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

(Yang et al. MMP8 in stem cell and arterial remodeling)

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Abstract

Objective: Emerging evidence has suggested that adventitia stem/progenitor cells (AdSPCs) migrate into the intima of arteries in response to injury, where they differentiate toward 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 TGF-β activity and a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10)/Notch1 signaling. 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 signaling.

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 provides 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.

Key Words: 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

Abbreviations: SMC, smooth muscle cell; AdSPCs, adventitia stem/progenitor cells; MSCs, mesenchymal stem cells; EC, endothelial cell; ES cells, embryonic stem cells; MMP8, matrix metalloproteinase-8; ADAM10, a disintegrin and metalloproteinase domain-containing protein 10; NICD, intracellular domain of the notch protein; CSL, CBF1, Suppressor of Hairless, and Lag-1; Sox, SRY-related HMG-box; TGF-β, transforming growth factor-β; Sca-1, stem cell antigen-1; qRT-PCR, quantitative reverse transcription polymerase chain reaction; shRNA, short hairpin RNA; CHIP, chromatin immunoprecipitation; SMαA, smooth muscle alpha-actin; SM22α, smooth muscle 22 alpha; SM-MHC, smooth muscle myosin heavy chain; SM-myh11, smooth muscle myosin-11; SRF, serum response factor; Myocd, myocardin; MEF2c, myocyte enhancer factor 2C.

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 remains 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 remodeling 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 signaling pathways including integrin/collagen IV axis[2], EGFR/$ERK_{1/2}$/β-catenin[29], c-Myb/myocardin[32], CXCR4[22], and DKK3 (dickkopf 3)/TGF-β (transforming growth factor-β)/ATF6/Wnt signaling[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 transforming growth factor-β (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[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.

Methods & Materials

Materials and Data Availability

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

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 grams) 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 (70μm), 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 bone marrow SPCs (BM_SPCs) according to the manufacturer's instructions as described in our previous study[40].

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 5ng/ml TGF-β) for 2 to 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 to 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 to 6 days. The CM was refreshed every day.

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. 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.

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.

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 bone marrow-derived macrophages (BMMs). Briefly, immediately after injury, 100μl Matrigel mixed with 20μl of cell culture medium containing 1x10^6 WT or MMP8_KO BMMs or culture

medium alone (vehicle) was applied perivascularly to injured carotid arteries. At day 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.

Statistical analysis. Results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Graphpad Prism5. 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 2 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 Online Supplement, include bone marrow-derived macrophage (BMM) culture; SMC differentiation from embryonic stem (ES) cells; shRNA 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.

Results

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 (**Figures S1A**) and protein (**Figures 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 (**Figures S1A & S1B**). Importantly, all four SMC differentiation genes were significantly down-regulated by MMP8 gene knockdown (**Figures S1C & S1D**), confirming an essential role for MMP8 in SMC differentiation

from ES cells.

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 (leukocyte), fibroblast-specific protein 1 (FSP1) (fibroblast), smooth muscle myosin heavy chain (SM-MHC) (SMC) and CD31 (EC) (**Figures S2A-S2D**). 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 (**Figures 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 5ng/ml TGF-β1, we observed a significant increase in SMC markers at both RNA (**Figures 1A**) and protein (**Figures 1B**) levels. Similarly, the gene expression of SMC differentiation transcription factor, serum response 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 (MEF2C) (**Figures 1C**). As expected, both Sox10 and Sox17 gene expression were dramatically down-regulated over the 6-day differentiation period (**Figures 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 (**Figures 1A**) and protein level (**Figures 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 (**Figure S3**). This unexpected observation prompted us to examine the expression levels of MMP8 in these AdSPCs. Indeed, data from RT-qPCR (**Figure S4A**) and Western blot (**Figure S4B**) analysis showed that while ES cells, ES cell-derived SMCs, as well as bone marrow-derived macrophages (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.

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. 1) AdSPCs belong to MSC family within arteries; 2) they express a similar pattern of cell markers to adipose tissue–derived MSCs; and 3) 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 (**Figure S5A & S5B**), and decreased expression levels of Sox10 and Sox17 (**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 (**Figure S6**), suggesting that secreted signals released by macrophages can induce SMC differentiation from AdSPCs.

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-2C**). A similar phenomenon was observed when MMP8_KO AdPSCs were incubated with the culture medium taken from MMP8_KO macrophages (**Figure 2D-2F**). This data demonstrates 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.

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 signaling 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 signaling may also responsible for macrophage-derived MMP8 mediated SMC differentiation from AdSPCs. As expected, an opposite effect for SMC gene 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 signaling.

ADAM10-Notch1 signaling 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 signaling concomitantly regulate SMC differentiation during VSMC phenotype switching[53]. Furthermore, data from the previous section suggests additional signal pathway may

play a role in macrophage-derived MMP8 mediated SMC differentiation from AdSPCs. Therefore, we wondered if ADAM10-Notch1 signaling 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 signaling) (**Figure 4C**). Consistently, the Notch signaling was significantly activated by addition of the recombinant MMP8 and ADAM10 activated proteins into the culture system (**Figure 4D and 4E**). Taken together, the above data demonstrates 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.

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 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 (**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-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 5C**), 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 5E**), revealing a dependence on this DNA element for Notch1-induced SMC gene expression. Finally, chromatin immunoprecipitation (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 5G** revealed that NICD1 over-expression 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.

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 remodeling. To this end, we first examined the gene expression profiles during arterial remodeling 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 remodeling 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 (**Figure S8**). Moreover, we observed MMP8 was mainly co-expressed with

CD68 in neointima (**Figure S8**). Furthermore, data shown in **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 remodeling in response to injury. These data indicate that the AdSPCs are activated and infer an involvement for AdSPC activation and/or differentiation during arterial remodeling, consistent with previous findings[8]. These data also suggest a potential role for MMP8 derived from activated macrophages in injury-induced arterial remodeling. 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 signaling 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 6G**), confirming a promotive effect of macrophages in neointimal lesion growth, and macrophage-derived MMP8 represents one of underlying mechanisms of macrophage-promoted neointimal SMC hyperplasia.

Discussion

The de-differentiation and proliferation of SMCs (SMC phenotype switching) has been widely established as the major contributor to vascular remodeling. 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 twofold. On the 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 summarised in an elegant review[41]. Through a series of studies we have comprehensively demonstrated that MMP8 plays a causal role in atherosclerosis pathogenesis and progression as well as injury-induced neointima formation through multiple mechanisms. MMP8 can 1) convert Ang I to Ang II which in turn increases the expression of VCAM1 on ECs and enhances recruitment of leukocytes into the vascular wall, leading to vascular inflammation and atherosclerotic plaque formation and growth[38]; 2) promote atherosclerotic angiogenesis by up-regulation of platelet/endothelial cell adhesion molecule-1 in ECs[62]; 3) 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 recruitement into atherosclerotic plaques, BM_SPCs can differentiate into inflammatory cells, further amplifying vascular wall inflammation and promoting atherosclerotic plaque growth[40]; 4) control macrophage differentiation and polarisation through activation of TGF-β1 signalling[35]; and 5) 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 signaling 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 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 remodeling. 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 signaling 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 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 4B**). Such a phenomenon was also observed in neointima cells during arterial remodelling (**Figure 6D and 6E**), indicating that macrophage-derived MMP8 promotes neointimal SMC hyperplasia by activating ADAM10/Notch1 signaling. Our data is 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 $c\text{-}Kit^+$/$NKX2.5^+$ 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 (**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 recognised 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 5C**). 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-5G**). 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 signaling axis. Thus, data from this study provides a new insight into the biological molecules and relevant mechanisms involved in SMC differentiation from AdSPCs particularly in the pathogenesis of post-angioplasty restenosis.

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Disclosures

None

Reference

1. Tigges U, Komatsu M, Stallcup WB. Adventitial pericyte progenitor/mesenchymal stem cells participate in the restenotic response to arterial injury. *J Vasc Res* 2013;**50**:134-144.
2. Chen Y, Wong MM, Campagnolo P, Simpson R, Winkler B, Margariti A, Hu Y, Xu Q. Adventitial stem cells in vein grafts display multilineage potential that contributes to neointimal formation. *Arterioscler Thromb Vasc Biol* 2013;**33**:1844-1851.
3. Passman JN, Dong XR, Wu SP, Maguire CT, Hogan KA, Bautch VL, Majesky MW. A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1+ smooth muscle progenitor cells. *Proc Natl Acad Sci U S A* 2008;**105**:9349-9354.
4. Corselli M, Chen CW, Sun B, Yap S, Rubin JP, Peault B. The tunica adventitia of human arteries and veins as a source of mesenchymal stem cells. *Stem Cells Dev* 2012;**21**:1299-1308.
5. Crisan M, Chen CW, Corselli M, Andriolo G, Lazzari L, Peault B. Perivascular multipotent progenitor cells in human organs. *Ann N Y Acad Sci* 2009;**1176**:118-123.
6. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Peault B. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**:301-313.
7. Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, Xu Q. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. *J Clin Invest.* 2004;**113**:1258-1265.
8. Tang Z, Wang A, Yuan F, Yan Z, Liu B, Chu JS, Helms JA, Li S. Differentiation of multipotent vascular stem cells contributes to vascular diseases. *Nat Commun* 2012;**3**:875.
9. Tavazoie M, Van der Veken L, Silva-Vargas V, Louissaint M, Colonna L, Zaidi B, Garcia-Verdugo JM, Doetsch F. A specialized vascular niche for adult neural stem cells. *Cell Stem Cell* 2008;**3**:279-288.
10. Colunga T, Dalton S. Building Blood Vessels with Vascular Progenitor Cells. *Trends Mol Med* 2018;**24**:630-641.
11. Torsney E, Xu Q. Resident vascular progenitor cells. *J Mol Cell Cardiol* 2011;**50**:304-311.
12. Psaltis PJ, Harbuzariu A, Delacroix S, Holroyd EW, Simari RD. Resident vascular progenitor cells--diverse origins, phenotype, and function. *J Cardiovasc Transl Res* 2011;**4**:161-176.
13. Majesky MW, Dong XR, Hoglund V, Mahoney WM, Jr., Daum G. The adventitia: a dynamic interface containing resident progenitor cells. *Arterioscler Thromb Vasc Biol* 2011;**31**:1530-1539.
14. Merkulova-Rainon T, Broqueres-You D, Kubis N, Silvestre JS, Levy BI. Towards the therapeutic use of vascular smooth muscle progenitor cells. *Cardiovasc Res* 2012;**95**:205-214.
15. Psaltis PJ, Simari RD. Vascular wall progenitor cells in health and disease. *Circ Res* 2015;**116**:1392-1412.
16. Zhang L, Issa Bhaloo S, Chen T, Zhou B, Xu Q. Role of Resident Stem Cells in Vessel Formation and Arteriosclerosis. *Circ Res* 2018;**122**:1608-1624.
17. Pasquinelli G, Tazzari PL, Vaselli C, Foroni L, Buzzi M, Storci G, Alviano F, Ricci F, Bonafe M, Orrico C, Bagnara GP, Stella A, Conte R. Thoracic aortas from multiorgan donors are suitable for obtaining resident angiogenic mesenchymal stromal cells. *Stem Cells* 2007;**25**:1627-1634.
18. Zengin E, Chalajour F, Gehling UM, Ito WD, Treede H, Lauke H, Weil J, Reichenspurner H, Kilic N, Ergun S. Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development* 2006;**133**:1543-1551.
19. Campagnolo P, Cesselli D, Al Haj Zen A, Beltrami AP, Krankel N, Katare R, Angelini G, Emanueli C,

Madeddu P. Human adult vena saphena contains perivascular progenitor cells endowed with clonogenic and proangiogenic potential. *Circulation* 2010;**121**:1735-1745.

20. Bearzi C, Leri A, Lo Monaco F, Rota M, Gonzalez A, Hosoda T, Pepe M, Qanud K, Ojaimi C, Bardelli S, D'Amario D, D'Alessandro DA, Michler RE, Dimmeler S, Zeiher AM, Urbanek K, Hintze TH, Kajstura J, Anversa P. Identification of a coronary vascular progenitor cell in the human heart. *Proc Natl Acad Sci U S A* 2009;**106**:15885-15890.
21. Dierick F, Hery T, Hoareau-Coudert B, Mougenot N, Monceau V, Claude C, Crisan M, Besson V, Dorfmuller P, Marodon G, Fadel E, Humbert M, Yaniz-Galende E, Hulot JS, Marazzi G, Sassoon D, Soubrier F, Nadaud S. Resident PW1+ Progenitor Cells Participate in Vascular Remodeling During Pulmonary Arterial Hypertension. *Circ Res* 2016;**118**:822-833.
22. Kramann R, Schneider RK, DiRocco DP, Machado F, Fleig S, Bondzie PA, Henderson JM, Ebert BL, Humphreys BD. Perivascular Gli1+ progenitors are key contributors to injury-induced organ fibrosis. *Cell Stem Cell* 2015;**16**:51-66.
23. Michelis KC, Nomura-Kitabayashi A, Lecce L, Franzen O, Koplev S, Xu Y, Santini MP, D'Escamard V, Lee JTL, Fuster V, Hajjar R, Reddy RC, Chikwe J, Stelzer P, Filsoufi F, Stewart A, Anyanwu A, Bjorkegren JLM, Kovacic JC. CD90 Identifies Adventitial Mesenchymal Progenitor Cells in Adult Human Medium- and Large-Sized Arteries. *Stem Cell Reports* 2018;**11**:242-257.
24. Hoshino A, Chiba H, Nagai K, Ishii G, Ochiai A. Human vascular adventitial fibroblasts contain mesenchymal stem/progenitor cells. *Biochem Biophys Res Commun* 2008;**368**:305-310.
25. Grudzinska MK, Kurzejamska E, Bojakowski K, Soin J, Lehmann MH, Reinecke H, Murry CE, Soderberg-Naucler C, Religa P. Monocyte chemoattractant protein 1-mediated migration of mesenchymal stem cells is a source of intimal hyperplasia. *Arterioscler Thromb Vasc Biol* 2013;**33**:1271-1279.
26. Klein D, Benchellal M, Kleff V, Jakob HG, Ergun S. Hox genes are involved in vascular wall-resident multipotent stem cell differentiation into smooth muscle cells. *Sci Rep* 2013;**3**:2178.
27. Pasquinelli G, Pacilli A, Alviano F, Foroni L, Ricci F, Valente S, Orrico C, Lanzoni G, Buzzi M, Luigi Tazzari P, Pagliaro P, Stella A, Paolo Bagnara G. Multidistrict human mesenchymal vascular cells: pluripotency and stemness characteristics. *Cytotherapy* 2010;**12**:275-287.
28. Le Bras A, Yu B, Issa Bhaloo S, Hong X, Zhang Z, Hu Y, Xu Q. Adventitial Sca1+ Cells Transduced With ETV2 Are Committed to the Endothelial Fate and Improve Vascular Remodeling After Injury. *Arterioscler Thromb Vasc Biol* 2018;**38**:232-244.
29. Wong MM, Winkler B, Karamariti E, Wang X, Yu B, Simpson R, Chen T, Margariti A, Xu Q. Sirolimus stimulates vascular stem/progenitor cell migration and differentiation into smooth muscle cells via epidermal growth factor receptor/extracellular signal-regulated kinase/beta-catenin signaling pathway. *Arterioscler Thromb Vasc Biol* 2013;**33**:2397-2406.
30. Majesky MW, Horita H, Ostriker A, Lu S, Regan JN, Bagchi A, Dong XR, Poczobutt J, Nemenoff RA, Weiser-Evans MC. Differentiated Smooth Muscle Cells Generate a Subpopulation of Resident Vascular Progenitor Cells in the Adventitia Regulated by Klf4. *Circ Res* 2017;**120**:296-311.
31. Sun Y, Wang K, Ye P, Wu J, Ren L, Zhang A, Huang X, Deng P, Wu C, Yue Z, Chen Z, Ding X, Chen J, Xia J. MicroRNA-155 Promotes the Directional Migration of Resident Smooth Muscle Progenitor Cells by Regulating Monocyte Chemoattractant Protein 1 in Transplant Arteriosclerosis. *Arterioscler Thromb Vasc Biol* 2016;**36**:1230-1239.
32. Shikatani EA, Chandy M, Besla R, Li CC, Momen A, El-Mounayri O, Robbins CS, Husain M. c-Myb Regulates Proliferation and Differentiation of Adventitial Sca1+ Vascular Smooth Muscle Cell

Progenitors by Transactivation of Myocardin. *Arterioscler Thromb Vasc Biol* 2016;**36**:1367-1376.

33. Karamariti E, Zhai C, Yu B, Qiao L, Wang Z, Potter CMF, Wong MM, Simpson RML, Zhang Z, Wang X, Del Barco Barrantes I, Niehrs C, Kong D, Zhao Q, Zhang Y, Hu Y, Zhang C, Xu Q. DKK3 (Dickkopf 3) Alters Atherosclerotic Plaque Phenotype Involving Vascular Progenitor and Fibroblast Differentiation Into Smooth Muscle Cells. *Arterioscler Thromb Vasc Biol* 2018;**38**:425-437.
34. Van Lint P, Libert C. Matrix metalloproteinase-8: cleavage can be decisive. *Cytokine Growth Factor Rev* 2006;**17**:217-223.
35. Wen G, Zhang C, Chen Q, Luong le A, Mustafa A, Ye S, Xiao Q. A Novel Role of Matrix Metalloproteinase-8 in Macrophage Differentiation and Polarization. *J Biol Chem* 2015;**290**:19158-19172.
36. Van Den Steen PE, Wuyts A, Husson SJ, Proost P, Van Damme J, Opdenakker G. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *Eur J Biochem* 2003;**270**:3739-3749.
37. Cox JH, Dean RA, Roberts CR, Overall CM. Matrix metalloproteinase processing of CXCL11/I-TAC results in loss of chemoattractant activity and altered glycosaminoglycan binding. *J Biol Chem* 2008;**283**:19389-19399.
38. Laxton RC, Hu Y, Duchene J, Zhang F, Zhang Z, Leung KY, Xiao Q, Scotland RS, Hodgkinson CP, Smith K, Willeit J, Lopez-Otin C, Simpson IA, Kiechl S, Ahluwalia A, Xu Q, Ye S. A role of matrix metalloproteinase-8 in atherosclerosis. *Circ Res* 2009;**105**:921-929.
39. Xiao Q, Zhang F, Grassia G, Hu Y, Zhang Z, Xing Q, Yin X, Maddaluno M, Drung B, Schmidt B, Maffia P, Ialenti A, Mayr M, Xu Q, Ye S. Matrix metalloproteinase-8 promotes vascular smooth muscle cell proliferation and neointima formation. *Arterioscler Thromb Vasc Biol* 2014;**34**:90-98.
40. Xiao Q, Zhang F, Lin L, Fang C, Wen G, Tsai TN, Pu X, Sims D, Zhang Z, Yin X, Thomaszewski B, Schmidt B, Mayr M, Suzuki K, Xu Q, Ye S. Functional role of matrix metalloproteinase-8 in stem/progenitor cell migration and their recruitment into atherosclerotic lesions. *Circ Res* 2013;**112**:35-47.
41. Ye S. Putative targeting of matrix metalloproteinase-8 in atherosclerosis. *Pharmacol Ther* 2015;**147**:111-122.
42. Herman MP, Sukhova GK, Libby P, Gerdes N, Tang N, Horton DB, Kilbride M, Breitbart RE, Chun M, Schonbeck U. Expression of neutrophil collagenase (matrix metalloproteinase-8) in human atheroma: a novel collagenolytic pathway suggested by transcriptional profiling. *Circulation* 2001;**104**:1899-1904.
43. Turu MM, Krupinski J, Catena E, Rosell A, Montaner J, Rubio F, Alvarez-Sabin J, Cairols M, Badimon L. Intraplaque MMP-8 levels are increased in asymptomatic patients with carotid plaque progression on ultrasound. *Atherosclerosis* 2006;**187**:161-169.
44. Tuomainen AM, Nyyssonen K, Laukkanen JA, Tervahartiala T, Tuomainen TP, Salonen JT, Sorsa T, Pussinen PJ. Serum matrix metalloproteinase-8 concentrations are associated with cardiovascular outcome in men. *Arterioscler Thromb Vasc Biol* 2007;**27**:2722-2728.
45. Tang Z, Wang A, Wang D, Li S. Smooth muscle cells: to be or not to be? Response to Nguyen et Al. *Circ Res* 2013;**112**:23-26.
46. Margariti A, Xiao Q, Zampetaki A, Zhang Z, Li H, Martin D, Hu Y, Zeng L, Xu Q. Splicing of HDAC7 modulates the SRF-myocardin complex during stem-cell differentiation towards smooth muscle cells. *J Cell Sci* 2009;**122**:460-470.
47. Huang Y, Lin L, Yu X, Wen G, Pu X, Zhao H, Fang C, Zhu J, Ye S, Zhang L, Xiao Q. Functional

involvements of heterogeneous nuclear ribonucleoprotein A1 in smooth muscle differentiation from stem cells in vitro and in vivo. *Stem Cells* 2013;**31**:906-917.

48. Zhang L, Chen Q, An W, Yang F, Maguire EM, Chen D, Zhang C, Wen G, Yang M, Dai B, Luong LA, Zhu J, Xu Q, Xiao Q. Novel Pathological Role of hnRNPA1 (Heterogeneous Nuclear Ribonucleoprotein A1) in Vascular Smooth Muscle Cell Function and Neointima Hyperplasia. *Arterioscler Thromb Vasc Biol* 2017;**37**:2182-2194.
49. Zhang C, Chen D, Maguire EM, He S, Chen J, An W, Yang M, Afzal TA, Luong LA, Zhang L, Lei H, Wu Q, Xiao Q. Cbx3 inhibits vascular smooth muscle cell proliferation, migration, and neointima formation. *Cardiovasc Res* 2018;**114**:443-455.
50. Xiao Q, Zeng L, Zhang Z, Hu Y, Xu Q. Stem cell-derived Sca-1+ progenitors differentiate into smooth muscle cells, which is mediated by collagen IV-integrin alpha1/beta1/alphav and PDGF receptor pathways. *Am J Physiol Cell Physiol* 2007;**292**:C342-352.
51. Lee MJ, Kim MY, Heo SC, Kwon YW, Kim YM, Do EK, Park JH, Lee JS, Han J, Kim JH. Macrophages regulate smooth muscle differentiation of mesenchymal stem cells via a prostaglandin F(2)alpha-mediated paracrine mechanism. *Arterioscler Thromb Vasc Biol* 2012;**32**:2733-2740.
52. van Tetering G, van Diest P, Verlaan I, van der Wall E, Kopan R, Vooijs M. Metalloprotease ADAM10 is required for Notch1 site 2 cleavage. *J Biol Chem* 2009;**284**:31018-31027.
53. Tang Y, Urs S, Boucher J, Bernaiche T, Venkatesh D, Spicer DB, Vary CP, Liaw L. Notch and transforming growth factor-beta (TGFbeta) signaling pathways cooperatively regulate vascular smooth muscle cell differentiation. *J Biol Chem* 2010;**285**:17556-17563.
54. Noseda M, Fu Y, Niessen K, Wong F, Chang L, McLean G, Karsan A. Smooth Muscle alpha-actin is a direct target of Notch/CSL. *Circ Res* 2006;**98**:1468-1470.
55. Yang F, Chen Q, He S, Yang M, Maguire EM, An W, Afzal TA, Luong LA, Zhang L, Xiao Q. miR-22 Is a Novel Mediator of Vascular Smooth Muscle Cell Phenotypic Modulation and Neointima Formation. *Circulation* 2018;**137**:1824-1841.
56. Nguyen AT, Gomez D, Bell RD, Campbell JH, Clowes AW, Gabbiani G, Giachelli CM, Parmacek MS, Raines EW, Rusch NJ, Speer MY, Sturek M, Thyberg J, Towler DA, Weiser-Evans MC, Yan C, Miano JM, Owens GK. Smooth muscle cell plasticity: fact or fiction? *Circ Res* 2013;**112**:17-22.
57. Xiao Q, Pepe AE, Wang G, Luo Z, Zhang L, Zeng L, Zhang Z, Hu Y, Ye S, Xu Q. Nrf3-Pla2g7 interaction plays an essential role in smooth muscle differentiation from stem cells. *Arterioscler Thromb Vasc Biol* 2012;**32**:730-744.
58. Zhao H, Wen G, Huang Y, Yu X, Chen Q, Afzal TA, Luong le A, Zhu J, Ye S, Zhang L, Xiao Q. MicroRNA-22 regulates smooth muscle cell differentiation from stem cells by targeting methyl CpG-binding protein 2. *Arterioscler Thromb Vasc Biol* 2015;**35**:918-929.
59. Wang D, Wang A, Wu F, Qiu X, Li Y, Chu J, Huang WC, Xu K, Gong X, Li S. Sox10(+) adult stem cells contribute to biomaterial encapsulation and microvascularization. *Sci Rep* 2017;**7**:40295.
60. Wang D, Wu F, Yuan H, Wang A, Kang GJ, Truong T, Chen L, McCallion AS, Gong X, Li S. Sox10(+) Cells Contribute to Vascular Development in Multiple Organs-Brief Report. *Arterioscler Thromb Vasc Biol* 2017;**37**:1727-1731.
61. Yuan F, Wang D, Xu K, Wang J, Zhang Z, Yang L, Yang GY, Li S. Contribution of Vascular Cells to Neointimal Formation. *PLoS One* 2017;**12**:e0168914.
62. Fang C, Wen G, Zhang L, Lin L, Moore A, Wu S, Ye S, Xiao Q. An important role of matrix metalloproteinase-8 in angiogenesis in vitro and in vivo. *Cardiovasc Res* 2013;**99**:146-155.
63. Borggrefe T, Oswald F. The Notch signaling pathway: transcriptional regulation at Notch target genes.

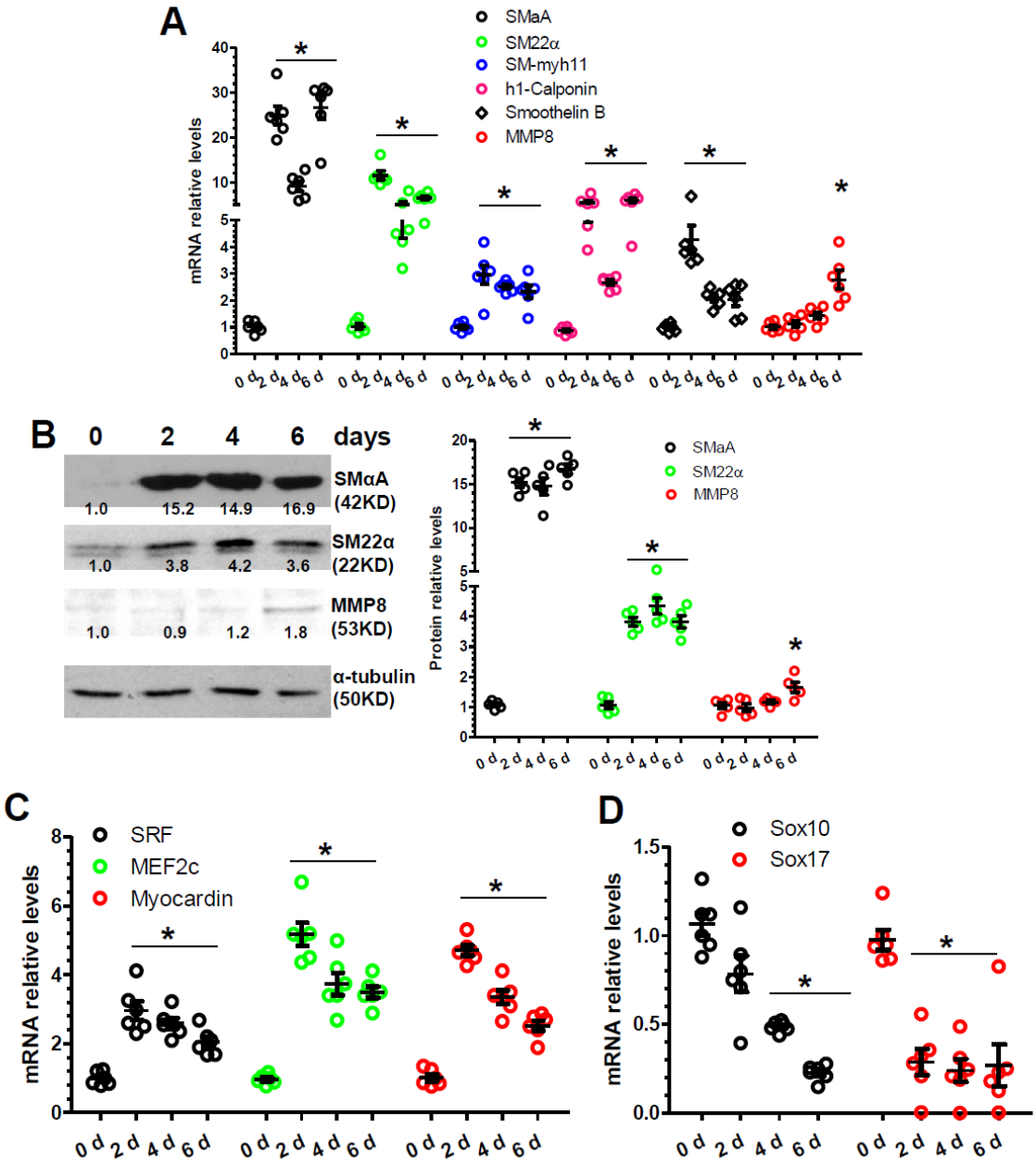
Cell Mol Life Sci 2009;**66**:1631-1646.

64. Bozkulak EC, Weinmaster G. Selective use of ADAM10 and ADAM17 in activation of Notch1 signaling. *Mol Cell Biol* 2009;**29**:5679-5695.
65. Dreymueller D, Pruessmeyer J, Groth E, Ludwig A. The role of ADAM-mediated shedding in vascular biology. *Eur J Cell Biol* 2012;**91**:472-485.
66. Zhang C, Tian L, Chi C, Wu X, Yang X, Han M, Xu T, Zhuang Y, Deng K. Adam10 is essential for early embryonic cardiovascular development. *Dev Dyn* 2010;**239**:2594-2602.
67. Glomski K, Monette S, Manova K, De Strooper B, Saftig P, Blobel CP. Deletion of Adam10 in endothelial cells leads to defects in organ-specific vascular structures. *Blood* 2011;**118**:1163-1174.
68. Alabi RO, Glomski K, Haxaire C, Weskamp G, Monette S, Blobel CP. ADAM10-Dependent Signaling Through Notch1 and Notch4 Controls Development of Organ-Specific Vascular Beds. *Circ Res* 2016;**119**:519-531.
69. van der Vorst EP, Jeurissen M, Wolfs IM, Keijbeck A, Theodorou K, Wijnands E, Schurgers L, Weber S, Gijbels MJ, Hamers AA, Dreymueller D, Rose-John S, de Winther MP, Ludwig A, Saftig P, Biessen EA, Donners MM. Myeloid A disintegrin and metalloproteinase domain 10 deficiency modulates atherosclerotic plaque composition by shifting the balance from inflammation toward fibrosis. *Am J Pathol* 2015;**185**:1145-1155.
70. Yang K, Lu L, Liu Y, Zhang Q, Pu LJ, Wang LJ, Zhu ZB, Wang YN, Meng H, Zhang XJ, Du R, Chen QJ, Shen WF. Increase of ADAM10 level in coronary artery in-stent restenosis segments in diabetic minipigs: high ADAM10 expression promoting growth and migration in human vascular smooth muscle cells via Notch 1 and 3. *PLoS One* 2013;**8**:e83853.
71. Morrow D, Guha S, Sweeney C, Birney Y, Walshe T, O'Brien C, Walls D, Redmond EM, Cahill PA. Notch and vascular smooth muscle cell phenotype. *Circ Res* 2008;**103**:1370-1382.
72. Gridley T. Notch signaling in vascular development and physiology. *Development* 2007;**134**:2709-2718.
73. Iso T, Hamamori Y, Kedes L. Notch signaling in vascular development. *Arterioscler Thromb Vasc Biol* 2003;**23**:543-553.
74. High FA, Zhang M, Proweller A, Tu L, Parmacek MS, Pear WS, Epstein JA. An essential role for Notch in neural crest during cardiovascular development and smooth muscle differentiation. *J Clin Invest* 2007;**117**:353-363.
75. Anderson LM, Gibbons GH. Notch: a mastermind of vascular morphogenesis. *J Clin Invest* 2007;**117**:299-302.
76. Chang L, Noseda M, Higginson M, Ly M, Patenaude A, Fuller M, Kyle AH, Minchinton AI, Puri MC, Dumont DJ, Karsan A. Differentiation of vascular smooth muscle cells from local precursors during embryonic and adult arteriogenesis requires Notch signaling. *Proc Natl Acad Sci USA* 2012;**109**:6993-6998.
77. Manderfield LJ, High FA, Engleka KA, Liu F, Li L, Rentschler S, Epstein JA. Notch activation of Jagged1 contributes to the assembly of the arterial wall. *Circulation* 2012;**125**:314-323.
78. Ding R, Jiang X, Ha Y, Wang Z, Guo J, Jiang H, Zheng S, Shen Z, Jie W. Activation of Notch1 signalling promotes multi-lineage differentiation of c-Kit(POS)/NKX2.5(POS) bone marrow stem cells: implication in stem cell translational medicine. *Stem Cell Res Ther* 2015;**6**:91.
79. Gude N, Joyo E, Toko H, Quijada P, Villanueva M, Hariharan N, Sacchi V, Truffa S, Joyo A, Voelkers M, Alvarez R, Sussman MA. Notch activation enhances lineage commitment and protective signaling in cardiac progenitor cells. *Basic Res Cardiol* 2015;**110**:29.

80. Garg V, Muth AN, Ransom JF, Schluterman MK, Barnes R, King IN, Grossfeld PD, Srivastava D. Mutations in NOTCH1 cause aortic valve disease. *Nature* 2005;**437**:270-274.
81. Koenig SN, Bosse KM, Nadorlik HA, Lilly B, Garg V. Evidence of Aortopathy in Mice with Haploinsufficiency of Notch1 in Nos3-Null Background. *J Cardiovasc Dev Dis* 2015;**2**:17-30.
82. Koenig SN, LaHaye S, Feller JD, Rowland P, Hor KN, Trask AJ, Janssen PM, Radtke F, Lilly B, Garg V. Notch1 haploinsufficiency causes ascending aortic aneurysms in mice. *JCI Insight* 2017;**2**.
83. Miano JM. Serum response factor: toggling between disparate programs of gene expression. *J Mol Cell Cardiol* 2003;**35**:577-593.

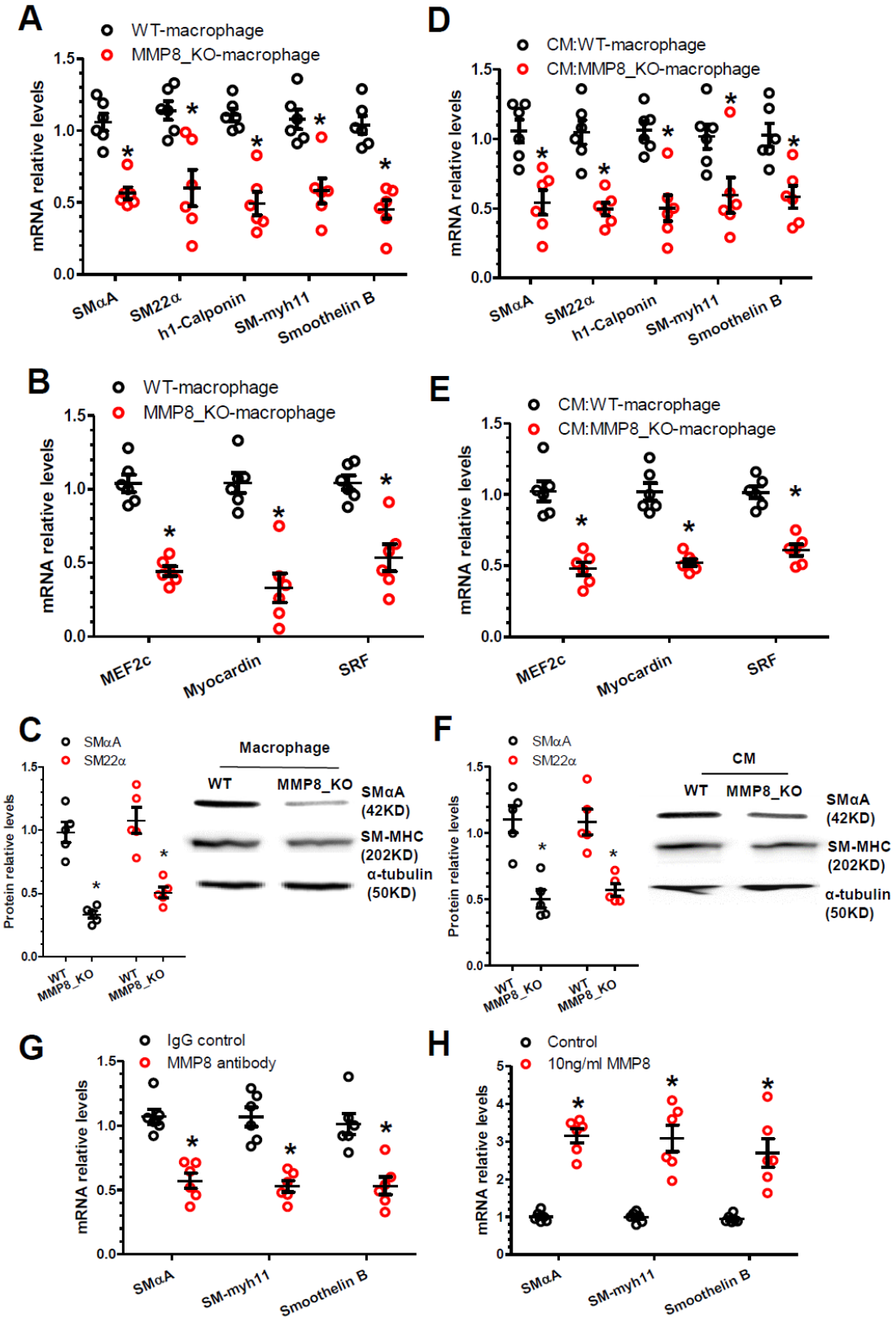
Figure Legends

Figure 1. SMC differentiation from AdSPCs.



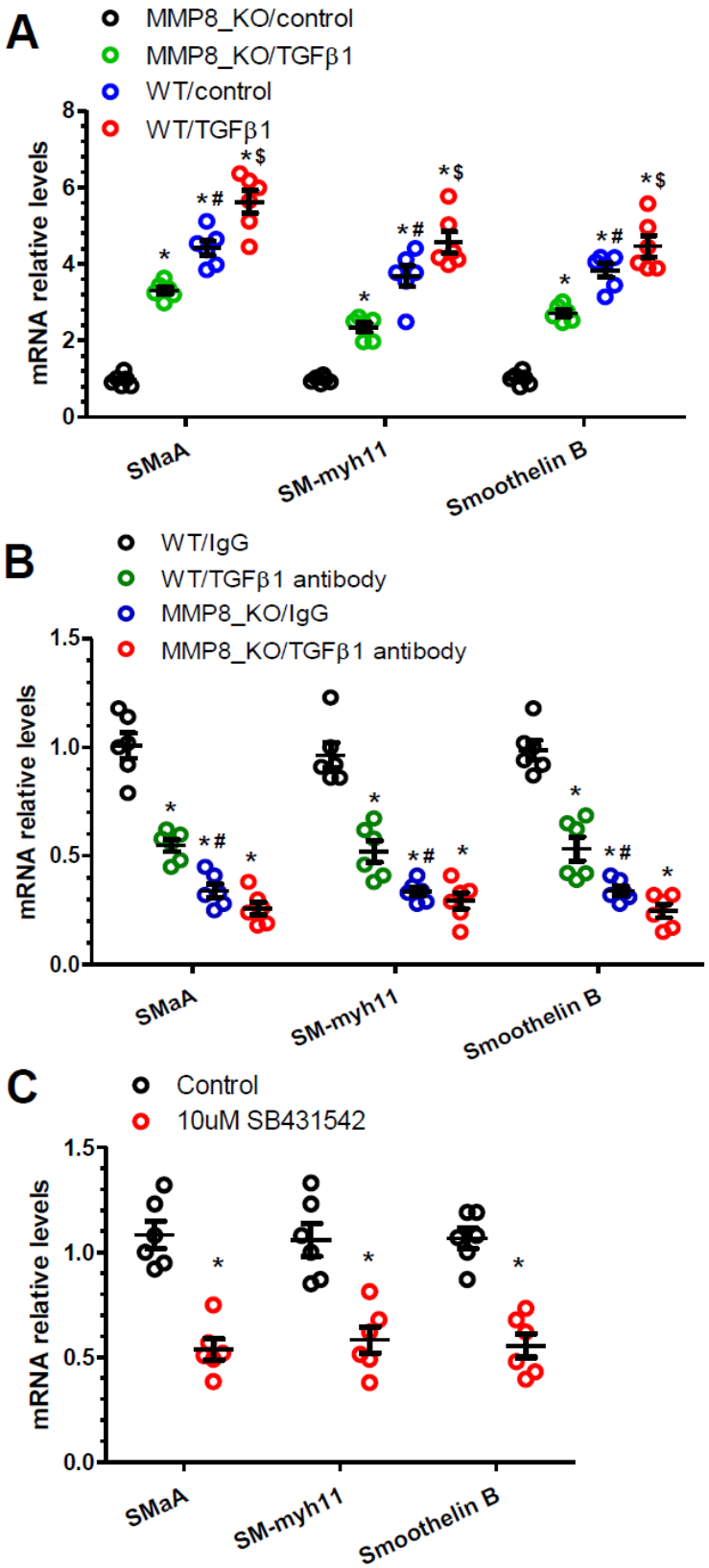
AdSPCs (p3~p10) were cultured in SMC differentiation induction medium (DMEM supplemented with 5% FBS and 5ng/ml TGF-β1) for the indicated times. Undifferentiated AdSPCs were used as control (0d). Total RNAs and proteins were harvested and subjected to RT-qPCR (A, C and D), and Western Blot analyses (B), 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±S.E.M of six independent experiments, respectively (n=6). *P<0.05 (versus 0d, one-way ANOVA with a post hoc test of LSD).

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 bone marrow derived macrophages (BMMs) isolated from wild type (WT) and MMP8_KO mice for 48 hours. (D-F) MMP8_KO AdSPCs (p3~p10) were incubated with the conditioned culture medium (CM) from WT and MMP8_KO BMMs for 48 hours. 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 (1μg/ml) at 37ºC for 30 minutes. 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 10ng/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±S.E.M of five or six independent experiments, respectively (n=5 or 6). *P<0.05 (versus WT-macrophage or CM:WT-macrophage, t-test).

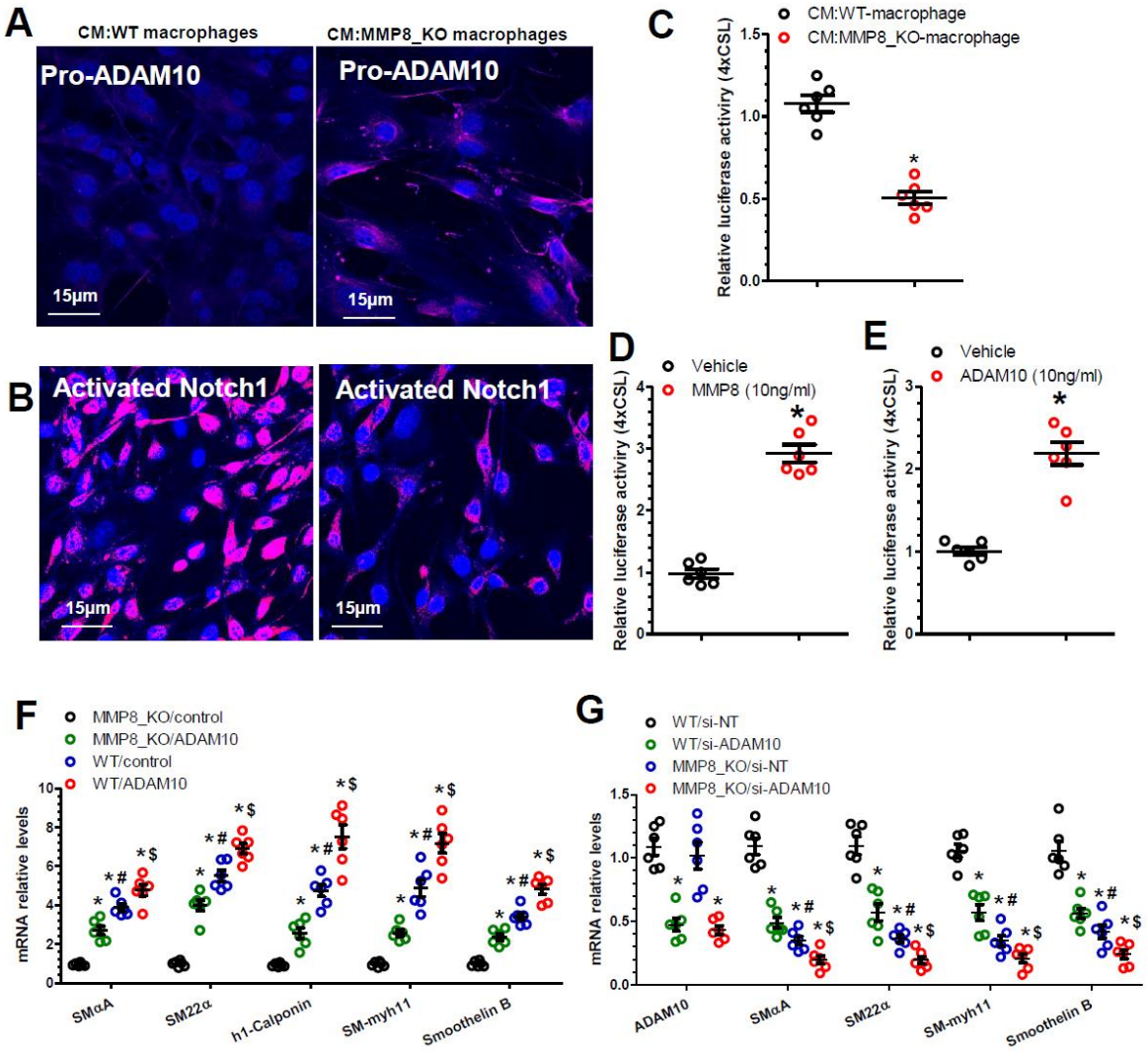
Figure 3. TGF-β1 secreted by BMMs is responsible for MMP8-mediated SMC differentiation from AdSPCs.



(A) Exogenous TGF-β1 protein increased SMC gene expression. WT or MMP8_KO BMMs conditioned culture medium (CM) contained 5ng/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 (1μg/ml) at 37ºC for 30 minutes. After then, the BMM CM was used to induce SMC differentiation from MMP8_KO AdSPCs. In (A & B), *P<0.05 (versus MMP8_KO/control or WT/IgG); #P<0.05 (versus MMP8_KO/TGFβ1 or WT/TGFβ1 antibody); $P<0.05 (versus 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 (versus control; t-test). Data presented here are Mean±S.E.M of six independent experiments (n=6).

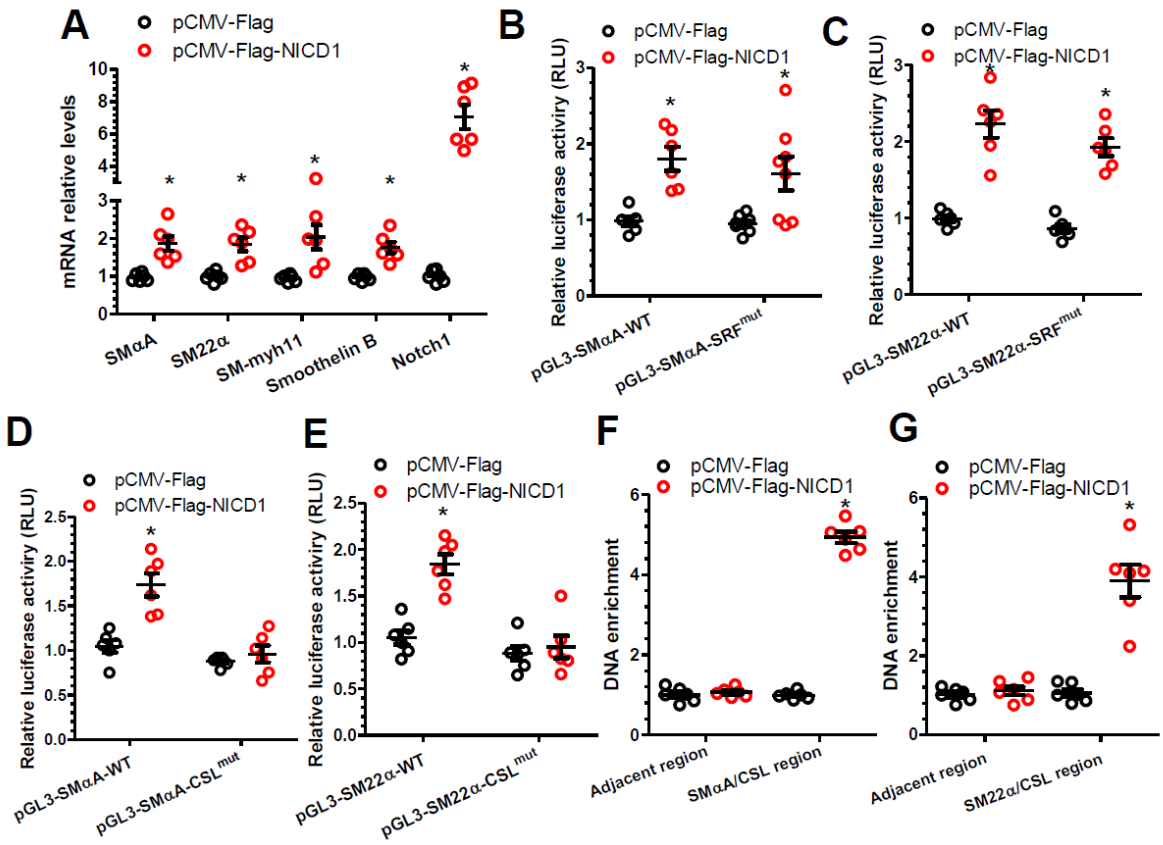
Figure 4. Importance of ADAM10-Notch1 signaling in macrophage-derived MMP8 mediated SMC differentiation from AdSPCs.



(A and B) Increased pro-ADAM10 protein abundance, but reduced Notch1 nuclear translocation in MMP8_KO AdSPCs treated with MMP8_KO BMMs conditional

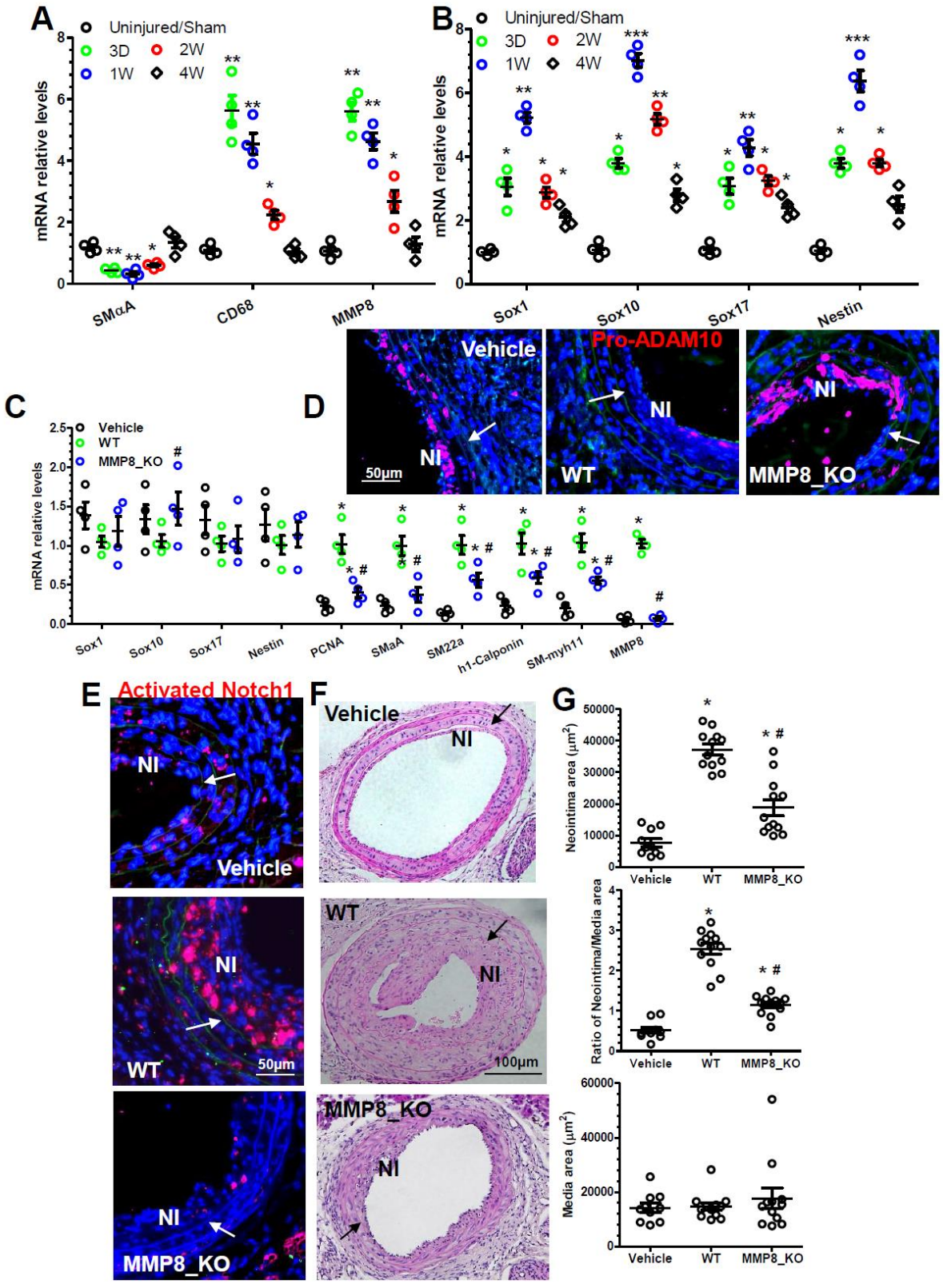
culture medium (CM). MMP8_KO AdSPCs were incubated with WT or MMP8_KO BMMs CM for two days. Cells were fixed and subjected to immunofluorescence staining with antibodies against the prodomain of ADAM10 (A) or activated Notch1 (B). Representative images from three experiments (n=3) were presented here. (C-E) Luciferase activity assays to examine Notch signaling. 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 10ng/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 & B) or Mean±S.E.M (C-G) of six independent experiments (n=6). In (C-E), *P<0.05 (versus CM:WT-macrophage, vehicle, control or control siRNA, t-test); in (F-G), *P<0.05 (versus MMP8_KO/control or WT/si-NT); $^{\#}$P<0.05 (versus MMP8_KO/ADAM10 or WT/si-ADAM10); $^{\$}$P<0.05 (versus WT/control or MMP8_KO/si-NT); one way ANOVA with a post hoc test of LSD.

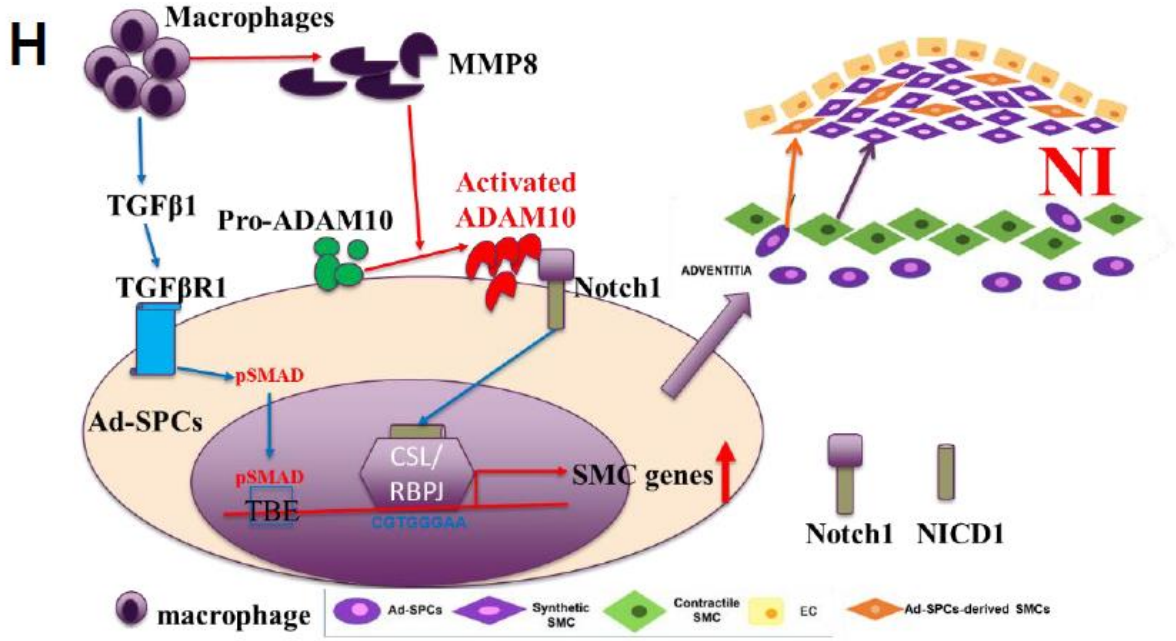
Figure 5. Notch1 promotes SMC differentiation from AdSPCs, and CSL binding site is required for Notch1-induced SMC gene expression.



(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 harboring 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 harboring 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±S.E.M of six independent experiments (n=6). *P<0.05 (versus pCMV-Flag; t-test).

Figure 6. Macrophage-derived MMP8 promotes injury-induced neointima SMC hyperplasia.





(A and B) Gene expression profiles during injury-induced arterial remodeling. Total RNAs were collected from uninjured/sham and injured carotid arteries at the indicated time points and subjected to RT-qPCR analyses. (C-G) Perivascular transplantation of macrophages and analysis. After balloon injury, 100μl of Matrigel containing vehicle or 10^6 bone marrow-derived macrophages (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±S.E.M. of four independent experiments (3~5 carotid arteries were pooled for each experiment). *P<0.05, **<0.01, ***<0.001 (versus uninjured vessels or Vehicle); $^{\#}$P<0.05 (versus 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 (versus vehicle) and $^{\#}$P<0.05 (versus 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.