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Circulation Research

ORIGINAL RESEARCH

PCSK6 Is a Key Protease in the Control of Smooth Muscle Cell Function in Vascular Remodeling

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RATIONALE: PCSKs (Proprotein convertase subtilisins/kexins) are a protease family with unknown functions in vasculature. Previously, we demonstrated PCSK6 upregulation in human atherosclerotic plaques associated with smooth muscle cells (SMCs), inflammation, extracellular matrix remodeling, and mitogens.

OBJECTIVE: Here, we applied a systems biology approach to gain deeper insights into the PCSK6 role in normal and diseased vessel wall.

METHODS AND RESULTS: Genetic analyses revealed association of intronic *PCSK6* variant rs1531817 with maximum internal carotid intima-media thickness progression in high-cardiovascular risk subjects. This variant was linked with PCSK6 mRNA expression in healthy aortas and plaques but also with overall plaque SMA+ cell content and pericyte fraction. Increased PCSK6 expression was found in several independent human cohorts comparing atherosclerotic lesions versus healthy arteries, using transcriptomic and proteomic datasets. By immunohistochemistry, PCSK6 was localized to fibrous cap SMA+ cells and neovessels in plaques. In human, rat, and mouse intimal hyperplasia, PCSK6 was expressed by proliferating SMA+ cells and upregulated after 5 days in rat carotid balloon injury model, with positive correlation to PDGFB (platelet-derived growth factor subunit B) and MMP (matrix metalloprotease) 2/MMP14. Here, PCSK6 was shown to colocalize and cointeract with MMP2/MMP14 by in situ proximity ligation assay. Microarrays of carotid arteries from Pcsk6$^{-/-}$ versus control mice revealed suppression of contractile SMC markers, extracellular matrix remodeling enzymes, and cytokines/receptors. Pcsk6$^{-/-}$ mice showed reduced intimal hyperplasia response upon carotid ligation in vivo, accompanied by decreased MMP14 activation and impaired SMC outgrowth from aortic rings ex vivo. PCSK6 silencing in human SMCs in vitro leads to downregulation of contractile markers and increase in MMP2 expression. Conversely, PCSK6 overexpression increased PDGFBB (platelet-derived growth factor BB)-induced cell proliferation and particularly migration.

CONCLUSIONS: PCSK6 is a novel protease that induces SMC migration in response to PDGFB, mechanistically via modulation of contractile markers and MMP14 activation. This study establishes PCSK6 as a key regulator of SMC function in vascular remodeling.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: atherosclerosis ■ carotid artery injuries ■ endarterectomy ■ extracellular matrix ■ vascular remodeling

Editorial, see p 586 | Meet the First Author, see p 568

Cardiovascular disease (CVD) is the leading cause of global mortality due to the complications of atherosclerosis, such as myocardial infarction and stroke. Late-stage disease is characterized by unstable atherosclerotic plaques with enhanced inflammation, enlarged and necrotic lipid core, bleeding from immature neovessels (intraplaque hemorrhage), and thinning of the fibrous cap from collagenolysis and smooth

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ORIGINAL RESEARCH

Novelty and Significance

What Is Known?

- Smooth muscle cells (SMCs) are the major cells responsible for vessel wall integrity, remodeling, and contribute to atherosclerotic plaque stability.
- SMC activation in vascular repair involves chemoattractants, mitogens and extracellular matrix (ECM) degradation controlled by various proteases.
- We previously reported that PCSK6 (proprotein convertase subtilisin/kexin 6) is one of the most significantly enriched molecules in human carotid atherosclerotic plaques.

What New Information Does This Article Contribute?

- PCSK6 is enriched in vascular tissues in association with clinical markers of vascular remodeling and symptomatic atherosclerosis and localized to SMCs.
- PCSK6 is of key importance for remodeling and SMC activation in response to vascular injury.
- Mechanistically, PCSK6 is implicated in MMP (matrix metalloprotease) 14-dependant SMC migration in response to PDGFB (platelet-derived growth factor subunit B) signaling in the vessel wall.

Recently, SMCs have been shown to contribute a higher proportion of the cells in vascular remodeling and atherosclerotic lesions than ever previously appreciated. Reports that SMC-restricted genes are causally related to development of vascular disease, imply that SMCs and related pathways could be attractive therapeutic targets. Apart from PCSK9, which is critical in the regulation of lipid metabolism in liver, other PCSKs have been poorly characterized in cardiovascular disease, especially in comparison with their well-documented functions in cancer. Our results reveal that PCSK6 is markedly enriched during the progression of vascular disease as well as in end-stage atherosclerotic plaques and linked with SMC-driven vascular remodeling. This study uncovers a new role for PCSK6, as a functional link that induces SMC migration in response to PDGFB, mechanistically via modulation of SMC contractile markers and MMP14 activation. These findings deepen our understanding of the SMC biology and offer clues to future therapeutic strategies.

Nonstandard Abbreviations and Acronyms

ACST	Asymptomatic Carotid Surgery Trial
AEGS1	AtheroExpress Genomics Study 1
BiKE	Biobank of Karolinska Endarterectomies
CASP8	caspase 8
cIMT	carotid intima-media thickness
CVD	cardiovascular disease
ECM	extracellular matrix
HDL	high-density lipoprotein
MMP	matrix metalloprotease
PCSK	proprotein convertase subtilisin/kexin
PCSK6	proprotein convertase subtilisin/kexin 6
SMA	smooth muscle α-actin
SMC	smooth muscle cell
SNP	single-nucleotide polymorphism
WT	wild type

muscle cell (SMC) apoptosis.[1] These processes weaken the fibrous cap, which may rupture and lead to clinical manifestations.[2,3]

SMCs have traditionally been recognized as the main cell type responsible for tissue integrity and lesion stability and have recently been shown to contribute a significantly higher proportion of the cell population in plaques than previously appreciated.[4] The capacity of SMCs to provide tissue integrity in atherosclerosis is based on their phenotypic plasticity and capacity to dedifferentiate into proliferative and synthetic cells that migrate into the intima, secrete extracellular matrix (ECM), and preserve the fibrous cap.[5] Clinically, the same reparative function with a burst of SMC proliferation is triggered after surgical or endovascular interventions leading to a healing response in the intima (intimal hyperplasia) that shares features with the formation of fibrous cap in atheroma.[1,6] This hyperproliferative healing response is also held responsible for the restenosis after angioplasty, therefore, it is important to increase understanding of the molecular pathways that control SMC function in vascular pathology.

SMC activation in vascular repair involves chemoattractants, mitogens, and ECM breakdown, which is partly controlled by MMPs (matrix metalloproteinases), a family of 20 secreted or membrane-associated enzymes, and their inhibitors.[7,8] Membrane-bound MMPs contribute to the invasion of SMCs into the intimal space by degradation of interstitial collagen but also to the regulation of SMC contractility and actomyosin cytoskeleton.[9] PCSKs (Proprotein convertase subtilisins/kexins) constitute a family of proteases, whose role in vascular disease was only recently recognized.[10] Apart from PCSK9, which plays a critical part in the regulation of lipid metabolism[11] other PCSKs have been poorly characterized in CVD, especially in comparison with their well-documented functions in cancer. In particular, PCSK6 (also called PACE4) has previously been linked to increased cancer

cell invasiveness by enhancing bioactivity of MMPs and cytokines.[12,13] With respect to CVD, the genetic region around *PCSK6* has been implicated in congenital heart disease by whole-genome linkage analyses[14] and DNA copy number alterations in this locus were linked to vessel wall instability and aortic dissection.[15] Adult Pcsk6$^{-/-}$ mice, without an obvious cardiovascular phenotype at baseline, were used to demonstrate a role for PCSK6 in corin activation and salt-sensitive blood pressure control.[16] We previously discovered PCSK6 is one of the most significantly enriched molecules in human carotid plaques compared with normal arteries and in plaque instability based on analyses of almost 400 patients, while other PCSKs did not show the same trend.[17] The finding that PCSK6 is upregulated in both cancers and atherosclerosis, extends the recently reported commonalities between these conditions,[18] and prompted us to further explore the role of PCSK6 in vascular disease.

We applied an integrative approach to investigate PCSK6, using several independent human biobanks, where: the IMPROVE cohort (Carotid Intima-Media Thickness [IMT] and IMT-Progression as Predictors of Vascular Events in a High-Risk European Population) of high-CVD risk patients was used to evaluate genetic association of *PCSK6* with clinical markers of vascular remodeling; AtheroExpress assessed genetic architecture of histologically phenotyped plaques; Biobank of Karolinska Endarterectomies (BiKE) encompassed carotid plaques from patients with end-stage atherosclerosis while SOKRATES contributed atheroprogression tissues. In a systems biology workflow, bioinformatic results were extended into functional investigations in human material in situ, animal models of vascular injury including Pcsk6$^{-/-}$ mice in vivo, and mechanistically by ex vivo and in vitro assays with perturbed PCSK6 expression. Our studies reveal that PCSK6 is markedly enriched during the progression of vascular disease and linked with SMC-driven vascular remodeling.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. The anonymized microarray data sets used in the study are available from Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/gds) with following permanent accession numbers: GSE21545 (for BiKE),[19] GSE40231,[20] and GSE43292[21].

Human Material and Cohorts

Patients undergoing surgery for symptomatic or asymptomatic, high-grade (>50% NASCET [North American Symptomatic Carotid Endarterectomy Trial])[22] carotid stenosis at the Department of Vascular Surgery, Karolinska University Hospital and Department of Surgery, Vascular section, Södersjukhuset, Stockholm, Sweden, were enrolled in the study and clinical data recorded on admission. Symptoms of plaque instability were defined as transient ischemic attack, minor stroke, and amaurosis fugax. Patients without qualifying symptoms within 6 months before surgery were categorized as asymptomatic and indication for carotid endarterectomy based on results from the ACST (Asymptomatic Carotid Surgery Trial).[23] Carotid endarterectomies (carotid plaques) and blood samples were collected at surgery and retained within the BiKE. The BiKE study cohort demographics, details of sample collection, processing, and large-scale analyses (genotyping, transcriptomic, and proteomic profiling) were as previously described.[19,24] Briefly, peripheral blood mononuclear cells were used for preparation of genomic DNA and high-density genotyping of n=127 BiKE patients was performed by Illumina 610w-QuadBead single-nucleotide polymorphism (SNP)-chips. For microarrays, plaques were divided transversally at the most stenotic part, the proximal half of the lesion used for RNA preparation while the distal half was fixed in 4% Zn-formaldehyde and processed for histology. Normal artery controls were obtained from 9 macroscopically disease-free iliac arteries and one aorta from organ donors without history of CVD. For LC-MS/MS (liquid chromatography–mass spectrometry and tandem mass spectrometry) proteomic analyses, atherosclerotic plaques from n=18 patients (n=9 symptomatic+9 asymptomatic; matched for sex, age, and statin medication) were collected and processed as previously described.[24] A central portion of the plaque corresponding to the maximum stenosis was separated from the respective downstream peripheral end (adjacent tissue) of the plaque and used in comparisons.[25] For immunohistochemistry, additional tissues were used: normal radial arteries obtained at coronary by-pass surgery, one internal carotid artery from a 61-year-old male treated for a neck tumor, and in-stent stenosis (intimal hyperplasia) tissue obtained from a patient after treatment of a traumatic aortic transection with a stent graft.

The database of IMPROVE, a large, multicenter, European longitudinal cohort study (acronym: carotid intima-media thickness [cIMT] and IMT-progression as predictors of vascular events in a high-risk European population) was used for studying SNP associations with various cIMT measures. IMPROVE was set up for the study of cIMT measures as predictors of incident coronary events and enrolled n=3711 subjects with at least 3 independent coronary artery disease risk factors. Detailed descriptions of IMPROVE, including the protocols for carotid ultrasound measures and SNP genotyping on Illumina CardioMetabochip and Immunochip arrays, have been reported.[26] In the present study, n=3378 subjects were used for genetic association analyses.

To study the impact of *PCSK6* variants on plaque histological characteristics, we used data from the AtheroExpress biobank (www.atheroexpress.nl). The methods of genotyping and imputation in AtheroExpress have been described previously.[27] Briefly, in 2 experiments n=1858 patients were genotyped using the Affymetrix SNP 5.0 (AEGS1 [AtheroExpress Genomics Study 1], n=836) and Affymetrix Axiom CEU arrays (AEGS2, n=1022); 1443 remained after community standard quality control.[28] Full immunohistochemical protocols used in the AtheroExpress were also detailed previously.[27] The presence of SMCs was assessed by smooth muscle α-actin (SMA).

The Sokrates study comprises progressive aortic atherosclerotic lesions collected during organ transplantation, covering all age groups, and the whole spectrum of atherosclerotic disease. Briefly, 2 centimeters of excessive aorta proximal and distal from the ostium of the renal artery was removed and lesions (n=28) were classified according to the American Heart Association classification[29] as adapted by Virmani et al.[30] Details of sample collection, demographics of the cohort along

with tissue processing, and full histological classification have been described previously.[31]

All samples were collected with informed consent from patients or organ donor's guardians. All human studies were approved by the regional Ethical Committees.

Statistical and Bioinformatic Analyses

For genetic analyses, all SNPs in the region ±200 000 kb around the *PCSK6* gene present on the Illumina CardioMetabochip and Immunochip were analyzed, after filtering out those with minor allele frequency <0.03 and linkage disequilibrium pruning for pairs with r^2>0.8. Linear regression analyses were performed between SNPs and different cIMT measures using PLINK (v1.07),[32] assuming an additive genetic model adjusting for age, sex, and population stratification, and results were corrected for multiple comparisons according to Bonferroni. All cIMT variables were logarithmically transformed before statistical analysis because of skewed distributions. Functional information about the variants was extracted using Haploreg v4.1 software (Broad Institute). Expression quantitative trait loci analyses from healthy tissues were performed using the public GTEx software (http://www.gtexportal.org). To enumerate the relative abundance of pericyte cell population in the plaque tissue, we applied the deconvolution strategy to BiKE microarrays using the Cibersort web software (https://www.ciber-sort.stanford.edu), based on recently published human plaque data.[33] Briefly, resident cell populations were deconvoluted using a signature markers file generated from single-cell RNA sequencing of atherosclerotic human coronary arteries. This gene-by-cell type matrix file contained expression levels for the top 26 genes specific for each of the major detected cell types.

Transcriptomic and proteomic data set analyses were performed with GraphPad Prism 6 and Bioconductor using a linear regression model adjusted for age and sex or a 2-sided Student *t* test assuming nonequal deviation, with correction for multiple comparisons according to Bonferroni. Distribution of the data was assessed using the Shapiro-Wilks normality test. For data from in vivo and in vitro experiments, comparative statistics between 2 groups was performed using the Student *t* test for parametric data and Mann-Whitney *U* test for nonparametric data. One-way ANOVA with Bonferroni multiple comparison test and Kruskal-Wallis with Dunn multiple comparison test was used for comparison of parametric and nonparametric data containing >2 groups, respectively. Pearson or Spearman rank correlations were calculated to determine the association between mRNA and protein expression levels from microarrays and LC-MS/MS, for parametric and nonparametric data, respectively.

A functional coupling network based on extended, experimentally validated PCSK6-MMP14-MMP2 protein-protein interactions was constructed using FunCoup software (http://www.funcoup.sbc.su.se). Gene set enrichment analysis was performed using GeneMania (http://www.genemania.org). Functional data about the genes were extracted using GeneCards (http:www.genecards.org) and PubMed literature search. Network matrices were constructed from the rat carotid injury microarray data set by calculation of the expression correlation coefficients using the Pearson method, and *P* values were corrected for multiple comparisons using Bonferroni. Clustering was performed with the Morpheus software (Broad Institute), where the distance matrix was created from dissimilarity index (1−gene correlation). Multiple sequence alignment of the PCSK6 amino acid sequence was plotted using the CLUSTAL and PRALINE softwares, and evolutionary conservation of the protein domain organization using the ENSEMBL database.

In all analyses, a *P* value<0.05 was considered to indicate statistical significance.

A detailed description of all other methods is provided in the Online Data Supplement.

RESULTS

PCSK6 Gene Variant Is Associated With Clinical Surrogate Markers of Vascular Remodeling and SMA+ Cells in Plaques

cIMT as determined by ultrasound is a predictive surrogate marker for atherosclerosis and pathological vascular remodeling.[34] Here, we first examined the impact of sequence variation in the *PCSK6* gene locus on severity and progression of cIMT in a large cohort of high-CVD risk subjects (IMPROVE, n=3378)[35] (Figure 1A; Online Table I). Of totally 23 variants tested, we found significant association with the maximum progression of internal carotid artery thickness for 2 variants rs1531817 and rs9972548 and tentatively for rs4965898. Next, by expression quantitative trait loci analyses, rs1531817 was shown to influence the expression of PCSK6 in healthy aortas (GTex resource, n=197 individuals, Figure 1B), while only tentative associations were found for rs9972548 and rs4965898 in coronary arteries (n=118, Online Table II). Moreover, rs1531817 associated with PCSK6 expression in late-stage carotid plaques (BiKE, n=127, proxy rs4965833, r^2=0.98, D′=1, Figure 1C). Of further interest, this variant emerged as associated with overall SMA+ cell content in lesions from patients with carotid stenosis (AtheroExpress, n=1443, *P*=0.045, minor allele frequency =0.32)[27,36] and also in particular with pericyte cell fraction in late-stage plaques (BiKE, n=127; cell quantitative trait loci; Figure 1D).[33] Functionally, this intronic *PCSK6* variant carries a transcriptional enhancer histone mark and represents the binding site for transcription factor BDP1 (B double prime 1), a general activator of RNA polymerase III (additional information in Online Table III). BDP1 is highly expressed in normal human smooth muscle compared to other tissues (source Human Protein Atlas, Online Figure IA). Extending on the link suggested by genetic association, in BiKE we found that BDP1 mRNA was significantly downregulated in plaques versus normal arteries and negatively correlated with PCSK6 expression (Online Figure IB).

PCSK6 Is Enriched in Plaques From Symptomatic Patients and Localized to SMA+ Cells in the Fibrous Cap and Neovessels

Next, we extended our previous findings demonstrating upregulation of PCSK6 mRNA in human carotid

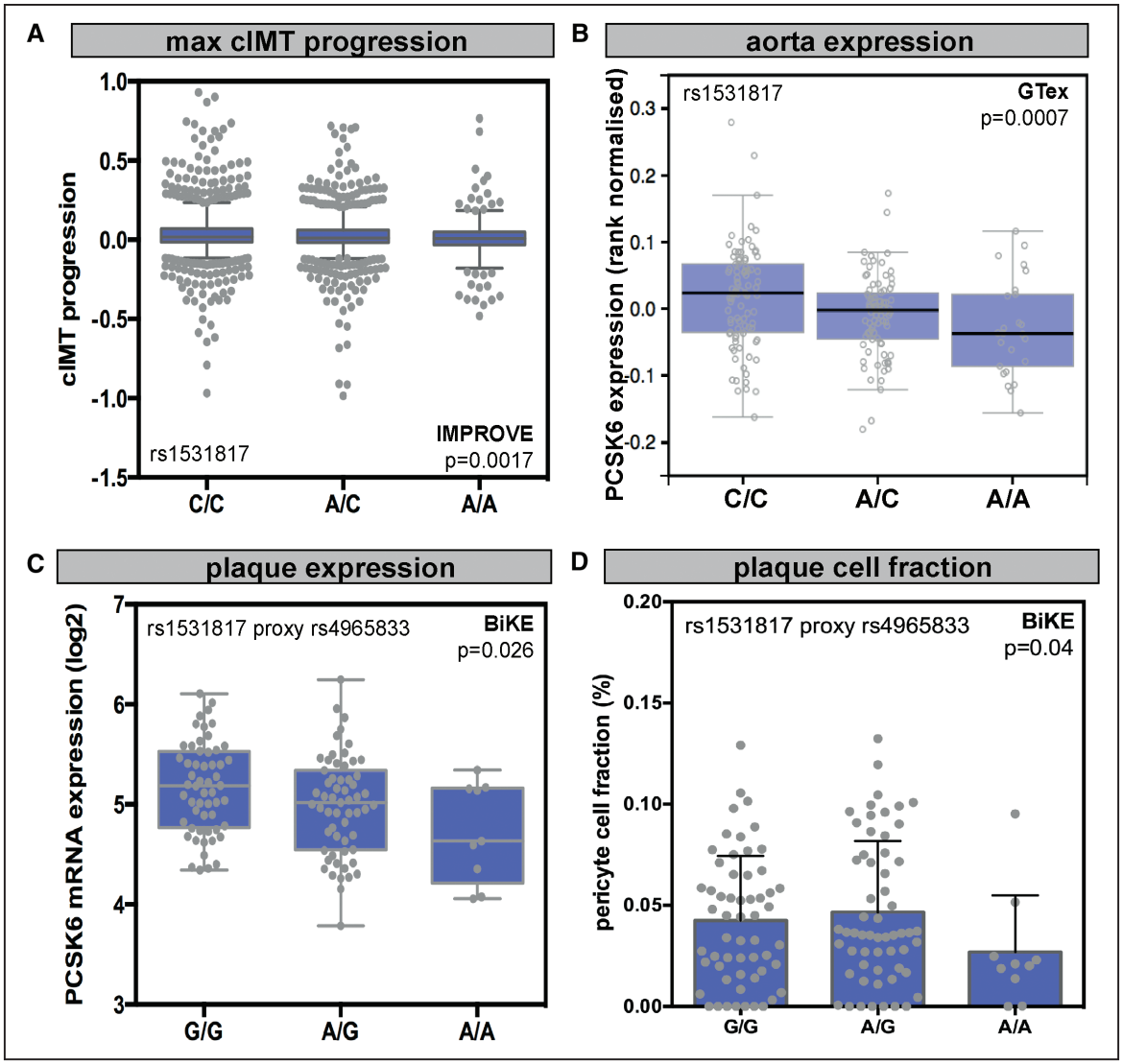

Figure 1. Integrative genetic analyses reveal association of the PCSK6 locus with atherosclerosis and vascular remodeling. Intronic *PCSK6* variant rs1531817 was associated with maximum carotid intima-media thickness (cIMT) progression in high-risk cardiovascular disease (CVD) subjects. By expression quantitative trait loci (QTL) analysis, this variant also significantly associated with PCSK (proprotein convertase subtilisins/kexin) 6 mRNA expression both in normal aortas (**B**) and in end-stage plaques (**C**). The same variant associated with pericyte cell fraction in end-stage plaques (**D**). Plots show median with 5th to 95th percentile in **A**, median with min to max in **B** and **C** and mean with SD in **C**. Raw *P* value from examination of n=23. variants found in the PCSK6 locus reported in A. *P* value in **D** was adjusted for multiple comparisons across n=15 major cell types

plaques,[17] using transcriptomic data from additional subsets of BiKE patients, as well as public microarray data sets (Figure 2A). Upregulation of PCSK6 transcript was confirmed: in carotid plaques (n=50) versus normal arteries (n=5) from BiKE (mean difference with SD=0.64±0.12); in a public dataset (GEO accession no. GSE40231) comparing coronary and carotid lesions (n=40) with normal arteries (n=40) (mean difference with SD=61.82±8.38), as well as in a public dataset (GEO accession no. GSE43292) comparing carotid plaques with matched adjacent tissue (n=32 patients, mean difference with SD=0.62±0.15). Importantly, PCSK6 protein was also enriched by mass spectrometry analysis comparing BiKE plaques central versus matched distal arterial tissue (n=18 patients, mean difference with SD=0.46±0.15) and in plaques from patients with symptoms compared with asymptomatic ones (mean difference with SD=0.41±0.14, Figure 2A).

To explore which cell types express PCSK6, we correlated PCSK6 protein levels from late-stage BiKE plaques with various cell-specific markers (Online Table IV). PCSK6 showed negative correlations with typical markers of quiescent, contractile SMCs (ie, Pcsk6/Myh11 Pearson r=−0.66, P<0.0001 and Pcsk6/Tagln r=−0.67, P<0.0001). Correlations with endothelial markers were nonsignificant or negative, whereas those with macrophage markers and lymphocytes were positive.

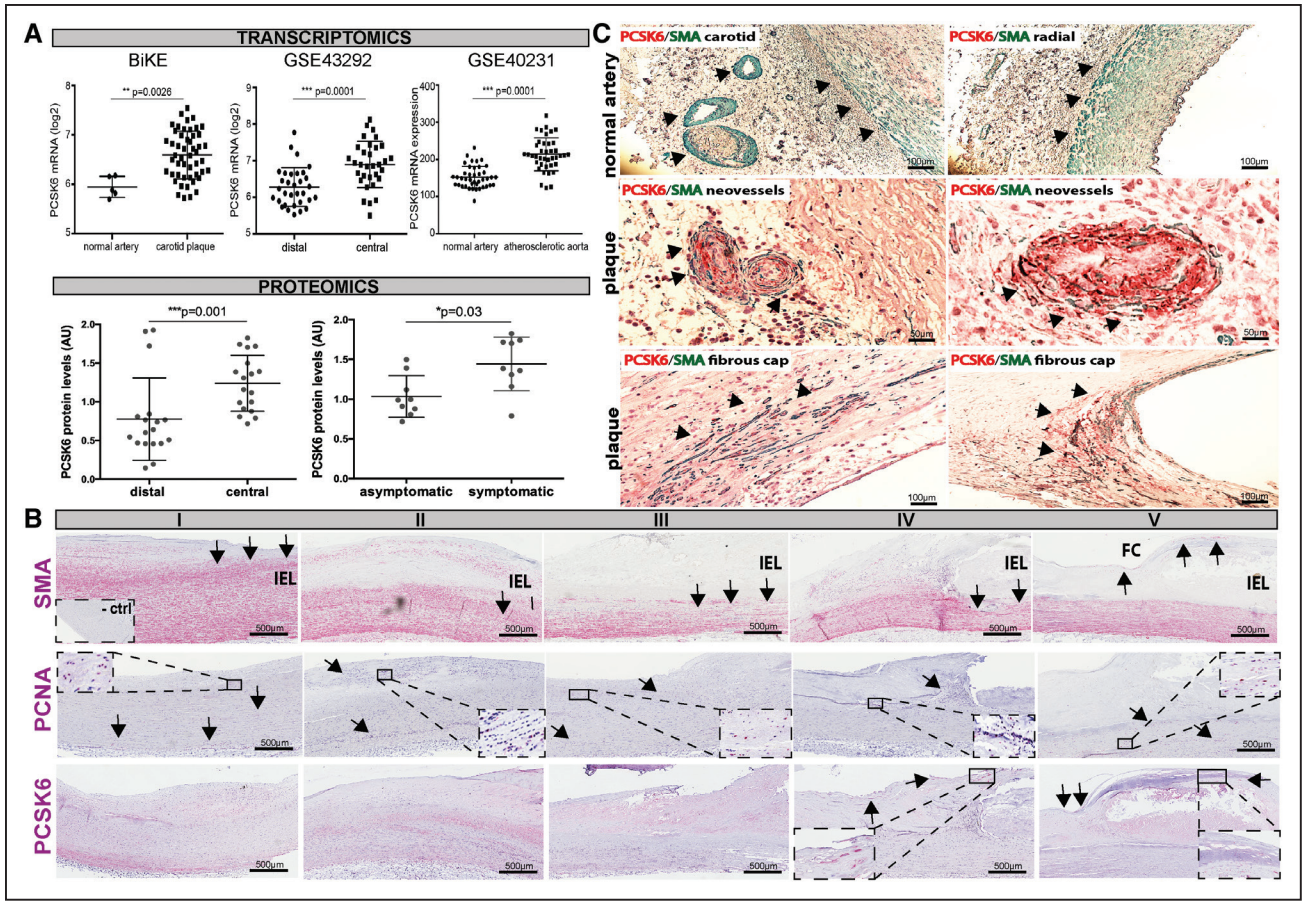

Figure 2. PCSK (Proprotein convertase subtilisins/kexin) 6 transcript and protein are upregulated in atherosclerotic lesions and localized to SMA+ cells.

PCSK6 mRNA expression was upregulated comparing plaques and normal arteries in the Biobank of Karolinska Endarterectomies (BiKE) microarray data set, which was validated in public microarrays from 2 additional cohorts comparing plaques with adjacent vascular tissue or normal arteries. Plots showing log2 mean±SD. In the proteomic analysis by mass spectrometry of n=18 matched patient samples (BiKE), PCSK6 was also enriched in plaques (central) compared with matched adjacent arterial tissue (distal samples) and in plaques from symptomatic vs asymptomatic individuals (**A**). By immunohistochemistry, smooth muscle α-actin (SMA; red) was continuously expressed in lesions from all stages of atherosclerotic disease (modified American Heart Association grading I–V; internal elastic laminae [IEL], arrows). Cells immunopositive for PCNA (proliferating cell nuclear antigen) were found both in media and intima in all tissues (red, enlarged insets, arrows show additional areas with positive signal). Signal for PCSK6 was detectable even in early lesions in subintimal SMA+ cells and in the fibrous cap (FC) in later stages (zoomed insets, arrows, **B**). Examination of end-stage plaques (**C**) showed strong PCSK6 expression in SMA+ cells in the fibrous cap and neovessels (arrows), while only a weak signal could be seen in media of normal arteries and none in adventitial microvessels. In all immunohistochemistry (IHC) experiments isotype rabbit or mouse Ig serum were used as negative controls. Images taken with 5× and 10× objective, insets with 40×.

Expression and localization of PCSK6 was analyzed by immunohistochemistry, using human aortic lesions from different stages of disease graded according to the modified American Heart Association criteria,[31] ranging from adaptive intimal thickening and xantomas (stages I and II), pathological intimal thickening (stage III), to early and thin-cap fibroatheromas (stages IV and V). PCSK6 was detectable in subintimal SMA+/PCNA+ (proliferating cell nuclear antigen) cells in stage I, and thereafter was gradually detected in fibrous cap SMA+ cells in late-lesions (Figure 2B). In end-stage plaques, PCSK6 displayed immunoreactivity in the fibrous cap overlapping with modified SMA+ cells[24] (Figure 2C), and SMA+ cells in plaque neovessels. In contrast, weak staining for PCSK6 was occasionally observed in the normal carotid artery media, while adventitial microvessels were negative. For comparison, PCSK6 staining was also absent in wild-type (WT) mouse carotid artery, whereas abundant expression was found in innominate plaques from ApoE$^{-/-}$ mice, in subintimal and fibrous cap SMA+ cells (Online Figure II).

Pcsk6 Is Induced in Activated SMCs During Intimal Hyperplasia Formation

Considering the genetic associations and expression of PCSK6 in activated SMA+ cells of plaques, we next investigated PCSK6 in the rat carotid artery injury model where intimal hyperplasia develops in response to SMC phenotypic modulation, migration, and proliferation. We have previously shown that typical markers of quiescent SMCs are gradually downregulated in the early phases

after injury, most prominently at day 5, while they reappear in mature neointima from 2 to 6 weeks after injury.[24] Time-dependent expression of Pcsk6 in balloon injured carotid artery microarrays showed an inverse profile compared with markers of differentiated, quiescent SMCs, with low expression directly after injury and upregulation from day 5 (Figure 3A). The results were verified by quantitative polymerase chain reaction, while no changes in gene expression pattern were observed for Pcsk6 in contralateral uninjured arteries (not shown). Expression of Pcsk6 was positively correlated with proliferation-related cytokines Pdgfb (platelet-derived growth factor subunit B) and Igf1 (Figure 3B).

The association between PCSK6 expression and SMC activation was corroborated by immunohistochemistry in human, rat, and mouse intimal hyperplasia tissue. In all examined species, proliferative SMA+/PCNA+ cells consistently expressed PCSK6, while control arteries were mostly negative for this protein (Figure 3C). Of note, PCNA labels cells undergoing DNA damage repair and tends to overestimate the cell replication per se, of which Ki67 is a more sensitive marker. However, due to the tissue preparation protocols, Ki67 antibodies did not work well in our immunohistochemistry.

Pcsk6-Mmp2-Mmp14 Network Controls SMC Activation in Vascular Injury

SMC activation, migration, and replication in the rat model of intimal hyperplasia starts during the first 2 days after injury[37,38] and between days 2 and 5 SMCs colonize the intimal surface, following activity related to chemoattractants and ECM degradation. Particularly, day 5 upon injury appears as a dynamic phase when ECM-receptor interactions and structure organization are especially affected through induction of MMPs.[24] Since members of the PCSK family have previously been associated with MMP activation,[39] we investigated the correlation of Pcsk6 with a panel of secreted and membrane-bound MMPs in our rat transcriptomic arrays (Online Table V). Significant positive correlations were found with Mmp12, Mmp2, and Mmp14 in injured arteries, whereas correlations with other Mmps were negative or nonsignificant both in injured and in uninjured arteries. In particular at day 5, when the expression of Pcsk6 was the lowest, positive correlations were restricted to Mmp14 and Mmp2, whereas that with Mmp12 was negative (Figure 4A).

Next, we constructed a functional network module connecting PCSK6-MMP2-MMP14 proteins via their direct and extended interaction partners.[40] This network

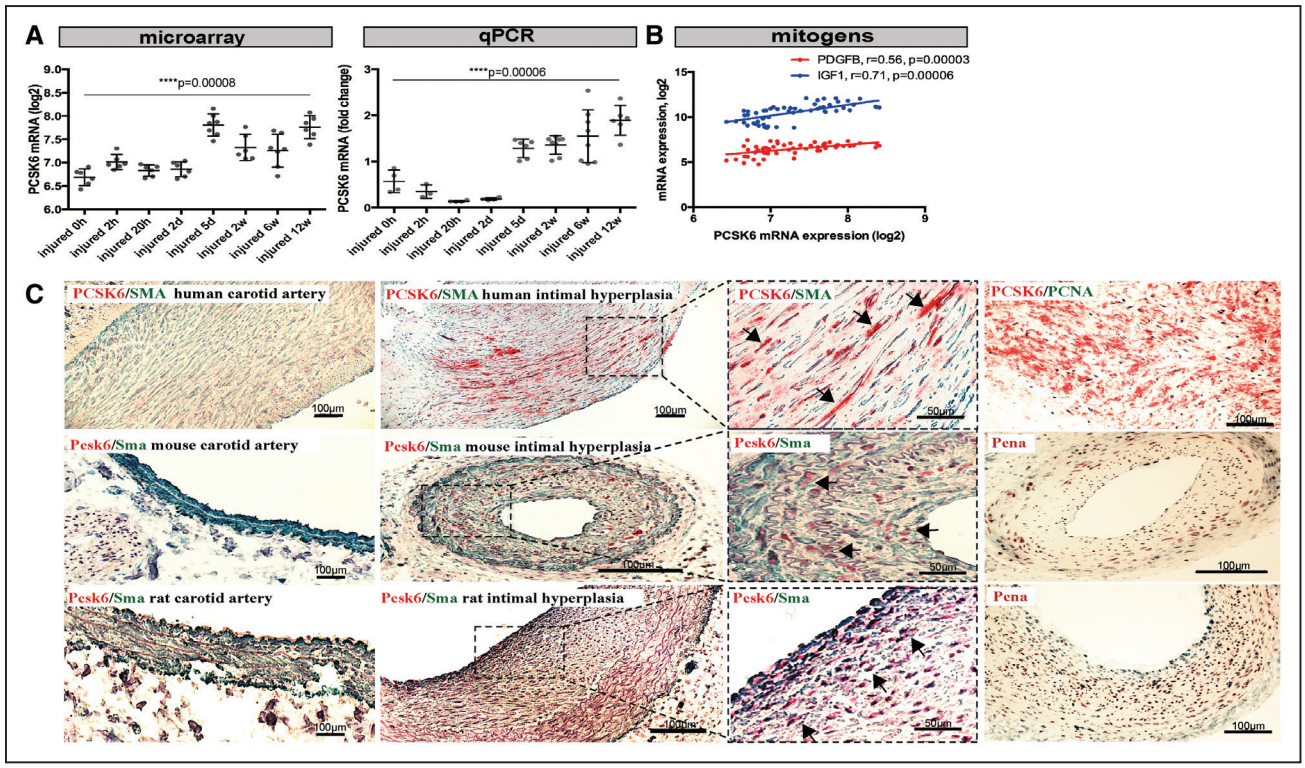

Figure 3. PCSK (proprotein convertase subtilisins/kexin) 6 is functionally related to smooth muscle cell (SMC) activation and intimal hyperplasia.

By microarray profiling and quantitative polymerase chain reaction (PCR) from rat carotid arteries after balloon injury, Pcsk6 was downregulated in early phases after injury, but upregulated from day 5 postinjury. ANOVA *P* value reported across groups (**A**). Positive expression correlations were found in this model between Pcsk6 and mitogens Pdgfb (platelet-derived growth factor subunit B) and Igf1 (**B**). By immunohistochemistry (IHC) in human and murine intimal hyperplasia (mouse carotid artery ligation 6 wk, rat carotid artery balloon injury 12 wk), PCSK6 (red signal) was strongly expressed by proliferative PCNA+ (proliferating cell nuclear antigen)/SMA+ (green signal) cells (enlarged images, arrows) and weekly expressed or absent in control vessels (**C**). In all IHC experiments isotype rabbit or mouse Ig serum was used as negative controls. Images taken with 20× and 40× objectives.

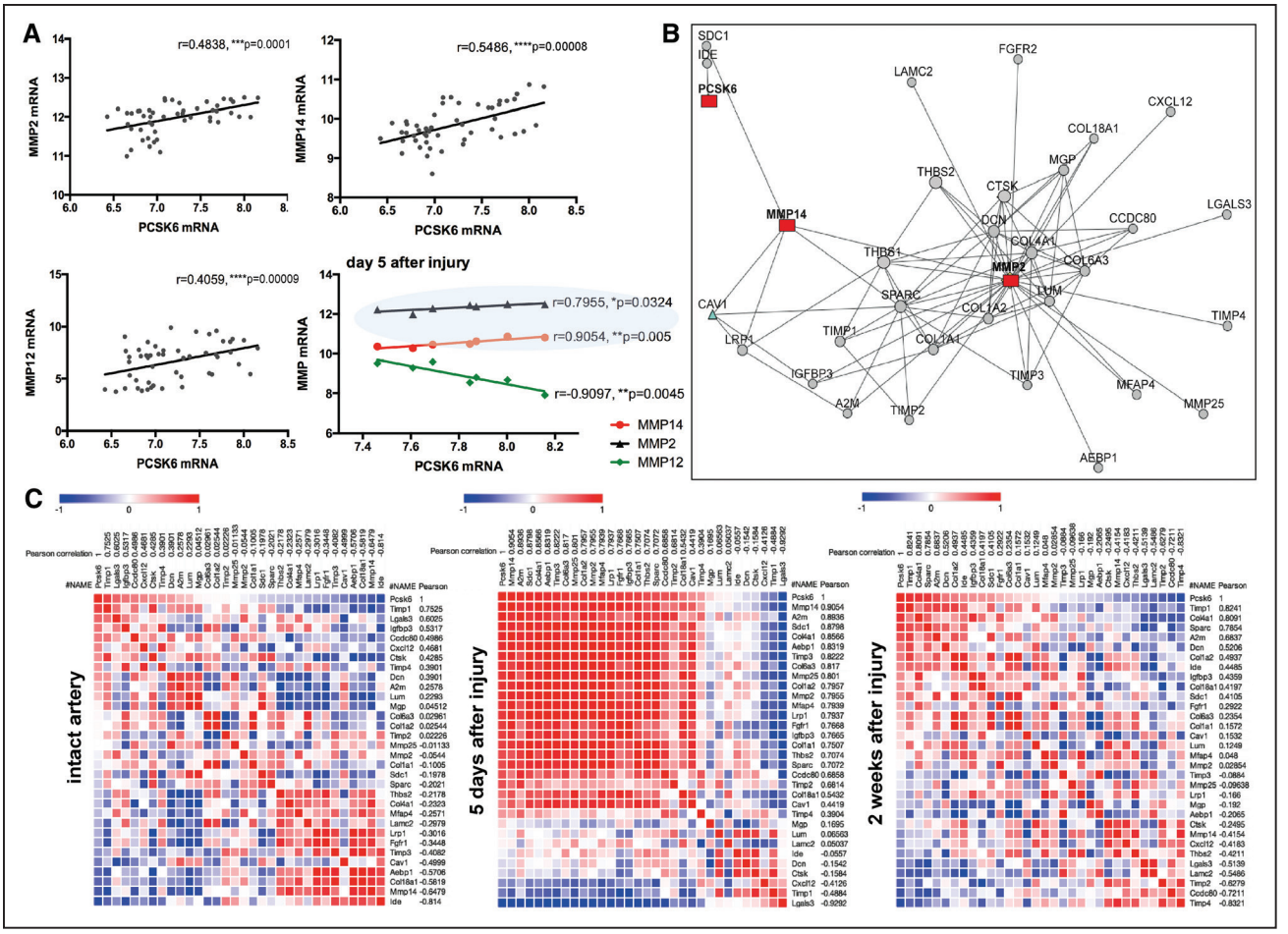

Figure 4. A network connecting Pcsk (proprotein convertase subtilisins/kexin) 6-Mmp (matrix metalloprotease) 2-Mmp14 is activated in rat carotid intimal hyperplasia.

Expression correlations of Pcsk6 during all time-points in the rat carotid artery injury model were significantly positive for Mmp2, Mmp14, and Mmp12. However, on day 5 after injury only correlations with Mmp2 and Mmp14 were positive, while the correlation with Mmp12 was significantly negative (**A**). The functional network constructed from direct and extended protein interaction partners of PCSK6, MMP2, and MMP14 (**B**). Network weighted for closeness in biological function based on publically available data. Expression correlations of the genes in this network were examined in rat carotid artery injury data sets and presented as matrices where the nearest neighbors were clustered based on the correlation index (**C**). Clustering of the Pcsk6-Mmp2-Mmp14 network is observed at 5 d after injury (red areas) compared with intact arteries and vessels 2 wk after injury. Positive Pearson correlation rho presented as shades of red, negative as shades of blue (legend). *P* values in **A** have been adjusted for n=13 comparisons taking into account the various MMPs.

contained 33 nodes (Figure 4B) and pathway analysis showed an enrichment of proteins involved in ECM organization (false discovery rate [FDR]=6.8×10^{-50}), collagen degradation (FDR=2.4×10^{-31}), and growth factor binding (FDR=2.6×10^{-13}) but also, that is, blood vessel development (FDR=0.049; Online Table VI). Correlation matrices were then built from rat intimal hyperplasia transcriptomic data sets and clustered based on the similarities of the gene expression profiles (Figure 4C). No significant clustering of the Pcsk6-Mmp2-Mmp14 network could be visualized using expression from intact arteries, where Pcsk6 was correlated with, that is, Timp1 (tissue inhibitor of metalloproteinases 1), suggesting that the network was inhibited in the steady state. However, a striking clustering was observed at day 5 after injury when Pcsk6 correlations with Mmp14, A2m, Aebp1 (adipocyte enhancer-binding protein 1), and Mmp2 became strongly positive. Two weeks after injury, when the neointima is established,[24] Pcsk6 was again positively correlated with Timp1 as well as, that is, Sparc (secreted protein acidic and rich in cysteine), whereas the correlation to Mmp14 was negative and the network appeared again uncoupled.

Coexpression of Pcsk6, Mmp2, and Mmp14 was then confirmed by immunohistochemistry in injured rat carotid arteries, where Pcsk6 and total Mmp14 protein were detected in deeper medial SMCs already at day 5 and in the neointima 2 weeks after injury. In contrast, immunoreactivity for Mmp2 was scarcely present at day 5 and more prominently after 2 weeks (Figure 5A). Moreover, proximity ligation assay demonstrated direct cointeraction between Pcsk6 and Mmp14 at day 5 but not between Pcsk6 and Mmp2. In tissues 2 weeks after injury, Pcsk6 was found to cointeract with both Mmp2 and Mmp14 (Figure 5B, Online Figure III). Together,

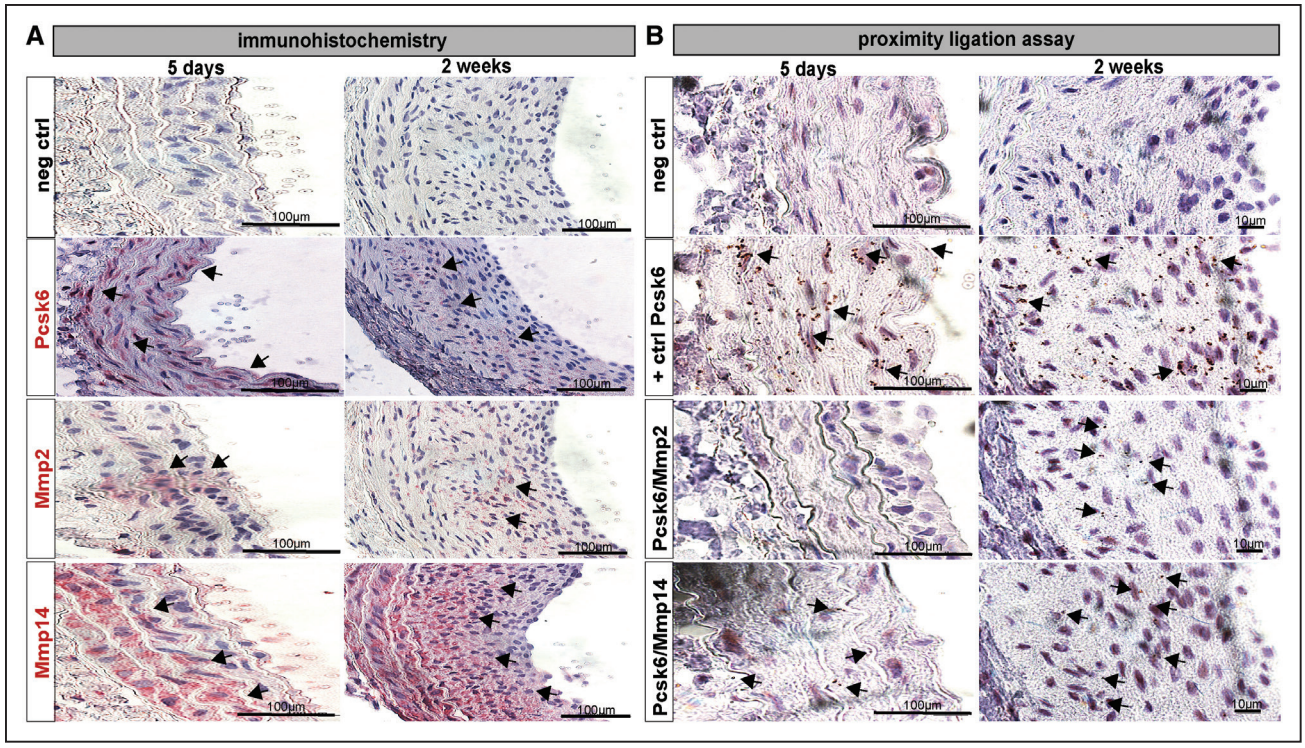

Figure 5. PCSK (proprotein convertase subtilisins/kexin) 6-MMP (matrix metalloprotease) 2-MMP14 interaction in situ. Immunohistochemistry (IHC) examination of rat carotid arteries after injury revealed that PCSK6 and MMP14 proteins were widely expressed in medial smooth muscle cells (SMCs) 5 days after injury, while MMP2 was not abundant at this stage. Two weeks after injury, all 3 proteins were widely expressed in the neointima (arrows, **A**). In all IHC experiments isotype rabbit or mouse Ig serum was used as negative controls. Using consecutive sections of injured arteries for proximity ligation assay (PLA), cointeraction between PCSK6 and MMP14 proteins could be detected already 5 days after injury and was even more prominent after 2 wk (**B**). The interaction between PCSK6 and MMP2 proteins could not be detected until 2 wk after injury in neointima. Arrows point to the PLA signal. Probe detecting PCSK6 was used as a positive control.

these studies from the rat model of intimal hyperplasia highlighted a mechanism where Pcsk6 interaction with Mmp14 and Mmp2 seems specifically associated with the mobilization of SMCs to build neointima.

Reduced Intimal Hyperplasia in Pcsk6$^{-/-}$ Mice due to Decreased Mmp14 Activation

From an evolutionary perspective, PCSK6 protein is highly conserved comparing human, rat, and mouse, both when it comes to the primary sequence (>90%) and domain organization structure (Online Figure IV), which implies a high level of functional conservation among species. Intriguingly, while 25% of the Pcsk6$^{-/-}$ mice are embryonic lethal, surviving adults are viable and do not present an obvious cardiovascular phenotype.[16] This prompted us to further study its role for SMC function using these knockouts and perform a global gene expression profiling of Pcsk6$^{-/-}$ versus WT carotid arteries (Online Figure VA). We found that there was a trend towards downregulation in the Pcsk6$^{-/-}$ arteries of many Pdgf and Tgf (transforming growth factor) cytokines including their receptors. There was also a trend towards upregulation of Mmp2 and Mmp15, but no significant change in mRNA expression was observed for Mmp14. Importantly, there was a downregulation of all SMC contractile markers and many ECM molecules (especially related to proteoglycans), and regulation of cell death/survival was also perturbed. Some of the most downregulated genes in Pcsk6$^{-/-}$ arteries were Tmem254b and Plac9b, both genetically associated with muscular myopathy.[41] Gene set enrichment analysis of significantly dysregulated genes showed the upregulation of lipid metabolism and other metabolic pathways, while ECM organization, elastic fibers/proteoglycan formation, and SMC contractility were downregulated along with regulation of necroptotic cell death and CASP8 (caspase 8) activity (Online Figure VB).

The Pcsk6$^{-/-}$ vasculature appeared functional at baseline despite these gene expression changes, and normal by gross morphology, hematoxylin, and SMA staining in carotid arteries (Online Figure VIA). Thus, we challenged the mice using carotid artery flow cessation, a model shown to generate reproducible intimal lesions attributable to SMC proliferation.[42] After 6 weeks, vessels from WT mice developed an increase in intimal area and intima-to-media ratio compared with Pcsk6$^{-/-}$ mice (mean difference with SD=6114.7±1893.5 and 0.08±0.05, respectively, Figure 6A and 6B). Although there was hardly any remaining proliferative activity in the media of WT mice 6 weeks after

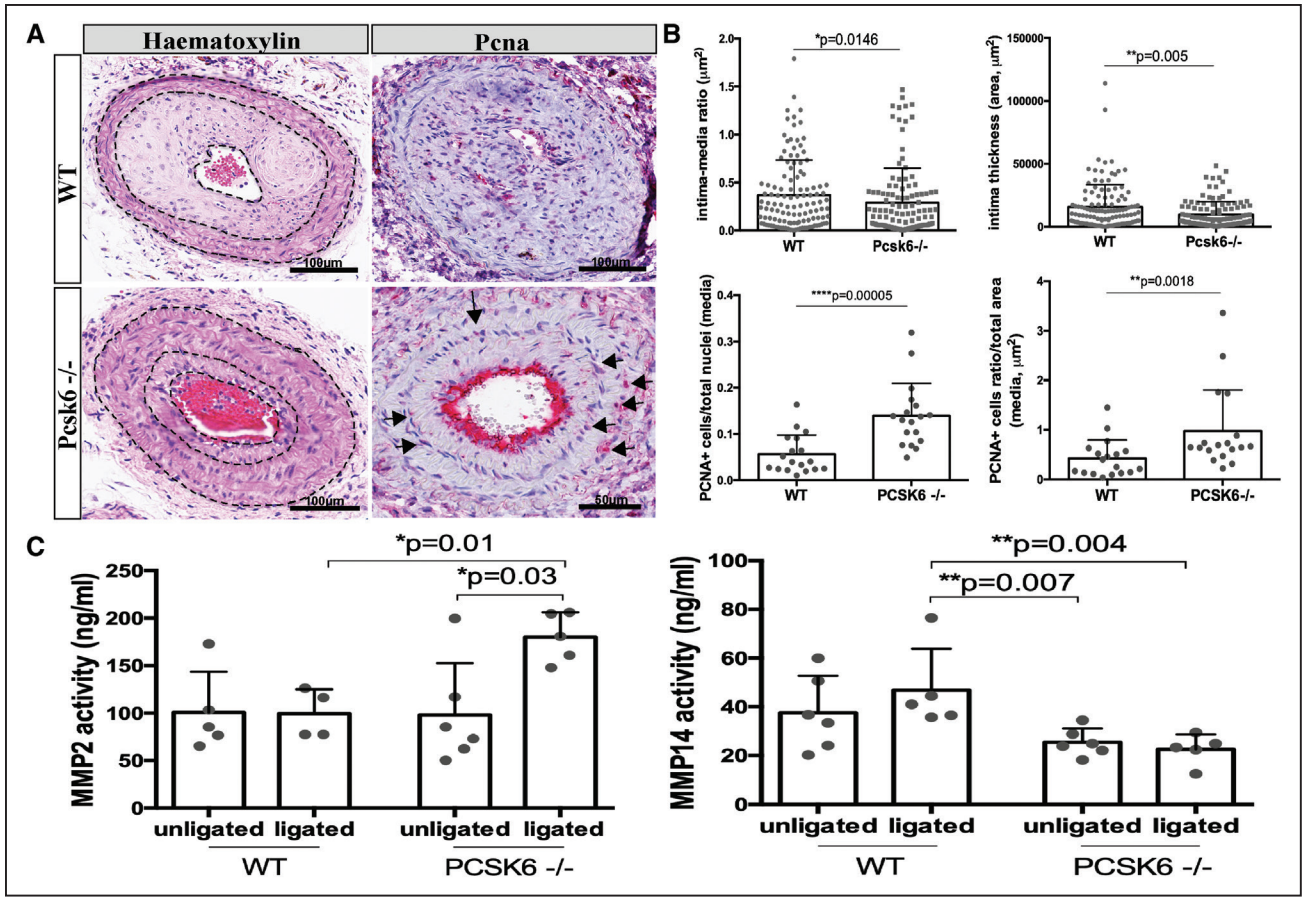

Figure 6. Reduced intimal hyperplasia after carotid artery ligation in Pcsk6−/− mice via impaired MMP (matrix metalloprotease) 14 activation.

Serial histological sections from carotid ligations in wild type (WT) and Pcsk6$^{-/-}$ mice were evaluated for intima and media thickness and number of medial PCNA+ (proliferating cell nuclear antigen) cells (**A**). Quantifications revealed significantly reduced intima-media ratio and intima thickness in Pcsk6$^{-/-}$ mice, with a higher number of proliferative cells trapped in the media of the ligated arteries (**B**). A quantitative assay from carotid tissue homogenates confirmed significantly less MMP14 activation in ligated arteries from Pcsk6$^{-/-}$ mice compared with WTs, while active MMP2 was increased (**C**). In all immunohistochemistry (IHC) experiments isotype rabbit or mouse Ig serum was used as negative controls. *P* values in plots under **C** are adjusted for multiple comparisons; **P*<0.05, ***P*<0.01

ligation, PCNA+ cells could still be detected in the media of Pcsk6$^{-/-}$ mice, as shown by a significantly higher replication index (mean difference PCNA+ cells/total nuclei with SD=0.08±0.02; mean difference PCNA+ cells ratio/total area with SD=0.55±0.21).

Upon carotid artery ligation, quantitative polymerase chain reaction showed induction of furin mRNA expression in WT mice but not in Pcsk6$^{-/-}$. Pcsk5 and Pcsk7 were also induced in response to ligation, both in WTs and even more in Pcsk6$^{-/-}$ mice. However, Pcsk2 showed a significant compensation capacity as it was specifically induced in ligated Pcsk6$^{-/-}$ mice and not in WTs (Online Figure VIB). Combined with the observed functional associations of Pcsk6, Mmp14, and Mmp2 in SMCs in rat intimal hyperplasia, we reasoned that the reduced neointimal response in Pcsk6$^{-/-}$ mice could be because of a defective SMC migration and possibly dependent on impaired Mmp enzymatic activation. Indeed, using a quantitative assay from tissue lysates, a reduction of Mmp14 activity was observed in ligated Pcsk6$^{-/-}$ versus WT arteries (mean difference with SD=−24.31±8.09) and a marginal compensatory increase in Mmp2 activity (mean difference with SD=80.69±17.28; Figure 6C).

PCSK6 Regulates SMC Migration and Proliferation in Response to PDGFBB

The role of PCSK6 in SMC function was thereafter studied in aortic explants from WT and Pcsk6$^{-/-}$ mice, where SMC outgrowth in collagen matrix takes place through a combination of ECM degradation, proliferation, and migration.[43] Upon stimulation with PDGFBB (platelet-derived growth factor BB), we observed significantly lower cellular outgrowth from Pcsk6$^{-/-}$ aortic rings compared with WT (mean difference with SEM=−34.79±15.82; Figure 7A). Morphologically, primary SMCs isolated from Pcsk6$^{-/-}$ aortas were spindle shaped with a filopodial type of spreading compared with WT SMCs that developed large lamellipodia on fibronectin, as observed by fluorescent cytoskeletal

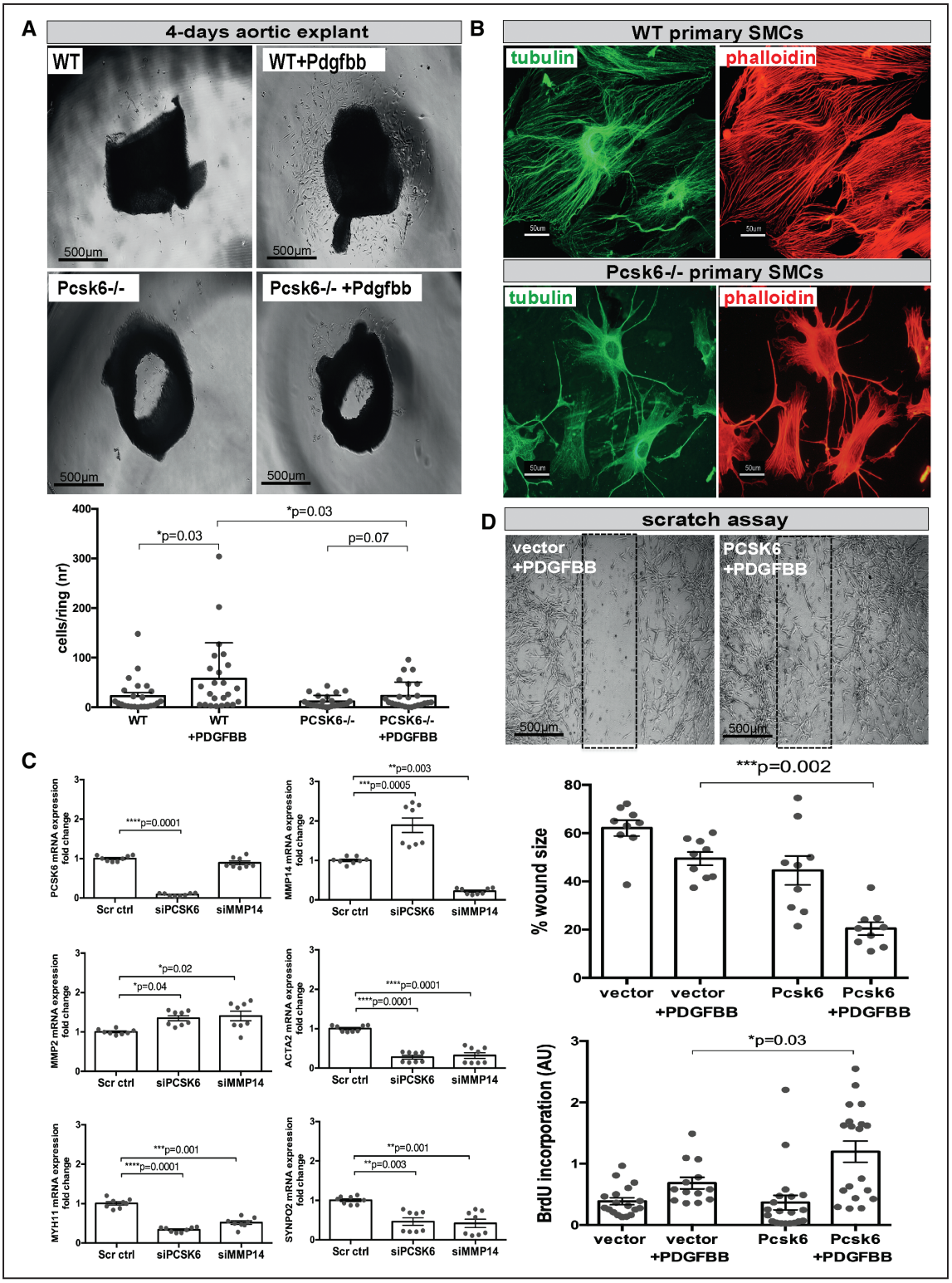

Figure 7. PCSK (Proprotein convertase subtilisins/kexin) 6 perturbation affects smooth muscle cells (SMCs) proliferation and migration in vitro.

Mouse aortic ring explants were grown in 3-dimensional-collagen matrix with or without PDGFBB (platelet-derived growth factor BB) stimulation. Significantly less cell outgrowth was quantified from Pcsk6$^{-/-}$ rings compared with wild types (WTs) in response to PDGFBB. Plots show mean ± SEM from 2 experiments (**A**). Primary SMCs isolated from Pcsk6$^{-/-}$ aortas displayed filopodial spreading in comparison with WT cells. Cytoskeletal staining for tubulin and actin by phalloidin (**B**). Silencing of PCSK6 or MMP (matrix metalloprotease) 14 mRNA in human SMCs lead to induction of MMP2 and repression of contractile markers (**C**). Overexpression of PCSK6 in human SMCs resulted in marginally increased proliferation (as evaluated by BrdU incorporation) but strongly enhanced cell migration in wound-healing assay by PDGFBB stimulation (**D**). Plots in **C** and **D** show mean ± SEM from 3 experiments. ANOVA with post hoc comparisons has been used in all plots.

staining (Figure 7B). Furthermore, these cells had a limited ability to propagate in culture compared with WT SMCs and were nonproliferative after passage 2 or 3.

We instead investigated these changes using primary human carotid artery SMCs with PCSK6 or MMP14 mRNA silencing (Figure 7C). As expected, silencing of both PCSK6 and MMP14 leads to strong downregulation of contractile SMC markers, replicating our in vivo data. Again, we confirmed that PCSK6 mRNA silencing had no effect on MMP14 mRNA expression, but interestingly MMP14 silencing resulted in 2-fold induction of PCSK6 (mean difference with SEM=0.89±0.18) while MMP2 was induced in response to both PCSK6 and MMP14 silencing (mean difference with SEM=0.35±0.07 and 0.40±0.12, respectively). Overexpression of PCSK6 in human SMCs, resulted in significantly higher proliferation and especially migration after PDGFBB stimulation compared with control cells (mean difference with SEM=0.51±0.23 and −29.03±3.81, respectively; Figure 7D; Online Figure VII).

DISCUSSION

Here, we applied an integrative approach to characterize the role of PCSK6 in vascular remodeling and show that PCSK6 is (1) enriched in association with clinical markers of vascular remodeling, symptomatic atherosclerosis, and particularly SMA+ cells in plaques; (2) involved in vascular remodeling in response to injury; and (3) mechanistically implicated in MMP14-dependant SMC migration in response to PDGFB. Moreover, our data show localization of PCSK6 to plaque fibrous cap and neovessels, which further corroborates the notion that PCSK6 participates in migration of SMA+ cell populations during pathological remodeling.

Several studies have established that cIMT changes over time are associated with vascular risk and prediction of adverse events both in subjects with plaques at baseline and in those without.[35] However, cIMT reflects not only early atherosclerosis but also nonprogressive compensatory remodeling with medial and intimal changes as a result of SMC activation, hyperplasia, and fibrocellular hypertrophy, which may be an adaptive response to changes in flow, wall tension, or lumen diameter.[34,44] Interestingly, *PCSK6* variant rs1531817 was associated with maximum cIMT progression in the internal carotid artery, where atherosclerotic plaques are more prevalent compared with the common carotid artery.[45] Here, we coupled PCSK6 genetics not only with cIMT progression but also with its expression and SMA+ cellular context in normal arteries and plaques (both SMCs and pericytes). In confirmation, we show by immunohistochemistry that PCSK6 becomes induced early during vascular remodeling and is abundant in SMCs already at the stage of intimal xantoma. Together, our findings imply that PCSK6 could be a causal factor contributing to predisposition towards vascular remodeling and atherosclerosis, driven by SMC-related changes.

Atherosclerotic lesions seem to undergo circles of remodeling with stabilization and destabilization events, which are dependant on ECM turnover and SMC migration and proliferation.[46] Changes in protease- and growth factor expression are considered to precede the invasiveness of SMCs in neointima formation and atherosclerosis.[47] The function of MMP14 in these processes has been demonstrated by decreased ligation-induced intimal hyperplasia in Mmp14$^{+/-}$ mice, and this protease has also been implicated in cancer metastasis, angiogenesis, wound healing, inflammation, and rheumatoid arthritis. It has been postulated that MMP14 localizes to membrane structures at the leading edge of migrating cells, where it activates other MMPs and degrades the pericellular matrix. MMP14-dependent MMP2 activation facilitates basement membrane collagen degradation by MMP2, as MMP14 cannot degrade type IV collagen.[48] Notably, the existence of a PCSK-MMP14-MMP2 axis has been suggested from previous in vitro studies, without specifying the exact PCSK involved and not excluding the possibilities of PCSK-independent MMP14 activation.[49] Consistent with our in vivo data showing that Pcsk6 deletion (similarly as the Mmp14$^{+/-}$ genotype) confers mice with resistance to neointimal hyperplasia by decreased MMP14 activation, our ex vivo findings highlight the importance of PCSK6 for collagenolytic and invasive properties of SMCs. In fact, since PCSK6 was also induced in response to MMP14 silencing, and MMP2 was induced in response to both PCSK6 and MMP14 silencing, this confirms that these 3 proteases are functionally linked in complex, partially redundant but also partially nonoverlapping mechanisms of regulation. However, direct crosstalk between membrane receptors for mechanical stretch, MMP2 production, intracellular signaling pathways via IGF (insulin-like growth factor) and PDGFB receptors, and subsequent SMC activation has also been demonstrated.[50] Thus, as MMP2 can also be activated independently from MMP14 that is impaired in the Pcsk6$^{-/-}$ mice, its increase in knockouts has a compensatory effect and likely attenuates some of the phenotypes observed in these mice. Moreover, the PCSK6-MMP14-MMP2 driven network in the rat vascular injury model offered broader insights into the mechanistic aspects of SMC regulation by PCSK6 through correlations with, that is, TIMP1 that inhibits protease activity, an AEBP1 transcription factor that plays a role in SMC differentiation and SPARC that inhibits SMC growth through interactions with ECM and PDGF.

In a mature artery, contractile SMCs display stable adhesive interactions with the surrounding basement membrane, which retains the cells in a differentiated state and prevents cell cycle entry in response to PDGFB and other mitogens.[51] Basement membrane disruption and altered cell-matrix interactions with type I collagen

and fibronectin, enhance responsiveness to mitogens and dedifferentiation at sites of injury.[51] In this context, MMP14 activity and PDGFR-β signaling promote the induction of a migratory and proliferating phenotype, to some extent through the intracellular domain of MMP14, which has been shown to directly modulate the SMC contractility markers.[9] Similarly, PCSK6 ablation leads to repression of contractile SMC markers, growth factors/receptors as well as proteoglycan ECM, which was further supported by the observation that primary SMCs isolated from PCSK6$^{-/-}$ mice displayed a decreased propagation capacity, whereas overexpression of PCSK6 in human SMCs was accompanied by increased migration and proliferation. Interestingly, MMP14 and PCSK6 were shown to be upregulated in SMCs in vitro by similar stimuli (eg, PDGFB, TGFB1, TNF [tumor necrosis factor]-α, and IFN [interferon]-γ[17]) and PCSK6 positively correlated with several mitogens and inflammatory markers in plaques and rat intimal hyperplasia. Thus, it is plausible that PCSK6 is induced in plaque SMCs by the enhanced inflammatory activity associated with plaque instability, also intimately coupled to other features of the unstable plaque, such as neovessel formation, SMC apoptosis and senescence, increased degradation of the ECM. Although our in vitro data supported a role for PCSK6 both in SMC migration and proliferation, the presence of PCNA+ cells in the media of ligated arteries in Pcsk6$^{-/-}$ mice suggests that PCSK6 is dispensable for SMC proliferation in vivo but not for migration. Taken together, this data strengthens the notion that PCSK6 and MMP14 cooperate and may also assist in the processing and release of growth factors from extracellular depots.

Human PCSKs are a family of 9 subtilisin-kexin like secretory serine proteases and represent some of the most abundant and diverse classes of enzymes.[52] With respect to CVD, PCSK3 (furin), and PCSK5 have been implicated in inflammation, MMP activation and integrin-processing in atherosclerosis, whereas PCSK7 and PCSK2 have been detected in SMCs and linked to myocardial infarction.[53–56] Polymorphisms in the PCSK3 and PCSK6 genes have been linked with hypertension and CVD risk and in PCSK5 with HDL (high-density lipoprotein) levels.[57–59] Because of the structural similarities within the PCSK family,[52] some limitations should be stated and functional redundancy cannot be excluded with respect to the findings in our study, as indicated by the increase in Pcsk2 in response to ligation in Pcsk6$^{-/-}$ mice. The lack of an obvious vascular phenotype in Pcsk6$^{-/-}$ mice indicates such compensatory mechanisms, although these were clearly not enough to fully restore SMC function. Furthermore, PCSK6 occurs in several isoforms with various subcellular localizations,[52] and we previously identified enrichment of the extracellular isoform in atherosclerotic plaques,[17] but detailed characterization of other PCSK6 isoforms remains necessary.

From a translational aspect, therapeutic properties of PCSK6 inhibitors have been studied in prostate cancer and rheumatoid arthritis,[60] the protein has been proposed as prostate cancer biomarker,[61] and investigations are ongoing into its potential for targeted molecular imaging.[62] The data presented in this study establish PCSK6 as a key modulator of SMC function in vascular remodeling and atherosclerosis through a novel mechanism implicating MMP14/MMP2 activation upon cytokine stimulation. Based on our findings, further investigations to determine how these mechanisms affect atherosclerotic plaque instability are warranted.

ARTICLE INFORMATION

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Disclosures

None.

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