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Research paper

Overexpression of α5β1 integrin and angiopoietin-1 co-operatively promote blood-brain barrier integrity and angiogenesis following ischemic stroke

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ABSTRACT

We previously demonstrated that cross-talk between α5β1 integrin and the angiopoietin-1 (Ang1) / Tie2 receptor plays an important role in regulating brain endothelial angiogenic responses in the ischemic penumbra following cerebral ischemic stroke (CIS). However, a recent study suggested that stimulation of the α5β1 integrin also has the potential of increasing blood-brain barrier (BBB) permeability after CIS, raising doubt about whether α5β1 integrin stimulation by itself will protect against ischemic injury. In light of these conflicting roles, the goal of this study was to evaluate the impact of co-overexpression of α5 integrin and Ang1 on vascular remodeling and repair under cerebral ischemic conditions both in vivo following 90 min of ischemia by temporary occlusion of the middle cerebral artery, and in vitro. Our results demonstrate that as compared to mock-transfected controls, overexpression of α5 integrin alone didn't improve the outcomes in neurological score and size of infarct and caused worse BBB breakdown in the ischemic hemisphere, offsetting its beneficial angiogenic effects during the early stages of CIS. However, co-overexpression of α5 integrin with Ang1 led to smaller infarcts and improved neurological deficits, which at the molecular level was underpinned by reduced BBB breakdown and increased expression of endothelial tight junction proteins in the ischemic penumbra during the early stages of CIS. Furthermore, co-overexpression of α5 integrin and Ang1 synergistically promoted BEC proliferation during the early stage of CIS, resulting in increased blood vessel density at later stages. Positive effects of α5 integrin and Ang1 co-overexpression on endothelial proliferation and tight junction protein expression were also confirmed in vitro. Collectively, these data indicate that co-overexpression of Ang-1 and α5 integrin in combination confers synergistic vascular protection against cerebral ischemic injury without the negative side effects on BBB permeability, suggesting a novel combinatorial approach for the treatment of CIS.

1. Introduction

Current evidence suggests that angiogenic remodeling after cerebral ischemia re-establishes functional microvasculature and creates a microenvironment more hospitable for neuronal plasticity, which can lead to improved functional recovery after cerebral ischemic stroke (CIS) (Chen et al., 2014). However, post-stroke angiogenic remodeling can be associated with transient loss of tight junctions (TJs) at the blood-brain barrier (BBB) as well as degradation of the extracellular matrix (ECM) during the early stages of angiogenesis (Sun et al., 2017), which has the potential to exacerbate vascular permeability.

The extracellular matrix (ECM) protein fibronectin and its integrin receptors are indispensible for angiogenesis, as displayed by failure of angiogenesis in mutant mice lacking fibronectin (George et al., 1993) or the fibronectin-specific receptor α5β1 integrin (Yang et al., 1993). We and others have shown that fibronectin and its receptors α5β1 and αvβ3 integrins are strongly upegulated on angiogenic vessels in the ischemic penumbra and that the α5β1 integrin plays an essential role in

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promoting angiogenesis under hypoxic conditions (del Zoppo and Milner, 2006; Li et al., 2012a; Li et al., 2012b; Pang et al., 2018; Sun et al., 2017). Interestingly, a recent study by Roberts et al. showed that endothelial cell-specific α5 integrin deficiency (α5-EC-KO mice) leads to reduced infarct volume and enhanced blood-brain barrier integrity in an experimental mouse model of CIS (Roberts et al., 2017), implying that activation of the fibronectin-α5β1 integrin signaling axis leads to increased BBB permeability and may exacerbate secondary injury after CIS.

In addition to ECM proteins, the angiopoietins are critical regulators of vascular stability and remodeling. When angiopoietin-1 (Ang1) binds to its receptor Tie2 on endothelial cells, it promotes endothelial cell survival and vascular remodeling, and studies have shown that Ang1 prevents plasma leakage into the ischemic brain and decreases ischemic lesion volume (Thurston et al., 2000; Zhang et al., 2002). More recently, we demonstrated that the α5β1 integrin co-localizes with phosphorylated Tie2 on cerebral vessels in the ischemic penumbra and that cross-talk between α5β1 integrin and Ang1 regulates brain endothelial angiogenic responses after CIS (Pang et al., 2018). In light of the recent study describing a potentially deleterious role for α5β1 integrin stimulation following CIS, the goal of this study was to evaluate the impact of overexpression of α5β1 integrin and Ang1, either alone, or together on vascular remodeling and repair under cerebral ischemic conditions both in vivo and in vitro.

2. Materials and methods

2.1. Experimental animals

Male C57Bl/6 mice (4–6-week-old) were used for all experiments. The present study was conducted in accordance with NIH guidelines for the care and use of animals in research and under protocols approved by the Animal Care and Use Committee of Gongli Hospital, Pudong New Area, Shanghai.

2.2. Adeno-associated viral vector construction and transduction

Adeno-associated viral vector (AAV) virions were produced by USEN Biological Technology Co. Ltd. (Shanghai, China). Briefly, murine Itga5 and Ang1 were PCR-amplified individually and cloned using standard methods (Wang et al., 2018) into AAV-PHP·B-3flag vector. AAV viral vectors were produced in HEK293 cells using three-plasmid co-transfection, and purified following previously published procedures (Zolotukhin et al., 1999). AAV-control, AAV-Itga5, AAV-Angl or AAV-Itga5/Ang1 (1×10^{12} vg/ml in 100 μl PBS) was injected into the male C57Bl/6 mice under anesthesia via the tail vein as previously described (Chan et al., 2017; Hordeaux et al., 2018; McCarty et al., 2009) and mice utilized in ischemic studies 3 weeks later.

2.3. MCAO model

Three weeks after AAV-pHB transduction, mice were anesthetized with pentobarbital anesthesia and underwent 90 min reversible right middle cerebral artery occlusion (MCAO) surgery followed by reperfusion as reported previously (Sun et al., 2017). Sham animals were subjected to the same procedure but did not receive MCAO.

2.4. Functional evaluation of neurological deficits

Neurobehavioral tests were assessed blindly before and 2, 4, 7, 14 days after MCAO using the modified neurological severity score (mNSS) ($n = 6$/group), as previously described (Chen et al., 2005). The severity score was graded at a scale from 0 to 14, in which 0 represents normal, and a higher score indicates a more severe injury (Tang et al., 2014).

2.5. Infarct volume assessment

Mice were euthanized at different time points of reperfusion. The brains were chilled at −80 °C for 4 min to slightly harden the tissue. For each brain, five, 2-mm coronal sections were made from the olfactory bulb to the cerebellum and then stained with 2% TTC at room temperature for 10 min. After staining, the slices were washed in PBS (three changes, 1 min each) and fixed in 0.1 mol/l-phosphate-buffered 4% formaldehyde for 6 h at room temperature. The stained brain sections were captured with a digital camera. The infarct area of each brain was measured in a blinded manner, using Sigmascan Pro5 image analysis software. The total volumes of both contralateral and ipsilateral hemisphere were measured and the infarct percentage was calculated as % contralateral structure to avoid mis-measurement secondary to edema (Liu et al., 2009).

2.6. Evaluation of blood-brain barrier permeability

BBB permeability in ischemic hemispheric tissue was blindly assessed by measuring Evans blue (EB) extravasation as previously described (Sun et al., 2017). Evans Blue (2% in 0.9% saline; 3 ml/kg) was injected via the tail vein 2 h prior to sacrifice at 2 and 4 days reperfusion after MCAO. Under deep anesthesia, the mice were transcardially perfused with saline to remove the intravascular EB dye. Brains were quickly removed, and the ischemic hemisphere including the cortex and striatum were weighed, followed by homogenization with 50% trichloracetic acid and centrifugation. The supernatants were collected and EB concentration was determined with a spectrophotometer at 620 nm for absorbance against a standard curve. EB leakage of each sample was expressed as micrograms per gram of wet tissue.

2.7. Immunohistochemistry studies and antibodies

Mice were euthanized by perfusion with ice-cold saline, and the brains rapidly dissected and stored at −80 °C. Immunofluorescent (IF) studies were performed as previously described (Sun et al., 2017) on 10um thick frozen coronal sections. The following monoclonal antibodies from BD Pharmingen (La Jolla, CA) were used in this study: FITC conjugated rat anti mouse CD31 (PECAM-1) (clone MEC13.3, 1:100), PE conjugated rat anti mouse α5 integrin (CD49e) (clone 5H10–27, 1:100) and FITC-conjugated rat anti-mouse Mac-1 (CD11b) (clone M1/70, 1:150). The rabbit monoclonal antibody to α5 integrin (CD49e) (ab150361,1:100) and Armenian hamster monoclonal antibody to CD31(ab119341, 1:250) were obtained from Abcam (Cambridge, MA, USA). The goat anti angiopoietin 1 polyclonal antibody (SC-6319, 1:100) was obtained from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antibodies against the following proteins were used in this study: Ki67 (ab15580, 1:800, Abcam), zonula occludens-1 (ZO-1) (1:200, Zymed Laboratories, San Francisco, CA), and claudin 5 (341,600, 1:200, Thermo Fisher Scientific, Rockford, IL). The AlexaFluor 488-conjugated goat anti-rat, anti-rabbit and Cy3-conjugated goat anti-rat, anti-rabbit secondary antibodies were obtained from EarthOx (Millbrae, CA, USA). Cy3-conjugated goat anti-Armenian Hamster secondary antibody and AlexaFluor 488-conjugated anti-goat were purchased from Jackson Immunoresearch (West Grove, PA). The negative controls for staining and confocal imaging were used to confirm a coexistence of the vessel proteins.

Quantification of the number of blood vessels positive for the different antigens was performed as previous reported (Huang et al., 2016; Sun et al., 2011). In brief, images of the region of interest were acquired using a × 20 objective on an Olympus fluorescence microscope (DP73) to determine the number of positive events per field of view (FOV). A minimum of three serial brain sections per mouse was selected for analysis of each antigen, and matched between mice so that the approximate position of sections used for IF staining was equivalent between different experimental conditions. Three images were taken from

the ischemic penumbra including cortex and striatum as well as ischemic core of each brain section and quantified by eye for the number of positive events per FOV. The number of antigen-positive events per FOV for each section was calculated as the mean of total numbers obtained from the three regions. These averages of three brain sections were used for statistical analysis for each mouse.

2.8. Cell culture

Immortalized mouse brain endothelial cells (BECs) of the bEnd3 cell line were obtained from Shanghai Bioleaf Biotech Co., Ltd. Cells were grown on six-well plates pre-coated with type I or IV collagen (10 μg/ml, Sigma, for 2 h at 37 °C) cultured in endothelial basal medium (EBM-2) (Lonza, CC-3156) supplemented with 10% FBS (Gibco), ascorbic acid, L-glutamine, penicillin/streptomycin, and human basic fibroblast growth factor (bFGF) (all from Sigma). Cells were maintained in a humidified incubator at 37 °C and 5% CO_2, and the medium was changed every 48 h.

2.9. Preparation of vectors and transfection

pLVX-IRES-ZsGreen1 vector was purchased from Takara Biomed Technology (Beijing, China). Murine Itga5 (NM_010577.4) or Ang1 (NM_009640.4) coding sequence containing the His-tag was cloned into the vector through *Xho*I and *Not* I sites to get pLVX-Itga5-IRES-ZsGreen1 and pLVX-Ang1-IRES-ZsGreen1 constructs respectively. BECs were transfected with sequencing-verified constructs using Lipofectamine 3000 (Invitrogen) as previously reported (Li et al., 2016). Briefly, 2.5 μg of pLVX-Itga5-IRES-ZsGreen1, pLVX-Ang1-IRES-ZsGreen1, both of pLVX-Itga5-IRES-ZsGreen1and pLVX-Ang1-IRES-ZsGreen1 or empty vector was mixed with 125 ul Opti-MEM® Medium followed by adding 5ul P3000 respectively. Meanwhile, 3.75 ul Lipofectamine 3000 was diluted in 125 ul Opti-MEM® Medium. The diluted Lipofectamine 3000 was then added into the diluted plasmids. After 5 mins incubation at room temperature, the mixture was added to the BEC cultures (5×10^5/well) in 6-well plates and mixed gently. After 48 h of transfection, BECs were harvested for the analysis of function and gene expression. Mock-transfected BECs were used as negative control.

2.10. Oxygen–glucose deprivation and restoration (OGD/R)

Forty-eight hours after transfection, BEC cultures were subjected to ischemia-like injury through oxygen glucose deprivation (OGD) for 4 h by placing cultures in an anaerobic chamber (Forma, Thermo Scientific, Asheville, NC, USA) with an atmosphere of 5% CO_2 and 95% N_2 in a deoxygenated glucose-free balanced salt solution (BSS0). After 4 h of OGD, cultures were returned to control conditions (restoration) by adding 5.5 mM glucose to the media under normoxic conditions. Control cultures (no injury) were incubated with a balanced salt solution containing 5.5 mM glucose (BSS5.5). All cultures were maintained in a humidified 37 °C incubator.

2.11. Western blot analysis

Western blot analysis was used to determine levels of α5 integrin, Ang1, claudin 5 and ZO-1 in brain tissue or/and cultured cells. The brain samples were taken from the ipsilateral ischemic cerebral cortex ($n = 4$/group) at different time points of reperfusion after MCAO and cell lysates were obtained from cultured bEnd3 cells at appropriate time period of restoration following OGD. Protein concentration was determined using the BCA protein assay kit (Eppendorf-Bio photometer, Germany). Western blotting, and semiquantitative analyses were performed as described previously (Li et al., 2008; Milner et al., 2007). Primary antibodies used were rabbit anti-angiopoietin-1 (AB10516, 1:1000, Merck Millipore, Darmstadt, Germany), rabbit anti-α5 integrin (AB1928, 1:1000, Merck Millipore, Darmstadt, Germany), mouse anti-claudin 5 monoclonal antibody (4C3C2, 35–2500, 1:200, Thermo Fisher Scientific, Rockford, IL), rat anti-ZO-1 (R40.76,MABT11, 1:500, Merck Millipore, Darmstadt, Germany), and β-actin (1:1000, Neomarker, Fremont, CA). Within each sample, levels of proteins were first normalised to the level of β-actin, and then expressed as the fold-increase over the level of NO-OGD/R control group.

2.12. Brain endothelial cell proliferation assay and immunocytochemistry

Cell proliferation was assayed as previously described (Yang et al., 2018). In brief, BECs were transfected with the indicated plasmid for 48 h, and then cultured on fibronectin-coated (10 μg/ml fibronectin (Sigma) for 2 h at 37 °C) glass coverslips until cells reached ~50% confluence. The confluent cells were subjected to 4 h of OGD and followed by 12 h of restoration (R) in the presence of 5-ethynyl-2′-deoxyuridine (EdU) in the last 2 h at 37 °C. The BECs were then fixed with 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, and processed for EdU assay according to the manufacturer's protocol (RiboBio, Guangzhou, China). BEC proliferation was assessed by quantifying the number of EdU-positive cells as a percentage of the total number of cells (Hoechst staining). Immunocytochemistry was performed using Armenian hamster monoclonal antibodies against endothelial-specific marker CD31 (ab119341, Abcam, Cambridge, MA, USA). Nuclei were counterstained with DAPI (Invitrogen Corporation, Carlsbad, CA).

2.13. Statistical analysis

All quantified data represent the mean ± standard deviation. Statistical significance was assessed by one- or two-way analysis of variance (ANOVA), and a Bonferroni post-hoc test was used to test multiple comparisons. All statistical analyses were performed with SPSS (version16.0; SPSS, Chicago, IL, USA) and significance was defined as $P < .05$.

3. Results

3.1. AAV-Itga5/Ang1 transduction simultaneously increases the expression of α5 integrin and Ang1 in the brain

As previously reported (Chan et al., 2017; Hordeaux et al., 2018), 3 weeks after intravenous administration of AAV-PHP·B to the mice, AAV-PHP·B can target the brain with high efficiency. To confirm that AAV-Itga5/Ang1 transduction induces the expression of α5 integrin and Ang1 in the brain, C57BL/6 mice were IV injected with or without AAV-pHB-mock, AAV-Itga5, AAV-Ang1, or AAV-Itga5/Ang1. Three weeks later the mice were subject to 90 min of ischemia by temporary occlusion of the middle cerebral artery followed by 2 or 4 days reperfusion. As shown in Fig. 1A&B, western blot analysis revealed that after 3 weeks AAV-pHB transduction, α5 integrin, Ang1 or both α5 integrin and Ang1 were markedly upregulated in the brain of AAV-Itga5, AAV-Ang1, or AAV-Itga5/Ang1 injected mice respectively, as compared to the normal or mock injected controls. Immunofluorescent (IF) staining confirmed that compared with mock injected mice, α5 integrin expression was significantly increased in the AAV-Itga5 and AAV-Itga5/Ang1 injected mice at pre-stroke and both at day 2 and 4 post-ischemia (early stages of CIS) (AAV-Itga5 vs. mock: $P < .05$ at pre-stroke, $P < .01$ at day 2, $P < .001$ at day 4; AAV-Itga5/Ang1 vs. mock: $P < .01$ at pre-stroke, $P < .01$ at day 2, $P < .001$ at day 4) (Fig. 1C-D). Similarly, Ang1 expression was significantly increased in the AAV-Ang1 and AAV-Itga5/Ang1 injected mice in the ischemic hemisphere at pre-stroke and both at day 2 and 4 post-ischemia (AAV-Ang1 vs. mock: $P < .05$ at pre-stroke, $P < .05$ at day 2, $P < .01$ at day 4; AAV-Itga5/Ang1 vs. mock: $P < .05$ at pre-stroke, $P < .05$ at day 2, $P < .05$ at day 4) (Fig. 1C,E).

To determine which cell type contributed to the overexpression of

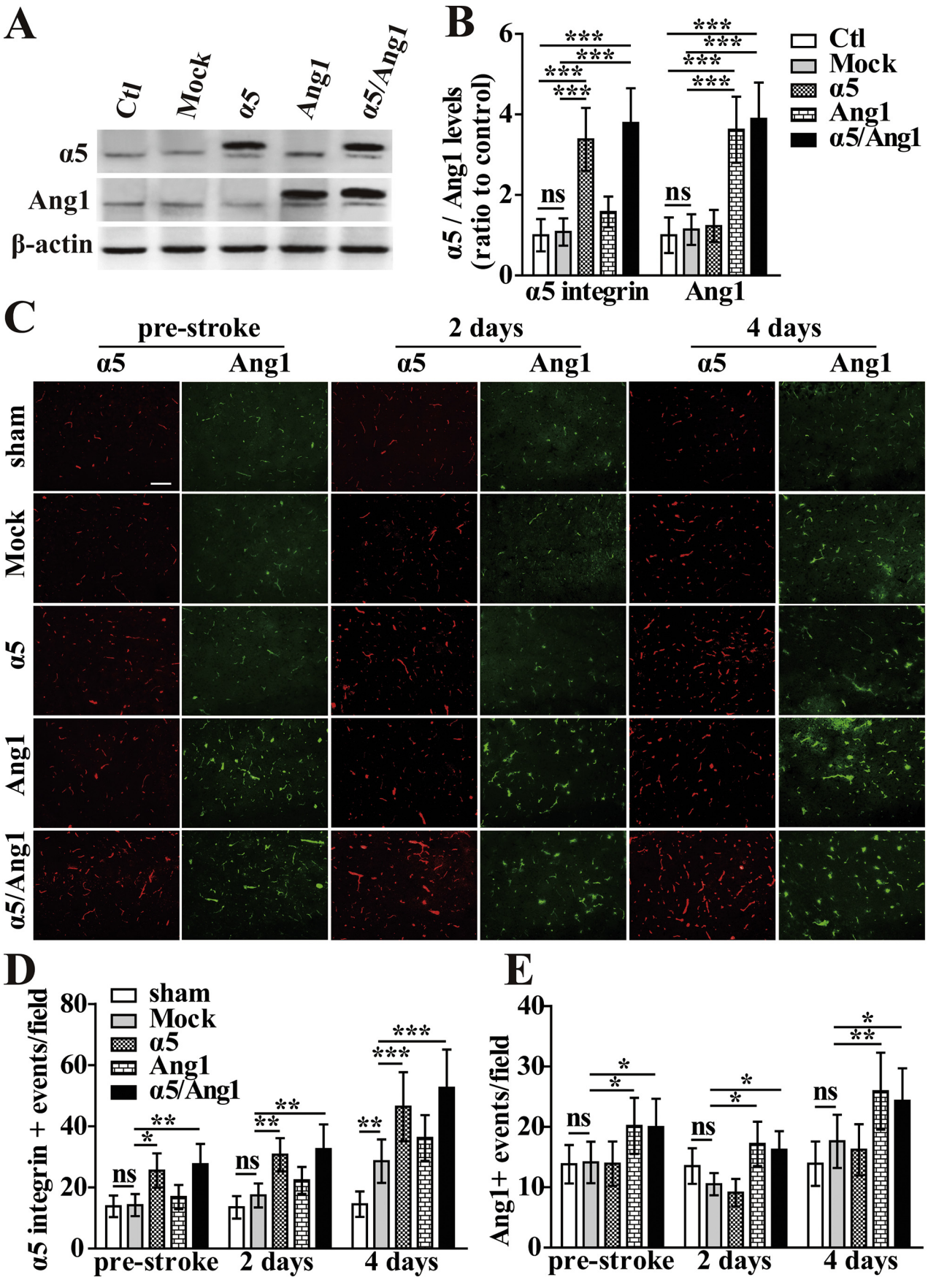

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Fig. 1. Upregulation of α5 integrin and Ang1 in the brain in adeno-associated viral vector (AAV)-α5 /Ang1 injected mice.
A. Confirmation of upregulation of both α5 integrin and Ang1 in brains of AAV-Itga5/Ang1 injected mice after 3 weeks transduction. Expression levels of α5 or Ang1 protein from brains of normal control (Ctl), AAV-mock injected (mock), AAV-Itga5 injected (α5), AAV-Ang1 injected (Ang1), and AAV-Itga5/Ang1 injected (α5/Ang1) mice were evaluated by western blotting. B. Quantitative analyses of Western blots for α5 integrin and Ang1. Data represent mean ± standard deviation and were analyzed by one-way ANOVA ($n = 4$ per experimental group). Note that relative to Ctl mice, the level of α5 integrin was significantly increased in the brains of the AAV-Itga5 and AAV-Itga5/Ang1 injected mice. Similarly, the level of Ang1 was markedly induced in the brains of AAV-Ang1 and AAV-Itga5/Ang1 injected mice. $^{***}P < .001$. C. Frozen sections of ischemic hemisphere taken from sham operated mice (sham), or mock, α5 integrin, Ang1 and α5/Ang1 mice at pre-stroke and day 2 or 4 post-ischemia were labeled with α5 integrin or Ang1 antibody. Scale bar = 100 μm. D and E. Quantification of α5 integrin (D) and Ang1 (E) expression. Results are expressed as the mean ± standard deviation of the number of positive events per field of view and analyzed by two-way ANOVA ($n = 6$ mice per experimental group). Note that compared with mock injected mice, α5 integrin expression was significantly increased in the AAV-Itga5 and AAV-Itga5/Ang1 injected mice at pre-stroke and day 2 and 4 post-ischemia. Similarly, Ang1 expression was markedly induced in the AAV-Ang1 and AAV-Itga5/Ang1 injected mice in the ischemic hemisphere at pre-stroke and day 2 and 4 post-ischemia. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

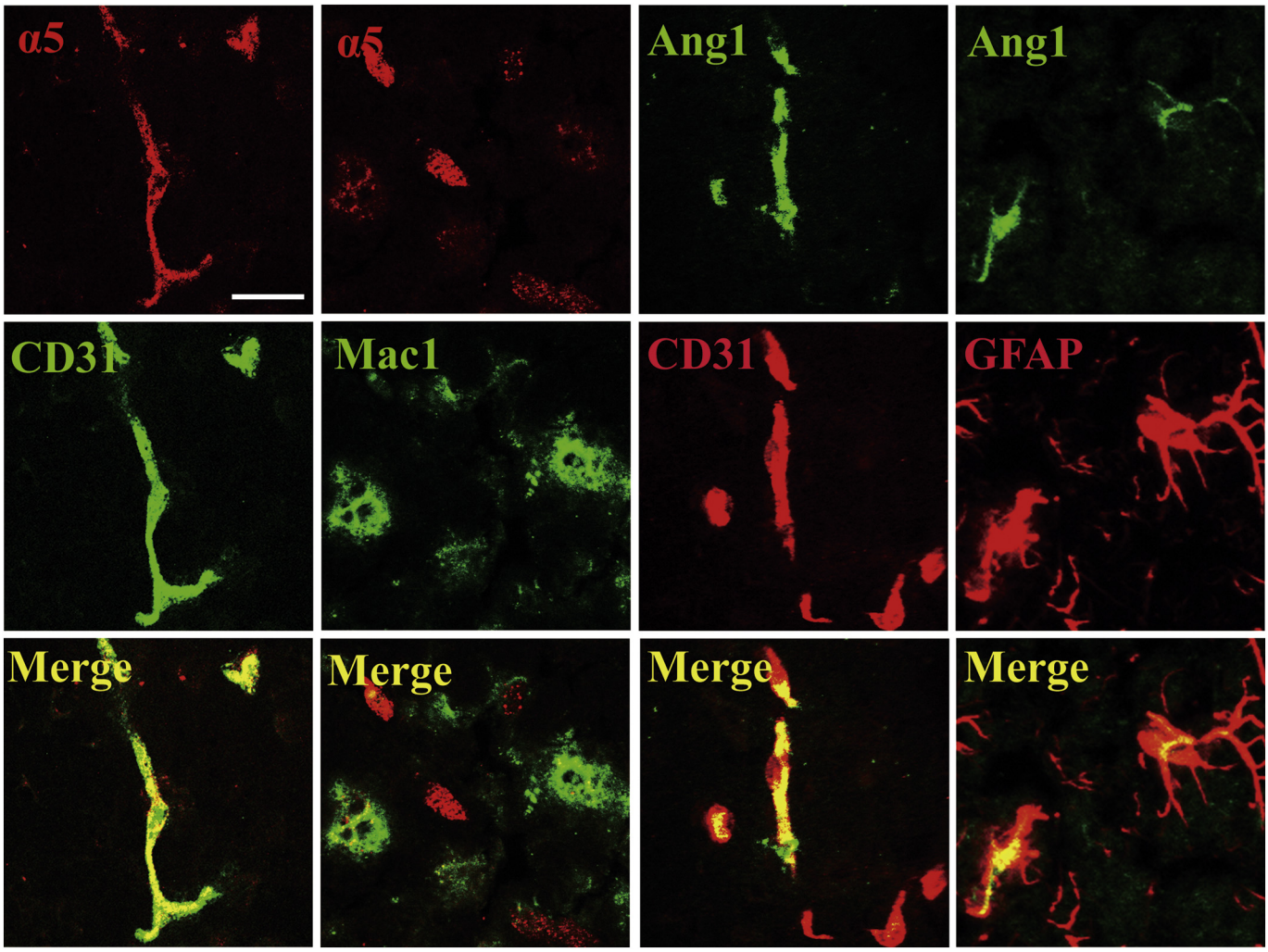

Fig. 2. Cellular localization of α5 integrin/Ang1 expression after focal cerebral ischemia.
Dual-IF was performed on frozen sections of ischemic hemisphere taken from AAV-Itga5/Ang1 injected (α5/Ang1) mice at day 4 post-ischemic reperfusion, using antibodies specific for α5 integrin (Cy-3), Ang1 (AlexaFluor-488), microglial marker Mac-1 (AlexaFluor-488), astrocyte marker GFAP (Cy-3), or the endothelial-specific marker CD31 (AlexaFluor-488). Scale bar = 25 μm. Note that α5 integrin expression co-localized with CD31-positive vessels and Mac-1 positive microglia/inflammatory macrophages in the ischemic hemisphere. Meanwhile, Ang1 co-localized with CD31-positive vessels and GFAP positive astrocytes in the ischemic hemisphere.

α5 integrin or Ang1 following cerebral ischemic stroke, dual-IF was performed on frozen sections of ischemic hemisphere taken from AAV-Itga5/Ang1 injected (α5/Ang1) mice at day 4 post-ischemic reperfusion, using antibodies specific for α5 integrin (Cy-3), Ang1 (AlexaFluor-488), microglial marker Mac-1 (AlexaFluor-488), astrocyte marker GFAP (Cy-3), or the endothelial-specific marker CD31 (AlexaFluor-488). As shown in Fig. 2, α5 integrin co-localized with CD31-positive vessels and Mac-1 positive microglia/inflammatory macrophages in the ischemic hemisphere. Meanwhile, Ang1 co-localized with CD31-positive vessels and GFAP positive astrocytes in the ischemic hemisphere.

3.2. Co-overexpression of α5 integrin and Ang1 reduces infarction and improves neurological function

Dr. Liu and his colleagues found that TTC staining can be accurately performed for measuring infarct damage as late as 7 d after stroke (Liu et al., 2009). To investigate how co-overexpression of α5 integrin and

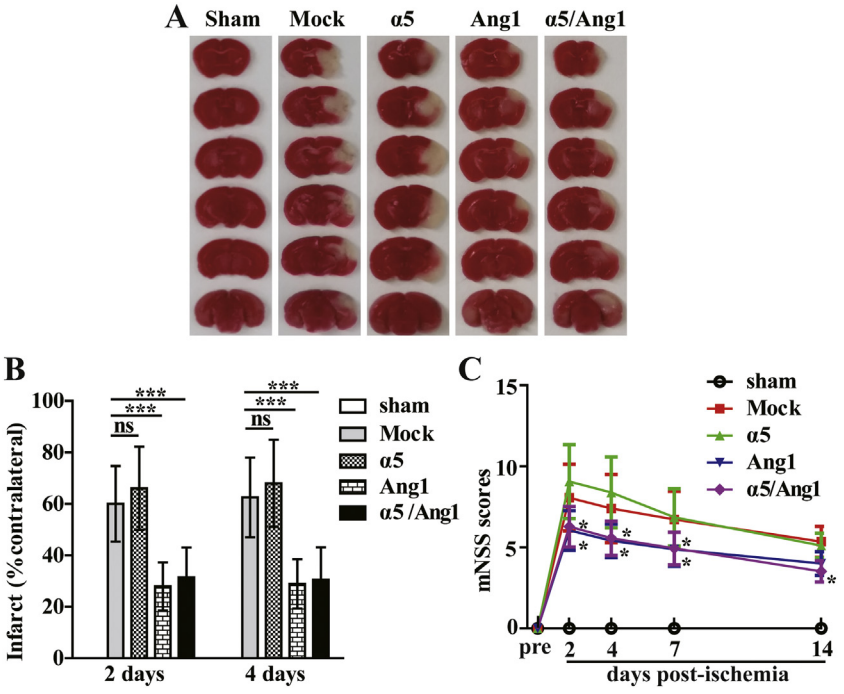

Fig. 3. Impact of co-overexpression of α5 integrin and Ang1 on the infarct volume and the neurological function after cerebral ischemic stroke.
A. Representative images of TTC staining from brain sections of sham operated mice or AAV-mock injected (mock), AAV-Itga5 injected (α5), AAV-Ang1 injected (Ang1), and AAV-Itga5/Ang1 injected (α5/Ang1) mice at day 2 post-ischemic reperfusion. C. Quantification of infarct volume after 2 or 4 days post-ischemic reperfusion. Results are expressed as the mean ± standard deviation and analyzed by two-way ANOVA ($n = 6$ mice per experimental group). Note that no infarct was seen in the control (sham) brain. Compared to the mock controls, mice receiving IV injection of α5 integrin showed slightly larger infarct volumes; however, mice injected with Ang1 alone or co-injected with α5 and Ang1 displayed significantly smaller infarct volumes 2 and 4 days post-ischemic reperfusion. ***$P < .001$. C. The neurological functional performance was assessed at pre-stroke and 2, 4, 7, 14 days after MCAO using modified Neurological Severity Score (mNSS) evaluation (n = 6 mice per experimental group). Note that mice injected with Ang1 alone or co-injected with α5 integrin and Ang1 had better functional recovery than did mock controls both at 2, 4 and 7 days post-ischemic reperfusion. In addition, co-overexpression of Ang-1 and α5 integrin in combination further improved functional recovery compared with the mock controls at day 14 post-ischemia. *$P < .05$ vs the mock injected mice.

Ang1 impacts the infarct volume following ischemic stroke, brain sections of sham operated mice or mock, α5 integrin, Ang1, and α5/Ang1 injected mice at day 2 or 4 post-ischemic reperfusion were stained with 2% TTC. This showed that no infarct was seen in the control (sham) brain. Compared to the mock injected mice, mice receiving IV injection of α5 integrin showed marginal though non-significant larger infarct volumes. However, mice injected with Ang1 alone or co-injected with α5 integrin and Ang1 exhibited significantly reduced infarct volumes both at day 2 and 4 reperfusion (AAV-Ang1 vs. mock: $P < .001$ at day 2 and day 4; AAV-Itga5/Ang1 vs. mock: $P < .001$ at day 2 and day 4) (Fig. 3A-B). Based on mNSS functional assessment, mice injected with α5 integrin didn't, but Ang1 alone exhibited lower mNSS scores (indicating a better functional performance) than did mock controls at early stages of stroke (≤7 days post-ischemia) (AAV-Ang1 vs. mock: $P < .05$ at day 2, 4, and 7); Furthermore, co-overexpression of Ang-1 and α5 integrin in combination significantly improved the neurological function from day 2 to day 14 post-ischemia compared with the mock injected group (AAV-Itga5/Ang1 vs. mock: $P < .05$ at day 2, 4, 7 and 14) (Fig. 3C).

3.3. Co-overexpression of α5 integrin and Ang1 promotes blood-brain barrier integrity following cerebral ischemic stroke

In a previous study we demonstrated that Evans Blue (EB) extravasation increases at day 1 post-ischemia, peaks at day 2, then decreases by day 7. We also demonstrated a tight temporal correlation between the angiogenic markers α5 integrin and Ang1 and the tight junction proteins (TJPs), suggesting a potential role for Ang1 and α5β1 integrin in promoting BBB integrity following ischemic stroke (Sun et al., 2017). In the current study we first wanted to determine how co-overexpression of α5 integrin and Ang1 impacts EB extravasation into the brain following cerebral ischemic stroke. To study this process, C57BL/6 mice were IV injected with AAV-mock, AAV-Itga5, AAV-Ang1, or AAV-Itga5/Ang1. Three weeks later the mice were subjected to 90 min MCAO and injected with EB via tail vein 2 h prior to sacrifice at 2 or 4 days reperfusion. As shown in Fig. 4A-B, no EB extravasation was detected in the control (sham) brain. IV injection of α5 integrin increased (AAV-Itga5 vs. mock: $P < .05$ at day 2 and day 4), while injection of Ang1 alone or co-injection with α5 integrin and Ang1

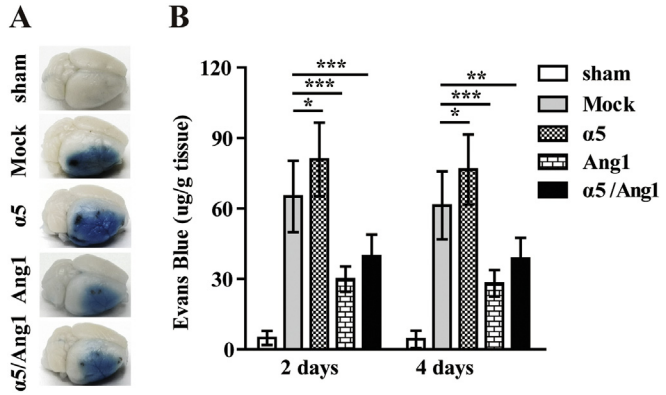

Fig. 4. Attenuated BBB permeability in AAV-Itga5/Ang1 injected mice during the early stages of ischemic stroke.
Mice were IV injected with AAV-mock (mock), AAV-Itga5 (α5), AAV-Ang1(Ang1), or AAV-Itga5/Ang1 (α5/Ang1). Three weeks later mice were subjected to 90 min MCAO, and further injected with Evans Blue (EB) 2 h prior to sacrifice at 2 or 4 days reperfusion. A. Representative images of EB leak into the ischemic brain from sham operated mice or mock, α5, Ang1 and α5/Ang1mice 2 days after ischemic reperfusion. B. Quantification of EB extravasation into the ischemic hemisphere. Results are expressed as the mean ± standard deviation and analyzed by two-way ANOVA ($n = 6$ mice per experimental group). Note that no EB extravasation was detected in the control (sham) brain. IV injection of α5 integrin increased, whereas injection of Ang1 alone or co-injection of α5 integrin and Ang1 significantly reduced EB extravasation into the ischemic hemisphere relative to mock injected controls both at 2 and 4 days post-ischemic reperfusion. *$P < .05$, **$P < .01$, ***$P < .001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly reduced EB extravasation into the ischemic hemisphere relative to the mock injection group both at day 2 and 4 post-ischemic reperfusion (AAV-Ang1 vs. mock: $P < .001$ at day 2, $P < .001$ at day 4; AAV-Itga5/Ang1 vs. mock: $P < .001$ at day 2, $P < .01$ at day 4).

We next examined how co-overexpression of α5 integrin and Ang1 altered endothelial expression of tight junction proteins (TJPs) after ischemic stroke by performing IF analysis of vascular expression of the TJPs ZO-1 and claudin-5 in the ischemic hemisphere of sham operated

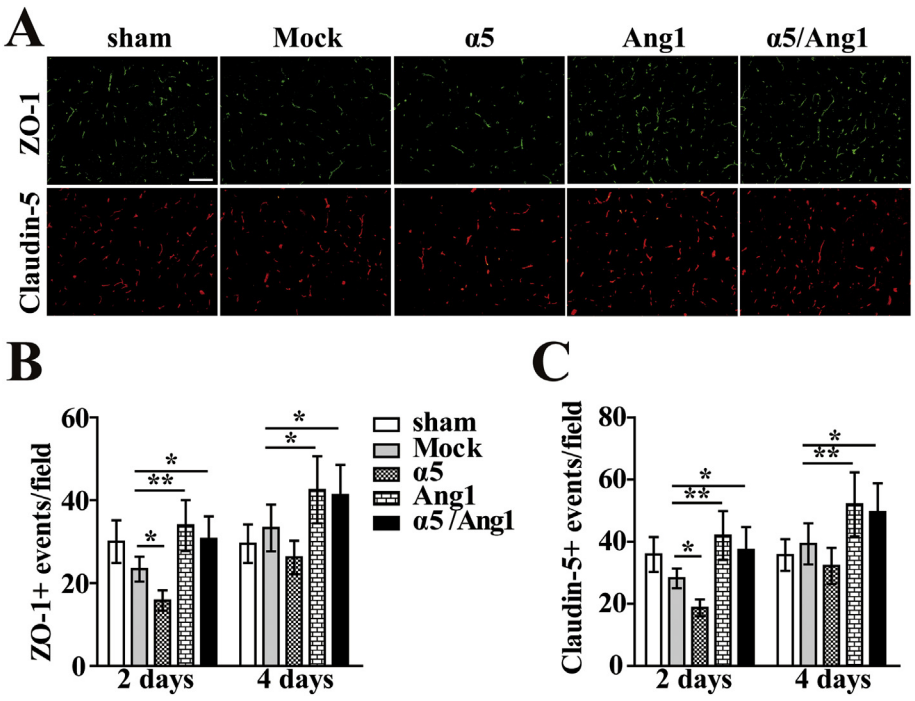

Fig. 5. Increased expression of tight junction proteins in AAV-Itga5/Ang1 injected mice during the early stages of ischemic stroke.
Frozen sections of ischemic hemisphere taken from sham operated mice (sham), or AAV-mock injected (mock), AAV-Itga5 injected (α5), AAV-Ang1 injected (Ang1), and AAV-Itga5/Ang1 injected (α5/Ang1) mice 2 or 4 days after post-ischemic reperfusion were examined for ZO-1 and claudin-5 expression. A. Representative images of IF staining for ZO-1 and claudin-5 at day 2 post-ischemic reperfusion. Scale bar = 100 μm. B and C. Quantification of ZO-1 (B) or claudin-5 (C) expression. Results are expressed as the mean ± standard deviation of the number of positive events per field of view and analyzed by two-way ANOVA (n = 6 mice per experimental group). Note that in the ischemic hemisphere, injection of α5 integrin reduced, but Ang1 alone or co-injection of α5 integrin and Ang1 significantly increased tight junction protein expression relative to the mock injection group both at 2 and 4 days post-ischemic reperfusion. *$P < .05$, **$P < .01$.

mice, or AAV-pHB-mock, AAV-Itga5 integrin, AAV-pHB-Ang1, and AAV-Itga5 integrin/Ang1 injected mice. This revealed that after 2 days reperfusion, compared to mock-infected control mice, levels of ZO-1 and claudin-5 were actually decreased in α5 integrin-infected mice, but interestingly, mice co-injected with α5 integrin and Ang1 showed significantly increased levels of the ZO-1 and claudin-5 both at the 2 and 4 day post-ischemic perfusion time-points (AAV-Itga5 vs. mock: $P < .05$ at day 2 for ZO-1 and claudin-5; AAV-Ang1 vs. mock: $P < .01$ at day 2 for ZO-1 and claudin-5, $P < .05$ at day 4 for ZO-1, $P < .01$ at day 4 for claudin-5; AAV-Itga5/Ang1vs. mock: $P < .05$ at day 2, $P < .05$ at day 4 for ZO-1 and claudin-5) (Fig. 5).

In parallel studies we also examined this regulation in vitro by transfecting brain endothelial cells (BECs) with α5 integrin, Ang1 and α5/Ang1 plasmids and then measured endothelial levels of claudin-5 and ZO-1 by Western blotting after 4 h oxygen glucose deprivation (OGD) followed by 12 h restoration (OGD/R). As shown in Fig. 6, α5 integrin-transfected BECs showed markedly reduced levels of TJs (α5-transfected group vs. mock: $P < .05$ for claudin-5 and ZO-1), but Ang1-transfected or α5/Ang1-co-transfected BECs displayed significantly higher levels of TJPs than mock-transfected BECs under OGD/R conditions (Ang1-transfected group vs. mock: $P < .001$ for claudin-5 and ZO-1; α5/Ang1-transfected group vs. mock: $P < .01$ for claudin-5, $P < .001$ for ZO-1).

3.4. Co-overexpression of α5 integrin and Ang1 promotes angiogenesis and vessel density following cerebral ischemic stroke

Because both Ang1 (Augustin et al., 2009) and integrin α5β1 (Wang et al., 2011) are key regulators of the angiogenic process, we next examined how co-overexpression of α5 integrin and Ang1 regulates post-stroke angiogenesis. CD31/Ki67 dual-IF on frozen sections of ischemic hemisphere taken from AAV-pHB injected mice revealed that injection of α5 integrin alone or co-injection with α5 integrin and Ang1 significantly increased the number of dual-positive CD31 + /Ki67 + cells in the ischemic penumbra relative to the mock injection group after 4 days reperfusion (AAV-Itga5 vs. mock: $P < .05$ at day 4; AAV-Itga5/Ang1 vs. mock: $P < .001$ at day 4). Of note, mice co-injected with α5 integrin and Ang1 showed significantly more numbers of CD31/Ki67 dual-positive cells in the ischemic penumbra than that of mice either injected with α5 integrin or Ang1 alone, suggesting a synergistic effect (AAV-Itga5/Ang1 vs. AAV-Itga5: $P < .05$ at day 4; AAV-Itga5/Ang1 vs. AAV-Ang1: $P < .001$ at day 4) (Fig. 7A and C). Consistent with this enhanced angiogenic response during the early stage of ischemic stroke, mice injected with α5 integrin, Ang1 alone or co-injected with α5 and Ang1 all showed increased blood vessel density in the ischemic penumbra relative to the mock injection group after 7 and 14 days reperfusion (AAV-Itga5 vs. mock: $P < .05$; AAV-Itga5/Ang1 vs. mock: $P < .01$ at day 7; AAV-Itga5 vs. mock: $P < .01$; AAV-Ang1 vs. mock: $P < .05$, AAV-Itga5/Ang1 vs. mock: $P < .001$ at day 14). Importantly, mice co-injected with α5 integrin and Ang1 displayed significantly more blood vessels in the ischemic penumbra than that of mice either injected with α5 integrin or Ang1 alone (AAV-Itga5/Ang1 vs. AAV-Itga5: $P < .05$; AAV-Itga5/Ang1 vs. AAV-Ang1: $P < .01$ at day 14) (Fig. 7B and E).

Our previous study showed that although no proliferating endothelial cells were detected in the contralateral hemisphere following 14 days ischemic reperfusion, vessel density gradually increased in this region and the blood vessel density in the contralateral hemisphere at day14 was significantly elevated over that of controls (Huang et al., 2015). In the current study, we got the similar results as before, and further found that combination treatment with α5 integrin and Ang1 significantly increased the vessel density in the contralateral (Contral) relative to the mock injected group at day 14 post-ischemia ($P < .01$) (Fig. 7B and D), suggesting co-overexpression of α5 integrin and Ang1 induces angiogenesis in the contralateral hemisphere.

Parallel in vitro studies using EdU incorporation revealed that brain endothelial cells (BECs) transfected with α5 integrin alone or co-transfected with α5 integrin and Ang1 showed increased proliferation relative to mock-transfected cells at 12 h of restoration after OGD (α5 integrin-transfected group vs. mock: $P < .05$; α5/Ang1-transfected group vs. mock: $P < .001$) (Fig. 8A-B). Collectively, these data suggest that α5β1 integrin and Ang1 synergistically promote cerebral angiogenesis post-stroke.

4. Discussion

In the current study, we examined how overexpression of α5 integrin and Ang-1 influences the severity and recovery following

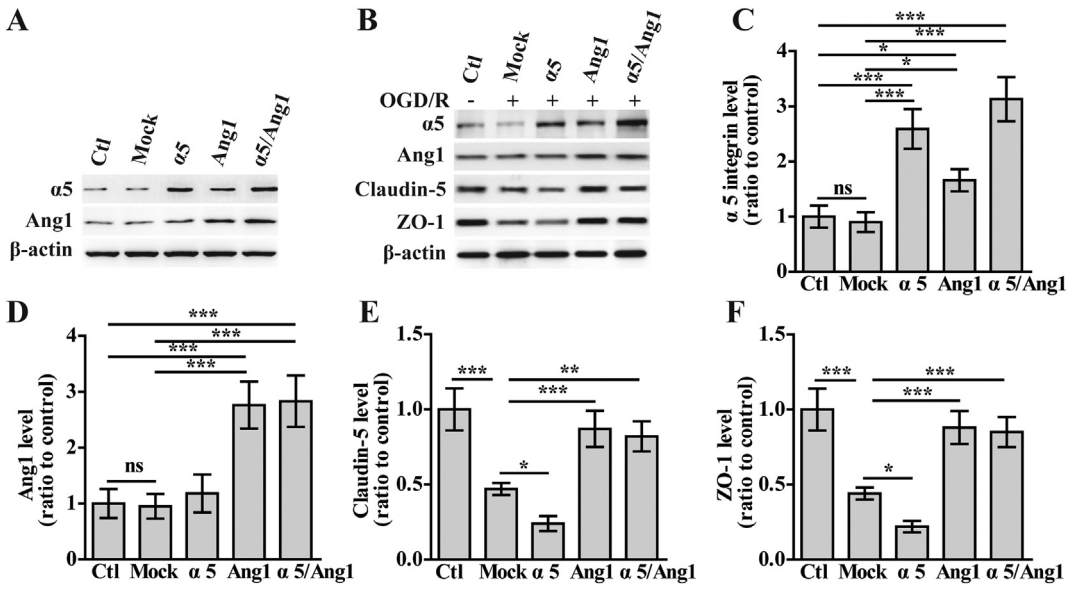

Fig. 6. The influence of co-overexpression of α5 integrin and Ang1 on tight junction protein expression in BECs under OGD/R conditions.
A. Brain endothelial cells (BECs) were transfected with control plasmid (mock) or α5, Ang1 and α5/Ang1 overexpression plasmid for 48 h. Levels of α5 integrin or Ang1 protein were measured in normal control (Ctl), mock, α5, Ang1 and α5/Ang1 treated cells by western blotting. BECs were transfected with mock or α5 integrin, Ang1 and α5 integrin/Ang1 overexpression plasmid for 48 h, then subject to either 4 h of OGD followed by 12 h of restoration or NO-OGD/R at 37 °C. B. Cell lysate protein levels were analyzed by western blotting using antibodies specific to α5 integrin, Ang1, ZO-1 and claudin-5. Densitometric analysis shows the relative amount of α5 integrin (C), Ang1 (D), claudin-5 (E) and ZO-1 (F) relative to β-actin. NO-OGD/R mock treated cells served as control (Ctl). Data represent mean ± standard deviation and were analyzed by one-way ANOVA ($n = 4$ per experimental group). Note that transfection with α5 integrin reduced, but transfection with Ang1 alone or co-transfection with α5 integrin and Ang1 significantly increased claudin-5 and ZO-1 expression in BECs relative to the mock transfected group under OGD/R conditions. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$; ns, not significant.

cerebral ischemic stroke (CIS). Our main findings were as follows: (i) although overexpression of α5 integrin alone stimulated angiogenesis compared to mock-transfected controls, it didn't improve the outcomes in neurological score and size of infarct and it caused worse BBB breakdown in the ischemic hemisphere, (ii) co-overexpression of α5 integrin with Ang1 led to smaller infarcts and improved neurological deficits relative to sham-infected mice, which at the molecular level was underpinned by reduced BBB breakdown and increased expression of endothelial tight junction proteins in the ischemic penumbra, (iii) co-overexpression of α5 integrin and Ang1 synergistically promoted BEC proliferation during the early stage of ischemic stroke, resulting in increased blood vessel density at later stages, and (iv) positive effects of α5 integrin and Ang1 co-overexpression on endothelial proliferation and tight junction protein expression were also confirmed in vitro. Taken together, these data indicate that co-overexpression of Ang-1 and α5 integrin in combination confers a synergistic vascular protection against cerebral ischemic injury without the negative side effects on BBB permeability, suggesting a novel approach for the treatment of CIS.

4.1. The influence of α5β1 integrin and Ang1 on structural integrity and vascular permeability following ischemic stroke

As the major components of the BBB, BECs play an important role in BBB function via the development of a highly selective barrier (Pan et al., 2016). Mounting evidence has shown that ischemia-induced BEC injury or death increases vascular permeability and BBB disruption (Sandoval and Witt, 2008; Yin et al., 2010), leading to the hemorrhagic transformation and edema formation in the brain. Thus, protecting the BECs against injury is a key step for the maintenance of the BBB integrity after CIS.

Angiogenesis is an endogenous protective response following CIS and a strong angiogenic response has been shown to correlate well with functional recovery. However, during the early stages of angiogenesis, degradation of the ECM and separation of neighboring endothelial cells are crucial initiating steps, which suggests that newly formed vessels may transiently increase vascular permeability (Vallon et al., 2014), which could result in aggravation of BBB damage caused by CIS. As described previously, α5β1 integrin has been identified as a potentially important mediator of beneficial angiogenic effects in treating experimental ischemic stroke (Lee et al., 2011). Interestingly, a recent study showed that α5-EC-KO mice develop accelerated vascular disruption and increased leukocyte infiltration into the spinal cord during the early stages of experimental autoimmune encephalomyelitis (EAE), suggesting that α5β1 integrin-mediated angiogenic remodeling represents an important repair mechanism that counteracts vascular disruption at an early stage of EAE development (Kant et al., 2019). However, in contrast to these findings, endothelial α5β1 integrin appears to play a potentially harmful role in ischemic stroke, as evidenced by reduced levels of BBB disruption in α5-EC-KO mice model following cerebral ischemia (Roberts et al., 2017). Consistent with this report, in the current study, we found that overexpression of α5 integrin alone increased EB extravasation into the ischemic hemisphere, implying that upregulation of α5β1 integrin by itself enhances vascular leakage during the early stages of CIS. This was further supported by the finding that overexpression of α5 integrin alone reduced endothelial expression of TJPs (claudin-5 and ZO-1) both in vivo and in vitro.

Our previous studies showed that Ang1 and α5β1 integrin are both upregulated in cerebral vessels after CIS (Huang et al., 2015; Li et al., 2012b; Sun et al., 2017). Moreover, extensive co-localization of Ang1 with α5 integrin was displayed on angiogenic blood vessels in the ischemic penumbra (Sun et al., 2017). Interestingly, vascular expression of TJPs followed the same time course as the expression of α5β1 and Ang1, suggesting a close correlation between α5 integrin and Ang1 in regulating BBB integrity after CIS. As an anti-permeability factor, Ang-1 inhibits leakage of cerebral vessels after focal cerebral embolic ischemia in mice analyzed 24 h after MCAO (Zhang et al., 2002). Others have shown that the Ang-1 anti-permeability effect is associated with upregulated expression of ZO-1 and occludin (Valable et al., 2005). We report here that overexpression of Ang1 alone or co-overexpression of α5 integrin and Ang1 significantly reduced BBB breakdown as well as

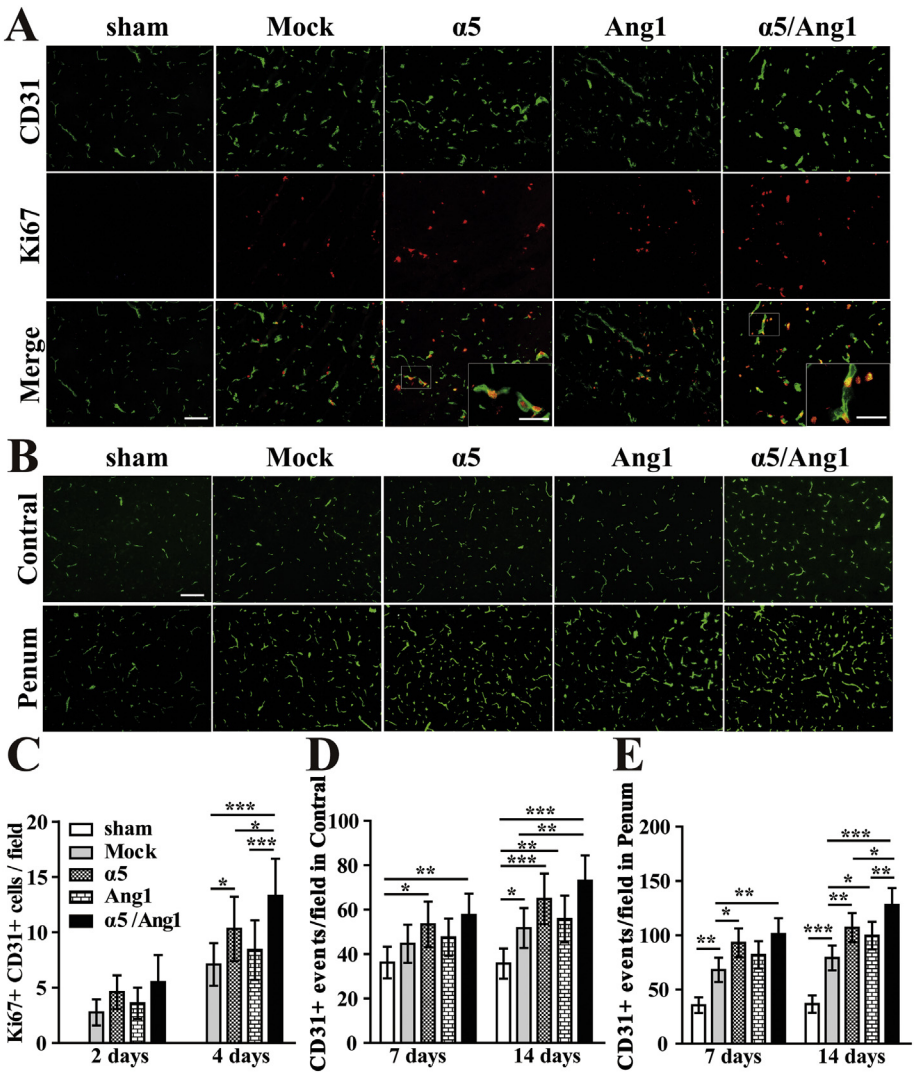

Fig. 7. Accelerated angiogenesis and increased vessel density in the cerebral ischemic penumbra in AAV-Itga5/Ang1 injected mice.

A. Frozen sections of ischemic hemisphere taken from sham operated mice (sham) or AAV-mock injected (mock), AAV-Itga5 injected (α5), AAV-Ang1 injected (Ang1), and AAV-Itga5/Ang1 injected (α5/Ang1) mice 4 days after post-ischemic reperfusion were dual-stained for the endothelial-specific marker CD31 (AlexaFluor-488) and the proliferation marker Ki67 (Cy-3). Scale bar = 100 μm (inserts = 40 μm). B. Frozen sections of ischemic hemisphere or contralateral hemisphere taken from sham operated mice (sham), or mock, α5 integrin, Ang1 and α5 integrin/Ang1 mice after 14 days post-ischemic reperfusion were stained for CD31 (AlexaFluor-488). Scale bar = 100 μm. C. Quantification of CD31/Ki67 dual-positive proliferating BECs. Results are expressed as the mean ± standard deviation of the number of CD31/Ki67 dual-positive cells per field of view (n = 6 per experimental group). Note that injection with α5 integrin alone or co-injection with α5 integrin and Ang1 significantly increased the number of proliferating endothelial cells relative to the mock injected group at day 4 post-ischemic reperfusion. Moreover, mice co-injected with α5 integrin and Ang1 showed significantly more proliferating endothelial cells per field of view than mice injected with α5 integrin or Ang1 alone. $^{*}P < .05$, $^{***}P < .001$. D&E. Quantification of vessel density. Results are expressed as the mean ± standard deviation of the number of CD31+ vessels per field of view (n = 4 per experimental group). Note that after 7 or 14 days reperfusion, injection with α5 integrin, Ang1 alone or combination treatment with α5 integrin and Ang1 all increased vessel density in the ischemic penumbra (Penum) relative to the mock injected group. Of note, mice co-injected with α5 integrin and Ang1 displayed higher vessel density than mice injected with α5 or Ang1 alone. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$; In addition, combination treatment with α5 integrin and Ang1 also significantly increased the vessel density in the contralateral (Contral) relative to the mock injected group. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

increased the expression of endothelial TJPs ZO-1 and claudin-5. This evidence suggests that Ang-1 counteracts the α5β1 integrin-induced permeability by increasing TJP expression on blood vessels during the early stage of CIS.

As previously reported, along with the new vessels, pericytes expressed ZO-1, astrocytes expressed ZO-1 and occludin, while endothelial cells expressed claudin-5 in the ischemic rat brain (Yang et al., 2013). As overexpression of Ang1 or α5 integrin/Ang1 therapy induced angiogenesis after CIS, the increase in ZO-1/claudin-5 on blood vessels with Ang1 or α5 integrin/Ang1 therapy is at least partially due to the increased number of vessels in the penumbra.

4.2. Co-overexpression of α5 integrin and Ang1 reduces ischemic injury and neurological deficit during the early stage of ischemic stroke

Following cerebral ischemia, α5-EC-KO mice showed much smaller infarcts and this correlated closely with reduced levels of BBB disruption in this stroke model (Roberts et al., 2017). Thus, the further increased BBB leakage induced by up-regulated expression of α5β1 integrin we observed in the current study may contribute to the continuously evolving infarct. Unexpectedly, we found that overexpression of α5 only slightly (but not significantly) increased infarct volume, and the infarct volume didn't increase proportionately with the increase of permeability, suggesting that cerebral edema caused by increased vascular permeability doesn't completely transform into cerebral infarction. Of note, these findings are at odds with the recent findings of Wu et al. (Wu et al., 2018) who showed that intraperitoneal injection of the synthetic α5β1 integrin ligand Pro-His-Ser-Arg-Asn (PHSRN) peptide reduces the infarct volume, and improves neurological function in MCAO rats by promoting vascular endothelial growth factor secretion through activation of integrin α5β1 and its downstream intracellular signaling pathways focal adhesion kinase, Ras, cRaf, and extracellular-signal-regulated kinase. The discrepancy between our two sets of results may be related to the different experimental conditions, such as the severity of ischemia employed, animal species of stroke model used, and the timing or stages of ischemic stroke observed.

In addition to promoting vascular stabilization (Suri et al., 1998) and preventing plasma leakage into the ischemic brain, transgenic overexpression of Ang1 has also been shown to decrease ischemic lesion volume (Thurston et al., 2000; Zhang et al., 2002). Although overexpression of α5 integrin alone is not sufficient to protect against ischemic injuries, our studies make the important point that co-overexpression of Ang1 with α5 integrin in the brain successfully overcomes the limitation of solely overexpressing α5 integrin, leading to

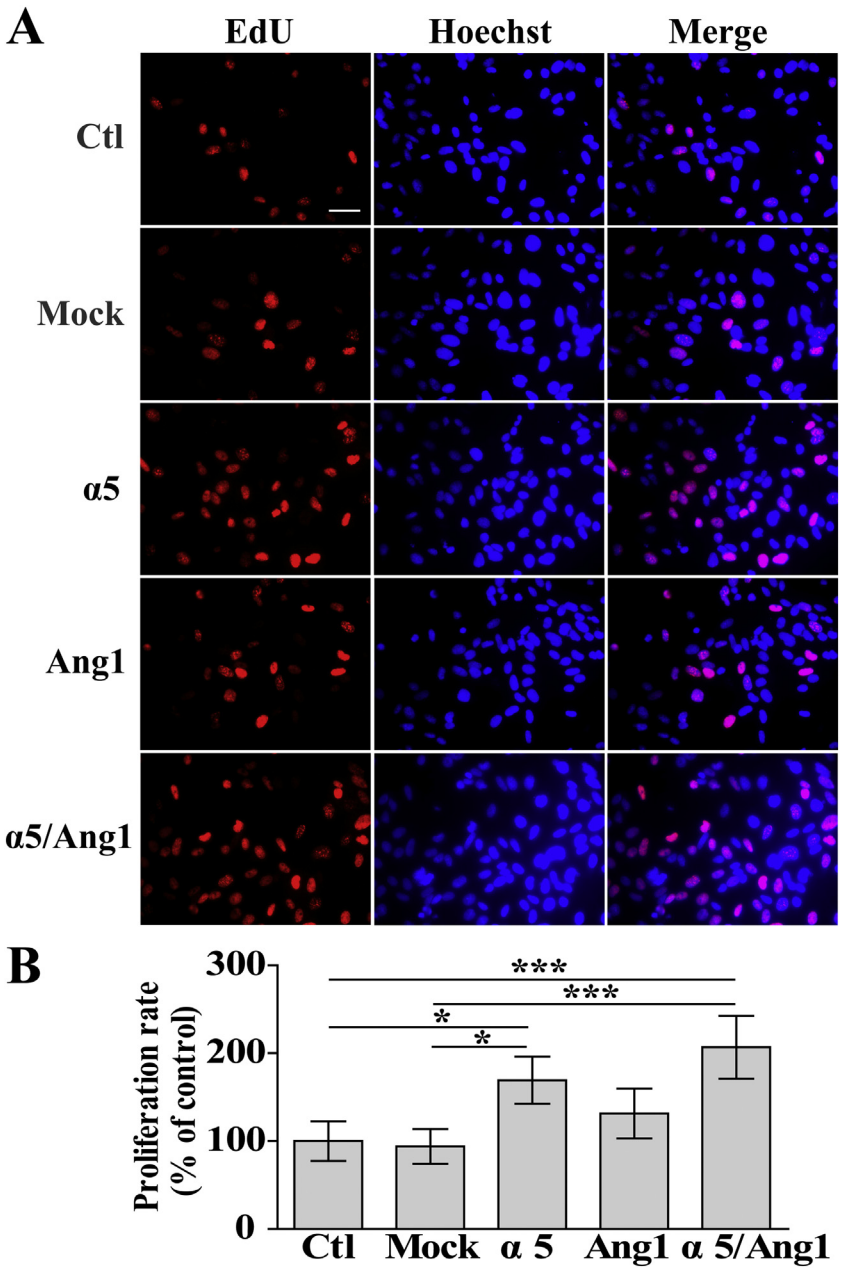

Fig. 8. The influence of co-overexpression of α5 integrin and Ang1 on brain endothelial cell (BEC) proliferation under OGD/R conditions.
A. BECs were transfected with mock or α5 integrin, Ang1 or α5 integrin/Ang1 overexpression plasmids for 48 h, then subject to either 4 h of OGD followed by 12 h of restoration or NO-OGD/R at 37 °C before cell proliferation rate was examined by EdU incorporation. Scale bar = 50 μm. B. Quantification of BEC proliferation. Proliferation was expressed as the mean ± standard deviation percentage of control levels (n = 4 per experimental group). Note that transfection with α5 integrin alone or co-transfection with α5 integrin and Ang1 significantly promoted BEC proliferation relative to mock transfected controls. $^{*}P < .05$, $^{***}P < .001$.

reductions in the size of infarct and neurological deficit at an early stage of CIS. One possible mechanism is that addition of Ang1 could counteract the vessel leakage and edema caused by α5 integrin and decrease the number of BBB phasic opening events, therefore decreasing the potential for secondary injury due to the massive infiltration of peripheral proinflammatory leukocytes, including T cells, B cells and neutrophils, and the release of proinflammatory mediators (Sandoval and Witt, 2008).

4.3. Co-overexpression of α5 integrin and Ang1 synergistically promotes vascular remodeling

In addition to a role in decreasing vascular permeability, Ang1 has been thought to promote endothelial cell/endothelial progenitor cell migration and sprouting by activating Tie2 signaling pathways and protect endothelial cells from apoptosis by phosphorylating the downstream effectors focal adhesion kinase (FAK) and Akt, a serine/threonine-specific protein kinase. (DeBusk et al., 2004; Kim et al., 2000; Moon et al., 2015), which support the concept that Ang1 contributes to the angiogenesis. In the current study, our in vivo and in vitro experiments both showed that although overexpression of Ang1 alone didn't significantly increased BECs proliferation relative to mock-treated group during the early stages of CIS, it induced remarkably increased vessel density relative to the mock injected group in the later stage, indicating that overexpression of Ang1 alone induces significant angiogenesis. Our previous studies and the work of others (Lee et al., 2011) showed that upregulation of α5β1 integrin plays an important role in promoting BEC proliferation following global hypoxia (Milner et al., 2008; Li et al., 2012b). In recent work we have extended this to show that α5β1 integrin regulates Ang1-induced endothelial cell migration and neovessel formation following cerebral ischemia (Pang et al., 2018). In the current study, we found that co-overexpression of α5 integrin and Ang1 co-operatively promoted BEC proliferation in the penumbra after 4 days post-ischemic reperfusion and in vitro after 12 h of restoration after OGD. This enhanced angiogenic response during the early stage of ischemic stroke resulted in increased blood vessel density

in the ischemic penumbra at later time-points (after 7 and 14 days reperfusion). Together, these results indicate that co-overexpression of α5 integrin and Ang1 synergistically promotes angiogenesis during the early stage of CIS, resulting in increased vascularization at the later stage. The angiogenic remodeling may enhance cerebral perfusion (Chen et al., 2014), and supply the oxygen and energy for growth and differentiation of neuronal stem/progenitor cells, thus the increased angiogenesis in the penumbra will lead to improved functional recovery after stroke.

In summary, our results demonstrate that co-overexpression of α5 integrin and Ang1 conferred vascular protection against cerebral ischemic injury. Our results suggest that co-overexpression of α5 integrin and Ang1 significantly reduces BBB leakage, improves the structural integrity of blood vessels, synergistically promotes the angiogenic response during the early stage of ischemic stroke and increases the vascularization in the later stage, leading to reduced infarction and consequently improved neurological deficits. These findings provide a proof of concept for the further translational validation of co-overexpress of α5 integrin and Ang1 for the treatment of cerebral ischemic stroke patients in clinical practice in the future. In the next set of experiments, we will directly test whether there might be a successful way by manipulating α5 integrin/Ang1expression therapeutically after CIS rather than using pre-stroke treatment.

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Authors' contributions

LW and XZ contributed equally to this work. Both of them conducted the experimental research and performed the statistical analysis and initial drafting of the manuscript. XL, GF, YF participated in the experimental research and also assisted in manuscript preparation. RM co-designed the study and helped to draft the manuscript. LL supervised the project and conceived of the study, and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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