Effects of erythropoietin priming on ECFCs homing and CD146 expression

A: Brain representative tomographic images and quantification (% of contralateral activity) of ^{99m}Tc-HMPAO-prelabelled ECFC$_{PBS}$ or ECFC$_{EPO}$ homing 12h after their injection.

B: Representative immunoblots of CD146 expression and quantification of ECFCs lysates after 24h incubation with erythropoietin (% of control).

C: Cytometry analysis quantifying CD146 expression on ECFCs after 24h incubation with erythropoietin (% of control).

*P<0.05, ***P<0.001

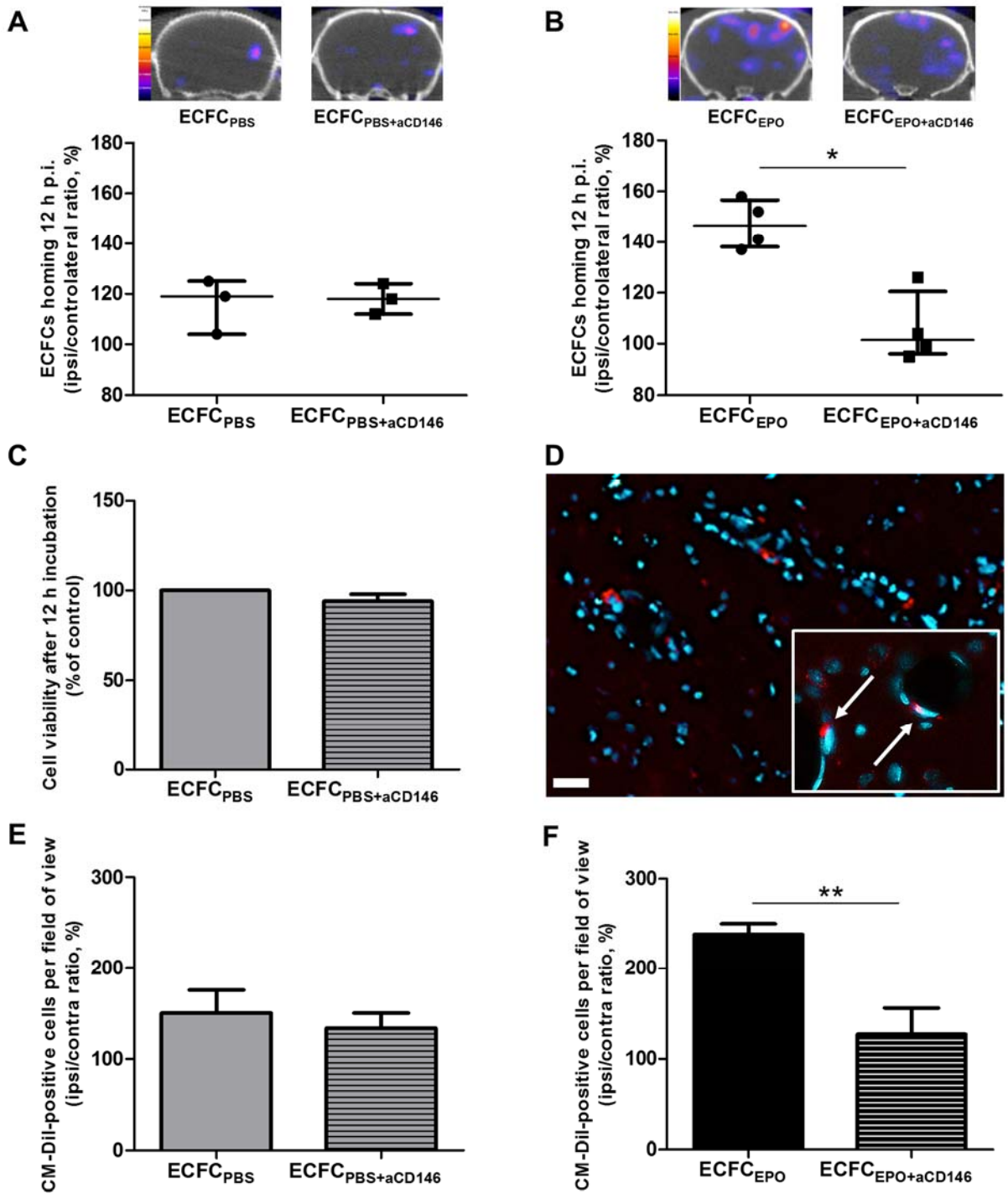

Fig. 5: Effects of CD146-immunoblocking on ECFCs homing

A, B: Brain representative tomographic images and quantification (% of contralateral activity) of ^{99m}Tc-HMPAO-prelabelled $ECFC_{PBS}$ or $ECFC_{PBS+aCD146}$ homing (A) and $ECFC_{EPO}$ or $ECFC_{EPO+aCD146}$ homing (B) 12h after their injection.

C: ECFC viability after CD146-immunoblocking by flow cytometry (% of control).

D: Brain representative immunofluorescence images (x20) of CM-DiI-labelled $ECFC_{EPO}$ homing 12h after their injection, DAPI counterstaining: blue, scale bar: 20µm. Insert: magnified image (x40) of CM-DiI-labelled $ECFC_{EPO}$ (white arrows) integrated to blood vessels.

E, F: Quantification of CM-DiI-positive cells in ischemic hemisphere 12h after $ECFC_{PBS}$ or $ECFC_{PBS+aCD146}$ injection (E) and after $ECFC_{EPO}$ or $ECFC_{EPO+aCD146}$ injection (F); % of contralateral activity.

*P<0.05, **P<0.01

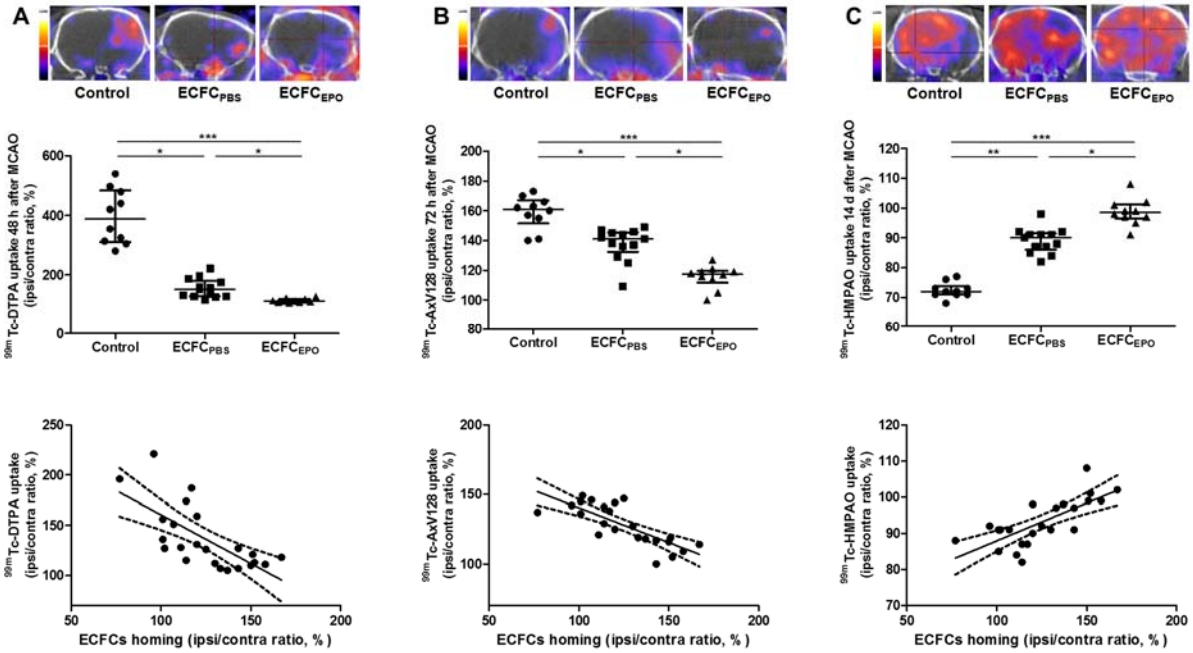

Fig. 6: Effects of ECFCs homing potentiation on BBBd, cerebral apoptosis and CBF SPECT/CT

Top: brain representative tomographic images of ^{99m}Tc-DTPA (A), ^{99m}Tc-Annexin-V-128 (B) and ^{99m}Tc-HMPAO (C) SPECT/CT, respectively assessing BBBd, apoptosis and CBF, 2 days, 3 days and 14 days after MCAO, in control rats, ECFC$_{PBS}$ or ECFC$_{EPO}$-treated rats.

Middle: quantification of ^{99m}Tc-DTPA (left), ^{99m}Tc-Annexin-V-128 (middle) and ^{99m}Tc-HMPAO (right) activity in ischemic hemisphere (% of contralateral activity).

Bottom: correlations between ECFCs homing and BBBd (left, *Pearson r*=-0.6930, *P*=0.0002), apoptosis (middle, *Pearson r*=-0.7251, *P*<0.0001) or CBF (right, *Pearson r*=0.7348, *P*<0.0001) SPECT/CT.

**P*<0.05, ***P*<0.01, ****P*<0.001