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Rationale: Accumulating evidence indicates that stem/progenitor cells (SPCs) represent an important source of cells in atheromas and contribute to lesion formation and progression.

Objective: We investigated whether matrix metalloproteinase-8 (MMP8) played a role in SPC migration and their recruitment into atheromas.

Methods and Results: We found that SPCs in atheromas expressed MMP8 and that MMP8 knockout significantly reduced SPC numbers in atherosclerotic lesions in apolipoprotein E (ApoE)–deficient mice fed a Western diet. Further in vivo experiments showed that ApoE$^{-/-}$/MMP8$^{-/-}$ mice injected with stem cells isolated from bone marrows of ApoE$^{-/-}$/MMP8$^{-/-}$ mice had fewer SPCs in atheromas and smaller lesions than ApoE$^{-/-}$/MMP8$^{-/-}$ mice injected with stem cells isolated from bone marrows of ApoE$^{-/-}$/MMP8$^{+/+}$ mice. Ex vivo experiments showed that MMP8 deficiency inhibited the ability of SPCs to migrate from the arterial lumen and the adventitia into atherosclerotic lesions. In vitro assays indicated that MMP8 facilitated SPC migration across endothelial cells and through Matrigel or collagen I. We also found that MMP8 cleaved a-disintegrin-and-metalloproteinase-domain-10 and that MMP8 deficiency reduced mature a-disintegrin-and-metalloproteinase-domain-10 on SPCs. Knockdown of MMP8 or incubation with the a-disintegrin-and-metalloproteinase-domain-10 inhibitor GI254023X decreased E-cadherin shedding on SPCs. The decrease in migratory ability of SPCs with MMP8 knockdown was reduced by incubation of such cells with culture supernatant from SPCs without MMP8 knockdown, and this compensatory effect was abolished by an antibody against soluble E-cadherin.

Conclusions: MMP8 plays an important role in SPC migration and their recruitment into atherosclerotic lesions. (_Circ Res_. 2013;112:35-47.)

Key Words: atherosclerosis ■ matrix metalloproteinase-8 ■ migration ■ progenitor cell ■ stem cell

The pathogenesis of atherosclerosis is a chronic inflammatory process initiated by biochemical and mechanical insults such as elevated level of low-density lipoprotein cholesterol and high blood pressure.[1] Major constituents of atherosclerotic lesions include lipids, macrophages, smooth muscle cells (SMCs), and extracellular proteins, especially collagen I and III. Traditionally, it is thought that the accumulation of SMCs in atheromas results from migration of resident SMCs from the arterial media into the intima, where SMCs undergo proliferation, and that the accumulation of monocytes/macrophages is the result of infiltration of circulating monocytes into the intima, where they differentiate into macrophages. Recent evidence, however, indicates that stem/progenitor cells (SPCs) represent another source of SMCs[2–5] and monocytes/macrophages[6–10] in atheromas and contribute to atherosclerotic lesion formation and progression.[2–13] Studies have identified 2 sources for SPCs in atherosclerotic lesions: (1) bone marrow–derived SPCs that are recruited into atherosclerotic lesions via the blood circulation and (2) local SPCs in the vascular wall that can migrate into

Non-standard Abbreviations and Acronyms	
ADAM10	a-disintegrin-and-metalloproteinase-domain-10
ApoE	apolipoprotein E
GFP	green fluorescence protein
MMP	matrix metalloproteinase
SMC	smooth muscle cell
SPC	stem/progenitor cell

atherosclerotic lesions.[3,10] However, the mechanisms by which SPCs are mobilized from their original sites and recruited into the atheroma are currently incompletely elucidated.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases with proteolytic activity on a variety of proteins. Collectively, the MMPs can degrade all proteins that constitute the interstitial extracellular matrix and the basement membrane. Moreover, a growing number of nonmatrix proteins have been shown to be susceptible to cleavage by MMPs. In some cases, such cleavage leads to increased activity of the proteins concerned, whereas in some other cases, the cleavage results in diminished activity.[14] There is evidence indicating that certain MMPs play important roles in SPC mobilization, homing, and migration into destination tissues. For example, studies have shown that MMP9, whose expression is induced by nitric oxide,[15] can cleave the membrane-bound kit ligand (also known as stem cell factor), leading to the release of this cytokine, which promotes transfer of SPCs from quiescent to proliferative niches in the bone marrow.[16] It also has been demonstrated that MMP9 facilitates the incorporation of bone marrow–derived endothelial progenitor cells into sites of ischemia-induced neovascularization.[17] Another MMP, namely MMP14, has been shown to play a role in granulocyte colony-stimulating factor–induced progenitor cell mobilization, such that MMP14, activated by this cytokine, cleaves off the protein CD44 on progenitor cells and thus disrupts CD44-mediated binding of progenitor cells to the basement membrane in the bone marrow, thereby promoting progenitor cell mobilization and egress.[18] Taken together, these studies indicate that different MMPs play divergent roles in SPC mobilization or migration.

MMP8 (also known as collagenase-2) has potent proteolytic activity on fibrillar collagens, particularly collagen I.[19] It also can degrade other matrix proteins such as laminin and fibronectin.[19] In addition, it has been shown to be able to cleave the chemokines CXCL5[20] and CXCL11.[21] Studies of polymorphonuclear cells have shown that a substantial proportion of MMP8 binds to the cell plasma membrane and that the membrane-bound MMP8 contributes importantly to collagen I degradation.[22]

Previous studies by other researchers have shown that macrophages, SMCs, and endothelial cells in atherosclerotic lesions express MMP8,[23] that lesion progression is associated with increased MMP8 expression,[24] and that increased plasma MMP8 levels correlate with increased risk of cardiovascular mortality,[25] indicating a connection between MMP8 and atherosclerosis. Further to those studies, we recently showed that MMP8 deficiency reduced atherosclerotic lesion formation in apolipoprotein E (ApoE) knockout mice fed a Western diet, with reduced lesional macrophage and SMC content,[26] suggesting a causal role of MMP8 in the pathogenesis of atherosclerosis. Because of the complexity of atherogenesis and potentially a wide range of proteins that might be susceptible to cleavage by MMP8, it is possible that multiple different mechanisms contribute to the role of MMP8 in atherosclerotic lesion formation and progression, and further work would be required to elucidate the mechanisms involved. In this study, we found evidence of an important role of MMP8 in SPC migration and their recruitment into atherosclerotic lesions.

Methods

Detailed Methods are provided in the Online Data Supplement. In brief, microscopic sections of aortic root atherosclerotic lesions from ApoE$^{-/-}$/MMP8$^{-/-}$ and ApoE$^{-/-}$/MMP8$^{+/+}$ mice fed[26] a Western diet for 12 weeks were subjected to hematoxylin and eosin staining and immunostaining for MMP8, Sca-1, CD34, c-kit, and Flk-1, respectively. In stem cell injection experiments, SPCs were isolated from bone marrows of ApoE$^{-/-}$/MMP8$^{-/-}$ and ApoE$^{-/-}$/MMP8$^{+/+}$ mice, respectively, and injected into ApoE$^{-/-}$/MMP8$^{-/-}$ mice via the tail vein (1×10^6 SPCs/mice). The mice were then fed a Western diet for 6 weeks or 12 weeks, and microscopic sections of aortic root atherosclerotic lesions were subjected to hematoxylin and eosin staining and immunostaining for Sca-1, c-kit, CD45, CD68, smooth muscle actin, and smooth muscle myosin heavy chain, respectively. In ex vivo transendothelial migration experiments, fluorescence-labeled embryonic Sca-1$^+$ SPCs that had been infected with MMP8 shRNA to knockdown MMP8 or nontarget shRNA to serve as controls were injected into the lumen of explants of aortas with atherosclerotic lesions; after closure of both ends of the vessels by ligation, the explants were cultured in the presence of stromal cell–derived factor-1α for 24 hours, followed by examination of fluorescence-labeled cells in the atherosclerotic lesions. In ex vivo transadventitia migration experiments, fluorescence-labeled embryonic Sca-1$^+$ SPCs that had been infected with MMP8 shRNA or nontarget shRNA were embedded in Matrigel and then patched around the adventitia of explants of atherosclerotic aortas connected to a microcirculation system with circulating progenitor cell culture medium supplemented with stromal cell-derived factor-1α; after 24 hours of incubation, fluorescence-labeled cells in the atherosclerotic lesions were examined.

Embryonic Sca-1$^+$ SPCs with or without exogenous MMP8 coating, embryonic Sca-1$^+$ SPCs that had been infected with MMP8 shRNA or nontarget shRNA (the efficiency and specificity of MMP8 shRNA knockdown in embryonic Sca-1$^+$ SPCs are shown in Online Figure I), and bone marrow–derived Sca-1$^+$ SPCs from MMP8$^{-/-}$ or MMP8$^{+/+}$ mice were subjected to assays for migration across an endothelial monolayer or through a variety of different types of matrix proteins as follows: Matrigel, collagen I, collagen IV, gelatin, fibronectin, and elastin, respectively.

Western blot analyses and flow cytometry assays were performed to examine pro–a-disintegrin-and-metalloproteinase-domain-10 (ADAM10) and mature ADAM10 on embryonic Sca-1$^+$ SPCs infected with MMP8 shRNA or nontarget shRNA. A recombinant ADAM10 (amino acids 19–673) consisting of the prodomain and the other ectodomains were incubated with or without active MMP8 and subjected to Western blot analysis. Western blot analyses and flow cytometry assays were performed to examine full-length E-cadherin and shed E-cadherin on embryonic Sca-1$^+$ SPCs that had been infected with MMP8 shRNA or nontarget shRNA and had been incubated with or without the ADAM10 inhibitor GI254023X.[27,28] Embryonic Sca-1$^+$ SPCs with MMP8 knockdown were cultured in the presence or absence of culture supernatant from embryonic Sca-1$^+$ SPCs without MMP8 knockdown, and in the presence or absence of an antibody against soluble E-cadherin, and then were subjected to assays for migration through collagen I. Levels of soluble E-cadherin in plasma samples from ApoE$^{-/-}$/MMP8$^{+/+}$ and ApoE$^{-/-}$/MMP8$^{-/-}$ mice were measured with the use of a commercially available ELISA kit.

Results

SPCs in Culture and Atherosclerotic Lesions Express MMP8

Previously, in an RNA microarray analysis of Sca-1+ SPCs derived from embryonic stem cells,[29,30] we noted that they expressed MMP8 mRNA (unpublished data). In this study, we performed Western blot analysis and detected substantial levels of MMP8 protein in cultured Sca-1+ SPCs derived from embryonic stem cells, bone marrow Sca-1+ SPCs, leukocytes, SMCs, and endothelial cells (Figure 1A). Immunocytochemical analysis further confirmed the expression of MMP8 in cultured Sca-1+ SPCs (Figure 1B). We found that MMP8 expression in embryonic Sca-1+ SPCs was increased after incubation with 4-hydroxynonenal and 7-ketocholesterol, components of oxidized low-density lipoprotein cholesterol (Figure 1C). Furthermore, immunohistochemical examination
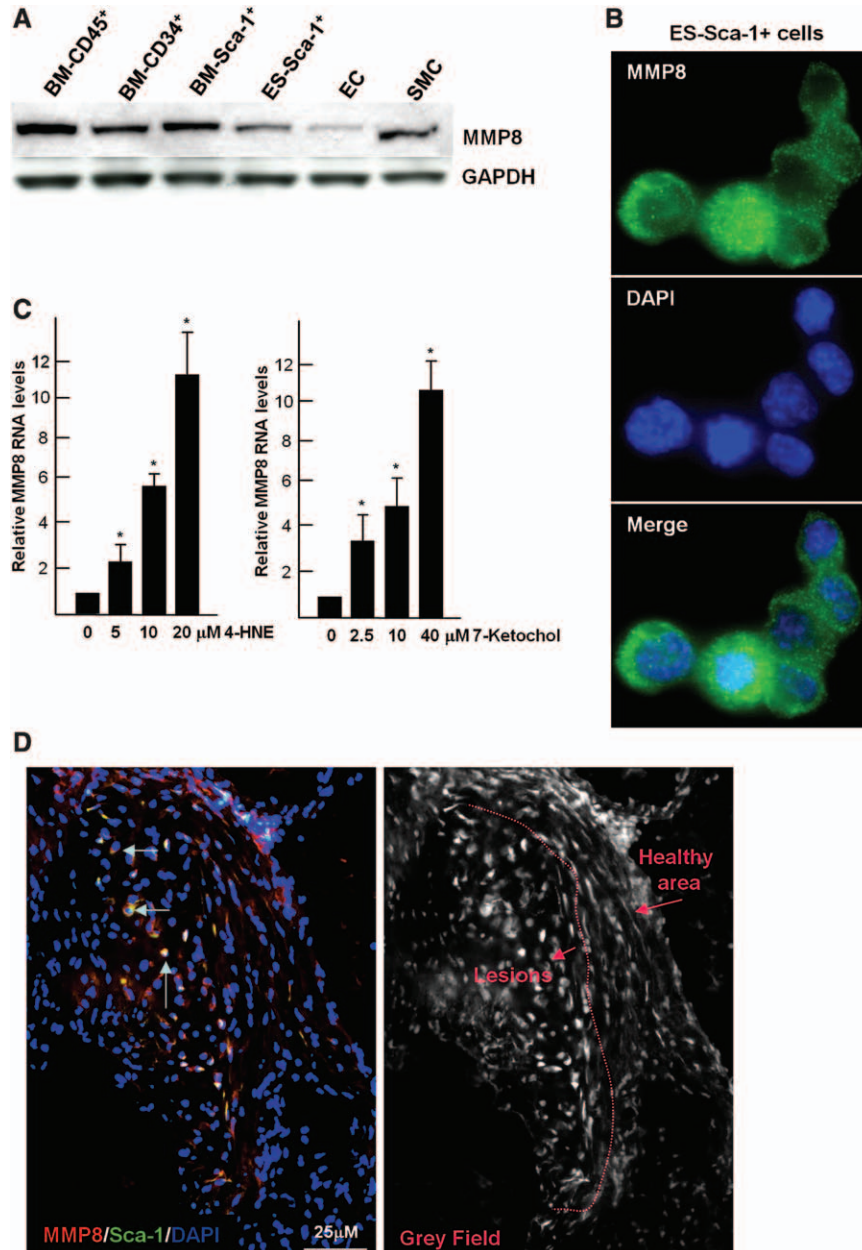
Figure 1. Stem/progenitor cells (SPCs) in culture and atherosclerotic lesions express matrix metalloproteinase-8 (MMP8).
A, Bone marrow (BM)–derived leukocytes (CD45+), BM-derived stem cells (CD34+ and Sca-1+), embryonic stem (ES) cell–derived Sca-1+ SPCs, endothelial cells (ECs), and smooth muscle cells (SMCs) express MMP8, as demonstrated by Western blot analysis. **B**, ES cell–derived Sca-1+ SPCs express MMP8, as demonstrated by immunofluorescence staining. **C**, Oxidized low-density lipoprotein constituents induce MMP8 expression. Cultured Sca-1+ SPCs were treated with various amounts of the oxidized low-density lipoprotein constituents 4-hydroxynonenal and 7-ketocholesterol for 24 hours, followed by reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of MMP8 gene expression. **D**, SPCs in atherosclerotic lesions express MMP8. ApoE−/−/MMP8+/+ mice were fed a Western diet for 12 weeks, and sections of the aortic roots were subjected to double immunofluorescence staining for MMP8 and Sca-1. **White arrows** in the immunofluorescence image indicate double-positive cells in the lesion. **Right**, Gray-field image of the same section. Red dotted line indicates the boundary between the lesion and healthy area.

in atherosclerotic lesions from ApoE-deficient (ApoE$^{-/-}$/MMP8$^{+/+}$) mice fed a Western diet for 12 weeks showed that Sca-1$^+$ SPCs in atherosclerotic lesions expressed MMP8 (Figure 1D).

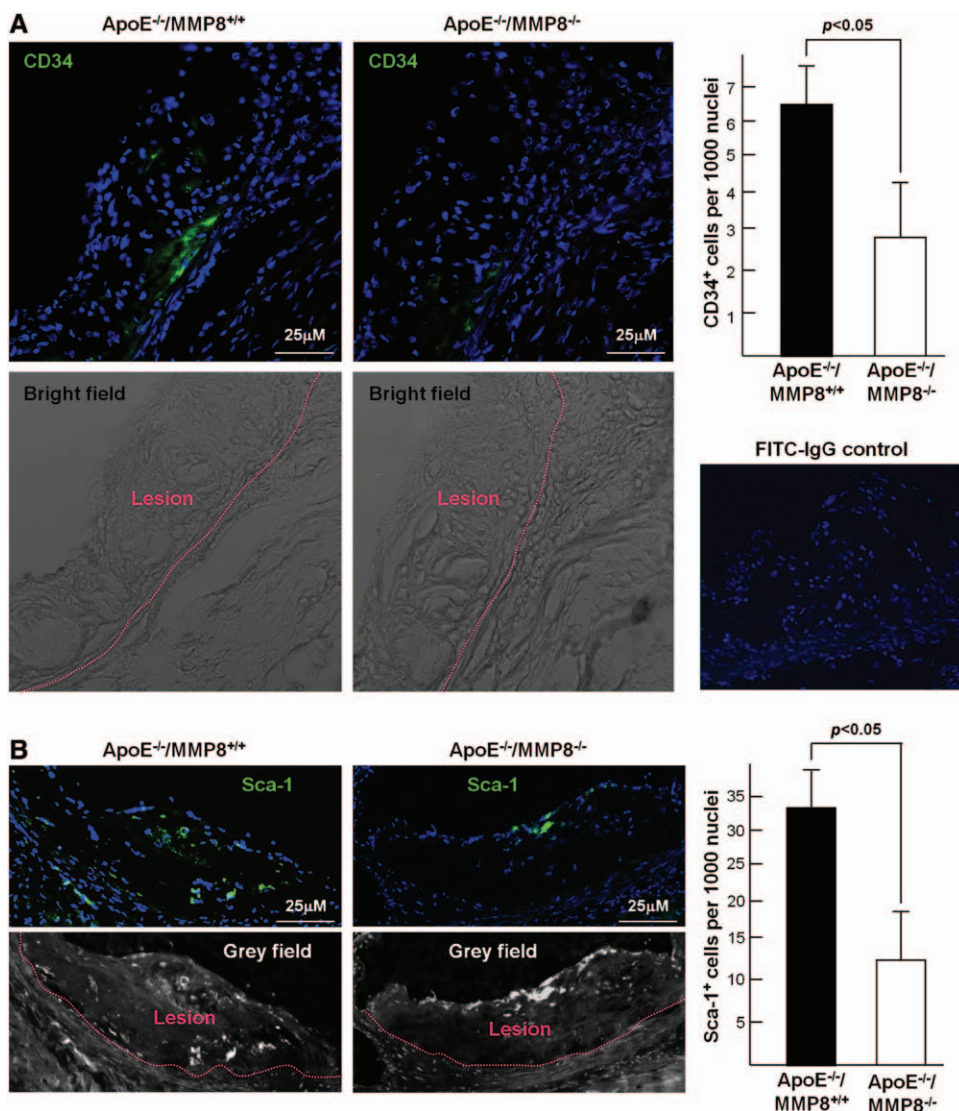## MMP8 Deficiency Reduces SPCs in Atherosclerotic Lesions

To begin to investigate whether MMP8 played a role in SPC recruitment into atherosclerotic lesions, we compared the numbers of SPCs in aortic root atherosclerotic lesions from ApoE-deficient mice with or without MMP8 knockout (ApoE$^{-/-}$/MMP8$^{-/-}$ and ApoE$^{-/-}$/MMP8$^{+/+}$, respectively). We used a panel of well-documented stem cell markers, including CD34, Sca-1, c-kit, and Flk-1, to characterize the SPCs in atherosclerotic lesions. We found that MMP8 knockout significantly reduced the numbers of CD34$^+$ cells (Figure 2A),

Sca-1$^+$ cells (Figure 2B), c-kit$^+$ cells (Online Figure IIA), and Flk-1$^+$ cells (Online Figure IIB) in atherosclerotic lesions.

MMP8 Plays a Role in SPC Migration and Recruitment Into Atherosclerotic Lesions

Having found that SPCs in culture and atherosclerotic lesions expressed MMP8 and that MMP8 knockout significantly reduced SPC numbers in atherosclerotic lesions, we examined whether MMP8 had an effect on SPC migration and their recruitment into atherosclerotic lesions.

To determine whether MMP8 had an effect on SPC recruitment from the arterial lumen into atherosclerotic lesions, we performed ex vivo experiments in which we injected fluorescence-labeled embryonic Sca-1$^+$ SPCs that had been infected with MMP8 shRNA to knockdown MMP8 or nontarget shRNA to serve as controls into the lumen of

Figure 2. Matrix metalloproteinase-8 (MMP8) deficiency reduces stem/progenitor cells (SPCs) in aortic root atherosclerotic lesions. Both ApoE$^{-/-}$/MMP8$^{+/+}$ and ApoE$^{-/-}$/MMP8$^{-/-}$ mice were fed a Western diet for 12 weeks. Aortic roots from ApoE$^{-/-}$/MMP8$^{+/+}$ and ApoE$^{-/-}$/MMP8$^{-/-}$ mice were harvested, sectioned, and subjected to immunofluorescence staining with antibodies against the stem cell markers CD34 (green) (**A**) and Sca-1 (green) (**B**). Shown on the **left** are representative and bright/gray-field images. **Far right** shows quantitative data of SPCs in atherosclerotic lesions of ApoE$^{-/-}$/MMP8$^{+/+}$ and ApoE$^{-/-}$/MMP8$^{-/-}$ mice, respectively. Columns represent mean±SD (n=5 mice in each group). Red dotted line indicates the boundary between the lesion and healthy area. A representative image of IgG control staining is also shown.

explants of mouse aortas with atherosclerotic lesions; after closing both ends of the vessels by ligation, we cultured the explants in the presence of stromal cell–derived factor-1α for 24 hours, followed by examination of fluorescence-labeled cells in the explants. We detected large numbers of fluorescence-labeled cells in the atherosclerotic lesions (and the arterial media) in the explants injected with nontarget shRNA–infected SPCs and found that the numbers of such cells were significantly smaller in the atherosclerotic lesions (and the arterial media) in the explants injected with MMP8 shRNA–infected SPCs (Figure 3A).

To assess whether MMP8 had an effect on SPC migration from the adventitia into the arterial media and atherosclerotic lesions, fluorescence-labeled embryonic Sca-1+ SPCs that had been infected with MMP8 shRNA or nontarget shRNA were embedded in Matrigel and then patched around the adventitia of explants of atherosclerotic aortas connected to a microcirculation system with circulating progenitor cell culture medium supplemented with stromal cell–derived factor-1α. After culturing the explants for 24 hours, we examined fluorescence-labeled cells in atherosclerotic lesions. Similar to the above experiments, we detected larger numbers
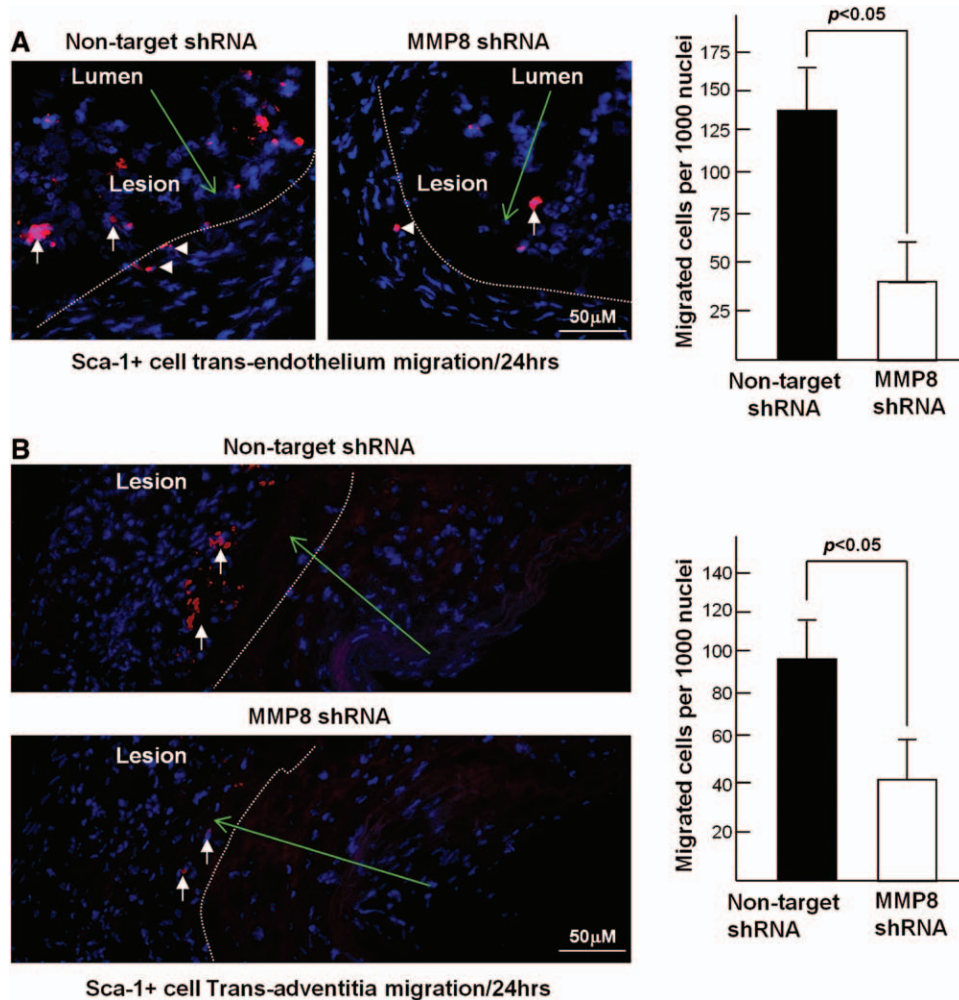
Figure 3. Matrix metalloproteinase-8 (MMP8) knockdown reduces stem/progenitor cell (SPCs) transadventitia migration and transendothelium migration in ex vivo experiments. A, MMP8 knockdown reduces Sca-1+ SPC transendothelium migration. Aortas with atherosclerotic lesions from ApoE$^{-/-}$/MMP8$^{+/+}$ mice were cut into several segments of 5 to 10 mm in length. Embryonic Sca-1+ SPCs (10^5 cells) infected with nontarget shRNA or MMP8 shRNA were labeled with PKH26, washed, and injected into the lumen of the aortas. After ligation of the ends, the aortic segments were cultured in progenitor cell culture medium supplemented with 100 ng/mL of stromal cell–derived factor-1α for 24 hours under normal cell culture conditions (5% CO_2, 21% O_2, pH 7.0–7.5, 37°C). After 24 hours of incubation, the aortic segments were harvested, cross-sectioned, and microscopically examined. **Green arrows** indicate SPC migration direction. **White arrows and arrowheads** indicate migrated SPCs in the lesion area and media layer, respectively. White dotted line indicates the boundary between the lesion and healthy area. **B,** MMP8 knockdown reduces Sca-1+ SPC transadventitia migration. Aortas (≈10 mm in length) with atherosclerotic lesions from ApoE$^{-/-}$/MMP8$^{+/+}$ mice were connected to minineedles of a microcirculation system filled with progenitor cell culture medium supplemented with 100 ng/mL of stromal cell–derived factor-1α. Embryonic Sca-1+ SPCs (10^5 cells) infected with nontarget shRNA or MMP8 shRNA were labeled with PKH26, washed, and mixed with 200 μL of Matrigel and then patched around the adventitia of the aortic segments. After 24 hours of incubation, the aortic segments were harvested, cross-sectioned, and microscopically examined. **Green arrows** indicate direction of SPC migration. **White arrows** highlight SPCs that have migrated into the lesions. White dotted line indicates the boundary between the lesion and healthy areas. **Left,** Representative images of aortic sections. **Right,** Quantitative data of Sca-1+ SPCs that have migrated into atherosclerotic lesions. Columns represent mean±SD (n=3 aortas, 10 sections per aortic segment, in each group).

of fluorescence-labeled cells in the atherosclerotic lesions (and the arterial media) in the explants patched with nontarget shRNA–infected cells than in the atherosclerotic lesions (and the arterial media) in the explants patched with MMP8 shRNA–infected cells (Figure 3B).

To further verify and characterize the effect of MMP8 on SPC migration, we performed in vitro migration assays using embryonic Sca-1+ SPCs that had been incubated with or without exogenous MMP8, embryonic Sca-1+ SPCs infected with MMP8 shRNA or nontarget shRNA, and bone marrow–derived SPCs from mice with or without MMP8 knockout. The assays showed that incubation with exogenous MMP8 enhanced, in a dose-dependent manner, the ability of embryonic Sca-1+ SPCs to migrate through thick Matrigel, which mimics the vascular matrix (Figure 4A) and their ability to transmigrate across an endothelial monolayer, which mimics vascular endothelium (Figure 4B). In contrast, depletion of endogenous MMP8 by shRNAs in embryonic Sca-1+ SPCs significantly reduced their migratory ability (Figure 4C). Consistently, Sca-1+ SPCs isolated from the bone marrow of MMP8 knockout mice had significantly lower migratory ability through matrix proteins than Sca-1+ SPCs isolated from the bone marrow of MMP8 wild-type mice (Figure 4D and 4E).

Because it was plausible that the effect of MMP8 on cell migration would likely be partly attributable its proteolytic activity to degrade proteins in the extracellular matrix, we further investigated which types of protein in the extracellular matrix were likely to primarily account for the effect of MMP8 on cell migration. We performed migration assays using transwells coated with collagen I, collagen IV, laminin, fibronectin, or elastin, and found that MMP8 had a significant effect on embryonic Sca-1+ SPC migration through collagen I but not the other protein types tested (Online Figure III).

Because adhesion of SPCs to the extracellular matrix could have an effect on their mobilization and migration, we further examined whether MMP8 affected SPC adhesion to Matrigel. We found that incubation of embryonic Sca-1+ SPCs with MMP8 dose-dependently inhibited their adhesion to Matrigel (Online Figure IVA).

We also examined whether MMP8 had an effect on SPC proliferation because this potentially could be another reason for the reduction in SPC numbers in atherosclerotic lesions of MMP8 knockout mice, but we detected no such effect (Online Figure IVB). Furthermore, we examined whether the numbers of hematopoietic SPCs (CD34+/CD45+ cells) in the bone marrow and peripheral
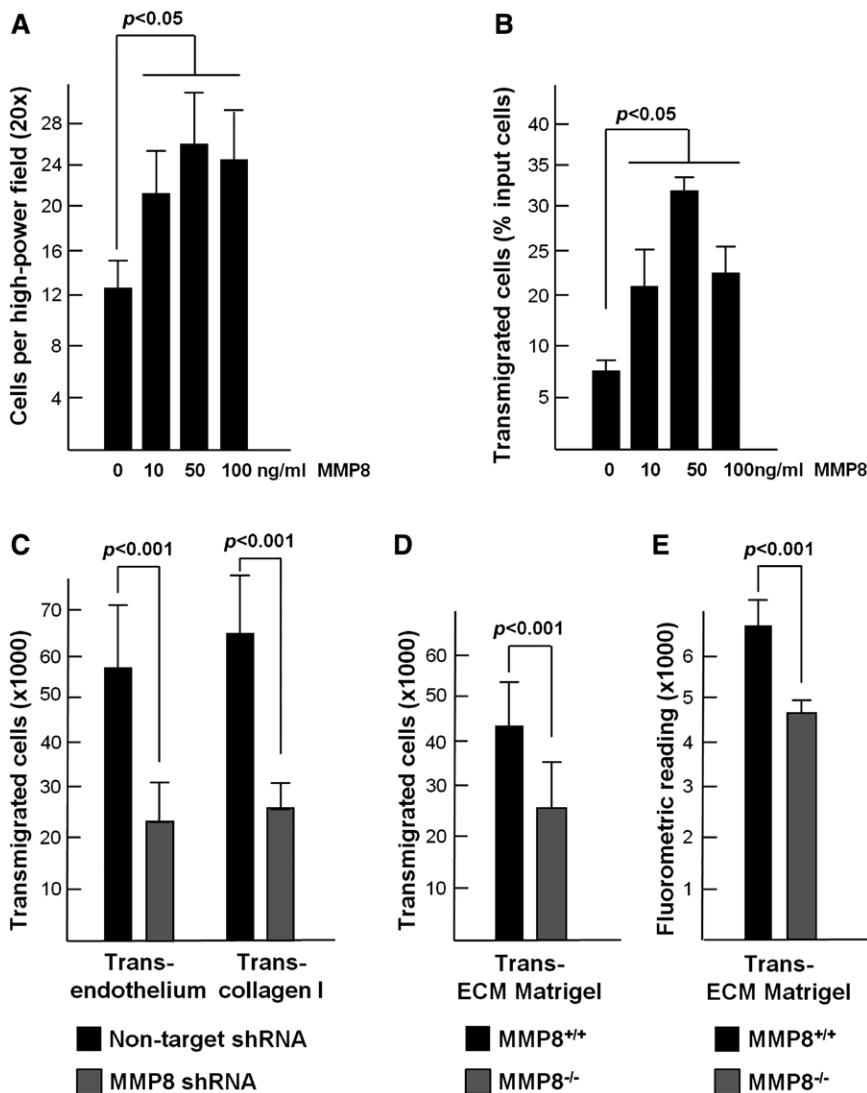
Figure 4. Matrix metalloproteinase-8 (MMP8) enhances stem/progenitor cell (SPC) migration in in vitro experiments. **A** and **B**, Incubation of SPCs with MMP8 enhances migration. Cultured embryonic Sca-1+ SPCs were pretreated with various amounts of MMP8 for 2 hours and then subjected to Matrigel migration assays (**A**) and transendothelial migration assays (**B**). **C**, Knockdown of MMP8 in SPCs reduces migration. Cultured embryonic Sca-1+ SPCs were infected with nontarget shRNA and MMP8 shRNA, followed by transendothelium and transcollagen I migration assays. **D** and **E**, MMP8 knockout reduces SPC migration. The migratory ability of Sca-1+ SPCs isolated from the bone marrow of ApoE−/−/MMP8+/+ and ApoE−/−/MMP8−/− mice was assessed with the use of Martigel-coated transwells (**D**) or extracellular matrix (ECM) invasion assay kit (**E**). Columns represent mean±SD (n=3 independent experiments).

blood were lower in MMP8 knockout mice than in MMP8 wild-type mice because this potentially could be another reason for reduced SPC numbers in atherosclerotic lesions of MMP8-deficient mice, but we observed no such difference (Online Figure V).

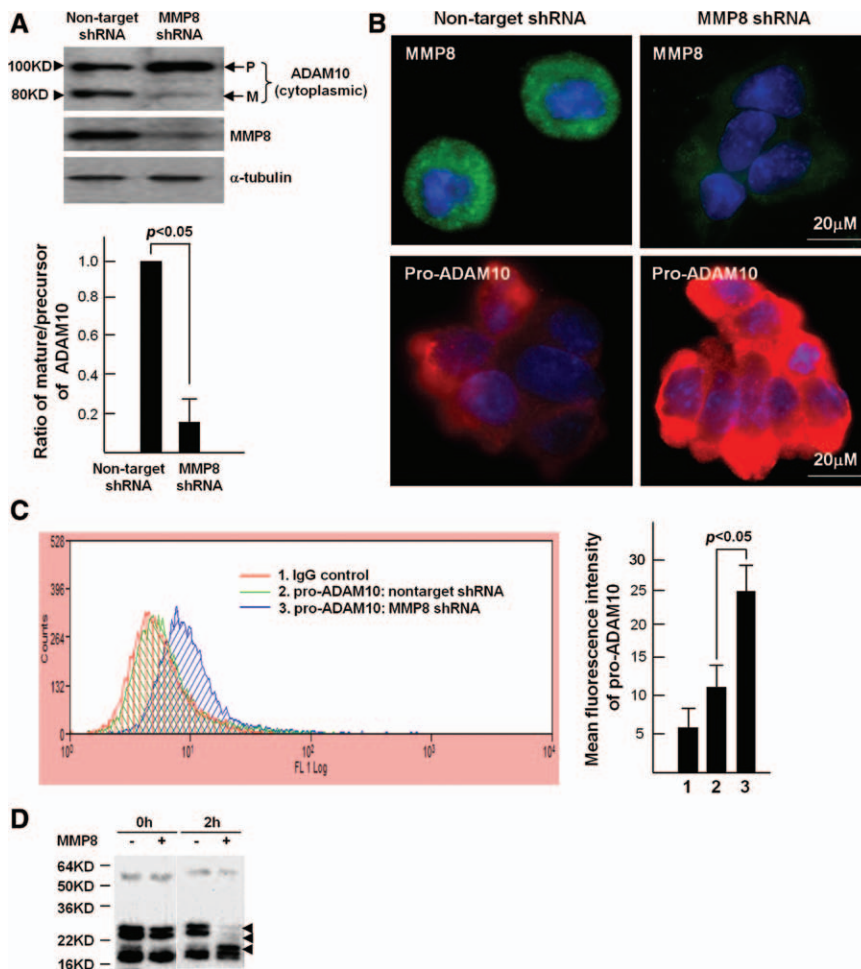## MMP8 Deficiency Reduces ADAM10 Maturation

In an ongoing study of vascular SMCs using proteomics techniques, we found a decrease of ADAM10 fragments in culture supernatants of vascular SMCs with MMP8 deficiency (unpublished data). Because ADAM10 had been shown to play a role in cell migration,[31] we examined whether MMP8 deficiency had an influence on ADAM10 on SPCs. Western blot analyses showed that the ratio of the amount of mature ADAM10 vs the amount of pro-ADAM10 in protein extracts from MMP8 shRNA–infected embryonic Sca-1^{+} SPCs was significantly lower than in protein extracts from nontarget shRNA–infected embryonic Sca-1^{+} SPCs (Figure 5A). Consistently, immunofluorescent staining and flow cytometry analyses using an antibody specifically against the ADAM10 prodomain showed that the levels of pro-ADAM10 on embryonic Sca-1^{+} SPCs were increased when MMP8 had been knocked-down by shRNA (Figure 5B and 5C).

To test whether MMP8 could cleave ADAM10, we incubated a recombinant mouse ADAM10 (amino acid residues 19–673, consisting of the prodomain and the other ectodomains) with active MMP8 and performed Western blot analysis using

an antibody against the prohormone convertase cleavage site (amino acid residues 157–221) of ADAM10. At the baseline, in the presence or absence of MMP8, the Western blot analysis detected a band for the uncleaved recombinant protein (60 kDa) and 3 strong bands corresponding to ≈28, ≈25, and ≈18 kDa fragments, respectively (Figure 5D; the ≈28, ≈25, and ≈18 kDa bands are indicated by arrowheads). After 2-hour incubation without MMP8, these 4 different bands of ADAM10 remained the same, whereas after 2-hour incubation with MMP8 the intensity of the 60-kDa band was reduced, the ≈28 and ≈25 kDa bands almost completely disappeared, and a new band of ≈20 kDa appeared, whereas the intensity of the ≈18 kDa band remained approximately the same (Figure 5D).

MMP8 Deficiency Reduces E-cadherin Shedding Likely by ADAM10

Our ongoing study of vascular SMCs using proteomics techniques also showed a reduction of E-cadherin fragments in culture supernatants of vascular SMCs with MMP8 deficiency (unpublished data). Because it had been reported that ADAM10 could shed E-cadherin on epithelial cells and thereby promote epithelial cell migration,[31] and because we had found a reduction in the amount of mature ADAM10 on MMP8-depleted SPCs, we examined whether MMP8 knockdown or ADAM10 inhibition could lead to a change in E-cadherin on SPCs. Western blot analyses showed that

Figure 5. Matrix metalloproteinase-8 (MMP8) depletion reduces a-disintegrin-and-metalloproteinase-domain-10 (ADAM10) maturation on stem/progenitor cells (SPCs). **A** to **C**, MMP8 knockdown reduces the amount of mature ADAM10 on SPCs. Cultured embryonic Sca-1^{+} SPCs were infected with nontarget shRNA or MMP8 shRNA and subjected to Western blot analysis with antibodies against ADAM10 (cytoplasmic domain), MMP8, or α-tubulin (**A**); immunofluorescence staining with antibodies against ADAM10 (prodomain) or MMP8 (**B**); and flow cytometric analysis with an antibody against ADAM10 (prodomain) (**C**). Representative images of Western blot, immunofluorescence staining, and flow cytometric analysis, respectively, are presented. Column chart in (**A**) shows quantitative data of the ratio of the amount of mature ADAM10 (M) vs the amount of pro-ADAM10 (P). Column chart in (**C**) shows mean fluorescence intensity for ADAM10 (prodomain) in Sca-1^{+} SPCs infected with nontarget shRNA or MMP8 shRNA (n=3 independent experiments). **D**, MMP8 cleaves ADAM10. Recombinant ADAM10 (amino acid residues 19–673, consisting of the prodomain and the other ectodomains, 500 ng; Sigma, A9975) was incubated with activated MMP8 (50 ng) in assay buffer for 0 and 2 hours, respectively, followed by Western blot analysis with an antibody against the prohormone convertase cleavage site (amino acid residues 157–221) of ADAM10 (Abcam, ab39153). **Arrowheads** indicate ADAM10 fragments affected by MMP8 incubation.

knockdown of MMP8 in embryonic Sca-1⁺ SPCs with MMP8 shRNA and incubation of Sca-1⁺ SPCs with the ADAM10-specific inhibitor GI254023X[27,28] increased the ratio of the amount of full-length E-cadherin vs the amount of E-cadherin

transmembrane cytoplasmic fragment in protein extracts from these cells (Figure 6A1 and 6B), suggesting a reduction in E-cadherin ectodomain shedding, probably by ADAM10, on MMP8-depleted SPCs. Consistently, flow cytometry analyses
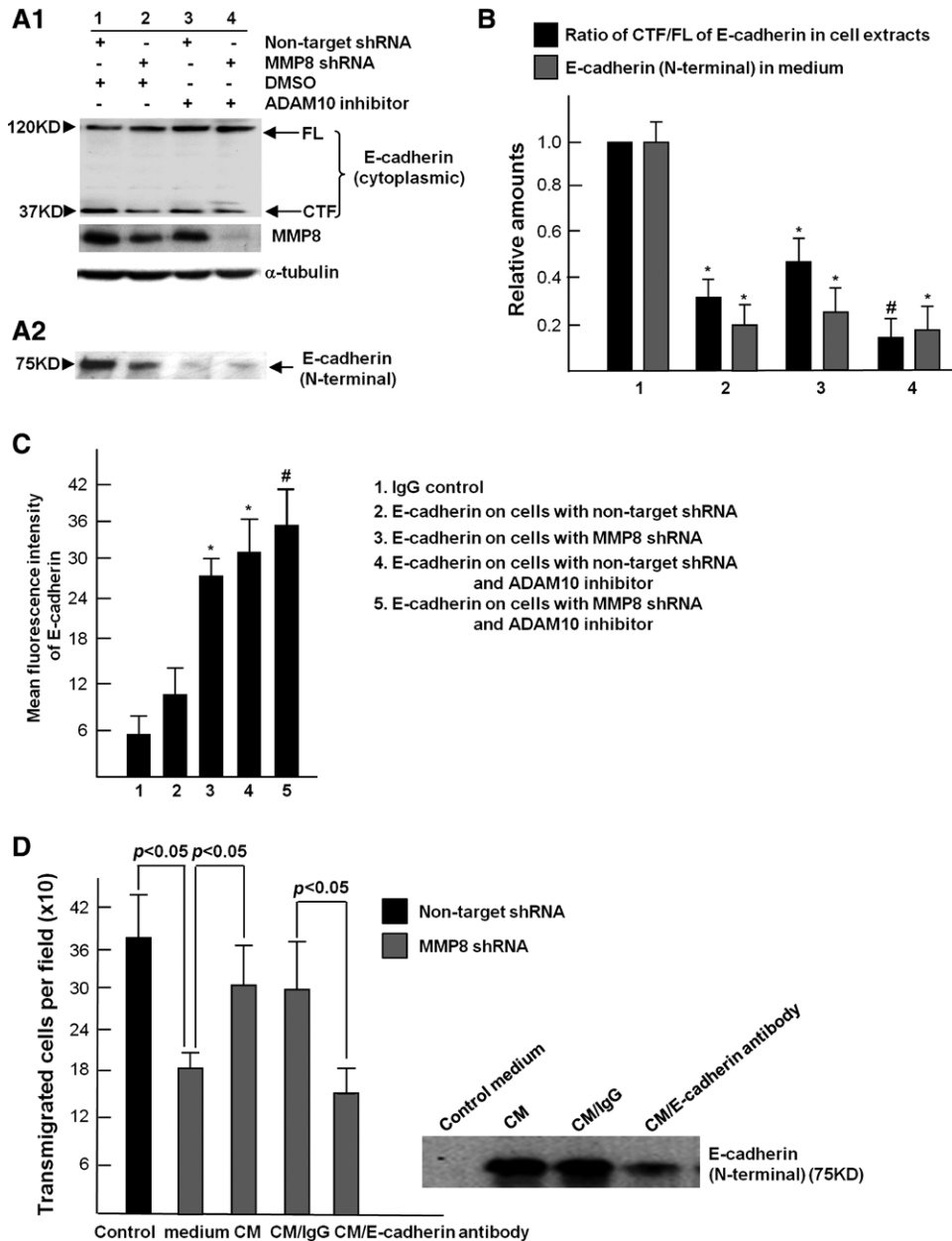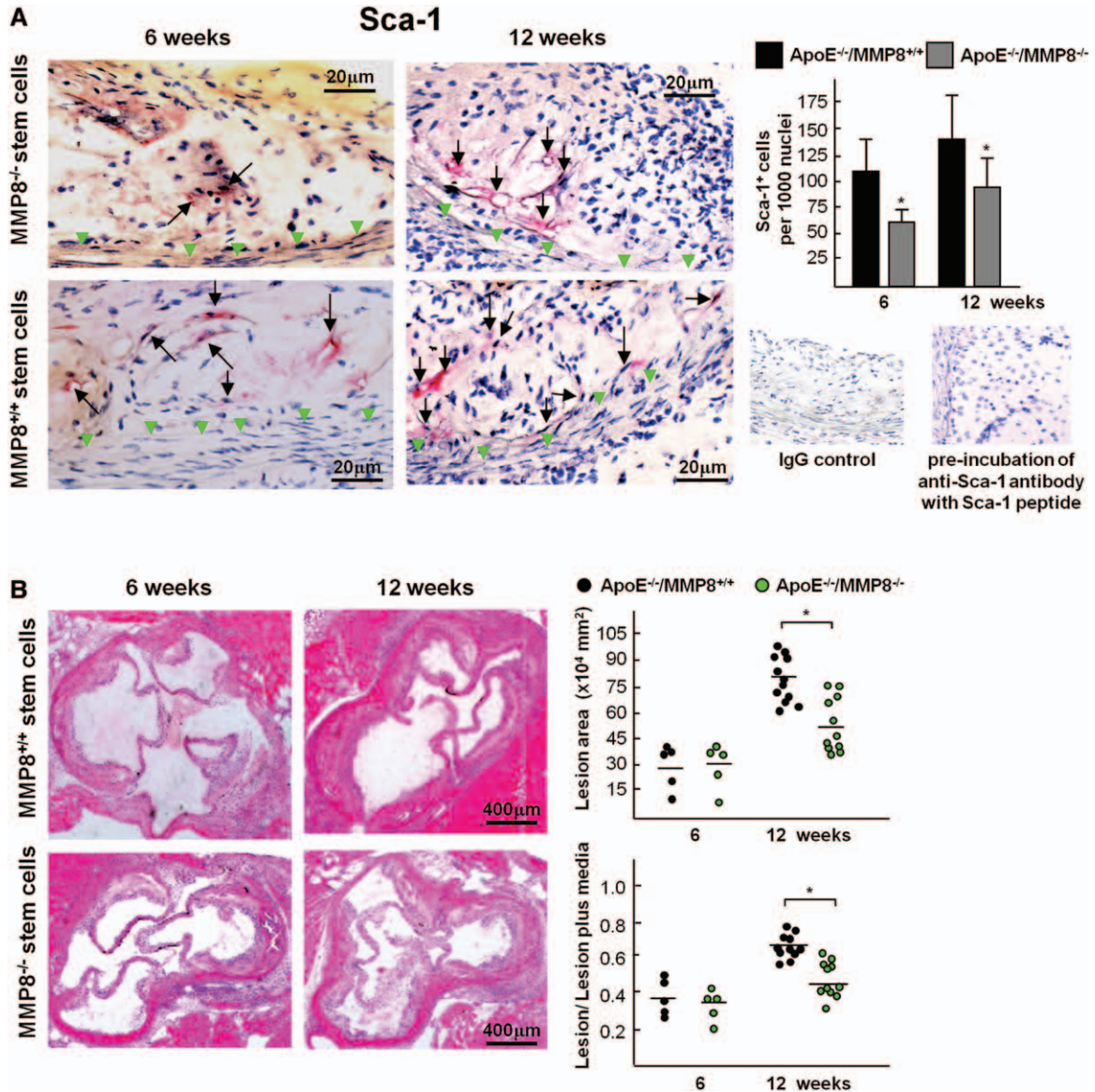

Figure 6. Matrix metalloproteinase-8 (MMP8) depletion or a-disintegrin-and-metalloproteinase-domain-10 (ADAM10) inhibition reduces E-cadherin shedding on stem/progenitor cells (SPCs) and decreases soluble E-cadherin–induced SPC migration. A to C, MMP8 knockdown or ADAM10 inhibition reduces E-cadherin shedding on SPCs. Cultured embryonic Sca-1⁺ SPCs were infected with nontarget shRNA or MMP8 shRNA, followed by 3 days of culture in the presence or absence of the ADAM10 inhibitor GI254023X (5 μmol/L). Cell lysates were harvested and subjected to Western blot analyses with antibodies against E-cadherin (cytoplasmic domain) or MMP8 (**A1**). Conditioned media were also harvested and subjected to Western blot analysis with an antibody against E-cadherin (*N*-terminal) (A2). **A1** and **A2**, Representative images of Western blot analyses. **B**, Western blot densitometric quantitative data (black columns show the ratios of the amount of C-terminal fragment [CTF] of E-cadherin vs the amount of full-length [FL] E-cadherin in cell lysates; gray columns show the relative amounts of *N*-terminal E-cadherin in conditioned media. n=3 independent experiments, *P<0.05 [treatment vs control], #P<0.05 [MMP8 shRNA plus GI254023X vs MMP8 shRNA or vs GI254023X]). **C**, Flow cytometric analyses of Sca-1⁺ SPCs that had been treated as in (**A**), with an antibody against E-cadherin (*N*-terminal). The column chart presentation of quantitative data of mean fluorescence intensity in the flow cytometric analyses (n=3 independent experiments, *P<0.05 [column 3 or 4 vs column 2], #P<0.05 [column 5 vs column 3]). **D**, Soluble E-cadherin induces SPC migration. Conditioned culture medium of Sca-1⁺ SPCs were concentrated 10×, incubated with rat IgG (CM/IgG) or an antibody against E-cadherin *N*-terminus (CM/DECAM-1) or neither (CM), and then transferred to cultures of MMP8 shRNA–infected Sca-1⁺ SPCs, followed by transcollagen I migration assays. Columns represent mean±SD (n=3 independent experiments). Also shown is a representative image of Western blot analysis of concentrated conditional medium, with the use of the E-cadherin *N*-terminus antibody.

Figure 7. Matrix metalloproteinase (MMP8) deficiency reduces stem/progenitor cell (SPC) recruitment and accumulation in atherosclerotic lesions in vivo. ApoE$^{-/-}$/MMP8$^{-/-}$ mice were injected with SPCs (1×10^6) isolated from the bone marrow of ApoE$^{-/-}$/MMP8$^{+/+}$ or ApoE$^{-/-}$/MMP8$^{-/-}$ mice and fed a Western diet for 6 or 12 weeks. Aortic roots from the recipient mice were harvested, sectioned, and subjected to immunohistochemical staining with an antibody against the stem cell marker Sca-1 (**A**) or hematoxylin and eosin staining (**B**). **A**, MMP8 deficiency reduces SPC accumulation in atherosclerotic lesions. Shown in the figure are representative images of immunohistochemical staining for Sca-1 and diagrammatic representation of quantitative data of Sca-1–positive cells in atherosclerotic lesions of ApoE$^{-/-}$/MMP8$^{-/-}$ mice injected with SPCs from ApoE$^{-/-}$/MMP8$^{+/+}$ and ApoE$^{-/-}$/MMP8$^{-/-}$ mice, respectively. **Black arrows** indicate Sca-1–positive cells in the lesions. **Green arrowheads** indicate the boundary between the lesion and healthy area. Also shown in the figure are representative staining images from IgG control or preincubation of Sca-1 antibody with a Sca-1 peptide. **B**, MMP8 deficiency results in smaller atherosclerotic lesions. Shown in the figure are representative images of hematoxylin and eosin staining and diagrammatic representation of quantitative data of the atherosclerotic lesions of aortic roots of ApoE$^{-/-}$/MMP8$^{-/-}$ mice injected with SPCs isolated from the bone marrow of ApoE$^{-/-}$/MMP8$^{+/+}$ or ApoE$^{-/-}$/MMP8$^{-/-}$ mice, respectively (black lines represent mean values in each group).

with an antibody specifically against E-cadherin ectodomain showed an increase in the amount of E-cadherin ectodomain on embryonic Sca-1$^+$ SPCs when infected with MMP8 shRNA or incubated with the ADAM10 inhibitor GI254023X (Figure 6C), whereas Western blot analysis showed a reduction in the amount of soluble E-cadherin in the culture supernatants (Figure 6A2).

Previous studies indicated that soluble E-cadherin could disrupt cell–cell adhesion and induce epithelial and cancer cell migration/invasion into collagen I gel.[32,33] Because we had

observed that MMP8 knockdown led to a reduction in soluble E-cadherin generation from Sca-1$^+$ SPCs, we investigated whether this could have an effect on SPC migration. We found that the decrease in migratory ability of embryonic Sca-1$^+$ SPCs infected with MMP8 shRNA was significantly reduced in the presence of supernatant from cultures of embryonic Sca-1$^+$ SPCs infected with nontarget shRNA (Figure 6D). Furthermore, we found that this rescuing effect of supernatant from cultures of Sca-1$^+$ SPCs infected with nontarget shRNA was abolished if an antibody against soluble E-cadherin had

been added to the supernatant to immunoprecipitate soluble E-cadherin (Figure 6D), suggesting that E-cadherin shedding, probably mediated by ADAM10, contributes to the effect of MMP8 on SPC migration.

To investigate whether MMP8 deficiency could have an effect on the level of soluble E-cadherin in vivo, we measured the plasma levels of soluble E-cadherin in mice with or without MMP8 knockout fed a Western diet for 12 weeks. We found that the plasma level of soluble E-cadherin was significantly lower in MMP8 knockout mice (226 ± 261 vs 1850 ± 2753 ng/mL; $P<0.01$; n=5 per each group).

MMP8 Deficiency Reduces SPC Recruitment and Accumulation in Atherosclerotic Lesions In Vivo

Having found that MMP8 played an important role in SPC migration and recruitment in ex vivo and in vitro experiments, we further examined the in vivo effects of MMP8 on SPC migration and recruitment into atherosclerotic lesions and on lesion formation and progression. To this end, we injected ApoE$^{-/-}$/MMP8$^{-/-}$ mice with SPCs isolated from the bone marrow of either green fluorescence protein (GFP) transgenic or ApoE$^{-/-}$/MMP8$^{-/-}$ or ApoE$^{-/-}$/MMP8$^{+/+}$ mice and fed the recipient mice a Western diet. In these experiments, MMP8-deficienct mice were chosen as recipients to exclude the effects of other cellular source of MMP8 on SPC migration and recruitment into atherosclerotic lesions. MagCellect Mouse Hematopoietic Cell Lineage Depletion Kit (MAGM209, R&D System), rather than single stem cell marker–positive selection, was used to isolate bone marrow SPCs for injection, because SPCs in atherosclerotic lesions are known to be heterogeneous and because no single stem cell marker can capture the entire SPC population. The suitability of using this method was confirmed by the finding that ≈90% of cells isolated using this kit were c-kit and CD34 single- or double-positive (Online Figure VI), and ≈27% of the c-kit/CD34 double-negative cells also were positive for another stem cell marker, Sca-1 (Online Figure VI).

In ApoE$^{-/-}$/MMP8$^{-/-}$ mice injected with SPCs isolated from the bone marrow of GFP transgenic mice and fed a Western diet for 12 weeks, we verified that injected SPCs (containing GFP as a marker) were recruited into and accumulated in atherosclerotic lesions (Online Figure VIIB), and we found that they were present also in the bone marrow, peripheral blood, thymus, spleen, liver, and lung (Online Figure VIA) but undetectable in the brain, kidneys, and skeletal muscles (data not shown). We found that the majority of GFP-positive cells in atherosclerotic lesions expressed the hematopoietic cell marker CD45 (35%) or the macrophage marker CD68 (29%; Online Figure VIIB), and some expressed the earlier SMC marker smooth muscle actin (5%; Online Figure VIIB) or the endothelial cell marker CD31 (<1%, data not shown), but none of them expressed smooth muscle myosin heavy chain, a marker for mature SMCs (Online Figure VIIB), indicating that the injected SPCs recruited into atherosclerotic lesions differentiate mainly into leukocytes/macrophages.

In ApoE$^{-/-}$/MMP8$^{-/-}$ mice injected with SPCs isolated from the bone marrow of either ApoE$^{-/-}$/MMP8$^{-/-}$ or ApoE$^{-/-}$/MMP8$^{+/+}$ mice and fed a Western diet for 6 or 12 weeks, we found that compared with the atherosclerotic lesions in

those mice injected with SPCs isolated from ApoE$^{-/-}$/MMP8$^{+/+}$ mice, the atherosclerotic lesions in those mice injected with MMP8-deficient SPCs (ie, isolated from ApoE$^{-/-}$/MMP8$^{-/-}$ mice) contained significantly fewer cells that were positive for Sca-1 (Figure 7A), c-kit (Online Figure VIIIA), CD45 (Online Figure VIIIB), or CD68 (Online Figure VIIIC), and had a trend toward fewer smooth muscle actin–positive cells (Online Supplement Figure VIIID), supporting that MMP8 plays an important role in SPC recruitment into and accumulation in atherosclerotic lesions in vivo. Furthermore, we found that at 12 weeks postinjection, the atherosclerotic lesions in the aortic roots of the mice injected with MMP8-deficient SPCs were significantly smaller than the atherosclerotic lesions in those mice injected with SPCs from ApoE$^{-/-}$/MMP8$^{+/+}$ mice (Figure 7B), further supporting an important role of MMP8 in SPCs in atherosclerotic lesion formation.

Discussion

Monocytes/macrophages and SMCs represent 2 major cell types in atherosclerotic lesions and play central roles in lesion formation and progression.[1] There is substantial evidence indicating that a proportion of SMCs in atherosclerotic lesions are derived from smooth muscle progenitor cells originally residing in the arterial adventitia.[2–5] There is also emerging evidence suggesting that a proportion of macrophages in atherosclerotic lesions are derived from hematopoietic SPCs, which might originate from the bone marrow and are recruited via the blood circulation into atherosclerotic lesions.[6–10] In agreement, our study shows the presence of SPCs in atherosclerotic lesions in ApoE-deficient mice fed a Western diet can induce atherosclerosis. Because SPCs are heterogeneous and no single marker can be used to capture the various types,[34,35] we used a panel of well-documented markers (CD34, Sca-1, c-kit, and Flk-1) to detect SPCs in atherosclerotic lesions in this study.

More importantly, results of our study indicate that MMP8 plays an important role in SPC migration and their recruitment into atherosclerotic lesions. We first found that compared with atherosclerotic lesions in ApoE$^{-/-}$/MMP8$^{+/+}$ mice, those in ApoE$^{-/-}$/MMP8$^{-/-}$ mice contained fewer SPCs (CD34, Sca-1, c-kit, and Flk-1–positive cells). To specifically test the importance of MMP8 in SPCs for the SPC recruitment into atherosclerotic lesions and for lesion formation, we injected ApoE$^{-/-}$/MMP8$^{-/-}$ mice with SPCs from bone marrows of GFP transgenic or ApoE$^{-/-}$/MMP8$^{-/-}$ or ApoE$^{-/-}$/MMP8$^{+/+}$ mice and fed the injected mice a Western diet. The experiment with SPCs isolated from GFP transgenic cells showed that at 12 weeks postinjection, bone marrows of the recipient mice contained GFP-positive SPCs, suggesting that the injected SPCs had been incorporated into the bone marrow. It also showed that atherosclerotic lesions in the recipient mice contained GFP-positive SPCs, most of which expressed markers of hematopoietic cells and macrophages, indicating that SPCs had been recruited into atherosclerotic lesions and differentiated into leukocytes/macrophages. The experiment with injection of SPCs from ApoE$^{-/-}$/MMP8$^{-/-}$ or ApoE$^{-/-}$/MMP8$^{+/+}$ mice showed that atherosclerotic lesions in recipient mice injected with SPCs from ApoE$^{-/-}$/MMP8$^{-/-}$ mice contained fewer SPCs and were smaller than atherosclerotic lesions in mice injected with SPCs from ApoE$^{-/-}$/MMP8$^{+/+}$ mice, corroborating

that MMP8 from SPCs is important for SPC recruitment to atherosclerotic lesions and for lesion development. Further to these findings, our ex vivo experiments on aorta explants demonstrated that the presence of MMP8 enhanced SPC migration from the arterial lumen and the adventitia into atherosclerotic lesions, whereas in vitro experiments showed that the presence of MMP8 increased the ability of SPCs to migrate across the endothelium or through extracellular matrix. Together, these in vivo, ex vivo, and in vitro findings indicate that MMP8 in SPCs plays an important role in SPC migration, which facilitates their recruitment into atherosclerotic lesions and increases lesion development.

Cell migration involves dissociation of the migrating cells from the extracellular matrix and degradation of extracellular matrix proteins in the migratory path. Our study shows that the presence of MMP8 inhibits SPC adhesion to extracellular matrix and enhances SPC migration through extracellular matrix proteins, particularly collagen I, the principal substrate of MMP8[19] and the major constituent of the interstitial matrix of the arterial wall.[36] This provides a likely mechanistic explanation for the role of MMP8 in SPC migration and their recruitment into atherosclerotic lesions.

SPC migration also entails disruption of their interactions with each other and with stromal cells, allowing them to be released from stem cell niches in, for example, the arterial adventitia.[2–5] The cell adhesion molecule E-cadherin, which is highly expressed in SPCs as shown in previous studies[37,38] and in our present study, mediates cell–cell adhesion by the formation of homodimers of the ectodomain of E-cadherin on adjacent cells.[31,39] Soluble E-cadherin generated by E-cadherin ectodomain shedding from the cell surface, however, can disrupt cell–cell adhesion and induce cell migration.[32,33] Results of our study indicate that MMP8 can promote SPC migration by indirectly (probably via ADAM10) or directly increasing E-cadherin ectodomain shedding and soluble E-cadherin generation, providing another likely mechanistic explanation for the role of MMP8 in SPC migration and their recruitment into atherosclerotic lesions. A previous study shows that ADAM10 can shed E-cadherin on epithelial cells, thereby reducing E-cadherin–mediated cell–cell adhesion and promoting epithelial cell migration.[31] In this study, we found that MMP8 depletion reduced the amount of mature ADAM10 on SPCs and that knockdown of MMP8 and the presence of the ADAM10-specific inhibitor GI254023X[27,28] resulted in reduced E-cadherin shedding on SPCs. In addition, we found that soluble E-cadherin levels in the plasma were decreased in MMP8 knockout mice. Furthermore, results of in vitro experiments indicate that soluble E-cadherin in SPC culture supernatant increased migration of SPCs with MMP8 knockdown. Therefore, it seems that future work to generate MMP8/ADAM10 and MMP8/E-cadherin double-knockout mice and to further characterize these effects would be warranted.

Some ADAMs, including ADAM10, when activated, possess proteolytic activity.[40,41] Their activation often involves the removal of their prodomain.[40,41] Although prodomain removal often occurs intracellularly, there is evidence indicating that the cell surface also contains pro-ADAMs (containing the prodomain) whose maturation (prodomain removal) can potentially take place extracellularly.[41–44] The results of the Western blot, immunocytochemical, and flow cytometry analyses of our study suggest the presence of pro-ADAM10 on Sca-1^{+} SPC surface and that the presence of MMP8 reduces the amount of pro-ADAM10 and increases the amount of mature ADAM10 on these cells. Our in vitro assays showed that incubation of a recombinant mouse ADAM10 (amino acid residues 19–673, consisting of the prodomain and the other ectodomains) with active MMP8 resulted in the generation of cleaved ADAM10 products detectable by an antibody against the ADAM10 prohormone convertase cleavage site (amino acid residues 157–221) in Western blot analysis. Although the exact cleavage sites are unknown and further studies are warranted, our data suggest that MMP8 can cleave ADAM10, which could potentially explain the finding of our study that the presence of MMP8 reduces pro-ADAM10 and increases mature ADAM10 on Sca-1^{+} SPCs.

Our recent study showed that MMP8 deficiency reduces atherosclerotic lesion formation in ApoE knockout mice fed a Western diet, with reduced lesional macrophage and SMC content,[26] indicating a causal role of MMP8 in the pathogenesis of atherosclerosis. This previous study also revealed evidence indicating that the role of MMP8 in atherogenesis is partly attributable to an MMP8-mediated increase in angiotensin II generation, leading to increased blood pressure and increased expression of vascular cell adhesion molecule-1 that plays an important role in leukocyte recruitment.[1] However, given the complexity of atherogenesis, and because MMP8 can potentially cleave multiple types of proteins with different functions, it is plausible that multiple mechanisms contribute to the role of MMP8 in atherogenesis. The finding of this study that MMP8 enhances SPC recruitment into atherosclerotic lesions indicates that this likely represents another mechanism for the role of MMP8 in atherogenesis. It is possible that reduced SPC recruitment into atherosclerotic lesions in MMP8-deficient mice is partly because of the reduced inflammatory surrounding and, therefore, attenuated chemotaxis of SPCs into the vascular wall. However, results of the in vivo experiments comparing MMP8-deficient mice injected with either MMP8-deficient SPCs or MMP8 wild-type SPCs indicate that MMP8 expression by SPCs themselves also plays an important role in their recruitment into atherosclerotic lesions.

In summary, our study shows that MMP8 plays a functional role in SPC migration and their recruitment into atherosclerotic lesions, and that MMP8 expression by SPCs contributes to atherosclerotic lesion formation in ApoE-deficient mice. The data from this study provide new insight into the biological molecules and related mechanisms involved in the regulation of SPC migration and in the pathogenesis of atherosclerosis.

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Disclosures

None.

References

1. Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868–874.
2. Xu Q. The impact of progenitor cells in atherosclerosis. *Nat Clin Pract Cardiovasc Med*. 2006;3:94–101.
3. Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, Xu Q. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. *J Clin Invest*. 2004;113:1258–1265.
4. Hu Y, Davison F, Ludewig B, Erdel M, Mayr M, Url M, Dietrich H, Xu Q. Smooth muscle cells in transplant atherosclerotic lesions are originated from recipients, but not bone marrow progenitor cells. *Circulation*. 2002;106:1834–1839.
5. Hu Y, Mayr M, Metzler B, Erdel M, Davison F, Xu Q. Both donor and recipient origins of smooth muscle cells in vein graft atherosclerotic lesions. *Circ Res*. 2002;91:e13–e20.
6. Zulli A, Buxton BF, Black MJ, Hare DL. CD34 Class III positive cells are present in atherosclerotic plaques of the rabbit model of atherosclerosis. *Histochem Cell Biol*. 2005;124:517–522.
7. Zulli A, Buxton BF, Black MJ, Hare DL. Embryonic stem cells markers are present within rabbit atherosclerotic plaques. *Histol Histopathol*. 2008;23:741–746.
8. Zulli A, Rai S, Buxton BF, Burrell LM, Hare DL. Co-localization of angiotensin-converting enzyme 2-, octomer-4- and CD34-positive cells in rabbit atherosclerotic plaques. *Exp Physiol*. 2008;93:564–569.
9. Febbraio M, Guy E, Silverstein RL. Stem cell transplantation reveals that absence of macrophage CD36 is protective against atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2004;24:2333–2338.
10. Zengin E, Chalajour F, Gehling UM, Ito WD, Treede H, Lauke H, Weil J, Reichenspurner H, Kilic N, Ergün S. Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development*. 2006;133:1543–1551.
11. Torsney E, Hu Y, Xu Q. Adventitial progenitor cells contribute to arteriosclerosis. *Trends Cardiovasc Med*. 2005;15:64–68.
12. Torsney E, Mandal K, Halliday A, Jahangiri M, Xu Q. Characterisation of progenitor cells in human atherosclerotic vessels. *Atherosclerosis*. 2007;191:259–264.
13. Xiao Q, Ye S, Oberhollenzer F, Mayr A, Jahangiri M, Willeit J, Kiechl S, Xu Q. SDF1 gene variation is associated with circulating SDF1alpha level and endothelial progenitor cell number: the Bruneck Study. *PLoS ONE*. 2008;3:e4061.
14. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol*. 2007;8:221–233.
15. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med*. 2003;9:1370–1376.
16. Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MA, Werb Z, Rafii S. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell*. 2002;109:625–637.
17. Iwakura A, Shastry S, Luedemann C, Hamada H, Kawamoto A, Kishore R, Zhu Y, Qin G, Silver M, Thorne T, Eaton L, Masuda H, Asahara T, Losordo DW. Estradiol enhances recovery after myocardial infarction by augmenting incorporation of bone marrow-derived endothelial progenitor cells into sites of ischemia-induced neovascularization via endothelial nitric oxide synthase-mediated activation of matrix metalloproteinase-9. *Circulation*. 2006;113:1605–1614.
18. Vagima Y, Avigdor A, Goichberg P, et al. MT1-MMP and RECK are involved in human CD34+ progenitor cell retention, egress, and mobilization. *J Clin Invest*. 2009;119:492–503.
19. Van Lint P, Libert C. Matrix metalloproteinase-8: cleavage can be decisive. *Cytokine Growth Factor Rev*. 2006;17:217–223.
20. Van Den Steen PE, Wuyts A, Husson SJ, Proost P, Van Damme J, Opdenakker G. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *Eur J Biochem*. 2003;270:3739–3749.
21. Cox JH, Dean RA, Roberts CR, Overall CM. Matrix metalloproteinase processing of CXCL11/I-TAC results in loss of chemoattractant activity and altered glycosaminoglycan binding. *J Biol Chem*. 2008;283:19389–19399.
22. Owen CA, Hu Z, Lopez-Otin C, Shapiro SD. Membrane-bound matrix metalloproteinase-8 on activated polymorphonuclear cells is a potent, tissue inhibitor of metalloproteinase-resistant collagenase and serpinase. *J Immunol*. 2004;172:7791–7803.
23. Herman MP, Sukhova GK, Libby P, Gerdes N, Tang N, Horton DB, Kilbride M, Breitbart RE, Chun M, Schönbeck U. Expression of neutrophil collagenase (matrix metalloproteinase-8) in human atheroma: a novel collagenolytic pathway suggested by transcriptional profiling. *Circulation*. 2001;104:1899–1904.
24. Turu MM, Krupinski J, Catena E, Rosell A, Montaner J, Rubio F, Alvarez-Sabin J, Cairols M, Badimon L. Intraplaque MMP-8 levels are increased in asymptomatic patients with carotid plaque progression on ultrasound. *Atherosclerosis*. 2006;187:161–169.
25. Tuomainen AM, Nyyssönen K, Laukkanen JA, Tervahartiala T, Tuomainen TP, Salonen JT, Sorsa T, Pussinen PJ. Serum matrix metalloproteinase-8 concentrations are associated with cardiovascular outcome in men. *Arterioscler Thromb Vasc Biol*. 2007;27:2722–2728.
26. Laxton RC, Hu Y, Duchene J, et al. A role of matrix metalloproteinase-8 in atherosclerosis. *Circ Res*. 2009;105:921–929.
27. Hundhausen C, Misztela D, Berkhout TA, Broadway N, Saftig P, Reiss K, Hartmann D, Fahrenholz F, Postina R, Matthews V, Kallen KJ, Rose-John S, Ludwig A. The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion. *Blood*. 2003;102:1186–1195.
28. Hoettecke N, Ludwig A, Foro S, Schmidt B. Improved synthesis of ADAM10 inhibitor GI254023X. *Neurodegener Dis*. 2010;7:232–238.
29. Xiao Q, Zeng L, Zhang Z, Margariti A, Ali ZA, Channon KM, Xu Q, Hu Y. Sca-1+ progenitors derived from embryonic stem cells differentiate into endothelial cells capable of vascular repair after arterial injury. *Arterioscler Thromb Vasc Biol*. 2006;26:2244–2251.
30. Xiao Q, Zeng L, Zhang Z, Hu Y, Xu Q. Stem cell-derived Sca-1+ progenitors differentiate into smooth muscle cells, which is mediated by collagen IV-integrin alpha1/beta1/alphav and PDGF receptor pathways. *Am J Physiol, Cell Physiol*. 2007;292:C342–C352.
31. Maretzky T, Reiss K, Ludwig A, Buchholz J, Scholz F, Proksch E, de Strooper B, Hartmann D, Saftig P. ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. *Proc Natl Acad Sci USA*. 2005;102:9182–9187.
32. Wheelock MJ, Buck CA, Bechtol KB, Damsky CH. Soluble 80-kd fragment of cell-CAM 120/80 disrupts cell-cell adhesion. *J Cell Biochem*. 1987;34:187–202.
33. Ryniers F, Stove C, Goethals M, Brackenier L, Noë V, Bracke M, Vandekerckhove J, Mareel M, Bruyneel E. Plasmin produces an E-cadherin fragment that stimulates cancer cell invasion. *Biol Chem*. 2002;383:159–165.
34. Yoder MC, Ingram DA. Endothelial progenitor cell: ongoing controversy for defining these cells and their role in neoangiogenesis in the murine system. *Curr Opin Hematol*. 2009;16:269–273.
35. Prater DN, Case J, Ingram DA, Yoder MC. Working hypothesis to redefine endothelial progenitor cells. *Leukemia*. 2007;21:1141–1149.
36. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation*. 1995;92:1355–1374.
37. Ueberham E, Aigner T, Ueberham U, Gebhardt R. E-cadherin as a reliable cell surface marker for the identification of liver specific stem cells. *J Mol Histol*. 2007;38:359–368.
38. Soncin F, Mohamet L, Eckardt D, Ritson S, Eastham AM, Bobola N, Russell A, Davies S, Kemler R, Merry CL, Ward CM. Abrogation of E-cadherin-mediated cell-cell contact in mouse embryonic stem cells results in reversible LIF-independent self-renewal. *Stem Cells*. 2009;27:2069–2080.
39. Turel KR, Rao SG. Expression of the cell adhesion molecule E-cadherin by the human bone marrow stromal cells and its probable role in CD34(+) stem cell adhesion. *Cell Biol Int*. 1998;22:641–648.

40. Seals DF, Courtneidge SA. The ADAMs family of metalloproteases: multidomain proteins with multiple functions. *Genes Dev*. 2003;17:7–30.
41. Edwards DR, Handsley MM, Pennington CJ. The ADAM metalloproteinases. *Mol Aspects Med*. 2008;29:258–289.
42. Lum L, Reid MS, Blobel CP. Intracellular maturation of the mouse metalloprotease disintegrin MDC15. *J Biol Chem*. 1998;273:26236–26247.
43. Mayer G, Hamelin J, Asselin MC, Pasquato A, Marcinkiewicz E, Tang M, Tabibzadeh S, Seidah NG. The regulated cell surface zymogen activation of the proprotein convertase PC5A directs the processing of its secretory substrates. *J Biol Chem*. 2008;283:2373–2384.
44. Charrier L, Yan Y, Driss A, Laboisse CL, Sitaraman SV, Merlin D. ADAM-15 inhibits wound healing in human intestinal epithelial cell monolayers. *Am J Physiol Gastrointest Liver Physiol*. 2005;288:G346–G353.

Novelty and Significance

What Is Known?

- Atherosclerotic lesions contain cells derived from stem/progenitor cells (SPCs).
- Matrix metalloproteinase-8 (MMP8) knockout reduces atherosclerosis in a mouse model.

What New Information Does This Article Contribute?

- MMP8 plays an important role in SPC migration and recruitment into atherosclerotic lesions.
- This contributes to atherosclerotic lesion progression.

Although previous research indicates that atherosclerotic lesions contain SPCs, and that these cells contribute to lesion formation and progression, the mechanisms by which they are recruited have remained incompletely understood. Because SPC migration requires degradation of proteins on the cell surface and the extracellular matrix, we investigated whether the protease MMP8 plays a role in SPC migration and their recruitment into atheromas. In a well-established mouse model, we observed that deletion of MMP8 reduced SPC levels in atherosclerotic lesions. We found that MMP8-null mice had development of larger atherosclerotic lesions if they had been injected with SPCs that expressed MMP8 compared with MMP8 knockout mice injected with MMP8-deficient SPCs. Furthermore, we found that MMP8-deficient SPCs had reduced ability to migrate across the endothelium, or through extracellular matrix (Matrigel or collagen I), or into atherosclerotic lesions from the arterial lumen or the adventitia. Our studies also indicate that the enhancing effect of MMP8 on SPC migration is partly because of its ability to degrade collagen I and to cleave a-disintegrin-and-metalloproteinase-domain-10, which in turn cleaves the cell surface protein E-cadherin. These novel findings contribute to our understanding of atherogenesis and may facilitate further translational stem cell research in this area.

A functional role of matrix metalloproteinase-8 in stem/progenitor cell migration and their recruitment into atherosclerotic lesions
(Xiao, et al. MMP8 and stem/progenitor cell migration)

Supplemental Data

Materials

Antibodies against MMP8 (ab53017), GAPDH (ab8245), ADAM10 (prodomain, ab39178; N-terminal, ab39153; and cytoplasmic domain, ab39177), E-cadherin (DECAM-1) (N-terminal, ab11512), MMP14 (ab51074) and CD34 (ab8158) were purchased from Abcam (Cambridge, UK). Antibody against E-cadherin (cytoplasmic domain, 24E10, 3195) was from Cell signaling Technology. Antibodies against α-tubulin (mouse) and smooth muscle α-actin (SMA) (Clone 1A4, A5228) were from Sigma. Antibodies against Sca-1 (Clone E13-161.7, 122516 and 122502) were from Biolegend, CD45 antibody (550539) was from BD Pharmingen, and CD68 antibody (DS-MB-03863) was from Raybiotech, Inc. All secondary antibodies were from Dako (Denmark). All other materials used in this study were purchased from Sigma except those indicated otherwise.

Animals and stem/progenitor cell injection experiments

ApoE$^{-/-}$/MMP8$^{-/-}$ double knockout mice (C57BL/6 background) and ApoE$^{-/-}$/MMP8$^{+/+}$ controls (littermates of ApoE$^{-/-}$/MMP8$^{-/-}$ double knockout mice) were generated in our previous study[1]. Six week old males of ApoE$^{-/-}$/MMP8$^{-/-}$ double knockout mice and controls were fed a Western diet (Research Diet, D12108, containing 21% fat, 1.25% cholesterol and 0% cholate) for 12 weeks. In stem/progenitor cell (SPC) injection experiments, eight week old males of ApoE$^{-/-}$/MMP8$^{-/-}$ double knockout mice were injected with 1x10^6 stem/progenitor cells (SPCs) isolated from the bone marrow of ApoE$^{-/-}$/MMP8$^{-/-}$ double knockout mice and ApoE$^{-/-}$/MMP8$^{+/+}$ controls through the tail vein and fed the Western diet for 6 or 12 weeks. In some experiments, SPCs were isolated from the bone marrow of GFP transgenic mice and injected into ApoE$^{-/-}$/MMP8$^{-/-}$ double knockout mice. All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals.

Characterization of atherosclerotic lesions

The extent of atherosclerotic lesions of aortic roots in ApoE$^{-/-}$/MMP8-/- double knockout mice received MMP8$^{-/-}$ or control SPCs was analyzed by hematoxylin/eosin staining as previously described[2]. The stained aortas were photographed using a digital camera, and the area of intima and media in a given image was highlighted and quantified (μm^2) by computer-assisted quantification using the Axiovision software.

Sca-1$^+$ stem/progenitor cell culture and shRNA mediated stable MMP8 gene silencing

Stem cell antigen 1-positive (Sca-1$^+$) SPCs isolated from differentiating embryonic stem cells were obtained from our previous studies[3, 4] and maintained/cultured in our laboratory as previously described[3, 4]. Lentiviral particles were produced using MISSION shRNA MMP8 plasmids DNA (NM_008611, Sigma) according to protocol provided. The shRNA Non-Targeting control vector (SHC002) was used as a negative control. Briefly, 293T cells were transfected with the lentiviral vector and the packaging plasmids, pCMV-dR8.2 and pCMV-VSV-G (both obtained from Addgene) using Fugene 6. The supernatant containing the lentivirus was harvested 48h later, filtered, aliquoted and stored at −80°C. p24 antigen ELISA (Zeptometrix) was used to determine the viral titre. The Transducing Unit (TU) was

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calculated using the conversion factor recommended by the manufacturer (10^4 physical particles per pg of p24 and 1 transducing unit per 10^3 physical particles for a VSV-G pseudotyped lentiviral vector), with 1pg of p24 antigen converted to 10 Transducing Units (TU). For lentiviral infection, mouse Sca-1⁺ SPCs were plated 24 hours prior to infection in 6 well-plates at 37°C. One transducing Unit per cell (or 2-3x10⁶/well) of shRNA or control virus were added with 10μg/ml hexadimethrine bromide (H9268; Sigma). Viral constructs were incubated 24 hours with the cells before the media was replaced with complete media containing 2μg/ml puromycin (P9620, Sigma). For selection of transductants, fresh media containing puromycin was added at 2-3 day intervals for 10 days. Stably infected cells were split and frozen for future experiments.

Isolation of Sca-1⁺ stem/progenitor cells from MMP8 knockout and wildtype mice
Sca-1⁺ SPCs were isolated from bone marrow of MMP8⁻/⁻/apoE⁻/⁻ and MMP8⁺/⁺/apoE⁻/⁻ mice by fluorescence cell sorter assay. Briefly, bone marrow was isolated from the tibias and femurs of mice 6-12 weeks of age. The bone marrow was triturated using an 18 gauge needle and passed through a 70 μm nylon mesh cell strainer (Becton Dickinson, Franklin Lakes, NJ) to make a single cell suspension in DMEM supplemented with 2%FCS/10mM Hepes. Red blood cells in bone marrow cells were lysed with a lysis buffer (8.3g NH_4CL, 1.0g $KHCO_3$ and 1.8ml of 5%EDTA in 1000ml). Bone marrow mononuclear cells were washed three times and counted with a hemacytometer and then resuspended in DMEM with 5%BSA at 1x10⁶ cells/100μl and stained with 1μg of rat IgG control-FITC or rat anti-mouse Sca-1 antibody-FITC, followed by fluorescence cell sorting assay with standard protocol. Sca-1-positive cells (>90%) were recovered in DMEM with 5% BSA and kept on ice prior to migration/invasion assays. In SPC injection experiments, MagCellect™ Mouse Hematopoietic Cell Lineage Depletion Kit (MAGM209, R&D System) was used to isolate bone marrow SPCs according to the manufacturer's instructions.

Immunoblotting
Cultured cells were harvested and lysed in a lysis buffer (50mM Tris-Cl pH 7.5, 150mM NaCl, 1 mM EDTA pH 8.0) supplemented with protease inhibitors and 0.5% Triton by sonication. Forty micrograms of protein was separated by SDS-PAGE with 4%~20% Tris-Glycine gel (Invitrogen, Carlsbad, CA, USA) and subjected to standard Western blot analysis.

Real-time RT-PCR
Real-time RT-PCR was performed as described before[5]. Briefly, total RNA was extracted from cells using RNeasy kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using an Improm-II™ RT kit (Promega, Madison, WI, USA) with RNase inhibitor (Promega) and Random primers (Promega). Simultaneous RT reactions were performed without the addition of reverse transcriptase to control the possible transcription of contaminating genomic DNA. Relative mRNA expression level was defined as the ratio of target gene expression level to 18S expression level with that of the control sample set as 1.0.

Indirect immunofluorescent staining of cells and frozen tissue sections
Indirect immunofluorescent assay was performed as described before[3, 5]. Briefly, cells or tissue sections were labeled with appropriate isotype IgG control or antibodies as indicated in the figures, and visualized with appropriate secondary antibodies conjugated with phycoerythrin (PE) or fluorescein isothiocyanate (FITC) (DAKO). Cells or sections were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma). Images were examined

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and acquired with SP5 confocal microscope with Leica TCS Sp5 software (Leica, Germany) at room temperature.

Immunohistochemical analysis
Microscopic sections of the heart with the aortic root harvested from mice injected with SPCs from GFP transgenic mice were subjected to double immunostaining for GFP together with either CD45, CD68, CD31 or vascular smooth muscle cell markers smooth muscle α-actin (SMA) or smooth muscle myosin heavy chain (SM-MHC). In brief, the sections were air dried and fixed in cold acetone at 4°C, then incubated at 75°C for 30 minutes to eliminate any potential endogenous alkaline phosphatise activity, followed by incubation with an avidin and biotin blocking solution (Avidin Biotin Blocking systems, VectorLab), and then a peroxidise blocking solution (3% H2O2), and then 10% goat serum (Dako). Thereafter, the sections were incubated with a primary antibody was either a rat anti-CD45 (Biolegend), rat anti-CD31 (Biolegend), rabbit anti-CD68 (Santa Cruz Biotechnology), rabbit anti-SMA (Sigma), or rabbit anti-SM-MHC (AbD Serotec) antibody. The sections were then incubated with a biotin-conjugated goat anti rabbit IgG or rat IgG antibody (Dako), followed by an incubation with avidin-conjugated horseradish peroxidise and then with 3,3′-diaminodbenzidine (Dako). The sections were then incubated with a mouse anti GFP antibody (Sigma), followed by an incubation with goat anti mouse IgG conjugated with alkaline phosphatase (Sigma), and then with Fast Red (Sigma). After counterstaining with hematoxylin and mounting, the slides were examined using an OLYMPUS BX61 microscope and images taken using a BX-PMTVC camera. Microscopic sections of other tissues/organs harvested from mice injected with SPCs from GFP transgenic mice were subjected to immunostaining for GFP. Similarly, microscopic sections of the heart with the aortic root harvested from mice injected with SPCs from MMP8 wildtype and knockout mice respectively were subjected to immunostaining for the either Sca-1, c-Kit, CD45, CD68 or SMA in atherosclerotic lesions.

Ex vivo assay stem/progenitor cell migration into atherosclerotic plaque
For *ex vivo* assessment of SPC trans-endothium migration into atherosclerotic plaque, aortas with atherosclerotic lesions were taken from ApoE$^{-/-}$/MMP8$^{+/+}$ mice and cut into several segments of 5 to 10 mm in length. Cultured embryonic Sca-1^{+} SPCs infected with non-target shRNA or MMP8 shRNA lentivirus were harvested, counted and labeled with PKH26 (Sigma, PKH26GL). Labeled cells (100,000) were washed three times and resuspended in 200µl of SPC culture medium and injected into the lumen of the aorta. After ligation of both ends, the aortic segments were cultured in normal SPC culture medium but supplement with 100ng/ml of SDF-1α for 24 hours, at 5% CO_2, 21% O_2, pH7.0-7.5, 37°C. For *ex vivo* assessment of SPC trans-adventitia migration into atherosclerotic plaque, aortas of approximately 10 mm in length with atherosclerotic lesions were prepared from ApoE$^{-/-}$/MMP8$^{+/+}$ mice and connected to mini-needles of a micro-circulation system filled with SPC culture medium supplemented with 100ng/ml of SDF-1α. Cultured embryonic Sca-1^{+} SPCs infected with non-target shRNA or MMP8 shRNA lentivirus were prepared and labeled with PKH26 as abovementioned and mixed with Martigel (BD Bioscience), then patched around the adventitia of aortic segments. After 24 hours of incubation (5% CO_2, 21% O_2, pH7.0-7.5, 37°C), the aortic segments were harvested, washed extensively and cross-sectioned. Sections were examined under a fluorescence microscope, images were taken by a digital camera, and migrated cells (red fluorescence in the vessel wall and atherosclerotic lesions) were counted. Migration activity was expressed as the mean number of migrated cell per 1000 nuclei in atherosclerotic lesions.

Stem/progenitor cell migration/invasion assay
Boyden chamber chemotaxis assay method. Boyden chamber chemotaxis assay were performed as described in previous study[6]. Briefly, 8 μm-pore polycarbonate filter (Nucleopore Corp.) pre-coated with Matrigel (50μg/ml, BD Bioscience) and 48-well chemotaxis chambers (Neuro Probe Inc.) were used. Sca-1^{+} SPCs cultured in complete media were trypsinized and suspended at a concentration of 4×10^6 cells/ml in 1% BSA DMEM. A volume of 50 μl cell suspensions with different dose of MMP8 was placed in the upper chamber, and 28 μl of DMEM supplemented with 1% BSA and SDF-1α (100 ng/ml, Miltenyi Biotec) or vehicle was placed in the lower chamber. The chambers were incubated at 37°C for 8h. After incubation, the filters were removed and the cells on the upper side of the filter were scraped off. The cells that had migrated to the lower side of the filter were fixed in methanol, stained with Mayer Hematoxylin and eosin, and quantified under a light microscope. Migration activity was expressed as the mean number of cells that had migrated per high power field (20x). All experiments were carried out in three independent triplicate experiments.

Cell migration assay with transwell. SPC trans-endothelium and/or trans-extracellular matrix (ECM) migration assays were performed on polycarbonate membrane inserts (8-μm pore size; Greiner Bio-One Inc., UK). Sca-1^{+} SPCs cultured in complete media were trypsinized and suspended at a concentration of 1×10^6 cells/ml in 1% BSA DMEM. 2.0×10^5 cells in 200 μl serum-free medium supplemented with vehicle or different dose of MMP8 were placed over the inner chamber of inserts pre-grown with monolayer of endothelial cell (for trans-endothelium migration) or pre-coated with different ECM (for trans-ECM migration, 50 μg/ml of Matrigel, Collagen I, Collagen IV, gelatin, fibronectin or elastin) in a 24-well tissue culture plate and 500 μl serum-free medium supplemented with 1% BSA and SDF-1α (100 ng/ml, Miltenyi Biotec) were added in the outer chamber of the insert. Plates were incubated at 37°C for 24 h. The cells that had migrated through to the lower surface of the insert were scraped off and mixed with the cells that had migrated into the bottom well. The total migrated cells were quantified by cell counter. Migration activity was either expressed as the mean number of cells that had migrated per well or the percentage (%) of migrated cells to input cells. All experiments were carried out in three independent triplicate experiments.

QCM ECMatrix Cell Migration/Invasion Assay. The CHEMICON® Cell Invasion Assay Kit (Millipore, ECM554) was used to evaluate the migratory and/or invasive ability of SPCs according to standard protocol provided with this kit. Cell preparation and incubation was similar to cell migration assay with transwell plate. The migrated cells were detached with cell detachment buffer and subsequently lysed and detected by CyQuant GR® dye with fluorescent plate reader. Migration/invasion activity of SPCs was expressed as the mean fluormetric reading per group. All experiments were carried out in three independent triplicate experiments.

Flow Cytometry Assay
Sca-1[+] SPCs infected with non-target shRNA or MMP8 shRNA were cultured in the presence or absence of the ADAM10 inhibitor GI254023X[7, 8] (5μM) for 48 hours. For flow cytometric analyses, the cells were harvested with Accutase (Sigma-Aldrich), washed and suspended in PBS containing 1% BSA at 10^7 cells per ml. The cell suspensions (100μl) were incubated for 60 minutes on ice with various antibodies (1μg/ml) as indicated in the Figures or an isotype-matched control antibody (1μg/ml). Subsequently, the cells were washed and incubated for 40 minutes on ice with appropriate secondary antibodies conjugated with FITC or PE (1μg/ml), and then analyzed by flow cytometry.

Mononuclear cells isolated from bone marrow and peripheral blood of MMP8$^{-/-}$/apoE$^{-/-}$ and MMP8$^{+/+}$/apoE$^{-/-}$ mice were analyzed by flow cytometry analysis to examine the SPC concentrations in bone marrow and peripheral blood, respectively.

Enzyme-linked immuno-sorbent assay (ELISA)
Commercially available ELISA kits (ab100678, Abcam, UK) were used to measure the concentrations of soluble E-cadherin in plasma samples and cell culture supernatants.

Statistical analyses
Data were analyzed by two-tailed student's *t*-test or one-way ANOVA. A value of *P* < 0.05 was considered statistically significant.

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Supplement figure legends:

Figure I. MMP8 shRNA can specifically knockdown MMP8 gene expression.
Total RNA samples and protein extracts were prepared from cultured embryonic Sca-1^{+} SPCs infected with non-target shRNA or MMP8 shRNA, and subjected to real-time RT-PCR and Western blot analyses. Left panel shows results of real-time RT-PCR. Right panel shows representative Western blot image.

Figure II. MMP8 deficiency reduces stem/progenitor cells in aortic root atherosclerotic lesions.
ApoE^{-/-}/MMP8^{+/+} and ApoE^{-/-}/MMP8^{-/-} mice were fed a Western diet for 12 weeks. Aortic roots from ApoE^{-/-}/MMP8^{+/+} and ApoE^{-/-}/MMP8^{-/-} mice were harvested, sectioned, and subjected to immunofluorescence staining with antibodies against the stem cell markers c-Kit (Red) (A) and Flk-1 (Red) (B). Upper panels are images of immunofluorescence staining and bottom panels are bright-field images. Red dotted line indicates the boundary between the lesion and healthy area. Far right panels show quantitative data of SPCs in atherosclerotic lesions of ApoE^{-/-}/MMP8^{+/+} and ApoE^{-/-}/MMP8^{-/-} mice respectively. Columns represent mean ± SD (n=5 mice in each group). A representative image with IgG control staining was also shown.

Figure III. MMP8 deficiency reduces ability of stem/progenitor cell to migrate through collagen I.
Sca-1[+] SPCs isolated from bone marrow of ApoE^{-/-}/MMP8^{+/+} and ApoE^{-/-}/MMP8^{-/-} mice were subjected to assays for migration through transwells coated with either collagen I, collagen IV, laminin, fibronectin or elastin. Columns represent mean ± SD (n=3 independent experiments).

Figure IV. MMP8 inhibits stem/progenitor cell adhesion to Matrigel but does not affect stem/progenitor cell proliferation.
(A) MMP8 inhibits SPC adhesion. Cultured Sca-1^{+} SPCs were incubated with various amounts of MMP8 for 2 hours, followed by cell adhesion assays.
(B) Aliquots of equal numbers (10,000) of Sca-1^{+} SPCs were seeded on Matrigel overnight and then incubated with various amounts of MMP8 for 48 hours. Cells were then harvested and counted. Columns represent mean ± SD (n=3 independent experiments).

Figure V. Concentrations of CD45^{+}/CD34^{+} cells in bone marrow and peripheral blood in MMP8 knockout mice do not significantly differ from those in MMP8 wildtype mice.
Cells in bone marrow samples and peripheral blood samples from ApoE^{-/-}/MMP8^{+/+} and ApoE^{-/-}/MMP8^{-/-} mice were harvested. Red blood cells were lysed, and the remaining cells were subjected to flow cytometer analysis to determine the percentages of hematopoietic SPCs, with the use of antibodies against CD45 and CD34, respectively. Up panels are the representative images of flow cytometer analysis. Columns represent mean ± SD (n=8 mice in each group).

Figure VI. Characterization of isolated bone marrow stem/progenitor cells.
Bone marrow cells were harvested from mice. Red blood cells were lysed, and the remaining cells were subjected to hematopoietic cell lineage depletion for 2 or 3 rounds to obtain high purity lineage-negative cells as described in the text. Isolated cells were subjected to flow cytometric analysis (A) and immunofluorescence staining (B) to determine the percentages of SPCs, with the use of antibodies against CD34, Sca-1 and CD34, respectively.

Figure VII. In vivo distributions and cell fates of transplanted stem/progenitor cells.

ApoE$^{-/-}$/MMP8$^{-/-}$ mice were injected with 1x10^6 SPCs isolated from the bone marrow of GFP transgenic mice, and fed a Western diet for 12 weeks. (A) Transplanted SPCs were homed to various tissues. Various tissues/organs were harvested and subjected to immunohistochemical staining for GFP with a primary mouse anti-GFP antibody and a secondary goat anti-mouse IgG antibody conjugated with alkaline phosphatase (AP), followed by an incubation with FAST Red. Black arrows indicate GFP-positive cells. (B) Transplanted SPCs were recruited into and accumulated in atherosclerotic lesions and differentiated into CD45 or CD68-positive cells. Aortic roots from recipient mice were harvested, sectioned, and subjected to double immunohistochemical staining with antibodies against GFP and CD45, CD68 or the vascular smooth muscle cell markers, SMA and SM-MHC. Black arrows indicate double positive cells. Representative images of single (A) or double (B) immunohistochemical staining are presented (n=3 mice). A representative image with IgG control staining is also shown in panel B.

Figure VIII. MMP8 deficiency in SPCs results in less stem and hematopoietic cell accumulation in atherosclerotic lesion.

ApoE$^{-/-}$/MMP8$^{-/-}$ mice were injected with 1x10^6 SPCs isolated from the bone marrow of ApoE$^{-/-}$/MMP8$^{+/+}$ or ApoE$^{-/-}$/MMP8$^{-/-}$ mice, and fed a Western diet for 12 weeks. Aortic roots from the recipient mice were harvested, sectioned, and subjected to immunohistochemical staining with antibodies against c-Kit (A), CD45 (B), CD68 (C) or SMA (D). Left panels are representative images of immunohistochemical staining for the respective markers, and right panels are quantitative data of c-Kit, CD45, CD68 or SMA-positive cells in atherosclerotic lesions of ApoE$^{-/-}$/MMP8$^{-/-}$ mice injected with SPCs from ApoE$^{-/-}$/MMP8$^{+/+}$ and ApoE$^{-/-}$/MMP8$^{-/-}$ mice respectively. Black arrows in left panels indicate c-Kit, CD45, CD68 and SMA-positive cells, respectively. Columns represent mean ± SD (n=5 mice in each group).

References:
1. Laxton RC, Hu Y, Duchene J, Zhang F, Zhang Z, Leung KY, Xiao Q, Scotland RS, Hodgkinson CP, Smith K, Willeit J, Lopez-Otin C, Simpson IA, Kiechl S, Ahluwalia A, Xu Q, Ye S. A role of matrix metalloproteinase-8 in atherosclerosis. *Circ Res*. 2009;105:921-929
2. Foteinos G, Hu Y, Xiao Q, Metzler B, Xu Q. Rapid endothelial turnover in atherosclerosis-prone areas coincides with stem cell repair in apolipoprotein e-deficient mice. *Circulation*. 2008;117:1856-1863
3. Xiao Q, Zeng L, Zhang Z, Hu Y, Xu Q. Stem cell-derived sca-1+ progenitors differentiate into smooth muscle cells, which is mediated by collagen iv-integrin alpha1/beta1/alphav and pdgf receptor pathways. *Am J Physiol Cell Physiol*. 2007;292:C342-352
4. Xiao Q, Zeng L, Zhang Z, Margariti A, Ali ZA, Channon KM, Xu Q, Hu Y. Sca-1+ progenitors derived from embryonic stem cells differentiate into endothelial cells capable of vascular repair after arterial injury. *Arterioscler Thromb Vasc Biol*. 2006;26:2244-2251
5. Xiao Q, Luo Z, Pepe AE, Margariti A, Zeng L, Xu Q. Embryonic stem cell differentiation into smooth muscle cells is mediated by nox4-produced h2o2. *Am J Physiol Cell Physiol*. 2009;296:C711-723
6. Bai X, Margariti A, Hu Y, Sato Y, Zeng L, Ivetic A, Habi O, Mason JC, Wang X, Xu Q. Protein kinase c{delta} deficiency accelerates neointimal lesions of mouse injured artery involving delayed reendothelialization and vasohibin-1 accumulation. *Arterioscler Thromb Vasc Biol*. 2010;30:2467-2474
7. Hundhausen C, Misztela D, Berkhout TA, Broadway N, Saftig P, Reiss K, Hartmann D, Fahrenholz F, Postina R, Matthews V, Kallen KJ, Rose-John S, Ludwig A. The disintegrin-like metalloproteinase adam10 is involved in constitutive cleavage of cx3cl1 (fractalkine) and regulates cx3cl1-mediated cell-cell adhesion. *Blood*. 2003;102:1186-1195
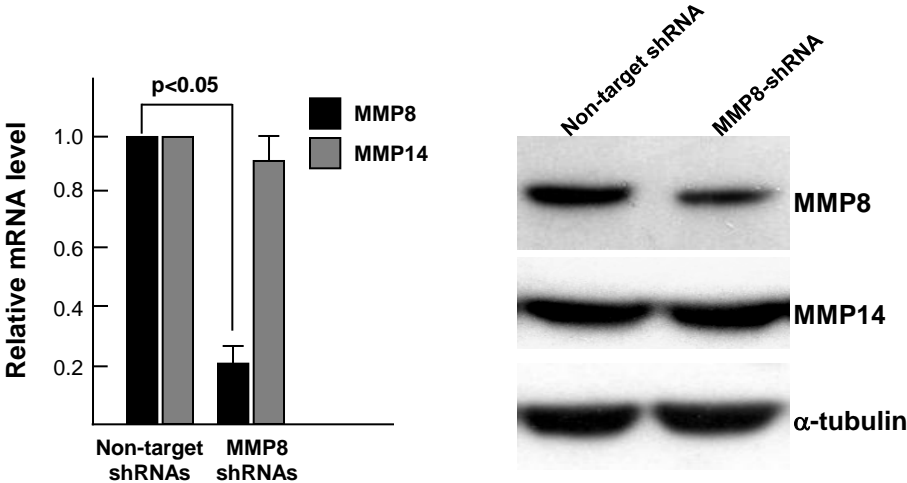8. Hoettecke N, Ludwig A, Foro S, Schmidt B. Improved synthesis of adam10 inhibitor gi254023x. *Neurodegener Dis*. 2010;7:232-238
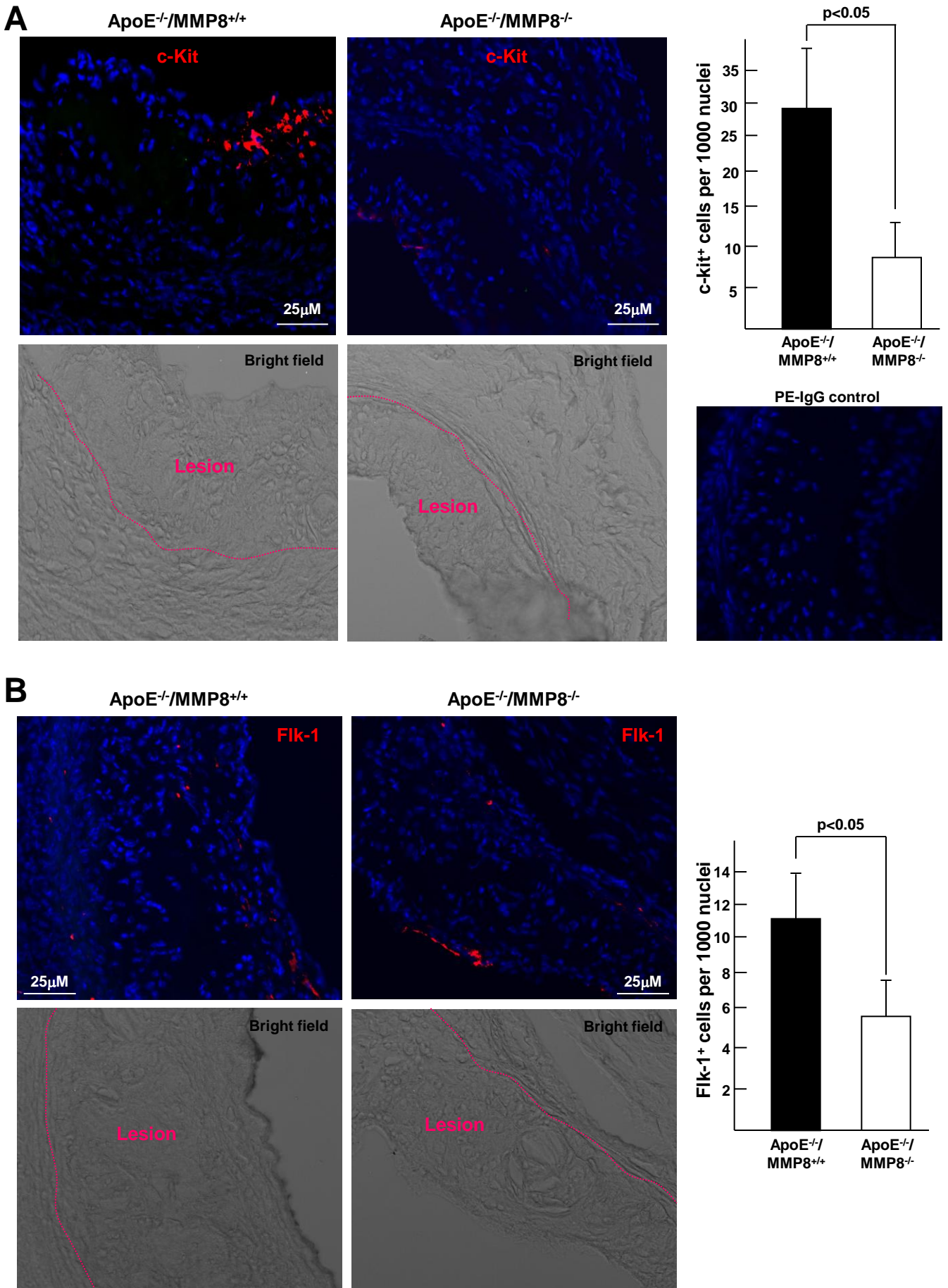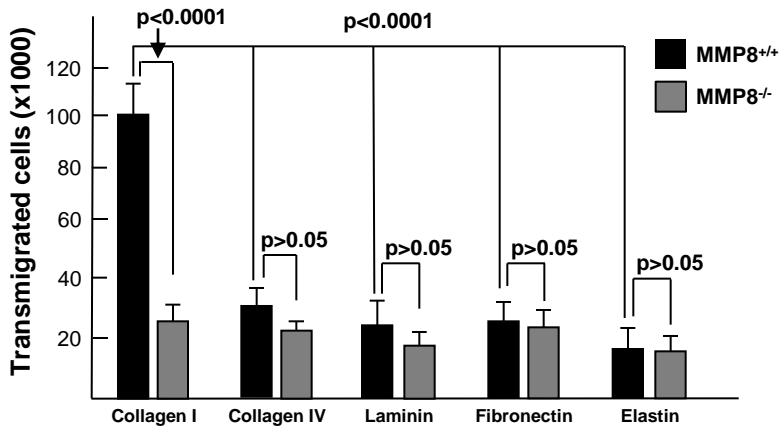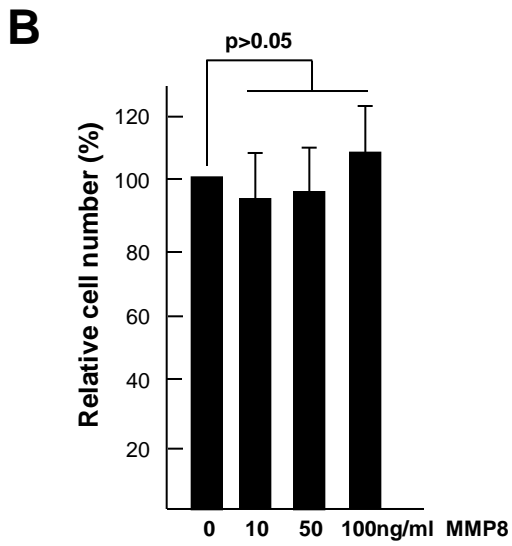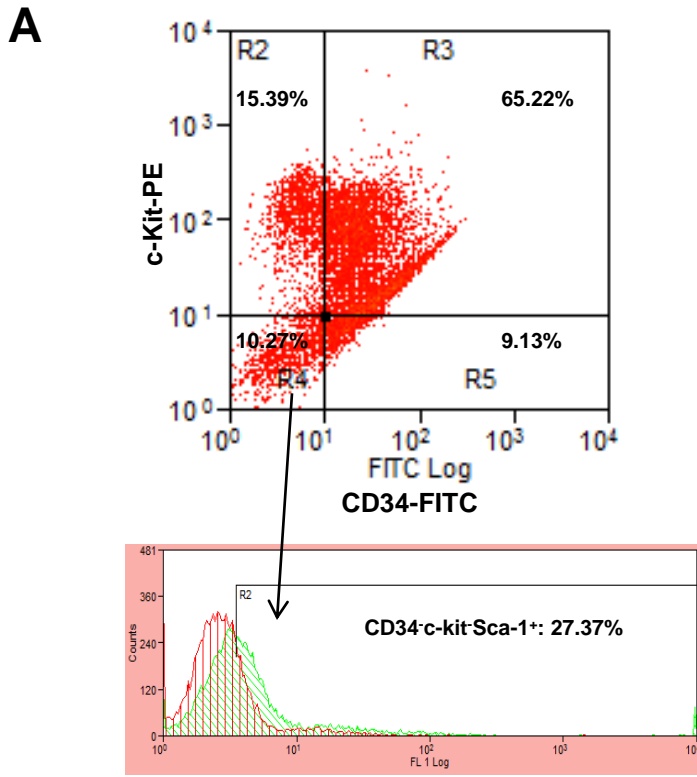
Figure I

Figure II

Figure III

Figure IV

A

p<0.001

Adherent cells per high-power field

175
150
125
100
75
50
25

0   10   50   100ng/ml   MMP8

B

p>0.05

Relative cell number (%)

120
100
80
60
40
20

0   10   50   100ng/ml   MMP8

Figure V

Figure VI

A

CD34⁻c-kit⁻Sca-1⁺: 27.37%

B

CD34/DAPI

c-Kit/DAPI

Sca-1/c-Kit/DAPI

Figure VII

A

Thymus

Spleen

Liver

Lung

B

GFP/CD45

GFP/CD68

IgG control

GFP/SMA

GFP/SM-MHC

Figure VIII