Hypoxia-Induced Endothelial Progenitor Cell Function Is Blunted in Angiotensinogen Knockout Mice

Jin-Hwa Choi1,2, Minh-Phuong Nguyen1,2, Dongjin Lee1,2, Goo-Taeg Oh3, and You-Mie Lee1,2,*

Angiotensinogen (AGT), the precursor of angiotensin I, is known to be involved in tumor angiogenesis and associated with the pathogenesis of coronary atherosclerosis. This study was undertaken to determine the role played by AGT in endothelial progenitor cells (EPCs) in tumor progression and metastasis. It was found that the number of EPC colonies formed by AGT heterozygous knockout (AGT+/−) cells was less than that formed by wild-type (WT) cells, and that the migration and tube formation abilities of AGT+/− EPCs were significantly lower than those of WT EPCs. In addition, the gene expressions of vascular endothelial growth factor (VEGF), Flk1, angiopoietin (Ang)-1, Ang-2, Tie-2, stromal derived factor (SDF)-1, C-X-C chemokine receptor type 4 (CXCR4), and of endothelial nitric oxide synthase (eNOS) were suppressed in AGT+/− EPCs. Furthermore, the expressions of hypoxia-inducible factor (HIF)-1α and -2α were downregulated in AGT+/− early EPCs under hypoxic conditions, suggesting a blunting of response to hypoxia. Moreover, the activation of Akt/eNOS signaling pathways induced by VEGF, epithelial growth factor (EGF), or SDF-1α were suppressed in AGT+/− EPCs. In AGT+/− mice, the incorporation of EPCs into the tumor vasculature was significantly reduced, and lung tumor growth and melanoma metastasis were attenuated. In conclusion, AGT is required for hypoxia-induced vasculogenesis.
Table 1. Primer sequences for PCR

| Name | Primer sequence | Application |
|------|----------------|-------------|
| SDF-1 Forward | 5'-CTGTAGGCCTGACGGGACCAAT-3' | real-time qPCR |
| SDF-1 Reverse | 5'-CCATTTCAGAGGACCAAT-3' | real-time qPCR |
| CXCR4 Forward | 5'-AGGCTCTGCTCATGGAGATT-3' | real-time qPCR |
| CXCR4 Reverse | 5'-GCCAAATGCAAAGCTCTGC-3' | real-time qPCR |
| Ang1 Forward | 5'-AACCTCACCCGCAAAAGATG-3' | real-time qPCR |
| Ang1 Reverse | 5'-CACAGATGCCCAGTGGTGG-3' | real-time qPCR |
| Ang2 Forward | 5'-CAAGGCACTGAGAGACAC-3' | real-time qPCR |
| Ang2 Reverse | 5'-GCCAAGTTCAAAAGCTCTGC-3' | real-time qPCR |
| Tie-2 Forward | 5'-CGTTGCTCTTCTCCTGGAAGAGT-3' | real-time qPCR |
| Tie-2 Reverse | 5'-GCCACCCGAGGACCAT-3' | real-time qPCR |
| VEGF Forward | 5'-GGGCAGAGCTGAGTGTTAGC-3' | real-time qPCR |
| VEGF Reverse | 5'-TCTCCTACGATGAGCTAG-3' | real-time qPCR |
| FLK1 Forward | 5'-ACGACCCCGGCAAAACAA-3' | real-time qPCR |
| FLK1 Reverse | 5'-ACGACCCCGGCAAAACAA-3' | semi-qPCR |
| eNOS Forward | 5'-CGGCATCACCAGGAGAGAC-3' | semi-qPCR |
| eNOS Reverse | 5'-CGGCATCACCAGGAGAGAC-3' | semi-qPCR |
| β-actin Forward | 5'-AAGTACCCTACCCATCACAAG-3' | semi-qPCR |
| β-actin Reverse | 5'-AAGTACCCTACCCATCACAAG-3' | semi-qPCR |
| AT1R Forward | 5'-AGCGGAGTGTACCTGCTTC-3' | semi-qPCR |
| AT1R Reverse | 5'-AGCGGAGTGTACCTGCTTC-3' | semi-qPCR |
| AT2R Forward | 5'-GCCATCTGGAAGGAGAGAC-3' | semi-qPCR |
| AT2R Reverse | 5'-GCCATCTGGAAGGAGAGAC-3' | semi-qPCR |
| GAPDH Forward | 5'-CTGTGCTCTACGTGAGGAGATG-3' | semi-qPCR |
| GAPDH Reverse | 5'-CTGTGCTCTACGTGAGGAGATG-3' | semi-qPCR |

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Table 1. Primer sequences for PCR

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|--------|----------------|-------------|
| SDF-1  | 5'-CTGTAGGCCTGACGGGACCAAT-3' | real-time qPCR |
| SDF-1  | 5'-CCATTTCAGAGGACCAAT-3' | real-time qPCR |
| CXCR4  | 5'-AGGCTCTGCTCATGGAGATT-3' | real-time qPCR |
| CXCR4  | 5'-GCCAAATGCAAAGCTCTGC-3' | real-time qPCR |
| Ang1   | 5'-AACCTCACCCGCAAAAGATG-3' | real-time qPCR |
| Ang1   | 5'-CACAGATGCCCAGTGGTGG-3' | real-time qPCR |
| Ang2   | 5'-CAAGGCACTGAGAGACAC-3' | real-time qPCR |
| Ang2   | 5'-GCCAAGTTCAAAAGCTCTGC-3' | real-time qPCR |
| Tie-2  | 5'-CGTTGCTCTTCTCCTGGAAGAGT-3' | real-time qPCR |
| Tie-2  | 5'-GCCACCCGAGGACCAT-3' | real-time qPCR |
| VEGF   | 5'-GGGCAGAGCTGAGTGTTAGC-3' | real-time qPCR |
| VEGF   | 5'-TCTCCTACGATGAGCTAG-3' | real-time qPCR |
| FLK1   | 5'-ACGACCCCGGCAAAACAA-3' | semi-qPCR |
| FLK1   | 5'-ACGACCCCGGCAAAACAA-3' | semi-qPCR |
| eNOS   | 5'-CGGCATCACCAGGAGAGAC-3' | semi-qPCR |
| eNOS   | 5'-CGGCATCACCAGGAGAGAC-3' | semi-qPCR |
| β-actin| 5'-AAGTACCCTACCCATCACAAG-3' | semi-qPCR |
| β-actin| 5'-AAGTACCCTACCCATCACAAG-3' | semi-qPCR |
| AT1R   | 5'-AGCGGAGTGTACCTGCTTC-3' | semi-qPCR |
| AT1R   | 5'-AGCGGAGTGTACCTGCTTC-3' | semi-qPCR |
| AT2R   | 5'-GCCATCTGGAAGGAGAGAC-3' | semi-qPCR |
| AT2R   | 5'-GCCATCTGGAAGGAGAGAC-3' | semi-qPCR |
| GAPDH  | 5'-CTGTGCTCTACGTGAGGAGATG-3' | semi-qPCR |
| GAPDH  | 5'-CTGTGCTCTACGTGAGGAGATG-3' | semi-qPCR |

Typical EC markers and myeloid/hematopoietic markers, but has functional improvement in ischemic diseases such as myocardial infarction (Hur et al., 2007; Kalka et al., 2000).

Interestingly, clinical evidence demonstrates that EPC level is elevated in a wide variety of cancer tissues (Dome et al., 2006; Igreja et al., 2007; Naik et al., 2008), and EPCs are being viewed as autologous vectors for the delivery of therapeutic cells and genes to sites of vascular growth in both regenerating tissues and tumors (Roncalli et al., 2011). However, it is still unclear that family history for cardiovascular diseases associates with reduced circulating EPCs (Fadini et al., 2012). Since RAS modulation is a major pharmacotherapeutic strategies for the treatment of cardiovascular diseases, its effects on EPCs are of importance in the context of optimizing RAS interventional or regenerative therapy. Angiotensin converting enzyme 2 (ACE2) priming enhances EPC function in vitro and in vivo using AGT transgenic mice (Chen et al., 2013). However, in contrast to the enormous number of studies conducted on tumor angiogenesis, few have addressed the regulatory role played by AGT in tumor vasculature, especially in hypoxic microenvironment. Using AGT deficient mice (Tanimoto et al., 1994), we analyzed function or nature of EPCs in response to hypoxia and suggested the possible relationship between innate low blood pressure and decreased EPCs function. In the present study, we cultured mouse BM-derived EPCs and examined the effects of AGT haplo-insufficiency on their proliferation, differentiation, and migration. In addition, we investigated the effects of AGT on tumor growth and metastasis, on EPC mobilization from BM to the peripheral circulation, and on the incorporation of EPCs into tumor vessels using a mouse tumor model using BM transplantation method.

MATERIALS AND METHODS

Mice
Animal experiments were performed using C57BL/6J mice (SLC, Japan), which were handled in strict compliance with the guidelines for care and use of laboratory animals issued by the institutional ethical animal care committee of Kyungpook National University Animal Care and Use Committee (Approved number: KNU2012-51, Korea) according to the guidelines from NIH guidelines (Guide for the care and use of laboratory animals). Mice were maintained under specific pathogen-free conditions and treated with CO2 by inhalation in chamber for anesthesia just before sacrifice. Transgenic mice expressing green fluorescent protein (GFP) under chick β-actin promoter (C57BL/6J) and AGT+/- mice generated as previously reported (Kim et al., 1995).
Isolation of mononuclear cells from mouse bone marrow and peripheral blood

Mice were treated with CO₂ by inhalation for anesthesia and all long bones were isolated from the mice. Mononuclear cells (MNCs) were isolated from WT or AGT⁻/⁻ mouse bone marrow (BM) or peripheral blood (PB) using a Histopaque-1083 density gradient based method (Sigma, USA). Freshly isolated MNCs were resuspended in EGM-2 media (Lonza, Switzerland) supplemented with 5% fetal bovine serum (FBS, Hyclone, USA), basic fibroblast growth factor (bFGF), VEGF, insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), ascorbic acid and heparin, and seeded onto 60 mm dishes (2 × 10⁷ cells/dish). After culture for 4 days, nonadherent cells were removed by washing with PBS and attached cells in gelatin-coated dishes were further cultured for 3 days. These cells were ‘early EPCs’ (Hur et al., 2007; Kalka et al., 2000).

EPC colony forming assay (EPC-CFA) (Masuda et al., 2011)

After isolation of c-kit⁺/Sca-1⁻/Lin⁻ (KSL) cells from mouse BM (Kwon et al., 2011), the frequencies of small or large colonies were determined after culturing KSL cells (500 cells/35 mm dish) for 10 days in methylcellulose-containing medium M3236 (Stem Cell Technologies) supplemented with 20 ng/ml stem cell factor (SCF), 50 ng/ml VEGF, 20 ng/ml interleukin-3 (IL-3) (R&D Systems), 50 ng/ml bFGF, 50 ng/ml EGF (PeproTech), 2 U/ml heparin (Sigma), 30% FBS, and antibiotics. Colony forming units (CFUs) were counted under an inverted microscope at 40x. In our previous study, we defined small- and large-CFUs for the expression of additional endothelial marker genes such as CD31, Flk-1, von Willebrand factor (vWF), VE-cadherin and eNOS (Kwon et al., 2011).

Early EPC migration assay

A polycarbonate filter (8-μm pore size) was placed between the upper and lower chambers of a transwell unit. Cell suspensions (5 x 10⁴ cells) were placed in the upper chamber, and the lower chamber was filled with EGM-2 basal medium containing VEGF, EGF or murine recombinant stromal-derived factor-1α (SDF-1α; PeproTech) and incubated for 6 h. Migrated cells on the lower side of filters were fixed with methanol, stained with hematoxylin and eosin (Sigma), and counted in five randomly selected microscopic fields (200x).

Tube formation assay

Human umbilical vein endothelial cells (HUVECs) (BD Biosciences) were mixed with ‘early EPCs’ (10⁴ HUVECs and 10⁴ early EPCs in 100 μl of 5% FBS/EGM-2) and seeded on a 96-well culture plate coated with Matrigel (BD Biosciences) for 30 min at 37°C. Plates were examined for tube formation after incubation for 8 h. Numbers of tubular formations were counted at 200x.

RNA isolation and real-time and semi-quantitative PCR

Total RNA was extracted from mouse ‘early EPCs’ using Trizol (Invitrogen) according to the manufacturer’s instructions. For real-time-RT-qPCR, cDNA synthesis was performed using 0.5 μg of total RNA and an ExScript RT reagent Kit (TaKaRa, Japan). Real-time PCR was performed using a SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers shown in Table 1. The thermal cycle used was 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s denaturation at 95°C, and 1 min annealing at 60°C. Relative gene expressions were calculated from mean cycle threshold (Ct) values with respect to β-actin (internal control).

For semi-quantitative RT-PCR, cDNA synthesis was performed using 2 μg of total RNA using M-MLV reverse transcriptase (Promega, USA). A cDNA sample (1 μl) was subjected to PCR amplification using gene-specific primers and Diaster Taq DNA polymerase (Solgent, Korea). Table 1 summarizes the sequences of the primers used in this study.

Western blot analysis

Western blot analysis was performed as previously described (Jung et al., 2012).

Flow cytometric analysis

To determine circulating EPC numbers, MNCs isolated from peripheral blood (PB) were stained with FITC-conjugated anti-CD34 monoclonal antibody (GeneTex, Inc., USA) and PE-conjugated anti-VEGFR2 monoclonal antibody (BD Biosciences). ‘Early EPCs’ were stained with FITC-conjugated anti-CD14 (BD Pharmingen) or AlexaFluor 647- anti-CD45.2 antibody (BioLegend). A FACScalibur unit (BD Biosciences) was used to detect fluorescent labeled cells.

Bone marrow transplantation (BMT) and tumor xenograft model

C57BL/6 wild type (WT) mice or AGT⁻/⁻ mice were exposed to a lethal dose of total body irradiation (10 Gy), and injected intravenously with 10⁷ BM-derived MNCs collected from GFP-expressing C57BL/6 or AGT⁻/⁻ mice. Mice were inoculated subcutaneously in the flank with 10⁶ Lewis lung carcinoma cells (LLC) or B16F10 melanoma cells at 4 weeks post-injection, and tumor bearing mice were sacrificed 2 weeks later.

Immunohistochemistry (IHC) and Immunofluorescence

Tumor masses or ‘early EPCs’ were fixed with 4% paraformaldehyde (PFA) in PBS. Sections were prepared from OCT embedded tumor tissues and washed in PBS. After blocking with 1% bovine serum albumin (BSA, Sigma) in PBS, sections were stained with rat anti-mouse CD31 antibody overnight at 4°C, and then with Alexa Fluor 647-conjugated anti-rat antibody (Invitrogen). Fixed cells were stained with FITC-conjugated anti-CD34 monoclonal antibody (GeneTex) and PE-conjugated anti-VEGFR2 monoclonal antibody (BD Biosciences).

Statistics

SPSS version 1 was used for the statistical analyses. ANOVA was used to compare the experimental groups. Statistical significance was accepted for p values < 0.05, and results are presented as means ± standard deviations (SD).

RESULTS

Characterization of ‘early EPCs’

After 7 days in culture, attached MNC cells changed from a round to a spindle-shaped morphology but did not form colonies yet during culture in the presence of endothelial cell (EC) growth supplements. These cells were characterized by the uptake of Dil-Ac-LDL and isocitrate B4 binding (96.1 ± 1.51 % of attached cells) (Fig. 1A). Immunofluorescent staining further demonstrated the expression of CD34 (a HSC marker) and of VEGFR2 (Flk1, an EC marker). The percentage of CD34 and VEGFR2 double positive cells among attached cells was 90.0 ± 3.8 % (Fig. 1A). Therefore, we further used these cells as ‘early EPCs’ (Hur et al., 2007; Kalka et al., 2000).
The proliferation and differentiation of EPCs were suppressed in AGT TT mice

An EPC-colony-forming assay (EPC-CFA) was used to investigate the proliferation and differentiation of EPCs lacking AGT. Haplo-insufficient AGT knockout cells from AGT TT mice were used in this experiment because AGT TT mice died within 8 days of birth (Fig. 1B). In this assay, KSL cells give rise to functional EPC progeny, which are identified as two types of EPCs for differentiation cascade in terms of physiological model of an EPC hierarchy (Masuda et al., 2011). EPC-colonies composed of relatively small and round cells (called small-CFUs) indicate the characteristics of primitive EPC, such as, a high proliferative phenotype and the preservation of immature properties. Whereas large-CFUs composed of spindle-shaped cells indicate late or mature EPC characteristics, including rapid tube formation ability and improved neovascularization (Kwon et al., 2011). In fact, we already characterized these CFUs for the expression of additional EC marker genes, such as VEGFR2, VE-cadherin, vWF, CD31, Ac-LDL, isolecin B4 and eNOS, suggesting that these CFUs are functional EPCs (Kwon et al., 2011).
As shown in Fig. 1C, the growth rate of AGT+/− EPCs was lower than that of the WT, and the number of large CFUs was reduced, indicating that the differentiation capacity of AGT+/− EPCs was lower than that of WT EPCs (Fig. 1D).

Inhibition of early EPC migration induced by growth factors or hypoxic conditions in AGT+/− early EPCs
To investigate the role of AGT in the migration ability of EPCs, migration assays were performed on WT and AGT+/− 'early EPCs'. Growth factors, such as, VEGF, EGF, and SDF-1α play important roles in the regulation of a variety of cellular functions of EPCs, including cell migration, proliferation, and survival (Aghila Rani and Kartha, 2010; Asahara et al., 1999; Fu et al., 2007). As shown in Fig. 2A, in the presence of VEGF (10 ng/ml), EGF (50 ng/ml), or SDF-1α (100 ng/ml), WT early EPCs migrated efficiently through the membrane. However, the growth factor-induced migrations of AGT+/− early EPCs were obviously diminished (Fig. 2A). Furthermore, hypoxia-induced migration was inhibited in AGT+/− EPCs as compared with WT EPCs (Fig. 2B).

Suppression of tube formation by AGT+/− EPCs co-cultured with HUVECs
To investigate the tube forming abilities of early EPCs, we used an in vitro assay of capillary tube formation on Matrigel matrix. WT or AGT+/− early EPCs were co-cultured with HUVECs for 8 h on Matrigel matrix. Figure 2C shows capillary formation by HUVECs and early EPCs. The number of incorporated AGT+/− EPCs into branches was markedly lower than that of WT early EPCs (Fig. 2D). Moreover, the number of GFP-expressing EPCs in tubes was lower for AGT+/− EPCs than for WT EPCs, implying that AGT depletion substantially reduced the contribution of EPCs to tubular networks.

Blunted expressions of hypoxia-induced factors or receptors in AGT+/− early EPCs
To investigate the expression levels of various cytokines and receptors in EPCs from mouse BM, real-time qPCR was used to measure their mRNA levels in WT or AGT+/− early EPCs cultured under hypoxic conditions. The results obtained revealed that AGT deletion attenuated the expressions of vasculogenesis-associated genes (Fig. 3). In hypoxia, WT early EPCs exhibit time-dependent elevated expressions of EPC-promoting factors and their receptors, such as, of VEGF, Flk1, SDF-1, CXCR4, angiopoietin-1 (Ang-1), Ang-2, Tie-2, and eNOS mRNA. However, hypoxic conditions did not increase these mRNA levels in AGT+/− early EPCs compared to WT. In addition, HIF-1α and HIF-2α mRNA levels were measured under hypoxic conditions, and both were found to be reduced in AGT+/− early EPCs compared to that of WT (Fig. 3A). Furthermore, the mRNA levels of AT1R and AT2R mRNA by RT-PCR. GAPDH served as an internal control. Three independent experiments were performed in all experiment. *Statistical significance (p < 0.05), ** Statistical significance (p < 0.01).

Suppression of growth factor-induced signaling pathways in AGT+/− early EPCs
The Akt/eNOS and MAPK signaling pathways control the migration, proliferation, survival, and differentiation of EPCs (Fu et al., 2007). Accordingly, we examined whether the growth factor-induced activation of these signaling pathways is responsible for the reduced EPC activity in AGT+/− mice. As shown in Fig. 4A, stimulation with VEGF, SDF-1α, or EGF strongly activated Akt, Erk, and eNOS in WT early EPCs but not AGT+/− early EPCs. Because VEGF is a key regulator of vasculogenesis (Asahara et al., 1999), we also measured the protein levels of VEGF and its receptor in WT and AGT+/− early EPCs. VEGF and Flk1 levels were clearly lower in AGT+/− early EPCs, but Flt1 expression was higher, suggesting that VEGF signaling via Flk1 (VEGFR2) is inhibited in AGT+/− early EPCs (Fig. 4B). In addition, we measured the protein levels of HIF-1α and integrin-linked kinase (ILK), which are important regulators of EPC function
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**Fig. 4.** VEGF- or SDF-1α-induced signaling pathways are blocked in AGT⁺⁻ EPCs. (A) After 24 h of starvation, WT EPCs or AGT⁺⁻ EPCs were stimulated with 10 ng/ml VEGF, 50 ng/ml EGF, or 100 ng/ml SDF-1α for 10 min. Levels of phosphorylated eNOS, eNOS, phosphorylated Akt, Akt, phosphorylated Erk, Erk or β-actin were determined with appropriate antibodies by immunoblotting. (B) After 7 days of culture, proteins were isolated from both WT and AGT⁺⁻ early EPCs. Level of VEGFR1, 2 or β-actin were determined with appropriate antibodies by immunoblotting. (C) After 4 h under hypoxic conditions, proteins were isolated from early EPCs. Expression of HIF-1α, ILK or β-actin was determined with appropriate antibodies by immunoblotting. Three independent experiments were performed in all experiment.

**Fig. 5.** Mobilization of EPCs from BM to the peripheral circulation in AGT⁺⁻ mice. MNCs were isolated from peripheral blood (PB) (n = 3) and stained with a FITC-labeled antibody against GFP, a PE-labeled antibody against anti-CD34 (BD Biosciences) and a conjugated anti-VEGFR2 antibody. Flow cytometric analysis was used to detect CD34⁺/VEGFR2⁺ cells in the circulating MNC population. (A) The number of MNCs in PB. (B) Ratio of CD34⁺/VEGFR2⁺ double positive cells in CD45⁻/CD14⁻ cells. Total number of circulating EPCs in PB. Three independent experiment were performed in all experiment. **Statistical significance (p < 0.01).

under hypoxic conditions (Lee et al., 2006). We found HIF-1α and ILK levels were far lower in AGT⁺⁻ early EPCs than in WT early EPCs both under normoxic and hypoxic conditions (Fig. 4C).

**Reduced mobilization of EPCs from the BM to the peripheral circulation in AGT⁺⁻ mice**

Numbers of circulating EPCs in WT and AGT⁺⁻ mice were also investigated. MNCs were isolated from the peripheral blood (PB) of WT or AGT⁺⁻ mice and the expressions of the EPC markers CD34 and VEGFR2 were detected by flow cytometry. But myeloid markers, CD11⁺/CD45⁺ cells were removed. As shown in Fig. 5A, MNC numbers were lower in AGT⁺⁻ mice (1418.9 ± 346.47 cells per 1 μl PB) than in WT mice (2634.8 ± 789.09 cells per 1 μl PB). The proportion of EPCs among PB-MNCs from AGT⁺⁻ mice (0.44% ± 0.032%) was also smaller than for WT mice (0.65% ± 0.035%) (Fig. 5B). Taken together, circulating EPCs counts in 1 μl of PB from AGT⁺⁻ mice (4.76 ± 0.35) were lower than those of WT mice (12.03 ± 0.64) (Fig. 5C).

**Xenograft tumor growth and metastasis were reduced in AGT⁺⁻ mice**

The effect of AGT deletion on the incorporation of EPCs into tumor neovessels was investigated by cross BM transplantation, that is, by exposing WT-GFP and AGT⁺⁻-GFP mice to γ-ray irradiation and then injecting them with GFP-expressing MNCs isolated from AGT⁺⁻-GFP or WT-GFP mice, respectively, and inoculating with lung cancer cells (LLCs) into BM transplanted mice. In WT mice injected with WT MNCs, rapid tumor growth was observed, however in AGT haploinsufficient mice as donors, recipients, or both, tumor growth was significantly inhibited (Fig. 6A).

To investigate metastasis, we inoculated B16F10 cells into a tail vein in WT or AGT⁺⁻ mice, and 10 days later counted numbers of lung nodules. As shown in Fig. 6B, the number of metastatic nodules in AGT⁺⁻ mice (72.0 ± 5.66) was significantly lower than in WT mice (219.5 ± 3.54). These results indicate that tumor growth and metastasis were suppressed in AGT⁺⁻ mice.

**Inhibition of the incorporation of EPCs into tumor neo-vessels in AGT⁺⁻ mice**

To assess the contribution of BM-derived EPCs to LLC tumor growth, we stained tumor tissues with CD31 (an endothelial cell marker). Marked recruitment of BM-derived GFP⁺ cells expressing CD31 (Fig. 6C) was observed in WT mice transplanted with WT BM-MNCs in AGT⁺⁻ mice transplanted with AGT⁺⁻ BM-MNCs, but not in WT mice transplanted with AGT⁺⁻ BM-MNCs or AGT⁺⁻ mice transplanted with AGT⁺⁻ BM-MNCs (Fig. 6D). Thus, GFP⁺CD31⁺ cells were clearly diminished in WT mice transplanted with AGT⁺⁻ BM-MNCs and in AGT⁺⁻ mice transplanted with AGT⁺⁻ BM-MNCs (Fig. 6E). These results suggest that AGT attenuation limits tumor progression by inhibiting the incorporation of EPCs into neovessels.

**DISCUSSION**

In the present study, we focused on the in vitro and in vivo functions of AGT⁺⁻ EPCs to determine the role played by AGT in EPCs during vasculogenesis. The results obtained during the course of the study suggest that AGT is importantly required for the participation of EPCs in tumor neovascularization and metastasis. Initially we found that the number of large EPC-CFUs...
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produced from the BM-derived KSL cells of AGT+/- mice was reduced (Fig. 1D). Because it has been shown that round- and spindle-shaped EPC-CFUs express EC markers (Kwon et al., 2011) whereas spindle shaped late EPC-CFUs exhibit a greater ability to form functional vessels in ischemia (Masuda et al., 2011), these results indicate that the ability of AGT+/- EPCs to differentiate into functional ECs was reduced. Next, we investigated the EC ability to migrate and form tubes as a means of measuring angiogenic potential in vitro. We found the growth factor- and hypoxia-induced migration activities of AGT+/- EPCs were less than those of WT early EPCs (Figs. 2A and 2B). Tube formation by AGT+/- early EPCs was also less than for WT early EPCs (Fig. 2D). These results indicate that the angiogenic potential of AGT+/- EPCs in vitro is smaller than that of WT EPCs. Furthermore, tumor growth was slower in AGT+/- mice, and the mobilization of EPCs from BM into PB (Figs. SB and 5C) and the incorporation of circulating EPCs into new tumor vessels (Fig. 6) were inhibited in AGT+/- mice.

HIF-1 is the master transcription factor of adaptive response to hypoxia. The primary mechanism of hypoxia-induced angiogenesis involves the upregulation of HIF-1 protein, which results in subsequent upregulations of growth factors and receptors, such as, VEGF, SDF-1, CXCR4, and other (Shweiki et al., 1992; Wu et al., 2003; Youn et al., 2011). It is well known that VEGF and SDF-1 promote neovascularization, and that they facilitate paracrine and hormone-like effects (Li et al., 2006; Youn et al., 2011). The immediate effect of VEGF on EPC mobilization is probably related to the prompt development of a chemotactic gradient and enhanced EPC differentiation (Li et al., 2006). Moreover, when acting in concert with VEGF, Ang-2 facilitates endothelial cell migration and proliferation, and thus, serves as a permissive angiogenic signal (Mandriota and Pepper, 1998). In a previous study, the up-regulation of VEGF receptor 2 (Fk1) was found to significantly enhance the migration of and tube formation by EPCs via a process dependent on integrin α6 subunit (Smadja et al., 2007). EPC mobilization and recruitment substantially depend on the ischemia-induced up-regulation of SDF-1α in tissues (Ceradini et al., 2004), and therefore, the SDF-1/CXCR4 signaling pathway plays an important role during EPC migration, proliferation, survival, and angiogenesis (Fu et al., 2007) in response to hypoxia. Furthermore, recent in vitro studies have shown that Ang II from AGT induces VEGF via AT1R signaling (Anandanadesan et al., 2008), suggesting lower levels of AGT in knockout mice reduce angiotensin II, and that this in turn contributes to reduced VEGF induction. Our results also indicate that decreased levels of HIF-1 in AGT+/- EPCs could lead to low VEGF and SDF-1 expression, and thus, reduced expressions of VEGF, Fk1, SDF-1 and CXCR4 might contribute to the suppression of vasculogenesis by AGT+/- EPCs under hypoxic conditions.

Previous studies have indicated that eNOS is critical for EPC function (Murohara et al., 1998). Indeed, angiogenesis in mouse ischemic hindlimbs was impaired in eNOS-deficient animals and capillary growth was stimulated in a rabbit administered with a NO donor (Murohara et al., 1998). In a previous study, it was observed that increased NO levels in BM resulted in the differentiation and mobilization of EPCs from BM niches to the circulation, and ultimately resulted in their participation in tissue-level vasculogenesis and wound healing (Aicher et al., 2003). The activation of growth factors is mediated by eNOS and the subsequent production of NO, as previously described in ischemia-induced angiogenesis (Fu et al., 2007; Silvestre et al., 2002), which suggests neovascularization or endothelization by AGT+/- EPCs in the present study was inhibited in part by reduced eNOS activation. Because AGT+/- mice have low plasma angiotensin II levels and are hypotensive (Babic et al., 1998), it is interesting that eNOS has an inhibitory effect on hypertension. In man, NO induced by vascular endothelium...
regulates vasodilator tone, and thus, blood pressure (Vallance et al., 1989). In a study of mice lacking the eNOS gene, it was found eNOS function is required for vascular and hemodynamic responses to acetylcholine, and thus, that disruption of eNOS gene leads to hypertension (Huang et al., 1995). Furthermore, recent reports demonstrate that whole-body NO production in patients with essential hypertension is reduced under basal conditions, as determined by measuring urinary and plasma nitrate levels (Forte et al., 1997). This is a systemic effect of NO on the regulation of blood pressure, and thus, the role of AGT on the synthesis and activation of eNOS in EPCs could be a different issue, for example, it might be a local effect on vasculogenesis in ischemic tissues.

Tumors are a cause of hypertension (Mendez et al., 2011; Pereira et al., 2004; Ziaja et al., 2008), but relationships between blood pressure and some cytokines related to tumor growth remains controversial. It was reported inhibition of the VEGF signaling pathway induced hypertension in patient with VEGF-targeted therapy (Robinson et al., 2010). On the other hand, in another study, a specific inhibitor of SDF-1/CCXCR4 signaling reduced hypoxia-induced pulmonary hypertension and vascular remodeling by decreasing BM-derived cell lung recruitment in chronic hypoxia in rat (Chen et al., 2000), which suggests that a reduction in hypoxia-induced SDF-1/CCXCR4 in AGT deficient mice reduces local blood pressure. Furthermore, plasma Ang-2 concentrations were found to be elevated in hypertensive patients, particularly in those with atherosclerosis (David et al., 2009). Unlike Ang-2, the Tie-2/Ang-1 pathway prevented pulmonary arterial hypertension in mice (Kugathasan et al., 2009). Because hypertensive phenotypes are represent- ed from complicated responses of a variety of tissues, such as, heart, microvessels in muscles, and kidneys, our results suggest that one of genes involved in blood pressure homeostasis regulates vascularity via BM-derived EPCs. Our findings are supported by other previous reports regarding RAS system and angiogenesis that angiotensin 1-7 improves endothelial function (Rentzsch et al., 2008), ameliorate angiotensin II-induced EC apoptosis (Yang et al., 2012) and EPCs function is increased by the introducing ACE2 genes in BM-derived EPCs (Chen et al., 2013).

In summary, the present study shows that AGT deficiency attenuates vasculogenesis and EPC function. This inhibitory effect observed in AGT+/- EPCs was attributed to a reduction in the differentiation and mobilization of EPCs from BM to hypoxic tissues and to a reduction in EPC function. Furthermore, because HIF-1 levels were reduced in AGT+/- EPCs, growth factors were down-regulated and the VEGF/FKt1, SDF-1/CCXCR4, and the Tie-2 pathways were inhibited. Additional studies are required to elucidate the mechanism of HIF-1 regulation in AGT+/- EPCs completely.

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