Bone Marrow AT<sub>1</sub> Augments Neointima Formation by Promoting Mobilization of Smooth Muscle Progenitors via Platelet-Derived SDF-1α


Objectives—Bone marrow (BM)-derived endothelial progenitor cells (EPCs) and vascular smooth muscle progenitor cells (VPCs) contribute to neointima formation, whereas the angiotensin II (Ang II) type 1 receptor (AT<sub>1</sub>)-mediated action on BM-derived progenitors remains undefined.

Methods and Results—A wire-induced vascular injury was performed in the femoral artery of BM-chimeric mice whose BM was repopulated with AT<sub>1</sub>-deficient (BM-Agtr1<sup>−/−</sup>) or wild-type (BM-Agtr1<sup>+/+</sup>) cells. Neointima formation was profoundly reduced by 38% in BM-Agtr1<sup>−/−</sup> mice. Although the number of circulating EPCs (Sca-1<sup>+</sup>Fk-1<sup>+</sup>) and extent of reendothelialization did not differ between the 2 groups, the numbers of both circulating VPCs (c-Kit<sup>+</sup>Sca-1<sup>+</sup>Lin<sup>−</sup>) and tissue VPCs (Sca-1<sup>+</sup>CD31<sup>+</sup>) incorporated into neointima were markedly decreased in BM-Agtr1<sup>−/−</sup> mice. The accumulation of aggregated platelets and their content of stromal cell–derived factor-1α (SDF-1α) were significantly reduced in BM-Agtr1<sup>−/−</sup> mice, accompanied by a decrease in the serum level of SDF-1α. Thrombin-induced platelets aggregation was dose-dependently inhibited (45% at 0.1 IU/mL, P<0.05) in Agtr1<sup>−/−</sup> platelets compared with Agtr1<sup>+/+</sup> platelets, accompanied by the reduced expression and release of SDF-1α.

Conclusions—The BM-A<sub>T</sub><sub>1</sub> receptor promotes neointima formation by regulating the mobilization and homing of BM-derived VPCs in a platelet-derived SDF-1α-dependent manner without affecting EPC-mediated reendothelialization. (Arterioscler Thromb Vasc Biol. 2010;30:60-67.)

Key Words: bone marrow progenitors □ angiotensin □ neointima formation □ stromal cell–derived factor-1α □ platelet

Bone marrow (BM)-derived progenitors have been shown to contribute to vascular repair and remodeling in both human and animals.\textsuperscript{1,2} BM-derived progenitors are mobilized from BM after vascular injury, home into the sites of healing, and differentiate into endothelial-like cells or vascular smooth muscle–like cells, thereby contributing to reendothelialization or neointima formation.\textsuperscript{3,4} Stromal cell-derived factor-1α (SDF-1α) and its receptor CXCR4 were shown to play a crucial role in the mobilization and homing of BM-derived progenitors after injury.\textsuperscript{5,6} However, the underlying mechanisms that regulate the serum level of SDF-1α and CXCR4 expression on BM-derived progenitors remain poorly understood.\textsuperscript{8}

Angiotensin II (Ang II)-mediated biological actions are involved in the pathogenesis of neointimal hyperplasia after vascular injury.\textsuperscript{9,10} Ang II type1 (AT<sub>1</sub>) receptor–deficient (Agtr1<sup>−/−</sup>) mice showed attenuated cuff-induced neointima formation.\textsuperscript{11} Ang II receptor blocker (ARB) also reduced neointimal hyperplasia in both animal experiments and clinical trials.\textsuperscript{12-14} Ohtani et al showed that peripheral blood mononuclear cells (MNCs) isolated from ARB-treated animals showed a decrease in transdifferentiation into smooth muscle-like progenitors.\textsuperscript{12} Yamada et al also reported that ARB treatment inhibited neointimal hyperplasia by reducing the accumulation of smooth muscle-like progenitors in neointima.\textsuperscript{13} However, the precise mechanisms for AT<sub>1</sub>-mediated actions on the mobilization/homing kinetics of BM-derived endothelial progenitor cells (EPCs) and vascular smooth muscle progenitor cells (VPCs) after injury remain poorly defined.

In this study, BM cells of wild-type (WT) were repopulated with Agtr1<sup>−/−</sup> or Agtr1<sup>+/+</sup> cells to elucidate the underlying

Received May 27, 2009; revision accepted September 29, 2009.

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mechanism for BM-AT1-mediated actions on vascular repair. The results demonstrated for the first time that BM-AT1 is closely involved in neointima formation by causing the mobilization and homing of VPCs rather than EPCs, in which aggregated platelet-derived SDF-1/H9251 plays a crucial role. These findings provide a novel understanding regarding the effect of BM-AT1 on the kinetics of BM-derived vascular-lineage progenitors after vascular injury especially through platelets AT1 receptor, and suggest that the BM renin–angiotensin system could be a potential therapeutic target for the vascular remodeling.

Methods
A full description of all methods can be found in the supplemental materials (available online at http://atvb.ahajournals.org).

Animal Preparation
Agtr1+/− mice (C57BL/6 background) were obtained from Tanabe Seiyaku Co Ltd (Osaka, Japan). Vascular injury was performed by inserting a spring-wire into the femoral artery of BM-chimeric mice whose BM was repopulated with AT1-deficient (BM-Agtr1−/−) or wild-type (BM-Agtr1+/+) cells. All animal experiments were conducted in accordance with the Guidelines for Animal Experiments at Kyoto Prefectural University School of Medicine.

Results
BM-AT1 Deficiency Attenuates Neointima Formation After Vascular Injury
The intimal area and intima/media ratio were significantly reduced in BM-Agtr1−/− mice (38% and 33%, respectively, P<0.05), and the lumen dimension was increased (47%, P<0.05) (Figure 1A and 1B). Hemodynamic data and peripheral blood counts data did not differ between the 2 groups (supplemental Tables I and II).

BM-AT1 Deficiency Does Not Affect Circulating EPCs or Reendothelialization
The number of circulating Sca-1+/Flk-1+ EPCs was similar between the 2 groups at day 3 after injury (Figure 1C). The extent of reendothelialization was also equivalent between the 2 groups at day 7 and day 14 after injury (Figure 1D and 1E), suggesting that attenuated neointima formation in BM-Agtr1−/− mice was not attributable to the accelerated re-endothelialization by BM-derived EPCs.

BM-AT1 Deficiency Inhibits the Mobilization of VPCs
BM-derived VPCs have been shown to contribute to neointima formation after arterial injury,3,4,12,13 in which
circulating VPCs were defined as c-Kit+/Sca-1+/Lin− cells. We found that the number of circulating VPCs (c-Kit+/Sca-1+/Lin−) was markedly increased by 117% in BM-Agtr1+/− mice at day 3 after injury (∗P<0.05 vs BM-Agtr1+/− mice before injury, #P<0.05 vs BM-Agtr1+/− mice 3 days after injury). The numbers of BM-VPCs at day 3 after injury were equivalent between BM-Agtr1+/− and BM-Agtr1+/− mice (Figure 2C and 2D), suggesting that the mobilization of VPCs from BM into the circulation was likely to be attenuated in BM-Agtr1+/− mice.

**BM−AT1 Deficiency Reduces Platelet Aggregation and SDF-1α Release**

SDF-1α/CXCR4 axis has been shown to play a crucial role in the mobilization of VPCs. The expression levels of CXCR4 on the surface of BM-VPCs did not differ between the 2 chimeric mice (supplemental Figure I). We next examined the vascular expression of SDF-1α and found that SDF-1α−positive staining was remarkably declined in BM-Agtr1+/− mice compared with BM-Agtr1+/− mice 3 days after injury (Figure 3A and 3E). Because aggregated platelets have been shown to secrete SDF-1α on the surface of injured arteries, the extent of aggregated platelets and their colocalization with SDF-1α were examined. One day after injury, the inner surface of the injured artery was uniformly covered by platelets, as indicated by CD41 (platelet integrin αIIb)-positive staining, which was almost equivalent between BM-Agtr1+/− and BM-Agtr1+/− mice (Figure 3B and 3F), suggesting that primary platelet adhesion was not affected by platelet AT1 deficiency. In contrast, fibrinogen-positive staining, which reflects the fibrinogen trapped by aggregated platelets, was broadly detected in BM-Agtr1+/− mice 3 days after injury, whereas it was apparently diminished in BM-Agtr1+/− mice (Figure 3C and 3G). The expression level of GPIIb mRNA was remarkably reduced by 36% in BM-Agtr1+/− mice compared with BM-Agtr1+/− mice (Figure 3H),
supporting the notion that platelet aggregation in the injured vessels is attenuated in BM-Agtr1/H11002 mice. Moreover, SDF-1/H9251-positive staining was mostly colocalized with fibrinogen-positive staining (Figure 3D), suggesting that diminished platelets aggregation contributes to the decreased content of SDF-1/H9251 at sites of injured vessel in BM-Agtr1/H11002 mice. We also examined the relationship between eNOS and SDF-1/H9251, because SDF-1/H9251 has been reported to have a deep relationship to NO synthase.8 Immunohistochemical analysis showed that CD31-positive endothelium was hardly observed in the inner layer of the injured vessels 3 days after injury, in which colocalization of aggregated platelets and SDF-1/H9251 was observed (supplemental Figure IIA). Consistent with this finding, the expression level of eNOS mRNA was much lower in the wire-injured vessels compared with the uninjured contralateral vessels, and the expression levels of eNOS mRNA in the wire-injured vessels did not differ between BM-Agtr1+/+ and BM-Agtr1−/− mice (supplemental Figure IIB). These findings suggest that eNOS is unlikely involved in the production of aggregated platelet-derived SDF-1α in our wire-mediated endothelium injury model, compared with the artery ligation model in which endothelium is preserved.8

**BM-AT1 Deficiency Decreases the Serum Level of SDF-1α**

To further elucidate the causal relationship between aggregated platelet-derived SDF-1α and the mobilization of VPCs, we examined the localization of SDF-1α in the injured vessels and the time course of serum levels of SDF-1α. The serum SDF-1α levels in both BM-Agtr1+/+ and BM-Agtr1−/− mice were significantly increased as rapid as 6 hours after injury and thereafter decreased (Figure 4A). Consistent with the findings demonstrated by Zernecke et al, our immunohistochemical analysis at 6 hours after injury showed that SDF-1α-positive staining was observed in the medial wall (data not shown), suggesting that medial smooth muscle cells were the major source of SDF-1α at the acute phase after injury. Thereafter, the serum SDF-1α level in BM-Agtr1+/+ mice gradually declined but was still higher than the baseline level 24 hours after injury and thereafter reverted to the baseline at 3 days. In contrast, the serum SDF-1α level in
BM-Agrt1⁻/⁻ mice declined more rapidly and normalized 24 hours after injury and thereafter further decreased. SDF-1α-positive staining at 3 days after injury was mostly colocalized with fibrinogen-positive staining at the inner surface of the injured artery but not the medial wall (Figure 3D). These findings suggest that a main source of serum SDF-1α after injury was medial smooth muscle cells in the early phase, and that in the late phase is derived from aggregated platelets.

We also examined the serum SDF-1α levels in sham operated animals 3 days after injury, and found that they were equivalent to those (baseline in Figure 4A) in unoperated animals (data not shown). These findings strongly support the notion that attenuated platelet aggregation followed by the reduced release of SDF-1α is closely involved in a rapid decline in the serum SDF-1α level, resulting in impaired mobilization of VPCs in BM-Agrt1⁻/⁻ mice.

**Impaired Mobilization of Agtr1⁻/⁻ VPCs Is Restored by SDF-1α**

We examined whether administration of SDF-1α restored the impaired mobilization of VPCs in BM-Agrt1⁻/⁻ mice. An injection of SDF-1α into vascular-injured BM-Agrt1⁻/⁻ mice increased the number of circulating VPCs by 44% (P<0.05), whereas vascular injury alone did not increase the number of circulating VPCs (Figure 4B). We also examined the effect of anti–SDF-1α antibody (to block CXCR4 axis) on the number of circulating VPCs. Three-day pretreatment with anti–SDF-1α antibody completely abolished the difference in the number of circulating VPCs between the 2 chimeric mice (supplemental Figure III). These findings strongly support the notion that the rapid decline in the serum SDF-1α level caused by the attenuated platelet aggregation seems to be attributable to the impaired mobilization of VPCs in BM-Agrt1⁻/⁻ mice.
BM-AT1 Deficiency Attenuates the Homing of VPCs

To examine the homing of VPCs, the vascular localization of VPCs was evaluated at day 7 after injury. As shown in Figure 4C and 4E, vascular Sca-1<sup>-</sup>/H11001 CD31<sup>-</sup>/H11002 cells (arrows), corresponding to VPCs, were colocalized with aggregated platelets which express SDF-1<sup>-</sup>/H9251. The number of VPCs was markedly reduced by 42% in BM-Agtr1<sup>-</sup>/H11002/H11002 mice compared with BM-Agtr1<sup>+/+</sup>/H11001/H11001 mice (P<0.05) (Figure 4D). In contrast, the number of Sca-1<sup>-</sup>/CD31<sup>-</sup> cells, corresponding to EPCs, did not differ between the 2 groups, suggesting that BM-AT1 deficiency attenuates the homing of BM-derived progenitor cells concomitant with the impaired mobilization of VPCs.

Platelet AT<sub>1</sub> Receptor Deficiency Inhibits Platelet Aggregation

Thrombin-stimulated Agtr1<sup>1+/+</sup> platelets showed an apparent increase in fibrinogen binding in a dose-dependent manner, whereas fibrinogen binding in Agtr1<sup>1−/−</sup> platelets was significantly reduced (45% at 0.1 IU/mL, P<0.05; supplemental Figure IVA and IVB), which was consistent with the previous data showing that ARB treatment significantly reduced platelet aggregation. Platelets produce reactive oxidative species (ROS) via NAD(P)H oxidase activation, and their function is tightly regulated by the redox state. We examined whether production of ROS is actually reduced in Agtr1<sup>1−/−</sup> platelets. Intracellular ROS production, which was detected by flow cytometric analysis with H<sub>2</sub>DCF-DA, showed a significant decrease in Agtr1<sup>1−/−</sup> platelets by 37% (P<0.01) compared with Agtr1<sup>1+/+</sup> platelets (supplemental Figure V). This finding strongly supports the notion that attenuated oxidative stress in Agtr1<sup>1−/−</sup> platelets is, at least in part, responsible for the impaired platelet aggregation.

Platelet AT<sub>1</sub> Receptor Deficiency Inhibits the Expression and Release of SDF-1α

Immunohistochemical analysis revealed that the number of CD41<sup>-</sup>/SDF-1α<sup>−</sup> platelets was markedly increased (3.2-fold) in the Agtr1<sup>1−/−</sup> platelets after thrombin stimulation, whereas it was severely inhibited (1.8-fold) in Agtr1<sup>1−/−</sup> platelets (Figure 5A). Furthermore, the SDF-1α protein in the conditioned medium after thrombin stimulation was significantly reduced in Agtr1<sup>1−/−</sup> platelets compared with Agtr1<sup>1+/+</sup> platelets (Figure 5B), suggesting that impaired platelets aggregation contributed to the decreased serum level of SDF-1α.
Discussion

The present study provides new evidence that deficiency of the BM-AT1 receptor inhibits neointimal formation after vascular injury by affecting the mobilization and homing of BM-derived VPCs (rather than EPCs) in a SDF-1α-dependent manner. Platelet-derived SDF-1α at the sites of injury and the serum level of SDF-1α were profoundly impaired in chimeric mice with Agtr1−/− BM cells, followed by the reduction of both circulating VPCs and vascular VPCs incorporated into neointima. Inhibition of platelet aggregation by ADP receptor blocker ticlopidine markedly suppressed platelet-derived SDF-1α production, which resulted in a decrease in circulating VPCs and attenuation of neointima formation (supplemental Figure VI). These findings provide a new insight into the action of BM-AT1 on the mobilization and homing kinetics of BM-derived vascular-lineage progenitors in the vascular repair.

The SDF-1α/CXCR4 axis has been shown to be implicated in the mobilization and homing of EPCs as well as VPCs.6 Stellos et al18 reported that platelet-derived SDF-1α enhanced the accumulation of CD34+ cells at sites of injury after intravenously injection of CD34+ cells. Likewise, Xiao et al2 reported that local transplantation of embryonic stem cell-derived EPCs inhibited neointimal hyperplasia after wire-induced femoral arterial injury. However, the direct effect of platelet-derived SDF-1α on the mobilization and homing of EPCs was not investigated in these experiments. Zemecke et al10 showed that blocking of SDF-1α after injury reduced the percentage of gated events of VPCs rather than those of EPCs. Taken together, SDF-1α appears to be preferentially involved in the mobilization and homing of VPCs rather than EPCs in the pathogenesis of neointima formation after vascular injury.

The effect of AT1 blockade on the kinetics of BM-derived VPCs after vascular injury remains poorly understood. Yamada et al13 recently investigated the effect of ARB on the homing of BM-derived smooth muscle–like cells using BM chimeric mice whose BM was repopulated with apoE−/−/GFP+ cells. ARB treatment has been shown to reduce vascular oxidative stress and the redox-sensitive gene expression of chemokines and other inflammation-promoting factors,9,10 all of which are critically involved in the mobilization and homing of BM-derived VPCs. In this study, BM chimeric mice were newly generated, which enabled the investigation of AT1-mediated effects on BM-derived progenitor cells independently of vascular AT1-mediated actions. Although the number of BM-VPCs did not differ between BM-Agrtr1−/− and BM-Agrtr1+/+ mice, circulating VPCs after injury were markedly reduced in BM-Agrtr1−/− mice. This finding suggests that the mobilization of VPCs after vascular injury was impaired by BM-AT1 deficiency independent of vascular AT1-mediated actions. Ohtani et al12 found that transdifferentiation of peripheral blood MNCs to smooth muscle progenitor cells was severely suppressed in ARB-treated animals, consistent with our present observation that ARB treatment significantly reduced the number of circulating VPCs after injury (supplemental Figure VII). Considering that platelet-derived SDF-1α causes the homing of BM-derived progenitor cells into injured arteries,15 these findings suggest for the first time that AT1 blockade attenuates the mobilization and homing of BM-derived VPCs into neointima by inhibiting platelet aggregation and the production of vascular SDF-1α without any effect on EPC-mediated reendothelialization.

Harada et al19 previously reported that neointima formation after vascular injury was not suppressed in AT1-deficient mice compared with wild-type mice, and they also described the possibility that growth factors and vasoactive peptides, such as FGF-2, PDGF-B, TGF-β1, and endothelin-1, etc., might substitute fully for AT1-mediated actions in AT1-deficient mice.19 We therefore compared the expression levels of FGF-2, PDGF-B, and TGF-β1 mRNAs between wild-type and AT1-deficient mice. We found that the relative expression levels of FGF-2 and PDGF-B mRNAs in the injured vessels were actually higher in AT1-deficient mice than wild-type mice, whereas TGF-β1 mRNA levels were equivalent between the 2 groups (supplemental Figure VIII). FGF-2 mRNA expression in BM-Agrtr1−/− mice was also significantly higher than BM-Agrtr1+/+ mice, however the extent was much lower than AT1-deficient mice. The mRNA expression levels of PDGF-B and TGF-β1 did not show any difference between BM-Agrtr1+/+ and BM-Agrtr1−/− mice. These findings confirm the involvement of growth factors in neointima formation after vascular injury in AT1-deficient mice, and provide evidence that BM-AT1 rather than vascular AT1 plays a more important role in the formation of neointima.

AT2 receptor has also been reported to play a crucial role in neointima formation after vascular injury,11 therefore we newly generated bone marrow chimeric mice whose bone marrow cells were repopulated with Agrtr2+/+ (BM-Agrtr2+/+) or Agrtr2−/− (BM-Agrtr2−/−) mice. In contrast to the effect of BM-AT1, neointimal area and neointima/media ratio were significantly increased by 40% and 39% in BM-Agrtr2−/− mice compared with BM-Agrtr2+/+ mice (supplemental Figure IXA and IXB), which was consistent with the previous findings that neointimal hyperplasia was exaggerated in Agrtr2−/− mice.13 Reendothelialization and the numbers of VPCs did not differ between BM-Agrtr2+/+ and BM-Agrtr2−/− mice (supplemental Figures IXC, IXD, and X). Similarly, the extent of aggregated platelets and their colocalization with SDF-1α showed no discernable difference between the two groups of mice (supplemental Figure XI). In agreement with these findings, serum concentrations of SDF-1α 3 days after injury and the number of Sca-1+CD31+ cells incorporating into the injured artery in BM-Agrtr2−/− mice were equivalent with those in BM-Agrtr2+/+ mice (supplemental Figure XII). These findings suggest that BM-AT2 receptor counteracts the effect of BM-AT1 on the development of neointimal formation, however underlying mechanisms for BM-AT2-mediated vasoprotective actions are different from those of BM-AT1, and further studies will be required to clarify it.

We also studied the effects of BM-AT1 and BM-AT2 on apoptosis in the development of neointimal formation. The
TUNEL index, which was calculated as the ratio of TUNEL-positive nuclei to total nuclei in the neointima and media, showed a slightly increase in BM-Agtr1−/− mice, whereas it was modestly, but not significantly, reduced in BM-Agtr2−/− mice compared with control mice (supplemental Figure XIII). Because all of the cells in neointima and media are not derived from bone marrow, the effects of BM-AT1 or BM-AT2 on apoptosis might be modest compared with the previous study in which total cells are AT1-deficient or AT2-deficient cells.\(^{11}\)

This study provides novel important evidence that BM-AT1 contributes to neointima formation after vascular injury by promoting the mobilization and homing of BM-derived VPCs in a platelet-derived SDF-1α-dependent manner, and that AT1 deficiency inhibits SDF-1α release by blocking aggregation of platelets. Such relationship between BM vascular-lineage progenitors and BM renin-angiotensin system, especially in terms of platelet-SDF-1α/VPCs interaction, may provide a new insight into the role of renin–angiotensin system in the pathogenesis of vascular repair.

Acknowledgments
We thank Prof T. Todo and J. Kobayashi, and the Radiation Biology Center Kyoto University (H18-17) for assistance with bone marrow transplantation.

Sources of Funding
This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (00240036).

Disclosures
No one.

References
Bone Marrow AT1 Muscle Progenitors via Platelet-Derived SDF-1 α


Arterioscler Thromb Vasc Biol. 2010;30:60-67; originally published online October 15, 2009; doi: 10.1161/ATVBAHA.109.192161

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplement Material
Supplement Figure I

A

BM-Agtr1^{+/+}  BM-Agtr1^{-/-}

![Flow cytometry plots]

B

BM-VPCs

![Bar chart]

CXCR4

Control  CXCR4

BM-Agtr1^{+/+}  BM-Agtr1^{-/-}
Supplement Figure Ⅱ

A

CD31

DAPI

Merged

B

eNOS mRNA level (fold)

Wire injury

(-)  (+)  (+)

BM-Agtr1+/+  BM-Agtr1-/-
Supplement Figure Ⅲ

Peripheral blood Sca-1+/c-Kit/Lin- cells (% control)

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(%)
Supplement Figure IV

A

Agtr1\(^{+/+}\)

Agtr1\(^{-/-}\)

Events

Relative fluorescence

B

Fibrinogen binding (MFI % control)

(%) Resting 0.01 0.05 0.1

Thrombin (IU/ml)

Resting 0.01 0.05 0.1

0 200 400 600 800

Thrombin (IU/ml)
Supplement Figure V

A

B

DCF

% of maximum

ROS production (fold)

Agtr1−/− Agtr1+/+

Platelets

Agtr1+/+ Agtr1−/−

*
Supplement Figure VI

A

BM-Agtr1**+/+**
BM-Agtr1**−/−**

B

Intimal area

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C

Peripheral blood Sca-1^+ c-Kit^− Lin^−^ cells

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Fibrinogen

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SDF-1α

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Merged

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Sca-1^+ CD31^− cells

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Sca-1^+ CD31^+ cells

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Supplement Figure VII

A  Saline  ARB

- Peripheral blood Sca-1+/c-Kit-/Lin- cells

B

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C

- Peripheral blood Sca-1+/c-Kit-/Lin- cells

D

- Merged Fibrinogen+ length/total lumen length

E

- Merged SDF-1α length/total lumen length

- Merged Sca-1+/CD31+ cells

- Merged Sca-1+/CD31- cells
Supplement Figure VII

**FGF-2 mRNA**

- Agtr1+/+
- Agtr1-/-
- BM-Agtr1+/+
- BM-Agtr1-/-

**PDGF-B mRNA**

- Agtr1+/+
- Agtr1-/-
- BM-Agtr1+/+
- BM-Agtr1-/-

**TGF-β1 mRNA**

- Agtr1+/+
- Agtr1-/-
- BM-Agtr1+/+
- BM-Agtr1-/-
Supplement Figure IX

A

BM-Agtr2^{+/+}  BM-Agtr2^{+-}

B

\[
\begin{array}{c|c|c|c}
& \text{Intimal area} & \text{Medial area} & \text{Intima/media ratio} \\
\hline
BM-Agtr2^{+/+} & 10 \times 10^3 \mu m^2 & 10 \times 10^3 \mu m^2 & 1.5 \\
BM-Agtr2^{+-} & 15 \times 10^3 \mu m^2 & 10 \times 10^3 \mu m^2 & 2.0 \\
\end{array}
\]

C

BM-Agtr2^{+/+}  BM-Agtr2^{+-}

day7  day14

D

\[
\begin{array}{c|c|c|c|c|c}
& \text{CD31}^+ \text{ length} & \frac{\text{CD31}^+ \text{ length}}{\text{total lumen length}} \\
\hline
BM-Agtr2^{+/+} & 0.2 & 0.6 \\
BM-Agtr2^{+-} & 0.4 & 0.8 \\
BM-Agtr2^{+/+} & 0.6 & 1.0 \\
BM-Agtr2^{+-} & 0.8 & 1.2 \\
\end{array}
\]
Supplement Figure X

A  

<table>
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<tr>
<th>Lin</th>
<th>FSC</th>
<th>c-Kit</th>
<th>Sca-1</th>
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<tr>
<td>BM-Agt2+/+</td>
<td>Specimen_001_Tube_004.fcsÉFSC-A, PE-Cy7-A subset</td>
<td>30.9</td>
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<td>BM-Agt2-/-</td>
<td>Specimen_001_Tube_004.fcsÉPerCP-Cy5-5-A subset</td>
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B  

<table>
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<tr>
<td>% of circulating VPCs (Sca-1+c-Kit-Lin- cells)</td>
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<tr>
<td>Day0</td>
</tr>
<tr>
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C  

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<th>Lin</th>
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<th>c-Kit</th>
<th>Sca-1</th>
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<td>BM-Agt2+/+</td>
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<td>APC-A</td>
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<tr>
<td>BM-Agt2-/-</td>
<td>Specimen_001_Tube_004.fcsÉFSC-A</td>
<td>PE-Cy7-A</td>
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D  

<table>
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<th>(%)</th>
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<tr>
<td>% of bone marrow VPCs (Sca-1+c-Kit-Lin- cells)</td>
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<tr>
<td>Day0</td>
</tr>
<tr>
<td>0</td>
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<tr>
<td>Day3</td>
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Legend:
- Lin: Lineage
- FSC: Forward Scatter
- Sca-1: Stem cell antigen-1
- c-Kit: c-Kit
- BM-Agt2+/+: Bone marrow Agtr2+/+
- BM-Agt2-/-: Bone marrow Agtr2-/-

% of circulating VPCs (Sca-1+c-Kit-Lin- cells)
Supplement Figure XI

A

BM-Agrt2^{+/+}  BM-Agrt2^{-/-}

CD41

SDF-1α

Fibrinogen

Merged

B

![Graph showing CD41+ length/total lumen length comparison between BM-Agrt2^{+/+} and BM-Agrt2^{-/-}](image)

C

![Graph showing SDF-1α+ length/total lumen length comparison between BM-Agrt2^{+/+} and BM-Agrt2^{-/-}](image)

D

![Graph showing Fibrinogen+ length/total lumen length comparison between BM-Agrt2^{+/+} and BM-Agrt2^{-/-}](image)
Supplement Figure XII

A

Serum level of SDF-1α (ng/ml)

BM-Agtr2+/+  BM-Agtr2−/

day3

B

Sca-1+CD31- cells (cells/section)

BM-Agtr2+/+  BM-Agtr2−/

day7
Supplement Figure XIII

A

TUNEL

DAPI

Merged

Transmitted

B

TUNEL Index (%)

BM-WT

BM-Agtr1−−

BM-Agtr2−−

n.s.
Supplement Figure XIV

FMO (Fluorescence-minus-one)
Full stain except Sca-1

All colors
Full stain including Sca-1

FSC
SSC
Lin
c-Kit
Sca-1

FSC
SSC
Lin
c-Kit
Sca-1
Supplement Table I
Body Weight and Hemodynamic Parameters

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<th>Systolic BP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
<th>Mean BP (mmHg)</th>
<th>Heart rate (bpm)</th>
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<td>BM-Agtr1\textsuperscript{++}</td>
<td>8</td>
<td>19.5 ± 0.3</td>
<td>97.7 ± 4.9</td>
<td>60.0 ± 5.1</td>
<td>72.5 ± 4.3</td>
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<tr>
<td>BM-Agtr1\textsuperscript{-/-}</td>
<td>7</td>
<td>19.1 ± 0.4</td>
<td>94.2 ± 7.2</td>
<td>58.6 ± 6.5</td>
<td>70.2 ± 6.0</td>
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Values are the mean ± SE. No significant differences were found between the genotypes using Fisher’s test. BP, blood pressure.
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<th>n</th>
<th>RBC (x10⁴/µl)</th>
<th>WBC (/µl)</th>
<th>Neutrophil (/µl)</th>
<th>Lymphocyte (/µl)</th>
<th>Monocyte (/µl)</th>
<th>Plt (x10⁴/µl)</th>
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<tr>
<td>BM-Agtr1⁺/⁺</td>
<td>8</td>
<td>807 ± 27</td>
<td>5,425 ± 1,185</td>
<td>1,000 ± 214</td>
<td>4,374 ± 999</td>
<td>301 ± 129</td>
<td>45 ± 8</td>
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<tr>
<td>BM-Agtr1⁻/⁻</td>
<td>8</td>
<td>774 ± 16</td>
<td>3,725 ± 653</td>
<td>653 ± 153</td>
<td>2,993 ± 602</td>
<td>258 ± 78</td>
<td>55 ± 7</td>
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</tbody>
</table>

Values are the mean ± SE. No significant differences were found between the genotypes using Fisher’s test. RBC, red blood cells; WBC, white blood cells; Plt, platelets.
Supplement Material

Bone Marrow AT₁ Augments Neointima Formation by Promoting Mobilization of Smooth Muscle Progenitors via Platelet-Derived SDF-1α

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Methods

Animal preparation: AT1a receptor-deficient (Agtr1\(^{-/-}\)) mice (C57BL/6 background) were obtained from Tanabe Seiyaku Co., Ltd. (Osaka, Japan). Two-month–old female wild type (Agtr1\(^{+/+}\)) recipient mice were lethally irradiated with 9 Gy using an X-ray source. BM cells were harvested from the femurs and tibias of donor male mice (Agtr1\(^{+/+}\) or Agtr1\(^{-/-}\)) by flushing with RPMI-1640 medium (Gibco BRL), and the recipients received 5\(\times\)10\(^6\) BM cells per mouse in 0.2 ml of medium by tail-vein injection. Six weeks after after BM transplantation, arterial injury was performed by insertion of a spring-wire into the left femoral artery. In another set of experiments, the administration of an adenosine diphosphate (ADP) receptor inhibitor, ticlopidine hydrochloride (50 mg/kg/day), into the chimeric mice was started from 5 days before arterial injury and continued until 7 days after injury. In ARB treatment experiments, the administration of Olmesartan (3.0 mg/kg/day) or saline into two-month-old female wild-type mice using an osmotic minipump was started from 5 days before injury and continued until 7 days or 28 days after injury. Another subgroup of mice were injected with blocking SDF-1\(\alpha\) mAb (clone 79014.111, R&D Systems) or IgG\(_1\) isotype control stating 1 day before injury (100\(\mu\)g i.p.) and 1 day after injury (50\(\mu\)g i.p.). To study the effect of SDF-1\(\alpha\) on
the number of circulating VPCs, SDF-1α (10μg i.p.) was injected into the BM-Agtr1<sup>−/−</sup> mice one day after injury. The animals were maintained at 22°C in a room with a 12-hour light/dark cycle and received drinking water ad libitum. All animal experiments were conducted in accordance with the Guidelines for Animal Experiments at Kyoto Prefectural University School of Medicine.

**Quantitative measurement of neointima formation and immunohistochemistry:**

Four weeks after injury, 4% paraformaldehyde was perfused under physiological pressure and histological analysis was performed. Neointima formation in the femoral artery was examined at 3 locations, each separated by 100 μm, after Elastica van Gieson staining. Three serial sections (6 μm thick) prepared from the middle portion of the femoral artery were stained immunohistochemically with antibodies against progenitor cells (Sca-1, Pharmingen), endothelial cells (CD31, Pharmingen), platelets (CD41, Pharmingen), SDF-1α (CXCL12/SDF-1, R&D Systems), and fibrinogen (Dako Cytomation), and then observed using a confocal microscope (LSM510, Carl Zeiss). As a negative control, non-immune IgG and FITC- or TRITC-conjugated secondary antibodies were used. Image analysis was performed with Scion Imaging Software.
Flow cytometry and cell sorting: Flow cytometric analysis was performed as described previously (1). For the staining of lineage antigens, biotinylated rat antibodies specific for CD3e (145-2C11), CD4 (RM4-5), CD8a (53-6.7), CD19 (6D5), B220 (RA3-6B2), and Gr-1 (RB6-8C5) were used followed by streptavidin-phycoerythrin-Cy7. For the staining of vascular potential progenitor cells and their expression of CXCR4, BM and peripheral blood cells were stained with allophycocyanin-conjugated anti-c-Kit (2b8), phycoerythrin-conjugated anti-Sca-1 (D7), and fluorescein isothiocyanate (FITC)-conjugated anti-CXCR4 (2B11/CXCR4). We evaluated background fluorescence by the method of fluorescence-minus-one (FMO) control (2). As shown in Supplement Figure XIV, phycoerythrin-conjugated anti-Sca-1 stain was omitted from the FMO control and included in the full stain to determine the boundary for Sca-1 positive cells. For the staining of circulating endothelial progenitor cells, antibodies against phycoerythrin-conjugated antimouse Flk-1 (AVAS 12α1) and fluorescein isothiocyanate (FITC)-conjugated anti-Sca-1 (D7) were used (3). Cells were sorted with a highly modified double-laser FACSria Cell Sorter. The intraday coefficient of variation was less than 15%, and mean intraday errors were less than 10%. FACS data were analyzed by two different observers to evaluate the interobserver variability. The percentage difference between the two independent observers blinded to genotype was less than 15%.

Enzyme-linked immunosorbent assay: The serum level of SDF-1α was measured by ELISA (Mouse CXCL12/SDF-1 alpha, Quantikine ELISA Kit, R&D systems).
Absorbance was read at 450 nm and background corrected, and the serum level of SDF-1α was calculated with a standard.

**Fibrinogen binding assay:** Washed platelets (1 x 10^8 platelets /ml) were incubated simultaneously with 0.01, 0.05, or 0.1 IU/ml thrombin, Oregon Green 488-conjugated fibrinogen (150 µg/ml), and Tyrode’s buffer in a final volume of 100 µl (4). After 10 minutes at 37°C without shaking, samples were fixed by the addition of formalin and then diluted 5 times with HEPES-Tyrode’s buffer. Samples were analyzed by flow cytometry using a FACSCalibur instrument and Cellquest software.

**Detection of apoptotic cells:** For the detection of DNA fragmentation, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining of the injured artery was performed. The TUNEL Index was calculated as the ratio of TUNEL-positive nuclei to total nuclei in the neointima and media. In addition, to examine the morphological changes of nuclear chromatin, chromatin dye staining was done with DAPI.
**Measurement of ROS production by FACS analysis:** Washed platelets (1 x 10^8 platelets /ml) from Agtr1^{+/+} or Agtr1^{-/-} mice were preincubated with H2DCF-DA (50 \mu M) for 5 minutes in PBS/5.5 mM glucose/1mM EDTA. Excessive dye was washed out, and platelets were resuspended in HEPES buffer. Samples were then analyzed for intracellular ROS production by flow cytometry (5).

**Statistical analysis:** All data are expressed as the mean ± SE. Mean values were compared using ANOVA. If a statistical significant effect was found, Fisher’s test was performed to detect the difference between the groups. P<0.05 was considered statistically significant.
Hemodynamic analysis

Mean blood pressure and heart rate were measured under conscious and unrestrained conditions using a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan). Hemodynamic parameters were analyzed 6 weeks after BM transplantation in conscious animals using tail-cuff measurements. Mean blood pressure and heart rate did not differ between the two groups before vascular injury (Supplement Table I).

Hematological analysis

Total leukocyte, red blood cell, and platelet counts were determined in peripheral blood using an automated counter (K-4500, CYSMEX Co., Ltd.). The manual 100-cell leukocyte differential counting of neutrophils and lymphocytes was performed with May-Grunwald/Giemsa-stained smears. There was no significant difference between the two groups before injury (Supplement Table II).
Supplement Figure Legend

Supplement Figure I

BM-AT₁ did not affect the CXCR4 expression on BM-VPCs. (A) Flow cytometry of CXCR4 expression in c-KitâŠ›Sca-1âŠ›lineage-negative (CD3, CD4, CD8, CD19, B220, and Gr-1) BM populations 3 days after injury. Histogram in graph shows CXCR4 surface expression in c-KitâŠ›Sca-1âŠ›lineage-negative BM cells from BM-Agtr1⁺/⁺ and BM-Agtr1⁺/⁻ mice (green line). PE-conjugated nonspecific mouse IgG was used to measure nonspecific binding (red line). (B) Quantitative analysis showing no difference in CXCR4 expression between the two groups. Values are the mean ± SE for at least 4 mice in each group.

Supplement Figure II

Endothelium was completely denuded in the wire-induced injury model 3 days after injury. (A) Immunofluorescence image showing CD31 (red) and DAPI (blue) 3 days after injury in BM-Agtr1⁺/⁺ mice. Arrows indicate inner surface of the injured vessels. L: lumen, M: media. (B) Quantitative analysis of eNOS mRNA expression levels by real-time PCR showing a significant decrease in BM-Agtr1⁺/⁺ mice after injury,
but no difference between BM-Agtr1\(^{+/+}\) and BM-Agtr1\(^{-/-}\) mice. Values are the mean ± SE for at least 6 mice in each group. \(* P<0.05\) vs. un-injured vessels.

**Supplement Figure III**

**Pretreatment with anti-SDF-1\(\alpha\) antibody abolished the difference in the number of circulating VPCs between BM-Agtr1\(^{-/-}\) and BM-Agtr1\(^{+/+}\) mice after vascular injury.**

The BM of 8-week-old wild-type mice was repopulated with AT\(_1\)-deficient (Agtr1\(^{-/-}\)) or wild-type (Agtr1\(^{+/+}\)) cells. Six weeks after BM transplantation, a wire-induced vascular injury was performed. Anti-SDF-1\(\alpha\) mAb was intraperitoneally injected one day before injury (100\(\mu\)g i.p.) and 1 day after injury (50\(\mu\)g i.p.). Flow cytometry of c-Kit and Sca-1 expression in lineage-negative (CD3, CD4, CD8, CD19, B220, and Gr-1) populations was performed on day 3 after injury. Quantitative analysis of the number of circulating VPCs showing no difference between BM-Agtr1\(^{-/-}\) and BM-Agtr1\(^{+/+}\) mice. The bar graphs represent the mean±SE relative to BM-Agtr1\(^{+/+}\) mice treated with anti-SDF-1\(\alpha\) mAb set at 100%. At least 5 mice were tested in each group.

**Supplement Figure IV**

**Attenuated platelet aggregation on ablation of marrow AT\(_1\).** (A) Fibrinogen binding
was analyzed by flow cytometry in Agtr1\textsuperscript{+/+} and Agtr1\textsuperscript{+/−} platelets under resting or stimulated conditions as indicated. (B) Quantitative analysis showing a significant decrease in fibrinogen binding in Agtr1\textsuperscript{−/−} platelets in a dose-dependent manner. Values are the mean ± SE for at least 4 mice in each group. *\(P<0.05\) vs. Agtr1\textsuperscript{+/+} platelets.

Supplement Figure V

**Intracellular ROS production was reduced in AT1 deficient platelets.** (A) Flow cytometry of ROS production assessed by H2DCF-DA. Histogram in graph shows fluorescence intensity of H2DCF-DA in Agtr1\textsuperscript{+/+} platelets (green line) and Agtr1\textsuperscript{−/−} platelets (red line). (B) Quantitative analysis showing a significant decreased production of ROS in Agtr1\textsuperscript{−/−} platelets. Values are the mean ± SE for at least 6 mice in each group. *\(P<0.05\) vs. Agtr1\textsuperscript{+/+} platelets.

Supplement Figure VI

**Short-term treatment with ADP receptor blocker ticlopidine abolished the inhibitory effect of BM-AT\textsubscript{1} deficiency on intima formation.** (A) Treatment with ticlopidine (50 mg/kg/day) was started 5 days before injury and continued until 7 days
after injury in both groups of mice. Representative Elastica van Gieson-stained femoral arteries 4 weeks after injury. (B) Quantitative analysis showing no significant difference between BM-Agtr1+/+ and BM-Agtr1−/− mice. Values are the mean ± SE for at least 8 mice in each group. (C) Quantitative analysis 3 days after injury showing no significant difference in the number of circulating VPCs (c-Kit−Sca-1−Lin−) between the two groups. Values are the mean ± SE for 5 mice in each group. (D) Immunofluorescence image showing fibrinogen and SDF-1α at day 3 after injury. Merged image showing fibrinogen (green) and SDF-1α (red). Quantitative analysis showing no difference in the extent of fibrinogen and SDF-1α-positive staining between BM-Agtr1+/+ and BM-Agtr1−/− mice. Values are the mean ± SE for at least 5 mice in each group. L: lumen, M: media. (E) The number of Sca-1+CD31− and Sca-1+CD31+ cells 7 day after injury. Values are the mean ± SE for at least 4 mice in each group.

**Supplement Figure VII**

**Short-term treatment with ARB inhibited neointima formation accompanied by the reduction of aggregated platelet-derived SDF-1α.** (A) Eight-week-old wild-type mice were treated with ARB, Olmesartan (3.0 mg/kg/day) from 5 days before injury and until 7 days after injury. Representative Elastica van Gieson-stained femoral arteries 4
weeks after injury. (B) Quantitative analysis showing significant decreases in neointima formation and intima/media ratio in ARB-treated mice. Values are the mean ± SE for at least 10 mice in each group. *P<0.05 vs. saline-treated mice. (C) Flow cytometry of c-Kit and Sca-1 expression in lineage-negative (CD3, CD4, CD8, CD19, B220, and Gr-1) circulating mononuclear cell populations 3 days after injury. Quantitative analysis showing a significant decrease in number of circulating VPCs (c-Kit^-Sca-1^-Lin^-) in ARB-treated mice. Values are the mean ± SE for 5 mice in each group. *P<0.05, vs. saline-treated mice. (D) Immunofluorescence image showing fibrinogen and SDF-1α 3 day after injury. Merged image showing fibrinogen (green) and SDF-1α (red). Quantitative analysis showing the decrease in fibrinogen and SDF-1α-positive staining in ARB-treated mice. Values are the mean ± SE for at least 5 mice in each group. *P<0.05, vs. saline-treated mice. L: lumen, M: media. (E) The number of Sca-1^-CD31^- and Sca-1^-CD31^+ cells 7 day after injury. The number of Sca-1^-CD31^- cells was significantly decreased in ARB-treated mice, while there was no significant difference in Sca-1^-CD31^+ cells between the two groups. Values are the mean ± SE for at least 4 mice in each group. *P<0.05, vs. saline-treated mice.
Supplement Figure VIII

Expression levels of FGF-2 and PDGF-B mRNAs were enhanced after vascular injury in AT$_1$ deficient mice but not in BM-Agtr$^{1/-}$ mice. (A) Quantitative analysis of the expression levels of FGF-2, PDGF-B, and TGF-$\beta$1 mRNAs by real-time PCR 7 days after injury in AT1 deficient mice (Agtr1$^{-/-}$), wild-type (Agtr1$^{+/+}$), BM-Agtr1$^{+/+}$, and BM-Agtr1$^{-/-}$ mice. Values are the mean±SE relative to the expression level of un-injured contralateral vessels set at 100% for at least 6 mice in each group. *$P<0.05$ vs. Agtr1$^{+/+}$ mice. # $P<0.05$ vs. BM-Agtr1$^{+/+}$ mice. †$P<0.05$ vs. Agtr1$^{-/-}$ mice.

Supplement Figure IX

Ablation of marrow AT$_2$ exaggerated neointima formation after vascular injury without affecting on EPC-mediated re-endothelialization. (A) Representative Elastica van Gieson-stained femoral arteries 4 weeks after injury. (B) Quantitative analysis showing significant decreases in neointima formation in BM-Agtr2$^{+/+}$ mice. Values are the mean ± SE for at least 8 mice in each group. *$P<0.05$ vs. BM-Agtr2$^{+/+}$ mice. (C) Representative immunohistochemical staining for CD31 at day 7 and day 14 after injury. L: lumen. (D) Quantitative analysis showing no difference in the extent of
re-endothelialization between the two groups. Values are the mean ± SE for at least 6 mice in each group. \( *P<0.05 \) vs. BM-Agtr1\(^{+/+}\) mice, \( \#P<0.05 \) vs. BM-Agtr2\(^{-/-}\) mice.

**Supplement Figure X**

**Ablation of marrow AT\(_2\) did not affect on the number of circulating and BM VPCs.**

(A) Flow cytometry of c-Kit and Sca-1 expression in lineage-negative circulating mononuclear cell populations of BM-Agtr2\(^{+/+}\) and BM-Agtr2\(^{-/-}\) mice. (B) Quantitative analysis showing no difference in the number of circulating VPCs (c-Kit\(^{+}\)Sca-1\(^{+}\)Lin\(^{-}\)) before and 3 days after injury between BM-Agtr2\(^{+/+}\) and BM-Agtr2\(^{-/-}\) mice. Values are the mean ± SE for 5 mice at each time point in each group.

(C) Flow cytometry of c-Kit and Sca-1 expression in lineage-negative BM populations at day 3 after injury. (D) Quantitative analysis showing no difference in the percentage of BM-VPCs of total cells between the two groups. Values are the mean ± SE for 5 mice in each group.

**Supplement Figure XI**

**Ablation of marrow AT\(_2\) did not affect on platelet aggregation and the content of SDF-1\(_\alpha\).** (A) Immunofluorescence image showing CD41 (1 day), SDF-1\(_\alpha\) (3 day), and fibrinogen (3 day) after injury. Positive staining was mainly detected on the inner
surface of the vessel wall. (B, C and D) Quantitative analysis showing no difference in CD41, SDF-1α and fibrinogen-positive staining between BM-Agtr2+/+ and BM-Agtr2−/− mice. Values are the mean ± SE for at least 5 mice in each group. L: lumen, M: media.

Supplement Figure XII

Ablation of marrow AT2 did not affect on the serum level of SDF-1α and the number of Sca-1+CD31− cells incorporated into neointima. (A) The serum SDF-1α level at 3 days after injury was equivalent between BM-Agtr2+/+ and BM-Agtr2−/−. Values are the mean ± SE for at least 4 mice in each group. (B) Quantitative analysis showing no difference in the number of Sca-1+CD31− cells between the two groups. Values are the mean ± SE for at least 4 mice in each group.

Supplement Figure XIII

In situ detection of apoptotic cells by TUNEL staining. (A) Immunofluorescence image showing TUNEL (red) and DAPI (blue) 28 days after injury. Arrows indicate TUNEL positive cells. L: lumen, I: intima, M: media, A: adventitia. (B) Quantitative analysis showing no difference in TUNEL Index among the three groups. TUNEL
index: the ratio of TUNEL-positive nuclei to total nuclei in the neointima and media. Values are the mean ± SE for at least 5 mice in each group.

**Supplement Figure XIV**

**Fluorescence-minus-one (FMO) staining revealed the boundary between Sca-1 positive and negative cells.** Peripheral blood cells were divided into lineage positive and negative cells, which were further divided into c-Kit−Sca-1− and c-Kit−Sca-1+ cells. Background fluorescence was evaluated by the method of fluorescence-minus-one (FMO) controls (2). Phycoerythrin-conjugated anti-Sca-1 stain was omitted from the FMO control and included in the full stain to determine the boundary for Sca-1 positive cells.


2. Tung JW, Parks DR, Moore WA, Herzenberg LA, Herzenberg LA. New approach to

