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# Macrophage-derived MMP-8 determines smooth muscle cell differentiation from adventitia stem/progenitor cells and promotes neointima hyperplasia

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**Aims** Emerging evidence has suggested that adventitia stem/progenitor cells (AdSPCs) migrate into the intima of arteries in response to injury, where they differentiate towards smooth muscle cells (SMCs) and participate in neointimal hyperplasia. We have previously identified matrix metalloproteinase-8 (MMP8) as a key player in atherogenesis. In this study, we aimed to investigate the functional roles of macrophage-derived MMP8 in AdSPC differentiation and injury-induced arterial remodelling.

**Methods and results** We first observed an important role for MMP8 in SMC differentiation from embryonic stem cells, but this effect was not seen in AdSPCs. Instead, through macrophages/AdSPCs co-culture and macrophage conditional culture medium studies, we have demonstrated that the MMP8 protein secreted from macrophages promotes SMC differentiation from AdSPCs. Mechanistically, we showed that macrophage-derived MMP8 promotes SMC differentiation from AdSPCs through modulating transforming growth factor-β activity and a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10)/Notch1 signalling. We further demonstrated that the binding site for CBF1, Suppressor of Hairless, and Lag-1 (CSL) within SMC gene promoters is responsible for Notch1 mediated SMC differentiation. Finally, we demonstrated that macrophage-derived MMP8 increased injury-induced neointimal SMC hyperplasia by activating ADAM10/Notch1 signalling.

**Conclusions** We have identified macrophage-derived MMP8 as a regulator in SMC differentiation from AdSPCs and neointimal SMC hyperplasia in response to injury. Our data provide new insights into the roles of MMP8 in AdSPC differentiation and the pathogenesis of neointima formation in the context of angiographic restenosis, and therefore may aid in the development of novel therapeutic agents for the prevention of this disease.

**Keywords** Adventitia stem cells • Progenitor cells • Arterial remodelling • Neointima formation • Atherosclerosis • Matrix metalloproteinase-8 • Smooth muscle cell differentiation • Notch signalling • A disintegrin and metalloproteinase domain-containing protein 10

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# 1. Introduction

Accumulating evidence has suggested that blood vessels throughout the body serve as a systemic reservoir of multipotent stem/progenitor cells (SPCs). Recently, a variety of SPCs have been identified that are both anatomically and functionally associated with adventitia/perivascular niches in various tissues, including adventitial SPCs (AdSPCs).[1–9] Moreover, their contributions to vascular regeneration,[10] development, homeostasis, health as well as diseases[2,7,8,11–16] has been gradually recognized and appreciated in the field of vascular biology. These AdSPCs, identified through distinct panels of cell markers, such as stem cell antigen-1 (Sca-1)[2,3,7] CD34[4,17–19] vascular endothelial growth factor receptor 2 (VEGFR2)/stem cell antigen c-Kit,[20] PW1,[21] Gli1,[22] CD90,[23] mesenchymal stem cell (MSC) markers (CD29, CD44, CD73, CD105, CD146, and/or CD166),[24–26] as well as one or more MSC markers in combination with neural crest (e.g. Sox1, Sox10, Sox17, and Nestin),[8] pericyte progenitor (NG2 and platelet-derived growth factor receptorβ[1]) or other stem (e.g. Stro-1, Notch-1, and Oct-4)[17,27] cell markers, are capable of differentiating into smooth muscle cells (SMCs), neural cells, chondrocytes, adipocytes, and/or osteoblasts. It has also been reported that the AdSPCs that are positive for Sca-1[28,29] CD34,[17–19] or VEGFR2/c-Kit,[20] but not for other stem cell markers, have the ability to differentiate towards endothelial cells (ECs), albeit to a lesser extent. Although the cellular origins of these AdSPCs remain to be elucidated, an elegant study has suggested some of them origin from the differentiated SMCs in the media.[30] Pathologically, these local residential AdSPCs have been suggested as one of the main cellular sources for neointima cells including SMCs during arterial remodelling in response to mechanical injury,[1,8] vascular grafting,[2,7,25,31] or acute/chronic inflammation,[13] through their differentiation into SMCs or SMC-like cells in intima. Despite several signalling pathways including integrin/collagen IV axis,[2] EGFR/$ERK_{1/2}$/β-catenin,[29] c-Myb/myocardin,[32] CXCR4,[22] and DKK3 (dickkopf 3)/transforming growth factor-β (TGF-β)/ATF6/Wnt signalling[33] have been implicated in SMC differentiation from these AdSPCs, a detailed description of how these AdSPCs are driven to differentiate into SMCs in intima is currently incomplete.

Matrix metalloproteinase-8 (MMP8), also known as collagenase-2, has potent proteolytic activity on matrix proteins such as fibrillar collagens, laminin, fibronectin,[34] and fibromodulin,[35] as well as a variety of other proteins [e.g. chemokines CXCL5[36] and CXCL11,[37] angiotensin I (Ang I),[38] a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10),[39,40] and TGF-β[35]]. Compelling evidence has suggested a role for MMP8 in the pathogenesis of atherosclerosis and related cardiovascular conditions such as myocardial infarction, heart failure, neointima formation following angioplasty, and abdominal aortic aneurysm.[41] Specifically, previous studies have shown that macrophages, SMCs, ECs,[42] and bone marrow-derived SPCs (BM_SPCs)[40] in atherosclerotic lesions express MMP8. Moreover, increased intraplaque MMP-8 levels are associated with carotid plaque progression lesion progression in asymptomatic patients,[43] and raised plasma MMP8 levels are an independent predictor for cardiovascular mortality in men,[44] highlighting a role for MMP8 in atherosclerosis and cardiovascular diseases. Indeed, by generating MMP8-deficient mice we are the first to confirm a causal role for MMP8 in the pathogenesis of atherosclerosis.[38] Importantly, we observed less SMC content within atherosclerotic plaques[38] and vascular injury-induced neointima[39] in MMP8-deficient mice, which may result from a lower level of SMC proliferation and migration from media, an impaired migratory ability of SPCs, and/or decreased capacity of the SPCs to differentiate into SMCs. We have recently proved that MMP8 gene deficiency results in decreased SMC migration and proliferation,[39] and MMP8-deficient SPCs exhibit an impaired ability to migrate into intima.[40] However, the importance of MMP8 in SMC differentiation from AdSPCs remains to be explored. In this study, we examined the functional importance of MMP8 in SMC differentiation from AdSPCs, and further elucidated the molecular mechanisms involved.

# 2. Methods

## 2.1 Materials and data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## 2.2 Cell isolation, culture, and treatments

The procedures for mouse aortic adventitia stem/progenitor cell (AdSPC) isolation and culture were similar to the protocols described in the previous study[8,45] with slight modifications. Briefly, mice (25–30 g) were euthanized by CO2 and the thoracic aortas were dissected to remove the surrounding connective tissues. Aortas were washed three times with PBS supplemented with 1% penicillin/streptomycin (P/S), and incubated in collagenase I for 15 min. Adventitia were carefully dissected away under a dissecting microscope, and cut into 1–2 mm size. Tissue blocks from 10 mice were pooled together and incubated with 3 mg/mL type II collagenase in DMEM with a 1/5 (w/v) ratio of tissue (g) to enzyme solution (mL). After incubation for 30 min, the same volume of 1 mg/mL elastase solution was added to the solution containing the tissue and collagenase. The tissues were incubated for another 1–2 h until all the tissues were digested. After filtering with a Cell Strainer (70 μm), single cell digestion solution was centrifuged to remove the digestion solution. Cells were re-suspended in AdSPC maintenance medium (DMEM with 2% chick embryo extract, 1% FBS, 1% N2, 2% B27, 100 nM retinoic acid, 50 nM 2-mercaptoethanol, 1% P/S, and 20 ng/mL bFGF) and transferred to six-well culture plate pre-coated with 1% CellStart (Invitrogen, A1014201). AdSPCs were maintained in the same medium for up to 10 passages. Every batch of AdSPCs at passage 3 was tested by AdSPC marker Sox10 and Nestin staining to ensure the purity of primary AdSPCs above 95%.

Detailed protocol for bone marrow-derived macrophage (BMM) culture was described in our previous study.[35] MagCellect™ Mouse Hematopoietic Cell Lineage Depletion Kit (MAGM209, R&D System) was used to isolate BM_SPCs according to the manufacturer's instructions as described in our previous study.[40]

## 2.3 Three protocols were used for SMC differentiation from AdSPCs

**TGF-β1 protocol**: Undifferentiated AdSPCs (p3–p10) were cultured in SMC differentiation induction medium (DMEM supplemented with 5% FBS and 5 ng/mL TGF-β) for 2–6 days. The medium was refreshed every other day.

**BMM co-culturing system:** Freshly prepared BMMs were co-cultured with AdSPCs (p3–p10) (1:1) in DMEM supplemented with 5% FBS for 2–6 days. The medium was refreshed every other day.

**BMM conditional culture medium**: Undifferentiated AdSPCs (p3–p10) were cultured in BMM conditional culture medium (CM) for 2–6 days. The CM was refreshed every day.

## 2.4 SMC gene promoters and CSL mutants

SMC gene promoters (pGL3-SMαA/SM22α-WT)[46] and their corresponding serum response factor (SRF) binding site mutants (pGL3-SMαA/SM22α-SRF$^{mut}$)[47] were generated in our previous studies. CBF1, Suppressor of Hairless, and Lag-1 (CSL) binding site mutation was introduced into pGL3-SMαA/SM22α-WT plasmids by using QuikChange™ site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions, and designated as pGL3-SMαA/SM22α-CSL$^{mut}$. All vectors were verified by DNA sequencing.

## 2.5 Animal experiments, anaesthesia, and euthanasia

All animal experiments were conducted according to the Animals (Scientific Procedures) Act of 1986 (United Kingdom). All the animal procedures were approved by Queen Mary University of London ethics review board (PPL number: 70/7216), and conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH guidelines (Guide for the care and use of laboratory animals). For mouse carotid artery denudation injury and macrophage transplantation, anaesthesia was induced using 100% $O_2$/4% isoflurane, and was maintained throughout the procedure by the administration of 100% $O_2$/2% isoflurane. At the end of protocol, all mice were euthanized by placing them under deep anaesthesia with 100% $O_2$/5% isoflurane, followed by decapitation.

## 2.6 Mouse carotid artery denudation injury and perivascular transplantation of macrophages

MMP8_KO mice on apolipoprotein E$^{-/-}$/C57BL/6 genetic background were generated in our previous studies.[38–40] The surgical procedure for carotid artery denudation injury was performed as previously described.[39,48,49] Removal of the endothelium of the left common carotid artery was achieved by 3–5 passages of a 0.38 mm curved flexible wire (Reference Part Number: C-SF-15-20, Cook Medical European Shared Services, Ireland). After the vascular injury, the injured carotid arteries were randomly embedded with Matrigel containing vehicle, WT, or MMP8_KO BMMs. Briefly, immediately after injury, 100 μL Matrigel mixed with 20 μL of cell culture medium containing $1 \times 10^6$ WT or MMP8_KO BMMs or culture medium alone (vehicle) was applied perivascularly to injured carotid arteries. At Days 3, 14, and 28 post-injury, the injured carotid arteries were harvested for gene expression, immunofluorescence staining, and morphometric analysis, respectively. For gene expression assay, 3–5 injured carotid femoral arteries (~5.0 mm) from each group were pooled for each independent experiment to ensure good quality of RNA samples. All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals.

## 2.7 Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Graphpad Prism5. The Shapiro–Wilk Normality test was used for checking the normality of the data. Two tailed unpaired Student's t-test was used for comparisons between two groups, or one-way analysis of variance with a *post hoc* test of LSD was applied when more than two groups were compared if the data display a normal distribution. Conversely, non-parametric Mann–Whitney *U* test or Kruskal–Wallis H test was applied for comparing two groups and three or more groups, respectively, if the data did not display normal distribution or if the number of observations from each group was smaller than 5 ($n < 5$). Alpha = 0.05 was chosen as the significance level, and a value of $P < 0.05$ was considered as statistically significant.

Additional materials and methods used in this study are described in detail in the Supplementary material online, include BMM culture; SMC differentiation from embryonic stem (ES) cells; short hairpin RNA lentiviral infection or siRNA transfection; plasmid transient transfection and luciferase assay; real-time quantitative PCR (RT-qPCR); immunoblotting; indirect immunofluorescent staining for cells; chromatin immunoprecipitation (ChIP) assays; morphometric analysis, quantification of lesion formation, and tissue immunofluorescence staining.

# 3. Results

## 3.1 MMP8 plays an important role in SMC differentiation from ES cells

We have previously established a simple but efficient approach to derive SMCs from ES cells,[50] and extensively proven that this model provides a powerful platform for us to uncover the potential important regulators governing SMC differentiation. To study the potential involvement of MMP8 in this model, we first detected if MMP8 expression is altered during SMC differentiation from ES cells. Indeed, both gene (Supplementary material online, *Figure S1A*) and protein (Supplementary material online, *Figure S1B*) expression levels of MMP8 were significantly up-regulated from Day 2 of differentiation and maintained at a higher expression level over the 8-day differentiation period compared to that of the undifferentiated (Day 0) ES cells. Interestingly, such up-regulation appears to precede SMC-related protein expression, inferring an involvement for MMP8 in SMC differentiation from ES cells (Supplementary material online, *Figure S1A* and *B*). Importantly, all four SMC differentiation genes were significantly down-regulated by MMP8 gene knockdown (Supplementary material online, *Figure S1C* and *D*), confirming an essential role for MMP8 in SMC differentiation from ES cells.

## 3.2 No significant difference was observed in SMC differentiation from control and MMP8-deficient AdSPCs

It worth mentioning that our ES-SMC differentiation model is an excellent platform to study SMC differentiation and maturation during embryonic development, but it is not bear close relevance to SMC differentiation in vascular disease and their contribution to vascular injury-induced restenosis. To establish such a disease-related SMC differentiation model, we first isolated and characterized AdSPCs using a previously reported protocol with modifications.[8,45] Double immunofluorescent staining confirmed that these AdSPCs were strongly stained positive for the reported stem cell markers including Sox1, Sox10, Sox17, and Nestin, but negative for CD45 (leucocyte), fibroblast-specific protein 1 (FSP1) (fibroblast), smooth muscle myosin heavy chain (SM-MHC) (SMC) and CD31 (EC) (Supplementary material online, *Figure S2A–D*). RT-qPCR data showed that the AdSPCs could be maintained *in vitro* for a long period (up to 10 passages, P10) without apparent change of gene expression. However, a decreased and increased expression for stem cell marker genes and SMC genes was observed in AdSPCs at later stage (P12), respectively (Supplementary material online, *Figure S2E*). Therefore, AdSPCs between P3 and P10 were used in this study. Importantly, when cultured in stem cell culture medium, these AdSPCs could maintain a high expression level of the abovementioned stem cell

markers with a very low expression level or even absence of SMC markers (SMαA and SM-MHC). However, when they were incubated with SMC differentiation medium containing 5 ng/mL TGF-β1, we observed a significant increase in SMC markers at both RNA (*Figure 1A*) and protein (*Figure 1B*) levels. Similarly, the gene expression of SMC differentiation transcription factor, SRF and its co-activator myocardin, were also significantly increased during SMC differentiation from AdSPCs, along with another SMC differentiation regulator, myocyte enhancer factor 2C (*Figure 1C*). As expected, both Sox10 and Sox17 gene expression were dramatically down-regulated over the 6-day differentiation period (*Figure 1D*), further confirming SMC differentiation from AdSPCs.

Unlike the finding from ES-SMC differentiation model, we unexpectedly observed a slight increased MMP8 expression at both RNA (*Figure 1A*) and protein level (*Figure 1B*) at a very late SMC differentiation stage (Day 6). To investigate whether MMP8 played a role in AdSPCs differentiation toward SMCs, we isolated AdSPCs from both MMP8 knockout (MMP8_KO) mice and their wildtype (WT) control littermates and compared their ability to differentiate into SMCs. Surprisingly, we found no significant effect of MMP8 gene inactivation on SMC differentiation from AdSPCs (Supplementary material online, *Figure S3*). This unexpected observation prompted us to examine the expression levels of MMP8 in these AdSPCs. Indeed, data from RT-qPCR (Supplementary material online, *Figure S4A*) and western blot (Supplementary material online, *Figure S4B*) analysis showed that while ES cells, ES cell-derived SMCs, as well as BMMs and BM-derived SPCs (BM_SPCs) isolated from WT mice exhibited varying degrees of MMP8 expression at both the RNA and protein levels, AdSPCs isolated from WT mice expressed little MMP8. On the other hand, BMMs, BM-derived SPCs and AdSPCs isolated from MMP8_KO mice expressed no MMP8, further validating MMP8 gene knockout in these cells. Taken together, the above data demonstrated that unlike BM-derived SPCs, AdSPCs express very little or no MMP8, while AdSPC-derived SMCs express MMP8 at later stage, albeit to a very low level. MMP8_KO AdSPCs were used in the rest of experiments to study the effect of macrophage-derived MMP8 on SMC differentiation from AdSPCs to minimalize the experimental variation and avoid potential influence of AdSPC-derived MMP8 on SMC differentiation.

## 3.3 The secretory molecules from macrophages are responsible for SMC differentiation from AdSPCs

As mentioned previously, macrophages are a major cellular source of MMP8 within atherosclerotic plaques. Indeed, a previous study showed that macrophages control SMC differentiation from human adipose tissue-derived MSCs.[51] Moreover, the following points prompted us to hypothesize that macrophage-derived MMP8 plays an important role in the regulation of SMC differentiation from AdSPCs. (i) AdSPCs belong to MSC family within arteries; (ii) they express a similar pattern of cell markers to adipose tissue–derived MSCs; (iii) importantly, as we have previously shown, MMP8 plays a critical role in the regulation of TGF-β1 production from macrophages, its bioavailability and biological activity.[35] To begin with, we first examined whether macrophages can control SMC differentiation. Our data revealed that co-culturing of macrophages with AdSPCs significantly promotes AdSPC differentiation towards SMCs as evidenced by an increased expression of SMC-related genes at both RNA and protein levels (Supplementary material online, *Figure S5A* and *B*), and decreased expression levels of Sox10 and Sox17 (Supplementary material online, *Figure S5C*). To investigate whether these findings are a result of direct, cell-to-cell interactions between macrophages and AdSPCs, or due to paracrine effects of macrophages, the culture medium of macrophages was collected and used. Following incubation of AdSPCs with macrophage culture medium, we observed a similar degree of SMC differentiation of AdSPCs to what has been described above (Supplementary material online, *Figure S6*), suggesting that secreted signals released by macrophages can induce SMC differentiation from AdSPCs.

## 3.4 Macrophage-derived MMP8 plays an important role in SMC differentiation from AdSPCs

Having determined that macrophage co-culture or conditioned culture medium can induce SMC differentiation from AdSPCs, we examined whether macrophage-derived MMP8 played a role in AdSPC differentiation towards SMCs. Incubation of MMP8_KO AdSPCs with macrophages isolated from MMP8_KO mice resulted in significantly decreased SMC marker expression in AdSPC, compared to AdSPCs incubated with macrophages from WT mice (*Figure 2A–C*). A similar phenomenon was observed when MMP8_KO AdPSCs were incubated with the culture medium taken from MMP8_KO macrophages (*Figure 2D–F*). These data demonstrate that macrophage-derived MMP8 plays an important role in promoting SMC differentiation from AdSPCs through a paracrine route.

To further examine the effects of MMP8 in the macrophage conditioned culture medium on AdSPC differentiation towards SMCs, the WT macrophage conditioned culture medium was incubated with an MMP8 neutralizing antibody prior to using in SMC differentiation. We observed a significant decrease of SMC gene expression in AdSPCs when they were cultured in the WT macrophage conditioned culture medium depleted of MMP8 protein (*Figure 2G*). Conversely, addition of exogenous activated MMP8 into the culture medium conditioned by MMP8_KO macrophages could dramatically promote SMC differentiation from AdSPCs (*Figure 2H*), further confirming an important role for macrophage-derived MMP8 in SMC differentiation from AdSPCs.

## 3.5 TGF-β activated by macrophage-derived MMP8 promotes SMC differentiation from AdSPCs

Since we have previously demonstrated that TGF-β1 production, as well as its bioavailability and biological activity in macrophages is regulated by MMP8,[35] and TGF-β1 is a powerful inducer for SMC differentiation from stem cells, we investigated whether TGF-β1 signalling is one of the underlying mechanisms through which macrophage-derived MMP8 mediates SMC differentiation from AdSPCs. Indeed, we observed that SMC gene expression was significantly up-regulated by addition of recombinant activated TGF-β1 protein into MMP8_KO macrophage conditional culture medium, and the gene expression was further increased when TGF-β1 was added into the WT macrophage conditional medium (*Figure 3A*). Importantly, compared to cells treated with TGF-β1 and MMP8_KO macrophage conditional medium we observed a higher level of SMC gene expression in AdSPCs incubated with WT macrophage conditional medium alone (*Figure 3A*), suggesting additional signalling may also responsible for macrophage-derived MMP8 mediated SMC differentiation from AdSPCs. As expected, an opposite effect for SMC gene

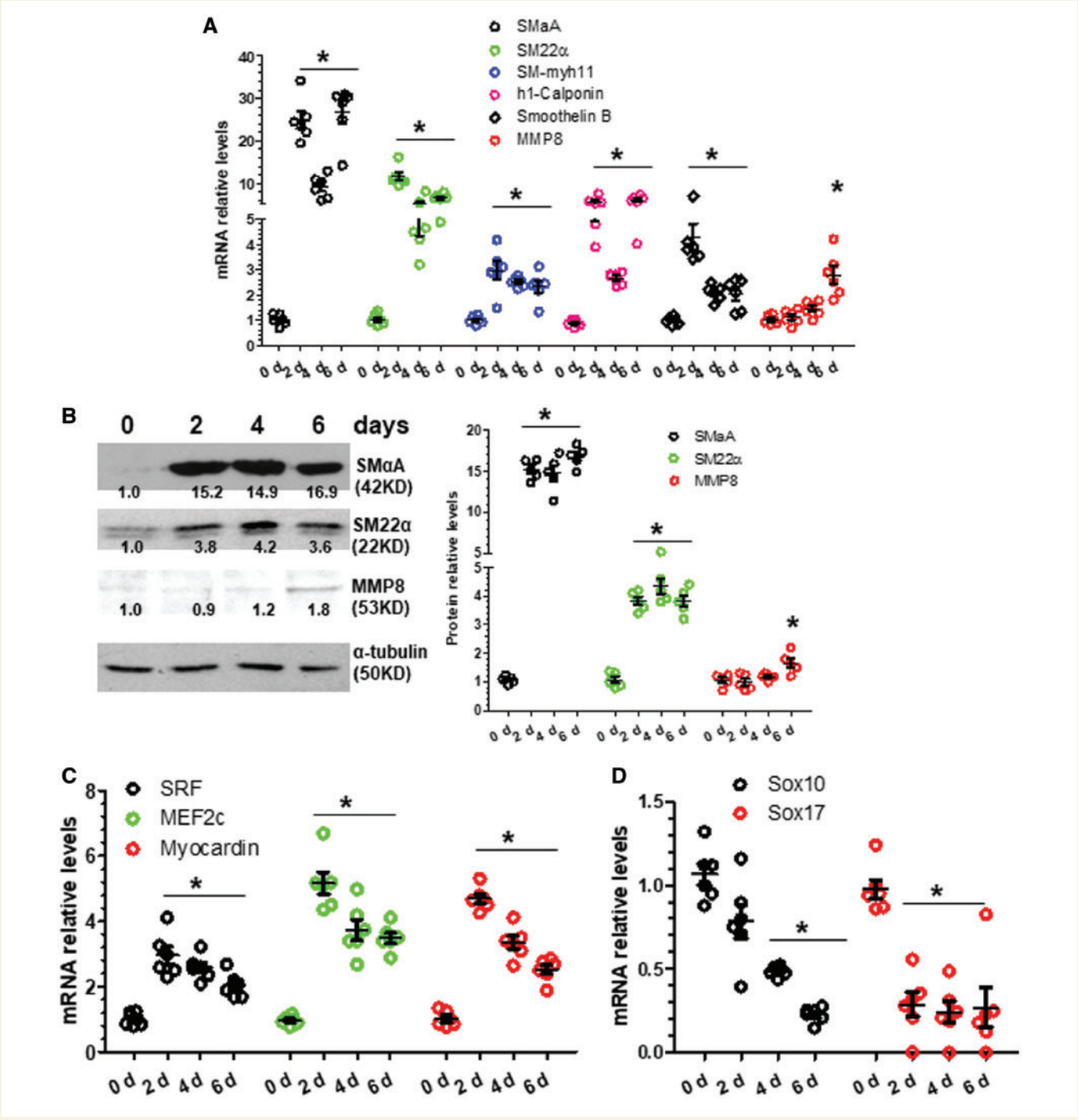


**Figure 1** SMC differentiation from AdSPCs. AdSPCs (p3–p10) were cultured in SMC differentiation induction medium (DMEM supplemented with 5% FBS and 5 ng/mL TGF-β1) for the indicated times. Undifferentiated AdSPCs were used as control (0 days). Total RNAs and proteins were harvested and subjected to RT-qPCR (*A*, *C*, and *D*), and western blot analyses (*B*), respectively. (*A*, *B*) SMC differentiation genes were significantly up-regulated in response to TGF-β1 treatment. (*C*) Activation of SMC-specific transcription factors during SMC differentiation. (*D*) Inhibition of AdSPCs specific genes. Data presented here are representative (*B*, left) or mean ± SEM of six independent experiments, respectively ($n = 6$). * $P < 0.05$ (vs. 0 days, one-way ANOVA with a *post hoc* test of LSD).

expression was observed when TGF-β1 was depleted from WT or MMP8_KO macrophage conditional culture medium using a neutralizing antibody (*Figure 3B*). Finally, the inhibitory effect of TGF-β1 depletion on SMC gene regulation was also mimicked by inhibiting TGF-β1 signal in WT macrophage conditional culture medium with a selective inhibitor SB431542 (*Figure 3C*). The above data suggest that macrophage-derived MMP8 promotes SMC differentiation from AdSPCs at least partially through modulating TGF-β1 signalling.

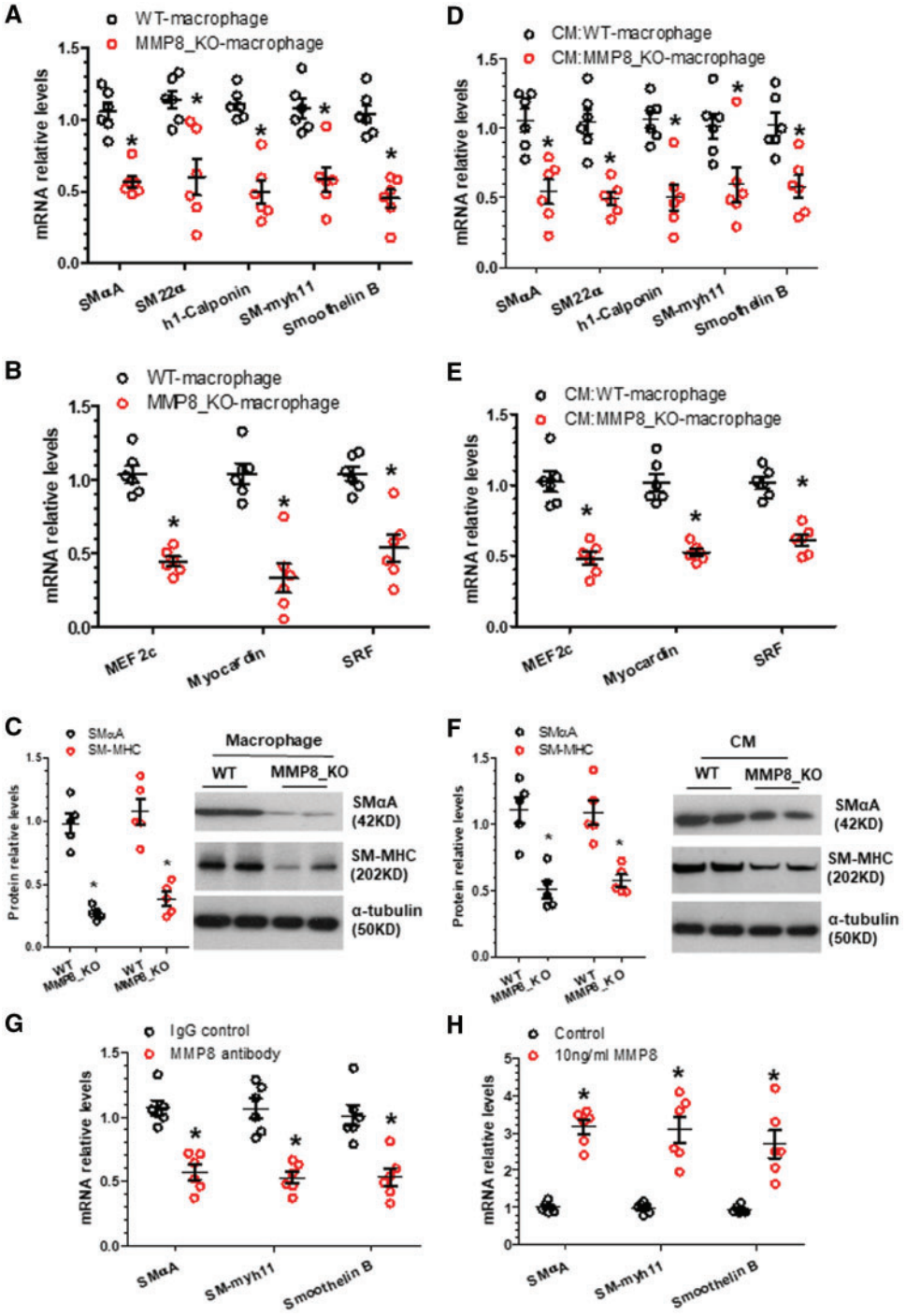


**Figure 2** An important role of macrophage-derived MMP8 in SMC differentiation from AdSPCs. (*A–C*) AdSPCs (p3–p10) isolated from MMP8_KO mice were co-cultured (1:1) with BMMs isolated from wild type (WT) and MMP8_KO mice for 48 h. (*D–F*) MMP8_KO AdSPCs (p3–p10) were incubated with the conditioned culture medium (CM) from WT and MMP8_KO BMMs for 48 h. Total RNAs and proteins were harvested and subjected to RT-qPCR and western blot (*C* and *F*) analyses, respectively. (*G*) Inhibition of MMP8 causes a decreased expression of SMC genes. WT BMM CM was incubated with MMP8 antibody or IgG control (1 μg/mL) at 37°C for 30 min. After then, BMM CM were used to induce SMC differentiation from MMP8_KO AdSPCs. (*H*) Activated MMP8 protein increased SMC gene expression. MMP8_KO BMM CM contained 10 ng/mL of activated MMP8 protein or vehicle was used to induce MMP8_KO AdSPC differentiation towards SMCs. Two days later, total RNAs were harvested and subjected to RT-qPCR analysis. Data presented here are representative (right panel in *C* and *F*) or mean ± SEM of five or six independent experiments, respectively ($n = 5$ or 6). *$P < 0.05$ (vs. WT-macrophage or CM:WT-macrophage, *t*-test).

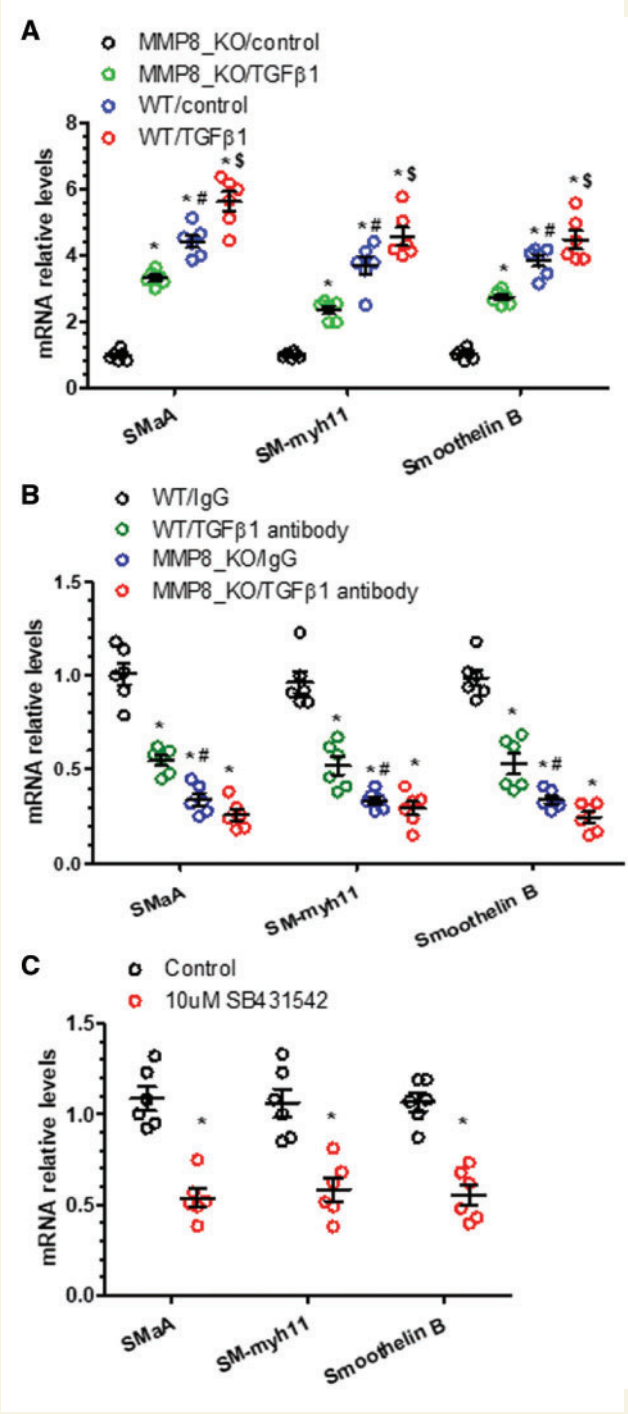


**Figure 3** TGF-β1 secreted by BMMs is responsible for MMP8-mediated SMC differentiation from AdSPCs. (*A*) Exogenous TGF-β1 protein increased SMC gene expression. WT or MMP8_KO BMMs conditioned culture medium (CM) contained 5 ng/mL of activated TGF-β1 protein or vehicle was used to induce MMP8_KO AdSPC differentiation towards SMCs. (*B*) Depletion of TGF-β1 results in decreased expression of SMC genes. WT or MMP8_KO BMMs CM was incubated with TGF-β1 antibody or IgG control (1 μg/mL) at 37°C for 30 min. After then, the BMM CM was used to induce SMC differentiation from MMP8_KO AdSPCs. In (*A*, *B*), $^{*}P < 0.05$ (vs. MMP8_KO/control or WT/IgG); $^{\#}P < 0.05$ (vs. MMP8_KO/TGFβ1 or WT/TGFβ1 antibody); $^{\$}P < 0.05$ (vs. WT/control or MMP8_KO/IgG); one-way ANOVA with a *post hoc* test of LSD. (*C*) SMC gene expressions were decreased by TGF-β1 inhibition. MMP8_KO AdSPCs were incubated with WT BMMs CM in the presence or absence of 10 μM SB431542, a selective TGF-β1 inhibitor. Two days later, total RNAs were harvested and subjected to RT-qPCR analysis. * $P < 0.05$ (vs. control; *t*-test). Data presented here are mean ± SEM of six independent experiments ($n = 6$).

## 3.6 ADAM10-Notch1 signalling pathway is activated by macrophage-derived MMP8 during SMC differentiation from AdSPCs

Our previous studies have shown that MMP8 promotes ADAM10 maturation by cleaving its prodomain.[39,40] Moreover, it has been reported that ADAM10 is required for Notch1 site 2 cleavage and activation,[52] and that Notch1 and TGF-β1 signalling concomitantly regulate SMC differentiation during VSMC phenotype switching.[53] Furthermore, data from the previous section suggest additional signal pathway may play a role in macrophage-derived MMP8 mediated SMC differentiation from AdSPCs. Therefore, we wondered if ADAM10-Notch1 signalling is another underlying mechanism of macrophage-derived MMP8 mediated SMC differentiation from AdSPCs. Immunofluorescence staining with an antibody against the prodomain of ADAM10 showed abundant ADAM10 containing its prodomain in AdSPCs incubated with MMP8_KO macrophage conditional culture medium (*Figure 4A*), indicating that the ADAM10 maturation and activity is inhibited in the absence of MMP8 in AdSPCs. Moreover, less activated Notch1 accumulated within the nuclei of AdSPCs treated with MMP8_KO macrophage conditional culture medium (*Figure 4B*), suggesting that the Notch1 signal is inhibited in AdSPCs when MMP8 protein was depleted from the culture system. Such inhibition was further confirmed in our luciferase activity assay using pGL2-4xCSL-luc (reporter for Notch signalling) (*Figure 4C*). Consistently, the Notch signalling was significantly activated by addition of the recombinant MMP8 and ADAM10 activated proteins into the culture system (*Figure 4D and E*). Taken together, the above data demonstrate that the ADAM10/Notch1 signal pathway is activated by macrophage-derived MMP8.

The importance of ADAM10 in macrophage-derived MMP8 mediated SMC differentiation was further examined by using the 'gain or loss of function' experiments. Addition of activated ADAM10 protein into the WT or MMP8_KO macrophage conditional culture medium significantly up-regulated the expression levels of all the SMC genes examined (*Figure 4F*). As expected the highest SMC gene expression was observed when activated ADAM10 protein was added into WT macrophage conditional medium. Interestingly, we observed a higher level of SMC gene expression in AdSPCs incubated with WT macrophage conditional medium alone compared to cells treated with ADAM10 and MMP8_KO macrophage conditional medium (*Figure 4F*). Importantly, an opposite effect for SMC gene expression was observed when the endogenous ADAM10 in AdSPCs was inhibited by siRNA (*Figure 4G*). Taken together, these data support an important role for ADAM10 in SMC differentiation from AdSPCs mediated by macrophage-derived MMP8.

## 3.7 Notch1 promotes SMC differentiation from AdSPCs, and CSL binding site is required for Notch1-induced SMC gene expression

To further explore the potential role of the Notch family in SMC differentiation from AdSPCs, the over-expression vectors for individual

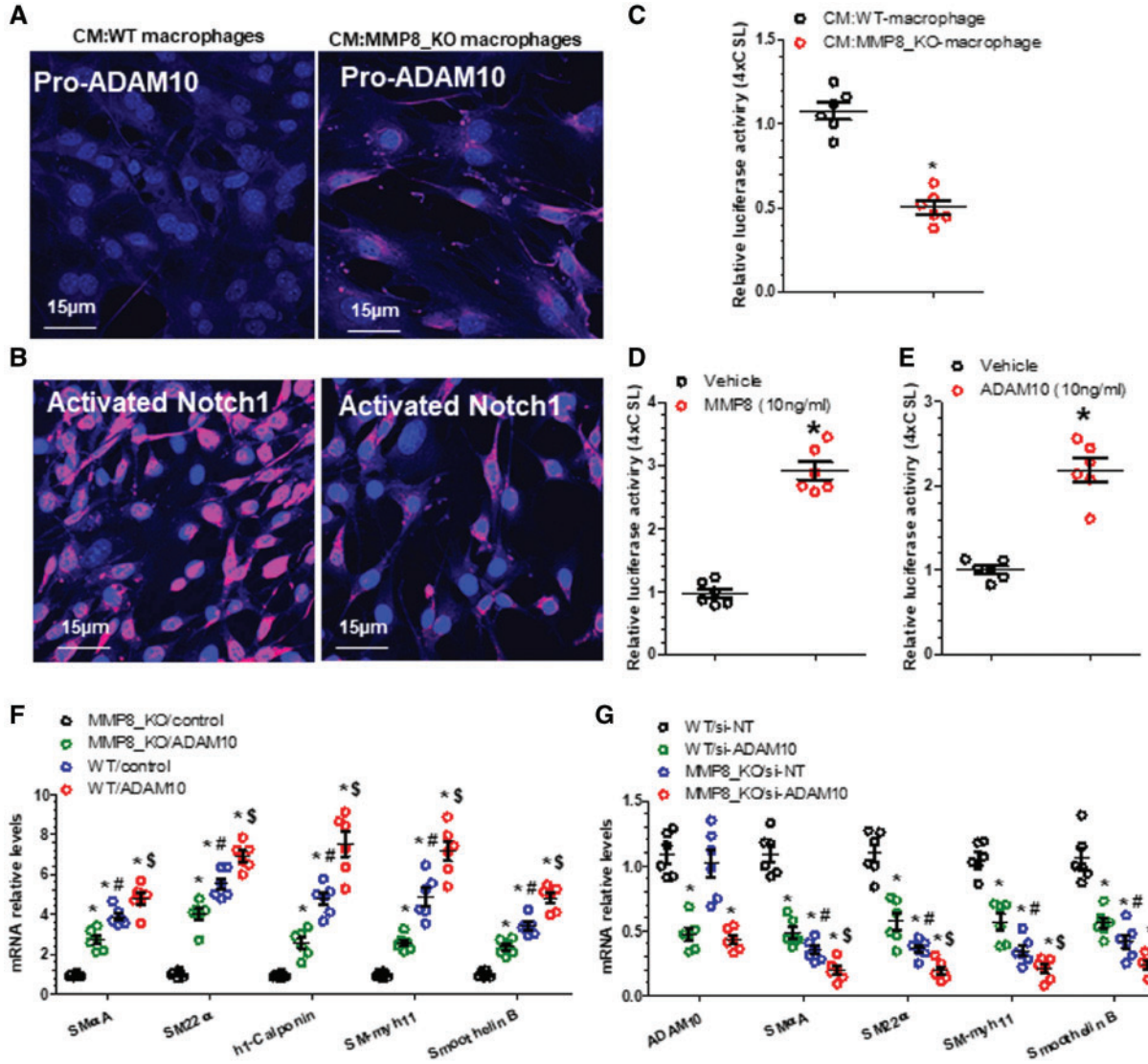


**Figure 4** Importance of ADAM10-Notch1 signalling in macrophage-derived MMP8 mediated SMC differentiation from AdSPCs. (*A* and *B*) Increased pro-ADAM10 protein abundance, but reduced Notch1 nuclear translocation in MMP8_KO AdSPCs treated with MMP8_KO BMMs conditional culture medium (CM). MMP8_KO AdSPCs were incubated with WT or MMP8_KO BMMs CM for 2 days. Cells were fixed and subjected to immunofluorescence staining with antibodies against the prodomain of ADAM10 (*A*) or activated Notch1 (*B*). Representative images from three experiments ($n = 3$) were presented here. (*C–E*) Luciferase activity assays to examine Notch signalling. MMP8_KO AdSPCs transfected with pGL2-4xCSL-luc reporter plasmid were incubated with CM from WT or MMP8_KO BMMs (*C*), or CM from MMP8_KO BMMs in the absence (vehicle) or presence of activated MMP8 (*D*)/ADAM10 (*E*) proteins, respectively. Two days later, total cell lysates were harvested and subjected to luciferase activity assays. (*F*) Activated ADAM10 protein increased SMC gene expression. CM from WT or MMP8_KO BMMs contained 10 ng/mL of activated ADAM10 protein or vehicle was used to induce MMP8_KO AdSPC differentiation towards SMCs. (*G*) Knockdown of ADAM10 inhibits SMC gene expression. MMP8_KO AdSPCs transfected with control (si-NT) or ADAM10 (si-ADAM10) specific siRNA were incubated with CM from WT or MMP8_KO BMMs. Two days later, total RNAs were harvested and subjected to RT-qPCR analysis. Data presented here are representative (*A* and *B*) or mean ± SEM (*C–G*) of six independent experiments ($n = 6$). In (*C–E*), $^{*}P < 0.05$ (vs. CM: WT-macrophage, vehicle, control, or control siRNA, *t*-test); in (*F–G*), $^{*}P < 0.05$ (vs. MMP8_KO/control or WT/si-NT); $^{\#}P < 0.05$ (vs. MMP8_KO/ADAM10 or WT/si-ADAM10); $^{\$}P < 0.05$ (vs. WT/control or MMP8_KO/si-NT); one-way ANOVA with a *post hoc* test of LSD.

Notch (pCMV-flag-NICD1, 2, 3, and 4) were introduced into MMP8_KO AdSPCs. We observed that over-expression of Notch1 (*Figure 5A*), but not other Notchs (Supplementary material online, *Figure S7*), significantly increased SMC-related gene expression, indicating that Notch1 is the main Notch responsible for macrophage-derived MMP8 mediated SMC differentiation from AdSPCs. It has been well recognized that SRF/Myocardin complex and its corresponding DNA binding element CArG boxes within the promoter of SMC-related genes is a central player in regulation of SMC gene expression and SMC differentiation from stem cells, we wondered if such a mechanism is behind Notch1-

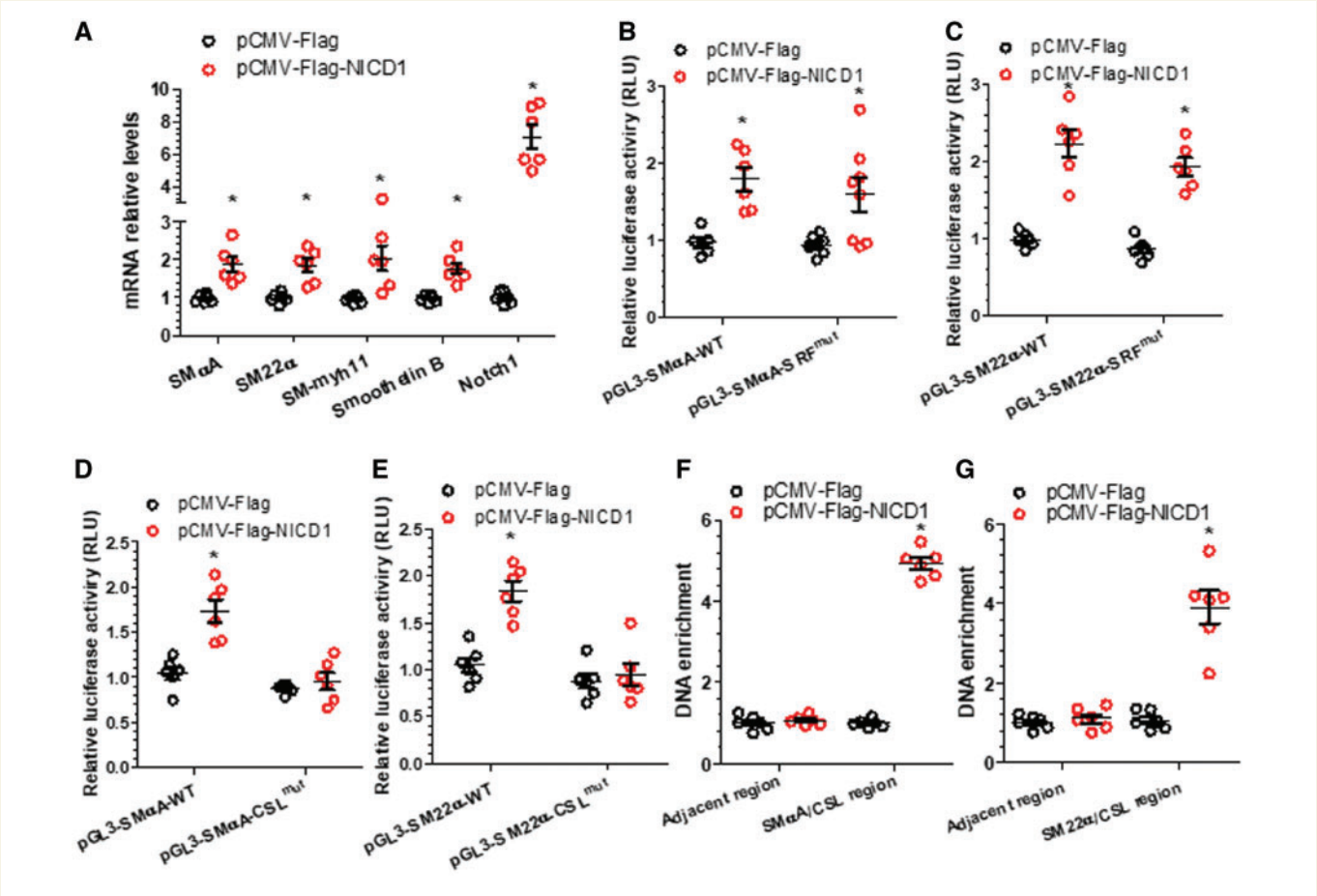


**Figure 5** Notch1 promotes SMC differentiation from AdSPCs, and CSL binding site is required for Notch1-induced SMC gene expression. (*A*) Over-expression of activated Notch1 (NICD1) in MMP8_KO AdSPCs increases SMC gene expression. MMP8_KO AdSPCs transfected with control (pCMV-flag) or activated Notch1 over-expression (pCMV-flag-NICD1) plasmids were incubated with CM from MMP8_KO BMMs. Two days later, total RNAs were harvested and subjected to RT-qPCR analysis. (*B* and *C*) SRF binding site (CArG) is not required for Notch1-induced SMC gene transcription. MMP8_KO AdSPCs co-transfected with pCMV-flag or pCMV-flag-NICD1 and reporter plasmids harbouring a native (WT) or CArG mutated ($SRF^{mut}$) promoter DNA sequence of SMαA (*B*)/SM22α (*C*) gene as indicated in the figures were incubated with CM from MMP8_KO BMMs. (*D* and *E*) CSL binding site (TGTGGGCA) within SMC gene promoters is critical for Notch1-induced SMC gene transcription. MMP8_KO AdSPCs co-transfected with pCMV-flag or pCMV-flag-NICD1 and reporter plasmids harbouring a native (WT) or CSL DNA binding motif mutated ($CSL^{mut}$) promoter DNA sequence of SMαA (*D*)/SM22α (*E*) gene as indicated in the figures were incubated with CM from MMP8_KO BMMs. Two days later, total cell lysates were harvested and subjected to luciferase activity assays. (*F* and *G*) Notch1 over-expression increases its enrichment within SMC gene promoter. ChIP assays were performed using antibodies against Flag (NICD1) or normal IgG, respectively, as described in online supplemental data. PCR amplifications of the adjacent regions were included as additional control for specific promoter DNA enrichment. Data presented here are Mean ± SEM of six independent experiments ($n = 6$). * $P < 0.05$ (vs. pCMV-Flag; *t*-test).

induced SMC gene regulation in AdSPCs. Data from luciferase activity assays, using the SMC gene promoter reporters containing the native (pGL3-SMαA/SM22α-WT) or CArG mutated (pGL3-SMαA/SM22α-$SRF^{mut}$) promoter DNA sequences of SMC genes, generated in our previous study,[47] showed that Notch1 significantly increased the WT gene promoter activity, and this was seen with the CArG mutated reporters as well (*Figure 5B and C*), suggesting that Notch1-induced SMC gene expression is independent of SRF/Myocardin complex.

Interestingly, a previous study has reported that SMαA is a direct target gene of Notch1, and human SMαA promoter contains a CSL consensus binding site (TGGGAA) beginning at ~64 from the translational starting site that is conserved in apes and rodents.[54] Indeed, after carefully searching the promoter sequences used for generating our mouse SMαA and SM22α gene reporters, we identified a DNA sequence, TGTGGGCA, which resembles but is not identical to the consensus binding site [(C/T)GTGGGAA] for CSL transcription factors, within both gene reporters. We therefore speculated that this DNA sequence serves as a functional CSL binding site. To confirm such a hypothesis, this sequence was mutated to generate CSL mutated gene reporters (pGL3-SMαA/SM22α-$CSL^{mut}$). Luciferase data with these gene reporters showed that while over-expression of Notch1 in AdSPCs significantly increased the WT gene promoter activity, this was almost abolished when the CSL binding site was mutated (*Figure 5D and E*), revealing a dependence on this DNA element for Notch1-induced SMC gene expression. Finally, ChIP assays were conducted using a Flag antibody (NICD1) in the differentiating AdSPCs to further verify if Notch1 activates specific

SMC gene transcription through its enrichment within SMC gene promoters. Data shown in *Figure 5F and G* revealed that NICD1 overexpression significantly increased its binding to the promoters of SMαA and SM22α. Taken together, the above findings demonstrate that Notch1 regulates SMαA and SM22α gene expressions during SMC differentiation from AdSPCs by increasing NICD1 enrichment within SMC gene promoters, and CSL binding site is required for SMC gene transcription regulated by Notch1.

### 3.8 Macrophage-derived MMP8 promotes neointima formation in response to vascular injury

Having established an important role for macrophage-derived MMP8 in SMC differentiation from AdSPCs, we further examined its involvement in arterial remodelling. To this end, we first examined the gene expression profiles during arterial remodelling in response to wire-induced vascular injury. Data shown in *Figure 6A* revealed that the gene expression level of SMαA was significantly decreased, while the macrophage gene CD68 along with MMP8 expression was dramatically increased during arterial remodelling induced by wire-injury. Importantly, compared to control arteries multiple AdSPC gene expression markers were significantly up-regulated following injury (*Figure 6B*). The increased expression of MMP8, CD68, Sox10, and Nestin in the injured arteries was further confirmed by immunostaining assays (Supplementary material online, *Figure S8*). Moreover, we observed MMP8 was mainly co-expressed with CD68 in neointima (Supplementary material online, *Figure S8*). Furthermore, data shown in Supplementary material online, *Figure S9* revealed that transplanted macrophages could migrate across the adventitia and media layer and infiltrate into intima, and that the transplanted macrophages was the major cellular source of MMP8 during arterial remodelling in response to injury. These data indicate that the AdSPCs are activated and infer an involvement for AdSPC activation and/or differentiation during arterial remodelling, consistent with previous findings.[8] These data also suggest a potential role for MMP8 derived from activated macrophages in injury-induced arterial remodelling. To investigate its role, vehicle (Matrigel) and BM-derived macrophages isolated from WT or MMP8_KO mice were perivascularly applied to carotid arteries immediately after injury similar to the protocol described in our previous studies.[48,49,55] Compared to WT macrophage transplantation, applying MMP8_KO macrophages onto the injured carotid arteries caused a significant decrease in the expression levels of SMC genes (SMαA, SM22α, h1-calponin, and SM-myh11) and cell proliferation marker gene PCNA, but this had no apparent effect on the AdSPC gene expression examined in this study (*Figure 6C*). However, compared with vehicle control a trend of decreased expression of stem cell genes but a significant up-regulation of PCNA and SMC genes was observed in mice transplanted with either WT or MMP8_KO macrophages (*Figure 6C*). Importantly, we observed an increased amount of inactivated ADAM10 (Pro-ADAM10) (*Figure 6D*), but a decreased level of activated Notch1 staining in the injured carotid arteries transplanted with MMP8_KO macrophages (*Figure 6E*), compared with the injured carotid arteries treated with WT macrophages or vehicle control, indicating that the ADAM10-Notch1 signalling is inhibited in the injured vessels in the absence of macrophage-derived MMP8. Consequently, the injury-induced neointimal SMC hyperplasia was significantly increased in the carotid arteries transplanted with either WT or MMP8_KO macrophages. Importantly, when compared with the mice transplanted with WT macrophages a much smaller neointima size was observed in the mice received a MMP8_KO macrophage transplantation (*Figure 6F and G*), confirming a promotive effect of macrophages in neointimal lesion growth, and macrophage-derived MMP8 represents one of the underlying mechanisms of macrophage-promoted neointimal SMC hyperplasia.

## 4. Discussion

The de-differentiation and proliferation of SMCs (SMC phenotype switching) has been widely established as the major contributor to vascular remodelling. However, increasing evidence[2,7,8,11–16] has also suggested an important involvement for a variety of vascular SPCs including AdSPCs, although the extent of their contribution remains to be elucidated and is source of great debate.[45,56] There is a growing consensus that in response to injury, AdSPCs migrate into the intima, where they differentiate toward SMCs, contributing to neointimal lesion growth. However, the underlying molecular mechanisms of SMC differentiation from AdSPCs in the intima remains to be determined before we can target this cellular differentiation in order to prevent angioplasty-induced restenosis. In this study, we provide evidence to suggest that macrophage-derived MMP8 is one of the driving forces behind SMC differentiation from AdSPCs and promotes neointimal SMC hyperplasia in response to injury. Macrophage-derived MMP8 exerts this function through activation of TGF-β1 signalling in parallel with the ADAM10/Notch1 signalling pathway (*Figure 6H*).

The reasons for selecting the MSC-like and Sox10/Nestin-positive AdSPCs, but not the Sca-1$^+$ cells described in our previous studies[57,58] in this study are two-fold. On one hand, although we have successfully isolated adventitial Sca-1$^+$ cells from mouse aortas and used them to validate the functional involvements of multiple SMC differentiation regulators, identified from our ES cell-SMC differentiation model, in SMC differentiation from vascular stem cells, we have found that the yield of the adventitia Sca-1$^+$ cells is very limited and only allowed us to conduct a few small validation experiments. Moreover, we experienced enormous difficulties in maintaining them in an undifferentiated state. Stem cell properties were lost quickly during *in vitro* cell culture regardless of which stem cell maintenance culture medium was used and whether or not repeated cell sorting was applied to them. On the other hand, we obtained a much higher yield of MSC-like and Sox10/Nestin-positive AdSPCs (30–50 times higher than that of Sca-1$^+$ cells from same amount of starting material), and found that culturing these AdSPCs is less laborious and time consuming. Importantly, these cells can be maintained *in vitro* for a long period (over 10 passages, P10) without any significant loss of stem cell properties, as evidenced by almost all of them expressing high levels of MSC markers (such as Sox10 and Nestin), and exhibiting the potential to differentiate toward multiple cell lineages (data not shown) including SMCs even at higher passages (P10). Moreover, new studies from Li's group has elegantly demonstrated that Sox10$^+$ MSCs can differentiate into SMCs to stabilize functional microvessels,[59] contribute to vascular pericytes/SMCs in most parts of the body,[60] and importantly established them as one of the cellular sources in the neointimal formation,[61] suggesting that these AdSPCs are a good cellular model to study the underlying molecular mechanisms of SMC differentiation from adult vascular SPCs and their contribution to neointimal SMC hyperplasia.

The importance of MMP8 in vascular diseases has been well-documented in several preclinical and clinical studies by others and our group, which has been nicely summarized in an elegant review.[41] Through a series of studies, we have comprehensively demonstrated

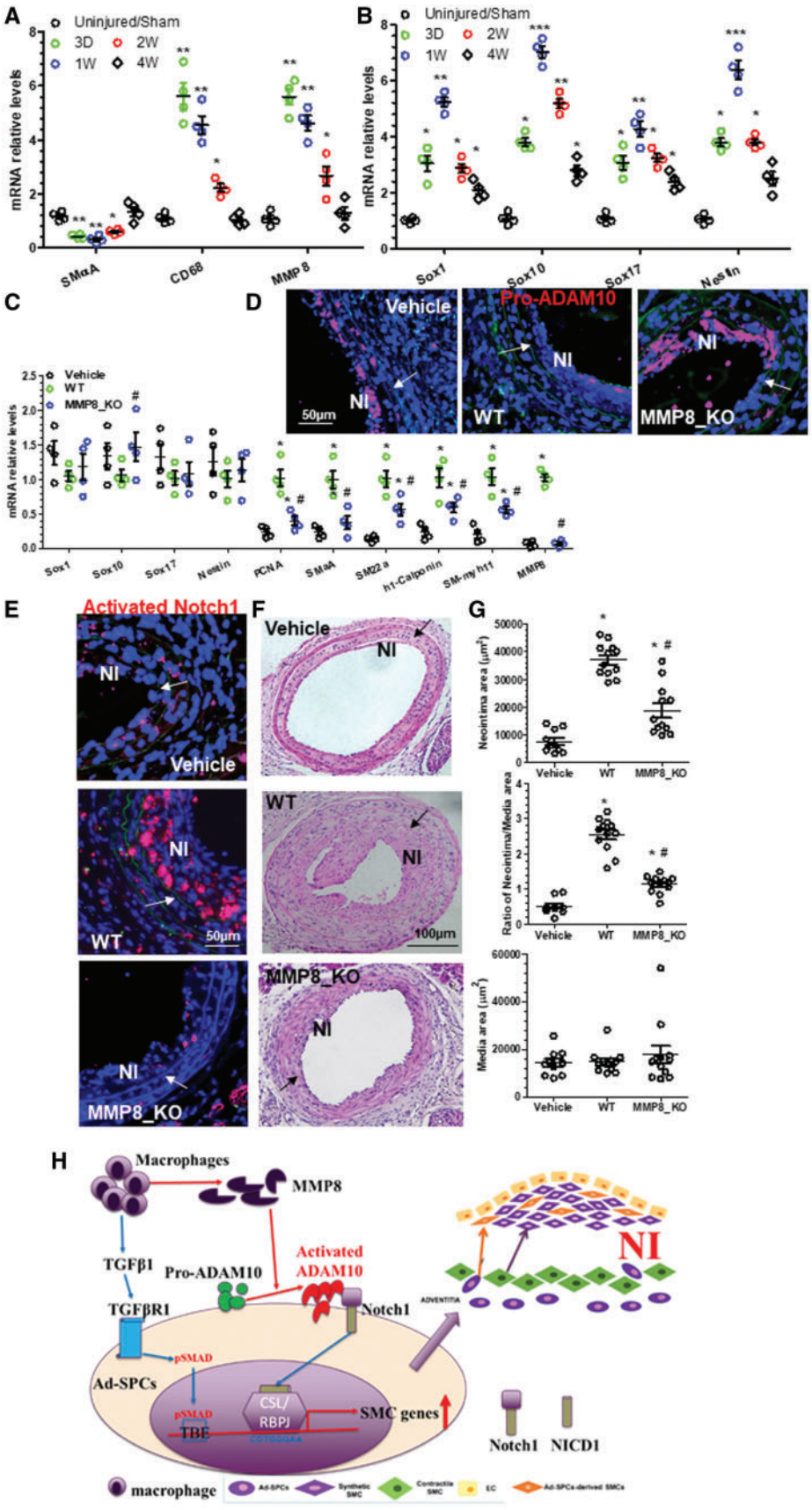


**Figure 6** Macrophage-derived MMP8 promotes injury-induced neointima SMC hyperplasia. (*A* and *B*) Gene expression profiles during injury-induced arterial remodelling. Total RNAs were collected from uninjured/sham and injured carotid arteries at the indicated time points and subjected to RT-qPCR

that MMP8 plays a causal role in atherosclerosis pathogenesis and progression as well as injury-induced neointima formation through multiple mechanisms. MMP8 can (i) convert Ang I to Ang II which in turn increases the expression of VCAM1 on ECs and enhances recruitment of leucocytes into the vascular wall, leading to vascular inflammation and atherosclerotic plaque formation and growth;[38] (ii) promote atherosclerotic angiogenesis by up-regulation of platelet/EC adhesion molecule-1 in ECs;[62] (iii) facilitate migration of BM_SPCs into the atherosclerotic lesions through its ability to degrade collagen I and to activate ADAM10 which in turn cleaves the intercellular protein E-cadherin that mediates the interaction between BM_SPCs and its niche. After recruitment into atherosclerotic plaques, BM_SPCs can differentiate into inflammatory cells, further amplifying vascular wall inflammation and promoting atherosclerotic plaque growth;[40] (iv) control macrophage differentiation and polarization through activation of TGF-β1 signalling;[35] (v) enhance SMC migration and proliferation through its ability to induce N-cadherin shedding by direct cleavage of N-cadherin ectodomains and/or via activating ADAM10 which in turn cleaves N-cadherin on SMCs. N-cadherin shedding from SMCs disrupts N-cadherin-mediated cell–cell adhesion and allows β-catenin to dissociate from cadherins and translocate into the nucleus, leading to SMC migration and proliferation. Increased SMC migration and proliferation contribute to neointimal SMC hyperplasia.[39] In this study, we reported another unrecognized role for MMP8 in SMC differentiation from AdSPCs, and further confirmed the functional importance of macrophage-derived MMP8 in vascular injury-induced neointimal SMC hyperplasia. Importantly, we found MMP8 regulates SMC differentiation through activation of the ADAM10/Notch1 signalling axis.

Unlike BM-derived SPCs, we unexpectedly found that AdSPCs express little MMP8 under physiological conditions, indicating these two SPCs are different from each other. This is certainly true in terms of their stem cell marker expression. The BM-derived SPCs express high levels of Sca-1 and c-Kit stem cell markers,[40] whereas the AdSPCs used in the current study are negative for both markers.[8] However, it would be inappropriate to dismiss the functional involvement of MMP8 in SMC differentiation from AdSPCs, particularly in an *in vivo* disease setting, in which multiple cells (e.g. adventitia macrophages and AdSPCs) interact with and regulate each other's functions through direct contact or in a paracrine manner. Indeed, we observed a significant increase in the numbers of macrophages but not the other MMP8 producing cells as well as AdSPCs within adventitia in response to injury. Moreover, it has been previously reported that macrophages control MSC differentiation towards SMCs in a paracrine fashion.[51] Furthermore, in our own previous study, we have demonstrated that MMP8 activates TGF-β1 signalling by increasing TGF-β production, as well as its bioavailability and biological activity in macrophages.[35] The above findings prompted us to hypothesize that macrophage-derived MMP8 may have a role in SMC differentiation. As expected, data from multiple experiments (macrophages-AdSPCs co-culturing, macrophage conditional culture medium, and MMP8 inhibition experiments using MMP8 neutralizing antibody) showed us that macrophage-derived MMP8 has a promotive effect on SMC differentiation from AdSPCs. Importantly, the expression levels of macrophage, MMP8 and AdSPC marker genes were significantly up-regulated during injury-induced arterial remodelling. Therefore, we have speculated that in response to injury, both macrophages and AdSPCs within adventitia are activated. The activated and increased macrophages produce and secrete MMP8 into the stem cell niche, where it promotes AdSPC differentiation towards SMCs. However, it is worth mentioning that one of the limitations in this study is that our current data do not allow us to differentially interpret the effect of the perivascularly added macrophages from the response of local macrophages, triggered by the vascular injury itself. Although we assumed that endogenous macrophages use the same mechanisms as transplanted ones, only additional data generated from the macrophage conditional knockout mice could address such a limitation.

Notch signalling is activated upon the binding of the corresponding Notch ligands to Notch receptors (Notch1–4), which in turn trigger two proteolytic cleavage events at the Notch receptor, catalyzed by the ADAM-family of metalloproteases and γ-secretase, respectively. Such cleavages releases the Notch intracellular domain (NICD) from plasma membrane, and allows it translocate to the nucleus, where they act as transcriptional coactivators to initiate down-stream signalling pathways.[63] Later studies confirmed the absolute requirement of ADAM10 for ligand-induced extracellular cleavage at site 2 (S2) of Notch1.[52,64] Importantly, both ADAM10 and Notch1 have been implicated in vascular development and diseases. ADAM10 has been reported to play a role in cell migration, adhesion, proliferation, survival, differentiation, angiogenesis, inflammation, and endothelium permeability through its capability to cleave many substrates with diverse function within the vasculature, such as Notch1, CD44, CD144, CX3CL1, CXCL16, VEGFRII, IL6R, and TNFα.[65] Global deletion of the ADAM10 gene is embryonically lethal due to multiple cardiac and vascular defects similar to Notch1 mutants.[66] ADAM10 conditional knockout experiments

**Figure 6** Continued

analyses. (*C*–*G*) Perivascular transplantation of macrophages and analysis. After balloon injury, 100 μL of Matrigel containing vehicle or $10^6$ BMMs per vessel per mice was immediately applied and packed around injured carotid arteries. Three days (*C*), 2 (*D* and *E*) or 4 weeks (*F* and *G*) later, injured segments of carotid arteries were harvested for analyses. (*C*) Perivascular transplantation of MMP8_KO BMMs decreased SMC and PCNA gene expression in injured arteries. Total RNAs were harvested from the injured arteries and subjected to RT-qPCR analyses with the indicated primers. The data presented in (*A*–*C*) are mean ± SEM of four independent experiments (3–5 carotid arteries were pooled for each experiment). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (vs. uninjured vessels or vehicle); $^{\#}P < 0.05$ (vs. WT); Kruskal–Wallis H test. (*D* and *E*) MMP8_KO BMMs transplantation inhibited ADAM10/Notch1 activation. Frozen sections from both groups ($n = 5$ mice) were prepared and subjected to immunofluorescence staining with antibodies against the pro-peptide domain of ADAM10 (Pro-ADAM10, Abcam, ab39178) and activated Notch1 (Abcam, ab8925), respectively. Note: white arrow indicates the autofluorescence (green) of the internal elastic lamina. (*F* and *G*) Locally transplantation of MMP8_KO BMMs reduced neointima formation in wire-injured carotid arteries. Paraffin sections from vehicle ($n = 10$) and two BMMs transplantation groups ($n = 12$ mice) were proceed for H&E staining and morphometric analysis. Representative images (*D*–*F*) and morphological characteristics including media area, neointimal area, and neointimal/media (N/M) ratio (*G*) at 28 days after injury were presented here. $^{*}P < 0.05$ (vs. vehicle) and $^{\#}P < 0.05$ (vs. WT BMMs); one-way ANOVA with a *post hoc* test of LSD. (*H*) Schematic illustration showing the model of action for macrophage-derived MMP8 in SMC differentiation from AdSPCs and injury-induced neointima SMC hyperplasia.

demonstrate that EC ADAM10 controls organ-specific vascular development,[67] mainly through its regulator role in Notch1 signalling.[68] Apart from its developmental role in the cardiovascular system, a later study also suggested a causal role for myeloid ADAM10 in modulating atherosclerotic plaque stability.[69] In the current study, we have shown that macrophage-derived MMP8 promotes ADAM10 cleavage and maturation in AdSPCs, leading to Notch1 activation (*Figure 4A and B*). Such a phenomenon was also observed in neointima cells during arterial remodelling (*Figure 6D and E*), indicating that macrophage-derived MMP8 promotes neointimal SMC hyperplasia by activating ADAM10/Notch1 signalling. Our data are consistent with previous findings which showed increased ADAM10 expression in coronary artery in-stent restenosis.[70]

The importance of Notch signalling in VSMC behaviour and phenotype,[71] vascular development and diseases[72,73] has been extensively explored. High *et al.*[74] used Cre-lox technology to activate a dominant-negative inhibitor of Notch signalling specifically in the neural crest lineage, and found the resulting mice displayed cardiac outflow tract defects, mainly due to the inhibition of Notch genes as well as decreased SMC differentiation from neural crest precursors. Accordingly, Notch has been hailed as a 'master regulator' of vascular morphogenesis.[75] Later studies further confirmed that Notch activity is required for the differentiation of a $Tie1^+$ local precursor to vascular SMCs in a spatiotemporal fashion across all vascular beds,[76] and that Notch activation in neural crest is required for SMC differentiation and aortic arch artery development.[77] Specifically, Notch1 was the predominant Notch receptor expressing in total and c-$Kit^+$/$NKX2.5^+$ BM-MSCs, and activation of Notch1 signalling contributed to SMC differentiation of BM-MSCs[78] and cardiac progenitor cells.[79] These findings are in alignment with our data showing that Notch1 (*Figure 5*), but not the other three Notchs (Supplementary material online, *Figure S7*), promotes SMC differentiation from AdSPCs, inferring a specific requirement for Notch1 in adult MSC differentiation toward SMCs. Apart from its critical role in vascular development, Notch1 has also been widely implicated in vascular disease. Particularly, Notch1 mutations have been widely reported in patients with bicuspid aortic valves and associated aortopathies such as ascending aortic aneurysm and aortic root dilation,[80] which has been further confirmed in mice with haploinsufficiency of Notch1.[81,82] Since inadequate arterial SMC repair capacity has been recognized as a fundamental underlying cause of aneurysm formation, it would be interesting to study if the MMP8/ADAM10/Notch1 signalling axis identified from the current study plays a positive role in this vascular disease through modulating SMC differentiation and phenotypes.

It has been well established that the SRF-CArG interaction is a critical convergence point for signals that either activates SMC gene expression to promote SMC differentiation under physiological environments or represses SMC gene expression during pathophysiological conditions.[83] However, this mechanism is not responsible for Notch1-mediated SMC differentiation from AdSPCs as evidenced by Notch1 over-expression in AdSPCs which could significantly increase the promoter activity of SMC genes with or without CArG binding sites to a similar extent (*Figure 5B and C*). Instead, we have now provided comprehensive evidence to suggest that both SMαA and SM22α are the direct transcriptional target genes of Notch1, and the DNA sequence (TGTGGGCA) which resembles but is not identical to CSL consensus binding site within SMαA and SM22α gene promoter acts as the functional binding site for Notch1/CSL transcriptional complex (*Figure 5D–G*). Such an interaction between Notch1/CSL and the DNA motif is essential for Notch1-mediated SMC gene transcription during SMC differentiation from AdSPCs.

In summary, although we provide no definitive evidence to discern the exact contribution of SMC differentiation from AdSPCs to intima formation in response to vascular injury, our study does show that macrophage-derived MMP8 plays a functional role in SMC differentiation from AdSPCs by activating both TGF-β1 and ADAM10/Notch1 pathways. Moreover, we also demonstrate that macrophage-derived MMP8 promotes vascular injury-induced neointimal SMC hyperplasia, partially through activation of ADAM10/Notch1 signalling axis. Thus, data from this study provide a new insight into the biological molecules and relevant mechanisms involved in SMC differentiation from AdSPCs particularly in the pathogenesis of post-angioplasty restenosis.

# Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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