

COMP-Ang1 Potentiates EPC Treatment of Ischemic Brain Injury by Enhancing Angiogenesis Through Activating AKT-mTOR Pathway and Promoting Vascular Migration Through Activating Tie2-FAK Pathway

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Successful recovery from brain ischemia is limited due to poor vascularization surrounding the ischemic zone. Cell therapy with strong angiogenic factors could be an effective strategy to rescue the ischemic brain. We investigated whether cartilage oligomeric matrix protein (COMP)-Ang1, a soluble, stable and potent Ang1 variant, enhances the angiogenesis of human cord blood derived endothelial progenitor cells (hCB-EPCs) for rescuing brain from ischemic injury. COMP-Ang1 markedly improved the tube formation of capillaries by EPCs and incorporation of EPCs into tube formation with human umbilical vein endothelial cells (HUVECs) upon incubation on matrigel *in vitro*. COMP-Ang1 stimulated the migration of EPCs more than HUVECs in a scratch wound migration assay. The transplanted EPCs and COMP-Ang1 were incorporated into the blood vessels and decreased the infarct volume in the rat ischemic brain. Molecular studies revealed that COMP-Ang1 induced an interaction between Tie2 and FAK, but

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AKT was separated from the Tie2-FAK-AKT complex in the EPC plasma membrane. Tie2-FAK increased pp38, pSAPK/JNK, and pERK-mediated MAPK activation and interacted with integrins $\alpha\beta3$, $\alpha4$, $\beta1$, finally leading to migration of EPCs. AKT recruited mTOR, SDF-1, and HIF-1 α to induce angiogenesis. Taken together, it is concluded that COMP-Ang1 potentiates the angiogenesis of EPCs and enhances the vascular morphogenesis indicating that combination of EPCs with COMP-Ang1 may be a potentially effective regimen for ischemic brain injury salvage therapy.

Key words: COMP-Ang1, angiogenesis, ischemia, Tie2-FAK-AKT pathway

INTRODUCTION

Cerebral stroke causes irreversible brain damage by inducing vascular occlusion in the adjacent tissue surrounding the ischemic zone. Neovascularization is a physiological response to ischemia that produces collateral vessels to resolve ischemic symptoms or signs, and therapeutic neovascularization has become one of the most important remedies to salvage tissue in critically ischemic patients [1, 2].

Endothelial progenitor cells (EPCs) have been known to present in peripheral human blood [3-5], bone marrow [6-8] and human umbilical cord blood (HUCB) [9]. Mobilization of EPCs and their differentiation into endothelial cells (ECs) and angiogenesis at ischemic penumbra zones are crucial steps for successful recovery of brain from ischemic insults. Endothelial cell (EC) migration is essential for angiogenesis which is closely influenced by cell adhesion, cytoskeletal dynamics, organization and related signal transduction [10]. Unfortunately, however, endogenous recruitment of EPCs from peripheral blood or bone marrow is limited and insufficient to salvage the brain from ischemic injury. It has been shown that an exogenous supply of HUCB derived EPCs into the ischemic brain augments neovascularization and salvages the ischemic insult [6, 11, 12]. It is highly probable that adding strong angiogenic factors to the exogenous supply of EPCs may potentiate the efficacy of EPCs to rescue the ischemic brain.

Angiopoietin (Ang)-Tie signaling is a key regulator of angiogenesis and adult vascular homeostasis. Ang1 has been identified as a secreted protein ligand of tyrosine kinase with Ig and epidermal growth factor (EGF) homology domain 2 (Tie2) [13], and the Ang1-Tie2-AKT system is a key regulator of angiogenic process including EC survival, migration, sprouting, and tube formation [13]. Ang1 overexpression in normal quiescent vasculature enhances circumferential proliferation of ECs resulting in vascular enlargement of a well-arranged EC architecture and intercellular junctions, which promote blood perfusion without increasing vascular leakage [5, 14]. It remains to be clarified how Ang1 induces such highly organized

vascular remodeling, although activation of Tie2, PI3-kinase/AKT, and the apelin-APJ signaling pathways have been reported to be involved [15]. In comparison, overexpression of Ang1 in ischemic and activated vasculature induces Tie2 translocation to cell-matrix contacts and promotes migration of endothelial cells by activating AKT, ERK, and Dok-R signaling, thereby inducing strong angiogenesis [15, 16]. This vascular remodeling is accompanied by attachment of pericytes and promotion of blood perfusion without increasing vascular leakage.

Ang1 tends to aggregate and is insoluble, which limit its production and clinical application. To overcome such problems, use of cartilage oligomeric matrix protein (COMP)-Ang1 has been proposed, in which the N-terminal portion of Ang1 is replaced with the short coiled-coil COMP domain, a non-collagenous extracellular matrix (ECM) protein comprised of five identical glycoprotein subunits [17]. COMP-Ang1 is a soluble, stable, and potent pentameric Ang1 variant that more effectively induces Tie2 phosphorylation than native Ang1, and is easier to produce than native Ang1 due to its stability [17].

Here, we report that COMP-Ang1 enhances capillary formation and migration of EPCs *in vitro* and that combined treatment with COMP-Ang1 and EPCs effectively mitigate ischemic brain injury by increasing angiogenesis.

MATERIALS AND METHODS

All experiments using humans or human products were conducted with informed consent and approved by the Institutional Review Board of Seoul National University Hospital. All animal experiments were performed with the approval from the Institutional Animal Care and Use Committee of the Biomedical Research Institute of the Seoul National University Hospital and complied with the National Research Council *Guidelines for the Care and Use of Laboratory Animals*. Methods are summarized in Supplementary Experimental Procedure.

Isolation of mononuclear cells (MNCs) derived from human umbilical cord blood (HUCB)

All human aspects of this study were approved by the institutional review board of Seoul National University Hospital (IRB No. 0802-039-234). For early EPC cultures, HUCB (50 ml) samples were collected from fresh placentas with attached umbilical cords by gravity flow. The MNCs were isolated by Ficoll density gradient centrifugation (Sigma-Aldrich, St. Louis, MO, USA) for 25 min at 2300 rpm/min, and then washed in phosphate-buffered saline (PBS, pH 7.4). The isolated MNCs were resuspended with the EGM-2-MV Bullet Kit system (Lonza, Walkersville, MD, USA) consisting of endothelial basal medium-2 (EBM-2), 5% fetal bovine serum (FBS), hEGF, vascular endothelial growth factor (VEGF), human fibroblast growth factor-B (hFGF-B), R3-IGF-1, ascorbic acid, and gentamicin. MNCs were seeded on 1.5% gelatin-coated (Sigma-Aldrich) 6-well plates (1×10^7 cells per well) and incubated in a 5% CO₂ incubator at 37°C.

Culture of putative hCB-EPCs and HUVECs

For this experiment, 2~5 passages of late EPCs and 2~6 passages of HUVECs were used. Unselected MNCs were plated on culture dishes coated with 1.5% gelatin and cultured using the EGM-2-MV Bullet Kit (Lonza) system. Non-adherent cells were removed after 5~6 d, and fresh culture medium was applied. Subsequently, the medium was changed every 2~3 d, and cultures were maintained for 8 weeks. As described by others, the endothelial phenotype occurred between d 4 and 7 [11]. A phenotypic analysis was performed on d 0, 1, 5, and 7 and at the end of weeks 2, 5, and 8. HUVECs, obtained from Lonza, were maintained with an EGM-2 Bullet Kit (Lonza) consisting of EBM-2, 2% FBS, hEGF, VEGF, hFGF-B, R³-IGF-1, ascorbic acid, heparin, and gentamycin in a 5% CO₂ incubator at 37°C.

Tube formation assay and HUVEC DiI-incorporation assay

A matrigel basement membrane matrix (BD Biosciences, San Diego, CA, USA) was added to Lab-Tek II chamber slide (NUNC, Roskilde, Denmark). hCB-EPC only (1×10^4 cell) or hCB-EPCs treated to silence Tie2, FAK, and AKT were added to matrigel coated chamber slides for 24 h at 37°C with EBM-2 medium containing COMP-Ang1 (200 ng/ml). The length of capillary tube formed was determined with ImageJ v1.38 (<http://rsb.info.nih.gov/ij/>). The ability of EPCs to incorporate into EC vascular structures was assed as follow. DiI-labeled HUVECs (1×10^4 cells) were seeded on a matrigel-coated chamber slide and incubated for 1 h. To the HUVECs, CFSE-labeled hCB-EPCs (1×10^4 cells) or CFSE-labeled hCB-EPCs in which Tie2, FAK, and AKT were silenced were added with EBM-2 medium containing COMP-Ang1 (200

ng/ml) and incubated for 24 h. The number of incorporated cells was determined using a confocal laser scanning microscope (Carl Zeiss LSM 510META, Carl Zeiss, Jena, Germany).

Scratch wound migration assay

Cells were seeded in 6-well tissue culture plates (2×10^4 cells per well), cultured to confluence, and the monolayers were wounded by scratching along the surface of the plastic dish with a razor blade. The blade was pressed down in the middle of the dish; thus, cutting the cell layer and concomitantly marking the wound boundary on the underlying plastic. Then, the blade was gently slid unidirectionally away from the wound boundary along the surface of the medium to remove half of the confluent cell layer. The remaining wounded monolayer was washed twice with PBS, re-fed with mitomycin C (1 mM, Sigma-Aldrich) containing serum-free EBM-2 or serum-free EBM-2 containing COMP-Ang1 (200 ng/ml), and incubated under standard culture conditions for selected times of 0~36 h, and analyzed using a IX51 microscope (cooled pe excitation system, Olympus, Japan).

Immunocytochemistry

Cells were grown on Lab-Tek II chamber slide (NUNC, Roskilde, Denmark). The cells were rinsed in PBS, fixed in methanol for 20 min, and rinsed again in PBS. The cells were incubated overnight with protein-specific antibodies and location markers at 4°C. The cells were rinsed with PBS and then incubated for 1 h at room temperature with secondary antibodies. The cells were incubated with DAPI (4'6-diamidino-2-phenylindole; 1 µg/ml, Sigma Aldrich) for 40 sec to counterstain nuclei. After washing with PBS, coverslips were mounted on the glass slides using Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA), and analyzed using a LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Proximity ligation assay (PLA)

PLA was performed in cells with and without the COMP-Ang1 treatment to visualize protein-protein interactions. The cells were washed with chilled PBS and incubated overnight with antibodies against specific proteins at 4°C. Proximity ligation was performed according to the manufacturer's protocol using the Duolink Detection Kit (Life Technologies, Carlsbad, CA, USA). Hoechst staining was performed during the detection reaction. Specimens were mounted with Vectashield mounting media (Vector Laboratories) and analyzed using a LSM 710 confocal microscope. The number of *in situ* PLA signals per cell was counted by semiautomated image analysis using BlobFinderV3.0.

qRT-PCR

RNA was isolated using TRIzol reagent (Invitrogen), pooled, and then subjected to first-strand cDNA synthesis using Reverse Transcription System (Takara) according to the manufacturer's protocol. qPCR primer sequences were designed by Primer Express (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was performed using the 7900 HT Fast Real-Time PCR system (Applied Biosystems). Threshold cycle number and reaction efficiency were determined using the software in the GeneAmp 7900 HT sequence detection system (Perkin-Elmer, Waltham, MA, USA). The following profile was used at 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 60s.

Western blot analysis

Whole cell lysates were prepared with RIPA buffer containing 4% CHAPS. Proteins from each group were separated on 4~12% polyacrylamide gels (Life Technologies) and transferred to nitrocellulose membranes. Cell lysates (30 µg protein) were subjected to immunoblotting using protein-specific antibodies with anti-p38, anti-ERK1/2, anti-SAPK/JNK, anti-pp38, anti-pERK1/2, anti-pSAPK/JNK (Cell Signaling Technology, Danvers, MA, USA), anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HIF-1α (BD Biosciences, San Jose, CA, USA).

Small inhibitory RNA (siRNA) injection

Cells were transiently transfected using the OneDrop Microporator MP kit (Invitrogen) according to the manufacturer's instructions after siRNA synthesis. Briefly, 1×10^5 cells were resuspended in 100 µl of resuspension buffer and transfected with 100 nM siRNA. After the transfection, the cells were transferred to 6-well plates (NUNC) containing fresh prewarmed DMEM and maintained for 72 h at 37°C in 5% CO₂. Then, the cells were subjected to migration assay, PLA assay, qRT-PCR, and immunoblotting.

ELISA

The amounts of SDF-1α in EPC and HUVEC cell lysates were determined by ELISA. The ELISA was performed according to the manufacturer's protocol using the SDF-1 Detection Kit. After washing the unbound HRP-conjugated secondary antibody, color was developed by an incubation with 3,3',5,5'-tetramethylbenzidine for 15 min, and the reaction was stopped with an equal volume of 2 M H₂SO₄. Absorbance in each well was measured at 450 nm using an ELISA plate reader (VERSA Max, Molecular Devices, Sunnyvale, CA, USA).

Animals

Adult male Sprague-Dawley rats (weight, 250~300 g; Orient Bio, Sungnam, Korea) were housed under a 12 h light/12 h dark cycle with food and water available *ad libitum* in the animal care facility of the Biomedical Research Institute of Seoul National University Hospital, Korea. Animal care and surgical procedures were carried out in accordance with guidelines on the ethical use of animals that were approved by the Experimental Animals Committee of Seoul National University Hospital. All efforts were made to minimize the number of animals and their suffering.

Surgical procedure

Rats were anesthetized by intraperitoneal injection of 1% Zoletil (30 mg/kg) and xylazine hydrochloride (4 mg/kg). Rectal temperature was maintained at 37°C using a thermostatically controlled heating blanket (Panlab, Heidelberg, Germany). Transient MCAO was induced for 2 h using an intraluminal vascular occlusion method, as described previously [18]. Rats were provided with free access to food and water after recovery from anesthesia and maintained in air-ventilated cages at 24±0.5°C for the duration of the experiment.

Intra-local administration of hCB-EPC or Adenovirus-COMP-Ang1 and PTD transfected hCB-EPCs

hCB-EPCs were labeled with CM-DiI (Invitrogen). hCB-EPCs were also transfected with with adenovirus-COMP-Ang1 and PTD (n=5) [19] and control group was 10 µL of 1X PBS only (n=10). Two days after MCAO, rats were anesthetized with an intraperitoneal injection of 1% Zoletil (30 mg/kg) and xylazine hydrochloride (4 mg/kg) and secured in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The skin was reflected and a burr hole was trephined at the right frontal bone using a microdrill. Using Hamilton syringe, 5×10^5 hCB-EPCs in 10 µL of 1X PBS were injected into the left striatum at a flow rate of 0.5 µL/min. The stereotaxic coordinates of the injection site were: anteroposterior (AP), -1 mm; lateral (L), -3 mm; and dorsoventral 5 mm according to the stereotaxic coordinates. The needle was slowly withdrawn (over 5 min) after completion of the injection and the wound was closed with 4-0-nylon sutures. After the injections, the animals were kept warm and allowed to recover from anesthesia. Subsequently, they were returned to the animal care facility for 4 weeks.

Histology

Four weeks after MCAO, rats were anesthetized with an intraperitoneal injection of 1% Zoletil (30 mg/kg) and xylazine hydrochloride (4 mg/kg) and perfused transcardially with ~50 ml of 0.9%

NaCl (heparin included) followed by ~50 ml of ice-cold (4°C) 4% paraformaldehyde. The rat brains were removed after perfusion, and placed in a 50 ml conical tube containing ice-cold 4% paraformaldehyde for 1 day. The rat brains were then transferred to 10%, 20%, and 30% sucrose sequentially every day until the brains sank to the bottom of the container. The treated rat brains were cut into 30 µm thick coronal sections on a freezing cryostat (Leica) throughout the whole brain.

limb placement test

Limb placement tests (LPTs) included eight subtests and were performed 2 days after ischemia as previously described [20]. Briefly, the test consists of three domains: (1) visual forward, (2) visual lateral, and (3) proprioception. The 'visual forward' is observation of forelimb flexion by holding up the tail. The stretch of the forelimbs towards the table is evaluated: normal stretch, 0 points; abnormal flexion, 1 point. The 'visual lateral' is observation of forelimb stretch by the stimulus to rat's whiskers while the examiner holds the rat's trunk. The visual lateral makes a score 0, 1, 2 and 3 points: normal lifting, 0 points; abnormal lifting, 1, 2, 3 points according to the times of normal stretch. The 'proprioception' is observation of stepping up of the forelimb and hindlimb on the table after pulling down the forelimb and hindlimb below the level of the table in three times each: normal lifting, 0 points; abnormal lifting, 1, 2, 3 points according to the times of normal stretch. Therefore the highest point is 10.

Rota-rod test

All rats were trained at a constant speed of 12 rpm/min for 100 sec before MCAO surgery. Then, the speed was increase from 4 to 40 rpm/min and measured the time at which they dropped down to the bottom of the Rota-rod machine. The test was performed at post cell transplantation 1, 2, and 4 weeks and the data evaluated after MCAO are presented as a percentage of the three Rota-rod trials compared with the control data before MCAO.

Measurement of infarct volume in rat brain slices

Every five brain sections in each group of animals stained with cresyl violet were observed. Each stained section were scanned by high resolution scanner (Epson), and then each hemisphere were compared each other using ImageJ, v1.38. Volume was presented as percentage of the corresponding intact brain.

Immunohistochemistry

Every tenth serial section was rinsed in PBS, permeabilized with PBS containing 0.1% (v/v) saponin and 4% (v/v) normal goat serum (NGS) for 15 min and then blocked with PBS containing 0.05%

(v/v) saponin and 5% (v/v) NGS for 15 min at room temperature. The sections were incubated overnight at 4°C with anti-laminin (Sigma Aldrich), anti-human nuclei (Millipore), anti-Tie2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) anti-FAK (Santa Cruz Biotechnology), anti-AKT (Cell Signaling Technology, Danvers, MA, USA), anti-pp38 (Cell Signaling Technology), anti-pERK1/2 (Cell Signaling Technology), anti-pSAPK/JNK (Cell Signaling Technology), anti-mTOR (Sigma Aldrich), anti-SDF1 (Santa Cruz Biotechnology), anti-FLAG (Sigma Aldrich). Subsequently, the sections were incubated for 1 h at room temperature with a fluorescent-labeled secondary antibody (FITC, Alexa488-labeled, raised in mouse; Jackson Immuno Research Laboratories, West Grove, PA, USA) and mounted with Vectashield medium containing DAPI (Vector Laboratories). Fluorescence staining was evaluated using the aforementioned confocal laser scanning microscope.

Statistical analysis

Statistical analysis was performed with Graph-Pad Prism version 5.0 (GraphPad Software, Inc, San Diego, CA, USA). Summary data are expressed as the means±standard error of mean (SEM). The significance of the differences between the means was determined with unpaired Student's *t*-tests and ANOVA. A difference with a *p*-value<0.05 was considered significant while a *p*-value<0.001 was considered highly significant. The quantification of data in the sham control and experimental groups were analyzed using Student's *T*-test or, if necessary, Mann-Whitney rank sum test. For the behavioral study, the results were analyzed with repeated ANOVA. The results were expressed as the means±SEM. A repeated ANOVA with post hoc Newman-Keuls tests was used to analyze the differences in behavior tests.

RESULTS

COMP-Ang1 enhances tube formation and migration of EPCs

Because tube formation and cell migration constitute an important process in vessel formation, we tested whether COMP-Ang1 enhances the tube formation and migration of EPCs and HUVECs (Fig. 1A, C). Considerable tube formation occurred when EPCs and HUVEC were incubated on matrigel for 24 h, and COMP-Ang1 significantly increased the tube formation. The greater increase for EPCs in tube formation by COMP-Ang1 was shown than HUVEC.

When CFSE-labeled EPCs (green) were co-cultured with DiI-labeled HUVECs (red), CFSE-labeled EPCs were incorporated into the DiI-labeled HUVECs tubes and COMP-Ang1 markedly increased the incorporation of CFSE-labeled EPCs into DiI-

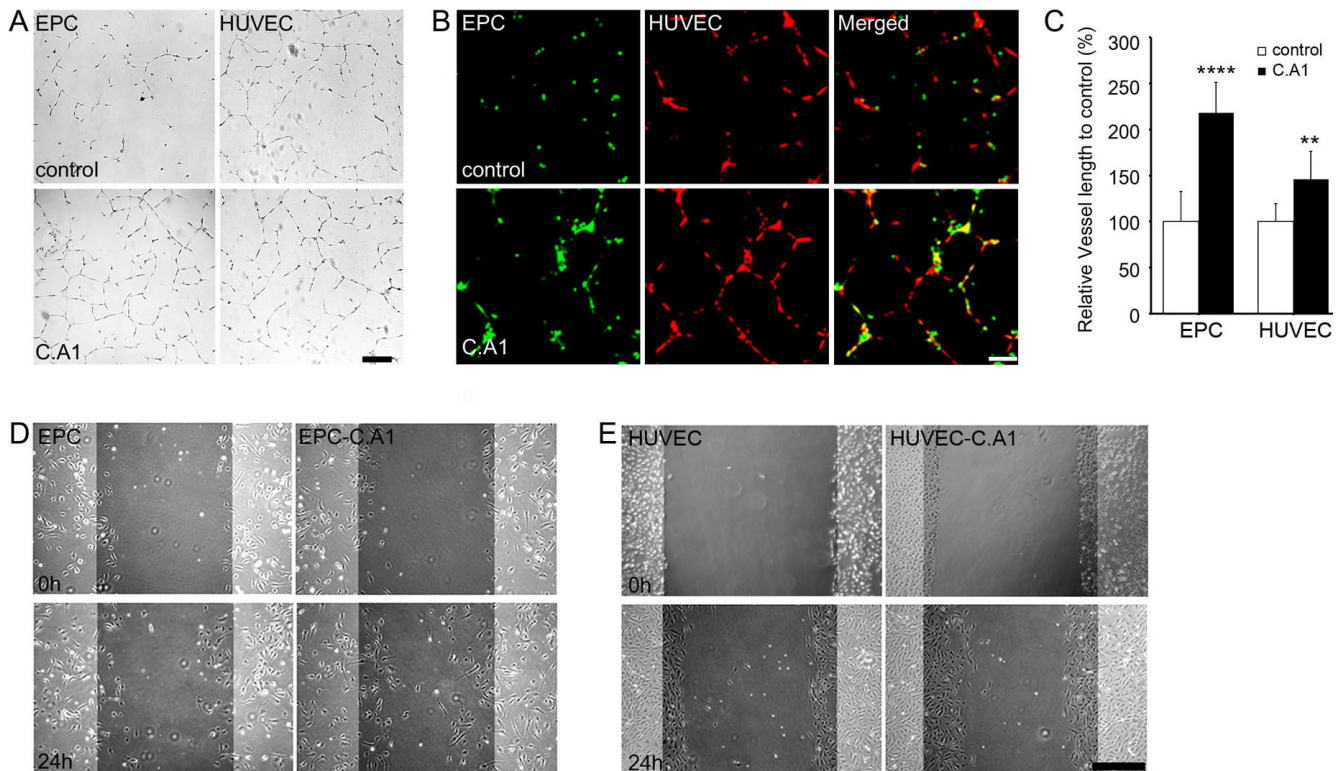


Fig. 1. COMP-Ang1 enhances tube formation and migration of hCB-EPCs. (A, C) COMP-Ang1 promoted tube formation, more in EPCs than in HUVECs. COMP-Ang1 (C.A1, 200 ng/mL) was incubated for 24 h (A) followed by densitometric analyses (C). Scale bar, 100 μ m. All values are the means \pm SEM. **** p < 0.0001, ** p < 0.01. (B) COMP-Ang1 promoted the number of EPC incorporated HUVECs. CFSE-labeled EPCs were incorporated on DiI-labeled HUVECs. COMP-Ang1 (200 ng/mL) was incubated for 24 h. Scale bar, 100 μ m. (D, E) COMP-Ang1 promoted cell migration in EPCs better than that in HUVECs. COMP-Ang1 (200 ng/mL) was incubated for 24 h. Scale bar, 50 μ m.

labeled HUVECs (Fig. 1B). The scratch wound migration assay demonstrated that COMP-Ang1 also increased the migration of cells, more for EPCs than HUVECs (Fig. 1D, E). These results indicated that COMP-Ang1 significantly enhances tube formation and migration of EPCs.

EPCs enhance angiogenesis and protect against brain damage in a rat ischemic model

EPCs were transplanted into the striatum of the middle cerebral artery occlusion (MCAO) rat model to investigate angiogenesis and brain protection capacities [21]. The size of infarct volume in the brains treated with EPCs was significantly smaller than that of control demonstrating that EPCs transplantation alone could significantly mitigate the ischemic damage of brain. The transplantation of EPCs together with COMP-Ang1 was far more effective than EPC transplantation alone to decrease the infarct size (Fig. 2A, B). The treatment with EPCs alone was not effective to improve the limb placement and combined treatment of EPCs and COMP-Ang1 slightly but not significantly improved the limb placement. On the other hand, EPC treatment improved the Rota-

Rod time, and COMP-Ang1 markedly increased the effect of EPCs to increase the Rota-Rod time (Fig. 2C, D).

Using Laminin (non-collagenous connective tissue glycoprotein) staining we examined the extent of angiogenesis in the brain. The laminin expression in the rat brains significantly increased by EPC transplantation and more so by the combined treatment of EPCs with COMP-Ang1 (Fig. 2E, F). These results clearly indicated that COMP-Ang1 significantly enhances the angiogenesis following EPCs treatment in rat brain.

COMP-Ang1 increases the Tie2/FAK interaction after translocation in EPCs

We investigated the effect of COMP-Ang1 on the location and interactions of Tie2 receptor, FAK and AKT, in EPCs to identify the molecular mechanism behind the increase in angiogenesis by COMP-Ang1 treatment. The location of Tie2 changed dramatically from cytoplasm to plasma membrane by COMP-Ang1 treatment (Fig. 3A). FAK also appeared more abundantly in cytoplasm and the plasma membrane after COMP-Ang1 treatment as compared to that in the control. However, the

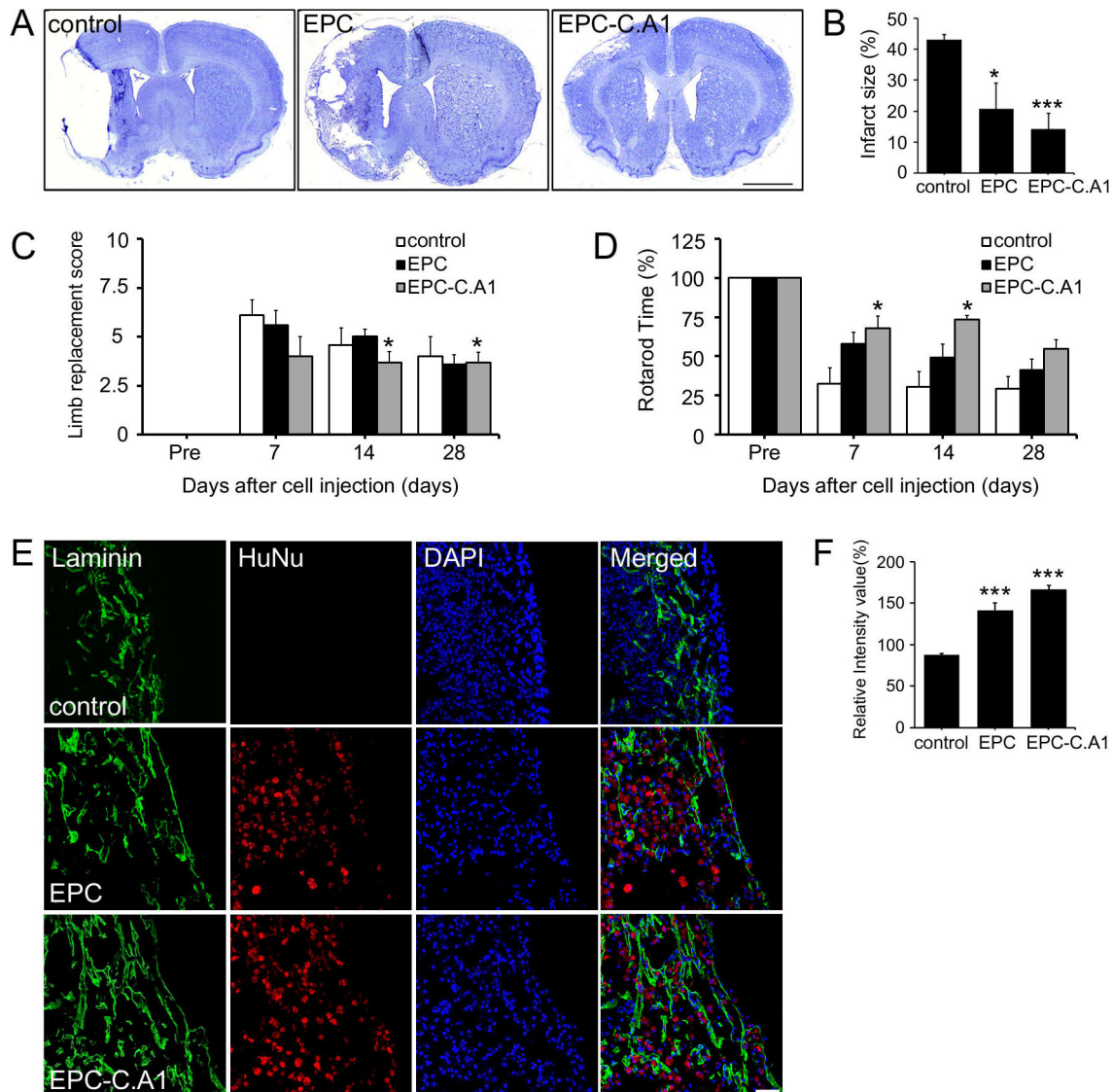


Fig. 2. hCB-EPCs enhance angiogenesis and protect against degeneration in a rat ischemic model. (A, B) Measurement of infarct size of the control, EPC and EPC-C.A1 in the rat model. (A) Densitometric analyses (B), Scale bar, 1000 μ m. All values are the means \pm SEM. *** p <0.001, * p <0.05. (C, D) The behavior test for the control, EPC and EPC-C.A1 in rat model. Limb placement test (C) and Rota-rod test (D). All values are the means \pm SEM. * p <0.05. (E, F) Confocal images of laminin staining (green) and HuNu staining (red) with the DAPI nuclear marker (blue) in ischemic rats with/without EPC treatment in the absence or presence of co-treatment with COMP-Ang1 (E) and densitometric analyses (F). Scale bar, 50 μ m. All values are the means \pm SEM. *** p <0.001.

location of AKT remained the same in cytoplasm and nucleus after COMP-Ang1 treatment (Fig. 3A). Next, we examined the interactions of these molecules after COMP-Ang1 treatment in EPCs using the proximity ligation assay. The interaction of Tie2 with FAK increased approximately 3 times after COMP-Ang1 treatment (Fig. 3B, C). However, the interactions between Tie2 and AKT and that between AKT and FAK decreased dramatically after COMP-Ang1 treatment (Fig. 3C). We further investigated the Tie2/FAK/AKT expression after COMP-Ang1 treatment in EPCs. Immunofluorescence staining indicated that the levels of Tie2, FAK

and AKT increased dramatically after COMP-Ang1 treatment in EPCs (Fig. 3D, E). Taken together, it is shown that COMP-Ang1 increases the interaction between Tie2 and FAK after translocation in EPCs.

COMP-Ang1 increases Tie2/FAK/ITG mediated migration and tube formation of EPCs

We examined the Tie2/FAK/ITG-centered molecular mechanism underlying the increase in migration and tube formation caused by COMP-Ang1 treatment in EPCs. The increase in tube formation by

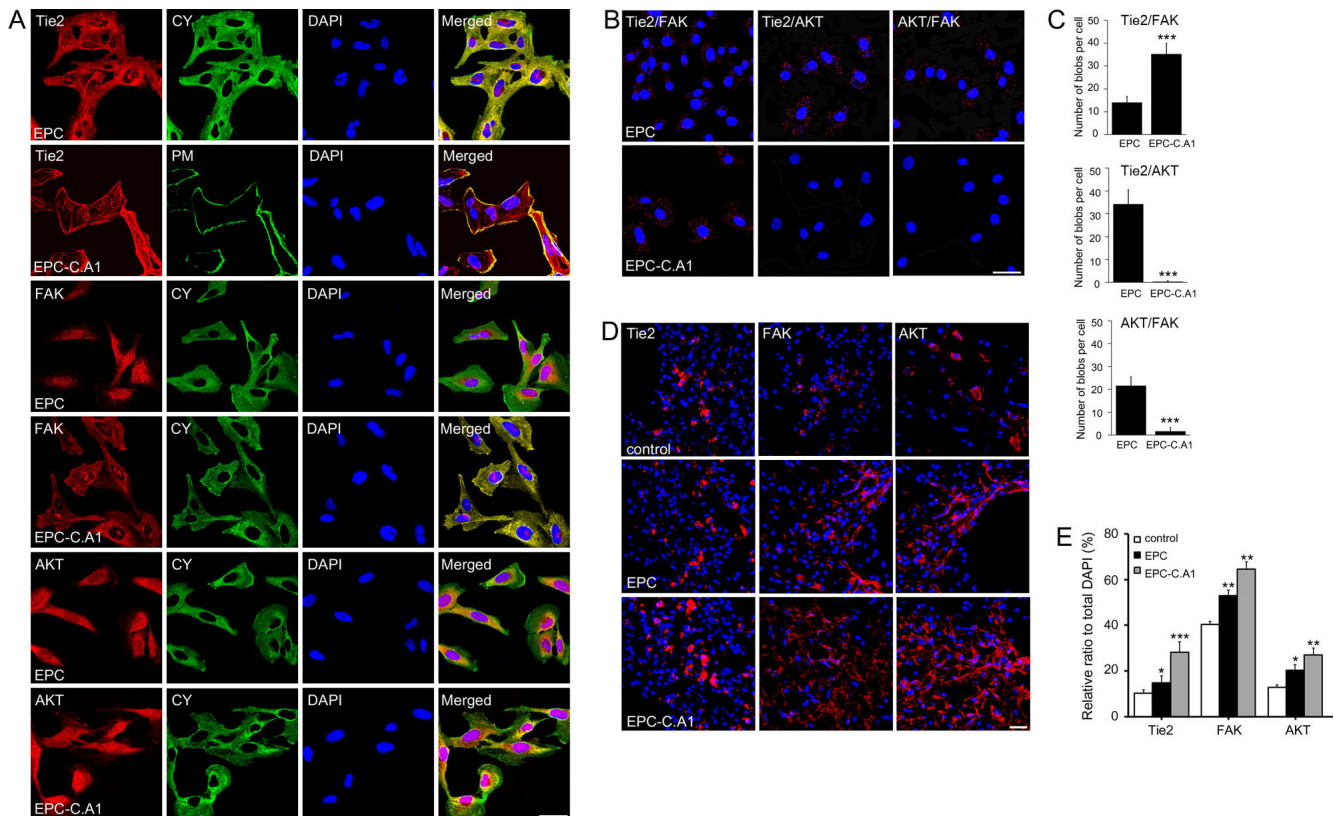


Fig. 3. COMP-Ang1 increase the Tie2/FAK interaction after translocation in hCB-EPCs. (A) Confocal images of Tie2, FAK, and AKT (red) with one of two location markers (CY or PM, green) and the DAPI nuclear marker (blue) with or without treatment of COMP-Ang1 in EPC. Yellow or cyan color indicate overlap between protein and the corresponding location markers. Scale bar, 200 μ m. (B, C) Interactions between Tie2: FAK (left), Tie2: AKT (middle), or AKT: FAK (right) with or without COMP-Ang1 treatment in EPCs. A proximity ligation assay (PLA) was used to measure the population of close physical interactions. Red spots indicate the physical proximity of the corresponding protein pair. Scale bar, 200 μ m. (B). Number of blobs (or interactions) per cell between Tie2 and FAK with or without COMP-Ang1 treatment in EPCs (C). Average number of “blobs” (spots or interactions) per cell for Tie2: FAK, Tie2: AKT, or AKT: FAK under the COMP-Ang1 treatment condition showing that the number of interacting pairs between Tie2: FAK increased dramatically, but interaction between Tie2: AKT and AKT: FAK decreased after COMP-Ang1 treatment. All values are the means \pm SEM. *** p <0.001. (D, E) Confocal images of Tie2, FAK, and AKT (red) with one of two location markers (CY or PM, green) and the DAPI nuclear marker (blue) in a rat model. (D) Densitometric analyses (E). Scale bar, 20 μ m. All values are the means \pm SEM. *** p <0.001, ** p <0.01, * p <0.05.

COMP-Ang1 treatment was completely inhibited by Tie2 silencing, but not by FAK silencing. The tubes were formed imperfectly when AKT was silenced (Fig. 4A, C). The incorporation of CFSE-labeled EPCs into Dil-labeled HUVECs was reduced by silencing Tie2 and AKT but not by silencing FAK (Fig. 4B).

We also performed the migration assay after silencing Tie2, FAK or AKT. Silencing Tie2 and FAK but not AKT decreased the migration of EPC treated with COMP-Ang1 (Fig. 4D).

We further investigated whether COMP-Ang1 increases the interaction of FAK with ITGs. COMP-Ang1 significantly increased the interaction of FAK with ITG β 1 (Fig. S1).

Next, we assessed the effect of COMP-Ang1 on the interactions among ITG molecules in EPCs. Specifically, the interactions between ITG α v β 3 and ITG α 4, ITG α v β 3 and ITG α v, or ITG α v β 3 and ITG β 1 were investigated in EPCs before or after Tie2 or FAK

silencing. The interactions between ITG molecules increased by COMP-Ang1 treatment, but Tie2 or FAK silencing decreased the interaction in both control EPCs and the EPCs treated with COMP-Ang1 (Fig. 4E, F).

We confirmed these results using various ITG subunits such as ITG α 4, α v, and β 3 in COMP-Ang1 treated EPCs and HUVECs by reverse transcription-polymerase chain reaction (RT-PCR). The expression of ITG α 4, α v and β 3 increased time dependently in EPCs but no clear changes were observed in HUVECs (Fig. S2A, B-C).

Because stress-activated MAPKs are critically important for initiating angiogenesis and apoptosis [22], we monitored the changes in the respective levels of MAPKs in EPCs after COMP-Ang1 treatment. Western blot analyses showed that the levels of pp38, pERK 1/2, and pSAPK/JNK increased significantly after

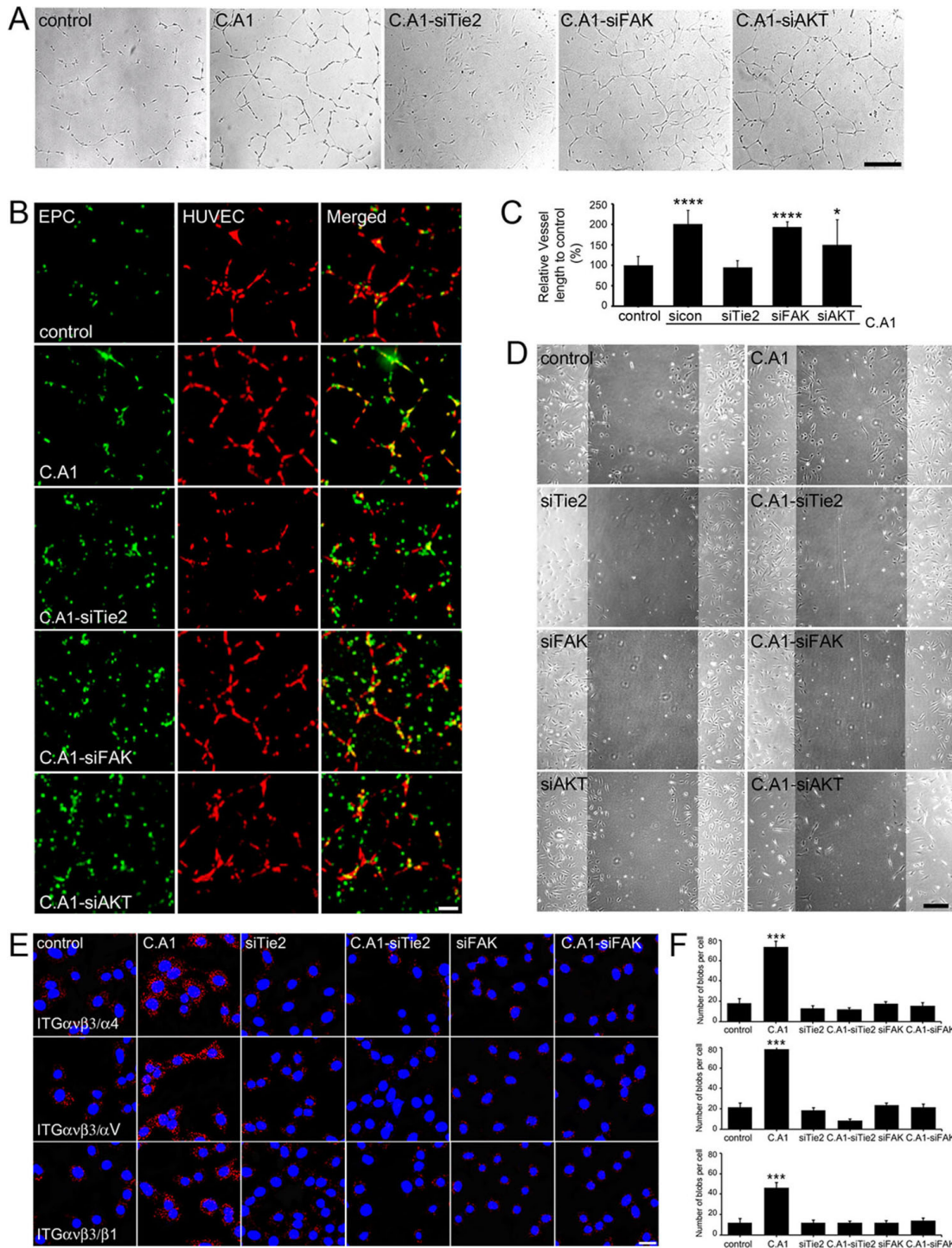


Fig. 4. COMP-Ang1 increases Tie2/FAK/ITG-mediated migration and tube formation. (A, C) Tube formation assay with COMP-Ang1 treated Tie2, FAK and AKT silencing (A). COMP-Ang1 (200 ng/mL) was incubated for 24 h followed by densitometric analyses (C). Scale bar, 100 μ m. All values are the means \pm SEM. **** p < 0.0001, * p < 0.05. (B) HUVEC-Dil incorporation assays with COMP-Ang1 treated Tie2, FAK, and Akt silencing. COMP-Ang1 (200 ng/mL) was incubated for 24 h. Scale bar, 100 μ m. (D) Scratch wound migration assay with COMP-Ang1 treated Tie2, FAK and AKT silencing. COMP-Ang1 (200 ng/mL) was incubated for 24 h. Scale bar, 50 μ m. (E, F) The numbers of interacting pairs between ITG α v β 3: ITG α 4, ITG α v β 3: ITG α v or ITG α v β 3: ITG β 1 did not change after Tie2, FAK or AKT silencing. Interactions between ITG α v β 3: ITG α 4 (top), ITG α v β 3: ITG α v (middle), or ITG α v β 3: ITG β 1 (bottom) with/without COMP-Ang1 treatment in EPCs before or after Tie2 or FAK silencing. Red spots indicate the physical proximity of the corresponding protein pair. Scale bar, 200 μ m. (E). Number of blobs (or interactions) per cell between ITG α v β 3: ITG α 4, ITG α v β 3: ITG α v or ITG α v β 3: ITG β 1 with/without COMP-Ang1 treatment in EPCs before or after Tie2 or FAK silencing (F). All values are the means \pm SEM. *** p < 0.001.

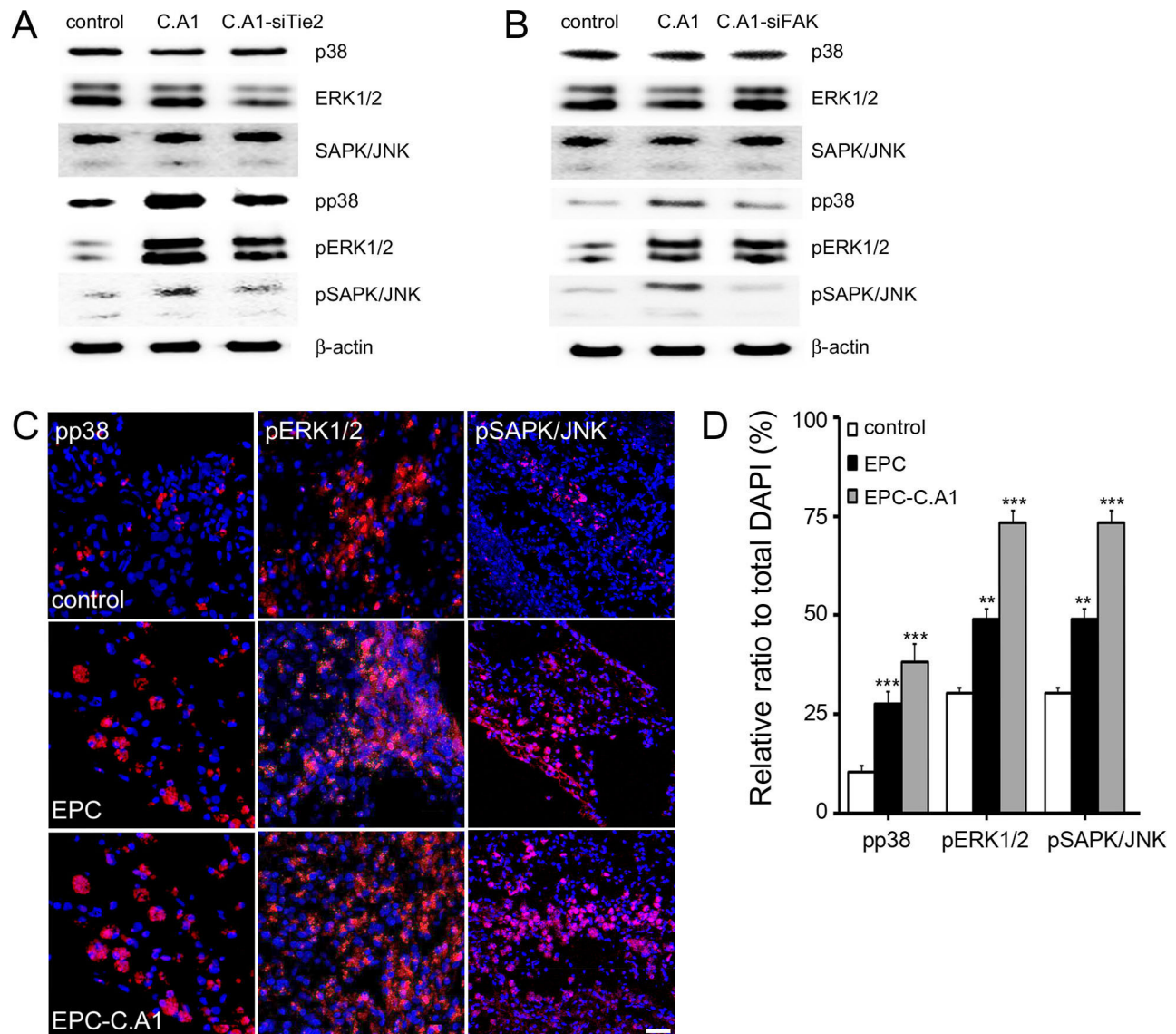


Fig. 5. COMP-Ang1 increases migration and tube formation through the MAPK pathway. (A, B) Relative protein expression levels of MAPK kinase with/without COMP-Ang1 treatment in EPCs before or after Tie2 or FAK silencing. Whole cell lysates (30 μ g protein/lane) were subjected to Western blot analyses to determine the levels of ERK1/2, pERK1/2, p38, pp38, and SAPK/JNK, pSAPK/JNK. β -actin was used as an internal control for equal protein loading of each lane. (C, D) Confocal images of pp38, pERK1/2, and pSAPK/JNK (red) with the DAPI nuclear marker (blue) in a rat model (C) followed by densitometric analyses (D). Scale bar, 20 μ m. All values are the means \pm SEM. *** p <0.001, ** p <0.01.

EPCs were exposed to COMP-Ang1 (Fig. 5A). The levels of pp38, pERK 1/2, and pSAPK/JNK decreased after silencing Tie2 (Fig. 5A). However, p38, ERK1/2, and SAPK/JNK levels remained the same after COMP-Ang1 treatment (Fig. 5A). Silencing FAK also decreased the expression of pp38 and pSAPK/JNK but the pERK 1/2 expression remained the same (Fig. 5B). We also studied the expression of pp38 and pSAPK/JNK in rat brain after treating with EPCs alone in combination with COMP-Ang1. The *in vivo* results were similar to those *in vitro* (Fig. 5C, D). These results indicate that COMP-Ang1 increases Tie2/FAK/ITG mediated migration

and tube formation.

COMP-Ang1 increases angiogenesis through an AKT/mTOR interaction

We investigated whether the separated AKT is involved in angiogenesis via an interaction with mTOR. PLA (Proximity ligation assay) revealed that the interaction between AKT/mTOR significantly increased after COMP-Ang1 treatment in EPCs, whereas it decreased after Tie2 silencing regardless of COMP-Ang1 treatment (Fig. 6A, B).

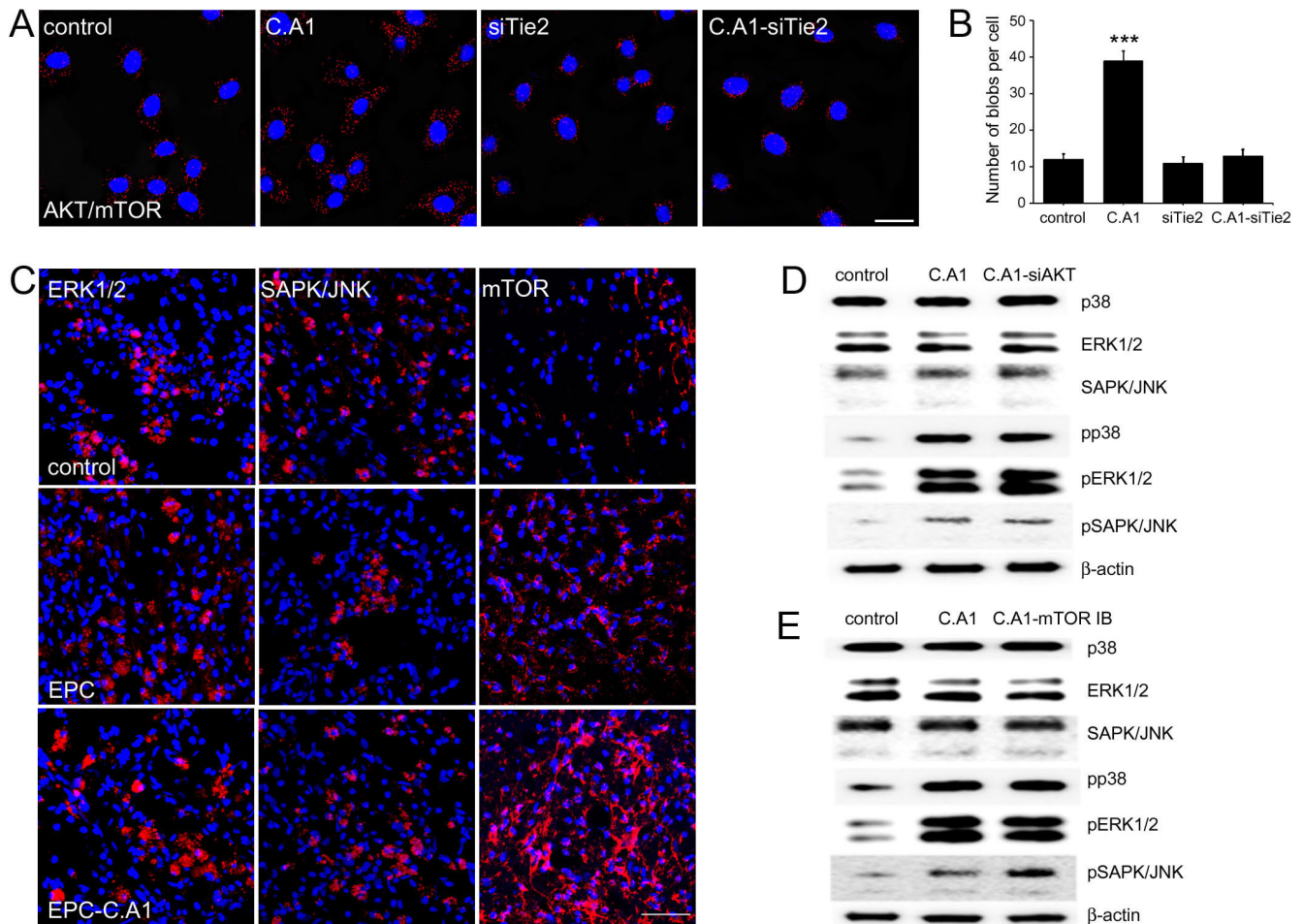


Fig. 6. COMP-Ang1 increase angiogenesis by AKT/mTOR interaction. (A,B) Interaction between AKT and mTOR with/without COMP-Ang1 treatment in EPCs before or after Tie2 silencing. Red spots indicate the physical proximity of the corresponding protein pair. Scale bar, 200 μ m. (A). Blobs showing interaction between AKT and mTOR with/without COMP-Ang1 treatment in EPCs before or after Tie2 silencing. The numbers of interacting pairs between AKT:mTOR no changed after Tie2 silencing (B). All values are the means \pm SEM. *** p <0.001. (C) Confocal images of ERK1/2, SAPK/JNK and mTOR (red) with DAPI of nucleus marker (blue) in rat model. Scale bar, 50 μ m. (D, E) The relative protein expression levels of MAPK kinase with/without COMP-Ang1 treatment in EPC before or after AKT silencing or mTOR inhibition. Whole cell lysates (30 μ g protein/lane) were subjected to Western blot analyses to determine the levels of ERK1/2, pERK1/2, p38, pp38, and SAPK/JNK, pSAPK/JNK. β -actin was used as an internal control for equal protein loading for each lane.

We also analyzed the MAPK molecules to determine whether the AKT/mTOR interaction induced angiogenesis via MAPK pathway in EPCs in rat brain. Immunohistochemistry and Western blot analyses of rat brain tissue showed that COMP-Ang1 treatment caused no changes in the levels of pp38, pERK 1/2, and pSAPK/JNK regardless of AKT silencing or mTOR inhibition (Fig. 6C, D-E). These results indicated that MAPK is not involved in the angiogenesis induced by Akt-mTOR interaction in rat brain.

COMP-Ang1 induces SDF-1/CXCR4/HIF-1 α mediated angiogenesis

We investigated CXCR4, SDF-1, and HIF-1 α expression before and after COMP-Ang1 treatment. CXCR4 expression in EPCs

in vitro increased significantly by COMP-Ang1 treatment, but decreased after silencing AKT or inhibiting mTOR. However, CXCR4 expression remained unchanged when FAK, ITG α 4, or ITG α V were inhibited (Fig. 7A, B, S3).

Confocal images of ischemic rat brain showed that the level of SDF-1 increased by EPC treatment and more markedly increased by EPC-COMP-Ang1 treatment (Fig. 7C, E). The expression of adenovirus-COMP-Ang1 was detected by FLAG antibody. The expression of FLAG was little increase by EPC treatment, but it was significantly increased by EPC-COMP-Ang1 treatment. The enzyme-linked immunosorbent assay (ELISA) showed that COMP-Ang1 treatment increased extracellular SDF-1 in both EPCs and HUVECs in time dependent manner (Fig. 7D).

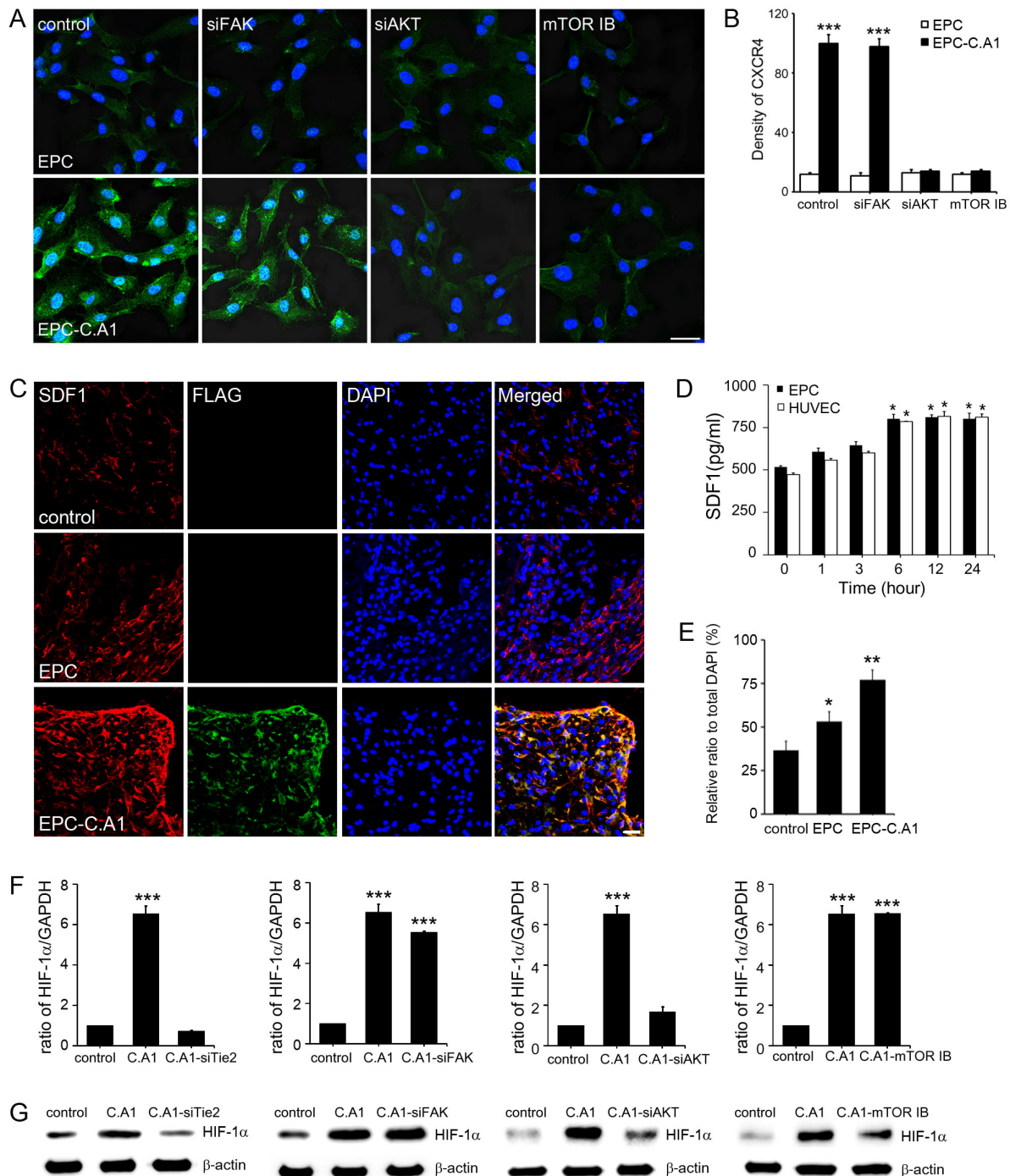


Fig. 7. COMP-Ang1 induces SDF-1/CXCR4/HIF-1 α mediated angiogenesis. (A, B) Confocal images of CXCR4 (green) with DAPI for nucleus marker (blue) with/without COMP-Ang1 treatment in EPC before or after FAK or AKT silencing or mTOR inhibition (A) and densitometric analyses (B). Scale bar, 200 μ m. All values are the means \pm SEM. *** p <0.001. (C, E) Confocal images of SDF-1 (red) and FLAG (green) with DAPI of nucleus marker (blue) in ischemia rat with/without EPC treatment in the absence or presence of co-treatment with COMP-Ang1 (C) followed by densitometric analysis (E). Scale bar, 20 μ m. All values are the means \pm SEM. ** p <0.01, * p <0.05. (D) The time-dependent changes of extracellular (supernatant) SDF-1 in EPC and HUVEC, treated with COMP-Ang1 for 1, 3, 6, 12, 24 h, were determined by ELISA. All values are the means \pm SEM. * p <0.05. (F) The relative mRNA expression level of HIF-1 α before and after COMP-Ang1 treatment in EPC with/without Tie2, FAK, or AKT silencing or mTOR inhibition. All values are the means \pm SEM. *** p <0.001. (G) Western blot analyses of HIF-1 α protein before or after COMP-Ang1 treatment in EPC with/without Tie2, FAK, or AKT silencing or mTOR inhibition. Whole cell lysates (30 μ g protein/lane) were subjected to Western blot analysis to determine the levels of HIF-1 α . β -actin was used as an internal control for equal protein loading for each lane. The mRNA and protein expression levels of HIF-1 α were increased dramatically after COMP-Ang1 treatment, but that of HIF-1 α no changed after Tie2 and AKT silencing.

Relative HIF-1 α mRNA expression level was also investigated before and after COMP-Ang1 treatment with or without Tie2, FAK, or AKT silencing or mTOR inhibition in EPCs *in vitro* (Fig. 7F, G). Western blot analyses showed that HIF-1 α expression increased dramatically after COMP-Ang1 treatment, but that HIF-1 α expression decreased significantly after Tie2 and AKT silencing or mTOR inhibition. No change in HIF-1 α expression was observed after FAK silencing (Fig. 7E, G). These results suggested that COMP-Ang1 induces SDF-1/CXCR4/HIF-1 α mediated angiogenesis.

DISCUSSION

In the present study we have demonstrated for the first time the feasibility of treating acute cerebral ischemia with umbilical cord blood EPCs in combination with COMP-Ang1. It has been shown that transplantation of mesenchymal stromal cells or angiopoietin-1 provides some therapeutic benefits for patients with cerebral, myocardial, or hindlimb ischemia [23-32]. Therapeutic benefits of Ang-1 gene-modified mesenchymal cell for cerebral ischemia have also been reported [33, 34]. However, the mechanism underlying the therapeutic effect of Ang-1 gene-modified cell therapy for cerebral ischemia has not been elucidated. Here, we report that cell therapy with umbilical cord blood EPCs potentiated by COMP-Ang1 gene provides therapeutic effects for patients with acute cerebral ischemia.

In this study, we isolated, examined, and characterized late EPCs derived from human CB. EPCs derived from adult peripheral blood consist of two populations, referred to as early and late EPCs, with distinct cell growth patterns and different angiogenic factor secreting abilities [11]. It has been reported that CD+34 mononuclear cells from human blood are EPCs and are involved in vasculogenesis [35]. On the other hand CD34-CD14+ cells from human blood have been demonstrated to possess characteristics of EPC [36]. Moreover, CD133⁻/CD14⁺ cells from HUCBs have been reported to have the potential to differentiate into EPCs [37]. However, the true origin of the heterogeneous populations of EPCs used in the above investigations is unclear. In contrast to adult peripheral blood- or bone marrow-derived progenitors, CB progenitors have distinctive proliferative advantages, including an ability to form greater numbers of colonies, a higher cell-cycle rate, and a longer telomere [9,38], indicating human CB is a valuable source of EPCs [39,40].

EPCs are believed to augment neovascularization not only by integrating into newly developing capillaries but also in a paracrine fashion through the secretion of angiogenic growth factors [41]. EPCs derived from CB show distinct expression of EPC-specific markers and functional differences compared with early EPCs (observed in the present study and unpublished data), suggesting

that late EPCs are homogenous and well differentiated.

COMP-Ang1 has previously been shown to possess numerous potential advantages over the native protein for stimulating formation of non-leaky neo-vasculatures [15, 17]. As shown in Figs. 1A, C-E, COMP-Ang1 markedly improved the endothelial characteristics of CB-EPCs *in vitro*: COMP-Ang1 significantly increased tube formation and migration of EPCs. Note that the effect of COMP-Ang1 to increase the angiogenic characteristics of EPCs was far greater than that of HUVECs.

Transplantation of EPCs significantly decreased the infarct volume in rat brain and such effect was further enhanced by concomitant treatment with COMP-Ang1. The Rota-rod test demonstrated that treatment of MACO rats with EPCs particularly together with COMP-Ang1 significantly improved the motor activity (Fig. 2D). Immunofluorescent staining showed higher laminin expression in the rat brains treated with EPCs together with COMP-Ang1 compared to that in the sham control rat brains. These observations strongly indicated that the combined treatment of EPCs and COMP-Ang1 promoted angiogenesis and repaired the infarcted volume, at least partially, in the rat brain. Our results are in agreement with previous report that EPC treatment induces neovascularization and improves blood flow, thereby providing clinical benefits to ischemic tissues [41].

We studied the effect of COMP-Ang1 on the location and interactions of Tie2 receptor, FAK, and AKT in EPCs to reveal the molecular mechanism behind the pro-angiogenic effect of COMP-Ang1. FAK, a 125-kDa protein kinase, is an important regulator of actin cytoskeletal changes that are prerequisite for cell migration [10]. In our study, COMP-Ang1 increased the levels of Tie2, FAK, and AKT (Fig. 3A). The interaction of Tie2/FAK/AKT was interesting. First, the interaction between Tie2 and FAK increased and induced MAPK-dependent migration activity in EPCs after COMP-Ang1 treatment. The silencing experiment confirmed that Tie2 is a key molecule for the enhancement of tube formation and endothelial cell migration caused by COMP-Ang1 treatment.

We also investigated the signaling molecules and their distinctive mechanisms involved in the various migratory effects of COMP-Ang1 in EPCs. Integrins are heterodimeric adhesion molecules consisting of 18 α and 8 β subunits capable of binding ECM components as well as the intracellular cytoskeleton. Particularly, ITG-mediated signaling induces cell migration and survival by activating various kinases such as FAK, AKT, ERK1/2, and p38 MAPK [42]. In this respect, in our study, COMP-Ang1 increased the expression of ITGs α v β 3, α 4 and β 1 in EPCs indicating that COMP-Ang1 enhanced the migration of EPCs (Fig. S2A, B-C). The neuronal functions of Ang1 might also be mediated by ITG

signaling [43]. Ang1 promotes adhesion and pro-survival signaling (AKT and MAPKp42/44) in skin cells through ITGβ1 [44]. Previous findings indicated that ITGβ1 is involved in cell adhesion promoted by Ang1 [42]. The Interaction of FAK with ITGβ1 also increased by COMP-Ang1 treatment (Fig. S1). However, the interactions of FAK/ITGαV or FAK/ITGαβ3 remained unchanged by COMP-Ang1 treatment.

We further investigated whether separated AKT is involved in angiogenesis via an interaction with mTOR. The PLA revealed that the interaction of AKT with mTOR significantly increased after COMP-Ang1 treatment in EPCs, whereas it decreased after Tie2 silencing regardless of COMP-Ang1 treatment (Fig. 6A). No interaction of Tie2 with mTOR was observed after AKT inhibition by COMP-Ang1 treatment, suggesting that COMP-Ang1 treatment induces separation of AKT from Tie2 and interacts with mTOR. AKT increased expression of SDF-1, CXCR4, and HIF-1α. This result was similar to a recent finding in bone marrow-derived progenitor cells [45]. In summary, AKT was separated from the Tie2/FAK/AKT complex after COMP-Ang1 treatment, and recruited the mTOR, SDF-1, CXCR4, and HIF-1α for angiogenesis *in vitro* and in a *in vivo* rat model (Fig. 7).

Taken together, our data show for the first time that COMP-Ang1 dramatically increases migration of CB-EPCs and angiogenesis. COMP-Ang1 increased the interaction of Tie2 with FAK from Tie2/FAK/AKT, and the ITG family downstream. The AKT separated from Tie2/FAK bound with mTOR, leading to overexpression of SDF-1 and HIF-1α. However, COMP-Ang1 treatment caused interaction of Tie2/FAK with ITG family, thereby activating MAPK (Fig. S4). These molecular events eventually induced prominent angiogenesis characterized by enhanced migration of endothelial cells, vascular formation, and endothelial proliferation. Our results revealed the mechanisms by which COMP-Ang1 promotes angiogenesis of hCB-EPCs providing strong evidence that COMP-Ang1 is an effective therapeutic candidate for mitigating ischemic injury such as stroke.

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