Am J Physiol Heart Circ Physiol 309: H1697–H1707, 2015.
First published September 18, 2015; doi:10.1152/ajpheart.00854.2014.

ACE2/Ang-(1–7)/Mas axis stimulates vascular repair-relevant functions of CD34$^+$ cells

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Submitted 2 December 2014; accepted in final form 4 September 2015

Singh N, Joshi S, Guo L, Baker MB, Li Y, Castellano RK, Raizada MK, Jarajapu YP. ACE2/Ang-(1–7)/Mas axis stimulates vascular repair-relevant functions of CD34$^+$ cells. *Am J Physiol Heart Circ Physiol* 309: H1697–H1707, 2015. First published September 18, 2015; doi:10.1152/ajpheart.00854.2014.—CD34$^+$ stem/progenitor cells have been identified as a promising cell population for the autologous cell-based therapies in patients with cardiovascular disease. The counter-regulatory axes of renin angiotensin system, angiotensin converting enzyme (ACE)/Ang II/angiotensin type 1 (AT_1) receptor and ACE2/Ang-(1–7)/Mas receptor, play an important role in the cardiovascular repair. This study evaluated the expression and vascular repair-relevant functions of these two pathways in human CD34$^+$ cells. CD34$^+$ cells were isolated from peripheral blood mononuclear cells (MNCs), obtained from healthy volunteers. Expression of ACE, ACE2, AT_1, and angiotensin type 2 and Mas receptors were determined. Effects of Ang II, Ang-(1–7), Norleu^3-Ang-(1–7), and ACE2 activators, xanthenone (XNT) and diminazene aceturate (DIZE) on proliferation, migration, and adhesion of CD34$^+$ cells were evaluated. ACE2 and Mas were relatively highly expressed in CD34$^+$ cells compared with MNCs. Ang-(1–7) or its analog, Norleu^3-Ang-(1–7), stimulated proliferation of CD34$^+$ cells that was associated with decrease in phosphatase and tensin homologue deleted on chromosome 10 levels and was inhibited by triciribin, an AKT inhibitor. Migration of CD34$^+$ cells was enhanced by Ang-(1–7) or Norleu^3-Ang-(1–7) that was decreased by a Rho-kinase inhibitor, Y-27632. In the presence of Ang II, XNT or DIZE enhanced proliferation and migration that were blocked by DX-600, an ACE2 inhibitor. Treatment of MNCs with Ang II, before the isolation of CD34$^+$ cells, attenuated the proliferation and migration to stromal derived factor-1α. This attenuation was reversed by apocynin, an NADPH oxidase inhibitor. Adhesion of MNCs or CD34$^+$ cells to fibronectin was enhanced by Ang II and was unaffected by Ang-(1–7). This study suggests that ACE2/Ang-(1–7)/Mas pathway stimulates functions of CD34$^+$ cells that are cardiovascular protective, whereas Ang II attenuates these functions by acting on MNCs. These findings imply that activation of ACE2/Ang-(1–7)/Mas axis is a promising approach for enhancing reparative outcomes of cell-based therapies.

CD34$^+$ cells; ACE-2; Ang-(1–7); Mas receptor; migration; proliferation

NEW & NOTEWORTHY

Angiotensin-(1–7) or its analog NorLeu3-Ang-(1–7) and the putative angiotensin converting enzyme (ACE)2 activators, xanthenone and diminazene aceturate, stimulate migration and proliferation - functional signatures of vasoreparative potential - of human CD34+ cells. Angiotensin II, by stimulating the generation of reactive oxygen species from mononuclear cells, attenuates the functions of CD34$^+$ cells.

CD34, A SIALOMUCIN-LIKE LIGAND for L-selectin, is long known to be a common cell-surface marker to many types of hematopoietic progenitor cells (7). Adult CD34$^+$ cells are bone marrow-derived multipotent stem cells and are capable of reconstituting hematopoietic systems (5). This is a major cell type for individuals needing hematopoietic reconstitution following chemotherapy or radiation therapy (9). Interestingly, CD34 is robustly expressed in small capillaries and in tissues that support early vascular development (48, 53). However, the role of CD34$^+$ cells in postnatal vasculogenesis was not known until recently. The groundbreaking studies by Asahara et al. (3) provided compelling evidence for a novel role of CD34$^+$ cells in the vascularization of ischemic tissue. Therefore, CD34$^+$ cells were quite often designated as endothelial progenitor cells. It has now been well documented that these cells in response to hypoxia-regulated factors such as stromal-derived factor-1 (SDF) and VEGF proliferate and migrate to the areas of ischemia, and accelerate the vascular repair thus preventing tissue damage (26). Autologous cell therapy using CD34$^+$ cell population has now emerged as a promising approach for the treatment of myocardial ischemic disease (32).

Classically, renin angiotensin system (RAS) consists of angiotensin converting enzyme (ACE), its product angiotensin (Ang) II and receptors for Ang II, angiotensin type 1 (AT_1), and angiotensin type 2 (AT_2) receptors. RAS has been expanded extensively in the recent years with the identification of ACE2, a monocarboxy peptidase that generates Ang-(1–7) from Ang II and the identification of the receptor, Mas, which mediates the biological actions of Ang-(1–7) (44). Although Ang II produces hypertensive, pro-oxidative, hypertrophic and pro-fibrotic effects in cardiovascular system, Ang-(1–7) elicits counter-regulatory effects on ACE/Ang II pathway by producing vasodilatory, antihypertensive, antihypertrophic, antifibrotic, and antithrombotic effects (11, 23).

Local or tissue expression of ACE and ACE2 systems has been identified and was shown to have paracrine, autocrine, and intracrine effects (33, 37). Evidence for the local RAS in bone marrow has been provided by studies that have shown hematopoietic functions of angiotensin peptides in human and murine bone marrow cells (39, 43). With respect to the functions of reparative and regenerative functions of CD34$^+$ cells, role of local RAS is yet to be investigated. We have recently reported that ACE2 and Mas receptor are expressed in these

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cells and that Ang-(1–7) support migratory function (24). In this study we have characterized the expression of ACE and ACE2 axes in total mononuclear cells, Lineage-negative (Lin^-) cells that are enriched for primitive stem/progenitor cells and $CD34^+$ cells. Effects of Ang II, Ang-(1–7) or its analog $NorLeu^3$-Ang-(1–7), and the putative ACE2 activators diminazene aceturate (DIZE) (45) and xanthenone (XNT) (12, 15) were evaluated on proliferation, migration, and adhesion, which are in vitro indicators of vasoreparative functions of $CD34^+$ cells.

MATERIALS & METHODS

Characteristics of subjects. This study was approved by Institutional Biosafety Committee of North Dakota State University (protocol No. B12017). The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Human leucocyte samples were obtained from United Blood Services (Fargo, ND). Healthy volunteers included both males and females of age ranging from 48 to 75 years. All volunteers are Caucasians. Leucocytes were collected in chambers of Leucoreduction system (LRS chambers) following apheresis carried out by using Trima Accel system (80440).

Isolation of cells. $CD34^+$ cells were isolated from leucocytes as described before (25). Peripheral blood mononuclear cells (MNCs) were separated from total leucocytes by gradient centrifugation using Ficoll (Ficoll-Paque; GE Healthcare Biosciences). Plasma was completely excluded from the cell fraction by a series of washings using PBS with 2% FBS and 1 mM EDTA and centrifugation at 120 *g*. These cells were enriched for lineage negative (Lin^-) cells by using a negative selection kit (StemCell Technologies) as per supplier's instructions. Lin^- cells were then enriched for $CD34^+$ cells by using an immunomagnetic selection kit (Easysep, Human CD34 positive selection kit; StemCell Technologies) as per the manufacturer's instructions. The purity of enriched $CD34^+$ cells was assessed by flow cytometry. Cells were stained by incubating with anti-CD45 and anti-CD34 antibodies (R&D systems) or isotype control antibodies in the presence of FcR blocking reagent (Milteny Biotech) for 45 min at 4°C. Cells were analyzed by a cytometer (C6 Accuri, BD). The purity ranged from 86% to 92%, when evaluated randomly during this study.

MNCs, Lin^- cells, or $CD34^+$ cells were used for different experimental protocols. Freshly isolated cells were either used for assays or preserved by snap-freezing cell pellets in liquid nitrogen and storing at −80°C for later use. When needed, freshly isolated Lin^- or $CD34^+$ cells were plated in U-bottom 96-well plates (Nunc) in StemSpan containing cytokine cocktail (StemCell Technologies) for not more than 48 h. In some experiments, MNCs were treated with 100 nM Ang II alone or in combination with 300 μM apocynin for 2 h at 37°C in PBS containing 2% FBS and glucose (1 mg/ml). In all assays, cells from the same donor were used as untreated control and treatment groups to account for the donor-to-donor variation during comparison.

Real-time PCR. Total RNA was isolated by using the RNeasy Plus Mini Kit according to the manufacturer's protocol (Qiagen). The concentration and purity of RNA was determined by a spectrophotometer (NanoDrop Technologies). cDNA was synthesized by using 50 ng of RNA using iScript cDNA Synthesis kit (Bio-Rad).

Taqman gene expression assays were used for evaluating the gene expression of Mas receptor (MAS1) (Hs00267157_s1) and ACE (Hs00174179_m1) with β-actin (Hs99999903_m1) as a housekeeping gene by using Taqman universal PCR mastermix (Applied Biosystems). Gene expression of ACE2, AT_1 receptor (AT_1R), and AT_2 receptor (AT_2R) was evaluated by SYBR-Green Real-time RT-PCR by using All-in-One qPCR Mix (GeneCopoeia) and optimal primer concentrations in a quantitative PCR System (Applied Biosystems 7500). All primers (Table 1) were synthesized by Invitrogen. Real-time RT-PCR reaction conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. The relative gene expression was quantified by ΔCt relative to β-actin.

Western blotting. The protein expression of ACE, ACE2, and AT_1 receptor were evaluated in MNCs, Lin- cells, and $CD34^+$ cells by Western Blot analysis. Briefly, equal concentrations of proteins from whole cell lysates were resolved by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane and stained with primary antibodies, mouse anti-human ACE (GTX11737; Genetex), rabbit anti-human ACE2 (sc-20998; Santa Cruz Biotechnology), mouse antihuman Mas1, or rabbit antihuman AT_1 (ab124734; Abcam). β-Tubulin was used as a loading control and stained with rabbit antihuman antibody (Santa Cruz BioTechnology). IgG-HRP conjugated, goat anti-mouse, or goat anti-rabbit secondary antibodies were obtained from Bio-Rad. Blots were processed by using ECL Prime (GE Healthcare).

Recent studies (4, 21) have questioned the specificity of commercially used antibodies of mouse or rat AT_1 receptor; therefore, we sought to confirm the specificity of the antibody used in the current studies. This was accomplished by RNA silencing approach by using small interference RNA (siRNA) (Catalog No. sc-29750; Santa Cruz BioTechnology). MNCs were transfected with 5 μM siRNA duplex with transfection medium as per the manufacturer's protocol for 72 h. MNCs treated with serum-free medium or scrambled siRNA (Catalog No. sc-37007) served as a control and as a negative control, respectively. Expression of AT_1 receptor mRNA and protein were quantified as described above by real-time PCR and by Western blotting, respectively.

Flow cytometric analysis. Surface expression of Mas receptor in $CD34^+$ cells was evaluated by flow cytometry by using two different protocols. The first method was performed by staining the $CD34^+$ cells (1×10^5) with primary rabbit anti-MAS1 antibody (NLS1531; Novus Biologicals) for 45 min at room temperature after blocking with FcR blocking reagent (Milteny Biotech). The cells were then washed three times with washing buffer, and a Dylight 488 anti-rabbit secondary antibody (Vector laboratories) was added to cells for 30 min at room temperature. The cells were analyzed for green fluorescence by using Accuri C6 flow cytometer (BD Biosciences). For the second method, a fluorescent self-conjugated MAS1 antibody was used to detect the receptor presence on $CD34^+$ cells. The conjugation was performed by using Zenon-APC Rabbit IgG labelling kit (Life Technologies) as per the manufacturer's protocol. Briefly, 5 μl of the Zenon rabbit IgG labeling reagent was added to 1 μg of primary rabbit anti-MAS1

Table 1. *List of primer sets used for the real-time PCR studies*

Gene	Primer Sequence	
	Forward	Reverse
Angiotensin converting enzyme 2	5′-CATTGGAGCAAGTGTTGGATCTT−3′	5′-GAGCTAATGCATGCCATTCTCA−3′
Angiotensin type 1 receptor	5′-GACGCACAATGCTTGTAGCCA−3′	5′-CTGCAATTCTACAGTCACGTATG−3′
Angiotensin type 2 receptor	5′-GGGCTTGTGAACATCTCTGG−3′	5′-GTAAATCAGCCACAGCGAGG−3′
β-Actin	5′-GACAGGATGCAGAAGGAGATTACT−3′	5′-TGATCCACATCTGCTGGAAGGT−3′

antibody (Novus Biologicals) antibody in PBS and incubated for 5 min at room temperature. Then, 5 μl of the Zenon blocking reagent was added to the reaction mixture and incubated for 5 min at room temperature. The complexes were then applied immediately to CD34$^+$ cell suspension in staining buffer treated with FcR blocking reagent and incubated for 45 min at 4°C. The cells were analyzed for APC fluorescence by flow cytometry.

To assess the expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), an intracellular protein, CD34$^+$ cells were treated with FcR blocking reagent, and then fixed and permeabilized with BD Cytofix/Perm Reagent (BD Biosciences). Cells were then stained with Alexa Fluor 647 Mouse anti human-PTEN (BD PhosFlow) or isotype control antibody for 45 min at 4°C. Cells were analyzed by flow cytometry. Effect of treatments on PTEN levels was determined by enumerating PTEN-expressing cells and by quantifying mean fluorescence intensity.

ACE2 activity assay. ACE-2 activity was determined by using the intramolecularly quenched synthetic ACE2 specific substrate 7-Mca-YVADAPK(Dnp) (R&D systems) (20). Protein (10 μg) from cell lysates was used for the assay in a buffer containing 50 mM 4-morpholineethanesulphonic acid, 300 mM NaCl, 10 μM $ZnCl_2$, and 0.01% Triton X-100 at pH 6.5. Reaction was initiated by the addition of 50 μM substrate, and the fluorescence was monitored for 3 h by using a plate reader (SpectraMax M5; Molecular Devices) at an excitation wavelength 320 nm and emission wavelength 405 nm. ACE 2 activity was defined as ACE2 inhibitor (MLN4760, 10 μM)-sensitive fluorescence and was expressed as percentage of total fluorescence.

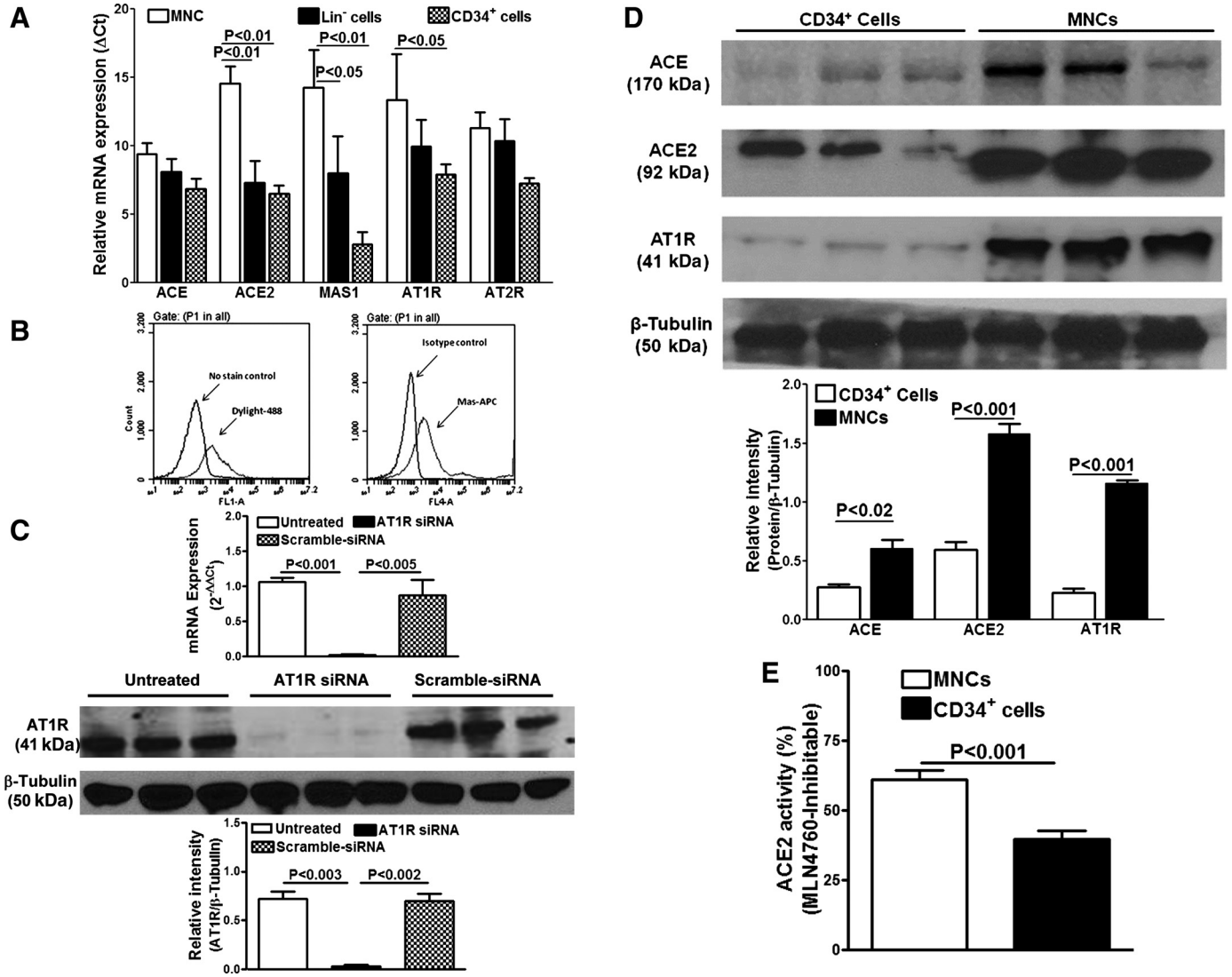


Fig. 1. Comparative expression of mRNA and protein of angiotensin converting enzyme (ACE), ACE2, MAS1, Ang II type 1 receptor (AT$_1$R), and Ang II type 2 receptor (AT$_2$R) in human mononuclear cells (MNCs), lineage-negative cells (Lin$^-$ cells), and CD34$^+$ cells. *A*: expression of mRNA was represented by ΔCt values relative to the expression of β-actin (n = 5 to 7). *B*: representative dot plots of Mas receptor expression in CD34$^+$ cells detected by using primary and a Dylight 488-labeled secondary antibody (*left*) or by using Zenon-APC labeling kit of primary antibody (*right*) (n = 4). Shown were CD34$^+$ cell number versus fluorescence intensity, and the rightward shift indicates the surface expression of Mas receptor. *C*: specificity of AT$_1$R antibody: treatment of MNCs with small interference RNA (siRNA) resulted in robust decrease in the expression of mRNA or protein of AT$_1$R. Shown were representative Western blot of AT$_1$R in MNCs, untreated control, and siRNA- or scramble-siRNA-treated. Levels of mRNA were expressed relative to the untreated control. Expressions of mRNA or protein following treatments were compared by 1-way ANOVA (n = 6). *D*: representative Western blots of ACE, ACE2, and AT$_1$R proteins in human CD34$^+$ cells in comparison with the expression in MNCs. Expression of proteins was quantified relative to the β-tubulin and was lower in CD34$^+$ cells compared with MNCs (n = 6). *E*: ACE2 activity in MNCs and CD34$^+$ cells: activity was defined as MLN4760-inhibitable fluorescence of the fluorogenic substrate and was expressed as percentage of total fluorescence. Activity observed in MNCs was significantly higher than the activity in CD34$^+$ cells (n = 6; t-test).

Proliferation assay. Proliferation of CD34$^+$ cells was evaluated by determining BrdU incorporation using a kit (Cell Proliferation ELISA; Roche Bioscience) as per the manufacturer's instructions. The assay was performed by using 10,000 cells per condition, and each sample was tested in duplicate. Cells were plated in basal medium StemSpan (Stemcell Technologies) with or without drug treatments, and the proliferation was evaluated after 48 h. Absorbance was quantified by using Spectramax plate reader. Proliferation was expressed as fold increase relative to the effect of mitomycin (1 μM), which inhibits proliferation.

Migration assay. As described before (25), migration of CD34$^+$ cells was evaluated by the QCM Chemotaxis cell migration assay kit (EMD Millipore) as per the manufacturer's instructions. Assay was carried out by using 20,000 cells per treatment in a basal medium, HBSS (Mediatech), and each sample was tested in duplicate. Cells were allowed to migrate in response to the treatments for 5 h, and the response was expressed as arbitrary fluorescence units.

Adhesion assay. Adhesion assay was carried out as described in a previous study (27). Briefly, peripheral blood MNCs (1 million) or CD34$^+$ cells (5×10^4) were suspended in EBM-2 medium supplemented with EGM-2MV SingleQuots and plated in each well of a 6-well or a 24-well plate, respectively. The plates were coated with fibronectin (Sigma) at least an hour before plating the cells. In case of MNCs, after 4 h of plating, the nonadherent cells were collected and were replated on another fibronectin-coated well. Cells were treated with 100 nM Ang II once a day for 4 days. On *day 4*, nonadherent cells were removed by PBS wash before acquiring bright field images. In some experiments, adhesion was evaluated 4 h following replating of MNCs. In case of CD34$^+$ cells, which are nonadherent by nature, treated with Ang II once a day for 4 days following plating on fibronectin. On *day 4*, nonadherent cells were removed by using PBS before acquiring bright field images of adhered cells. Images of adhered cells were obtained by using a 4× objective lens Olympus microscope. Adhered cells were enumerated by using ImageJ software (National Institutes of Health).

Drugs. Norleu^{3}Angiotensin-(1–7), Angiotensin-(1–7), Angiotensin II, and A779 were obtained from Bachem Americas (Torrance, CA). Losartan and apocynin were purchased from Sigma (St. Louis, MO). DIZE and DX600 were purchased from LKT Laboratories (St. Paul, MN) and Anaspec (Fremont, CA), respectively. MLN-4760 was purchased from EMD Millipore. Xanthenone Derivative, 1-[[2-(dimethylamino)ethyl]amino]-4-(hydroxymethyl)-7-[[(4-methylphenyl)sulfonyl]oxy]-9H-xanthen-9-one (XNT), and MLN was synthesized in the Core Synthesis Facility, North Dakota State University (Fargo, ND) according to the method reported by Archer et al. (2) with major modifications (see Supplemental Material).

Data analysis and statistics. Results were expressed as means ± SE, and *n* represents the number of donors used. Results were analyzed for statistical significance by using the software program GraphPad (GraphPad Prism). Either *t*-test or one-way ANOVA with Bonferroni's post-test for multiple comparisons was used where appropriate. Power analysis was carried out post hoc by using Minitab software program, and all experiments are powered at 0.8 or higher.

RESULTS

Expression of ACE2/Mas is higher in lin$^-$ cells and CD34$^+$ cells. Evaluation of the expression of RAS components by real-time PCR identified mRNA transcripts of ACE, ACE2, AT$_1$R, AT$_2$R, and Mas receptor in MNCs, Lin$^-$ cells, and CD34$^+$ cells. The data were analyzed for comparing the expression of a member of RAS in three different cell populations. The expression of Mas was found to be higher (low ΔCt) in Lin$^-$ ($P<0.05$) or CD34$^+$ ($P < 0.01$) cells compared with MNCs (Fig. 1*A*). ACE2 expression showed similar trend as Mas, higher expression in Lin$^-$ or CD34$^+$ ($P < 0.01$) cells compared with MNCs (Fig. 1*A*). In contrast, ACE expression is similar among all three different cell populations (Fig. 1*A*); however, the expression of AT$_1$R or AT$_2$R is not significantly different among different cell populations (Fig. 1*A*). In MNCs, expression of ACE is higher than ACE2 ($P < 0.001$). In CD34$^+$ cells, expression of Mas is higher than AT$_1$R or AT$_2$R ($P < 0.01$). Furthermore, protein expression of ACE, ACE2, AT$_1$, and Mas were confirmed by either flow cytometry (Fig. 1*B*), or by activity assay (Fig. 1*E*) or by Western blotting (Fig. 1, *C* and *D*) in MNCs and CD34$^+$ cells. Specificity of AT$_1$R antibody was confirmed by RNA silencing approach. Treatment with siRNA resulted in robust decrease in the mRNA expression of AT$_1$R compared with the untreated control ($P < 0.001$) or the scramble-siRNA-treated cells ($P < 0.005$; $n = 6$)

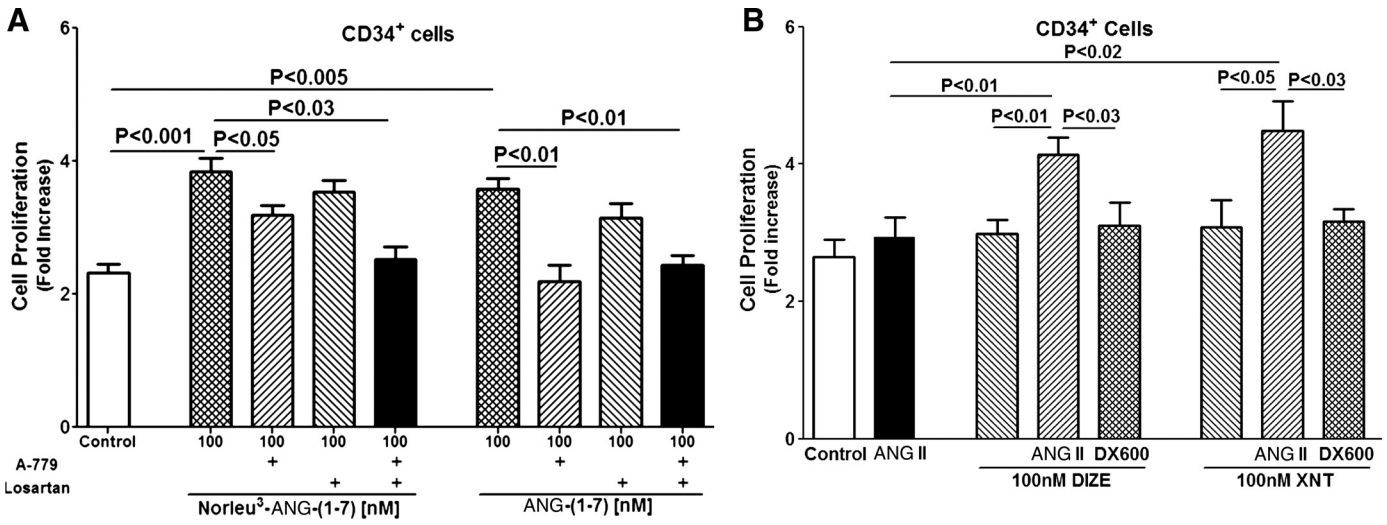


Fig. 2. *A*: effect of Ang-(1–7), Norleu^3-Ang-(1–7), or ACE2 activators on proliferation of CD34$^+$ cells. Norleu3-Ang-(1–7) and Ang-(1–7) stimulated proliferation of CD34$^+$ cells ($n = 8$). Proliferation induced by Norleu^3-Ang-(1–7) (100 nM) was inhibited by A779 ($n = 8$) or by combination of A779 and losartan ($n = 5$). Along similar lines, the effect of Ang-(1–7) was inhibited by A779 ($n = 8$), which was not affected by losartan, alone or in combination with A-779 ($n = 8$; 2-sample *t*-test). *B*: Ang II has no effect on proliferation. Diminazene aceturate (DIZE) and xanthenone (XNT) have stimulated proliferation in the presence of Ang II, and this response was blocked by DX-600 ($n = 5$; paired *t*-test).

(Fig. 1*C*). In the siRNA-treated MNCs, detection of the AT$_1$R protein by the antibody, ab124734, was almost absent, whereas protein expression was apparent in the scramble-siRNA-treated cells, compared with the untreated control ($n = 6$) (Fig. 1*C*).

Western blotting and ACE2-activity assay indicated no correlation between protein or activity and mRNA levels. Despite the higher mRNA expression in CD34$^+$ cells, the protein expression of ACE, ACE2, and AT$_1$R were lower than those observed in MNCs (Fig. 1*D*). Consistent with this, ACE2 activity was higher in MNCs compared with CD34$^+$ cells ($P < 0.001$; $n = 6$) (Fig. 1*E*).

Activation of Mas receptor or ACE2 promotes proliferation of CD34$^+$ cells. Both Ang-(1–7) and Norleu^3-Ang-(1–7) stimulated proliferation of CD34$^+$ cells (Fig. 2*A*). The response was comparable with that produced by 100 nM SDF. The effect of Norleu^3-Ang-(1–7) was partially antagonized by 1 μM A779. Losartan (1 μM), an AT$_1$R antagonist, alone did not affect this response; however, to our surprise, with the combination of A779 and losartan, the effect was completely antagonized (Fig. 2*A*). The effect of Ang-(1–7) was sensitive to blockade by Mas receptor antagonist A779 and was not affected by losartan. In agreement with these findings activators of ACE2 stimulated proliferation in CD34$^+$ cells. In the presence of 100 nM Ang II, 100 nM DIZE or 100 nM XNT increased the proliferation of CD34$^+$ cells compared with Ang II alone (Fig. 2*B*). The effect of either XNT or DIZE was inhibited by an ACE2 blocker 1 μM DX600. Neither DIZE nor XNT affected proliferation in the absence of Ang II, a substrate for ACE2 (data not shown). Along the similar lines, Ang II alone did not affect the proliferation of CD34$^+$ cells (Fig. 2*B*).

PTEN has been shown to be a major regulator of human and murine hematopoietic stem cell functions (30). PTEN opposes the proliferation of human CD34$^+$ cells by negatively regulating phosphatidylinositol-3,4,5 trisphosphate/AKT signaling

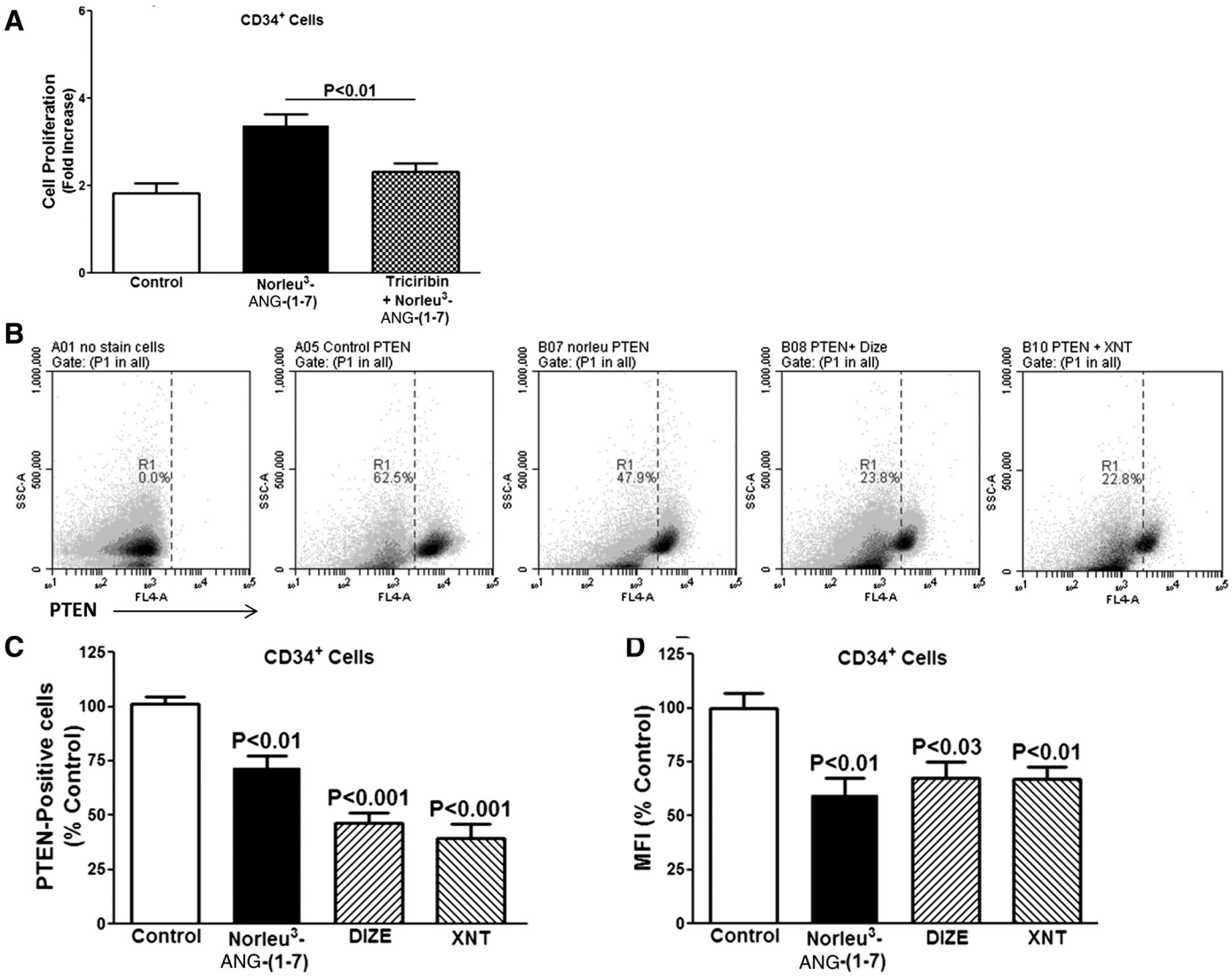
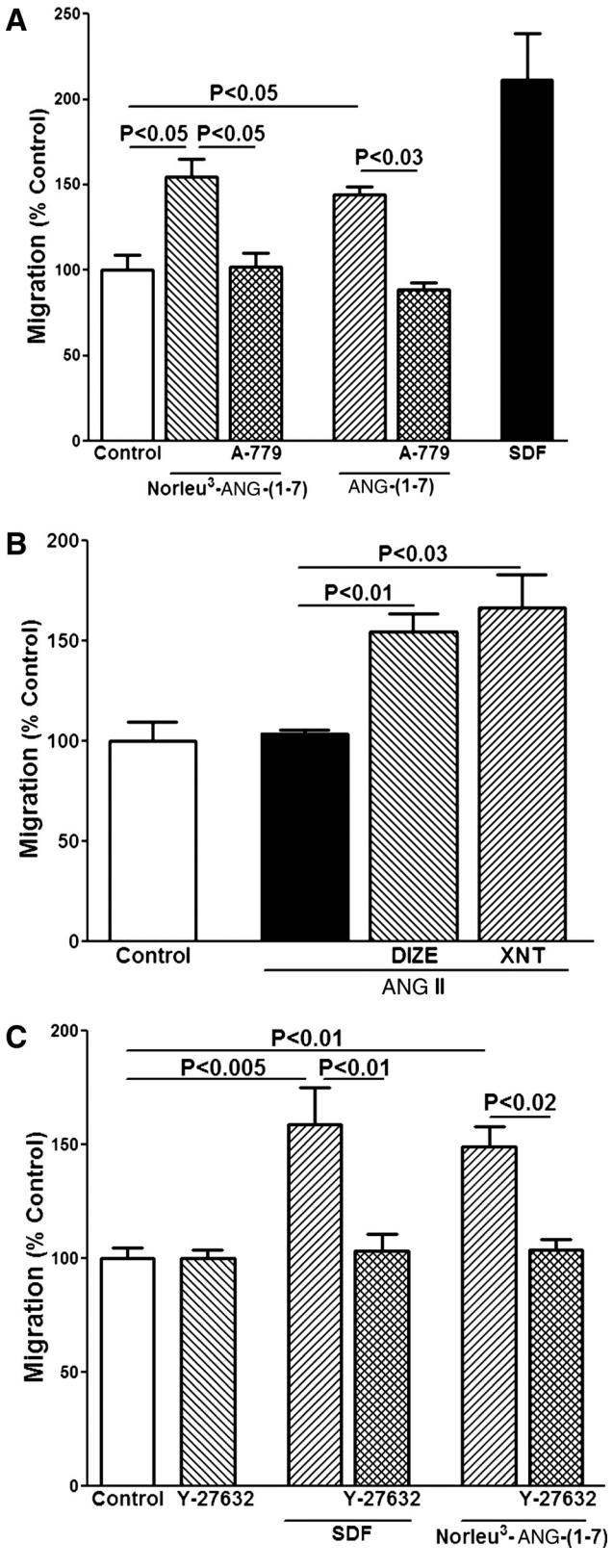


Fig. 3. Proliferation of CD34$^+$ cells by Norleu^3-Ang-(1–7) involves modulation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-Akt pathway. *A*: Norleu^3-Ang-(1–7)-induced proliferation was inhibited by Akt-inhibitor Triciribin (1 μM; $n = 5$). *B*: a representative flow cytometric dot plots of PTEN-positive CD34$^+$ cells with and without different treatments. Decreased number of PTEN-expressing cells (*C*) and decreased PTEN-fluorescence (*D*) in CD34$^+$ cells were observed following treatments with Norleu^3-Ang-(1–7), DIZE, or XNT ($n = 7$–9; 1-way ANOVA). MFI, mean fluorescence intensity.

pathway (30). We have previously shown that proliferation and survival of CD34$^+$ cells induced by Ang-(1–7) is phosphatidylinositol-3-kinase (PI3K)/Akt-dependent (24). In the presence of an AKT inhibitor, triciribin, Norleu^3-Ang-(1–7)-induced proliferation was significantly inhibited ($P < 0.01$; $n = 5$) (Fig. 3*A*). We then checked the levels of PTEN in cells proliferating in response to Norleu^3-Ang-(1–7). Flow cytometric evaluation of PTEN in CD34$^+$ cells indicated significant decrease in the number of cells expressing PTEN-expressing cells ($P < 0.01$) as well as the mean fluorescence intensity ($P < 0.01$; $n = 9$) following treatment with Norleu^3-Ang-(1–7) compared with the untreated (Fig. 3, *B–D*). Furthermore, the effects of DIZE or XNT were evaluated on PTEN levels in CD34$^+$ cells. As predicted, both of these molecules produced significant decrease in the number of PTEN-expressing cells and the fluorescence intensity ($n = 7$–9) (Fig. 3, *B–D*).



Activation of Mas receptor or ACE2 promotes migration of CD34$^+$ cells. Both Ang-(1–7) and Norleu^3-Ang-(1–7) stimulated migration of CD34$^+$ cells ($P < 0.05$; $n = 7$–9; Fig. 4*A*). However, the effects were not concentration dependent (not shown). The responses are comparable with that produced by SDF (Fig. 4*A*). Effects of both Ang-(1–7) and Norleu^{3}Ang-(1–7) were inhibited by A779 (Fig. 4*A*). XNT or DIZE, on their own, did not affect the migration of CD34$^+$ cells (data not shown). In the presence of Ang II, migration was significantly enhanced by DIZE ($P < 0.01$; $n = 5$) or XNT ($P < 0.03$; $n = 5$) (Fig. 4*B*). Ang II alone did not affect migratory response. We further observed that migration induced by Norleu^3-Ang-(1–7) was significantly inhibited by Rho-kinase (ROCK) inhibitor Y-27632 (10 μM; $P < 0.02$; $n = 6$) (Fig. 4*C*). Similar blockade was also observed in SDF-induced migration ($P < 0.01$; $n = 6$; Fig. 4*C*).

Ang II attenuates migration and proliferation of CD34$^+$ cells by acting on MNCs. As shown above, Ang II, on its own, did not affect basal migration or proliferation of CD34$^+$ cells (Figs. 2 and 3). We then tested whether Ang II could modulate CD34$^+$ cell functions indirectly via acting on MNCs. This was accomplished by the treatment of MNCs with 100 nM Ang II, as described above, followed by isolation of CD34$^+$ cells. Pretreatment with Ang II before isolation significantly attenuated SDF-mediated migration (Fig. 5*A*) or proliferation (Fig. 5*B*) in CD34$^+$ cells. Ang II is known to activate NADPH-oxidase and to increase the generation of reactive oxygen species (ROS), such as superoxide, (35) which could in turn impair physiological functions of CD34$^+$ cells including migration (25). Therefore, we tested whether the impaired migration and proliferation were due to the activation of NADPH-oxidase by Ang II in MNCs. This was achieved by treatment of MNCs with apocynin (300 μM), an inhibitor of NADPH-oxidase, during Ang II treatment. Treatment with apocynin reversed Ang II-induced impairment in SDF-migration (Fig. 5*A*) or proliferation (Fig. 5*B*) in CD34$^+$ cells. To our surprise, in the presence of apocynin, basal and stimulated migration

Fig. 4. Effects of Ang-(1–7), Norleu^3-Ang-(1–7), or ACE2 activators on migration of CD34$^+$ cells. *A*: migration was expressed as percentage of response observed in untreated/control cells. Migration induced by 100 nM Ang-(1–7) or Norleu^3-Ang-(1–7) was inhibited by A779 ($n = 5$–9; 1-way ANOVA). *B*: ACE2 activators, DIZE or XNT (100 nM), have stimulated migration in the presence of Ang II ($n = 5$; paired *t*-test). *C*: migration induced by Norleu^3-Ang-(1–7) or stromal derived factor-1α (SDF) is Rho-kinase-dependent: Rho-kinase inhibitor Y-27632 (10 μM) did not affect basal migration on its own. Migration induced by SDF or Norleu^3-Ang-(1–7) ($n = 6$; 2-sample *t*-test) was inhibited by Y-27632.

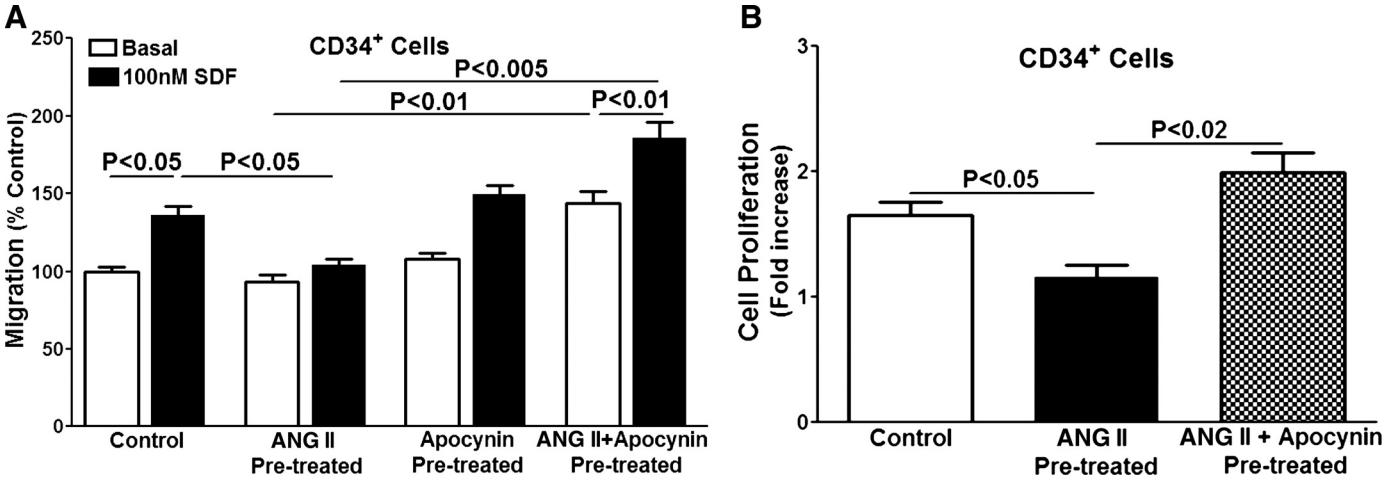


Fig. 5. Ang II attenuates migration and proliferation of CD34$^+$ cells via acting on MNCs. CD34$^+$ cells were isolated from MNCs that were treated with 100 nM Ang II. This pretreatment resulted in decreased migration of CD34$^+$ cells to SDF (n = 6–10; *A*) and decreased proliferation (n = 6; 2-sample t-test; *B*). Migration was expressed as percentage of response observed in untreated/control cells. The effect of Ang II on migration (n = 9; *A*) and proliferation (n = 5; *B*) was reversed by apocynin-treatment of MNCs.

was increased in Ang II-pretreated cells, compared with the responses in cells-treated by apocynin only.

Ang II promotes adhesion of both MNCs and CD34$^+$ cells. Finally, we tested whether Ang II or Ang-(1–7) alters the adhesion of MNCs or CD34$^+$ cells. We tested the adhesion of MNCs or CD34$^+$ cells to fibronectin, an extracellular matrix protein extensively used for studying vasoreparative functions of stem/progenitor cells. The integrin $\alpha_4\beta_1$ is known to mediate CD34$^+$ cell adhesion to endothelial cells and to extracellular matrix proteins such as fibronectin (28, 52). Ang-(1–7) did not modify adhesion of CD34$^+$ cells or MNCs on fibronectin (data not shown). Ang II increased the adhesion of MNCs to fibronectin following treatment for 4 days ($P < 0.05$; $n = 6$) of plating, compared with the untreated cells (Fig. 6*A*). Interestingly, adhesion of CD34$^+$ cells was also enhanced by Ang II, when evaluated after 4-day treatment ($P < 0.04$ vs. untreated) (Fig. 6*B*).

DISCUSSION

This study reports several novel findings. ACE2/Mas expression is higher in primitive Lin$^-$ or CD34$^+$ cells compared with MNCs. Norleu^3-Ang-(1–7) is as potent as Ang-(1–7) in inducing migration or proliferation in human CD34$^+$ cells. Activation of MNCs by Ang II decreased proliferation and migration of CD34$^+$ cells most likely by stimulating the generation of ROS by NADPH-oxidase. Adhesion of both MNCs and CD34$^+$ cells was enhanced by Ang II. Thus, ACE2/Ang-(1–7) pathway produces vascular repair-relevant functions of CD34$^+$ cells, whereas Ang II attenuates these functions indirectly by acting on MNCs.

Local RAS in CD34$^+$ cells. This is the first study to evaluate the expression of local RAS in human CD34$^+$ cells at mRNA and protein levels. CD34$^+$ cells are primitive cells and are enriched in Lin$^-$ cells. According to the present study, among all the five members of RAS, gene expression of Mas and ACE2 are higher in these primitive cells that have reparative functions, compared with the expression in MNCs, a fully differentiated cell population. On the other hand, ACE is expressed to a similar extent in both primitive and differentiated cells though the expression is lesser than ACE2. Our findings are in agreement with a recent study by Uz et al. (50) that characterized the mRNA expression of RAS members in bone marrow CD34$^+$ cells derived from healthy individuals and patients with multiple myeloma. Interestingly, despite the higher mRNA expression, the steady-state protein levels were lower in CD34$^+$ cells compared with MNCs.

ACE2/Ang-(1–7)/Mas axis stimulates CD34$^+$ cell functions that are relevant for cardiovascular repair. Proliferation and migration of CD34$^+$ cells are functional signatures of the reparative properties. In this study, we show that activation of Mas receptor by Ang-(1–7) or Norleu^3-Ang-(1–7), or activation of ACE2 by DIZE or XNT stimulates proliferation of CD34$^+$ cells. Norleu^3-Ang-(1–7) is relatively resistant to degradation by proteases (40) and has been shown to accelerate wound healing in rodent models that was Mas receptor dependent (41, 42). These findings are in agreement with the previous study (19) that evaluated the effects of Ang-(1–7) in vitro and in vivo in cord blood-derived CD34$^+$ cells.

Proliferation induced by Norleu^3-Ang-(1–7) is higher than Ang-(1–7), although did not achieve significance. Although the effect of Ang-(1–7) was completely antagonized by A779, the effect of Norleu^3-Ang-(1–7) was partially antagonized. Combination of A779 with losartan resulted in complete blockade of Norleu^3-Ang-(1–7)-induced proliferation. Interestingly, Ang II did not induce proliferation, and losartan alone did not antagonize response to Norleu^3-Ang-(1–7). Collectively, these findings indicate a complex interaction between Mas and AT$_1$ receptors, possibly involving a binding site or intracellular mechanism that is not activated by Ang II. Previously, functional studies in murine myocardium provided evidence for interaction of these two receptors (6), although the intracellular mechanisms of this interaction were not characterized.

We then delineated the mechanisms involved in the increased proliferation in response to Norleu^3-Ang-(1–7). Inhibition of PTEN-mediated negative regulation of PI3K/AKT pathway by Norleu^3-Ang-(1–7) appears to promote proliferation in response to Mas receptor activation. PTEN is a nonredundant phosphatase and a negative regulator of PI3K-AKT

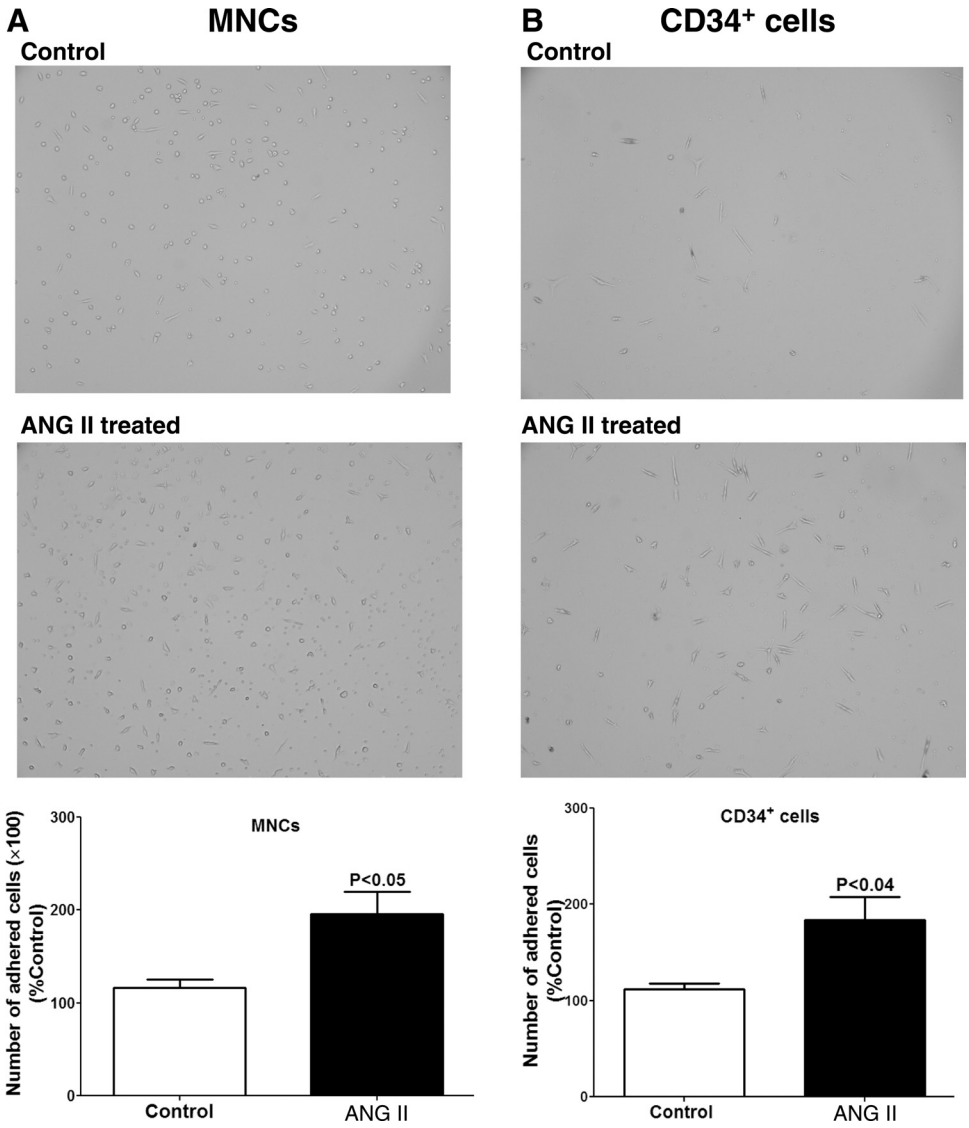


Fig. 6. Ang II increases adhesion of MNCs and CD34$^+$ cells. *A*: representative bright field images of MNCs adhered to fibronectin with or without 100 nM Ang II treatment, and the bar graph depicting the effect of Ang II. Number of cells adhered were higher compared with control following Ang II treatment ($n = 6$). *B*: representative bright field images of CD34$^+$ cells adhered to fibronectin with or without 100 nM Ang II treatment, and the bar graph depicting the effect of Ang II. Number of cells adhered were increased by Ang II treatment compared with control ($n = 6$).

pathway, which in turn regulates HSC proliferation and differentiation (56). Our findings suggested that Norleu^3-Ang-(1–7)-induced proliferation was associated with a decrease in PTEN levels. A decrease in PTEN enhances accumulation of AKT, which promotes proliferation and self-renewal without inducing leukemic response, whereas complete deficiency of PTEN increases the risk for leukemia (54, 56). Thus PTEN is cell-autonomously required for the maintenance of stem/progenitor cells. The current study suggests that activation of Mas receptor does not induce leukemic response in human CD34$^+$ cells, and thus modification of CD34$^+$ cell functions by Ang-(1–7) or by ACE2 activators for cell-based therapies would most likely be free from myeloproliferative side effects.

Migration to the areas of ischemia is an important property of CD34$^+$ cells that determines the reparative function of these cells. This function is modulated mainly by hypoxia-regulated factors such as SDF and VEGF (34). Current study shows that activation of ACE2 or Mas receptor induces migration of these cells that is comparable with the response produced by SDF. Effects of both Ang-(1–7) and Norleu^3-Ang-(1–7) on migration were mediated by the activation of Mas receptor. This is in contrast with the effect on proliferation, which was partly mediated by AT$_1$ receptor (see above). This study further confirmed the beneficial effects of putative ACE2 activators, XNT and DIZE, on migration of CD34$^+$ cells, and the presence of Ang II is necessary for their effects. Importantly, the effects were sensitive to blockade by a known ACE2 inhibitor, DX600 (22). ACE2 activation by these novel molecules has been confirmed in several in vitro and in vivo studies (12, 14, 38, 45, 49), whereas a recent study proposed ACE2-independent effects on cardiovascular hemodynamics (18). The in vitro experimental approach that was used in the current study, and the use of selective blocker of ACE2 confirms that the effects produced by these molecules in CD34$^+$ cells are ACE2 dependent. Furthermore, the present study shows that migration induced by Mas receptor activation was partially sensitive to blockade by Rho-kinase inhibitor Y-27632. ROCK promotes migration of cells by actin polymerization (46), which is the mediator of migratory response to SDF (51), and other growth factors, such as VEGF and PDGF (17, 55). Sensitivity of Mas-receptor-mediated migration to

the ROCK blockade suggests that Mas receptor, in part, acts through mechanisms that are activated by growth factors in $CD34^+$ cells. However, involvement of alternative pathways in the response mediated by $Norleu^3$-Ang-(1–7) cannot be ruled out since the blockade by Y-27632 is partial.

Ang II attenuates $CD34^+$ cell functions by acting on MNCs. In the current study, Ang II did not affect the proliferation or migration of $CD34^+$ cells in vitro, although the mRNA transcript and protein for AT_1 receptor were identified. Earlier studies have shown evidence for hematopoietic functions of Ang II in murine and human primitive progenitor cells (43). In vitro treatment with Ang II robustly enhanced differentiation of human $CD34^+$ cells to Colony Forming Units via activating AT_1 receptor; however, these studies did not evaluate proliferation without differentiation.

This study further evaluated the indirect effects of Ang II on the functions of $CD34^+$ cells by activating other cell types. Ang II is known to modulate functions of different subsets of mononuclear leucocytes and lymphocytes via activating AT_1 receptor and largely by inducing NADPH-dependent generation of ROS and oxidative stress (13). Reparative functions of $CD34^+$ cells were shown to be attenuated by increased oxidative stress (25). In the current study, treatment of the whole MNC population with Ang II before isolation of $CD34^+$ cells resulted in significant attenuation of proliferation and migration. Given the fact that $CD34^+$ cells circulate with several of MNCs in the near vicinity, sustained elevation in the circulating Ang II would attenuate $CD34^+$ reparative functions by increasing local oxidative stress in MNC-dependent mechanism. Although this study focused at the interaction between MNCs and $CD34^+$ cells, the role of other inflammatory cells that are reactive to the Ang II cannot be ruled out and would likely amplify the attenuation of $CD34^+$ cell functions.

Taken together, findings from studies involving apocynin and ROCK inhibitor imply either no interaction between ROS and RhoA/ROCK pathway or negative modulation of RhoA/ROCK activation by ROS. Inhibiting ROS increased migration of $CD34^+$ cells, which is consistent with our previous study (25). Another study in monocytes showed compelling evidence for the attenuation of RhoA/ROCK activation and decreased migration by ROS (1). Therefore, in the current study, NADPH-derived ROS attenuated $CD34^+$ cells migration very likely via attenuating ROCK activation, which is in contrast with several studies in vascular preparations that have consistently shown that ROS activates RhoA/ROCK pathway leading to increased vascular smooth muscle contraction (31, 35).

Adhesion is another important characteristic of $CD34^+$ cells, which determines homing of cells to the areas needing vascular repair (29, 57). We have tested the property of adhesion on fibronectin because it is a ligand for the integrin $\alpha_4\beta_1$, which is highly expressed in $CD34^+$ cells (29). Fibronectin is highly expressed on proliferating endothelial cells (10) and in endothelial cells very early following vascular injury (8). Thus the selective expression of fibronectin in the areas of injury promotes homing of vasoreparative cells and accelerates vascular or tissue repair. $CD34^+$ cells use α_4 integrin to adhere to fibronectin-producing endothelium that facilitates extravasation of cells into damaged/inflamed tissue (16, 36). Although Ang-(1–7) did not have any effect on adhesion of $CD34^+$ cells or MNCs, Ang II enhanced adhesion of both MNCs and $CD34^+$ cells, likely by functional modification of integrins. Although this study did not characterize functional modification of integrins that were specifically affected by Ang II, we did observe that the effect of Ang II could be reversed by inhibiting ROS generation. Inhibition of NADPH oxidase by apocynin reversed Ang II-mediated increase in the adhesion of MNCs or $CD34^+$ cells, suggesting that Ang II-stimulated ROS generation increased the adhesion of cells, likely by modifying the integrin-fibronectin interactions. Unlike migration and proliferation, adhesion was enhanced by direct actions of Ang II on $CD34^+$ cells. It is important to note that adhesion assay involved treatment of Ang II for a longer period, once a day for 4 days, and that the expression of AT_1 receptor is very low. Although the adhesion of $CD34^+$ cells to fibronectin is beneficial for vascular repair, adhesion of MNCs will not promote vascular repair and may even attenuate this process (28, 47). These findings are in agreement with the previous report that MNCs from hypertensive patients or those activated by Ang II adhere more compared with the MNCs from healthy individuals (47).

Conclusions

Overall, the current study shows that both cardiovascular protective and deleterious arms of RAS modulate the vasoreparative functions of $CD34^+$ cells. ACE2/Ang-(1–7)/Mas axis activates vasoprotection-relevant functions of these cells. In contrast, ACE/Ang II/AT_1 axis either directly or indirectly via acting on MNCs attenuates $CD34^+$ cell functions. Therefore, relative expression of the two axes of local RAS in $CD34^+$ cells could be a good measure of their vasoreparative functions. Along the similar lines, an imbalance toward increased expression of ACE/Ang II/AT_1 axis would promote dysfunction of $CD34^+$ cells and could be a prognostic marker for an increased risk for the development of cardiovascular disease.

ACKNOWLEDGMENTS

We thank the staff at the United Blood Systems, Fargo, ND, for kindly providing leucocyte samples for the study.

GRANTS

This study is partly supported by a Scientist Development Grant from the American Heart Association. The Core Synthesis facility at North Dakota State University is supported by P30 GM103332-01 from the National Center for Research Resources (National Institutes of Health).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.S., S.J., L.G., M.B., and Y.L. performed experiments; N.S., S.J., and L.G. analyzed data; N.S. prepared figures; N.S. and Y.P.J. drafted manuscript; M.B., R.C., and Y.P.J. interpreted results of experiments; R.C., M.K.R., and Y.P.J. approved final version of manuscript; M.K.R. and Y.P.J. edited and revised manuscript; Y.P.J. conception and design of research.

REFERENCES

1. **Aflaki E, Balenga NA, Luschnig-Schratl P, Wolinski H, Povoden S, Chandak PG, Bogner-Strauss JG, Eder S, Konya V, Kohlwein SD, Heinemann A, Kratky D.** Impaired Rho GTPase activation abrogates cell polarization and migration in macrophages with defective lipolysis. *Cell Mol Life Sci* 68: 3933–3947, 2011.

2. **Archer S, Pica-Mattoccia L, Cioli D, Seyed-Mozaffari A, Zayed AH.** Preparation and antischistosomal and antitumor activity of hycanthone and some of its congeners. Evidence for the mode of action of hycanthone. *J Med Chem* 31: 254–260, 1988.
3. **Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM.** Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275: 964–967, 1997.
4. **Benicky J, Hafko R, Sanchez-Lemus E, Aguilera G, Saavedra JM.** Six commercially available angiotensin II AT_1 receptor antibodies are nonspecific. *Cell Mol Neurobiol* 32: 1353–1365, 2012.
5. **Berenson RJ, Bensinger WI, Hill RS, Andrews RG, Garcia-Lopez J, Kalamasz DF, Still BJ, Spitzer G, Buckner CD, Bernstein ID.** Engraftment after infusion of $CD34^+$ marrow cells in patients with breast cancer or neuroblastoma. *Blood* 77: 1717–1722, 1991.
6. **Castro CH, Santos RA, Ferreira AJ, Bader M, Alenina N, Almeida AP.** Evidence for a functional interaction of the angiotensin-(1–7) receptor Mas with AT_1 and AT_2 receptors in the mouse heart. *Hypertension* 46: 937–942, 2005.
7. **Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH.** Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol* 133: 157–165, 1984.
8. **Coito AJ, Brown LF, Peters JH, Kupiec-Weglinski JW, van de Water L.** Expression of fibronectin splicing variants in organ transplantation: a differential pattern between rat cardiac allografts and isografts. *Am J Pathol* 150: 1757–1772, 1997.
9. **Demirer T, Bensinger WI, Buckner CD.** Peripheral blood stem cell mobilization for high-dose chemotherapy. *J Hematother* 8: 103–113, 1999.
10. **Elices MJ, Tsai V, Strahl D, Goel AS, Tollefson V, Arrhenius T, Wayner EA, Gaeta FC, Fikes JD, Firestein GS.** Expression and functional significance of alternatively spliced CS1 fibronectin in rheumatoid arthritis microvasculature. *J Clin Invest* 93: 405–416, 1994.
11. **Ferreira AJ, Santos RA, Bradford CN, Mecca AP, Sumners C, Katovich MJ, Raizada MK.** Therapeutic implications of the vasoprotective axis of the renin-angiotensin system in cardiovascular diseases. *Hypertension* 55: 207–213, 2010.
12. **Ferreira AJ, Shenoy V, Yamazato Y, Sriramula S, Francis J, Yuan L, Castellano RK, Ostrov DA, Oh SP, Katovich MJ, Raizada MK.** Evidence for angiotensin-converting enzyme 2 as a therapeutic target for the prevention of pulmonary hypertension. *Am J Respir Crit Care Med* 179: 1048–1054, 2009.
13. **Fortuno A, Olivan S, Beloqui O, San Jose G, Moreno MU, Diez J, Zalba G.** Association of increased phagocytic NADPH oxidase-dependent superoxide production with diminished nitric oxide generation in essential hypertension. *J Hypertension* 22: 2169–2175, 2004.
14. **Fraga-Silva RA, Costa-Fraga FP, Murca TM, Moraes PL, Martins Lima A, Lautner RQ, Castro CH, Soares CM, Borges CL, Nadu AP, Oliveira ML, Shenoy V, Katovich MJ, Santos RA, Raizada MK, Ferreira AJ.** Angiotensin-converting enzyme 2 activation improves endothelial function. *Hypertension* 61: 1233–1238, 2013.
15. **Fraga-Silva RA, Sorg BS, Wankhede M, Dedeugd C, Jun JY, Baker MB, Li Y, Castellano RK, Katovich MJ, Raizada MK, Ferreira AJ.** ACE2 activation promotes antithrombotic activity. *Mol Med* 16: 210–215, 2010.
16. **Gigant C, Latger-Cannard V, Bensoussan D, Feugier P, Bordigoni P, Stoltz JF.** Quantitative expression of adhesion molecules on granulocyte colony-stimulating factor-mobilized peripheral blood, bone marrow, and cord blood $CD34^+$ cells. *J Hematotherapy Stem Cell Res* 10: 807–814, 2001.
17. **Gong C, Stoletov KV, Terman BI.** VEGF treatment induces signaling pathways that regulate both actin polymerization and depolymerization. *Angiogenesis* 7: 313–321, 2004.
18. **Haber PK, Ye M, Wysocki J, Maier C, Haque SK, Batlle D.** Angiotensin-converting enzyme 2-independent action of presumed angiotensin-converting enzyme 2 activators: studies in vivo, ex vivo, and in vitro. *Hypertension* 63: 774–782, 2014.
19. **Heringer-Walther S, Eckert K, Schumacher SM, Uharek L, Wulf-Goldenberg A, Gembardt F, Fichtner I, Schultheiss HP, Rodgers K, Walther T.** Angiotensin-(1–7) stimulates hematopoietic progenitor cells in vitro and in vivo. *Haematologica* 94: 857–860, 2009.
20. **Hernandez Prada JA, Ferreira AJ, Katovich MJ, Shenoy V, Qi Y, Santos RA, Castellano RK, Lampkins AJ, Gubala V, Ostrov DA, Raizada MK.** Structure-based identification of small-molecule angiotensin-converting enzyme 2 activators as novel antihypertensive agents. *Hypertension* 51: 1312–1317, 2008.
21. **Herrera M, Sparks MA, Alfonso-Pecchio AR, Harrison-Bernard LM, Coffman TM.** Lack of specificity of commercial antibodies leads to misidentification of angiotensin type 1 receptor protein. *Hypertension* 61: 253–258, 2013.
22. **Huang L, Sexton DJ, Skogerson K, Devlin M, Smith R, Sanyal I, Parry T, Kent R, Enright J, Wu QL, Conley G, DeOliveira D, Morganelli L, Ducar M, Wescott CR, Ladner RC.** Novel peptide inhibitors of angiotensin-converting enzyme 2. *J Biol Chem* 278: 15532–15540, 2003.
23. **Iusuf D, Henning RH, van Gilst WH, Roks AJ.** Angiotensin-(1–7): pharmacological properties and pharmacotherapeutic perspectives. *Eur J Pharmacol* 585: 303–312, 2008.
24. **Jarajapu YP, Bhatwadekar AD, Caballero S, Hazra S, Shenoy V, Medina R, Kent D, Stitt AW, Thut C, Finney EM, Raizada MK, Grant MB.** Activation of the ACE2/angiotensin-(1–7)/Mas receptor axis enhances the reparative function of dysfunctional diabetic endothelial progenitors. *Diabetes* 62: 1258–1269, 2013.
25. **Jarajapu YP, Caballero S, Verma A, Nakagawa T, Lo MC, Li Q, Grant MB.** Blockade of NADPH oxidase restores vasoreparative function in diabetic $CD34^+$ cells. *Invest Ophthalm Vis Sci* 52: 5093–5104, 2011.
26. **Jarajapu YP, Grant MB.** The promise of cell-based therapies for diabetic complications: challenges and solutions. *Circ Res* 106: 854–869, 2010.
27. **Jarajapu YP, Hazra S, Segal M, LiCalzi S, Jhadao C, Qian K, Mitter SK, Raizada MK, Boulton ME, Grant MB.** Vasoreparative dysfunction of $CD34^+$ cells in diabetic individuals involves hypoxic desensitization and impaired autocrine/paracrine mechanisms. *PloS One* 9: e93965, 2014.
28. **Jin H, Aiyer A, Su J, Borgstrom P, Stupack D, Friedlander M, Varner J.** A homing mechanism for bone marrow-derived progenitor cell recruitment to the neovasculature. *J Clin Invest* 116: 652–662, 2006.
29. **Jin H, Su J, Garmy-Susini B, Kleeman J, Varner J.** Integrin $\alpha_4\beta_1$ promotes monocyte trafficking and angiogenesis in tumors. *Cancer Res* 66: 2146–2152, 2006.
30. **Kim I, Kim YJ, Metais JY, Dunbar CE, Larochelle A.** Transient silencing of PTEN in human $CD34^+$ cells enhances their proliferative potential and ability to engraft immunodeficient mice. *Exp Hematol* 40: 84–91, 2012.
31. **Knock GA, Ward JP.** Redox regulation of protein kinases as a modulator of vascular function. *Antioxid Redox Signal* 15: 1531–1547, 2011.
32. **Mackie AR, Losordo DW.** CD34-positive stem cells: in the treatment of heart and vascular disease in human beings. *Texas Heart Inst J* 38: 474–485, 2011.
33. **Miyazaki M, Takai S.** Tissue angiotensin II generating system by angiotensin-converting enzyme and chymase. *J Pharmacol Sci* 100: 391–397, 2006.
34. **Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L.** Regulation of transendothelial migration of hematopoietic progenitor cells. *Ann New York Acad Sci* 872: 176–185, 1999.
35. **Nguyen Dinh Cat A, Montezano AC, Burger D, Touyz RM.** Angiotensin II, NADPH oxidase, and redox signaling in the vasculature. *Antioxid Redox Signal* 19: 1110–1120, 2013.
36. **Papayannopoulou T, Priestley GV, Nakamoto B, Zafiropoulos V, Scott LM.** Molecular pathways in bone marrow homing: dominant role of $\alpha_4\beta_1$ over β_2-integrins and selectins. *Blood* 98: 2403–2411, 2001.
37. **Paul M, Poyan Mehr A, Kreutz R.** Physiology of local renin-angiotensin systems. *Phys Rev* 86: 747–803, 2006.
38. **Qi Y, Zhang J, Cole-Jeffrey CT, Shenoy V, Espejo A, Hanna M, Song C, Pepine CJ, Katovich MJ, Raizada MK.** Diminazene aceturate enhances angiotensin-converting enzyme 2 activity and attenuates ischemia-induced cardiac pathophysiology. *Hypertension* 62: 746–752, 2013.
39. **Rodgers K, Xiong S, DiZerega GS.** Effect of angiotensin II and angiotensin(1–7) on hematopoietic recovery after intravenous chemotherapy. *Cancer Chemother Pharmacol* 51: 97–106, 2003.
40. **Rodgers KE, Ellefson DD, Espinoza T, Maulhardt H, Roda N, Maldonado S, Dizerega GS.** Fragments of Nle-angiotensin(1–7) accelerate healing in dermal models. *J Peptide Res* 66, *Suppl* 1: 41–47, 2005.
41. **Rodgers KE, Espinoza T, Felix J, Roda N, Maldonado S, diZerega G.** Acceleration of healing, reduction of fibrotic scar, and normalization of tissue architecture by an angiotensin analogue, NorLeu3-A(1–7). *Plastic Reconstruct Surg* 111: 1195–1206, 2003.
42. **Rodgers KE, Roda N, Felix JE, Espinoza T, Maldonado S, diZerega G.** Histological evaluation of the effects of angiotensin peptides on wound repair in diabetic mice. *Exp Dermatol* 12: 784–790, 2003.

43. **Rodgers KE, Xiong S, Steer R, diZerega GS.** Effect of angiotensin II on hematopoietic progenitor cell proliferation. *Stem Cells* 18: 287–294, 2000.
44. **Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T.** Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Nat Acad Sci USA* 100: 8258–8263, 2003.
45. **Shenoy V, Gjymishka A, Jarajapu YP, Qi Y, Afzal A, Rigatto K, Ferreira AJ, Fraga-Silva RA, Kearns P, Douglas JY, Agarwal D, Mubarak KK, Bradford C, Kennedy WR, Jun JY, Rathinasabapathy A, Bruce E, Gupta D, Cardounel AJ, Mocco J, Patel JM, Francis J, Grant MB, Katovich MJ, Raizada MK.** Diminazene attenuates pulmonary hypertension and improves angiogenic progenitor cell functions in experimental models. *Am J Respirat Crit Care Med* 187: 648–657, 2013.
46. **Sit ST, Manser E.** Rho GTPases and their role in organizing the actin cytoskeleton. *J Cell Sci* 124: 679–683, 2011.
47. **Syrbe U, Moebes A, Scholze J, Swidsinski A, Dorffel Y.** Effects of the angiotensin II type 1 receptor antagonist telmisartan on monocyte adhesion and activation in patients with essential hypertension. *Hypertension Res* 30: 521–528, 2007.
48. **Tavian M, Coulombel L, Luton D, Clemente HS, Dieterlen-Lievre F, Peault B.** Aorta-associated $CD34^+$ hematopoietic cells in the early human embryo. *Blood* 87: 67–72, 1996.
49. **Thatcher SE, Zhang X, Howatt DA, Yiannikouris F, Gurley SB, Ennis T, Curci JA, Daugherty A, Cassis LA.** Angiotensin-converting enzyme 2 decreases formation and severity of angiotensin II-induced abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol* 34: 2617–2623, 2014.
50. **Uz B, Tatonyan SC, Sayitoglu M, Erbilgin Y, Hatirnaz O, Aksu S, Buyukasik Y, Sayinalp N, Goker H, Ozcebe OI, Ozbek U, Hazneda-roglu IC.** Local Renin-Angiotensin system in normal hematopoietic and multiple myeloma-related progenitor cells. *Turk J Haematol* 31: 136–142, 2014.
51. **Voermans C, Anthony EC, Mul E, van der Schoot E, Hordijk P.** SDF-1-induced actin polymerization and migration in human hematopoietic progenitor cells. *Exp Hematol* 29: 1456–1464, 2001.
52. **Wary KK, Vogel SM, Garrean S, Zhao YD, Malik AB.** Requirement of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin expression in bone-marrow-derived progenitor cells in preventing endotoxin-induced lung vascular injury and edema in mice. *Stem Cells* 27: 3112–3120, 2009.
53. **Wood HB, May G, Healy L, Enver T, Morriss-Kay GM.** CD34 expression patterns during early mouse development are related to modes of blood vessel formation and reveal additional sites of hematopoiesis. *Blood* 90: 2300–2311, 1997.
54. **Yilmaz OH, Valdez R, Theisen BK, Guo W, Ferguson DO, Wu H, Morrison SJ.** Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature* 441: 475–482, 2006.
55. **Yoshinaga K, Inoue H, Tanaka F, Mimori K, Utsunomiya T, Mori M.** Platelet-derived endothelial cell growth factor mediates Rho-associated coiled-coil domain kinase messenger RNA expression and promotes cell motility. *Ann Surg Oncol* 10: 582–587, 2003.
56. **Zhang J, Grindley JC, Yin T, Jayasinghe S, He XC, Ross JT, Haug JS, Rupp D, Porter-Westpfahl KS, Wiedemann LM, Wu H, Li L.** PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature* 441: 518–522, 2006.
57. **Zhang S, Shpall E, Willerson JT, Yeh ET.** Fusion of human hematopoietic progenitor cells and murine cardiomyocytes is mediated by alpha 4 beta 1 integrin/vascular cell adhesion molecule-1 interaction. *Circ Res* 100: 693–702, 2007.