diated effects on the differentiation/proliferation of HSCs and monocyte-lineage cells by flow cytometric analysis. The number of GMP was much lower in apoE$^{-/-}$/BM-Agtr1$^{-/-}$ mice (34%, $P<0.05$), whereas HSCs and CMP did not differ between the 2 groups (Figure 1B). The expression level of CCR2 on monocyte-lineage cells was not impaired in Agtr1$^{-/-}$ mice (supplemental Figure II).

M-CSF–Induced Macrophage-Colony–Forming Activity Is Attenuated in BM Cells From AT_1-Deficient Mice

We first compared the numbers of HSCs, CMP, and GMP between Agtr1$^{+/+}$ and Agtr1$^{-/-}$ mice under steady-state condition without hypercholesterolemia. There was no difference between the 2 genotypes of mice, suggesting that the steady-state development of monocyte-lineage cells is relatively well preserved in Agtr1$^{-/-}$ mice (supplemental Figure IIIA, supplemental Table III). We next performed a macrophage-colony–forming assay to investigate whether the response to M-CSF is attenuated in BM cells from Agtr1$^{-/-}$ mice. Stimulation by M-CSF markedly increased the number of macrophage-colony units in BM cells from Agtr1$^{+/+}$ mice, which was remarkably diminished in BM cells from Agtr1$^{-/-}$ mice ($P<0.01$; supplemental Figure IIIB), suggesting that BM-AT_1 is crucially implicated in M-CSF–induced differentiation/proliferation of HSCs into monocyte-lineage cells.

M-CSF–Induced Differentiation From HSCs to Monocyte-Lineage Cells Is Suppressed in HSCs From AT_1-Deficient Mice

We examined the time course of differentiation of HSCs from Agtr1$^{+/+}$ mice into monocyte-lineage cells with or without M-CSF. Stimulation by M-CSF preferentially increased the number of promonocytes (CD11b^{high}Ly-6G^{low}) terminally differentiated from myeloid progenitor (MP: c-Kit$^+$Sca-1$^-$Lin$^-$; supplemental Figure IV).

We next compared the differentiation potential of HSCs between Agtr1$^{+/+}$ and Agtr1$^{-/-}$ mice (Figure 2). In the absence of M-CSF, the numbers of myeloid progenitors and promonocytes (CD11b^{high}Ly-6G^{low}) did not differ between the 2 genotypes. In contrast, stimulation by M-CSF markedly increased the number of promonocytes in both groups, whereas the extent was significantly attenuated in HSCs from Agtr1$^{-/-}$ mice (38%, $P<0.01$).

c-Fms Expression Is Inhibited in AT_1-Deficient Mice

The expression of M-CSF receptor c-Fms was examined by flow cytometry. Consistent with the previous finding,[16] the expression level of c-Fms (CD115) was gradually upregulated during the developmental stage from HSCs to promonocytes in Agtr1$^{+/+}$ mice, whereas the expression was severely decreased in all developmental stages in Agtr1$^{-/-}$ mice (Figure 3). The mRNA expression of c-Fms was also suppressed by 71% in Agtr1$^{-/-}$ mice ($P<0.05$; supplemental Figure V).

We also examined the effect of hypercholesterolemia on the c-Fms expression. Four-week Western diet feeding significantly increased the expression level of c-Fms (CD115) in all populations of HSCs, myeloid progenitors, and promonocytes compared with chow diet feeding (supplemental Figure VI). In contrast, BM-AT_1 expression was not affected by the Western diet feeding (supplemental Figure VII). These findings suggest that in the hypercholesterolemic setting, M-CSF–mediated growth of monocyte-lineage cells is enhanced by an increase in its receptor c-Fms expression.

Phosphorylation of PKC-δ and JAK2 Is Inhibited in Monocyte-Lineage Cells From AT_1-Deficient Mice

To investigate the effect of reduced c-Fms expression on its downstream signals, we examined the phosphorylation of PKC-δ and JAK2, which are known to be essential in M-CSF–induced differentiation/proliferation of monocyte-lineage cells.[17,18] In c-Kit$^+$ Lin$^-$ population including HSCs (c-Kit$^+$Sca-1$^+$Lin$^-$) and myeloid progenitors (c-Kit$^+$Sca-1$^-$Lin$^-$) from Agtr1$^{+/+}$ mice, the peak phosphorylation levels of PKC-δ and JAK2 were observed at 5 and 30 minutes after M-CSF stimulation, respectively (supplemental Figure VIIIA). The M-CSF–induced phosphorylation levels of PKC-δ and JAK2 at each time point were dramatically diminished in HSCs and myeloid progenitors from Agtr1$^{-/-}$ mice (80% and 75%, respectively, $P<0.01$; supplemental Figure VIIIB and VIIIC). These findings were also confirmed by Western blot analysis (supplemental Figure IX). We further examined the effect of PKC-δ inhibitor (rottlerin) or JAK2 inhibitor (AG490) on M-CSF–induced differentiation/proliferation of BM monocyte-lineage cells. Administration of rottlerin (10μmol/L) or AG490 (50μmol/L) into the culture medium completely diminished the M-CSF–induced increase in the number of promonocytes (supplemental Figure X).

The Expression of c-Fms on Promonocytes Is Not Affected by Ang II or ARB

We next studied how AT_1 signals regulate the c-Fms expression on HSCs/promonocytes. The result from the in vitro culture assay showed that 4-day treatment with Ang II (1 μmol/L) or ARB (10 μmol/L) did not affect the expression levels of c-Fms on HSCs, myeloid progenitors, and promonocytes (only data in promonocytes shown in Figure 4), and also did not affect M-CSF–mediated growth of HSCs, myeloid progenitors, and promonocytes (supplemental Figure XIA), suggesting that AT_1 receptor–mediated signals are not directly involved in the expression of c-Fms on HSCs and BM monocyte-lineage cells nor the differentiation from HSCs to promonocytes.

TNF-α Restores the Impaired Expression of c-Fms on Promonocytes From Agtr1$^{-/-}$ Mice

To further elucidate the mechanism by which Ang II regulates the expression of c-Fms, we next focused on the BM stromal cells (CD45$^-$CD34$^-$) other than hematopoietic-lineage cells, and examined the expression of TNF-α, because TNF-α has been reported to regulate the c-Fms expression in various cell types.[19,20] Interestingly, the expression level of TNF-α was extremely higher in the purified CD45$^-$CD34$^-$ BM stromal cells compared with that in promonocytes (supplemental Figure XIIA). Immunohistochemical analysis also showed that TNF-α–positive staining was mostly colocalized with BM stromal cells (supplemental Figure XIIB). Furthermore, we found that the number of

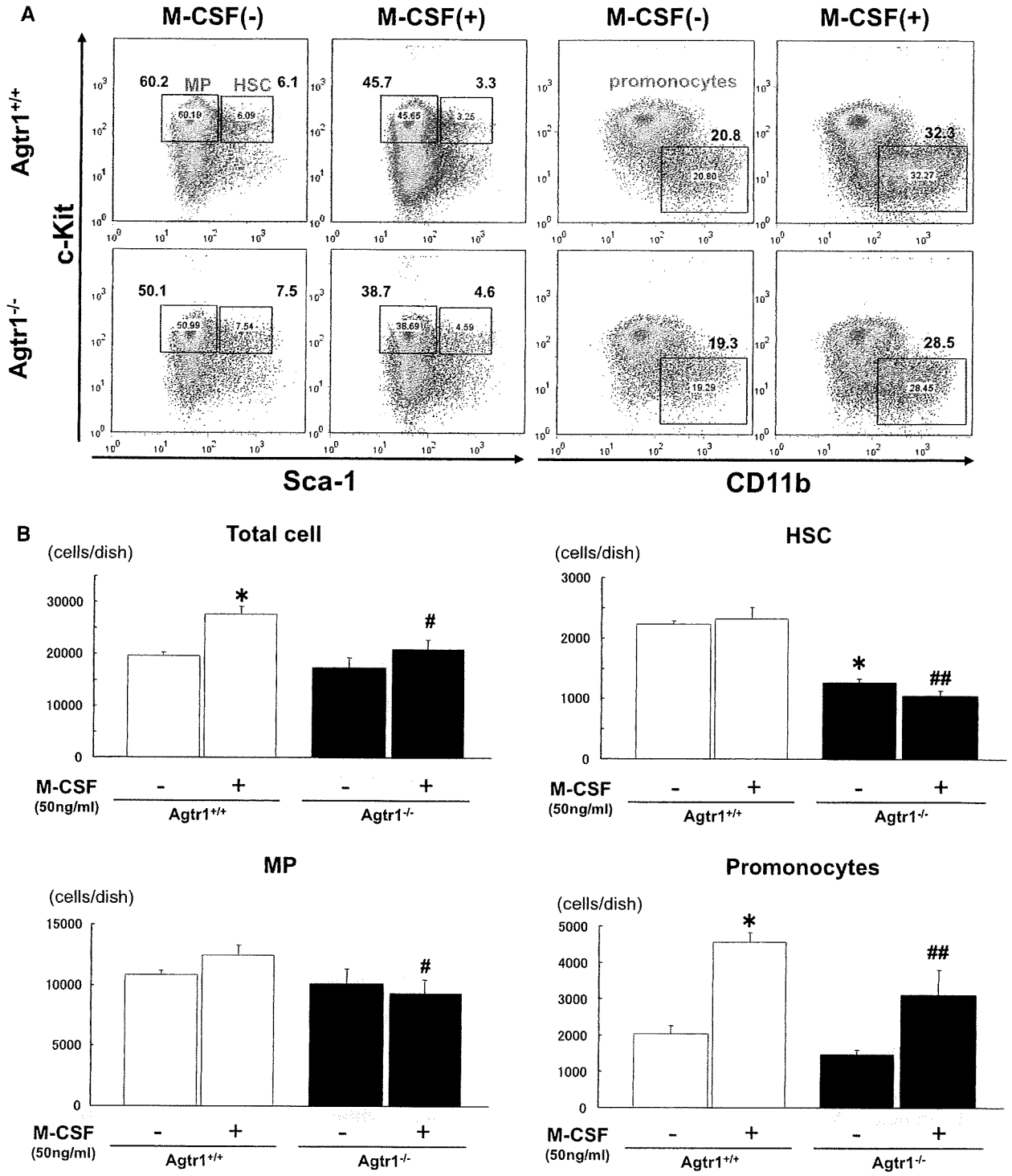

Figure 2. Inhibition of M-CSF–induced in vitro differentiation from HSCs into monocyte-lineage cells on ablation of marrow AT_1. A, Flow cytometry of c-Kit and Sca-1 expression in lineage-negative populations, and CD11b expression in c-Kit$^-$lineage-negative populations of HSCs from Agtr1$^{+/+}$ or Agtr1$^{-/-}$ mice cultured in the presence or absence of M-CSF (50 ng/mL) at day 4. B, Quantitative analysis showing an attenuated expansion of myeloid progenitors (MP:c-Kit$^+$Sca-1$^-$Lin$^-$) and promonocytes (CD11b^{high}Ly-6G^{low}) from Agtr1$^{-/-}$ mice after stimulation by M-CSF. HSCs indicates hematopoietic stem cells. Values are the mean±SE for at least 4 mice in each group. *P<0.01 vs Agtr1$^{+/+}$ cells cultured without M-CSF. #P<0.05, ##P<0.01 vs Agtr1$^{+/+}$ cells cultured with M-CSF.

CD45$^-$CD34$^-$ BM stromal cells was markedly diminished in Agtr1$^{-/-}$ mice compared with the Agtr1$^{+/+}$ mice (Figure 5A).

We further examined the effect of TNF-α on c-Fms expression in promoocytes from Agtr1$^{-/-}$ mice. Consistent with the previously reported data,[19,20] 4-day treatment with TNF-α (50 ng/mL) upregulated (65% versus control, P<0.01) the expression level of c-Fms in promonocytes from Agtr1$^{+/+}$ mice (Figure 4). Interestingly, the similar extent of

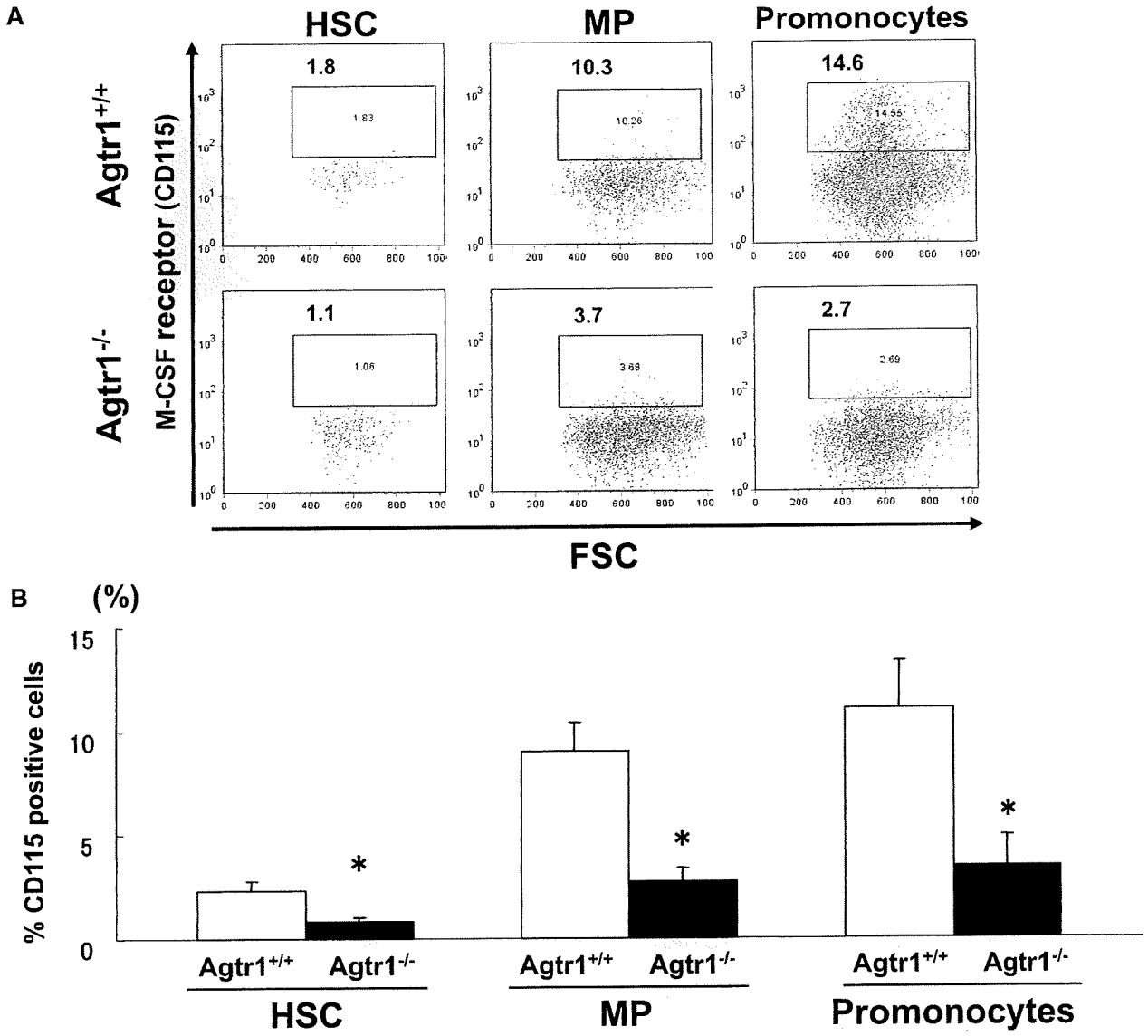

Figure 3. Inhibition of M-CSF receptor expression on ablation of marrow AT_1. A, Flow cytometry for c-Fms expression in HSCs, myeloid progenitors (MP:c-Kit$^+$Sca-1$^-$Lin$^-$), and promonocytes (CD11b^{high}Ly-6G^{low}) from Agtr1$^{+/+}$ and Agtr1$^{-/-}$ mice under steady-state condition without hypercholesterolemia. B, Quantitative analysis showing a significant inhibition of c-Fms expression in each fraction. Values are the mean±SE for at least 4 mice in each group. *$P<0.05$ vs Agtr1$^{+/+}$.

TNF-α–mediated induction of c-Fms was also observed in promonocytes from Agtr1$^{-/-}$ mice (Figure 4), indicating that TNF-α–mediated expression of c-Fms in HSCs and promonocytes is not impaired by AT_1 deficiency.

AT_1 Signals Regulate Growth of BM Stromal Cells and TNF-α Expression

The effects of AT_1 deficiency and ARB on the number of BM stromal cells and their expression of TNF-α were studied. Real-time PCR analysis showed that AT_1 mRNA expression is detectable in myeloid progenitors, promonocytes, and BM CD45$^-$CD34$^-$ stromal cells, whereas no significant expression is observed in HSCs (supplemental Figure XIII). One-week administration of ARB (Olmesartan: 3 mg/kg/d) into the wild-type mice profoundly reduced the percentage fraction of BM stromal cells, the extent of which was similar to that in Agtr1$^{-/-}$ mice (Figure 5A). Furthermore, ARB treatment significantly decreased the expression level of TNF-α mRNA in BM stromal cells (Figure 5C). Considering that Ang II did not affect the c-Fms expression (Figure 4) or the proliferation (supplemental Figure XIA) of HSCs, myeloid progenitors, and promonocytes, it is likely that the target of ARB is BM stromal cells, and Ang II directly regulates their growth and TNF-α synthesis/release, leading to the modulation of c-Fms expression on BM monocyte-lineage cells in a paracrine fashion.

ARB Reduces Atherosclerosis Accompanied by a Reduction of Monocyte-Lineage Cells Without Affecting Serum M-CSF Levels

We studied the effect of ARB on monocyte-lineage development in apoE$^{-/-}$ mice fed a Western diet. The atherosclerotic

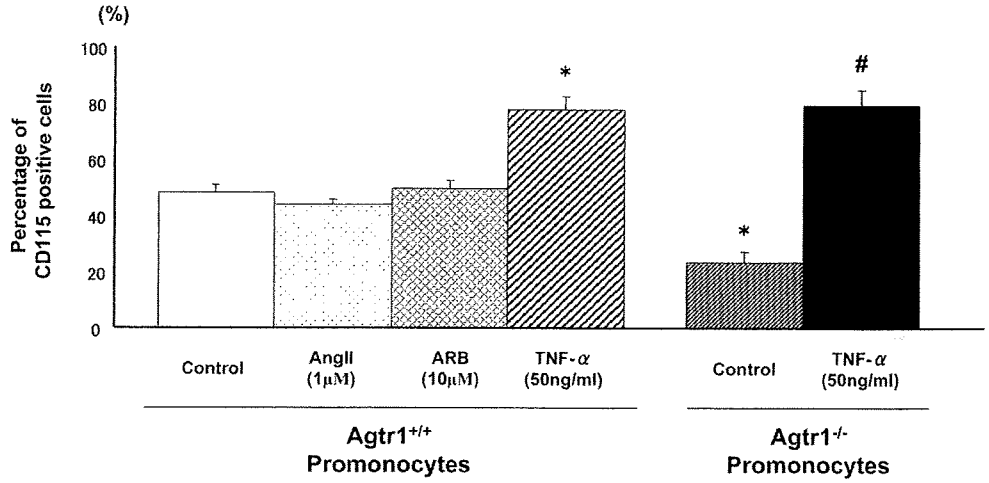

Figure 4. c-Fms expression on promonocytes is not affected by Ang II or ARB but augmented by TNF-α. Hematopoietic stem cells (HSCs) were isolated from Agtr1$^{+/+}$ mice and cultured with Ang II (1 μmol/L) or ARB (10 μmol/L) for 4 days. The expression levels of c-Fms on HSCs, myeloid progenitors, and promonocytes (only data in promonocytes were shown) were not affected by Ang II or ARB treatment. In contrast, treatment with TNF-α (50 ng/mL) upregulated c-Fms expression on promonocytes. Whereas the c-Fms expression was significantly impaired in promonocytes from Agtr1$^{-/-}$ mice, the similar extent of TNF-α–mediated induction are observed. Values are the mean±SE for at least 4 mice in each group. *P<0.05 vs promonocytes from Agtr1$^{+/+}$ mice. #P<0.05 vs promonocytes from Agtr1$^{-/-}$ mice.

lesion area showed a significant reduction in ARB-treated mice compared with hydralazine- and saline-treated mice (supplemental Figure XIVA). Whereas the number of myeloid progenitors was significantly increased by 4-week Western diet feeding, it was completely reduced in ARB-treated mice (supplemental Figure XIVB), consistent with the results from BM chimeric mice. Furthermore, the frequency of circulating monocytes (CD11b^{high}Ly-6G^{low}Ly-6C^{high}) in saline- and hydralazine-treated mice was completely diminished in ARB-treated mice (supplemental Figure XIVB). The serum M-CSF concentration was significantly elevated by 4-week Western diet feeding but was not suppressed by ARB treatment (supplemental Figure XIVC). These findings suggest that the decreased number of monocyte-lineage cells in ARB-treated hypercholesterolemia mice is not attributable to a decrease in serum M-CSF levels but to the direct actions of ARB on BM cells.

Discussion

The present study demonstrated that Ang II affects the expression profile of the M-CSF receptor c-Fms on HSCs and monocyte-lineage cells through BM stromal cell–derived TNF-α, and thereby regulates M-CSF–mediated differentiation/proliferation of BM monocyte-lineage cells followed by the mobilization of monocytes, which contributes to the AT_1-mediated proatherogenic actions. These findings provide novel information on the BM renin–angiotensin system and a unique opportunity to develop therapeutic strategies targeting BM stem cells for the prevention of atherosclerotic cardiovascular disease.

In contrast with Ang II–induced atherosclerosis, the role of BM-AT_1 on hypercholesterolemia-induced atherosclerosis is controversial. Fukuda et al[8] demonstrated that apoE$^{-/-}$ mice repopulated with Agtr1$^{-/-}$ marrow showed a modest but significant reduction of atherosclerotic lesion development, whereas ablation of BM-AT_1 receptor in LDLr$^{-/-}$ mice had no effect on atherosclerosis,[7,21] suggesting that the different models used may differ in their consequence of BM-AT_1 on atherosclerosis. Indeed, Strawn et al[12] demonstrated that native LDL significantly upregulated AT_1 receptor expression on CD34$^+$ cells, which was completely diminished by treatment with a neutralizing LDL receptor antibody, suggesting that hypercholesterolemia-induced expression of AT_1 receptor is comparatively higher in apoE$^{-/-}$ mice than LDLr$^{-/-}$ mice. In addition, Daugherty et al[2] demonstrated that hypercholesterolemia extensively increased the plasma Ang II concentration in LDLr$^{-/-}$ mice, which was completely abolished in Agtr1$^{-/-}$ mice. Taken together, it is quite likely that BM-AT_1 receptor activation is more implicated in the hypercholesterolemia-induced atherosclerosis in apoE$^{-/-}$ mice rather than LDLr$^{-/-}$ mice.

BM stem cells are primed for multilineage gene expression and can differentiate into all types of blood cells.[14,15] M-CSF is the principal regulator of proliferation and terminal differentiation of monocyte-lineage cells.[22] We found that M-CSF–induced colony forming activity was dramatically attenuated in BM cells from Agtr1$^{-/-}$ mice, and that in vitro differentiation of HSCs from Agtr1$^{-/-}$ mice was significantly reduced in the presence of M-CSF. We further demonstrated that M-CSF receptor c-Fms expression and its downstream signaling were impaired. In hypercholesterolemia, activated endothelial cells, vascular smooth muscle cells, and inflammatory leukocytes have been shown to secrete a variety of cytokines, chemokines, and growth factors, including M-CSF.[23] In this study, we showed that serum M-CSF levels were significantly elevated in apoE$^{-/-}$ mice fed a Western diet (supplemental Figure XIVC). Accordingly, Ang II–mediated action in the differentiation/proliferation of monocyte-lineage cells is considered to be more augmented in various pathological conditions[24,25] as well as atherosclerosis, in which serum M-CSF levels were elevated.

The M-CSF receptor c-Fms is encoded by the *c-fms* protooncogene,[26] whose expression is predominantly regulated by the transcription factor Pu.1.[27] Agtr1$^{-/-}$ mice do not show any phenotype of low growth rate, tooth deficiency, severe osteopetrosis, reduced bone marrow cellularity, or

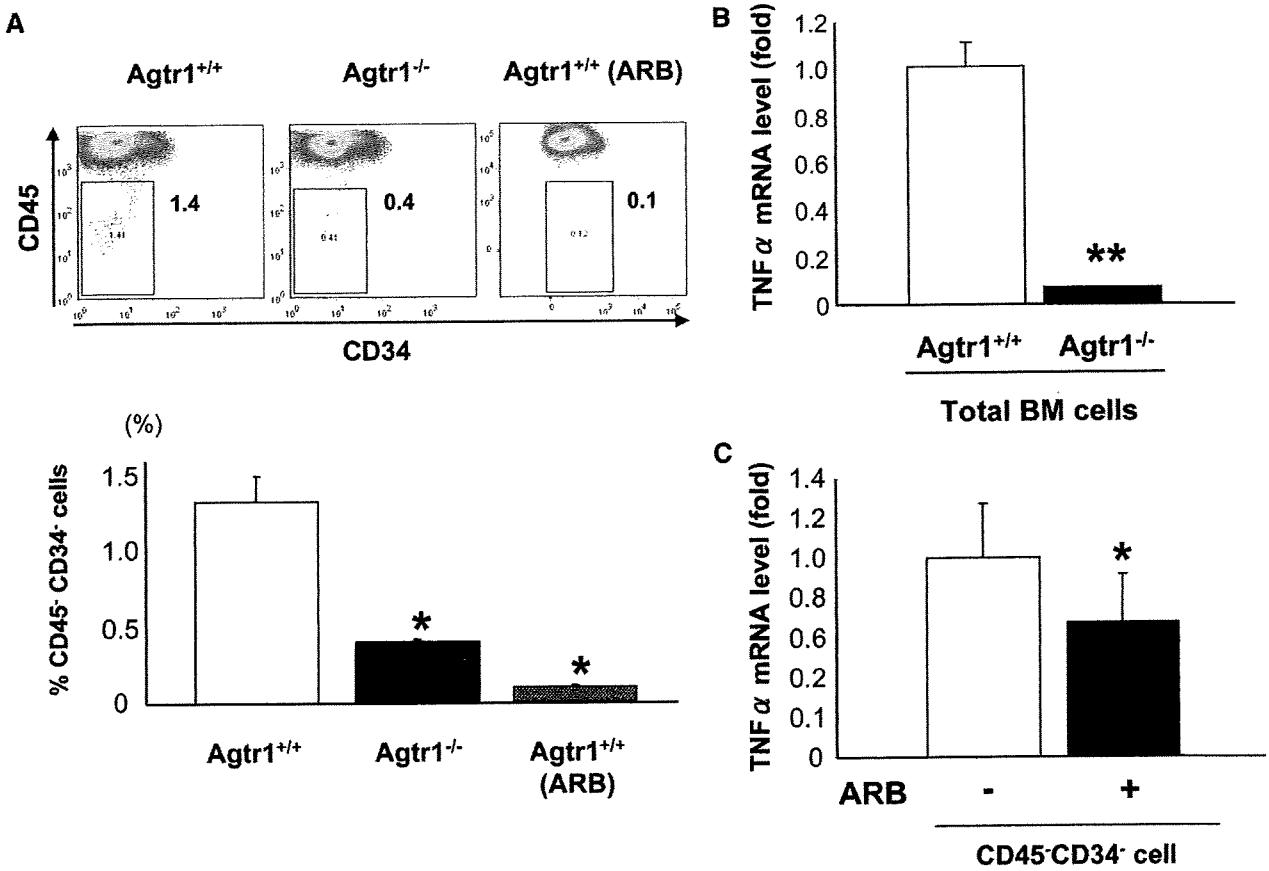

Figure 5. AT_1 signals regulate growth of BM stromal cells and TNF-α expression. A, The percentage fraction of BM $CD45^-CD34^-$ cells was reduced in $Agtr1^{-/-}$ mice. One-week administration of ARB (Olmesartan: 3 mg/kg/d) into $Agtr1^{+/+}$ mice also reduced the percentage fraction of BM $CD45^-CD34^-$ cells. Values are the mean±SE for at least 4 mice in each group. *$P<0.05$ vs saline-treated $Agtr1^{+/+}$ mice. B, Real-time PCR analysis showing the reduced expression of TNF-α in total BM cells from $Agtr1^{-/-}$ mice. C, One-week treatment with ARB (Olmesartan: 3 mg/kg/d) on $Agtr1^{+/+}$ mice significantly decreased the expression level of TNF-α mRNA in BM stromal cells. Total RNA was isolated from the same cell numbers of BM cells (B) or $CD45^-CD34^-$ cells (C), and TNF-α mRNA levels was shown as values relative to the control. Values are the mean±SE for at least 4 mice in each group. *$P<0.05$, **$P<0.01$ vs control $Agtr1^{+/+}$ mice.

depletion of circulating monocytes, all of which were observed in $Csf1r^{-/-}$ mice,[28] in which the c-Fms gene is genetically disrupted. Likewise, $Agtr1^{-/-}$ mice do not show any of the phenotypes observed in $PU.1^{-/-}$ mice.[29] TNF-α directly stimulates BM blood osteoclast precursor genesis by enhancing c-Fms expression.[19,20] TNF-α increased the expression level of c-Fms on promonocytes from $Agtr1^{-/-}$ mice to the same extent as $Agtr1^{+/+}$ mice (Figure 4), suggesting that TNF-α–mediated expression of c-Fms is not impaired by AT_1 deficiency. Given the reduced expression of TNF-α in BM cells from $Agtr1^{-/-}$ mice (Figure 5B), it is conceivable that decreased expression of c-Fms in monocyte-lineage cells from $Agtr1^{-/-}$ mice is primarily attributable to the impaired TNF-α–mediated actions.

Bone marrow niche plays an important role in the differentiation and proliferation of HSCs, in which BM stromal cells and mesenchymal stem cells (MSCs) regulate localization, self-renewal, and differentiation of HSCs through the secretion of cytokines and growth factor, cell-to-cell interactions, and the influence of extracellular matrix proteins.[30] Recently, Matsushita et al reported that BM-MSCs expressed AT_1 receptor and secreted Ang II.[31] BM stromal cells and BM-MSCs have been reported to secrete TNF-α as well as M-CSF.[32] Our present study demonstrates that TNF-α derived from BM stromal cells upregulates the c-Fms expression on HSCs and BM monocyte-lineage cells, and that AT_1 deficiency is indirectly involved in the regulation of c-Fms expression by inhibiting the proliferation of BM stromal cells. Considering that ARB treatment of the wild-type mice inhibits the proliferation of BM stromal cells (Figure 5), and that AT_1 signals activate ERK1/2 and Akt pathways in mesenchymal stem cells,[33] it is likely that Ang II plays an important role in the proliferation of BM stromal stem cells rather than HSCs. In fact, the mRNA expression level of AT_1 is much higher in BM stromal cells, whereas no expression was detected in HSCs (supplemental Figure XIII). Further studies will be needed to elucidate how Ang II differentially regulates the proliferation and differentiation of BM stem cells.

ARB treatment significantly attenuated macrophage-colony-forming activity in a dose-dependent manner (supplemental Figure XIB). Ang II has been shown to augment the number of macrophage-colony forming units.[5] In contrast with these findings, neither Ang II nor ARB treatment affected the M-CSF–induced differentiation of monocyte-

lineage cells in vitro culture assay. Colony forming unit assays were performed using total BM cells that include nonhematopoietic lineage cells such as BM stromal cells and BM-MSCs. The discrepant result from in vitro culture assay seems to be attributable to the effects of Ang II or ARB on BM stromal cells and BM-MSCs. Thus, the target of ARB is BM stromal cells, and Ang II directly regulates their growth and TNF-α synthesis/release, leading to the modulation of c-Fms expression on BM monocyte-lineage cells.

In conclusion, our findings demonstrate that Ang II promotes the M-CSF–mediated differentiation/proliferation of BM monocyte-lineage cells through TNF-α–mediated up-regulation of c-Fms expression, and that the TNF-α is mainly derived from BM stromal cells growth-controlled by Ang II and specifically regulates the c-Fms expression on monocyte-lineage cells, thus leading to the increased numbers of circulating monocytes that modulate AT_1-mediated proatherogenic activities.

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Disclosures

None.

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Bone Marrow AT_1 Augments Neointima Formation by Promoting Mobilization of Smooth Muscle Progenitors via Platelet-Derived SDF-1α

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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_1)-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_1-deficient (BM-Agtr1$^{-/-}$) or wild-type (BM-Agtr1$^{+/+}$) cells. Neointima formation was profoundly reduced by 38% in BM-Agtr1$^{-/-}$ mice. Although the number of circulating EPCs (Sca-1$^+$Flk-1$^+$) and extent of reendothelialization did not differ between the 2 groups, the numbers of both circulating VPCs (c-Kit$^-$Sca-1$^+$Lin$^-$) and tissue VPCs (Sca-1$^+$CD31$^-$) incorporated into neointima were markedly decreased in BM-Agtr1$^{-/-}$ mice. The accumulation of aggregated platelets and their content of stromal cell–derived factor-1α (SDF-1α) were significantly reduced in BM-Agtr1$^{-/-}$ 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$^{-/-}$ platelets compared with Agtr1$^{+/+}$ platelets, accompanied by the reduced expression and release of SDF-1α.

Conclusions—The BM-AT_1 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.[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.[1,2,3,4] 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.[3–7] However, the underlying mechanisms that regulate the serum level of SDF-1α and CXCR4 expression on BM-derived progenitors remain poorly understood.[8]

Angiotensin II (Ang II)-mediated biological actions are involved in the pathogenesis of neointimal hyperplasia after vascular injury.[9,10] Ang II type1 (AT_1) receptor–deficient (Agtr1$^{-/-}$) mice showed attenuated cuff-induced neointima formation.[11] Ang II receptor blocker (ARB) also reduced neointimal hyperplasia in both animal experiments and clinical trials.[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.[12] Yamada et al also reported that ARB treatment inhibited neointimal hyperplasia by reducing the accumulation of smooth muscle-like progenitors in neointima.[13] However, the precise mechanisms for AT_1-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$^{-/-}$ or Agtr1$^{+/+}$ cells to elucidate the underlying

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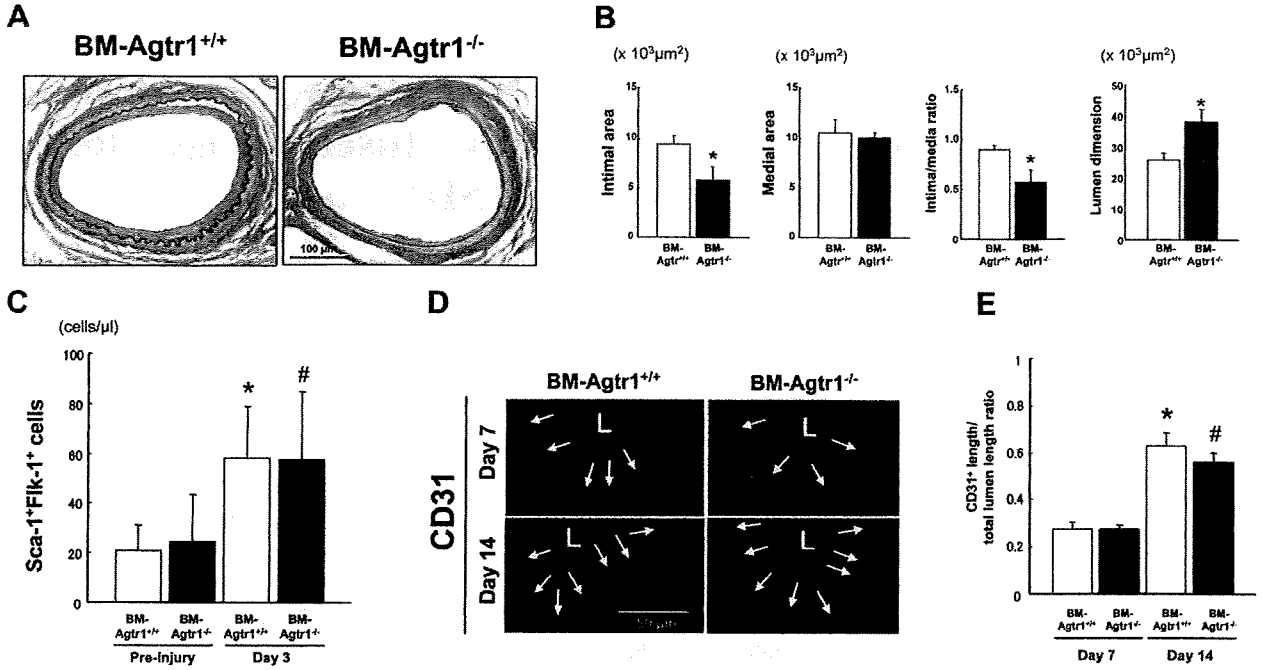

Figure 1. Ablation of marrow AT_1 attenuated neointima formation after vascular injury without affecting on EPC-mediated reendothelialization. A, Representative Elastica van Gieson–stained femoral arteries 4 weeks after injury. B, Quantitative analysis showing significant decreases in neointima formation in BM-Agtr1$^{-/-}$ mice. Values are the mean±SE for at least 8 mice in each group. *P<0.05 vs BM-Agtr1$^{+/+}$ mice. C, Flow cytometry of Sca-1 and Flk-1 expression in circulating mononuclear cell populations 3 days after injury. Quantitative analysis showing no difference in the number of EPCs between the 2 groups before and 3 days after injury. 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-Agtr1$^{-/-}$ mice. D, Representative immunohistochemical staining for CD31 at day 7 and day 14 after injury. L indicates lumen. E, Quantitative analysis showing no difference in the extent of reendothelialization between the 2 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-Agtr1$^{-/-}$ mice.

mechanism for BM-AT_1-mediated actions on vascular repair. The results demonstrated for the first time that BM-AT_1 is closely involved in neointima formation by causing the mobilization and homing of VPCs rather than EPCs, in which aggregated platelet-derived SDF-1α plays a crucial role. These findings provide a novel understanding regarding the effect of BM-AT_1 on the kinetics of BM-derived vascular-lineage progenitors after vascular injury especially through platelets AT_1 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 AT_1-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-AT_1 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-AT_1 Deficiency Does Not Affect Circulating EPCs or Reendothelialization

The number of circulating Sca-1$^+$Flk-1$^+$ EPCs was similarly increased ≈2-fold in both BM-Agtr1$^{+/+}$ and BM-Agtr1$^{-/-}$ mice 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-AT_1 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

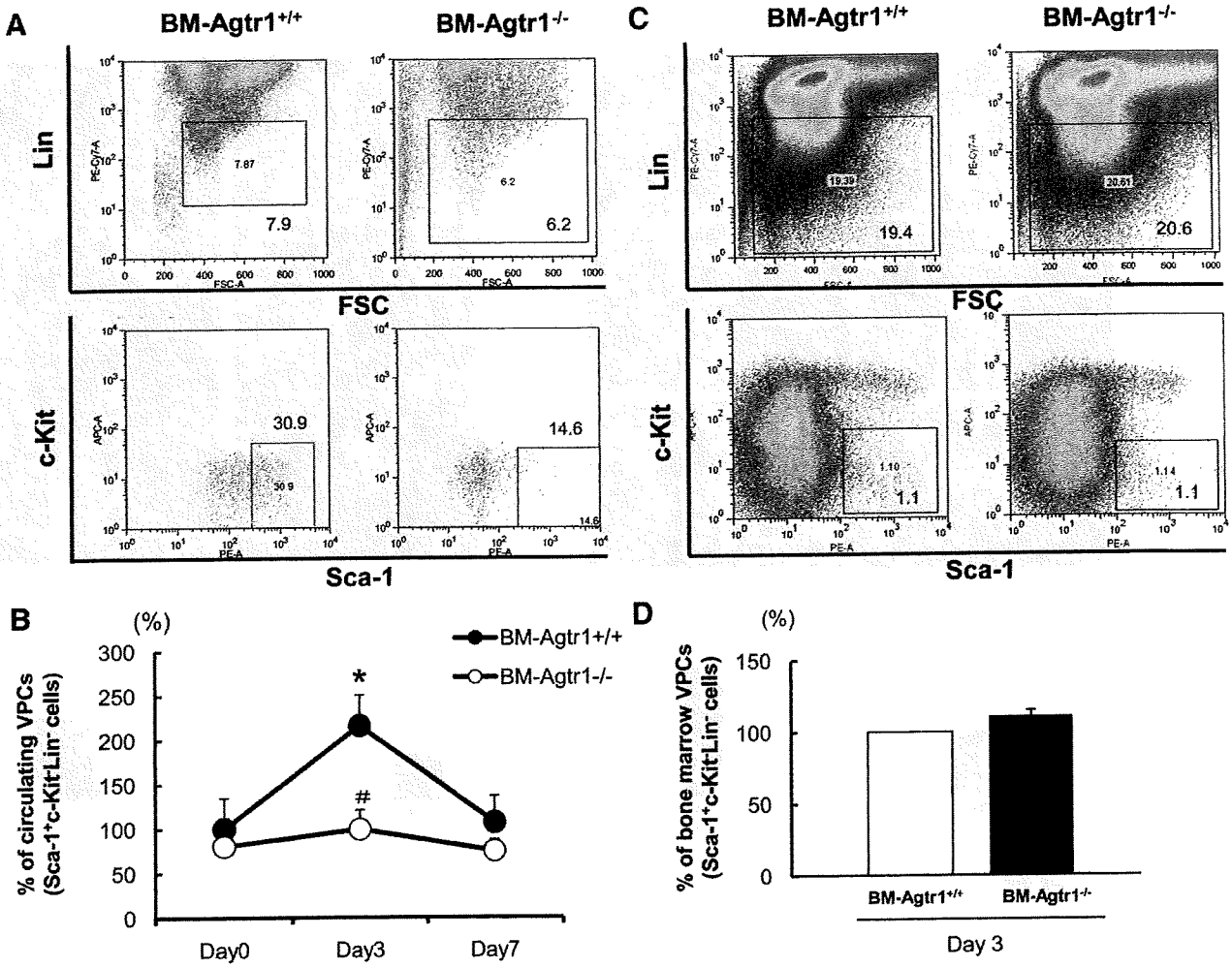

Figure 2. Decreased number of circulating VPCs on ablation of marrow AT_1. A, Flow cytometry of c-Kit and Sca-1 expression in lineage-negative circulating mononuclear cell populations of BM-Agtr1$^{+/+}$ and BM-Agtr1$^{-/-}$ mice before and after injury. B, Quantitative analysis showing the marked increase in the number of circulating VPCs (c-Kit$^-$Sca-1$^+$Lin$^-$) in BM-Agtr1$^{+/+}$ mice at day 3 after injury, whereas a modest increase was observed in BM-Agtr1$^{-/-}$ mice. Values are the mean±SE for 5 mice at each time point in each group. *P<0.05 vs BM-Agtr1$^{+/+}$ mice before injury, #P<0.05 vs BM-Agtr1$^{+/+}$ mice 3 days after injury. 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 2 groups. Values are the mean±SE for 5 mice in each group.

circulating VPCs were defined as c-Kit$^-$Sca-1$^+$Lin$^-$ cells.[4] 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), whereas it was completely diminished in BM-Agtr1$^{-/-}$ mice (Figure 2A and 2B). 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-AT_1 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.[4] 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,[15] 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 the BM-Agtr1$^{-/-}$ and BM-Agtr1$^{+/+}$ mice (Figure 3B and 3F), suggesting that primary platelet adhesion was not affected by platelet AT_1 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),

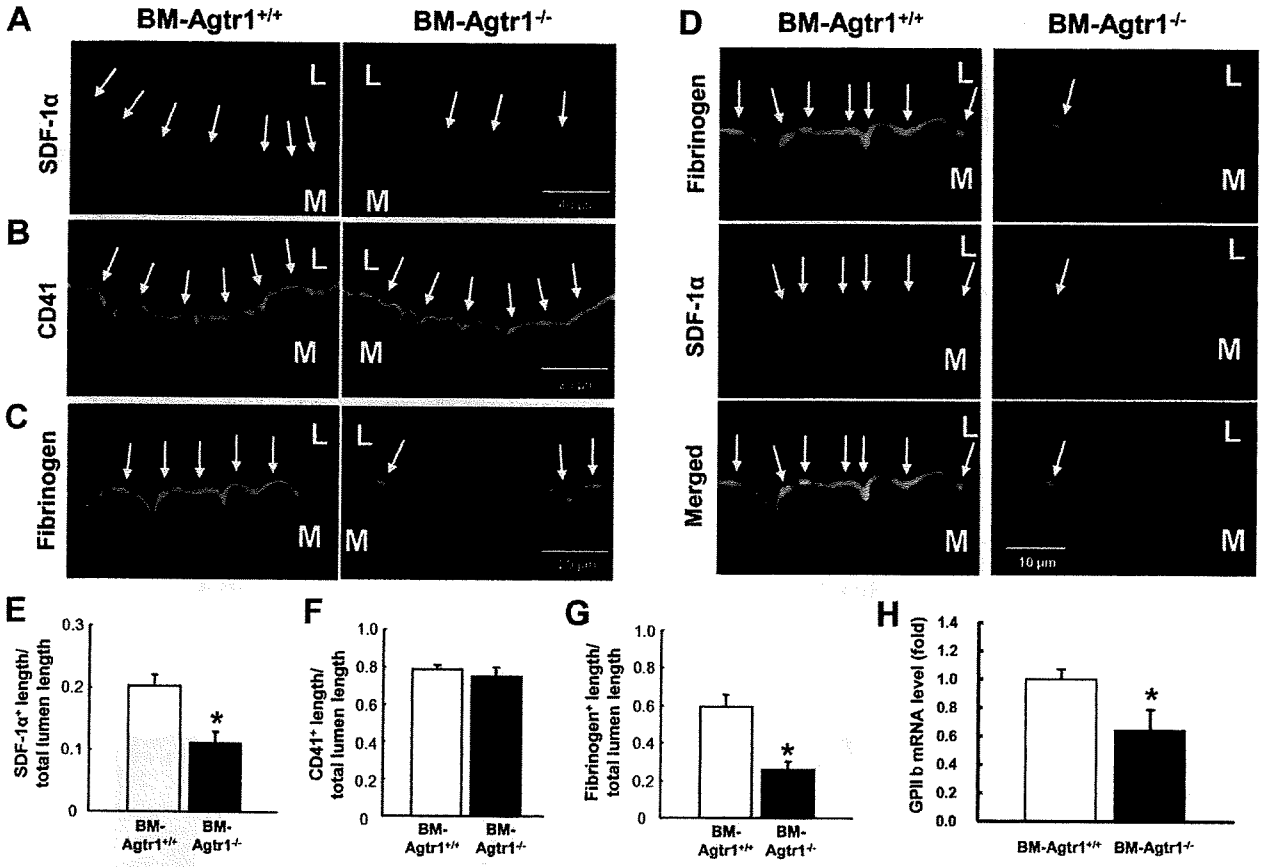

Figure 3. Attenuated platelet aggregation and the content of SDF-1α on ablation of marrow AT_1. A, Immunofluorescence image showing SDF-1α 3 day after injury. SDF-1α–positive staining was mainly detected on the inner surface of the vessel wall. B and C, Immunofluorescence image showing CD41 and fibrinogen at day 1 and day 3 after injury, respectively. D, Merged image showing fibrinogen (green) and SDF-1α (red). E, F, and G, Quantitative analysis showing a decrease in SDF-1α and fibrinogen-positive staining but not in CD41-positive staining in BM-Agtr1$^{-/-}$ mice. Values are the mean±SE for at least 5 mice in each group. *P<0.01 vs BM-Agtr1$^{+/+}$ mice. L indicates lumen; M, media. H, Real-time PCR analysis showing the reduced expression of GPIIb in BM-Agtr1$^{-/-}$ mice. Total RNA was isolated from the injured arteries at day 3, and GPIIb mRNA levels were shown as values relative to BM-Agtr1$^{+/+}$ mice. Values are the mean±SE for at least 5 mice in each group. *P<0.05 vs control BM-Agtr1$^{+/+}$ mice.

supporting the notion that platelets aggregation in the injured vessels is attenuated in BM-Agtr1$^{-/-}$ mice. Moreover, SDF-1α–positive staining was mostly colocalized with fibrinogen-positive staining (Figure 3D), suggesting that diminished platelets aggregation contributes to the decreased content of SDF-1α at sites of injured vessel in BM-Agtr1$^{-/-}$ mice. We also examined the relationship between eNOS and SDF-1α, because SDF-1α 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-1α 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-AT_1 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

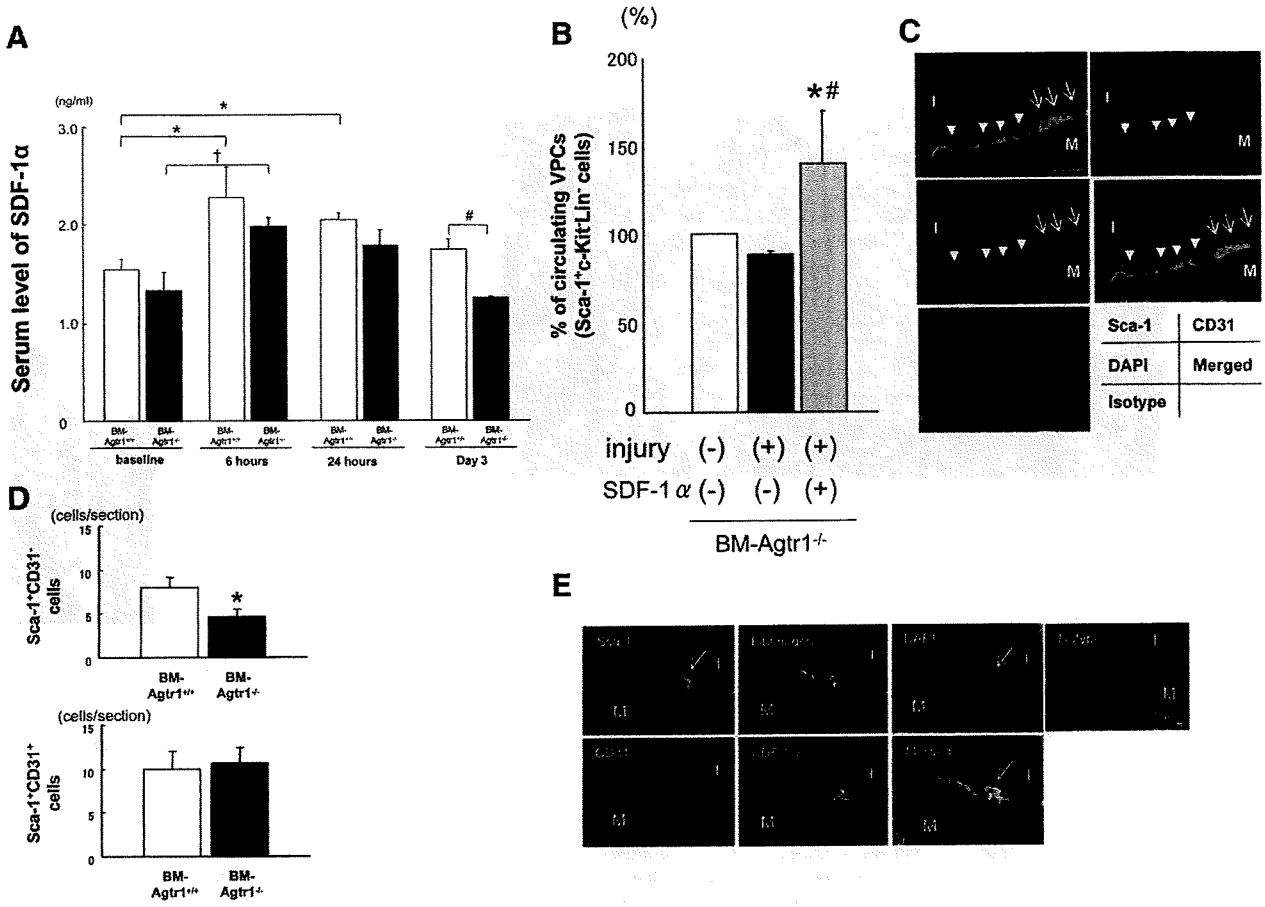

Figure 4. Decrease in the serum level of SDF-1α accompanied by the decreased number of Sca-1$^+$CD31$^-$ cells incorporated into neointima on ablation of marrow AT_1. A, The serum SDF-1α level was markedly increased after injury with a peak of 6 hours in both groups. In the BM-Agtr1$^{-/-}$ mice, the serum SDF-1α level rapidly declined and normalized at 3 days after injury, which was significantly lower than that in BM-Agtr1$^{+/+}$ mice. Values are the mean±SE for at least 4 mice in each group. *P<0.01 vs BM-Agtr1$^{+/+}$ mice before injury. †P<0.01 vs BM-Agtr1$^{-/-}$ mice before injury, #P<0.05 vs BM-Agtr1$^{+/+}$ mice 3 days after injury. B, BM-Agtr1$^{-/-}$ mice were injected with SDF-1α (10 μg i.p.) 1 day after injury. Flow cytometry of c-Kit and Sca-1 expression in lineage-negative populations was performed on day 3 after injury. The bar graphs represent the mean±SE relative to BM-Agtr1$^{-/-}$ before injury set at 100%. At least 5 mice were tested in each group. *P<0.05 vs BM-Agtr1$^{-/-}$ mice before injury. #P<0.05 vs BM-Agtr1$^{-/-}$ mice after injury. C, Immunofluorescence image showing Sca-1 (green), CD31 (red), and DAPI (blue) 7 days after injury. Arrows indicate Sca-1$^+$CD31$^-$ cells, and arrowheads indicate Sca-1$^+$CD31$^+$. D, Quantitative analysis showed that the number of Sca-1$^+$CD31$^-$ cells was significantly reduced in BM-Agtr1$^{-/-}$ mice, whereas no difference was observed in Sca-1$^+$CD31$^+$ cells between the 2 groups. Values are the mean±SE for at least 4 mice in each group. *P<0.05 vs BM-Agtr1$^{+/+}$ mice. I indicates intima; M, media. E, Immunofluorescence image showing VPC (arrow) which was colocalized with fibrinogen-positive staining (cyan blue), that reflects the fibrinogen trapped by aggregated platelets, and SDF-1α (yellow).

BM-Agtr1$^{-/-}$ 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-Agtr1$^{-/-}$ 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-Agtr1$^{-/-}$ mice. An injection of SDF-1α into vascular-injured BM-Agtr1$^{-/-}$ 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-Agtr1$^{-/-}$ mice.

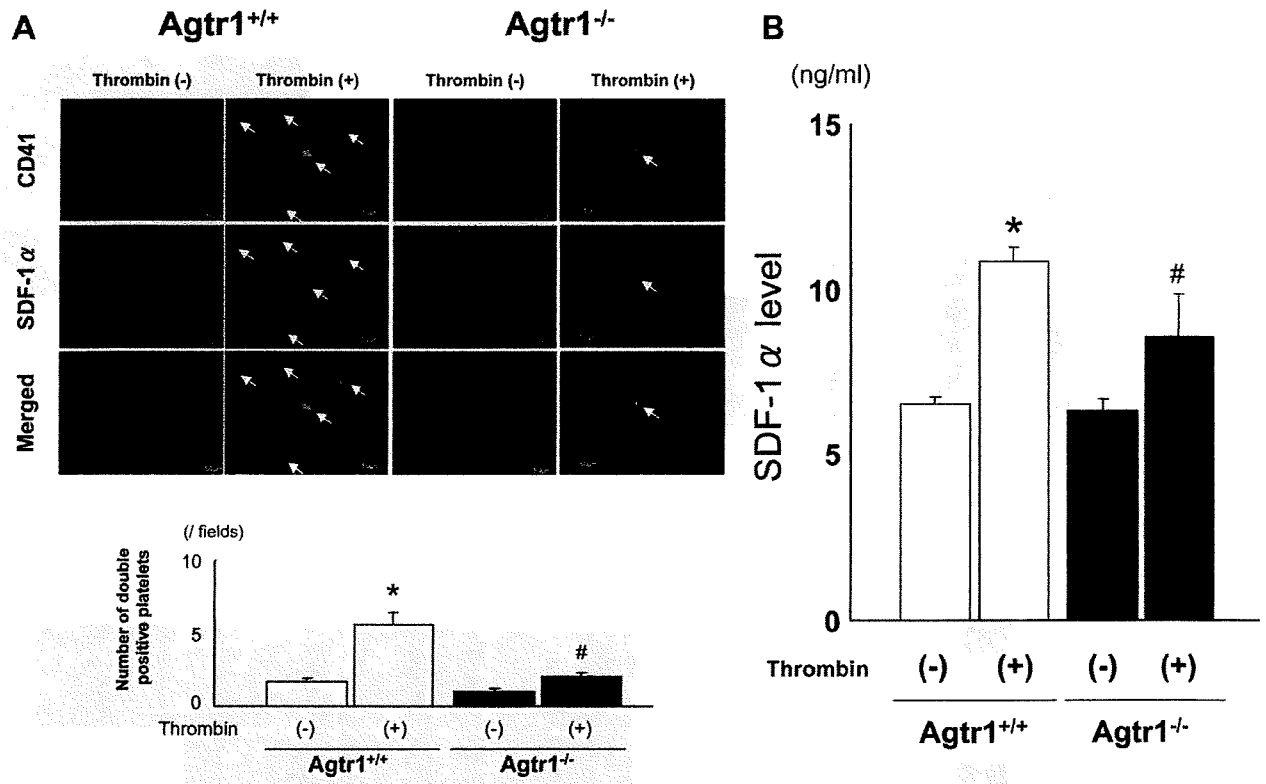

Figure 5. Attenuated platelet aggregation and thrombin-activated platelets-derived SDF-1α expression and release on ablation of marrow AT_1. A, Mice platelets were stimulated with 0.2 U/mL thrombin, fixed, and stained with FITC-labeled anti-CD41 and anti–SDF-1α. Arrows indicate $CD41^+SDF\text{-}1\alpha^+$ platelets. $CD41^+SDF\text{-}1\alpha^+$ platelets indicated by arrows were aggregated cells but not a single platelet. Quantitative analysis showed that the number of double positive platelets was significantly increased in $Agtr1^{+/+}$ platelets after stimulation with thrombin, whereas it was significantly diminished in $Agtr1^{-/-}$ platelets. *$P<0.01$ vs $Agtr1^{+/+}$ resting platelets, #$P<0.05$ vs $Agtr1^{+/+}$ activated platelets. B, Measurement of SDF-1α released from $Agtr1^{+/+}$ platelets or $Agtr1^{-/-}$ platelets after thrombin stimulation by the ELISA method. SDF-1α protein in the conditioned medium was significantly reduced in $Agtr1^{-/-}$ platelets after thrombin stimulation. Values are the mean±SE for at least 5 mice in each group. *$P<0.05$ vs $Agtr1^{+/+}$ resting platelets. #$P<0.05$ vs $Agtr1^{+/+}$ activated platelets.

BM-AT_1 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 $Sca\text{-}1^+CD31^-$ cells (arrows), corresponding to VPCs,[8] were colocalized with aggregated platelets which express SDF-1α. The number of VPCs, was markedly reduced by 42% in BM-$Agtr1^{-/-}$ mice compared with BM-$Agtr1^{+/+}$ mice ($P<0.05$) (Figure 4D). In contrast, the number of $Sca\text{-}1^+CD31^+$ cells, corresponding to EPCs,[7] did not differ between the 2 groups, suggesting that BM-AT_1 deficiency attenuates the homing of BM-derived progenitor cells concomitant with the impaired mobilization of VPCs.

Platelet AT_1 Receptor Deficiency Inhibits Platelet Aggregation

Thrombin-stimulated $Agtr1^{+/+}$ platelets showed an apparent increase in fibrinogen binding in a dose-dependent manner, whereas fibrinogen binding in $Agtr1^{-/-}$ 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.[16] Platelets produce reactive oxidative species (ROS) via NAD(P)H oxidase activation, and their function is tightly regulated by the redox state.[17] We examined whether production of ROS is actually reduced in $Agtr1^{-/-}$ platelets. Intracellular ROS production, which was detected by flow cytometric analysis with H_2DCF-DA, showed a significant decrease in $Agtr1^{-/-}$ platelets by 37% ($P<0.01$) compared with $Agtr1^{+/+}$ platelets (supplemental Figure V). This finding strongly supports the notion that attenuated oxidative stress in $Agtr1^{-/-}$ platelets is, at least in part, responsible for the impaired platelet aggregation.

Platelet AT_1 Receptor Deficiency Inhibits the Expression and Release of SDF-1α

Immunohistochemical analysis revealed that the number of $CD41^+SDF\text{-}1\alpha^+$ platelets was markedly increased (3.2-fold) in the $Agtr1^{+/+}$ platelets after thrombin stimulation, whereas it was severely inhibited (1.8-fold) in $Agtr1^{-/-}$ platelets (Figure 5A). Furthermore, the SDF-1α protein in the conditioned medium after thrombin stimulation was significantly reduced in $Agtr1^{-/-}$ platelets compared with $Agtr1^{+/+}$ 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-AT_1 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-AT_1 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.[7] Stellos et al[18] 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 al[2] 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. Zernecke et al[4] 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 AT_1 blockade on the kinetics of BM-derived VPCs after vascular injury remains poorly understood. Yamada et al[13] 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 AT_1-mediated effects on BM-derived progenitor cells independently of vascular AT_1-mediated actions. Although the number of BM-VPCs did not differ between BM-Agtr1$^{-/-}$ and BM-Agtr1$^{+/+}$ mice, circulating VPCs after injury were markedly reduced in BM-Agtr1$^{-/-}$ mice. This finding suggests that the mobilization of VPCs after vascular injury was impaired by BM-AT_1 deficiency independent of vascular AT_1-mediated actions. Ohtani et al[12] found that trasnsdifferentiation 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 AT_1 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 al[19] previously reported that neointima formation after vascular injury was not suppressed in AT_1-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 endotheline-1, etc, might substitute fully for AT_1-mediated actions in AT_1-deficient mice.[19] We therefore compared the expression levels of FGF-2, PDGF-B, and TGF-β1 mRNAs between wild-type and AT_1-deficient mice. We found that the relative expression levels of FGF-2 and PDGF-B mRNAs in the injured vessels were actually higher in AT_1-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-Agtr1$^{-/-}$ mice was also significantly higher than BM-Agtr1$^{+/+}$ mice, however the extent was much lower than AT_1-deficient mice. The mRNA expression levels of PDGF-B and TGF-β1 did not show any difference between BM-Agtr1$^{+/+}$ and BM-Agtr1$^{-/-}$ mice. These findings confirm the involvement of growth factors in neointima formation after vascular injury in AT_1-deficient mice, and provide evidence that BM-AT_1 rather than vascular AT_1 plays a more important role in the formation of neointima.

AT_2 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 Agtr2$^{+/+}$ (BM-Agtr2$^{+/+}$) or Agtr2$^{-/-}$ (BM-Agtr2$^{-/-}$) cells. In contrast to the effect of BM-AT_1, neointimal area and neointima/media ratio were significantly increased by 40% and 39% in BM-Agtr2$^{-/-}$ mice compared with BM-Agtr2$^{+/+}$ mice (supplemental Figure IXA and IXB), which was consistent with the previous findings that neointimal hyperplasia was exaggerated in Agtr2$^{-/-}$ mice.[11] Reendothelialization and the numbers of VPCs did not differ between BM-Agtr2$^{+/+}$ and BM-Agtr2$^{-/-}$ 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-Agtr2$^{-/-}$ mice were equivalent with those in BM-Agtr2$^{+/+}$ mice (supplemental Figure XII). These findings suggest that BM-AT_2 receptor counteracts the effect of BM-AT_1 on the development of neointimal formation, however underlying mechanisms for BM-AT_2-mediated vasoprotective actions are different from those of BM-AT_1, and further studies will be required to clarify it.

We also studied the effects of BM-AT_1 and BM-AT_2 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-AT_1 or BM-AT_2 on apoptosis might be modest compared with the previous study in which total cells are AT_1-deficient or AT_2-deficient cells.[11]

This study provides novel important evidence that BM-AT_1 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 AT_1 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.

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Disclosures

None.

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Future Prospect of RNA Interference for Cancer Therapies

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Abstract: RNA interference (RNAi) is a phenomenon of sequence-specific gene silencing in mammalian cells and its discovery has lead to its wide application as a powerful tool in post-genomic research. Recently, short interfering RNA (siRNA), which induces RNAi, has been experimentally introduced as a cancer therapy and is expected to be developed as a nucleic acid-based medicine.

Selection of appropriate gene targets is an important parameter in the potential success of siRNA cancer therapies. Candidate targets include genes associated with cell proliferation, metastasis, angiogenesis, and drug resistance. Importantly, silencing of such genes must not affect the functions of normal cells. Development of suitable drug delivery systems (DDSs) is also an important issue. Numerous methods to transfect siRNAs into cells have been developed, and the use of non-viral DDSs is preferred because it offers greater safety for clinical application than does the use of viral DDSs. Currently, atelocollagen and cationic liposomes represent the most advantageous non-viral DDSs available.

In this article, we briefly review the mechanism of RNAi and non-viral DDSs. Next we discuss in detail some of the most recent findings concerning the administration of potential nucleic acid-based drugs against polo-like kinase-1 (PLK-1), which regulates the mitotic process in mammalian cells. These promising results demonstrate that PLK-1 is a suitable target for cancer therapy. Finally, several current clinical trials of RNAi therapies against cancers are discussed. Results of current studies and clinical trials demonstrate that manipulation of RNAi mechanism by use of targeted siRNA offers promising strategies for cancer therapies.

Keywords: RNAi, siRNA, cancer, PLK-1, DDS, liposome, atelocollagen.

INTRODUCTION

RNA interference (RNAi) is a process of sequence specific post-transcriptional gene silencing induced by double-strand RNA (dsRNA). This phenomenon was discovered in the nematode *Caenorhabditis elegans* (*C. elegans*) by Drs. Fire and Mello [1], and for this condition they were awarded the 2006 Nobel Prize in medicine (http://nobelprize.org/nobel_prizes/medicine/laureates/2006). RNAi has been shown to function in higher organisms including mammals, and methods that exploit RNAi mechanisms have been developing rapidly. RNAi plays important roles in vital responses against the infection of microorganisms as well as post-transcriptional endogenous gene regulation [2, 3]. Aberrant expression of endogenous normal or mutant genes occurs in pathological conditions, resulting in alterations in signal pathways, cellular proliferation, and apoptosis. Post-transcriptional gene regulation by RNAi controls these alterations positively or negatively, and consequently RNAi has now been well-established as a method for experimental analyses of gene function *in vitro* as well as in high-throughput screening [4, 5].

Cytotoxic compounds or antibodies are widely used as strategies for targeting cellular functions or structures of cancerous cells. Recently the application of nucleic acid-based inhibitors such as antisense oligonucleotides (ODNs), RNA aptamars, small interfering RNAs (siRNAs), and locked nucleic acids (LNAs) have been investigated worldwide, and many researchers are searching for ways to use them in therapeutic approaches. To apply the RNAi phenomenon to therapeutics, it is important to select suitable targets for the inhibition of cancer progression and also to develop effective drug delivery systems (DDSs). Polo-like kinase- 1 (PLK-1) belongs to the family of serine/threonine kinases and regulates cell division as several points in the mitotic phase [6]. Because PLK-1 is overexpressed in many types of malignancies and its overexpression is associated with poor prognosis of cancer patients [7, 8], we have developed RNAi therapy against PLK-1 using orthotopic mouse models [9, 10]. In this review, we discuss possible targets as RNAi therapies against cancers and options for non-viral DDSs.

MECHANISMS OF RNAi

Post-transcriptional gene-silencing small RNAs are roughly categorized into two subgroups, siRNAs and microRNAs (miRNAs). siRNAs have been considered to defend the genome in response to the invasive nucleic acids of viruses and transgenes only in lower eukaryotes including *C. elegans* [11, 12] and *Drosophila melanogaster* [13]. However, recent study has demonstrated that mRNA expressions of murine endogenous retrovirus-L and intracisternal A particle, two autonomous long terminal repeat retrotransposons, increased in 2-cell or 8-cell embryo when mouse Dicer siRNAs were microinjected in 1-cell embryo [14]. Moreover, siRNAs were discovered also in mouse oocytes and these siRNAs degraded retrotransposon-derived mRNAs [15]. These findings suggest that siRNAs exist in mammalians and that retrotransposons are suppressed through the

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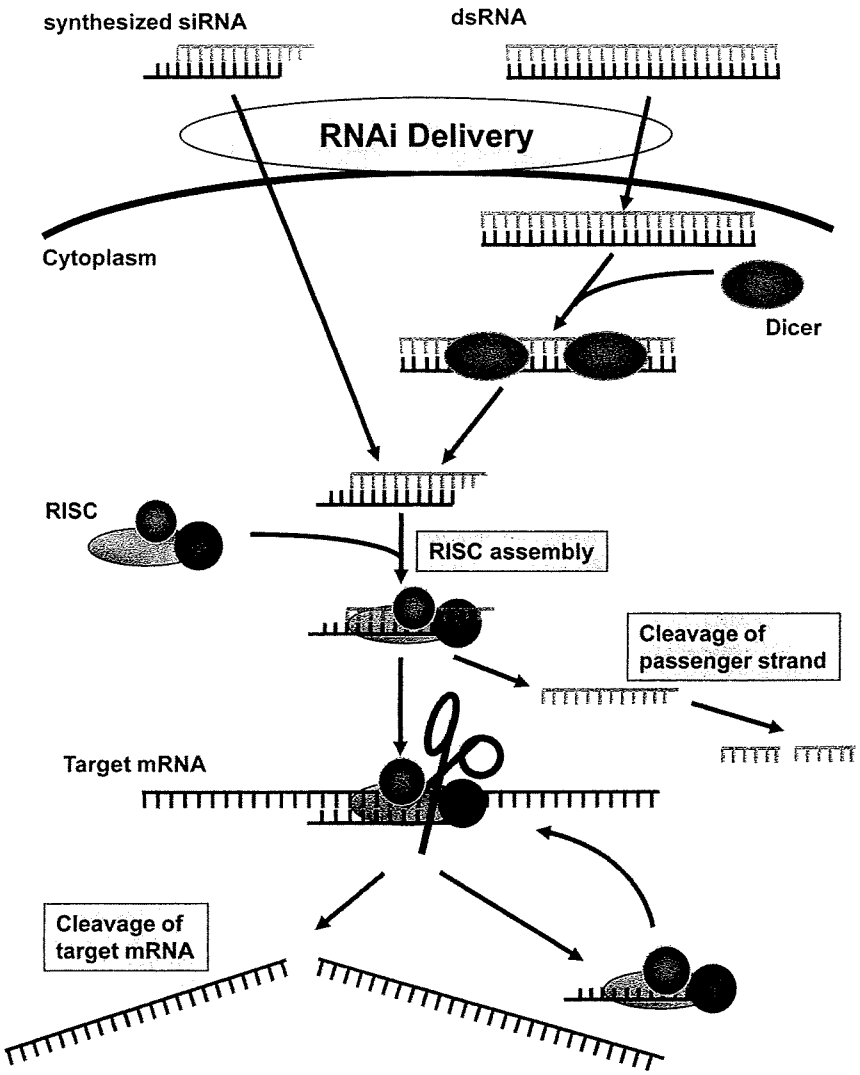

Fig. (1). Mechanisms of RNA interference

After the introduction of dsRNA into a target cell (See Fig. (**2**)), the dsRNA is processed into siRNA length of 21-23 nucletides by Dicer siRNA then enters an RNA-induced silencing complex (RISC) assembly pathway. The dsRNA unwinds to form two single-strands of RNA The passenger strand rapidly degrades and the guide strand binds and cleaves the target mRNA, resulting in mRNA degradation.

RNAi pathway in the early stage of embryonic development and mouse oocytes. On the contrary, hundreds of miRNAs, which regulate endogenous gene expression, have been identified in mammalians, but not in lower eukaryotes.

The precise mechanisms of RNAi are discussed in several reviews [5, 16, 17]. RNAi processes can be roughly divided into the initiation phase and the effector phase (Fig. **1**). In the initiation phase, following introduction of dsRNA into a target cell, dsRNA is processed into shorter lengths of 21-23 nucleotides (nts) dsRNAs, termed siRNAs by the ribonuclease activity of a dsDNA-specific RNAse III family ribonuclase Dicer. Dicer consists of the following domains: an N-terminal helicase domain, an RNA-binding Piwi/ Argonaute/Zwille (PAZ) domain, two tandem RNAse III domains, and a dsRNA-binding domain [18, 19]. The number of Dicers present in a cell varies depending on the species of the organism. Mammals and nematodes have only a single Dicer, which acts to produce both siRNAs and miRNAs [20-22], while other organisms have multiple Dicers which perform separate, specialized function *Drosophila* has two Dicers: *Drosophila* Dicer-1 is require for generating miRNAs, whereas *Drosophila* Dicer-2 pro duces siRNAs [17, 23]. dsRNA precursors are sequentiall processed by the two RNAse III domains of Dicer, an cleaved into smaller dsRNAs with 3' dinucleotide overhang [18, 24].

Smaller dsRNAs enter into an RNA-induced silencin complex (RISC) assembly pathway [25]. RISC contain Argonaute (Ago) proteins, a family of proteins characterize by the presence of a PAZ domain and a PIWI domain [26 The PAZ domain recognizes the 3' terminus of RNA, an the PIWI domain adopts an RNAse H-like structure that ca catalyze the cleavage of the guide strand. Most species hav multiple Ago proteins, but only Ago2 can cleave its RN target in humans. The dsRNA is unwound by ATP dependent RNA helicase activity to form two single-strand of RNA. The strand that directs silencing is called the guid (antisense) strand, and the other is called the passenge

(sense) strand. Ago2 protein selects the guide strand and cleaves its RNA target at the phosphodiester bond positioned between nucleotides 10 and 11 [24, 27]. The resulting products are rapidly degraded because of the unprotected ends, and the passenger strand is also degraded [28, 29]. The targeted RNA dissociates from the siRNA after the cleavage, and the RISC cleaves additional targets, resulting in decrease of expression of the target gene.

Since the discovery of the first miRNA *lin-4* [30, 31], miRNAs, some of which are non-coding RNA species, have been shown to regulate diverse biological processes in eukaryotes. miRNAs are derived from stem-loop-structured primary miRNAs (pri-miRNAs) by the cleavage activity of Drocha, a nuclear-localized member of the RNAse III family, to yield short precursor miRNAs called pre-miRNAs. Pre-miRNAs comprising 60-70 nts exhibit a hairpin structure with a 5'-phosphate and a 3'-2 nts overhang. After translocation from the nucleus to the cytoplasm by Exportin-5, pre-miRNAs are processed by Dicer into miRNAs of 20-24 nts. miRNAs as well as siRNAs enter into RISC assembly pathway. Once associated with Ago, the guide strands of miRNAs bind to the sequence-specific basepairs of target RNAs, resulting in translational repression or mRNA degradation (Fig. 2) [32]. Unlike siRNAs, the complimentarity of miRNAs are most often imperfect. Bioinformatic studies reveal that a single miRNA might bind to as many as 200 gene targets which have diverse functions [33-35]. In case of the perfect complimentarity, the target mRNA can be cleaved by an Ago protein. When complimentarity is imperfect, the translation is suppressed without RISC-mediated cleavage [36-38]. Early studies indicated that repression of animal miRNAs occurred without corresponding decrease of mRNA [31, 36]. However, recent studies have demonstrated that miRNAs can reduce the mRNA levels in mammalian cells and *C. elegans* [39, 40]. Furthermore, mRNA degradation is controlled by cytoplasmic processing body (P-body) [41-43]. Although the complete components of cytoplasmic P-body are not identified yet, some proteins involving in nonsense-mediated decay, target mRNA, and miRNAs are observed. miRNAs colocalize with their target transcripts in sites of mRNA degradation, resulting in repression of mRNA translation [41, 44, 45].

While synthesized siRNAs that are directly transfected into cells, short-hairpin RNAs (shRNAs) are synthesized in the nucleus, and they are transcribed by either RNA polymerase II or III [2, 46]. shRNAs have a stem-loop structure and are processed through the endogenous RNAi machinery similar to miRNA maturation pathway (Fig. 2) [32]. shRNAs are usually expressed from plasmids or viral-based expression vectors [47]. Adenoviral vectors are widely used both *in vitro* and *in vivo*, however, the main drawback associated with their use is vector-mediated immunogenicity. Adeno-associated viral (AAV) vectors have low inflammatory potential and they have an advantage in terms of safety for *in vivo* application [48]. Therefore, clinical application of AAV vectors to RNAi is expected. Retroviral vectors can induce long-term gene silencing and have low inflammatory potential. However, they can transduce shRNAs into only

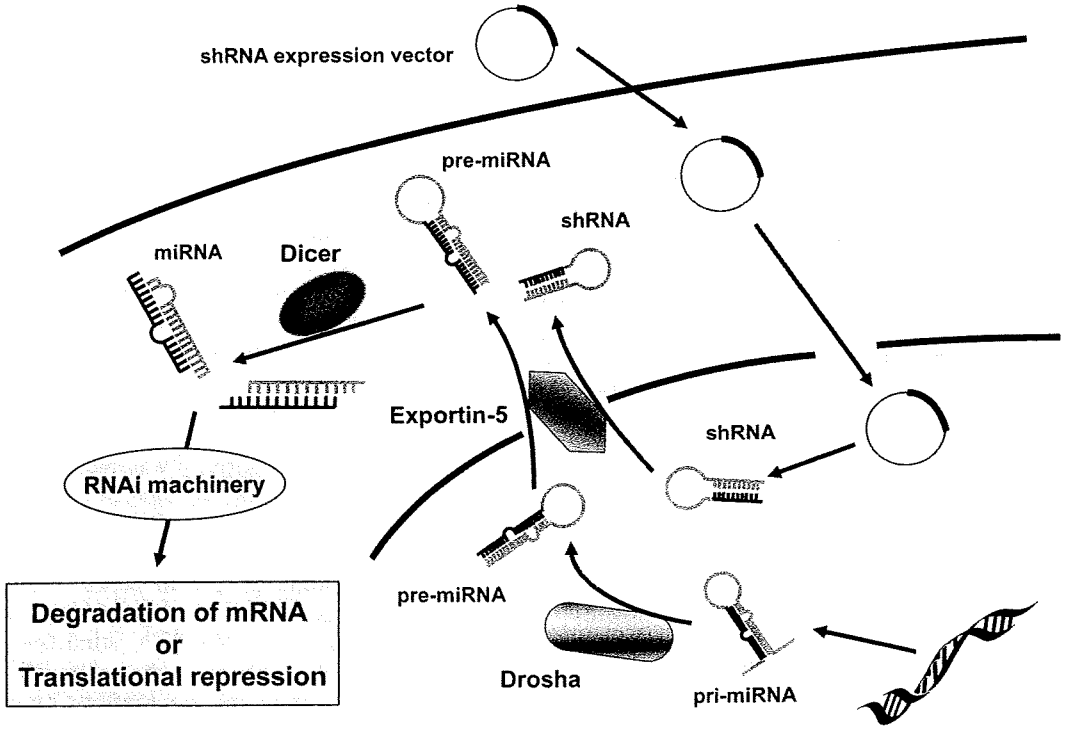

Fig. (2). Processing of miRNAs and shRNAs

pri-miRNAs are cleaved by Drosha and pre-miRNAs are produced. pre-miRNAs are translocated from nucleus to cytopolam by Exprotin-5, and are processed by Dicer into miRNAs. Through the RNAi machinery, miRNAs repress translation or degrade mRNAs (See Fig. (1)). After shRNA expression vector is delivered into the cytoplasm, the vector is transported into the nucleus for transcription. Thereafter, primary shRNA is generated and this has a hairpin-like stem-loop structure similar to pri-miRNA. The primary shRNA transcript is translocated into the cytpoplasm by Exportin-5. Then, shRNA is processed by Dicer and enter the endogenous RNAi machinery.

dividing cells. On the contrary, lentiviral vectors can transduce into non-dividing cells and can induce persistent gene-silencing in most tissues. Lentiviral vectors will be important delivery systems for wide range of diseases including hematopoitic disorders. However, these integrating viral vectors have major concerns of insertional mutagenesis [49, 50]. The development of site-specific insertional machinery [51, 52] is expected to prevent the vector-insertional tumorigenesis.

TARGETING CANCER-ASSOCIATED GENES BY RNAi

Because of its high specificity and high efficiency in binding gene products, RNAi has recently been evaluated as a therapeutic strategy for cancer treatment. To develop nuclear medicine against cancers, it is essential that suitable gene targets are selected. Such targets include antiapoptotic proteins, cell cycle regulators, transcription factors, signal transduction proteins, and factors associated with malignant biological behaviors of cancer cells, all of these genes are associated with the poor prognosis of cancer patients.

siRNA's candidate targets include molecules of apoptosis/cell cycle regulation. The antiapoptotic Bcl-2 protein contributes to resistance to apoptosis against external stimuli, including cytotoxic agents. Bcl-2 is upregulated in a variety of cancers and participates in tumorigenesis and progression [53-58]. Overexpression of Bcl-2 in tumor cells correlates with the poor prognosis of the patients [59-61]. Many studies have demonstrated that siRNA treatment against Bcl-2 inhibited the proliferation of tumor cells. Administration of synthetic Bcl-2 siRNA, using a cationic liposome, suppressed tumor progression in a xenograft mouse model [62-64], indicating that Bcl-2 is a suitable target for cancer therapy. Survivin is a member of the inhibitors of apoptosis proteins (IAPs) family and plays a role in chromatin cleavage associated with spindle formation in cell division [65]. Survivin is almost undetectable in adult tissues [66]. However, overexpression of survivin has been shown in cancers [66, 67] and to correlate with poor prognosis of cancer patients [68-73]. Moreover, survivin is associated with resistance to chemotherapy and radiotherapy [74-77]. Therefore, a siRNAs-mediated decrease of the expression of survivin represents a potential target for siRNA anticancer therapy and may improve the clinical outcomes of cancer patients.

Another category of siRNA candidate targets are molecules of signal transduction. Members of the signal transducer and activator of transcription (STAT) family act as key components of cytokine signaling pathways that regulate gene expression [78, 79]. Among seven types of STATs, STAT3 is the most strongly implicated in carcinogenesis. Whereas STAT3 is transiently activated in normal cells, a constitutively active form is detected in a variety of cancers [80] and dysregulates the downstream target genes of cell proliferation and survival [81-83]. Knockdown of STAT3 protein expression inhibited tumor growth and invasion [84-86]. Bcr-Abl fusion protein, which is created by the molecular consequence of the t(9;22) translocation, is a constitutively active tyrosine kinase that causes leukemias [87]. Imatinib mesylate (IM; Gleevec™, Glivec™) was developed as a first generation tyrosine kinase inhibitor (TKI) and its emergence has dramatically changed th outcomes of therapies against Philadelphia (Ph)–positiv leukemia, especially chronic myelogenous leukemia (CML [88-91]. Moreover, the use of several second generatio TKIs developed to overcome resistance to IM have yielde excellent outcomes [92-95]. These findings indicate tha targeting Bcr-Abl protein is a promising strategy to eliminat Bcr-Abl-positive leukemic cells. The approach to downregu late the expression of Bcr-Abl mRNA by RNAi was invest gated *in vitro* [96-98], and further studies are needed for it introduction to the clinical field. β-catenin is a downstrean protein of the canonical Wnt signaling pathway that has bee shown to play an important role in the process of deve lopment, proliferation, and differentiation [99]. In th absence of Wnt, intracellular levels of β-catenin are regu lated by multiple proteins, such as glycogen synthase kinase 3, casein kinase 1 and adenomatous polyposis coli, and β catenin is phosphorylated and is degraded by the ubiquitin proteasome pathway. In the presence of the Wnt ligand intracellular β-catenin is stabilized in the cytoplasm and th subsequent intracellular accumulation of β-catenin results i its translocation to the nucleus. In the nucleus, β-cateni binds to the T cell factor transcription factor and target gene such as *c-Myc* and *Cyclin D1*, which are upregulated resulting in cell proliferation [100]. Recently, this pathwa has been focused on as it is involved in cancer developmen Overexpression of β-catenin was detected in many types o cancers [101-105]. Treatment of siRNAs against β-cateni successfully suppressed the proliferation of colon cance cells and myeloma cells by inducing caspase-dependen apoptosis [105, 106]. Thus, β-catenin represents a suitabl target for RNAi therapy.

Other candidate siRNA targets are molecules that defin the behavior of cancerous cells, such as metastasis or resis tance to cytotoxic agents. P-glycoprotein (P-gp) is a produc of *multidrug resistance* (*MDR1*) gene, and the overexpre ssion of P-gp induces cross-resistance against structurall unrelated cytotoxic agents [107]. Depletion of P-gp b knockdown of MDR1 mRNA reversed the sensitivity t adriamycin or vincristine in cancer cell lines [108, 109]. I addition, chemosensitivity was increased in cancer cell line by treatment with siRNAs against other molecules associate with chemoresistance, including multidrug resistance protei 1 (MRP1), ABCG2, and MRP7/ABCC10 [110-113]. Th vascular endothelial growth factor (VEGF)/VEGF recepto (VEGFR) axis plays an important role in angio- an lymphangiogenesis. VEGF-A stimulates anginogenesis i tumor vessels, enhances the permeability of the bloo vessels, and promotes the motility of cancer cells, whic results in metastases [114-116]. Treatment with VEGF-A siRNAs successfully prevented metastases of cancers [117 119]. VEGF-C is associated with tumor lymphangiogenesi and lymph node metastasis, and the overexpression o VEGF-C/D in cancer cells increased metastases via lympl vessels [120-123]. VEGF-C siRNA inhibited metastasis o breast cancer in a mouse xenograft model [124].

POLO-LIKE KINASE-1

PLKs belong to the family of serine/threonine kinase and are highly conserved among eukaryotes. PLK family ha identified PLK-1, PLK-2 (SNK), PLK-3 (FNK), and PLK-4

(SAK) in mammalians so far and PLKs function as regulators of both cell cycle progression and cellular response to DNA damage [6, 125-127]. PLK-1 is the best characterized among the four PLKs identified to date. PLK-1 has an N-terminal serine/threonine protein kinase domain and two polo box domains at the C-terminal region. Polo box domains regulate the kinase activity of PLK-1 [7, 128]. PLK-1 regulates cell division at several points in the mitotic phase: mitotic entry through CDK1 activation, bipolar spindle formation, chromosome alignment, segregation of chromosomes, and cytokinesis [6, 129]. PLK-1 gene expression is regulated during cell cycle progression, with a peak level occurring at M phase [130, 131]. Similar to its gene expression, PLK-1 protein expression and its activity are low in G0, G1, and S phases, and begin to increase in G2 phase with peak in M phase [130-133]. When cells exit from mitosis, PLK-1 is degraded through the ubiquitin-proteasome pathway as a substrate of ubiquitin-ligase, known as the anaphase-promoting complex/cyclosome [134, 135]. PLK-1 is highly expressed in tissues with actively proliferating cells, placenta, spleen, and testis, whereas PLK-1 is scarcely detectable in most other adult tissues [130, 136, 137].

Many reports have described that PLK-1 is overexpressed in cancerous tissues and that PLK-1 expression levels were tightly correlated with histological grades of tumors, clinical stages, and prognosis of the patients. PLK-1 mRNA levels were elevated in non-small cell lung cancer (NSLC) tissues and this transcript levels were correlated with the survivals of cancer patients [138]. Moreover, the immunohistoligical study showed that PLK-1 protein was overexpressed in NSLC tissues in patients at progressed stages of cancer (postsurgical stage ≥II) and in patients with poorly differentiated NSLCs [10]. Patients with urinary bladder cancers expressing high levels of PLK-1 have a poor prognosis compared with patients with its low expression. Moreover, the histologically high-grade, deeply invasive, lymphatic-invasive, and venous-invasive bladder cancers demonstrated significantly higher PLK-1 expression [9]. Elevated expression of PLK-1 is observed in colorectal cancers. Overexpression of PLK-1 correlated positively with clinicopathological features including Dukes stage, tumor stage, and nodal status. Additionally, PLK-1 expression was a prognostic marker in univariate survival analysis [139]. PLK-1 overexpression is detected in other various cancers and, therefore, PLK-1 overexpression is a prognostic biomarker for cancer patients (Table **1**).

Inhibition of PLK-1 activity induces mitotic arrest and tumor cell apoptosis [140-142]. Depletion of PLK-1 mRNA also inhibits the functions of PLK-1 protein in DNA damages and spindle formation and causes the inhibition of the cell proliferation in a time- and a dose-dependent manner. PLK-1 siRNA treatment induces an arrest at the G2/M phase in the cell cycle with the increase of CDC2/Cyclin B1 [9, 10, 117, 143-145]. PLK-1 siRNA-transfected cells had dumbbell-like and misaligned nuclei, indicating that PLK-1 depletion induced abnormalities of cell division during M phase, and these cells were shown to yield to caspase-dependent apoptosis [9, 10, 143]. PLK-1 silencing inhibits the cell proliferation of other various malignancies including prostate cancer [145], esophageal cancer [146], hepatocellular carcinoma [144], breast cancer [147], gastric cancer [148], and leukemia [149, 150]. Moreover, preclinical studies using xenograft mouse models demonstrated that the administration of PLK-1 siRNA suppressed the growth of cancers [10, 144 , 151-154].

Interestingly, depletion of PLK-1 increases the susceptibility of anticancer agents [147, 155]. Polymerized microtubles attach to kinetochores and their dynamics carry out cell division [156]. Paclitaxel targets microtubule dymanics [156, 157]. PLK-1 monitors the tension, and its depletion by siRNA reduced expression of some spindle checkpoint proteins including Mad2, Cenp-E, Hec/Ndc80, and Spc [158]. Consequently, PLK-1 siRNA acts synergistically with paclitaxel to inhibit the proliferation of breast cancer cells [147, 153]. Cyclin-dependent kinase inhibitor $p27^{kip1}$ is an atypical tumor suppressor that regulates G0 to S phase transition, and reduced $p27^{kip1}$ expression is associated with poor prognosis of cancer patients [159, 160]. Herceptin is a humanized monoclonal antibody agent against Her2-positive

Table 1. Cancers whose PLK-1 Overexpression is Correlated with the Prognosis of Patients

Cancer	Authors
Oropharyngeal carcinoma	Knecht, *et al.* [235, 236]
Esophageal carcinoma	Tokumitsu, *et al.* [237], Feng, *et al.* [238]
Non-small cell lung cancer	Wolf, *et al.* [138]
Breast cancer	Wolf, *et al.* [239], Ahar, *et al.*[240], Weichert, *et al.* [241], Loddo, *et al.*[242]
Gastric cancer	Weichert, *et al.* [243], Kanaji, *et al.* [244]
Hepatoblastoma	Yamada, *et al.* [245]
Colorectal cancer	Takahashi, *et al.* [246], Weichert, *et al.* [139], Macmillan, *et al.* [247]
Urinary bladder cancer	Nogawa, *et al.* [151], Yamamoto, *et al.* [248]
Ovarian cancer	Weichert, *et al.* [249]
Melanona	Strebhart, *et al.* [250]
Non-Hodgkin's lymphoma	Mito, *et al.* [251], Liu, *et al.* [252]

cancers. Herceptin-resistant breast cancer cells decreased the $p27^{kip1}$ expression, moreover, exogenous addition of $p27^{kip1}$ increased the sensitivity of herceptin [161]. Because PLK-1 siRNA treatment induced $p27^{kip1}$ expression and seems to facilitate the induction of apoptosis by the cleavage of caspases in HER2-positive breast cancer cells, PLK-1 siRNA acts synergistically with herceptin [147].

Because PLK family cooperatively acts in mitosis, depletion of PLK-1 alters the expressions of other PLKs. PLK-2 and PLK-3 transcripts are increased after the PLK-1 siRNA treatment [10]. Unlike PLK-1, PLK-2 and PLK-3 play inhibitory roles in cell proliferation. PLK-2 is transcriptionally regulated by p53 in response to DNA damage by irradiation and PLK-3 is activated by DNA damage checkpoint [126]. These observations suggest that PLK-1 depletion induces mitotic catastrophe and activation of DNA damage checkpoint and spindle checkpoint, resulting in increase of PLK-2 and PLK-3 transcripts. Although PLK-1 plays an important role in cell division, depletion of PLK-1 does not affect the proliferation normal cells [10, 140, 162]. This suggests that some ot kinases compensate loss of PLK-1 function during mitosis normal cells [10, 162]. Because depletion of PLK-1 indu mitotic catastrophe and cell death in cancer cells specifica PLK-1 could be an excellent target for cancer therapy.

DEVELOPMENT OF NON-VIRAL DDSs

Although siRNA target molecules are overexpressed cancer cells, most of them are essential to maintain meostasis of physiological functions in humans. Therefo siRNAs must be delivered selectively into cancer ce Moreover, naked siRNAs are degraded by endogenous n leases when administered *in vivo*, so that delivery meth that protect siRNAs from such degradation are essential. these reasons, safer and more effective DDSs must developed. DDSs are divided into two categories: v vector based carriers, and non-viral based carriers. V

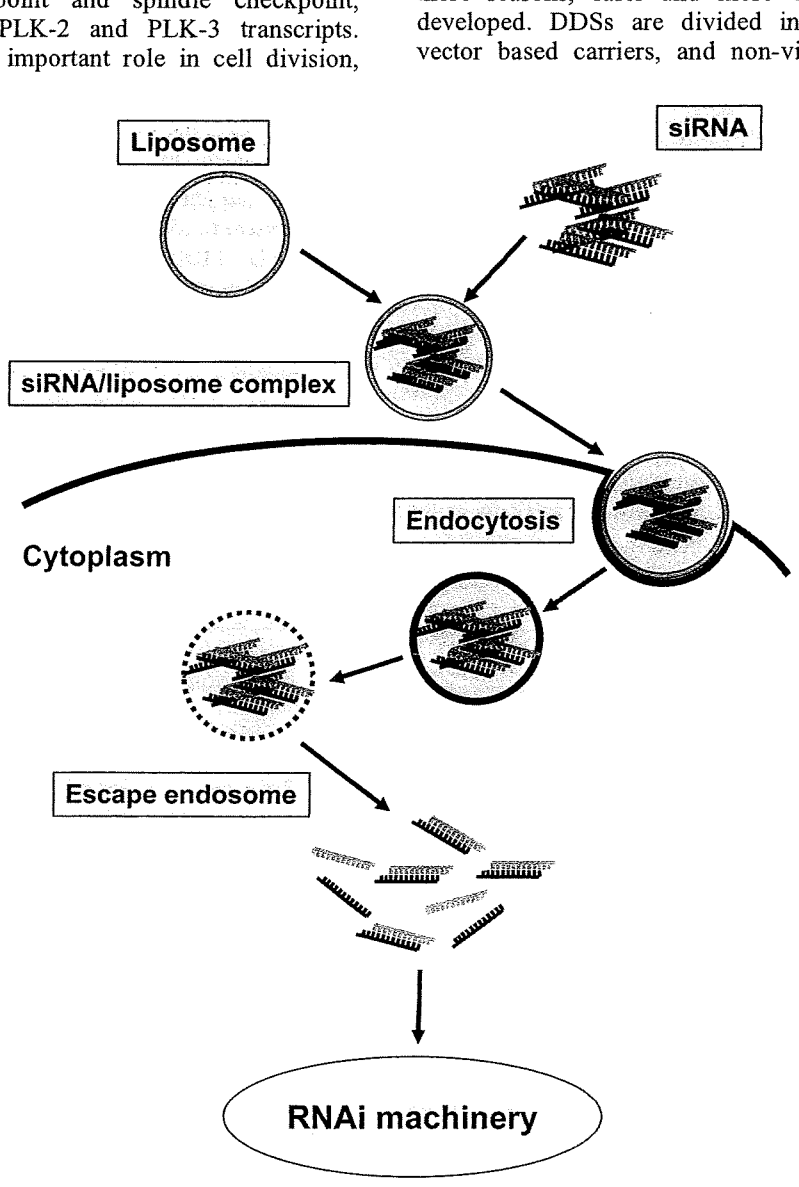

Fig. (3). Delivery of siRNA/liposome complex

Positively charged liposomes facilitate complex formation with negatively charged siRNAs and binding to cell membrane. After binding the cell membrane, the siRNA/liposome complex enters the cytoplasm of the cell via endocytosis. The complex then escapes the endoson and releases its siRNA to the RNAi machinery (See Fig. **1**).