Matrix Metalloproteinase-8 Promotes Vascular Smooth Muscle Cell Proliferation and Neointima Formation

Qingzhong Xiao,* Feng Zhang, * Gianluca Grassia, Yanhua Hu, Zhongyi Zhang, Qiuru Xing, Xiaoke Yin, Marcella Maddaluno, Binia Drung, Boris Schmidt, Pasquale Maffia, Armando Ialenti, Manuel Mayr, Qingbo Xu, Shu Ye

Objective—We investigated the role of matrix metalloproteinase-8 (MMP8) in neointima formation and in vascular smooth muscle cell (VSMC) migration and proliferation.

Approach and Results—After carotid artery wire injuring, MMP8+/−/apoE−/− mice had fewer proliferating cells in neointimal lesions and smaller lesion sizes. Ex vivo assays comparing VSMCs isolated from MMP8 knockout and wild-type mice showed that MMP8 knockout decreased proliferation and migration. Proteomics analysis revealed that a disintegrin and metalloproteinase domain–containing protein 10 (ADAM10) had lower concentrations in MMP8 knockout VSMC culture media than in MMP8 wild-type VSMC culture media. Western blot, flow cytometric, and immunocytochemical analyses showed that MMP8 knockout VSMCs contained more pro-ADAM10 but less mature ADAM10, more N-cadherin, and β-catenin in the plasma membrane but less β-catenin in the nucleus and less cyclin D1. Treatment of MMP8 wild-type VSMCs with an ADAM10 inhibitor, GI254023X, or siRNA knockdown of ADAM10 in MMP8 wild-type VSMCs inhibited proliferation and migration, increased N-cadherin and β-catenin in the plasma membrane, reduced β-catenin in the nucleus, and decreased cyclin D1 expression. Incubation of MMP8 knockout VSMCs with a recombinant ADAM10 rescued the proliferative and migratory ability of MMP8 knockout VSMCs and increased cyclin D1 expression. Furthermore, immunohistochemical analyses showed colocalization of ADAM10 with VSMCs and N-cadherin, and nuclear accumulation of β-catenin in the neointima in apoE−/−/MMP8+/− mice.

Conclusions—MMP8 enhances VSMC proliferation via an ADAM10, N-cadherin, and β-catenin–mediated pathway and plays an important role in neointima formation. (Arterioscler Thromb Vasc Biol. 2014;34:90-98.)

Key Words: matrix metalloproteinase-8 ■ myocytes, smooth muscle ■ neointima

The pathogenesis of postangioplasty restenosis involves migration and proliferation of vascular smooth muscle cells (VSMCs), which constitute a major component of postangioplasty neointimal lesions.1–4 VSMC migration and proliferation are regulated by growth factors, adhesion molecules, proteases, and intracellular proteins. Among them, the cadherin–β-catenin complex and its cognate intracellular pathway have been increasingly appreciated as important regulators of these processes.5–8

Matrix metalloproteinase-8 (MMP8) was once thought to be produced exclusively by polymorphonuclear leukocytes, but more recent studies have shown that various other cell types including stem/progenitor cells express this protease.5 Compared with some other members of the MMP family, MMP8 has been less investigated for its proteolytic substrates and biological roles. Herman et al10 were the first to reveal that VSMCs, endothelial cells, and macrophages in atherosclerotic plaques express MMP8. Subsequently other investigators showed a correlation between increased MMP8 expression and rapid atherosclerotic lesion progression.11,12 A causal role of MMP8 in atherosclerosis development was demonstrated by our recent study, which showed that in apolipoprotein E (apoE)-deficient mice fed a Western diet for 12 weeks, MMP8 knockout resulted in a significant reduction of atherosclerotic lesions with decreased macrophage and VSMC contents.13 The study also revealed a role of MMP8 in vascular recruitment of leukocytes,13 providing a mechanistic explanation for the effect of MMP8 knockout on macrophage content in atherosclerotic lesions.

In the present study, we sought to investigate whether MMP8 also plays a role in neointima formation after vessel
injury and its effect on VSMC migration and proliferation, which are key processes in neointima formation.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Impact of MMP8 on Cell Proliferation and Neointima Formation After Vessel Injury**

To investigate whether MMP8 played a role in neointima formation, we used an established in vivo vascular injury model in which the endothelium of a segment of the carotid artery is removed with the use of a wire.\textsuperscript{14–16} The study showed that at day 28, neointimal lesions in MMP8 knockout mice were significantly smaller than those in MMP8 wild-type mice (Figure 1A). We also examined cell proliferation in the injured carotid arteries at days 7 and 28, and found that there were significantly fewer proliferating cells in injured carotid arteries in MMP8 knockout mice than in MMP8 wild-type mice at day 7 (5.3±0.6% [n=9] versus 11.9±1.0% [n=9]; P<0.01) as well as at day 28 (10.2±1.8% [n=7] versus 20.2±2.1% [n=8]; P<0.01; Figure 1B). Moreover, the number of neointimal VSMCs was significantly reduced in MMP8\textsuperscript{-/-}/apoE\textsuperscript{-/-} mice compared with MMP8\textsuperscript{+/-}/apoE\textsuperscript{-/-} mice (63.8±5.7 [n=10] versus 117.7±18.6 [n=7], respectively; P<0.05) without significant differences in the cellular density (number of VSMCs/mm\textsuperscript{2}; 574.8±131.4 [n=10] versus 479.1±129.4 [n=7]). Immunostaining of the injured arteries showed an increase in MMP8 protein in the MMP8\textsuperscript{-/-}/apoE\textsuperscript{-/-} mice during neointima formation (Figure I in the online-only Data Supplement).

**Effect of MMP8 on Ex Vivo VSMC Proliferation and Migration**

To verify that MMP8 has a positive effect on VSMC proliferation, we compared the rates of proliferation of cultured VSMCs isolated from aortas of MMP8 knockout and wild-type mice. Immunocytochemical staining and immunoblotting confirmed that cultured VSMCs from MMP8 wild-type mice expressed MMP8, whereas VSMCs from MMP8 knockout mice did not (Figure 2A and 2B). Proliferation assays showed that MMP8 knockout VSMCs had a significantly lower rate of proliferation than MMP8 wild-type VSMCs (Figure 2C). In agreement, immunostaining for the proliferation marker Ki-67 showed that the percentage of Ki-67 positive cells was significantly lower among MMP8

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADAM10</td>
<td>a disintegrin and metalloproteinase domain–containing protein 10</td>
</tr>
<tr>
<td>MMP8</td>
<td>matrix metalloproteinase-8</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
</tr>
<tr>
<td>WISP1</td>
<td>WNT1 inducible signaling pathway protein 1</td>
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**Figure 1.** Matrix metalloproteinase-8 (MMP8) knockout reduces neointima formation and cell proliferation. The endothelium of the left common carotid artery of MMP8\textsuperscript{-/-}/apoE\textsuperscript{-/-} and MMP8\textsuperscript{+/-}/apoE\textsuperscript{-/-} mice was damaged by passing a 0.35-mm-diameter flexible nylon wire through the lumen of the artery 3 times. Carotid arteries were collected 28 days after wire injury and sectioned and subjected to hematoxylin/eosin staining or immunostaining with an antibody against the proliferation marker proliferating cell nuclear antigen (PCNA). A, Representative photomicrographs of hematoxylin/eosin staining at day 28 postinjury, and column charts showing quantitative data (means±SEM) of neointima areas, media areas, lumen areas, and neointima/media ratio, respectively (n=7 for MMP8\textsuperscript{+/-} and n=10 for MMP8\textsuperscript{-/-}). B, Representative photomicrographs of PCNA staining at day 28 postinjury, and column chart showing percentages (±SEM) of neointimal and medial PCNA-positive cells (n=8 for MMP8\textsuperscript{+/-} and n=7 for MMP8\textsuperscript{-/-}). \*P<0.05, \**P<0.01, \***P<0.001.
knockout VSMCs than that among MMP8 wild-type VSMCs (Figure 2D). In contrast, no significant difference was observed in terms of cell apoptosis between MMP8 wild-type and knockout VSMCs (data not shown).

To investigate whether MMP8 had an effect on VSMC migration, we performed in vitro scratch wound healing assays and Boyden chamber invasion assays on cultured VSMCs. The assays showed that compared with MMP8 wild-type VSMCs, MMP8 knockout VSMCs had significantly reduced migratory ability (Figure 2E–2G).

Proteomic Analysis of MMP8 Knockout and Wild-Type VSMC–Conditioned Culture Media

To investigate into molecular pathways involved in MMP8 regulation of VSMC proliferation and migration, we performed a mass spectrometry–based quantitative proteomic analysis on MMP8 knockout and wild-type VSMC–conditioned media. The assay detected 6 secreted/membrane proteins whose concentrations significantly differed in MMP8 knockout versus MMP8 wild-type VSMC–conditioned media (Table 1). Among these proteins, a disintegrin and metalloproteinase domain–containing protein 10 (ADAM10) is of particular interest because there has been evidence indicating that it can promote cell proliferation and migration via the cadherin–β-catenin pathway. ADAM10 has several structural domains including a signal peptide, a propeptide domain, a metalloproteinase domain, a disintegrin-like domain, a cystein-rich domain, a transmembrane domain, and a cytoplasmic domain. The propeptide domain is cleaved off during ADAM10 maturation and activation. ADAM10 has several structural domains including a signal peptide, a propeptide domain, a metalloproteinase domain, a disintegrin-like domain, a cystein-rich domain, a transmembrane domain, and a cytoplasmic domain. In the proteomics analysis, we found that MMP8 wild-type VSMC–conditioned media had significantly higher concentrations of propeptide domain fragments than MMP8 knockout VSMC–conditioned media (Table 2). In addition, we detected

Table 1. Secreted/Membrane Proteins Whose Concentrations Significantly Differed in MMP8 Wild-Type and Knockout VSMC–Conditioned Media

<table>
<thead>
<tr>
<th>UniProt ID</th>
<th>Protein Name</th>
<th>MW, kDa</th>
<th>t Test (P Value)</th>
<th>Normalized Spectra Count</th>
<th>WT (n=4)</th>
<th>KO (n=4)</th>
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</thead>
<tbody>
<tr>
<td>ADA10_MOUSE</td>
<td>A disintegrin and metalloproteinase domain-containing protein 10</td>
<td>84</td>
<td>0.028</td>
<td>3.7±2.4</td>
<td>0.2±0.5</td>
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<tr>
<td>ATL2_MOUSE</td>
<td>ADAMTS-like protein 2</td>
<td>106</td>
<td>0.00096</td>
<td>0</td>
<td>1.4±0.5</td>
<td></td>
</tr>
<tr>
<td>CGRE1_MOUSE</td>
<td>Cell growth regulator with EF hand domain protein 1</td>
<td>31</td>
<td>0.027</td>
<td>2.3±0.9</td>
<td>0.5±0.9</td>
<td></td>
</tr>
<tr>
<td>CO5A2_MOUSE</td>
<td>Collagen α-2(V) chain</td>
<td>145</td>
<td>0.039</td>
<td>74.5±29.3</td>
<td>32.5±12.6</td>
<td></td>
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<tr>
<td>RISC_MOUSE</td>
<td>Retinoid-inducible serine carboxypeptidase</td>
<td>51</td>
<td>0.027</td>
<td>1.5±1.0</td>
<td>0</td>
<td></td>
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<tr>
<td>SSP0_MOUSE</td>
<td>SCO-spondin</td>
<td>535</td>
<td>0.04</td>
<td>0</td>
<td>1.6±1.3</td>
<td></td>
</tr>
</tbody>
</table>

KO indicates knockout; MMP8, Matrix Metalloproteinase-8; MW, molecular weight; VSMC, vascular smooth muscle cell; and WT, wild type.
fragments of other ectodomains of ADAM10 in MMP8 wild-type VSMC–conditioned media but not in MMP8 knockout VSMC–conditioned media (Table 2).

**Effect of MMP8 on ADAM10**

Immunoblot analysis confirmed that MMP8 wild-type VSMC–conditioned media had significantly higher concentrations of ADAM10 propeptide than MMP8 knockout VSMC–conditioned media (Figure 3A), suggesting the possibility of an effect of MMP8 on ADAM10 propeptide domain cleavage. Therefore, we investigated whether there was a difference in the amount of mature ADAM10 (lacking the propeptide domain) versus the amount of pro-ADAM10 (full length, containing the propeptide domain) in MMP8 knockout and wild-type VSMCs. Immunoblot analysis showed that the amount of mature ADAM10 versus the amount of pro-ADAM10 was indeed greater in MMP8 wild-type VSMC extracts than in MMP8 knockout VSMC extracts (Figure 3B). The total intensity of the pro-ADAM10 band plus mature ADAM10 band was similar between the MMP8 wild-type and knockout VSMC extracts (Figure 4B), suggesting that MMP8 knockout did not lead to a change in ADAM10 expression. In agreement, flow cytometric and immunocytochemical analyses with an antibody against the ADAM10 propeptide domain both showed greater signals on MMP8 knockout VSMCs than on MMP8 wild-type VSMCs (Figure 3C and 3D), consistent with the notion that MMP8 knockout VSMCs have larger amounts of unprocessed ADAM10 (containing the propeptide domain) than MMP8 wild-type VSMCs.

Table 2. ADAM10 Fragments Detected by Mass Spectrometry in MMP8 Wild-Type and Knockout VSMC–Conditioned Media

<table>
<thead>
<tr>
<th>Amino Acid Sequences of ADAM10 Fragments Detected</th>
<th>Index</th>
<th>Domain</th>
<th>Times Detected* in MMP8 Wild-Type VSMC–Conditioned Media (n=4)</th>
<th>Times Detected* in MMP8 Knockout VSMC–Conditioned Media (n=4)</th>
</tr>
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<tbody>
<tr>
<td>GGTYEIPAER</td>
<td>132–142</td>
<td>Propeptide</td>
<td>++++++</td>
<td>+</td>
</tr>
<tr>
<td>YGPQGCADHSVFER</td>
<td>167–181</td>
<td>Propeptide</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>ACTYQTTDFSGIR</td>
<td>253–267</td>
<td>Metalloproteinase</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MAPSTCASTGSLWKS</td>
<td>603–618</td>
<td>Cysteine rich</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>TITLOPSFCDFR</td>
<td>624–637</td>
<td>Cysteine rich</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

ADAM10 indicates a disintegrin and metalloproteinase domain–containing protein 10; and VSMC, vascular smooth muscle cell.

*Peptides with SEQUEST Xcorr scores >2 were counted.

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**Figure 3.** Matrix metalloproteinase-8 (MMP8) knockout reduces a disintegrin and metalloproteinase domain–containing protein 10 (ADAM10) propeptide domain cleavage. **A**, A representative image of immunoblot analysis of ADAM10 propeptide in MMP8 wild-type and knockout vascular smooth muscle cell (VSMC)–conditioned media, from 3 independent experiments. **B**, Immunoblot analysis of whole cell lysates from MMP8 wild-type and knockout VSMCs with antibodies against ADAM10 (cytoplasmic domain) or α-tubulin (as a protein loading control); the representative immunoblot image shows bands for full-length (FL) and mature (M) ADAM10, and the column charts show the means (±SEM; n=4) of the ratio of FL ADAM10 band intensity vs M ADAM10 band intensity (**left**) and the means (±SEM; n=4) of FL ADAM10 band intensity plus mature ADAM10 band intensity (**right**). **C**, Flow cytometric analysis of MMP8 wild-type and knockout VSMCs with an antibody against the ADAM10 propeptide domain; the column chart shows mean fluorescence intensity (±SEM) in 4 independent experiments. **D**, Representative images of immunofluorescent staining of MMP8 wild-type and knockout VSMCs with antibodies against the ADAM10 propeptide domain, from 3 independent experiments. *P<0.05.
To investigate whether ADAM10 might be involved in MMP8 regulation of VSMC proliferation and migration, we treated VSMCs with GI254023X, an ADAM10 specific inhibitor that binds the S1’ specificity pocket of ADAM10 and prevents ADAM10 from shedding surface molecules on cells.32 In these experiments, we found that GI254023X treatment did not alter ADAM10 protein level or propeptide cleavage (data not shown). The experiment showed that GI254023X significantly reduced proliferation and migration of MMP8 wild-type VSMCs but had no such effect on MMP8 knockout VSMCs (Figure 4A–4C). An additional experiment further demonstrated that knockdown of the endogenous ADAM10 with specific ADAM10 siRNA also reduced proliferation and migration of MMP8 wild-type VSMCs (Figure 4D–4E), supporting the notion that ADAM10 plays a role in MMP8 regulation of VSMC proliferation and migration.

We then performed proliferation and migration in the absence or presence of recombinant mouse ADAM10. The experiments showed that the addition of the recombinant ADAM10 rescued the proliferative and migratory ability of MMP8 knockout VSMCs (Figure 5A and 5B), further supporting that ADAM10 plays a role in MMP8 regulation of VSMC proliferation and migration.

Effect of MMP8 on N-Cadherin, β-Catenin, Cyclin D1, and WNT1 Inducible Signaling Pathway Protein 1
ADAM10 can cleave and shed cell surface N-cadherin,17–21 an adhesion molecule that can exert an effect on VSMC...
proliferation via the cadherin–β-catenin complex. After the finding that MMP8 wild-type VSMCs had higher levels of mature ADAM10 than MMP8 knockout VSMCs, we investigated whether there were differences in the concentrations of N-cadherin and β-catenin between MMP8 wild-type and knockout VSMCs. We found that plasma membranes of MMP8 wild-type VSMCs contained less N-cadherin than plasma membranes of MMP8 knockout VSMCs, (Figure 6A) and that incubation of VSMCs with the ADAM10 inhibitor GI254023X increased the amounts of N-cadherin on plasma membranes (Figure 6A). In contrast, MMP8 wild-type VSMC–conditioned media had higher concentrations of N-cadherin than MMP8 knockout VSMC–conditioned media (Figure 6B).

Knockdown of endogenous ADAM10 by siRNA resulted in a marked decrease in the amount of N-cadherin in conditioned media of MMP8 wild-type VSMCs (Figure 6B), whereas incubation of VSMCs with a recombinant ADAM10 led to a significant increase in N-cadherin concentration in conditioned media of MMP8 knockout VSMCs (Figure 6B).

It has been shown that N-cadherin shedding can result in dissociation of the cadherin–β-catenin complex and translocation of β-catenin into the nucleus where β-catenin interacts with a transcription factor complex to upregulate the expression of cell proliferation proteins such as cyclin D1 and WNT1 inducible signaling pathway protein 1 (WISP1).

In our study, we found that nuclei of MMP8 wild-type VSMCs had a higher concentration of β-catenin than nuclei of MMP8 knockout VSMCs (Figure 6C), and that its concentration was reduced if VSMCs were transfected with ADAM10 specific siRNA (Figure 6C). In addition, we found that compared with MMP8 knockout VSMCs, MMP8 wild-type VSMCs expressed the higher levels of the cell proliferation proteins cyclin D1 and WISP1 (Figure 6D and 6E), and that their levels were reduced when VSMCs were treated with GI254023X (Figure 6D) or transfected with ADAM10 specific siRNA (Figure II in the online-only Data Supplement). In contrast, their expression levels in MMP8 knockout VSMCs were significantly increased if cells were cultured in the presence of a recombinant ADAM10 (Figure 6E).

**ADAM10 and β-Catenin in Vessel Injury-Induced Neointima Lesions**

To study the relationship of MMP8 with ADAM10, N-cadherin, and β-catenin in neointima formation, we examined the cellular location and temporal expression of ADAM10, N-cadherin, and β-catenin in carotid arteries of apoE−/−/MMP8+/+ and apoE−/−/MMP8−/− mice at various time points after vessel injury. The experiment showed that ADAM10 colocalized with VSMCs

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**Figure 6.** Matrix metalloproteinase-8 (MMP8) knockout and/or a disintegrin and metalloproteinase domain–containing protein 10 (ADAM10) inhibition reduces N-cadherin shedding and β-catenin signaling. MMP8 wild-type and knockout vascular smooth muscle cells (VSMCs) were cultured in the presence or absence of the ADAM10 specific inhibitor GI254023X (5 µmol/L) or ADAM10 siRNA or recombinant ADAM10 (Rec-ADAM10; 20 ng/mL). A–D, Protein preparations from plasma membranes, nuclei, cell lysates, or conditioned media were subjected to immunoblot analyses and band intensities quantified. Shown are representative images each from 3 independent experiments, and column charts of relative protein levels (mean±SEM; n=3). A, N-cadherin and β-catenin values were standardized against values of the membrane protein loading control platelet-derived growth factor receptor-β (PDGFR-β); B, equal amounts of protein were loaded in every lane; C, β-catenin values were standardized against values of the nuclear protein loading control histone 4 (H4); D, values of cyclin D1 and WNT1 inducible signaling pathway protein 1 (WISP1) were standardized against values of the cell lysate protein loading control α-tubulin. E, Total cellular RNA was subjected to real-time reverse-transcriptase polymerase chain reaction. Column charts show relative RNA levels (mean±SEM; n=3). *P<0.05.
and N-cadherin in the media and neointima, with noticeable accumulation in the arteries of apoE−/−/MMP8−/− mice subjected to injury (Figures III and IV in the online-only Data Supplement). Importantly, there was substantial nuclear accumulation of β-catenin in the neointima in apoE−/−/MMP8−/− mice (Figure V in the online-only Data Supplement), supporting a relationship of MMP8 with ADAM10 and β-catenin in vessel injury-induced neointima formation.

Discussion

VSMC migration and proliferation is a key process in neointima formation responsible for postangioplasty restenosis.1–4 Our study shows that MMP8 promotes VSMC proliferation and migration. Using an established mouse model, we found that knockout of MMP8 significantly deterred neointima formation after vessel injury. Ex vivo experiments of VSMCs isolated from MMP8 knockout and wild-type mice, respectively, showed that MMP8 knockout resulted in VSMCs having decreased proliferative and migratory ability, indicating a positive effect of MMP8 on VSMC proliferation and migration. By proteomic analysis and follow-up experiments, we identified a molecular pathway mediating such effects of MMP8 on VSMCs. Specifically, we found that MMP8 knockout resulted in a reduction of the amount of mature ADAM10 versus the amount of pro-ADAM10, an increase of N-cadherin and β-catenin on the plasma membrane, a decrease in β-catenin translocation to the nucleus, and lower expression of the cell proliferation proteins cyclin D1 and WISP1. To our knowledge, this is the first report of a role of MMP8 in VSMC proliferation and migration and in neointima formation.

In this study, we found that MMP8 knockout VSMC–conditioned media had significantly less ADAM10 propeptide and ectodomains than MMP8 wild-type VSMC–conditioned media. ADAM10 contains several structural domains including a signal peptide, a propeptide domain, a metalloproteinase domain, a disintegrin-like domain, a cysteine-rich domain, a transmembrane domain, and a cytoplasmic domain.29 The metalloproteinase domain has proteolytic activity, whereas the propeptide domain blocks the proteolytic active site in the metalloproteinase domain and is removed during ADAM10 maturation.29,29 The results of our study indicate that MMP8 has an effect on ADAM10 maturation in VSMCs. Activated ADAM10 can mediate shedding of some transmembrane proteins including N-cadherin.17–21 Interestingly, ADAM10 itself is also subjected to shedding, which releases ADAM10 ectodomains from the plasma membrane.30,31 and the shedded ADAM10 ectodomains can function as a soluble protease with increased mobility.30,31 There is evidence indicating that ADAM10 ectodomain shedding in different tissues/cells requires different proteases; however, their identities are still unknown for most tissues/cells, apart from neuronal cells where ADAM10 can be shed by ADAM9 and ADAM15.32 The results of our study implicate MMP8 in ADAM10 shedding in VSMCs.

We found that plasma membranes of MMP8 wild-type VSMCs contained substantially less N-cadherin than plasma membranes of MMP8 knockout VSMCs, whereas MMP8 wild-type VSMC–conditioned media had higher concentrations of N-cadherin than MMP8 knockout VSMC–conditioned media, and that incubation of VSMCs with the ADAM10 inhibitor GI254023X increased the amounts of N-cadherin on plasma membranes whereas knockdown of ADAM10 in VSMCs reduced the amount of N-cadherin in conditioned media of MMP8 wild-type VSMCs. These findings suggest that the effect of MMP8 on N-cadherin shedding is mediated, at least in part, by ADAM10. Interestingly, a previous study showed that MMP9 and MMP12, but not MMP2 or MMP14, can cause N-cadherin shedding on VSMCs.33 Thus, it seems that MMP9 and MMP12 can shed N-cadherin, whereas MMP8 can promote N-cadherin shedding by ADMA10.

An important role of N-cadherin in modulating VSMC proliferation via the β-catenin signaling pathway has been elegantly demonstrated by other investigators.34 On the plasma membrane, N-cadherin interacts with β-catenin, forming cadherin–β-catenin complexes. Cleavage of N-cadherin results in dissociation of these 2 proteins, allowing β-catenin to be translocated into the nucleus, where β-catenin interacts with the transcription factor T cell–specific transcription factors5,6,35–37 and thereby regulates the transcription of several genes that encode proteins involved in cell proliferation, such as cyclin D1 and WISP1.5,6,17–27,33,35–37 In this study, we found that MMP8 wild-type VSMCs contained substantially less β-catenin in the plasma membrane than MMP8 knockout VSMCs, whereas nuclei of MMP8 wild-type VSMCs had more β-catenin than nuclei of MMP8 knockout VSMCs. In agreement, the expression levels of the cell proliferation proteins cyclin D1 and WISP1 were higher in MMP8 wild-type VSMCs. We also found that inhibition of ADAM10 activity by GI254023X substantially increased the amount of β-catenin in the plasma membrane and that knockdown of ADAM10 by siRNA markedly reduced the amount of β-catenin in the nucleus of MMP8 wild-type VSMCs and the expression levels of cyclin D1 and WISP1 in these cells. These findings are consistent with an effect of MMP8 on β-catenin signaling, mediated by ADAM10. It is noteworthy that such an effect has also been observed in stem/progenitor cells as we reported recently.9

Some other members of the MMP family have also been reported to have important roles in VSMC proliferation. It has been shown that VSMCs isolated from MMP9 knockout mice have a significantly lower proliferation rate than VSMCs from wild-type mice,38 and that MMP9 and MMP12 promote VSMC proliferation by shedding N-cadherin and activating the β-catenin signaling pathway.38 Meanwhile, MMP2 and MMP14 have been shown to play a role in oxidized low density lipoprotein–induced VSMC proliferation involving a sphingomyelin/ceramide signaling pathway.39 The difference in VSMC proliferation between MMP8 wild-type and knockout is not attributable to a change in the expression of those MMPs because we showed in our previous study that there is no difference in their expression between MMP8 wild-type and knockout.33

Migration of VSMCs from the arterial media to the intima is also an important process in the development of vascular lesions, especially neointima formation underlying restenosis or in stent-restenosis after angioplasty or stent implantation. Our study showed that MMP8 wild-type VSMCs had greater migratory ability than MMP8 knockout VSMCs, and
that this difference was abolished by the ADAM10 inhibitor GI254023X or ADAM10 knockdown by siRNA, whereas the reduced migratory ability of MMP8 knockout VSMCs could be rescued by incubating cells with a recombinant ADAM10. These findings suggest that MMP8 has an effect on VSMC migration via its effect on ADAM10. This is consistent with the findings of other studies showing that ADAM10 can disrupt cell–cell adhesion attributable to its effect on cadherins and thereby facilitate cell migration.17,22,26

In addition to ADAM10, several other proteins also showed significantly different concentrations between MMP8 knock-out and wild-type VSMC–conditioned media in the proteomic analysis, including ADAMTS-like protein 2, cell growth regulator with EF hand domain protein 1, collagen α2(V) chain, retinoid-inducible serine carboxypeptidase, and SCO-spondin. However, the biological functions of these proteins are largely unknown.

In summary, our study demonstrates that MMP8 plays an important role in neointima formation after vessel injury. Although it is likely that the overall contribution of MMP8 to its pathogenesis would involve multiple cell types and mechanisms, the results of our study indicate that MMP8 modules VSMC proliferation and migration and that this is at least partly via its effect on ADAM10 and consequently on N-cadherin and the β-catenin signaling pathway.

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Disclosures
None.

References


**Significance**

Smooth muscle cells are the main constituents of neo-intima lesions responsible for restenosis after angioplasty. The pathogenesis of neo-intima formation involves smooth muscle cell proliferation and their migration from the arterial media into the intima. In this study, we found that matrix metalloproteinase-8 plays an important role in vascular smooth muscle cell migration and proliferation, via activating the protease a disintegrin and metalloproteinase domain–containing protein 10, which in turn breaks down the cell adhesion molecular N-cadherin and also releases the protein beta-catenin, allowing the latter to translocate to the cell nucleus to activate the expression of proteins required for cell proliferation. These findings are useful for understanding the molecular pathways involved in vascular smooth muscle cell migration and proliferation, and suggest that matrix metalloproteinase-8 could be a potential target for development of therapeutics for preventing postangioplasty restenosis.
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Matrix metalloproteinase-8 promotes vascular smooth muscle cell proliferation and neointima formation
Qingzhong Xiao, Feng Zhang, Gianluca Grassia, Yanhua Hu, Zhongyi Zhang, Qiuru Xing, Xiaoke Yin, Marcella Maddaluno, Binia Drung, Boris Schmidt, Pasquale Maffia, Armando Ialenti, Manuel Mayr, Qingbo Xu, Shu Ye

Online Supplement
Figure I. An increase of MMP8 protein co-localized with SMCs in the media and neointima of carotid arteries after vessel injury

Immunofluorescence staining of α-SMA (green) and MMP8 (red) with DAPI nucleus staining (blue) in apoE−/− mouse carotid arteries at day 3, 7 and 28 after injury. Yellow color indicates co-localization of MMP8 and α-actin. Bar = 50μm. Arrowheads indicate autofluorescence; Arrows indicate MMP8-positive cells.
Figure II. Effects of ADAM10 knockdown on β-catenin signaling

MMP8 wildtype and knockout VSMCs were transfected with control siRNA or ADAM10 siRNA (30nM of final concentration) and cultured for additional 72 hours. Total RNA were isolated and subjected to real time quantitative RT-PCR. Shown in the figure are quantitative RT-PCR results from three independent experiments.
Figure III. ADAM10 protein colocalized with SMCs in the media and neointima of carotid arteries after vessel injury

Serial sections of carotid arteries harvested at day 3, 7 and 28 after injury were subjected to double immunofluorescence staining with antibodies against ADAM10 (red) and α-SMA (green). DAPI (blue) was used to staining nuclei. Yellow color indicates co-localization of ADAM10 and α-SMA. Bar = 50μm. Arrowheads indicate autofluorescence; Arrows indicate ADAM10-positive cells.
Figure IV. ADAM10 protein colocalized with N-cadherin in the neointima and media of carotid arteries after vessel injury

Serial sections of carotid arteries harvested at day 3, 7 and 28 after injury were subjected to double immunofluorescence staining with antibodies against ADAM10 (red) and N-cadherin (green). DAPI (blue) was used to staining nuclei. Yellow color indicates co-localization of ADAM10 and N-cadherin. Bar = 50μm.
Serial sections of carotid arteries harvested at day 28 after injury were subjected to immunofluorescence staining with antibodies against β-catenin (red) to examine their cellular location in the vessel wall. DAPI (blue) was used to stain nuclei. Violet color indicates nuclear co-localization of β-catenin (white arrows). Bar = 50μm.
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Materials and Methods

Animals
MMP8<sup>−/−</sup>/apoE<sup>−/−</sup> mice (C57BL/6 background) and MMP8<sup>+/+</sup>/apoE<sup>−/−</sup> controls (generated from littermates of MMP8<sup>−/−</sup>/apoE<sup>−/−</sup> mice) were generated in our previous study. All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals.

Vascular injury and characterization of neointimal lesions
A carotid injury procedure was carried out as described previously. In brief, MMP8<sup>−/−</sup>/apoE<sup>−/−</sup> and MMP8<sup>+/+</sup>/apoE<sup>−/−</sup> mice were fed a Western diet (21% fat, 0.15% cholesterol, 19.5% casein, Mucedola TD88137) from 1 week before and until 4 weeks after the carotid injury procedure. In the procedure, mice were anaesthetized and endothelial injury of the left common carotid artery was performed with a 0.35 mm diameter flexible nylon wire introduced through the left external carotid artery and advanced to the aortic arch. The endothelium was damaged by passing the wire through the lumen of the artery three times. Perfusion fixed carotid arteries were collected 3, 7 and 28 days after wire injury. Some mice were subjected to all the surgical procedure except the endothelial damage (Sham). Sections from the carotid artery collected at 7 and 28 days after injury were subjected to
immunohistochemical staining with an antibody against the proliferation marker proliferating cell nuclear antigen (PCNA, Sigma-Aldrich, P-8825, followed by Biotin-SP-conjugated F(ab')2fragment goat anti-mouse IgG, Jackson ImmunoResearch). Sections incubated with no primary antibody were used as negative control. The proliferating cell number was scored in 5-10 sections from each carotid artery, and expressed as the percentage of total medial and neointimal cells positive for PCNA. Ten sections from each paraffin embedded carotid artery collected at 28 days after injury were stained with haematoxylin and eosin. The cross-sectional areas of the vessel and the lumen, the area circumscribed by the external elastic lamina (EEL), and the area circumscribed by the internal elastic lamina (IEL) were calculated. The neointimal and media areas were computed as follows: neointimal area = IEL area - lumen area; media area = EEL area - IEL area. Additionally, 10 sections from the middle portion of the carotid artery collected at 28 days after injury were subjected to immunohistochemical staining with an antibody against SMαA (Sigma-Aldrich, A5228) and the number of SMαA-positive cells in the neointima was counted. Finally, the serial sections prepared from carotid arteries 3, 7, and 28 days after injury were processed as described above and incubated with polyclonal rabbit anti-ADAM10 antibody (1:200, Abcam), polyclonal rabbit anti-MMP8 (1:250, Abcam), monoclonal mouse anti-N Cadherin (1:200, Novus Biologicals) or polyclonal rabbit anti-β-catenin (1:50, H-102, Santa Cruz) diluted in blocking buffer/0.3% Triton X-100 (MP Biomedicals) in PBS overnight before being washed in TNT wash buffer. Sections incubated with non-immune rabbit or mouse serum were used as negative controls. Subsequently, the sections were incubated with Texas Red donkey anti-rabbit IgG (1:250, Jackson ImmunoResearch Laboratories) and/or with FITC donkey anti-mouse IgG. Monoclonal anti-α-SMA FITC (1:100, clone 1A4, Sigma) was added in blocking buffer for 1h before washing as described above. DAPI was used to identify nuclei.
Cell culture

VSMCs were isolated by autogrowth of explant culture from the thoracic aortas of MMP8−/−/apoE−/− and MMP8+/+/apoE−/− mice as described previously. Briefly, mouse thoracic aortas were removed and washed with Dulbecco’s modified Eagle’s medium (DMEM). The intima and inner two thirds of the media were carefully dissected from the vessels and cut into pieces (∼1 mm³). The tissue pieces were then explanted onto a 0.02% gelatin-coated flask, and cultured in DMEM supplemented with 20% fetal bovine serum (FBS), penicillin and streptomycin. Cells were allowed to autogrow for 2 weeks and then passaged. Cells were then maintained in DMEM supplemented with 10% FBS, penicillin and streptomycin. Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Proteomics analysis

VSMCs from 4 MMP8 knockout mice and 4 MMP8 wildtype mice were cultured in DMEM with 10% FBS to 80% confluence. Cells were washed 3 times with serum-free DMEM and cultured for another 3 hours in serum-free DMEM at 37°C in a humidified atmosphere with 5% CO₂. Cells were washed again with serum-free DMEM and cultured in serum-free DMEM for another 24 hours. The culture media (10mL from each sample) were collected and centrifuged at 3000g for 20 minutes. The supernatants were concentrated into 50μL per sample with the use of centrifugal concentrators (Millipore Centriprep YM-10, 10 kDa NMWL), and proteins precipitated using ReadyPrep 2-D cleanup kit (Bio-Rad). The proteins were re-suspended in 30μL loading buffer (100mM Tris pH 6.8, 0.2% SDS, 40% glycerol, 0.02% bromophenol blue and 2%β-mercaptoethanol) and denatured at 97°C for 5 minutes. The samples were then loaded on each lane of a Bis-Tris discontinuous 4-12% polyacrylamide gradient gel (NuPAGE, Invitrogen) and electrophoresis was performed at 160V constant voltage for 2 hours. Prestained protein standards (All blue, Precision Plus,
Bio-Rad) were run alongside the samples to allow molecular mass estimation of proteins. The gel was placed in a fixation solution (50% methanol, 5% acetic acid and 45% deionized distilled water (ddH2O)) at room temperature for 30 minutes and then washed with ddH2O for 5 minutes. The gel was then incubated in a staining solution (0.025% Coomassie Blue R-250, 10% acetic acid and 90% ddH2O) at room temperature for 2 hours, and the background destained with a destaining solution (10% acetic acid and 90% ddH2O) at room temperature for 2 hours.

Each lane in the gel was divided into 10 parts without any gap and the gel bands were excised in identical parallel positions. The gel pieces were cut into about 1mm³ size and placed into each well of a 96 well plate and subjected to in-gel digestion with trypsin in an Investigator ProGest (Genomic Solutions) robotic digestion system. The plate was then lyophilized and digestion products re-suspended in 20μL of 0.05% trifluoroacetic acid. The samples were separated by liquid chromatography on a reverse-phase column (Dionex PepMap C18, 25cm length, 75μm internal diameter, 3μm particle size) and applied to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific). The peptides were eluted using an acetonitrile/0.1% formic acid gradient. Acetonitrile concentration was increased gradually from 5% to 80%. Spectra were collected from the mass analyzer using full ion scan mode over the mass to charge (m/z) range 450-1600. MS-MS scans were performed on top 6 most abundant ions using dynamic exclusion. Database search was performed using SEQUEST program (BioWorks Browser 3.3.1 SP1, Thermo Fisher Scientific) against UniProt mouse database (version 15.13). Scaffold (version 2.6, Proteome Software Inc., Portland, OR) was used to calculate the spectral counts and to validate MS/MS-based peptide and protein identifications.
**Cell proliferation assays**

VSMCs were seeded on 0.02% gelatin-coated 6-well tissue culture plates (50,000 cells/well) and cultured in the presence or absence of the ADAM10 specific inhibitor GI254023X (5µM), or the presence or absence of recombinant mouse ADAM10 (20ng/ml, Sigma), for 4 days. Cells were then harvested by trypsinization and cell numbers counted with the use of a Vi-CELL cell viability analyzer.

**Migration assays**

Scratch wound healing assays were carried out using a previously described method. In brief, VSMCs were cultured to confluence on 6-well plates. A linear wound per wall was made by scraping, and photomicrographic images of the wounds were obtained using an Olympus inverted microscope equipped with a digital camera. Cells were then cultured in the presence or absence of the ADAM10 specific inhibitor GI25323X (5µM) or the vehicle DMSO for 24 hours, and photomicrographic images taken again. ImageJ software was used to measure the distance between two edges of each wound, and the migrated distance calculated by comparing the distance between the two edges of the wound at 0 and 24 hours.

Cell invasion assays were performed with the use of cell invasion assay kit (QCM™ 24-Well Cell Invasion Assay, Chemicon) containing 24 Boyden chambers on each plate. In brief, VSMCs were cultured in FBS-containing DMEM to 80% confluence and then in FBS-free DMEM for 24 hours, and harvested by trypsinization. An aliquot (250,000 cells) of the cells in FBS-free DMEM was dispensed into the upper chamber, and DMEM with 10% FBS was placed in the lower chamber. The invasion chambers were incubated at 37°C in a 5% CO₂ incubator for 16 hours. Invaded cells on the lower-outer surface of the upper chamber were collected by incubating the chamber with a cell detachment solution, incubated with a
fluorescent dye, and lysed by incubating with a lysis buffer. The fluorescence intensity was measured with the use of a fluorometer.

In additional cell invasion assays, cells (50,000 cells/insert) were cultured in transwells (8-µm pore size; Greiner Bio-One Inc.), in the presence of the mouse ADAM10 (20ng/ml, Sigma) for 24hrs. Migrated cells were then stained with hematoxylin and counted.

**Flow cytometry**

VSMCs were cultured to 80% confluence, harvested by incubation with Accutase (Sigma-Aldrich), incubated with an ADAM10 pro-peptide domain antibody (Abcam, ab39178) or an isotype-matched control antibody, then incubated with a fluorescein isothiocyanate-conjugated secondary antibody, fixed in 2% paraformaldehyde, and analyzed by flow cytometry.

**Immunocytochemistry**

VSMCs were cultured on 0.02% gelatin-coated coverslips on 6-well plates. When cells reached 80% confluence, the culture medium was removed and cells fixed by incubation with 4% paraformaldehyde for 10 minutes. For MMP8 and Ki-67 staining but not for pro-ADAM10 staining, cells were permeabilized by incubation with 1% Triton-100 for 10 minutes. Fixed cells were incubated with antibodies against MMP8 (Abcam, ab78423), Ki-67 (Abcam, ab15580), or the ADAM10 pro-peptide domain (Abcam, ab39178), then a fluorophore conjugated secondary antibody, and subsequently 4’-6-Diamidino-2-phenylindole. The coverslips were then mounted on slides with fluorescence mounting medium. Slides were visualized using a fluorescence microscope, and photomicrographs taken using a digital camera.
**Immunoblot analyses**

Cell lysates were prepared by incubating cells with a lysis buffer (1% SDS, 62.5mM Tris-HCl, pH 7.8) containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Sigma Aldrich). Nuclear protein extracts were prepared by incubating cells with a subcellular fractionation buffer (250mM Sucrose, 20mM HEPES pH7.4, 10mM KCl, 1.5mM MgCl2, 1mM EDTA, 1mM EGTA, 1mM DTT, and 10% protein inhibitor cocktail) with scrapping, passing the lysates through a 25G needle, incubating on ice for 20 minutes, centrifuging the lysates at 720g for 15 minutes to collect the nuclei, and incubating the nuclei with a lysis buffer (1M Tris pH 7.8, 10% SDS, and 10% protease inhibitor cocktail), followed by sonication to shear nuclear DNA. Plasma membrane protein extracts were prepared by incubating cells with the subcellular fractionation buffer described above, centrifuging the lysates at 720g for 15 minutes to remove the nuclei, and then centrifuging the supernatants at 10,000g for 20 minutes to remove other organelles, followed by centrifugation of the supernatants at 100,000g for 1 hour, re-suspending the pellets in the subcellular fractionation buffer, centrifuging the suspensions at 100,000g for 45 minutes, and re-suspending the pellets with the lysis buffer described above. Proteins in conditioned media were collected and concentrated with the use of centrifugal concentrators (Millipore, UFC901024).

Protein concentrations in the cell lysates, plasma membrane extracts, nuclear extracts and concentrated conditioned media, respectively, were measured by Bradford assay. Proteins was separated by Tris-glycine, sodium-dodecyl-sulfate, polyacrylamide gel electrophoresis, followed by standard immunoblot analysis with antibodies against MMP8 (Abcam, ab78423), the ADAM10 pro-peptide domain (Abcam, ab39178), the ADAM10 cytoplasmic domain (Abcam, ab39177), N-cadherin (Abcam, ab18203), β-catenin (Santa Cruz, Sc-7199), Cyclin
D1 (Santa Cruz, Sc-8396), WISP1 (Santa Cruz, Sc-25441), α-tubulin (Sigma-Aldrich, T6074), PDGFR-β (Santa Cruz, sc-80991), or histon H4 (Santa Cruz, sc-10810).

Densitometric analyses of blots were carried out with the use of Image J software. Relative protein expression level was defined as the ratio of target protein band intensity to internal control (PDGFR-β, histone 4 or α-tubulin) protein band intensity, with that of the control sample set as 1.0.

**siRNA experiments**

ADAM10 siRNA (SASI_Mm01_00197171) and non-targeting control siRNA (SIC001-10NMOL) were purchased from Sigma. VSMCs were transfected with 20 μl (per 10^6 cells) of 10μM siRNA using siIMPORTER transfection reagents (Millipore) according to the manufacturer’s protocol provided. Cells were harvested at 72 hours after transfection and real-time RT-PCR and Western blot analysis were performed.

**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.

**References**


