20-HETE Regulates the Angiogenic Functions of Human Endothelial Progenitor Cells and Contributes to Angiogenesis In Vivo

Li Chen, Rachel Ackerman, Mohamed Saleh, Katherine H. Gotlinger, Michael Kessler, Lawrence G. Mendelowitz, John R. Falck, Ali S. Arbab, A. Guillermo Scicli, Michal L. Schwartzman, Jing Yang, and Austin M. Guo

Department of Pharmacology, School of Medicine, Wuhan University, Wuhan, PR China (L.C., J.Y., A.M.G.); Department of Pharmacology, New York Medical College, Valhalla, New York (L.C., R.A., M.S., K.H.G., M.L.S., A.M.G.); Department of Obstetrics and Gynecology, Westchester Medical Center, Valhalla, New York (M.K.); Obstetrics and Gynecology, Phelps Memorial Hospital Center, Sleepy Hollow, New York (L.G.M.); University of Texas Southwestern Medical Center, Dallas, Texas (J.R.F.); and Henry Ford Hospital, Detroit, Michigan (A.S.A., A.G.S.)

Received October 1, 2013; accepted January 6, 2014

ABSTRACT

Circulating endothelial progenitor cells (EPC) contribute to postnatal neovascularization. We identified the cytochrome P450 4A/F-20-hydroxyeicosatetraenoic acid (CYP4A/F-20-HETE) system as a novel regulator of EPC functions associated with angiogenesis in vitro. Here, we explored cellular mechanisms by which 20-HETE regulates EPC angiogenic functions and assessed its contribution to EPC-mediated angiogenesis in vivo. Results showed that both hypoxia and vascular endothelial growth factor (VEGF) induce CYP4A11 gene and protein expression (the predominant 20-HETE synthases in human EPC), and this is accompanied by an increase in 20-HETE production by ~1.4- and 1.8-fold, respectively, compared with the control levels. Additional studies demonstrated that 20-HETE and VEGF have a synergistic effect on EPC proliferation, whereas 20-HETE antagonist 20-HEDGE or VEGF-neutralizing antibody negated 20-HETE- or

VEGF-induced proliferation, respectively. These findings are consistent with the presence of a positive feedback regulation on EPC proliferation between the 20-HETE and the VEGF pathways. Furthermore, we found that 20-HETE induced EPC adhesion to fibronectin and endothelial cell monolayer by 40 ± 5.6 and $67 \pm 10\%$, respectively, which was accompanied by a rapid induction of very late antigen-4 and chemokine receptor type 4 mRNA and protein expression. Basal and 20-HETE-stimulated increases in adhesion were negated by the inhibition of the CYP4A-20-HETE system. Lastly, EPC increased angiogenesis in vivo by 3.6 ± 0.2 -fold using the Matrigel plug angiogenesis assay, and these increases were markedly reduced by the local inhibition of 20-HETE system. These results strengthened the notion that 20-HETE regulates the angiogenic functions of EPC in vitro and EPC-mediated angiogenesis in vivo.

Introduction

Recent developments in stem cell biology suggest that endothelial progenitor cells (EPC) contribute to postnatal neovascularization, which is an important adaptation for pathologic conditions, including wound healing, ischemia, and tumor development (Asahara et al., 1999; Kalka et al., 2000; Kocher et al., 2001; Lyden et al., 2001; Jarajapu and Grant,

2010). 20-Hydroxyeicosatetraenoic acid (20-HETE) is the ω -hydroxylation metabolite of arachidonic acid formed by cytochrome P450 enzymes, most notably the CYP4A and CYP4F families (Williams et al., 2010). It is primarily found in the microcirculation of the kidney, liver, and brain (Carroll et al., 1997; Miyata and Roman, 2005). Studies in our laboratory identified the CYP4A11-20-HETE system as a novel regulator of several EPC functions associated with the neovascularization response (Guo et al., 2011). It promotes EPC proliferation, migration, and secretion of proangiogenic molecules, such as hypoxia-inducible factor-1- α (HIF-1 α), vascular endothelial growth factor (VEGF), and stroma-derived factor (SDF)-1, as well as EPC-mediated endothelial cell tube formation (Guo et al., 2011). Furthermore, 20-HETE increases EC proliferation and migration;

This study was supported by the American Heart Association [Grant 11SDG6870004] (to A.M.G.); the National Institutes of Health National Cancer Institute [Grant 1R01-CA160216] (to A.S.A.); the National Institutes of Health [Grants HL34300 (to M.L.S.), DK38226 (to J.R.F.)]; the Robert A. Welch Foundation [Grant GL625910] (to J.R.F.); and the National Natural Science Foundation of China [Grants 30973552, 81173089] (to J.Y.).

A.M.G. and J.Y. contributed equally to this article.
dx.doi.org/10.1124/jpet.113.210120.

ABBREVIATIONS: 20-HEDGE, *N*-(20-hydroxyeicosa-6(Z),15(Z)-dienoyl) glycine; 20-HETE, 20-hydroxyeicosatetraenoic acid; CXCR4, chemokine receptor type 4; DDMS, dibromo-dodecenyl-methylsulfonamide; EC, endothelial cells; EPC, endothelial progenitor cells; FN, fibronectin; HET0016, *N*-hydroxy-*N'*-(4-*n*-butyl-2-methylphenyl) formamidine; HIF-1 α , hypoxia-inducible factor-1- α ; hMVEC, human microvascular endothelial cells; PBS, phosphate-buffered saline; SDF, stroma-derived factor; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; VLA-4 ($\alpha 4 \beta 1$), very late antigen-4 (integrin $\alpha 4 \beta 1$).

both are essential steps in the angiogenic cascade (Guo et al., 2007; Jiang et al., 2004; Guo et al., 2009). However, the underlying cellular mechanisms by which 20-HETE regulates EPC angiogenic functions and its contribution to physiologic and pathologic neovascularization in vivo is largely unknown.

The VEGF pathway is an important signaling mechanism of neovascularization. Accumulating evidence has implicated the existence of interactions between the VEGF signaling pathway and the CYP4A–20-HETE system. 20-HETE induces the expression of HIF-1 α and its downstream target VEGF in EPC and EC (Guo et al., 2007, 2009, 2011). Production of SDF-1 α and VEGF is regulated by upstream HIF-1 α (Ceradini et al., 2004; De Falco et al., 2004; Hoenig et al., 2008), and 20-HETE increases the expression of both SDF-1 and VEGF (Guo et al., 2011), suggesting that 20-HETE may be upstream of VEGF. On the other hand, Amaral et al. (2003) found that 20-HETE lies downstream of the VEGF signaling pathway for angiogenesis in skeletal muscles. In addition, the selective 20-HETE synthesis inhibitor HET0016 blocks VEGF-induced EPC proliferation and migration (Guo et al., 2011) and VEGF-mediated corneal neovascularization (Chen et al., 2005). These data are consistent with 20-HETE being downstream of the VEGF pathway. Consequently, we postulated that a positive feedback loop regulatory mechanism exists between the VEGF pathway and the 20-HETE system in EPC.

EPC contributes to the neovascularization process, which consists of four crucial steps: mobilization, homing, migration, and differentiation into endothelial cells (EC). The major chemokines and corresponding cell surface receptors that regulate EPC mobilization, adhesion, and chemotaxis are SDF-1 and chemokine receptor type 4 (CXCR4) (Hidalgo et al., 2001; De Falco et al., 2004; Guo et al., 2011). VEGF plays a critical role in the regulation of EPC function by increasing mobilization of EPC from the bone marrow and mediating their migration into the circulation (Li et al., 2006; Rosti et al., 2007). Upon entering the circulation, one of the key integrin-mediated EPC adhesion factors to fibronectin (FN), a major component of extracellular matrix (ECM) and endothelial lining of the blood vessels, is known as very late antigen 4 (VLA-4; also known as $\alpha 4\beta 1$ integrin) (Chan et al., 1992; Ruegg et al., 1992). VLA-4 regulates EPC homing to the target sites of neovascularization, where it can promote vascular growth and repair by increasing production of VEGF and other growth factors within ischemic tissues (Tepper et al., 2005).

The present study was undertaken to explore the cellular mechanisms by which 20-HETE regulates EPC angiogenic functions and assess the contribution of 20-HETE to EPC-mediated angiogenesis in Matrigel plug in vivo. Here, we demonstrate that: 1) hypoxia and VEGF induce the expression of CYP4A11, the predominant 20-HETE synthase, and increase 20-HETE production in human EPC; 2) 20-HETE synergizes with VEGF to increase EPC proliferation, VEGF neutralizing antibody, or 20-HETE antagonist (20-HEDGE) negates VEGF- or 20-HETE-induced EPC proliferation, respectively, suggesting a positive feedback loop regulatory mechanism between VEGF and 20-HETE; 3) 20-HETE induces EPC adhesion to both FN and EC monolayer via an upregulation of CXCR4 and VLA-4 expression, which may collectively lead to an increase in EPC homing, an essential component of EPC-mediated neovascularization; 4) inhibition of 20-HETE synthesis or action locally diminishes

EPC-induced angiogenesis in vivo. These findings provide strong evidence that 20-HETE is a key regulator of EPC functions and their contributions to postnatal neovascularization.

Materials and Methods

Isolation of AC133+ EPC and Cell Culture. AC133+ EPC were collected and isolated from donated human umbilical cord blood under the guidelines of the Institutional Review Board for human subject research approval. EPC cell isolation, culturing, and characterization studies were performed as previously described (Arbab et al., 2008; Janic et al., 2010; Guo et al., 2011). For hypoxia studies, cells were incubated under 2% O₂, while normoxia was maintained at 20% O₂. Human microvascular endothelial cells (hMVECs) were grown in Medium 131 containing microvascular growth supplement (5% fetal bovine serum, hydrocortisone, recombinant human fibroblast growth factor, heparin, recombinant human EGF, and dibutyryl cyclic AMP; Invitrogen, Carlsbad, CA). hMVECs were plated and allowed to form a cell monolayer before EPC were introduced for the adhesion assay. Passages 3–5 were used for all experiments using hMVECs. All cells were maintained at 37°C in a humidified incubator containing 5% CO₂.

Cell Proliferation Assay. EPC proliferation studies were performed as previously described (Guo et al., 2011). Cells were incubated with either VEGF (R&D Systems, Minneapolis, MN; 250 pg/ml), 20-HETE (1 nM), or both for 48 hours. The effects of VEGF and 20-HETE on proliferation were assessed by cell counting, using EPC treated with solvent (EtOH, 0.1%) as control. In separate experiments, EPC were treated for 48 hours as follows: 1) control (EtOH, 0.1%); 2) 20-HETE (10 nM); 3) VEGF-neutralized antibody (5 ng/ml) (Sigma-Aldrich, St. Louis, MO); 4) 20-HETE (10 nM) in the presence of VEGF-neutralized antibody (5 ng/ml); 5) VEGF alone (20 ng/ml); 6) *N*-[20-hydroxyeicosa-6(Z),15(Z)-dienoyl]glycine (20-HEDGE, a 20-HETE antagonist; 10 nM); 7) 20-HEDGE alone (100 nM); 8) VEGF (20 ng/ml) in the presence of 20-HEDGE (10 nM); and 9) VEGF (20 ng/ml) in the presence of 20-HEDGE (100 nM). Cell numbers were counted and normalized to EtOH-treated cultures and expressed as a percentage of control at the end of these experiments.

EPC Adhesion Assay. EPC (5×10^5) were treated with vehicle (EtOH, 0.1%), 1 nM 20-HETE, 10 μ M dibromo-dodeceny-methylsulfonamide [DDMS, a 20-HETE synthesis inhibitor (Wang et al., 1998)], 10 nM 20-HEDGE, and 20-HETE in the presence of 20-HEDGE for 2 hours. 20-HEDGE was added 15 minutes prior to 20-HETE addition. In dose-response adhesion assay, 5×10^5 EPC were treated with vehicle (0.1% EtOH), or 20-HETE (0.1, 1, 5, and 10 nM), or DDMS (10 μ M), or 20-HEDGE (10 nM) for 2 hours. After pretreatment, cells were spun down, washed with phosphate-buffered saline (PBS), resuspended in adhesion media (RPMI 1640 with 0.2% bovine serum albumin) (Peled et al., 2000), and then transferred to 12-well plates (1.5×10^5 /0.5 ml per well) coated with either 1 μ g/cm² human fibronectin (BD Bioscience, San Jose, CA) or hMVEC monolayer, and incubated at 37°C for 2 hours. After removing excess cells and media, the wells were gently washed three times with adhesion media, and then adherent cells were fixed with ice-cold methanol and stained with 0.5% crystal violet for 30 minutes. Fifteen randomly selected fields per well were counted and adherent cell numbers were normalized and expressed as percent of control.

RNA Extraction and Real-Time Polymerase Chain Reaction. RNA was extracted from EPC using TRIzol reagent, treated with DNase, and concentration was measured by reading absorbance at 260 nm. RNA (1 μ g) was reverse-transcribed using a BluePrintTM1st Strand cDNA Synthesis Kits (RR 6115A; TaKaRa Biotechnology, Dalian, China). Real-time polymerase chain reaction was performed using the Mx3000p Real-Time PCR System (Stratagene/Agilent Technologies, Santa Clara, CA), including 2 μ l of reverse transcription product, 12.5 μ l of TaKaRa SYBR Premix Ex Taq, 0.5 μ l of ROX Reference Dye II (50 $\times$, RR420A; TaKaRa

Biotechnology), and a 0.2 μ M concentration of various primers (GeneLink, Hawthorne, NY). Primers used for PCR were CYP4A11 forward 5'-AATTTGCCATGAACGAACGAGCTGA-3' and reverse 5'-TGTTCCAAAGGCCACAAGG-3'; CXCR4 forward 5'-GAAACCCTCAGCGTCTCACT-3' and reverse 5'-TAGTGGGCTAAGGG-CACAAG-3'; VLA-4 forward 5'-TGGATCCATCGTGAAGTGTG-3' and reverse 5'-TCTTTCGTAAATCAGGGGGG-3'; β -actin forward 5'-AAGATCATTGCTCCTC-CTGA-3' and reverse 5'-CTCGTCA-TACTCTGCTTGCT-3'; 18S rRNA forward 5'-GATGGGC-GGCGGAAAATAG-3; 18S rRNA reverse 5'-GCGTGGATTCTG-CAT AATGGT-3'. The reactions were incubated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 55°C for 5 seconds, and 72°C for 15 seconds. The threshold cycle (Ct) data were determined using default threshold settings. The Ct was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative changes in the gene expression.

Western Blot Analysis. Homogenates were prepared from EPC using radioimmunoprecipitation assay buffer as previous described (Guo et al., 2005). Equal amounts of protein (5–10 μ g) were separated on a 10% Tris-glycine gel, transferred to a polyvinylidene difluoride membrane, and incubated with anti-cytochrome P450 CYP4A11 antibody (1:500 dilution; Abcam, Cambridge, UK), anti-CXCR4 (1: 500 dilution; Abcam, Cambridge, UK), anti-VLA-4 antibody (1:1000 dilution; Cell Signaling Technology, Beverly, MA), and anti- β -actin antibody (1:1000, Cell Signaling Technology, Danvers, MA) at 4°C overnight. The membrane then was incubated with anti-rabbit IRDye800 CW secondary antibody (LI-COR, Lincoln, NE) at 1: 10,000 at room temperature for 2 hours, and scanned with LI-COR Odyssey Infrared Imaging system. Human β -actin was used as loading control.

Matrigel Plug Angiogenesis Assay. Twelve-week-old Balb/c female mice were purchased from Charles River Laboratory (Wilmington, MA). All experiments conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. All animal experimental procedures were approved by the New York Medical College Institutional Animal Care and Use Committee. Before the Matrigel injection, mice were anesthetized, shaved, and depilated. High concentration Matrigel (cat. no. 354248; BD Biosciences) containing various treatments were administered as follows: control, 20-HETE (20 μ g), EPC (3×10^6), and EPC in the presence of 25 μ M DDMS or 20-HEDGE (a comparable dose of 10 mg/kg per day used by others in vivo) were injected subcutaneously into the flanks of the mice (600 μ l per animal). After 7 days, the plugs were removed, washed with PBS, and separated into two portions. One portion of the plugs were digested with 0.3 ml of 0.1 mg/ml Dispase I (Sigma-Aldrich) at 4°C overnight and spun down; the supernatants were subjected to the measurement of hemoglobin levels using Drabkin's reagent (Sigma-Aldrich) and were normalized to the weight of Matrigel based on a previously published protocol (Qin et al., 2010). Corresponding remaining portions of plugs were embedded with paraffin and saved for microvessel density assay.

Microvessel Density Assay. The quantification of the microvessel formation was conducted by staining for the endothelial markers using tomato lectin–fluorescein isothiocyanate. In brief, the samples were incubated with 10 μ g/ml tomato lectin–fluorescein isothiocyanate (Vector Laboratories, Burlingame, CA) at room temperature for 30 minutes, followed by 0.1 μ g/ml DAPI (Sigma-Aldrich) counterstained for 10 minutes. The immunofluorescence staining was acquired with the Zeiss AXIO Imager M1 fluorescence microscope. Six to seven fields were chosen randomly and blindly from various section levels to ensure the objectivity of the sampling. The tomato lectin positive stains were counted for the numbers of microvessels. Microvessel density data were shown as the number of microvessels in each field.

Liquid Chromatography–Tandem Mass Spectrometry Analysis of 20-HETE Formation in EPC. For 20-HETE measurement, d9 fresh healthy EPC were spun down and washed two times with

PBS, then 4×10^6 cells were cultured in EPC growth media overnight to maximize the effects of hypoxia as well as VEGF. Both cells and media were harvested for analysis. Cells were homogenized in oxygenated Krebs buffer followed by incubation with 1 mM NADPH (Calbiochem, San Diego, CA) and 2 μ M indomethacin (Sigma-Aldrich) for 1 hour at 37°C, while conditioned media were extracted without incubation. Both the cell lysates and the conditioned media were acidified to pH 4.0, and the lipids were extracted two times with ethyl acetate in the presence of d6-20-hydroxyeicosatetraenoic acid (1 ng). The organic phase was collected, dried under nitrogen, and eicosanoids identification and quantification were performed with a QTRAP 3200 linear ion trap quadrupole mass spectrometer (Foster City, CA) as previously described (Inoue et al., 2009).

Statistical Analysis. Data are expressed as means $\pm$ S.D. in in vitro experiments and means $\pm$ S.E.M. in in vivo experiments. Significance of difference in mean values was determined using *t* test and one-way analysis of variance (ANOVA), followed by the Newman-Keuls post hoc test. *P* < 0.05 was considered to be significant.

Results

A Positive Feedback Regulation Exists between the VEGF Pathway and the CYP4A11–20-HETE System.

The VEGF pathway is one of the essential signaling mechanisms in regulating neovascularization. 20-HETE has been shown to interact with this important signaling pathway (Guo et al., 2011). We postulated that a positive feedback loop regulatory mechanism may exist between the VEGF and the 20-HETE pathway as shown in Fig. 1A. We first examined the effects of hypoxia (a VEGF inducer) and recombinant VEGF on the expression of the CYP4A11, the predominant human 20-HETE synthase in EPC (Guo et al., 2011). VEGF induced CYP4A11 gene expression by 3.5 ± 0.4 -fold after 4 hours of treatment (Fig. 1B), whereas hypoxia induced the CYP4A11 expression by 2.4 ± 0.6 - and 1.8 ± 0.5 -fold at 2 and 6 hours, respectively (Fig. 1C). Likewise, hypoxia and VEGF also induced CYP4A11 protein expression by 1.88 ± 0.1 - and 2.04 ± 0.08 -fold after 4-hour exposure, respectively (Fig. 1D). Next, we assessed the production of 20-HETE by EPC after 12-hour incubation under hypoxia or in the presence of VEGF (20 ng/ml). Liquid chromatography–tandem mass spectrometry analysis confirmed that 20-HETE synthesis was significantly increased under hypoxia or VEGF stimulation (Fig. 1E), consistent with the finding that the CYP4A11–20-HETE system is activated by the VEGF pathway.

20-HETE and VEGF have both been shown to induce one of the EPC angiogenic functions in vitro, the proliferation of the cells. We examined whether 20-HETE and human recombinant VEGF have synergistic or additive stimulatory effects on EPC proliferation. 20-HETE (1 nM) or VEGF (250 pg/ml) alone increased EPC proliferation by only $12 \pm 6.2\%$ and $8.4 \pm 3.2\%$, respectively. These increases in EPC proliferation are statistically significant but may not be biologically relevant. However, 20-HETE together with VEGF increased EPC proliferation by $46 \pm 5.5\%$ (Fig. 2A), supporting the existence of a synergistic effect of 20-HETE and VEGF on EPC proliferation.

To further strengthen the potential regulatory relationship between 20-HETE and VEGF on EPC proliferation, we first blocked the action of 20-HETE with 20-HEDGE in the presence or absence of VEGF. 20-HEDGE (10 and 100 nM) markedly abolished VEGF-induced EPC proliferation as well as EPC basal proliferation, suggesting that 20-HETE may be

Fig. 1. Effects of hypoxia and VEGF on the CYP4A11 expression and 20-HETE production in EPC. (A) Proposed scheme of a positive feedback regulatory mechanism between the CYP4A11–20-HETE system and the VEGF pathway. The effects of human recombinant VEGF (20 ng/ml, 4-hour treatment) (B) and hypoxia (2-hour or 6-hour exposure) (C) on CYP4A11 expression ($n = 3$; $*P < 0.05$ versus control) were assessed by real-time PCR as described. (D) CYP4A11 protein expressions were measured by Western blot analysis after 4-hour exposure to human recombinant VEGF (20 ng/ml) or hypoxia. Densitometry analysis was performed to quantitate these changes in comparison with the controls. (E) Measurement of 20-HETE formation (pg/mg protein) after exposing EPC to hypoxia and VEGF (20 ng/ml) for 12 hours ($n = 3$ –4 in triplicates; $*P < 0.05$ versus control). Data were shown as mean $\pm$ S.D.

necessary for VEGF-induced cell proliferation (Fig. 2B). On the other hand, addition of anti-VEGF antibody to neutralize the effects of VEGF completely negated 20-HETE-mediated EPC proliferation, indicating a role for VEGF in this process (Fig. 2C). Taken together, these data strongly implicate the presence of a positive feedback loop regulatory mechanism between the HIF-1 α –VEGF and the CYP4A11–20-HETE pathways.

20-HETE Increases Cell Adhesion and Key Adhesion Molecule Expression in Human EPC. During the homing processes of EPC to sites of neovascularization, increased cell adhesion plays a critical role in regulating EPC-mediated angiogenesis (Caiado and Dias, 2012). We examined the effects of 20-HETE on EPC adhesion *in vitro* and found that exogenously added 20-HETE induced EPC adhesion to FN

in a concentration-dependent manner with the maximal effects at 1 nM (Fig. 3A). Therefore, we chose 1 nM as the concentration for the cell adhesion assay. 20-HETE induced EPC adhesion to fibronectin-coated surfaces by $40 \pm 5.6\%$; this increase was completely abolished in the presence of the 20-HEDGE (10 nM) (Fig. 3B). Moreover, 20-HEDGE (a 20-HETE antagonist) and DDMS (a 20-HETE synthase inhibitor) (Wang et al., 1998) significantly decreased the basal adhesion of EPC to FN, suggesting that endogenous 20-HETE participates in the regulation of EPC adhesion.

Under physiologic conditions, circulatory EPC adhere to the EC lining of the blood vessel at the target sites of neovascularization (Vajkoczy et al., 2003). To mimic the physiologic environment of EPC homing, EPC adhesion to hMVEC monolayer was also examined. As seen in Fig. 3C, similar to

Fig. 2. A positive feedback regulation exists between the VEGF pathway and the CYP4A11–20-HETE system on EPC proliferation. EPC were cultured and treated with 20-HETE (1 nM), VEGF (250 ng/ml), 20-HEDGE (10 and 100 nM), VEGF-neutralizing antibody (5 ng/ml), or combinations of these agents. Cell proliferation was analyzed using cell counts 48 hours after various treatments. (A) Synergistic effects of 20-HETE and VEGF on EPC proliferation ($n = 3$ in triplicates; * $P < 0.05$ versus control, † $P < 0.05$ versus 20-HETE or VEGF alone). The effects of 20-HETE inhibition with 20-HEDGE on VEGF-induced EPC proliferation (B) and VEGF neutralizing on 20-HETE-induced EPC proliferation ($n = 3$ –4; * $P < 0.05$ versus control, † $P < 0.05$ versus VEGF or 20-HETE alone) (C).

data shown for EPC adhesion to FN (Fig. 3B), 20-HETE increased EPC adhesion to EC monolayer by $67 \pm 10\%$, and this increase was completely abolished by cotreatment with 20-HEDGE (Fig. 3C). Similar to cell adhesion to FN, both 20-HEDGE and DDMS inhibited basal EPC-EC adhesion (Fig. 3C), again suggesting that the EPC-derived CYP4A–20-HETE system regulates adhesion during the homing processes.

Besides EPC adhesion, the interactions between SDF-1/CXCR4 and VLA-4/FN, two major well-known mediators in EPC mobilization and homing, are also known to positively regulate EPC contribution to neovascularization. Our results demonstrate that 20-HETE significantly increased VLA-4 (Fig. 4A) and CXCR4 (Fig. 4B) gene expressions by ~ 3 - and 2-fold, respectively. Addition of 20-HEDGE significantly inhibited the 20-HETE-induced increases in VLA-4 and CXCR4 expression.

Moreover, 20-HEDGE decreased the expression of CXCR4 by $\sim 60\%$ below the control levels (Fig. 4B), suggesting an important role for EPC-derived 20-HETE in the regulation of EPC homing involving the signaling of CXCR4-SDF axis. Western blot analyses were also performed to confirm the induction of VLA-4 and CXCR4 proteins by 20-HETE. Data shown in Fig. 4C demonstrate that 20-HETE treatment increased VLA-4 and CXCR4 protein expressions by 2.6 ± 0.2 and 1.9 ± 0.2 -fold, respectively.

Local 20-HETE Inhibition Decreases EPC-Induced Angiogenesis in Matrigel Plug Angiogenesis Assay. 20-HETE regulates multiple EPC functions that are associated with angiogenesis (Guo et al., 2011). To determine whether 20-HETE plays a role in the regulation of EPC angiogenic functions in vivo, the Matrigel plug angiogenesis assay was

Fig. 3. Effects of the 20-HETE system on EPC adhesion in vitro. EPC (5×10^5) were cultured and treated with various agents accordingly before placement on fibronectin-coated plates or EC monolayer. EPC cell adhesion was quantified 2 hours later. The dose response effects of 20-HETE on EPC adhesion to fibronectin (A) and effects of DDMS (10 μ M) and 20-HEDGE (10 nM) on basal and 20-HETE (1 nM)-stimulated EPC adhesion to fibronectin ($n = 5$ in triplicates; * $P < 0.05$ versus control, † $P < 0.05$ versus 20-HETE) (B), and to human microvascular endothelial cell monolayers ($n = 3$ in triplicates; * $P < 0.05$ versus control, † $P < 0.05$ versus 20-HETE) (C). All results were normalized against the controls and expressed as the percent of control.

performed. Figure 5A shows representative Matrigel plugs from each experimental group. We found that the negative control plugs were opaque in color, while the EPC- (3×10^6) and 20-HETE- (20 μ g) containing plugs were significantly more yellow/red in color than the negative controls, indicating more angiogenesis. Furthermore, EPC-induced angiogenesis was abolished by either DDMS (25 μ M) or 20-HEDGE (25 μ M) (Fig. 5A), suggesting that the EPC-induced angiogenesis is 20-HETE-dependent. To confirm our visual observations, we quantified the level of angiogenesis by measuring both hemoglobin contents and microvascular density in the Matrigel plugs. Hemoglobin measurements showed that

EPC induced angiogenesis by 3.6 ± 0.2 -fold over the controls and this increase was markedly inhibited by DDMS (1.5 ± 0.6 -fold versus control) and 20-HEDGE (1.5 ± 0.5 -fold versus control) (Fig. 5B). The hemoglobin measurements were further validated by assessing changes in actual angiogenesis in the plugs via measurements of microvessel density as shown in Fig. 5C.

Discussion

Over the past decade, multiple studies were conducted investigating the role of EPC in postnatal neovascularization

Fig. 4. Effects of the 20-HETE system on key cell adhesion molecule expressions. EPC were harvested and grew for 9 days in culture and treated with 20-HETE (1 nM) in the presence and absence of 20-HEDGE (10 nM) for 2 hours. RNAs were extracted using TRIzol. Real-time PCR were performed as described in *Materials and Methods* to quantify the expression of (A) VLA-4 and (B) CXCR4 ($n = 3$ in triplicates; * $P < 0.05$ versus control, and † $P < 0.05$ versus 20-HETE). (C) EPC were treated with 20-HETE for 2 hours and VLA-4 and CXCR4 protein expressions were also measured by Western blot analysis, and densitometry analysis was used to quantitate the changes ($n = 3$; * $P < 0.05$ versus control). All results were normalized against the controls and expressed as the percent of control.

in vascular disease. Cord blood-derived AC133+ cells, a major source of EPC other than bone marrow, represent a pluripotent adult progenitor cell population (Harris and Rogers, 2007; Murohara, 2001). The contribution of EPC to new vessel formation consists of four interrelated events: First, mobilization of EPC from bone marrow to peripheral blood in response to chemoattractant gradients, such as SDF-1 and (VEGF); second, homing to the target site by adhering to the EC lining; third, transendothelial migration through the blood vessel wall; and fourth, differentiation into EC and forming neovessels under the influence of multiple growth factors such as VEGF and SDF-1 in an autocrine or paracrine manner (Urbich and Dimmeler, 2004; Urbich et al., 2005; Caiado and Dias, 2012). Thus, to contribute to neovascularization, EPC must be released from the bone marrow, enter the peripheral circulation, and migrate across the blood vessel and home to targeted tissues (Caiado and Dias, 2012). A better understanding of the regulators of the

angiogenic functions of EPC is needed to advance cell-based therapy of vascular abnormalities.

Our previous study demonstrated that CYP4A11, the predominant human EPC 20-HETE synthase, was expressed in different batches of human umbilical cord blood-derived EPC, indicating the presence of a functionally active 20-HETE system (Guo et al., 2011). We have shown that 20-HETE induces HIF-1 α and VEGF expression in EPC. Since the VEGF signaling pathway is important in neovascularization, we postulate that a positive feedback regulatory loop exists between the VEGF pathway and the 20-HETE system. Hypoxia and VEGF stimulated CYP4A11 expression at both the mRNA and protein levels and, consistent with the increase in CYP4A11 expression, 20-HETE synthesis was also increased under these conditions. It is well known that EPC proliferation is a crucial step in postnatal neovascularization by maintaining the number and functionality of EPC (Kirtan and Xu, 2010) and by enhancing the effects of EPC-

Fig. 5. Local inhibition of the CYP4A–20-HETE system decreases EPC-mediated angiogenesis in Matrigel plug angiogenesis assay. Matrigel plugs containing EPC (3×10^6) in the presence or absence of DDMS (25 μ M) and 20-HEDGE (25 μ M) were subcutaneously implanted in 12-week-old Balb/C mice. 20-HETE (20 μ g)-containing plugs were used as positive controls. The plugs were extracted after 7 days for analysis of angiogenesis. (A) Images of the representative Matrigel plugs extracted at the end of 7-day experiments. Quantification of the angiogenesis in Matrigel plug were carried out by (B) measurement of hemoglobin levels and (C) microvessel density (MVD) counts ($n = 4$; * $P < 0.05$ versus vehicle control, † $P < 0.05$ versus EPC).

derived growth factors that promote differentiation into EC (Caiado and Dias, 2012). Both VEGF and 20-HETE have been previously shown to independently induce EPC proliferation (Guo et al., 2011). In turn, they positively regulate EPC contribution to the neovascularization process. In this study, we demonstrated that 20-HETE and VEGF exert a synergistic effect on EPC proliferation. The presence of a strong regulatory relationship between 20-HETE and VEGF was further substantiated by demonstrating that VEGF or 20-HETE stimulation of EPC proliferation was abolished by adding a VEGF-neutralizing antibodies or 20-HETE antagonist, respectively. These findings reinforce the notion that cross-talk

exists between these two autacoids and that 20-HETE is crucial to VEGF-induced proliferation and vice versa. Taken together, our results implicate the presence of a positive feedback regulatory loop between the VEGF and the CYP4A11–20-HETE pathways in human EPC.

Within the multistep process of postnatal neovascularization, EPC homing has been identified as playing a crucial role (Takahashi et al., 1999; Urbich et al., 2003; Ceradini et al., 2004; Urbich and Dimmeler, 2004; Ceradini and Gurtner, 2005; Tepper et al., 2005; Caiado and Dias, 2012). During EPC homing, two major types of cellular interactions are critical: 1) the interaction between VLA-4 and the extracellular matrix

component FN or the EC cell surface molecule vascular cell adhesion molecule-1 (VCAM-1) and 2) the interaction between SDF-1 and its specific chemokine receptor CXCR4. Namely, chemokine axis SDF-1/CXCR4-mediated chemotaxis/adhesion and integrin interaction-mediated adhesion may contribute to EPC-induced neovascularization. Moreover, SDF-1 can induce chemotaxis/adhesion through activation of specific integrin molecules, such as modulating VLA-4-dependent adhesion to FN and VCAM-1 on primitive progenitor cells (Peled et al., 2000; Hidalgo et al., 2001; De Falco et al., 2004). These interactions are the major regulators of EPC chemotaxis and adhesion (Caiado and Dias, 2012). Additionally, we showed that 20-HETE increases EPC adhesion to FN in a dose-dependent manner, and that the CYP4A-20-HETE system in EPC contributes to the regulation of the adhesion process. Besides interacting with the extracellular matrix component FN, circulating EPC is also required to interact with vascular EC during the homing and invasion steps. We mimicked the physiologic environment of EPC homing by studying EPC adhesion to hMVEC monolayers. 20-HETE stimulated EPC adhesion to EC monolayers, and inhibition of the CYP4A-20-HETE system suppressed it. Interestingly, the effect of 20-HETE on EPC adhesion to hMVEC monolayers was more potent than to FN. In the present study, we found that 20-HETE induced CXCR4 as well as VLA-4 gene and protein expression in EPC. Since SDF-1 is constitutively expressed by vascular EC (Salvucci et al., 2002; De La Luz Sierra et al., 2004), a possible explanation for this observation is that 20-HETE may amplify adhesion signals through activation of the interaction between SDF-1 and CXCR4, and then further enhancing VLA-4-mediated EPC adhesion to VCAM-1 in hMVEC monolayer. Furthermore, 20-HETE antagonist 20-HEDGE not only negated the 20-HETE-mediated increase in CXCR4 expression but also lowered the basal CXCR4 expression level. On the other hand, 20-HEDGE only partially reduced the increases in VLA-4 expression. The expression and affinity of VLA-4 as well as the G-protein-coupled receptor CXCR4 are different from primitive and committed progenitor cells and regulated by a more complicated signal network (Oostendorp and Dormer, 1997). CXCR4 may be sensitized and expressed on the cell surface or desensitized and internalized (Kucia et al., 2004; Burger and Kipps, 2006; Cencioni et al., 2012). Thus, 20-HETE may be differentially regulating these two adhesion components. Collectively, these observations suggest that the primary mechanism by which 20-HETE enhances EPC homing and adhesion into sites of neovascularization is through activation of the SDF-1/CXCR4 axis, rather than its involvement through integrin regulation. These observations are intriguing, but future studies are needed to further investigate the precise molecular mechanisms that are involved. Taken together, inhibition of 20-HETE biosynthesis or blockade of its actions inhibit not only the 20-HETE-induced but also basal adhesion of EPC to FN and EC, thus strongly suggesting that the CYP4A-20-HETE pathway in EPC is a key regulator of these functions and that 20-HETE of EPC origin contributes to adhesion and homing in an autocrine and paracrine manner.

We had previously established that the CYP4A-20-HETE system promotes EPC function associated with angiogenesis in vitro (Guo et al., 2011). These findings are consistent with other reports that 20-HETE is proangiogenic (Amaral et al.,

2003; Jiang et al., 2004; Chen et al., 2005, 2012; Miyata and Roman, 2005; Guo et al., 2007, 2009). In this study, we further demonstrated that 20-HETE contributes to the angiogenic functions in vivo. Local inhibition of the 20-HETE system markedly abolished EPC-induced angiogenic effects in a Matrigel plug angiogenesis assay, clearly implicating an important role for 20-HETE in this process. However, since EPC were mixed with Matrigel to mimic physiologic angiogenesis, the homing process was obviated in this model. Thus, the antiangiogenic effects of DDMS and 20-HEDGE may be due mainly to their effects on the abilities of EPC to produce multiple growth factors that are necessary for angiogenesis locally.

In summary, we conclude that a positive feedback regulation exists between 20-HETE and the VEGF pathway. 20-HETE activates EPC functions associated with angiogenesis through synergism with VEGF. 20-HETE also increases chemokine- and integrin-mediated EPC chemotaxis and adhesion. These processes can ultimately lead to EPC homing to neoangiogenic sites. Local inhibition of the 20-HETE production or action decreased EPC-induced angiogenesis in vivo, suggesting that the CYP4A-20-HETE system may be a key regulator of EPC-mediated neovascularization.

Acknowledgments

The authors thank Dr. Alberto Nasjletti for intellectual mentoring and editorial contributions to this study, the entire Labor and Delivery nursing staff at Westchester Medical Center and the Maternity nursing staff at Phelps Memorial Hospital for their time and effort in collecting human umbilical cord blood. This study would not have been possible without their seminal contributions.

Authorship Contributions

Participated in research design: Chen, Arbab, Scicli, Schwartzman, Yang, Guo.

Conducted experiments: Chen, Ackerman, Saleh, Gotlinger, Guo.

Contributed new reagents or analytic tools: Falck, Kessler, Mendelowitz, Arbab.

Performed data analysis: Chen, Ackerman, Gotlinger, Guo.

Wrote or contributed to the writing of the manuscript: Chen, Scicli, Schwartzman, Yang, Guo.

References

- Amaral SL, Maier KG, Schippers DN, Roman RJ, and Greene AS (2003) CYP4A metabolites of arachidonic acid and VEGF are mediators of skeletal muscle angiogenesis. *Am J Physiol Heart Circ Physiol* **284**:H1528–H1535.
- Arbab AS, Janic B, Knight RA, Anderson SA, Pawelczyk E, Rad AM, Read EJ, Pandit SD, and Frank JA (2008) Detection of migration of locally implanted AC133+ stem cells by cellular magnetic resonance imaging with histological findings. *FASEB J* **22**:3234–3246.
- Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, and Isner JM (1999) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* **85**:221–228.
- Burger JA and Kipps TJ (2006) CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. *Blood* **107**:1761–1767.
- Caiado F and Dias S. (2012) Endothelial progenitor cells and integrins: adhesive needs. *Fibrogenesis Tissue Repair* **5**:4.
- Carroll MA, Balazy M, Huang DD, Rybalova S, Falck JR, and McGiff JC (1997) Cytochrome P450-derived renal HETEs: storage and release. *Kidney Int* **51**: 1696–1702.
- Cencioni C, Capogrossi MC, and Napolitano M (2012) The SDF-1/CXCR4 axis in stem cell preconditioning. *Cardiovasc Res* **94**:400–407.
- Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, and Gurtner GC (2004) Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med* **10**:858–864.
- Ceradini DJ and Gurtner GC (2005) Homing to hypoxia: HIF-1 as a mediator of progenitor cell recruitment to injured tissue. *Trends Cardiovasc Med* **15**:57–63.
- Chan BM, Elices MJ, Murphy E, and Hemler ME (1992) Adhesion to vascular cell adhesion molecule 1 and fibronectin. Comparison of alpha 4 beta 1 (VLA-4) and alpha 4 beta 7 on the human B cell line JY. *J Biol Chem* **267**:8366–8370.

- Chen P, Guo M, Wygle D, Edwards PA, Falck JR, Roman RJ, and Scicli AG (2005) Inhibitors of cytochrome P450 4A suppress angiogenic responses. *Am J Pathol* **166**: 615–624.
- Chen L, Ackerman R, and Guo AM (2012) 20-HETE in neovascularization. *Prostaglandins Other Lipid Mediat* **98**:63–68.
- De Falco E, Porcelli D, Torella AR, Straino S, Iachininoto MG, Orlandi A, Truffa S, Biglioli P, Napolitano M, and Capogrossi MC, et al. (2004) SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells. *Blood* **104**:3472–3482.
- De La Luz Sierra M, Yang F, Narazaki M, Salvucci O, Davis D, Yarchoan R, Zhang HH, Fales H, and Tosato G (2004) Differential processing of stromal-derived factor-1 α and stromal-derived factor-1 β explains functional diversity. *Blood* **103**: 2452–2459.
- Guo M, Roman RJ, Falck JR, Edwards PA, and Scicli AG (2005) Human U251 glioma cell proliferation is suppressed by HET0016 [N-hydroxy-N'-(4-butyl-2-methylphenyl)formamidin], a selective inhibitor of CYP4A. *J Pharmacol Exp Ther* **315**:526–533.
- Guo AM, Arbab AS, Falck JR, Chen P, Edwards PA, Roman RJ, and Scicli AG (2007) Activation of vascular endothelial growth factor through reactive oxygen species mediates 20-hydroxyeicosatetraenoic acid-induced endothelial cell proliferation. *J Pharmacol Exp Ther* **321**:18–27.
- Guo AM, Scicli G, Sheng J, Falck JC, Edwards PA, and Scicli AG (2009) 20-HETE can act as a nonhypoxic regulator of HIF-1 α in human microvascular endothelial cells. *Am J Physiol Heart Circ Physiol* **297**:H602–H613.
- Guo AM, Janic B, Sheng J, Falck JR, Roman RJ, Edwards PA, Arbab AS, and Scicli AG (2011) The cytochrome P450 4A/F-20-hydroxyeicosatetraenoic acid system: a regulator of endothelial precursor cells derived from human umbilical cord blood. *J Pharmacol Exp Ther* **338**:421–429.
- Harris DT and Rogers I (2007) Umbilical cord blood: a unique source of pluripotent stem cells for regenerative medicine. *Curr Stem Cell Res Ther* **2**:301–309.
- Hidalgo A, Sanz-Rodriguez F, Rodriguez-Fernández JL, Albella B, Blaya C, Wright N, Cabañas C, Prósper F, Gutierrez-Ramos JC, and Teixidó J (2001) Chemokine stromal cell-derived factor-1 α modulates VLA-4 integrin-dependent adhesion to fibronectin and VCAM-1 on bone marrow hematopoietic progenitor cells. *Exp Hematol* **29**:345–355.
- Hoernig MR, Bianchi C, and Sellke FW (2008) Hypoxia inducible factor-1 α , endothelial progenitor cells, monocytes, cardiovascular risk, wound healing, cobalt and hydralazine: a unifying hypothesis. *Curr Drug Targets* **9**:422–435.
- Inoue K, Sodhi K, Puri N, Gotlinger KH, Cao J, Rezzani R, Falck JR, Abraham NG, and Laniado-Schwartzman M (2009) Endothelial-specific CYP4A2 overexpression leads to renal injury and hypertension via increased production of 20-HETE. *Am J Physiol Renal Physiol* **297**:F875–F884.
- Janic B, Guo AM, Iskander AS, Varma NR, Scicli AG, and Arbab AS (2010) Human cord blood-derived AC133+ progenitor cells preserve endothelial progenitor characteristics after long term in vitro expansion. *PLoS ONE* **5**:e9173.
- Jarajapu YP and Grant MB (2010) The promise of cell-based therapies for diabetic complications: challenges and solutions. *Circ Res* **106**:854–869.
- Jiang M, Mezentssev A, Kemp R, Byun K, Falck JR, Miano JM, Nasjletti A, Abraham NG, and Laniado-Schwartzman M (2004) Smooth muscle-specific expression of CYP4A1 induces endothelial sprouting in renal arterial microvessels. *Circ Res* **94**: 167–174.
- Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, and Asahara T (2000) Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci USA* **97**: 3422–3427.
- Kirton JP and Xu Q (2010) Endothelial precursors in vascular repair. *Microvasc Res* **79**:193–199.
- Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, and Itescu S (2001) Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* **7**:430–436.
- Kucia M, Jankowski K, Reza R, Wysoczynski M, Bandura L, Allendorf DJ, Zhang J, Ratajczak J, and Ratajczak MZ (2004) CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. *J Mol Histol* **35**:233–245.
- Li B, Sharpe EE, Maupin AB, Teleron AA, Pyle AL, Carmeliet P, and Young PP (2006) VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *FASEB J* **20**: 1495–1497.
- Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, and Witte L, et al. (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* **7**:1194–1201.
- Miyata N and Roman RJ (2005) Role of 20-hydroxyeicosatetraenoic acid (20-HETE) in vascular system. *J Smooth Muscle Res* **41**:175–193.
- Murohara T (2001) Therapeutic vasculogenesis using human cord blood-derived endothelial progenitors. *Trends Cardiovasc Med* **11**:303–307.
- Oostendorp RA and Dörmer P (1997) VLA-4-mediated interactions between normal human hematopoietic progenitors and stromal cells. *Leuk Lymphoma* **24**:423–435.
- Peled A, Kollet O, Ponomarev T, Petit I, Franitza S, Grabovsky V, Slav MM, Nagler A, Lider O, and Alon R, et al. (2000) The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood* **95**: 3289–3296.
- Qin J, Chen X, Xie X, Tsai MJ, and Tsai SY (2010) COUP-TFII regulates tumor growth and metastasis by modulating tumor angiogenesis. *Proc Natl Acad Sci USA* **107**:3687–3692.
- Rosti V, Massa M, Campanelli R, De Amici M, Piccolo G, and Perfetti V (2007) Vascular endothelial growth factor promoted endothelial progenitor cell mobilization into the peripheral blood of a patient with POEMS syndrome. *Haematologica* **92**:1291–1292.
- Rüegg C, Postigo AA, Sikorski EE, Butcher EC, Pytela R, and Erle DJ (1992) Role of integrin α 4 β 7/ α 4 β 1 in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. *J Cell Biol* **117**:179–189.
- Salvucci O, Yao L, Villalba S, Sajewicz A, Pittaluga S, and Tosato G (2002) Regulation of endothelial cell branching morphogenesis by endogenous chemokine stromal-derived factor-1. *Blood* **99**:2703–2711.
- Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Wagner M, Isner JM, and Asahara T (1999) Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* **5**: 434–438.
- Tepper OM, Capla JM, Galiano RD, Ceradini DJ, Callaghan MJ, Kleinman ME, and Gurtner GC (2005) Adult vasculogenesis occurs through in situ recruitment, proliferation, and tubulization of circulating bone marrow-derived cells. *Blood* **105**: 1068–1077.
- Urbich C and Dimmeler S (2004) Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res* **95**:343–353.
- Urbich C, Heeschen C, Aicher A, Dernbach E, Zeiher AM, and Dimmeler S (2003) Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. *Circulation* **108**:2511–2516.
- Urbich C, Aicher A, Heeschen C, Dernbach E, Hofmann WK, Zeiher AM, and Dimmeler S (2005) Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. *J Mol Cell Cardiol* **39**:733–742.
- Vajkozy P, Blum S, Lamparter M, Mailhammer R, Erber R, Engelhardt B, Vestweber D, and Hatzopoulos AK (2003) Multistep nature of microvascular recruitment of ex vivo-expanded embryonic endothelial progenitor cells during tumor angiogenesis. *J Exp Med* **197**:1755–1765.
- Wang MH, Brand-Schieber E, Zand BA, Nguyen X, Falck JR, Balu N, and Schwartzman ML (1998) Cytochrome P450-derived arachidonic acid metabolism in the rat kidney: characterization of selective inhibitors. *J Pharmacol Exp Ther* **284**:966–973.
- Williams JM, Murphy S, Burke M, and Roman RJ (2010) 20-hydroxyeicosatetraenoic acid: a new target for the treatment of hypertension. *J Cardiovasc Pharmacol* **56**: 336–344.

Address correspondence to: Dr. Austin M. Guo, New York Medical College, 15 Dana Road, BSB 546A, Valhalla, NY 10595. E-mail: austin_guo@nymc.edu; and Dr. Jing Yang, School of Medicine, Wuhan University, Donghu Road, Wuhan 430071, P.R. China. E-mail: yangjingliu2013@163.com