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## Angiotensin Converting Enzyme 2 Priming Enhances the Function of Endothelial Progenitor Cells and their Therapeutic Efficacy

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### Abstract

Angiotensin converting enzyme 2 (ACE2) is a lately discovered enzyme catalyzing Angiotensin II into Angiotensin (1-7). Angiotensin II has been reported to impair endothelial progenitor cell (EPC) function and is detrimental to stroke. Here, we studied the role of ACE2 in regulating EPC function *in vitro* and *in vivo*. EPCs were cultured from human renin and angiotensinogen transgenic (R+A+) mice and their controls (R-A-). In *in vitro* experiments, EPCs were transduced with lentivirus-ACE2 (Lenti-ACE2) or Lenti-GFP. The effects of ACE2 over-expression on EPC function and endothelial oxide synthase (eNOS)/NADPH oxidase (Nox) expression were determined. ACE2, eNOS and Nox inhibitors were used for pathway validation. In *in vivo* studies, the therapeutic efficacy of EPCs over-expressing ACE2 was determined at day 7 after ischemic stroke induced by middle cerebral artery occlusion. We found that 1) Lenti-ACE2 transduction resulted in a four-fold increase of ACE2 expression in EPCs. This was accompanied with an increase in eNOS expression and nitric oxide production, and a decrease in Nox2, 4 expression and reactive oxygen species production. 2) ACE2 over-expression improved the abilities of EPC migration and tube formation which were impaired in R+A+ mice. These effects were inhibited by ACE2 or eNOS inhibitor and further enhanced by Nox inhibitor. 3) Transfusion of Lenti-ACE2 primed EPCs reduced cerebral infarct volume and neurologic deficits, increased cerebral microvascular density and angiogenesis. Our data demonstrate that ACE2 improves EPC function via regulating eNOS and Nox pathways and enhances the efficacy of EPC-based therapy for ischemic stroke.

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## Keywords

ACE2; EPCs; ROS; NO; ischemic stroke

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## Introduction

Angiotensin converting enzyme 2 (ACE2) is a newly discovered enzyme of the renin-angiotensin system (RAS), which catalyses the conversion of angiotensin I (Ang I) into Ang (1-9) and Ang II into Ang (1-7) (1). Accumulating evidence demonstrate that ACE2 provides vascular protective effects by counteracting the deleterious effects of Ang II and possesses great potential for developing new avenues to treat vascular diseases (2;3). Endothelial cell (EC) dysfunction/injury is well known as the common pathological basis for vascular diseases. It has been reported that ACE2 alleviates the development of early atherosclerotic lesions by improving EC function (4). The antiatherosclerotic effect of ACE2 involves in down-regulation of the Ang II-activated reactive oxygen species (ROS). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) derived ROS plays a key role in Ang II-induced endothelial damage (5). Oxidative stress induced EC dysfunction is accompanied by the reduction of nitric oxide (NO) bioavailability and plays an important role in cerebrovascular disease (6).

Endothelial progenitor cells (EPCs) which are defined as bone marrow (BM) derived immature cells with the ability to differentiate into mature ECs are suggested to play important roles in vascular homeostasis and angiogenesis (7). Reduced number and impaired function of circulating EPCs are associated with poor cardiovascular outcomes in patients with coronary artery disease (8). It is recently reported that Ang II through type 1 angiotensin (AT1) receptor activation and oxidative stress impairs EPC function *in vitro* and *in vivo* (9). Ang II induces EPC senescence via activation of NADPH oxidase (10). Our previous study shows that the BM derived EPCs are reduced and dysfunctional in human renin and angiotensinogen transgenic (R+A+) mice, and that blockade of Ang II/AT1 signaling with losartan is able to improve these defeats (11). Endothelial nitric oxide synthase (eNOS) down-regulation and decreased NO generation can also induce impairment of EPC function and senescence (12). Although the role of ACE2 in counteracting the effects of Ang II has been well detailed, the role of ACE2 in EPC function and the specific mechanism have not been explored.

In this study, we determined the role of ACE2 in regulating EPC function. We analyzed the eNOS/NO and Nox/ROS pathways in order to explore the underlying mechanisms. To further investigate the possible implication of ACE2 over-expression in EPCs, we evaluated the therapeutic efficiency of EPCs over-expressing ACE2 on experimental ischemic stroke.

## Methods and Materials

### Culture and characterization of early outgrowth EPCs

BM derived EPCs were generated from R+A+ mice (R+A+ EPCs) and their controls (R-A-) (R-A- EPCs) and characterized as we previously reported (13). After 7 days of culture, cells

double-positive for Di-LDL and Bs-Lectin staining were considered as early outgrowth EPCs (14). In addition, EPCs were stained with PE-conjugated CD31 (10  $\mu$ l, BD Bioscience, San Jose, CA, USA), PE-conjugated CD34 (10  $\mu$ l, AbD Serotec), FITC-conjugated VE-Cadherin (1  $\mu$ l, eBioscience), PE-Cy7-conjugated vascular endothelial growth factor receptor-2 (VEGFR-2, 5  $\mu$ l, BD Bioscience), PE-conjugated von Willebrand Factor (vWF, 10  $\mu$ l, BD Bioscience), FITC-conjugated CD133 (5  $\mu$ l, BD Bioscience), FITC-conjugated CD45 (1  $\mu$ l, eBioscience) or FITC-conjugated CD146 (1  $\mu$ l, eBioscience) antibodies. The phenotype of EPCs was analyzed by flow cytometry (Accuri C6 flow cytometer) as we described before (13).

### **EPCs transduction with lentivirus-ACE2 and pathway blocking studies**

The lentivirus containing murine ACE2 cDNA (Lenti-ACE2) and lentivirus containing green fluorescence protein (Lenti-GFP) were produced as previously described (15). EPCs were transduced with Lenti-ACE2 (Lenti-ACE2-EPCs) or Lenti-GFP (Lenti-GFP-EPCs) as previously reported (16). In brief, after cultivation for 7 days, EPCs were cultured in six-well plates ( $1 \times 10^5$ /well) and incubated with serum-free EPC culture medium containing the lentivirus (at  $5 \times 10^6$  infection-forming units) for 4 hrs, after which the serum-free medium was replaced with 10% (v/v) FBS medium for 24 hrs. Transduction efficiency (the percentage of GFP expressing cells) was quantified by direct counting using an optical grid.

For blocking experiments, after transduction with Lenti-GFP or Lenti-ACE2, EPCs were incubated with ACE2 inhibitor (DX600, 1  $\mu$ mol/L), eNOS inhibitor (L-NAME, 1 mmol/L) or Nox inhibitor (apocynin, 10  $\mu$ mol/L) for 24 hrs. Then, the EPCs were harvested for the analyses of function and gene expression.

### **EPC proliferate assay**

The EPC proliferation was performed according to the manufacturer's protocol (Cell proliferation enzyme-linked immunosorbent assay, bromodeoxyuridine [BrdU; colorimetric], Roche) as previous reported (17). Briefly, EPCs were seeded on 96-well plates ( $1 \times 10^4$  cells/well). The cells were grown in complete culture medium which was changed after 12 hrs with complete media supplemented with 10  $\mu$ M BrdU. BrdU incorporation was measured after 24 hrs using a microtiterplate reader (Packard) at 450 nm with a reference wavelength of 690 nm. BrdU uptake was calculated as the percentage of the untransduced EPCs.

### **Real-time RT PCR**

The levels of ACE2 in EPCs transduced with Lenti-ACE2 were determined using real-time RT-PCR method (18). EPC total mRNAs were isolated using RNeasy Mini kit and reverse-transcribed with the high capacity cDNA archive kit (Qiagen). The real-time PCR was run using SYBR Green reagents (Qiagen). The primer sequences for ACE2 were 5'-AAGCTAGCATAGCCAGGTCCTCCTGGCTCCTTC-3' and 5'-AAGTCGACCTAAAAGGAAGTCTGAGCATCATCACTG-3'.  $\beta$ -actin was chosen as the housekeeping gene for normalizing the data of gene expression. The mRNA level of ACE2 in EPCs from R-A- mice transduced with Lenti-GFP was defined as 100%.

### EPC function assays

The migration and tube formation abilities of EPCs were evaluated by using Boyden chamber (Chemicon) and tube formation assay kit (Chemicon) methods as we previously described (13).

### Measurement of ROS generation

Intracellular ROS generation in EPCs was determined by dihydroethidium (DHE) staining as previously described (19). Cells were incubated with DHE (2  $\mu\text{mol/L}$ ) in dark for 30 min. After washing with phosphate buffer saline (PBS), cells were then lysed with 50  $\mu\text{l}$  lysis buffer on ice. The lysates were transferred into black 96-well plates for fluorescence measurement using a spectrofluorometer.

### Determination of NO production

The membrane-permeable indicator diaminofluorescein (DAF-FM) diacetate (Invitrogen, Grand Island, NY, USA) was used to assess NO production released by EPCs (20). Briefly, the EPCs were loaded with 2  $\mu\text{mol/L}$  DAF-FM diacetate in serum free EBM-2 (37°C for 30 min), washed twice with PBS and incubated with DAF-FM diacetate-free in EBM-2 (20 min) for deesterification of the indicator. DAF-FM fluorescence was measured using a spectrofluorometer.

### Animals and procedures

**Animals**—Male adult (8-10 weeks of age, weight ranges from 25 g to 32 g) R+A+ mice and their age-matched controls (R-A-) with C57BL/6J genetic background were used for all experiments. The strains were maintained in our laboratory (founders were from Dr. Curt D. Sigmund's lab at the University of Iowa) (21;22). Mice were maintained in a 22°C room with a 12-hr light/dark cycle and fed with standard chow and drinking water ad libitum. All experimental procedures were approved by the Wright State University Laboratory Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health (NIH).

**Middle cerebral artery occlusion (MCAO) surgery and EPC transfusion**—Focal ischemic stroke was induced in animals by MCAO surgery under anesthesia by inhaling 2.5% isoflurane as we reported previously (13). Two hours after MCAO, mice were injected via the tail vein with Lenti-GFP-EPCs or Lenti-ACE2-EPCs ( $2 \times 10^5$  cells/100  $\mu\text{l}$  in PBS) or the same volume of PBS as previously described (13;23). EPCs were donated from R-A- mice. Pain and discomfort was minimized by an initial injection of Buprenorphine (0.1mg/kg, s.c) followed with another two injection every 12 hrs.

**BrdU labeling**—To label the new generated cells, mice were injected with BrdU (i.p., 65  $\mu\text{g/g/day}$ ) immediately after EPC infusion for continuous 7 days (24).

**Functional evaluation of neurological deficits**—On day 7, the neurological deficit score of each mouse was evaluated by using the 5-point scale method as previously described (22).

## Measurements of cerebral infarct volume and microvascular density

On day 7 after EPC transfusion, the brains were immediately collected and fixed in 4% paraformaldehyde (PFA) overnight and in 4% PFA plus 30% sucrose for 3 days. The brains were then cut into coronal sections (20  $\mu$ m). As we previously described (13;22), cerebral ischemic damage and cerebral microvascular density (cMVD) in the peri-infarct area were revealed by Fluoro-Jade (0.001%, Histo-chem) and CD31 (1:50, Invitrogen) staining, respectively.

## Immunofluorescence analysis

Brain coronal sections were incubated with BrdU (1:50, Abcam), CD31 (1:50, BD Biosciences) or GFP (1:50, Santa Cruz) antibody overnight at 4°C. Next, brain sections were reacted with Cy5 (blue, for BrdU), Cy3 (red, CD31) or FITC (green, for GFP) conjugated secondary antibodies (1:250, Invitrogen) for 30 min at room temperature in the dark. The positive cells in the peri-infarct area of each section were visualized using confocal microscopy (Leica TCS SP2). Angiogenesis was determined as BrdU+CD31+ cells according to previous reports (24;25). In vivo tracking of transfused EPCs were recognized as GFP+ cells. Cell counting was performed from photographs in 6 random microscopic fields (200x) by an investigator who was unaware of grouping. The average of five sections from rostral to caudal represented the data for sample.

## Western blot analysis

Proteins from EPCs were isolated with lysis buffer (Roche Diagnostic). The antibodies used were anti-ACE2 (1:1000, Cell Signaling Technology), eNOS (1:1000, Abcam), Nox2 (1:1000, Abcam) or Nox4 (1:250, Abcam) at 4°C overnight.  $\beta$ -actin (1:4000, Sigma) was used to normalize protein loading.

## Statistical analysis

All data, excepting neurologic deficit scores, are presented as mean $\pm$ SE. The neurologic deficit scores were expressed as median (range). The neurological deficit scores among different groups were compared by the Kruskal–Wallis test. When the Kruskal–Wallis test showed a significant difference, the Mann–Whitney U-tests were applied. For the rest measurements, comparisons for two groups were performed by the student's t test. Multiple comparisons were analyzed by one- or two-way ANOVA. For all tests, a *P*-value <0.05 was considered significant.

## Results

### Characterization of EPCs

As we reported previously, BM derived EPCs were defined as the cells up-taking Di-LDL and binding with Bs-Lectin (13), as well as expressing specific surface markers. The EPCs showed positive to CD34, VEGFR2, CD133, and negative to CD31, VE-Cadherin, vWF, CD45 and CD146 (Figure 1). There were no difference regarding cell markers expressed on the EPCs from the R-A- and R+A+ mice.

### The Effects of Lentivirus Transduction on EPC proliferation

The transduction efficiency was about  $94 \pm 2\%$  (Figure 2A). Lentiviral transduction decreased EPC proliferation rate by 15%. The proliferation rate of Lenti-ACE2-EPCs was increased when compared to Lenti-GFP-EPCs (by 35%) or untransduced EPCs (by 20%) (Figure 2B).

### Lenti-ACE2 Transduction Increases ACE2 Expression in EPCs

The basal levels of ACE2 mRNA and ACE2 protein expression were not different in the EPCs derived from R-A- and R+A+ mice. Lenti-ACE2 transduction induced a four-fold up-regulation of ACE2 in EPCs at both mRNA ( $P < 0.01$ ; Figure 2D) and protein levels ( $P < 0.01$ ; Figure 2C and E).

### Transduction of EPCs with Lenti-ACE2 Increases eNOS Expression and Decreases Nox2, 4 Expressions

At basal, the level of eNOS expression was lower ( $0.24 \pm 0.02$  versus  $0.18 \pm 0.01$ ,  $P < 0.05$ ; Figure 3A), whereas levels of Nox2 ( $0.36 \pm 0.03$  versus  $0.48 \pm 0.03$ ,  $P < 0.05$ ; Figure 3B) and Nox4 ( $0.34 \pm 0.02$  versus  $0.46 \pm 0.03$ ,  $P < 0.01$ ; Figure 3C) were higher in the EPCs generated from R+A+ mice. Transduction of Lenti-ACE2 increased eNOS, and decreased Nox2 and Nox4 expressions in EPCs from both R+A+ (by 61%, 27% and 28%, respectively) and R-A- (by 62%, 44% and 50%, respectively) mice ( $P < 0.01$ ). In addition, DX600 (ACE2 inhibitor) was able to partially block those effects of Lenti-ACE2 on EPCs ( $P < 0.05$  or  $0.01$ ; Figure 3).

### Transduction of EPCs with Lenti-ACE2 Increases NO and Decreases ROS Production

The NO production was higher (versus  $P < 0.05$ ; Figure 4A), whereas the ROS production was lower ( $P < 0.05$ ; Figure 4B) in the EPCs generated from R+A+ mice. Lenti-ACE2 transduction increased 25% NO production, and decreased 48% ROS production in EPCs derived from R-A- mice, and induced 32% increase of NO and 28% decrease of ROS production from R+A+ mice ( $P < 0.01$ ). Again, DX600 partially blocked those effects of Lenti-ACE2 on EPCs ( $P < 0.05$ ). In addition, APO (Nox inhibitor) decreased the ROS production, while L-NAME (eNOS inhibitor) blocked the NO production in Lenti-ACE2 primed EPCs ( $P < 0.05$ ; Figure 4).

### Lenti-ACE2 Transduction Enhances EPC Function via Both eNOS and Nox Pathways

As shown in Figure 5, the abilities of EPC migration and tube formation were attenuated in EPCs from R+A+ mice by 31% and 26%, respectively ( $P < 0.01$  or  $0.05$ ). Lenti-ACE2 transduction enhanced those functions of EPCs from both R+A+ (by 47% and 52%, respectively) and R-A- (by 34% and 50%, respectively) mice ( $P < 0.01$ ). Moreover, DX600 abolished and L-NAME partially blocked Lenti-ACE2 induced enhancement of those functions in EPCs ( $P < 0.01$ ). APO further enhanced those functions in Lenti-ACE2 primed EPCs from R+A+ mice ( $P < 0.05$ ), but not in Lenti-ACE2 primed EPCs from R-A- mice.



### Infusion of Lenti-ACE2-EPCs Enhances the Efficacy in Decreasing Ischemic Injury and Neurologic Deficit Score

The infarct volume of MCAO-induced stroke was exaggerated in R+A+ mice ( $P<0.05$ ; Figure 6A and B). Lenti-GFP-EPC transfusion decreased infarct volume in both R-A- (by 33%,  $P<0.01$ ) and R+A+ mice (by 20%,  $P<0.05$ ; Figure 6A and B) and in neurologic deficit score ( $P<0.05$ ; Figure 6C). Furthermore, transfusion of Lenti-ACE2-EPCs was able to further decrease the infarct volume in R-A- (by 30%,  $P<0.05$ ) and R+A+ mice (by 40%,  $P<0.05$ ; Figure 6A and B) and further improve neurologic motor function ( $P<0.05$  or  $0.01$ ; Figure 6C).

### Infusion of Lenti-ACE2-EPCs Enhances the Efficacy in Increasing cMVD in the Peri-Infarct Area

At basal, the cMVD in the peri-infarct area was decreased by 41% in R+A+ mice ( $P<0.05$ ; Figure 7A and B). Infusion of Lenti-GFP-EPCs was able to increase the cMVD in peri-infarct area by 22% in R-A- mice and 30% in R+A+ mice ( $P<0.01$ ; Figure 7B). Transfusion of Lenti-ACE2-EPCs could further induce a 17% increase in cMVD in the peri-infarct area in R-A- mice ( $P<0.01$ ; Figure 7B), and 55% in R+A+ mice ( $P<0.05$ ).

### Infusion of Lenti-ACE2-EPCs Enhances the Efficacy in Promoting Angiogenesis in the Peri-Infarct Area

Figure 8A shows representative images of *in vivo* tracking of transfused EPCs (GFP+ cells) in the peri-infarct area in different groups on day 7 after MCAO surgery. The number of GFP+ cells was more in Lenti-ACE2-EPCs transfusion group than in Lenti-GFP-EPC transfusion group ( $P<0.01$ ; Figure 8C).

Figure 8B shows representative micrographs of angiogenesis (BrdU+CD31+ cells) in the peri-infarct area. Lenti-GFP-EPC transfusion promoted angiogenesis on day 7 in R-A- mice (by 30%;  $P<0.01$ ). Transfusion of Lenti-ACE2-EPCs further promoted angiogenesis in both R-A- (by 81%;  $P<0.01$ ) and R+A+ mice (by 300%;  $P<0.01$ ; Figure 8D).

## Discussion

EPCs have emerged as a pivotal type of cells for maintaining endothelial homeostasis and replenishing injured ECs, and have been shown to substantially contribute to endothelial regeneration and functional restoration (7;26). It has been recently documented that Ang II reduces EPC number and induces EPC dysfunction *in vitro* and *in vivo* (9). In addition, studies indicated that ACE inhibitors and AT1 receptor blockers have protective effects on EPCs (27;28). A recent study showed that over-expressing ACE2 in blood vessels improves EC function (29). However, whether ACE2 counteracts the effects of Ang II on EPCs has not been explored.

In this study, we demonstrated that the abilities of migration and tube formation of BM derived EPCs from R+A+ mice are impaired. This result is consistent with previous studies showing that Ang II via AT1 receptor decreases the differentiation and accelerates the senescence of BM derived EPCs (11;30). Here, we also found that the eNOS expression and

NO production are lower, whereas Nox2, 4 expression and ROS production are higher in the EPCs generated from R+A+ mice. These findings are supported by previous evidence demonstrating that the RAS plays a key role in modulating EC function through regulating NO and ROS production (31). Recently, it has been suggested that Ang II inhibits Akt-induced eNOS activation and NO release in ECs (32) and that Ang II infusion decreases NO production in aorta by causing eNOS uncoupling (33).

One of the major findings in the present study is that ACE2 over-expression in EPCs generated from both R-A- and R+A+ mice enhances their abilities of tube formation and migration. Interestingly, those effects are more evident in the EPCs from R+A+ mice and can be partially abolished by ACE2 inhibitor (DX600) suggesting that ACE2 might counteract the effect of Ang II on EPCs. We assume that the changes with ACE2 over-expression might mainly reflect the elevation of Ang (1-7) in addition to the reduction of Ang II. This is supported by a recent report showing that ACE2 over-expression in myocytes and fibroblasts induces Ang II reduction and Ang (1-7) elevation in cell lysates (34). DX600 is a large peptide which would not traverse the cell membrane. Since the presence of intracellular RAS peptidases (35), the incomplete reversal with DX600 would reflect that intracellular expression of ACE2 regulates the intracellular ratio of Ang II to Ang (1-7).

At the same time, our data show that ACE2 over-expression down-regulates the expression of Nox2/Nox4 and alleviates the ROS production, whereas, up-regulates the expression of eNOS and elevates the NO production. These findings are in agreement with other studies. ACE2/Ang (1-7) counterregulates Ang II induced Nox activation in ECs (36), and prevents ROS over-production and senescence in EPCs (37). The eNOS also has been reported to regulate the mobilization and function of EPCs (38). We speculate that the dual effects of ACE2 over-expression on stimulating NO and reducing ROS production may be beneficial from the reduced formation of peroxynitrite. There is a study reported that ACE2 deficiency enhances Ang II-mediated peroxynitrite production (39). In addition, both the decreased ROS formation and enhanced ROS scavenging enzymes might account for the reduction of ROS production (40;41). To further confirm whether reduction of oxidative stress and up-regulation of eNOS associated with the ACE2 mediated improvement of EPC function, we conducted the pathway block studies. As we expected, the beneficial effects of ACE2 over-expression on EPCs were partially blocked by NOS inhibition and further enhanced by a Nox inhibitor. These data add new information to previous reports showing the protective role of ACE2 in cells. For examples, ACE2 over-expression in the human monocyte cell line attenuates Ang II-induced release of inflammatory factors (1). Transduction of cardiac fibroblasts with lenti-ACE2 results in a significant attenuation of both basal and hypoxia/re-oxygenation-induced collagen production and inflammatory cytokine production (16).

To explore the function of EPCs over-expressing ACE2 *in vivo*, we investigated the therapeutic efficacy of Leni-ACE2 primed EPCs on experimental ischemic stroke mice. Transfused EPCs were tracked *in vivo* suggesting recruitment of transfused EPCs to the peri-infarct area. Our data demonstrate for the first time that transfusion of ACE2 primed EPCs is able to enhance the efficacy of non-primed EPCs in attenuating cerebral damage (decreasing the infarct volume, improving neurologic deficits) and promoting cerebral repair (increasing cMVD and angiogenesis). Angiogenesis is a vital component of wound repair. EPCs are



believed to play an important role in maintaining endothelial integrity and vascular homeostasis, and to participate in angiogenesis which represents an important endogenous tissue repair mechanism. Introduction or mobilization of EPCs can restore tissue vascularization after ischemic stroke and re-establish endothelial integrity (13;42). In this study, we double stained the brain slides with CD31 and BrdU to measure the newly generated ECs for the index of angiogenesis, which is commonly used in the similar studies (24;25). Our results showed that infusion of Lenti-GFP-EPCs increased angiogenesis in R-A- mice, but not in R+A+ mice. Although the detailed mechanism has not been explored in this study, we tentatively attribute this to the high level of Ang II in the R+A+ mice (22), which impairs the function of EPC (9;43). Of note, we further discovered that infusion of EPCs over-expressing ACE2 promotes angiogenesis in both R-A- and R+A+ mice, which might benefit from the ACE2 catalyzing Ang II into Ang (1-7). This is supported by other reports showing that Ang (1-7) improves endothelial function (29) and ameliorates Ang II-Induced endothelial cell apoptosis (44). Although it is unclear whether the improvement of angiogenesis is exclusively mediated by EPC integration or paracrine effects, our *in vivo* results imply that over-expression of ACE2 in EPCs could enhance the beneficial effect of EPC-based therapy for ischemic stroke. Future studies are warranted to determine the fate of transfused EPC populations by analyzing the dynamics of transfused cells in living animals.

In summary, both the *in vitro* functional and *in vivo* therapeutic findings of present study demonstrate that over-expression of ACE2 in EPCs enhances EPC function via eNOS/NO and Nox/ROS signaling pathways. Transfusion of Lenti-ACE2 primed EPCs might provide a novel approach for treating ischemic stroke, especially in those with Ang II activation.

## Perspectives

Our findings show that ACE2 enhances EPC function via regulating eNOS/NO and Nox/ROS pathways and improves EPC-based therapeutic effects on ischemic stroke. This study depicts the new aspect of ACE2 gene function and the underlying mechanisms. Our data provide a strong rationale for using ACE2 target to develop vasoprotective drugs and to enhance the efficacy of EPC-based therapy. In addition, further study on the effect of ACE2 on EPCs should provide important insights into the pathogenesis of vascular disease and development of novel therapeutic interventions.

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## Novelty and Significance

### What Is New?

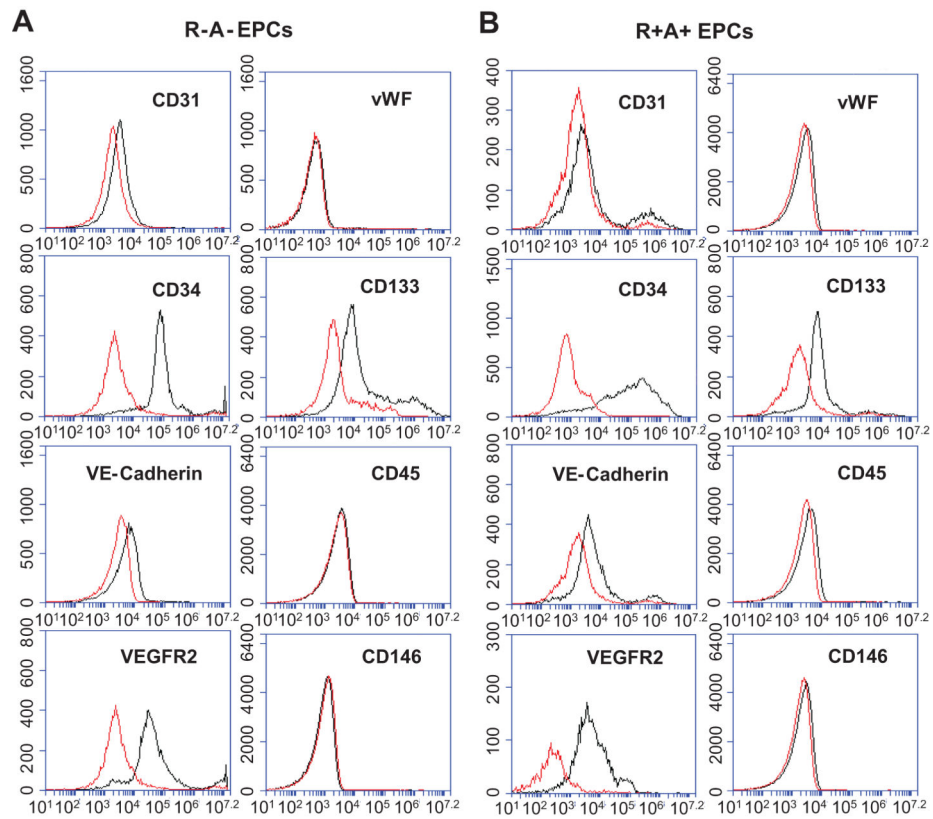
This study demonstrates the protective role of ACE2 in EPC function in vitro and the beneficial effects of ACE2 primed EPCs on treating ischemic stroke in vivo.

### What Is Relevant?

The present study supports the hypothesis that overexpressing ACE2 could improve EPC function and enhance the efficacy of EPC-based therapy for ischemic stroke via counteracting the detrimental effects of Ang II.

### Summary

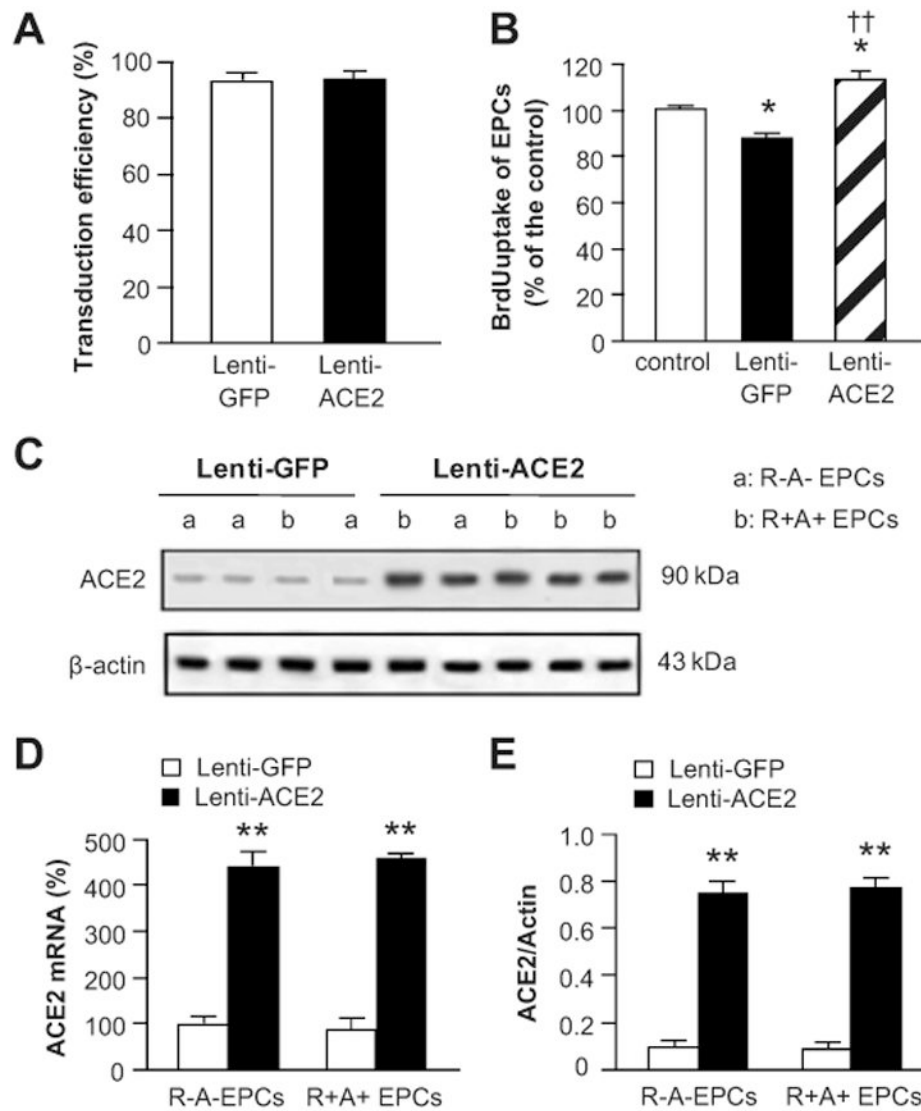
ACE2 over-expression enhances EPC function via modulating Nox/ROS and eNOS/NO signaling pathways, and improves the therapeutic efficacy of EPC transfusion for ischemic stroke by reducing ischemic injury and promoting recovery. Our findings provide novel insight into ACE2 as a potential target for new drug development.



**Figure 1.**

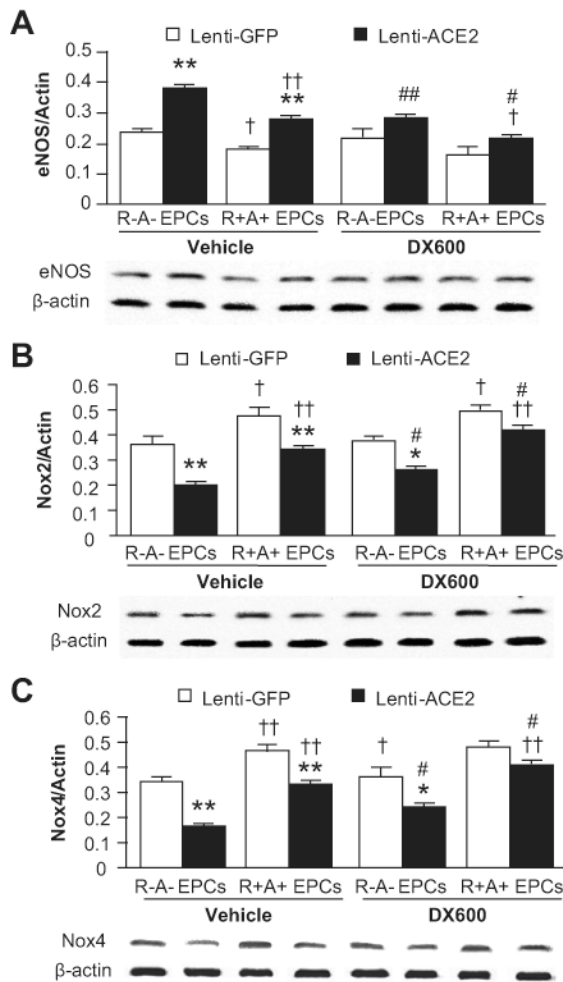
The phenotype of cultured EPCs. Representative flow plot showing the expression of CD34, VEGFR2, CD133, CD31, VE-Cadherin, vWF, CD45 and CD146 on the EPCs cultured from R-A- (A) and R+A+ mice (B).





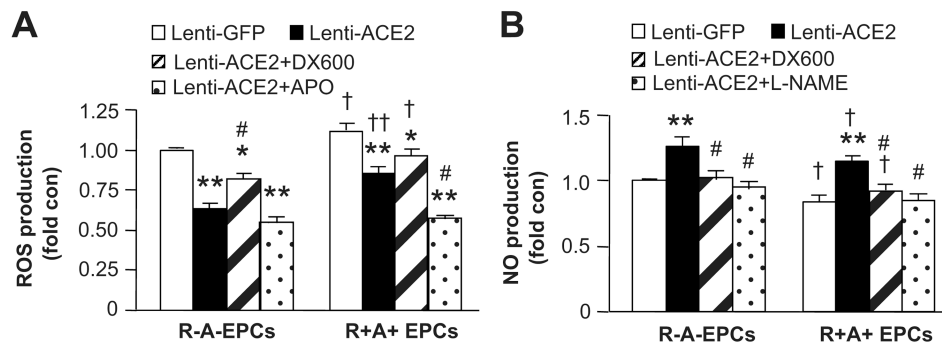
**Figure 2.**

Transduction efficiency, proliferation rate and ACE2 expression in EPCs. A, Transduction efficiency of lentivirus in EPCs. B, Proliferation rate of control, and EPCs transduced with Lenti-GFP or Lenti-ACE2. \* $P < 0.05$  versus control; <sup>††</sup> $P < 0.01$  versus Lenti-GFP. C, Representative Western blot bands showing the ACE2 expression in EPCs. Summarized data of ACE2 expression in EPCs at mRNA (D) and protein (E) levels. \*\* $P < 0.01$  versus Lenti-GFP,  $n = 6$ /group. Control: untransduced EPCs.



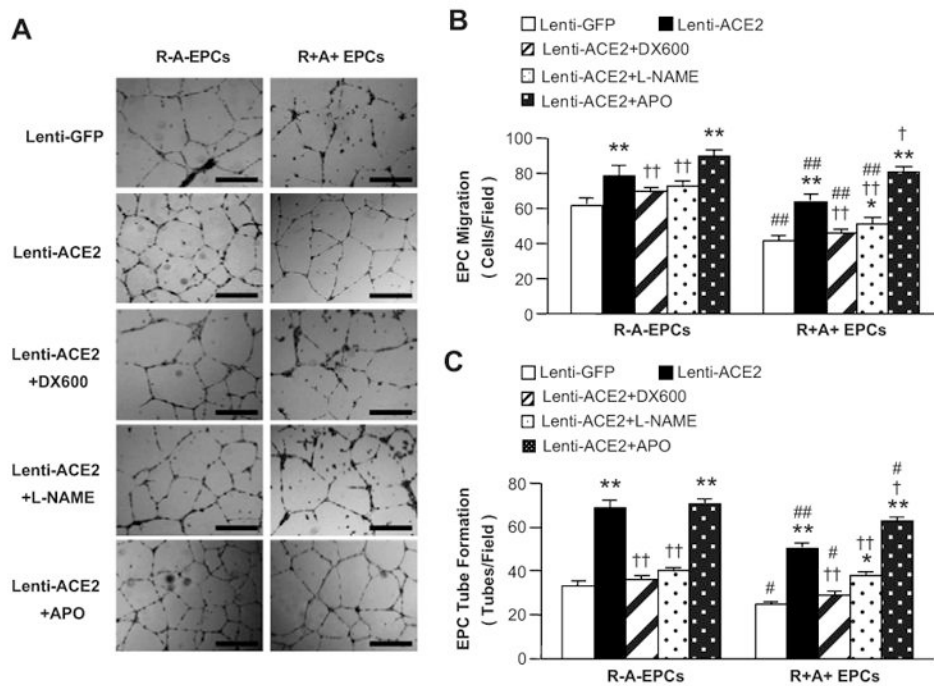
**Figure 3.**

The effects of ACE2 over-expression on gene expression. The eNOS (A), Nox2 (B) and Nox4 (C) expression in EPCs. EPCs were transduced with Lenti-GFP or Lenti-ACE2, and then incubated with or without DX600 (1  $\mu\text{mol/L}$ ). The protein expression was normalized to the expression of  $\beta$ -actin. \* $P < 0.05$ , \*\* $P < 0.01$  versus Lenti-GFP; † $P < 0.05$ , †† $P < 0.01$  versus R-A-EPCs; # $P < 0.05$  versus vehicle,  $n = 6/\text{group}$ . eNOS: endothelial nitric oxide synthase; Nox: nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.



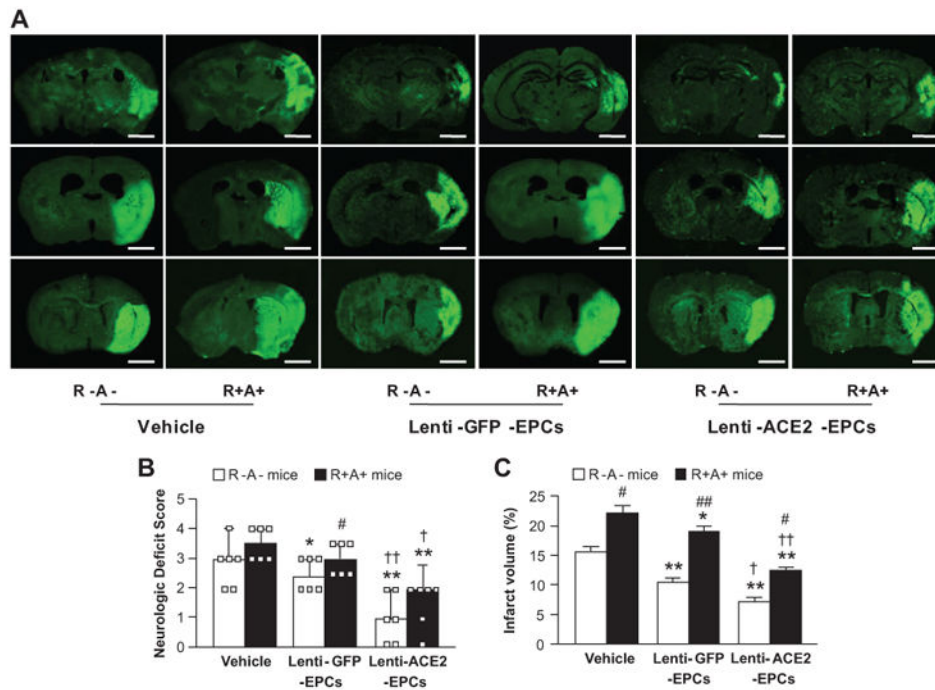
**Figure 4.**

The effects of ACE2 over-expression on ROS and NO production of EPCs. The ROS (A) and NO (B) production in EPCs transduced with Lenti-GFP or Lenti-ACE2. Cells were incubated with DX600 (1  $\mu$ mol/L), APO (10  $\mu$ mol/L) or L-NAME (1 mmol/L). \* $P$ <0.05, \*\* $P$ <0.01 versus Lenti-GFP; † $P$ <0.05, †† $P$ <0.01 versus R-A- EPCs; # $P$ <0.05 versus Lenti-ACE2, n=6/group. ROS: reactive oxygen species; NO: nitric oxygen.



**Figure 5.**

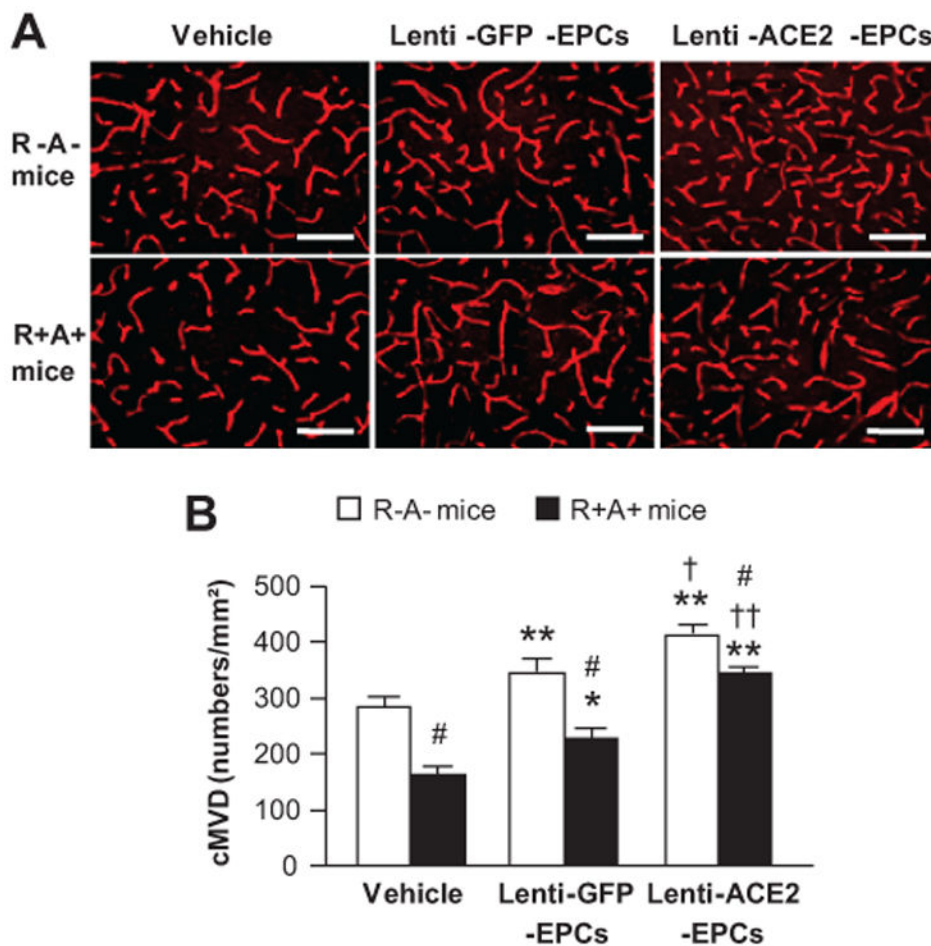
ACE2 over-expression enhances the function of EPCs. A, Representative images of EPC tube formation. Scale bar: 600  $\mu$ m. The abilities of EPC migration (B) and tube formation (C) in EPCs transduced with Lenti-GFP or Lenti-ACE2. Cells were incubated with DX600 (1  $\mu$ mol/L), APO (10  $\mu$ mol/L) or L-NAME (1 mmol/L). \* $P$ <0.05, \*\* $P$ <0.01 versus Lenti-GFP; † $P$ <0.05, †† $P$ <0.01 versus Lenti-ACE2; # $P$ <0.05, ## $P$ <0.01 versus R-A- EPCs, n=6/group.



**Figure 6.**

Effects of Lenti-ACE2-EPC infusion on infarct volume and neurologic deficit score. A, Representative pictures of Fluoro-J staining 7 days after EPC infusion. Scale bar: 2 mm. Summarized data on neurologic deficit scores (B) and infarct volume (C).

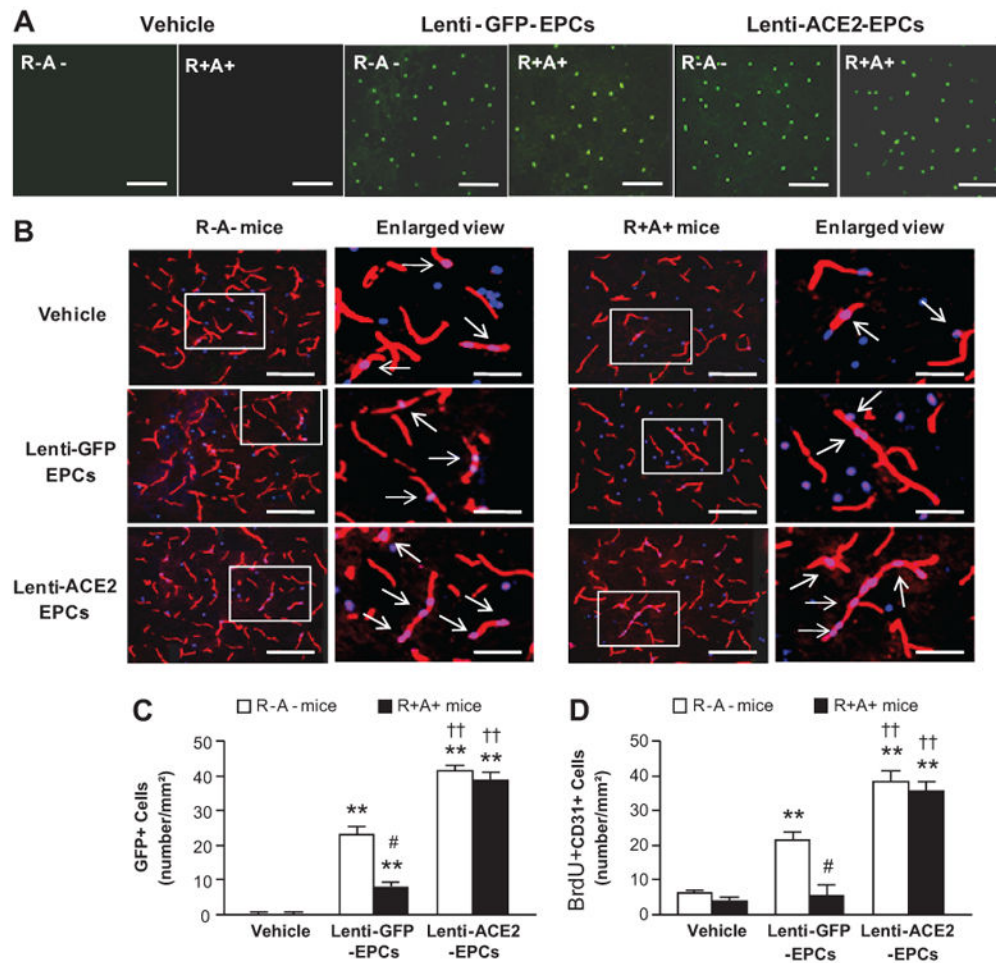
Transfusion of Lenti-ACE2-EPCs has better efficacy than Lenti-GFP-EPCs in decreasing infarct volume and improving neurologic deficit. \* $P < 0.05$ , \*\* $P < 0.01$  versus vehicle; † $P < 0.05$ , †† $P < 0.01$  versus Lenti-GFP-EPCs; # $P < 0.05$ , ## $P < 0.01$  versus R-A- mice,  $n = 6$ /group.



**Figure 7.**

Effects of Lenti-ACE2-EPC infusion on cMVD in cerebral microvasculature in the peri-infarct area. A, Representative pictures of cMVD (CD31 immunostaining) on day 7 following MCAO and EPC infusion. Scale bar: 50  $\mu$ m. B, Summarized data of cMVD. \* $P$ <0.05, \*\* $P$ <0.01 versus vehicle;  $\dagger P$ <0.05,  $\dagger\dagger P$ <0.01 versus Lenti-GFP-EPCs; # $P$ <0.05 versus R-A- mice,  $n=6$ /group. cMVD: cerebral microvascular density.





**Figure 8.**

Effect of Lenti-ACE2-EPC infusion on angiogenesis in the peri-infarct area. A, Representative pictures of *in vivo* tracking of transfused EPCs 7 days after EPC infusion. Green: GFP for transfused EPCs. Scale bar: 50  $\mu$ m. B, Representative pictures of angiogenesis (BrdU+CD31+ cells). Red: CD31 for vessel; Blue: BrdU for new generated cells; Pink: double staining. Scale bar: 50  $\mu$ m. The enlarged view of double staining cells is in the left panel of each image. The arrows indicate the double staining cells. Scale bar: 20  $\mu$ m. Histogram showing the number of GFP+ cells (C) and BrdU+CD31+ cells (D). \*\* $P$ <0.01 versus vehicle; †† $P$ <0.01 versus Lenti-GFP-EPCs; # $P$ <0.05 versus R-A- mice, n=6/group.