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PCSK6 is a key protease in the control of smooth muscle cell function in vascular remodeling

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Abstract

Rationale: Proprotein convertase subtilisins/kexins (PCSKs) 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 (ECM) remodeling and mitogens. 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 vs. healthy arteries, using transcriptomic and proteomic datasets. By immunohistochemistry, PCSK6 was localised 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 and MMP2/MMP14. Here, PCSK6 was shown to co-localise and co-interact with MMP2/MMP14 by *in situ* proximity ligation assay. Microarrays of carotid arteries from Pcsk6$^{-/-}$ *vs.* control mice revealed suppression of contractile SMC markers, ECM 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* lead to downregulation of contractile markers and increase in MMP2 expression. Conversely, PCSK6 overexpression increased PDGFBB-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.

Key Words: proprotein convertase subtilisins/kexins, vulnerable plaque, human biobank, rat carotid artery injury

Non-standard Abbreviations and Acronyms

PCSK6 – proprotein covertase subtilisin/kexin 6
CEA – carotid endarterectomy
TIA – transient ischemic attack
AF – amaurosis fugax
MS – minor stroke
BiKE – **Bi**obank of **K**arolinska **E**ndarterectomies
NA – normal arteries
CP – carotid plaques
S – symptomatic
AS – asymptomatic
IHC – immunohistochemistry
IFL – immunofluorescence
PLA – proximity ligation assay
WT - wild type
KO – knockout
CVD - cardiovascular disease
MMP - matrix metalloprotease
PCSK - proprotein convertase subtilisin/kexin
ECM – extracellular matrix
cIMT – carotid intima-media thickness
SNP - single nucleotide variant

Introduction

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 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 matrix metalloproteinases (MMPs), 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]. Proprotein convertase subtilisins/kexins (PCSKs) constitute a family of proteases, whose role in vascular disease was only recently recognised[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 nymber 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 as 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, utilizing several independent human biobanks, where: the IMPROVE cohort of high-CVD-risk patients was used to evaluate genetic association of *PCSK6* with clinical markers of vascular remodeling; AtheroExpress assessed genetic architecture of histologicaly phenotyped plaques; 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.

Materials and Methods

Human material and cohorts

Patients undergoing surgery for symptomatic (S) or asymptomatic (AS), high-grade (>50% NASCET)[19] 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 transitory ischemic attack (TIA), minor stroke (MS) and amaurosis fugax (AF). Patients without qualifying symptoms within 6 months prior to surgery were categorized as AS and indication for carotid endarterectomy (CEA) based on results from the Asymptomatic Carotid Surgery Trial (ACST)[20]. Carotid endarterectomies (carotid plaques, CP) and blood samples were collected at surgery and retained within the **Bi**obank of **K**arolinska **E**ndarterectomies **(BiKE)**. The BiKE study cohort demographics, details of sample collection, processing and large-scale analyses (genotyping, transcriptomic and proteomic profiling) were as previously described[21, 22]. Briefly, peripheral blood mononuclear cells were used for preparation of genomic DNA and high-density genotyping of n=127 BiKE patients was performed by Ilumina 610w-QuadBead 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 (NA) were obtained from nine macroscopically disease-free iliac arteries and one aorta from organ donors without history of cardiovascular disease. The microarray dataset is available from Gene Expression Omnibus (GSE21545). For LC-MS/MS proteomic analyses, atherosclerotic plaques from n=18 patients (n=9S+9AS; matched for gender, age and statin medication) were collected and processed as previously described[22]. 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[23]. 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 **I**ntima **M**edia Thickness (IMT) and IMT-**PR**ogression as Predictors of **V**ascular **E**vents in a High-Risk European Population) was used for studying single nucleotide polymorphism (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 three independent CAD 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[24]. 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 (AE, www.atheroexpress.nl). The methods of genotyping and imputation in AE have been described previously[25]. Briefly, in two experiments n=1858 patients were genotyped using the Affymetrix SNP 5.0 (AtheroExpress Genomics Study 1, AEGS1, n=836) and Affymetrix Axiom CEU arrays (AtheroExpress Genomics Study 2, AEGS2, n=1022); 1443 remained after community standard quality control[26]. Full immunohistochemical protocols used in the AE were also detailed previously[25]. 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, two centimetres 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 (AHA) classification[27] as adapted by Virmani et al[28]. Details of sample collection, demographics of the cohort along with tissue processing and full histological classification have been described previously[29].

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 analysed, after filtering out those with minor allele frequency (MAF)<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)[30], assuming an additive genetic model adjusting for age, gender 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 (eQTL) analyses from healthy tissues were performed using the public GTEx software (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://cibersort.stanford.edu), based on recently published human plaque data[31]. 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 dataset analyses were performed with GraphPad Prism 6 and Bioconductor using a linear regression model adjusted for age and gender or a two-sided Student's t-test assuming non-equal 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 two groups was performed using Students t-test for parametric data and Mann-Whitney U-test for non-parametric data. One-way ANOVA with Bonferroni multiple comparison test and Kruskal-Wallis with Dunn's multiple comparison test was used for comparison of parametric and non-parametric data containing more than two groups, respectively. Pearson or Spearman's rank correlations were calculated to determine the association between mRNA and protein expression levels from microarrays and LC-MS/MS, for parametric and non-parametric data respectively.
A functional coupling network based on extended, experimentaly validated PCSK6-MMP14-MMP2 protein-protein interactions was constructed using FunCoup software (http://funcoup.sbc.su.se). Gene set enrichment analysis was performed using GeneMania (www.genemania.org). Functional data about the genes was extracted using GeneCards (www.genecards.org) and PubMed literature search. Network matrices were constructed from the rat carotid injury microarray dataset 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 p-value <0.05 was considered to indicate statistical significance.

A detailed description of all other methods is provided in the Supplemental Data file.

Results

PCSK6 gene variant is associated with clinical surrogate markers of vascular remodeling and SMA+ cells in plaques

Carotid artery intima media thickness (cIMT) as determined by ultrasound is a predictive surrogate marker for atherosclerosis and pathological vascular remodeling[32]. 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)[33] (Figure 1A, Supplementary 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 (expression QTL) 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, Supplementary 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, MAF 0.32)[25, 34] and also in particular with pericyte cell fraction in late-stage plaques (BiKE, n=127) (cell QTL) (Figure 1D)[31]. Functionally, this intronic *PCSK6* variant carries a transcriptional enhancer histone mark and represents the binding site for transcription factor BDP1, a general activator of RNA polymerase III (additional information in Supplementary Table III). BDP1 is highly expressed in normal human smooth muscle compared to other tissues (source Human Protein Atlas, Supplementary Figure IA). Extending on the link suggested by genetic association, in BiKE we found that BDP1 mRNA was significantly downregulated in plaques vs. normal arteries and negatively correlated with PCSK6 expression (Supplementary 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 plaques[17], using transcriptomic data from additional subsets of BiKE patients, as well as public microarray datasets (Figure 2A). Upregulation of PCSK6 transcript was confirmed: in carotid plaques (n=50) vs. normal arteries (n=5) from BiKE (mean difference with SD=0.64±0.12); in a public dataset (GEO accession nr. 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 (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 vs. 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).

In order to explore which cell types express PCSK6, we correlated PCSK6 protein levels from late-stage BiKE plaques with various cell-specific markers (Supplementary Table IV). PCSK6 showed negative correlations with typical markers of quiescent, contractile SMCs (i.e. Pcsk6/Myh11 Pearson r=-0.66, p<0.0001 and Pcsk6/Tagln r=-0.67, p<0.0001). Correlations with endothelial markers were non-significant or negative, whereas those with macrophage markers and lymphocytes were positive.

Expression and localisation of PCSK6 was analysed by immunohistochemistry, using human aortic lesions from different stages of disease graded according to the modified American Heart Association criteria[29], 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+ 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[22] (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 mouse carotid artery, whereas abundant expression was found in innominate plaques from ApoE$^{-/-}$ mice, in subintimal and fibrous cap SMA+ cells (Supplementary 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 2-6 weeks after injury[22]. 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 qPCR, while no changes in gene expression pattern were observed for Pcsk6 in contralateral un-injured arteries (not shown). Expression of Pcsk6 was positively correlated with proliferation-related cytokines Pdgfb 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 two days after injury[35, 36] and between days 2 and 5 SMCs colonise 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 organisation are especially affected through induction of MMPs[22]. Since members of the PCSK family have previously been associated with MMP activation[37], we investigated the correlation of Pcsk6 with a panel of secreted and membrane-bound MMPs in our rat transcriptomic arrays (Supplementary Table V). Significant positive correlations were found with Mmp12, Mmp2 and Mmp14 in injured arteries, while correlations with other Mmps were negative or non-significant both in injured and in un-injured 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[38]. This network contained 33 nodes (Figure 4B) and pathway analysis showed an enrichment of proteins involved in ECM organisation (FDR=6.8E-50), collagen degradation (FDR=2.4E-31) and growth factor binding (FDR=2.6E-13), but also i.e. blood vessel development (FDR=0.049) (Supplementary Table VI). Correlation matrices were then built from rat intimal hyperplasia transcriptomic datasets 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 i.e. Timp1, 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 and Mmp2 became strongly positive. Two weeks after injury, when the neointima is established[22], Pcsk6 was again positively correlated with Timp1 as well as i.e. Sparc, whereas the correlation to Mmp14 was negative and the network appeared again un-coupled.

Co-expression of Pcsk6, Mmp2 and Mmp14 was then confirmed by IHC 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 co-interaction between Pcsk6 and Mmp14 at day 5, but not between Pcsk6 and Mmp2. In tissues 2 weeks after injury, Pcsk6 was found to co-interact with both Mmp2 and Mmp14 (Figure 5B, Supplementary Figure III). Together, these studies from the rat model of intimal hyperplasia highlighted

a mechanism where Pcsk6 interaction with Mmp14 and Mmp2 seems specifically associated with the mobilisation 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 organisation structure (Supplementary 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$^{-/-}$ vs. WT carotid arteries (Supplementary Figure VA). We found that there was a trend towards downregulation in the Pcsk6$^{-/-}$ arteries of many Pdgf and Tgf 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[39]. 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 activity (Supplementary 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 (Supplementary Figure VIA). Thus, we challenged the mice using carotid artery flow cessation, a model shown to generate reproducible intimal lesions attributable to SMC proliferation[40]. 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, B). While there was hardly any remaining proliferative activity in the media of WT mice 6 weeks after 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, qPCR 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 (Supplementary 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 due to 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$^{-/-}$ vs. 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[41]. Upon stimulation with PDGFBB, 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 to WT SMCs that developed large lamellapodia on fibronectin, as observed by fluorescent cytoskeletal staining (Figure 7B). Furthermore, these cells had a limited ability to propagate in culture compared to WT SMCs and were non-proliferative 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 lead 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, Supplementary Figure VII).

Discussion

Here we applied an integrative approach to characterise 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 shows localisation 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[33]. However, cIMT reflects not only early atherosclerosis, but also nonprogressive compensatory remodeling with medial and/or 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[32, 42]. Interestingly, *PCSK6* variant rs1531817 was associated with maximum cIMT progression in the internal carotid artery, where atherosclerotic plaques are more prevalent compared to the common carotid artery[43]. 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 appear to undergo circles of remodeling with stabilisation and destabilisation events, which are dependant on ECM turnover and SMC migration and proliferation[44]. Changes in protease- and growth factor expression are considered to precede the invasiveness of SMCs in neointima formation and atherosclerosis[45]. 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[46]. 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[47]. 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 three proteases are functionally linked in complex, partially redundant but also partially non-overlapping mechanisms of regulation. However, direct crosstalk between membrane receptors for mechanical stretch, MMP2 production, intracellular signaling pathways *via* IGF and PDGFB receptors, and subsequent SMC activation has also been demonstrated[48]. 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 i.e. TIMP1 that inhibits protease activity, 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[49]. Basement membrane disruption and altered cell-matrix interactions with

type I collagen and fibronectin, enhance responsiveness to mitogens and dedifferentiation at sites of injury[49]. In this context, MMP14 activity and PDGFRβ signalling 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 lead 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, TNFα, IFNγ[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 nine subtilisin-kexin like secretory serine proteases and represent some of the most abundant and diverse classes of enzymes[50]. 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[51-54]. Polymorphisms in the PCSK3 and PCSK6 genes have been linked with hypertension and CVD risk, and in PCSK5 with HDL levels[55-57]. Because of the structural similarities within the PCSK family[50], 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 localisations[50], 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[58], the protein has been proposed as prostate cancer biomarker[59] and investigations are ongoing into its potential for targeted molecular imaging[60]. 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 these findings, further investigations to determine how these mechanisms affect atherosclerotic plaque instability are warranted.

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Disclosures

None.

References

1. Finn AV, Nakano M, Narula J, Kolodgie FD and Virmani R. Concept of vulnerable/unstable plaque. *Arteriosclerosis, thrombosis, and vascular biology*. 2010;30:1282-92.
2. Libby P, Ridker PM and Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature*. 2011;473:317-25.
3. Pasterkamp G and van der Steen AF. Intraplaque hemorrhage: an imaging marker for atherosclerotic plaque destabilization? *Arteriosclerosis, thrombosis, and vascular biology*. 2012;32:167-8.
4. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, Swiatlowska P, Newman AA, Greene ES, Straub AC, Isakson B, Randolph GJ and Owens GK. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. *Nature medicine*. 2015;21:628-37.
5. Gomez D and Owens GK. Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovascular research*. 2012;95:156-64.
6. Schwartz SM, Virmani R and Rosenfeld ME. The good smooth muscle cells in atherosclerosis. *Current atherosclerosis reports*. 2000;2:422-9.
7. Takawale A, Sakamuri SS and Kassiri Z. Extracellular matrix communication and turnover in cardiac physiology and pathology. *Compr Physiol*. 2015;5:687-719.
8. Hu J, Van den Steen PE, Sang QX and Opdenakker G. Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat Rev Drug Discov*. 2007;6:480-98.
9. Lehti K, Rose NF, Valavaara S, Weiss SJ and Keski-Oja J. MT1-MMP promotes vascular smooth muscle dedifferentiation through LRP1 processing. *J Cell Sci*. 2009;122:126-35.
10. Turpeinen H, Raitoharju E, Oksanen A, Oksala N, Levula M, Lyytikainen LP, Jarvinen O, Creemers JW, Kahonen M, Laaksonen R, Pelto-Huikko M, Lehtimaki T and Pesu M. Proprotein convertases in human atherosclerotic plaques: the overexpression of FURIN and its substrate cytokines BAFF and APRIL. *Atherosclerosis*. 2011;219:799-806.
11. King A. Lipids. Antibodies against PCSK9--a new era of therapy. *Nature reviews Cardiology*. 2013;10:1.
12. Tsuji A, Sakurai K, Kiyokage E, Yamazaki T, Koide S, Toida K, Ishimura K and Matsuda Y. Secretory proprotein convertases PACE4 and PC6A are heparin-binding proteins which are localized in the extracellular matrix. Potential role of PACE4 in the activation of proproteins in the extracellular matrix. *Biochimica et biophysica acta*. 2003;1645:95-104.
13. Bassi DE, Fu J, Lopez de Cicco R and Klein-Szanto AJ. Proprotein convertases: "master switches" in the regulation of tumor growth and progression. *Molecular carcinogenesis*. 2005;44:151-61.
14. Flaquer A, Baumbach C, Pinero E, Garcia Algas F, de la Fuente Sanchez MA, Rosell J, Toquero J, Alonso-Pulpon L, Garcia-Pavia P, Strauch K and Heine-Suner D. Genome-wide linkage analysis of congenital heart defects using MOD score analysis identifies two novel loci. *BMC Genet*. 2013;14:44.
15. Suh JH, Yoon JS, Kwon JB, Kim HW and Wang YP. Identification of genomic aberrations by array comparative genomic hybridization in patients with aortic dissections. *The Korean journal of thoracic and cardiovascular surgery*. 2011;44:123-30.
16. Chen S, Cao P, Dong N, Peng J, Zhang C, Wang H, Zhou T, Yang J, Zhang Y, Martelli EE, Naga Prasad SV, Miller RE, Malfait AM, Zhou Y and Wu Q. PCSK6-mediated corin activation is essential for normal blood pressure. *Nature medicine*. 2015;21:1048-53.
17. Perisic L, Hedin E, Razuvaev A, Lengquist M, Osterholm C, Folkersen L, Gillgren P, Paulsson-Berne G, Ponten F, Odeberg J and Hedin U. Profiling of atherosclerotic lesions by gene and tissue microarrays reveals PCSK6 as a novel protease in unstable carotid atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology*. 2013;33:2432-43.
18. DiRenzo D, Owens GK and Leeper NJ. "Attack of the Clones": Commonalities Between Cancer and Atherosclerosis. *Circulation research*. 2017;120:624-626.
19. Naylor AR, Rothwell PM and Bell PR. Overview of the principal results and secondary analyses from the European and North American randomised trials of endarterectomy for symptomatic

carotid stenosis. *European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery*. 2003;26:115-29.
20. Halliday A, Harrison M, Hayter E, Kong X, Mansfield A, Marro J, Pan H, Peto R, Potter J, Rahimi K, Rau A, Robertson S, Streifler J, Thomas D and Asymptomatic Carotid Surgery Trial Collaborative G. 10-year stroke prevention after successful carotid endarterectomy for asymptomatic stenosis (ACST-1): a multicentre randomised trial. *Lancet*. 2010;376:1074-84.
21. Perisic L, Aldi S, Sun Y, Folkersen L, Razuvaev A, Roy J, Lengquist M, Akesson S, Wheelock CE, Maegdefessel L, Gabrielsen A, Odeberg J, Hansson GK, Paulsson-Berne G and Hedin U. Gene expression signatures, pathways and networks in carotid atherosclerosis. *Journal of internal medicine*. 2016;279:293-308.
22. Perisic Matic L, Rykaczewska U, Razuvaev A, Sabater-Lleal M, Lengquist M, Miller CL, Ericsson I, Rohl S, Kronqvist M, Aldi S, Magne J, Paloschi V, Vesterlund M, Li Y, Jin H et al. Phenotypic Modulation of Smooth Muscle Cells in Atherosclerosis Is Associated With Downregulation of LMOD1, SYNPO2, PDLIM7, PLN, and SYNM. *Arteriosclerosis, thrombosis, and vascular biology*. 2016;36:1947-61.
23. Matic LP, Jesus Iglesias M, Vesterlund M, Lengquist M, Hong MG, Saieed S, Sanchez-Rivera L, Berg M, Razuvaev A, Kronqvist M, Lund K, Caidahl K, Gillgren P, Ponten F, Uhlen M et al. Novel Multiomics Profiling of Human Carotid Atherosclerotic Plaques and Plasma Reveals Biliverdin Reductase B as a Marker of Intraplaque Hemorrhage. *JACC Basic Transl Sci*. 2018;3:464-480.
24. Baldassarre D, Hamsten A, Veglia F, de Faire U, Humphries SE, Smit AJ, Giral P, Kurl S, Rauramaa R, Mannarino E, Grossi E, Paoletti R, Tremoli E and Group IS. Measurements of carotid intima-media thickness and of interadventitia common carotid diameter improve prediction of cardiovascular events: results of the IMPROVE (Carotid Intima Media Thickness [IMT] and IMT-Progression as Predictors of Vascular Events in a High Risk European Population) study. *Journal of the American College of Cardiology*. 2012;60:1489-99.
25. van der Laan SW, Foroughi Asl H, van den Borne P, van Setten J, van der Perk ME, van de Weg SM, Schoneveld AH, de Kleijn DP, Michoel T, Bjorkegren JL, den Ruijter HM, Asselbergs FW, de Bakker PI and Pasterkamp G. Variants in ALOX5, ALOX5AP and LTA4H are not associated with atherosclerotic plaque phenotypes: the Athero-Express Genomics Study. *Atherosclerosis*. 2015;239:528-38.
26. Laurie CC, Doheny KF, Mirel DB, Pugh EW, Bierut LJ, Bhangale T, Boehm F, Caporaso NE, Cornelis MC, Edenberg HJ, Gabriel SB, Harris EL, Hu FB, Jacobs KB, Kraft P, Landi MT, Lumley T, Manolio TA, McHugh C, Painter I, Paschall J, Rice JP, Rice KM, Zheng X, Weir BS and Investigators G. Quality control and quality assurance in genotypic data for genome-wide association studies. *Genet Epidemiol*. 2010;34:591-602.
27. Stary HC. Natural history and histological classification of atherosclerotic lesions: an update. *Arteriosclerosis, thrombosis, and vascular biology*. 2000;20:1177-8.
28. Virmani R, Kolodgie FD, Burke AP, Farb A and Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arteriosclerosis, thrombosis, and vascular biology*. 2000;20:1262-75.
29. van Dijk RA, Virmani R, von der Thusen JH, Schaapherder AF and Lindeman JH. The natural history of aortic atherosclerosis: a systematic histopathological evaluation of the peri-renal region. *Atherosclerosis*. 2010;210:100-6.
30. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ and Sham PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. *American journal of human genetics*. 2007;81:559-75.
31. Wirka RC, Wagh D, Paik DT, Pjanic M, Nguyen T, Miller CL, Kundu R, Nagao M, Coller J, Koyano TK, Fong R, Woo YJ, Liu B, Montgomery SB, Wu JC, Zhu K, Chang R, Alamprese M, Tallquist MD, Kim JB and Quertermous T. Atheroprotective roles of smooth muscle cell phenotypic modulation and the TCF21 disease gene as revealed by single-cell analysis. *Nature medicine*. 2019;25:1280-1289.
32. Finn AV, Kolodgie FD and Virmani R. Correlation between carotid intimal/medial thickness and atherosclerosis: a point of view from pathology. *Arteriosclerosis, thrombosis, and vascular biology*. 2010;30:177-81.

33. Baldassarre D, Veglia F, Hamsten A, Humphries SE, Rauramaa R, de Faire U, Smit AJ, Giral P, Kurl S, Mannarino E, Grossi E, Paoletti R, Tremoli E and Group IS. Progression of carotid intima-media thickness as predictor of vascular events: results from the IMPROVE study. *Arteriosclerosis, thrombosis, and vascular biology*. 2013;33:2273-9.
34. Verhoeven BA, Velema E, Schoneveld AH, de Vries JP, de Bruin P, Seldenrijk CA, de Kleijn DP, Busser E, van der Graaf Y, Moll F and Pasterkamp G. Athero-express: differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. *European journal of epidemiology*. 2004;19:1127-33.
35. Majesky MW, Schwartz SM, Clowes MM and Clowes AW. Heparin regulates smooth muscle S phase entry in the injured rat carotid artery. *Circulation research*. 1987;61:296-300.
36. Thyberg J, Blomgren K, Hedin U and Dryjski M. Phenotypic modulation of smooth muscle cells during the formation of neointimal thickenings in the rat carotid artery after balloon injury: an electron-microscopic and stereological study. *Cell and tissue research*. 1995;281:421-33.
37. Remacle AG, Rozanov DV, Fugere M, Day R and Strongin AY. Furin regulates the intracellular activation and the uptake rate of cell surface-associated MT1-MMP. *Oncogene*. 2006;25:5648-55.
38. Schmitt T, Ogris C and Sonnhammer EL. FunCoup 3.0: database of genome-wide functional coupling networks. *Nucleic acids research*. 2014;42:D380-8.
39. Kuhl A, Melberg A, Meinl E, Nurnberg G, Nurnberg P, Kehrer-Sawatzki H and Jenne DE. Myofibrillar myopathy with arrhythmogenic right ventricular cardiomyopathy 7: corroboration and narrowing of the critical region on 10q22.3. *Eur J Hum Genet*. 2008;16:367-73.
40. Tran PK, Tran-Lundmark K, Soininen R, Tryggvason K, Thyberg J and Hedin U. Increased intimal hyperplasia and smooth muscle cell proliferation in transgenic mice with heparan sulfate-deficient perlecan. *Circulation research*. 2004;94:550-8.
41. Baker M, Robinson SD, Lechertier T, Barber PR, Tavora B, D'Amico G, Jones DT, Vojnovic B and Hodivala-Dilke K. Use of the mouse aortic ring assay to study angiogenesis. *Nat Protoc*. 2011;7:89-104.
42. Stein JH, Korcarz CE, Hurst RT, Lonn E, Kendall CB, Mohler ER, Najjar SS, Rembold CM, Post WS and American Society of Echocardiography Carotid Intima-Media Thickness Task F. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force. Endorsed by the Society for Vascular Medicine. *J Am Soc Echocardiogr*. 2008;21:93-111; quiz 189-90.
43. Selwaness M, van den Bouwhuijsen Q, van Onkelen RS, Hofman A, Franco OH, van der Lugt A, Wentzel JJ and Vernooij M. Atherosclerotic plaque in the left carotid artery is more vulnerable than in the right. *Stroke; a journal of cerebral circulation*. 2014;45:3226-30.
44. Libby P. How does lipid lowering prevent coronary events? New insights from human imaging trials. *European heart journal*. 2015;36:472-4.
45. Filippov S, Koenig GC, Chun TH, Hotary KB, Ota I, Bugge TH, Roberts JD, Fay WP, Birkedal-Hansen H, Holmbeck K, Sabeh F, Allen ED and Weiss SJ. MT1-matrix metalloproteinase directs arterial wall invasion and neointima formation by vascular smooth muscle cells. *J Exp Med*. 2005;202:663-71.
46. Itoh Y. Membrane-type matrix metalloproteinases: Their functions and regulations. *Matrix Biol*. 2015;44-46:207-23.
47. Yana I and Weiss SJ. Regulation of membrane type-1 matrix metalloproteinase activation by proprotein convertases. *Molecular biology of the cell*. 2000;11:2387-401.
48. Seo KW, Lee SJ, Kim YH, Bae JU, Park SY, Bae SS and Kim CD. Mechanical stretch increases MMP-2 production in vascular smooth muscle cells via activation of PDGFR-beta/Akt signaling pathway. *PloS one*. 2013;8:e70437.
49. Hedin U, Roy J and Tran PK. Control of smooth muscle cell proliferation in vascular disease. *Current opinion in lipidology*. 2004;15:559-65.
50. Seidah NG and Prat A. The biology and therapeutic targeting of the proprotein convertases. *Nature reviews Drug discovery*. 2012;11:367-83.
51. Stawowy P and Fleck E. Proprotein convertases furin and PC5: targeting atherosclerosis and restenosis at multiple levels. *Journal of molecular medicine*. 2005;83:865-75.

52. Stawowy P, Meyborg H, Stibenz D, Borges Pereira Stawowy N, Roser M, Thanabalasingam U, Veinot JP, Chretien M, Seidah NG, Fleck E and Graf K. Furin-like proprotein convertases are central regulators of the membrane type matrix metalloproteinase-pro-matrix metalloproteinase-2 proteolytic cascade in atherosclerosis. *Circulation*. 2005;111:2820-7.
53. Stawowy P, Marcinkiewicz J, Graf K, Seidah N, Chretien M, Fleck E and Marcinkiewicz M. Selective expression of the proprotein convertases furin, pc5, and pc7 in proliferating vascular smooth muscle cells of the rat aorta in vitro. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*. 2001;49:323-32.
54. Fujimaki T, Kato K, Yokoi K, Yoshida T, Oguri M, Watanabe S, Metoki N, Yoshida H, Satoh K, Aoyagi Y, Nozawa Y and Yamada Y. Genetic risk for myocardial infarction in Japanese individuals with or without chronic kidney disease. *International journal of molecular medicine*. 2010;25:743-9.
55. Li JP, Wang XB, Chen CZ, Xu X, Hong XM, Xu XP, Gao W and Huo Y. The association between paired basic amino acid cleaving enzyme 4 gene haplotype and diastolic blood pressure. *Chinese medical journal*. 2004;117:382-8.
56. Ganesh SK, Tragante V, Guo W, Guo Y, Lanktree MB, Smith EN, Johnson T, Castillo BA, Barnard J, Baumert J, Chang YP, Elbers CC, Farrall M, Fischer ME, Franceschini N et al. Loci influencing blood pressure identified using a cardiovascular gene-centric array. *Human molecular genetics*. 2013.
57. Iatan I, Dastani Z, Do R, Weissglas-Volkov D, Ruel I, Lee JC, Huertas-Vazquez A, Taskinen MR, Prat A, Seidah NG, Pajukanta P, Engert JC and Genest J. Genetic variation at the proprotein convertase subtilisin/kexin type 5 gene modulates high-density lipoprotein cholesterol levels. *Circulation Cardiovascular genetics*. 2009;2:467-75.
58. Byun S, Tortorella MD, Malfait AM, Fok K, Frank EH and Grodzinsky AJ. Transport and equilibrium uptake of a peptide inhibitor of PACE4 into articular cartilage is dominated by electrostatic interactions. *Archives of biochemistry and biophysics*. 2010;499:32-9.
59. Klee EW, Bondar OP, Goodmanson MK, Dyer RB, Erdogan S, Bergstralh EJ, Bergen HR, 3rd, Sebo TJ and Klee GG. Candidate serum biomarkers for prostate adenocarcinoma identified by mRNA differences in prostate tissue and verified with protein measurements in tissue and blood. *Clinical chemistry*. 2012;58:599-609.
60. Couture F, Levesque C, Dumulon-Perreault V, Ait-Mohand S, D'Anjou F, Day R and Guerin B. PACE4-based molecular targeting of prostate cancer using an engineered (6)(4)Cu-radiolabeled peptide inhibitor. *Neoplasia*. 2014;16:634-43.

Figure Legends

Figure 1. Integrative genetic analyses reveal association of the *PCSK6* locus with atherosclerosis and vascular remodeling. Intronic *PCSK6* variant rs1531817 was associated with maximum cIMT progression in high-risk CVD subjects. By expression QTL analysis, this variant also significantly associated with PCSK6 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 5-95 percentile in A, median with min to max in B and C and mean with standard deviation 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. cIMT-carotid intima-media thickness, QTL-quantitative trait locus.

Figure 2. PCSK6 transcript and protein are upregulated in atherosclerotic lesions and localised to SMA+ cells. PCSK6 mRNA expression was upregulated comparing plaques and normal arteries in the BiKE microarray dataset, which was validated in public microarrays from 2 additional cohorts comparing plaques with adjacent vascular tissue or normal arteries. Plots showing log2 mean±standard deviation (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, SMA (red) was continuosly expressed in lesions from all stages of atherosclerotic disease (modified American Heart Association grading I-V; IEL- internal elastic laminae, arrows). Cells immunopositive for PCNA 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 IHC experiments isotype rabbit or mouse Ig serum were used as negative controls. Images taken with 5x and10x objective, insets with 40x.

Figure 3. PCSK6 is functionally related to SMC activation and intimal hyperplasia. By microarray profiling and quantitative PCR from rat carotid arteries after ballon injury, Pcsk6 was downregulated in early phases after injury, but upregulated from day 5 post injury. ANOVA p-value reported across groups (A). Positive expression correlations were found in this model between Pcsk6 and mitogens Pdgfb and Igf1 (B). By IHC in human and murine intimal hyperplasia (mouse carotid artery ligation 6 weeks, rat carotid artery balloon injury 12 weeks), PCSK6 (red signal) was strongly expressed by proliferative PCNA+/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 20x and 40x objectives.

Figure 4. A network connecting Pcsk6-Mmp2-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 datasets and presented as matrices where the nearest neighbours were clustered based on the correlation index (C). Clustering of the Pcsk6-Mmp2-Mmp14 network is observed at 5 days after injury (red areas) compared with intact arteries and vessels 2 weeks 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.

Figure 5. PCSK6-MMP2-MMP14 interaction *in situ*. IHC examination of rat carotid arteries after injury revealed that PCSK6 and MMP14 proteins were widely expressed in medial 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), co-interaction between PCSK6 and MMP14 proteins could be detected already 5 days after injury and was even more prominent after 2 weeks (B). The interaction between PCSK6 and MMP2 proteins could not be detected until 2 weeks after injury in neointima. Arrows point to the PLA signal. Probe detecting PCSK6 was used as a positive control.

Figure 6. Reduced intimal hyperplasia after carotid artery ligation in Pcsk6$^{-/-}$ mice *via* impaired MMP14 activation. Serial histological sections from carotid ligations in WT and Pcsk6$^{-/-}$ mice were evaluated for intima and media thickness and number of medial PCNA+ 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 IHC experiments isotype rabbit or mouse Ig serum was used as negative controls. WT-wild type. P-values in plots under C are adjusted for multiple comparisons; *p<0.05, **p<0.01

Figure 7. PCSK6 perturbation affects SMCs proliferation and migration *in vitro*. Mouse aortic ring explants were grown in 3-dimmensional-collagen matrix with or without PDGFBB stimulation. Significantly less cell outgrowth was quantified from Pcsk6$^{-/-}$ rings compared with WTs in response to PDGFBB. Plots show mean ± SEM from 2 experiments (A). Primary SMCs isolated from Pcsk6$^{-/-}$ aortas displayed fillopodial spreading in comparison with WT cells. Cytoskeletal staining for tubulin and actin by phalloidin (B). Silencing of PCSK6 or MMP14 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. Unpaired T-test with Welch's correction has been used in all comparisons.

Visual Abstract. Proposed mechanism for smooth musle cell activation upon vascular injury and extracellular matrix remodeling *via* interaction between PCSK6 and MMP14.

Novelty and Significance

What is known?

- Smooth muscle cells (SMCs) are the major cells responsible for vessel wall integrity, remodeling and atherosclerotic plaque stability.
- SMC activation in vascular repair involves chemoattractants, mitogens and extracellular matrix (ECM) degradation controlled by various proteases.
- We previously reported that Proprotein convertase subtilisin/kexin 6 (PCSK6) 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 MMP14-dependant SMC migration in response to PDGFB 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. Teports 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 uunderstanding of the SMC biology and offer clues to future therapeutic strategies.

Figure 1

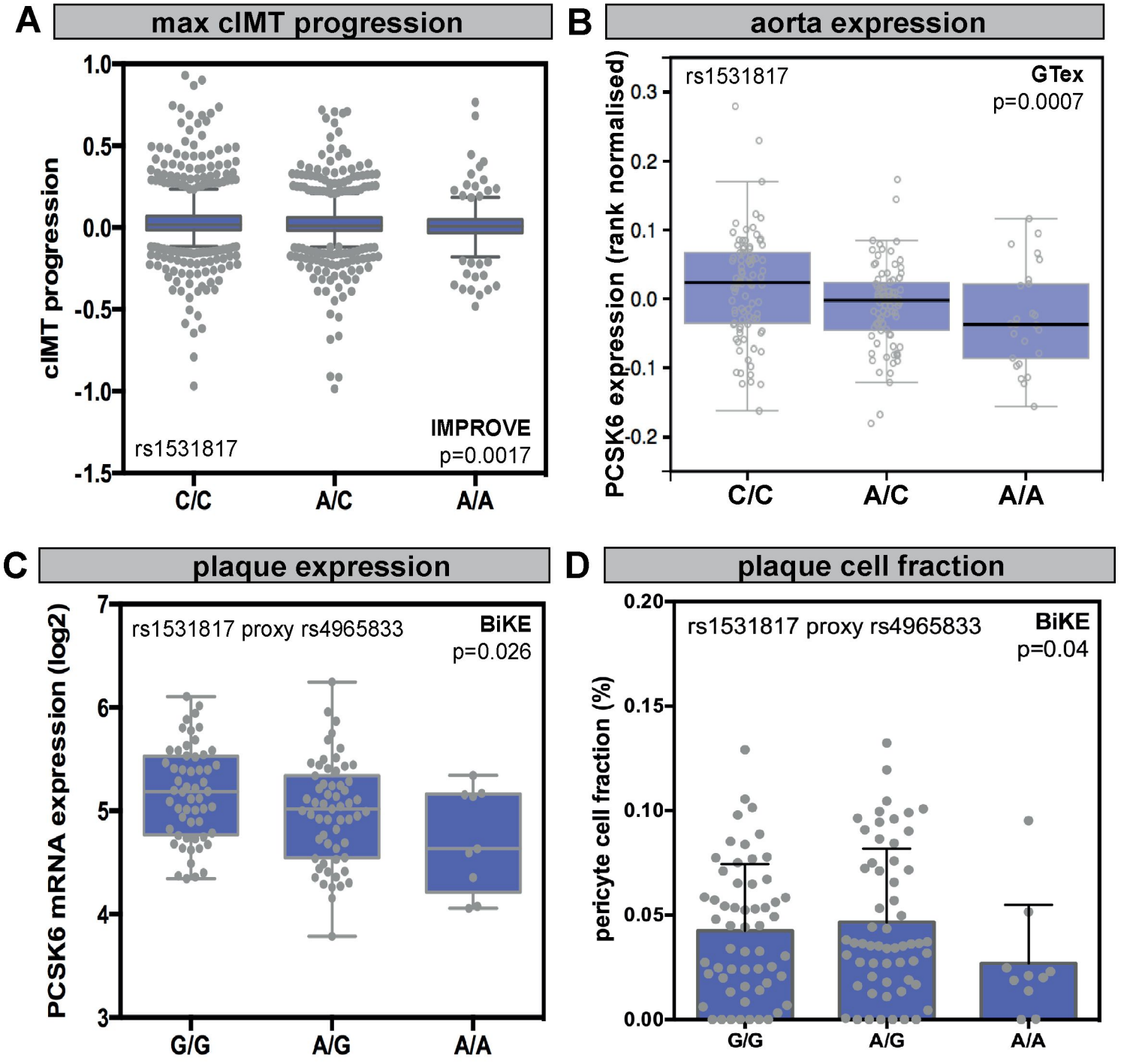

A
max cIMT progression
cIMT progression
1.0
0.5
0.0
-0.5
-1.0
-1.5
rs1531817
IMPROVE
p=0.0017
C/C
A/C
A/A
B
aorta expression
PCSK6 expression (rank normalised)
0.3
0.2
0.1
0.0
-0.1
-0.2
rs1531817
GTex
p=0.0007
C/C
A/C
A/A
C
plaque expression
PCSK6 mRNA expression (log2)
7
6
5
4
3
rs1531817 proxy rs4965833
BiKE
p=0.026
G/G
A/G
A/A
D
plaque cell fraction
pericyte cell fraction (%)
0.20
0.15
0.10
0.05
0.00
rs1531817 proxy rs4965833
BiKE
p=0.04
G/G
A/G
A/A

Figure 2

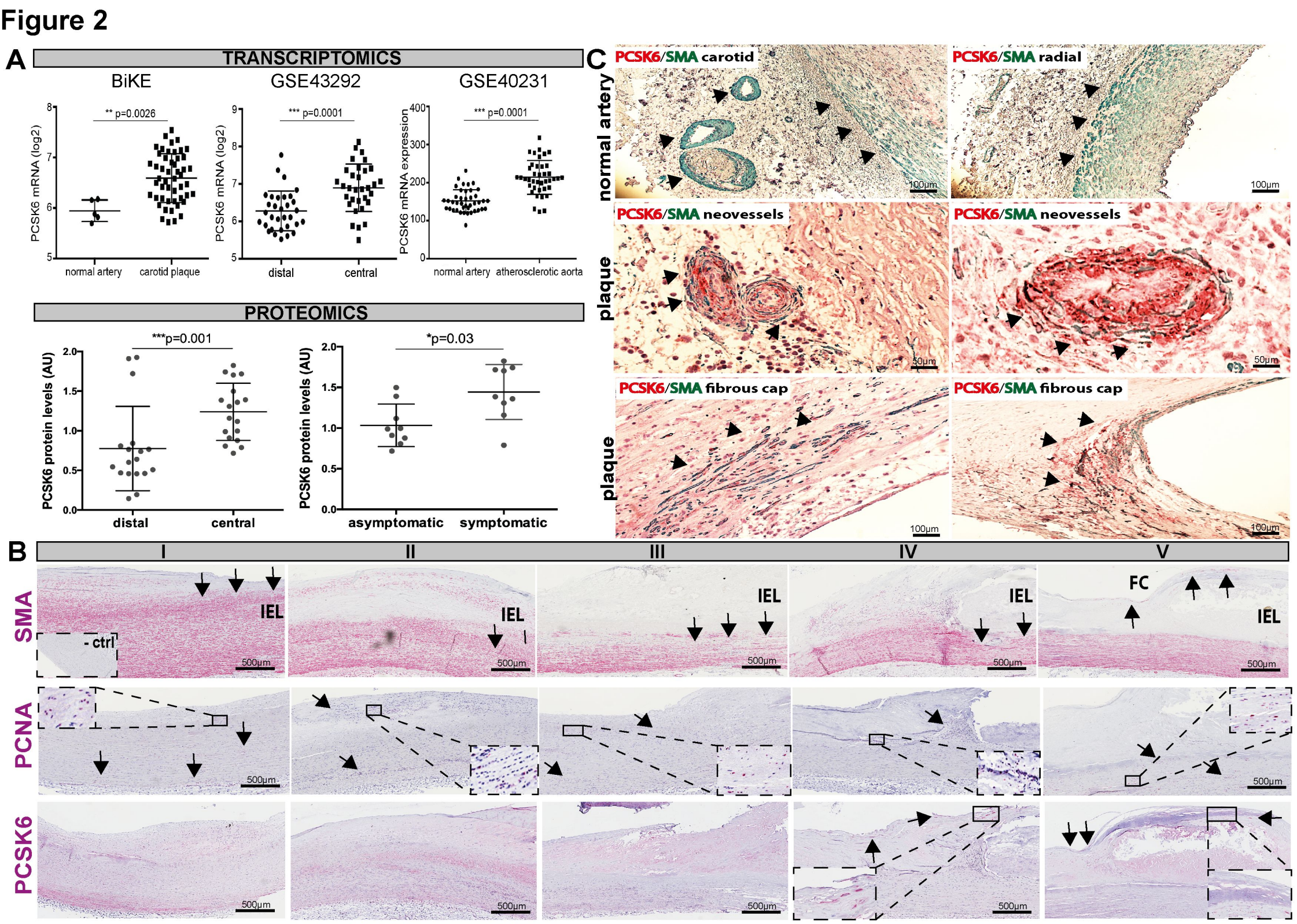

A
TRANSCRIPTOMICS
BiKE
GSE43292
GSE40231
PCSK6 mRNA (log2)
** p=0.0026
normal artery
carotid plaque
*** p=0.0001
distal
central
PCSK6 mRNA expression
normal artery
atherosclerotic aorta
PROTEOMICS
***p=0.001
PCSK6 protein levels (AU)
distal
central
*p=0.03
asymptomatic
symptomatic
C
PCSK6/SMA carotid
PCSK6/SMA radial
normal artery
PCSK6/SMA neovessels
PCSK6/SMA neovessels
plaque
PCSK6/SMA fibrous cap
PCSK6/SMA fibrous cap
plaque
100µm
50µm
B
I
II
III
IV
V
SMA
PCNA
PCSK6
IEL
- ctrl
FC
500µm

Figure 3

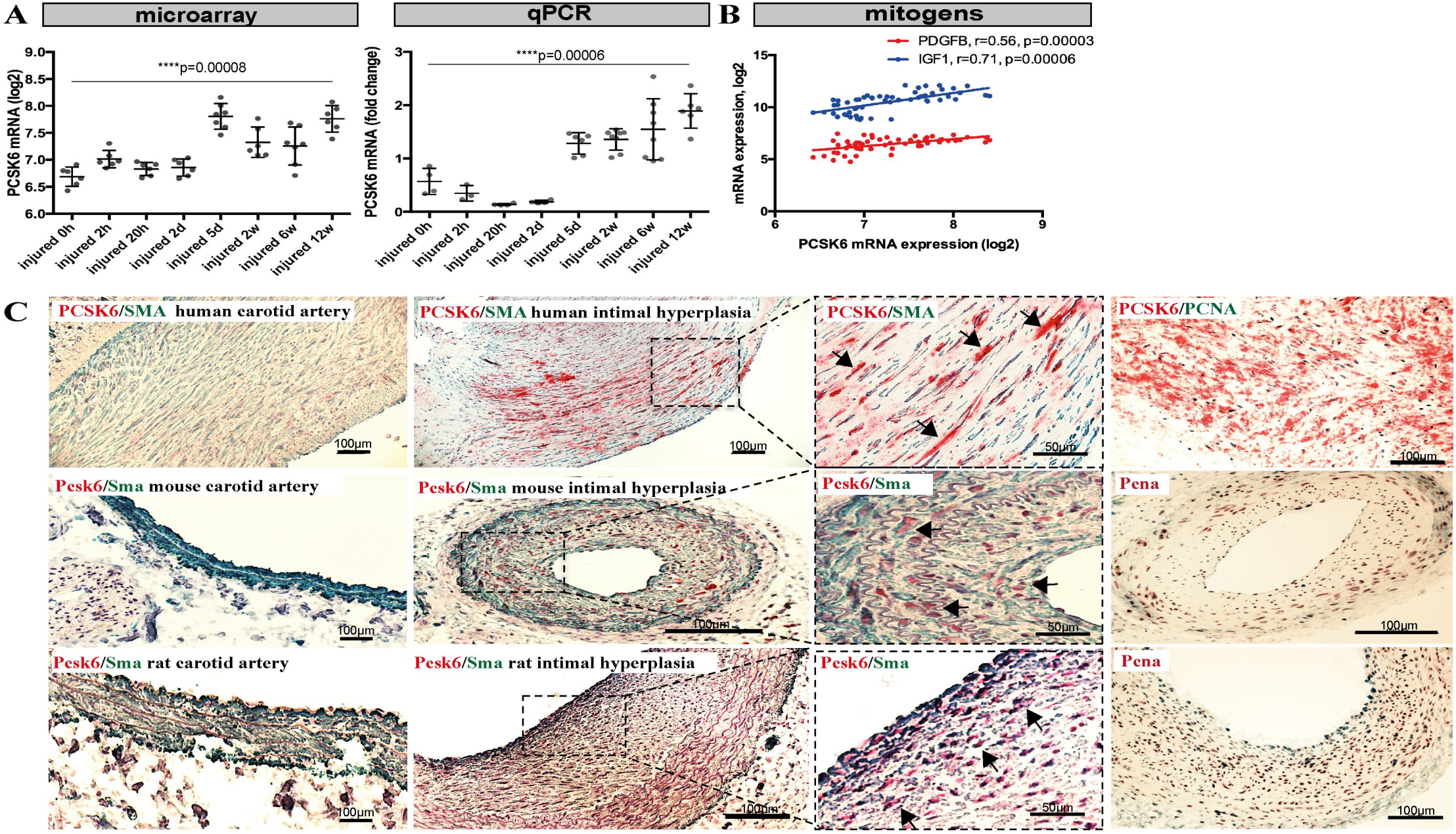

A
microarray
PCSK6 mRNA (log2)
****p=0.00008
injured 0h
injured 2h
injured 20h
injured 2d
injured 5d
injured 2w
injured 6w
injured 12w
qPCR
PCSK6 mRNA (fold change)
****p=0.00006
B
mitogens
PDGFB, r=0.56, p=0.00003
IGF1, r=0.71, p=0.00006
mRNA expression, log2
PCSK6 mRNA expression (log2)
C
PCSK6/SMA human carotid artery
PCSK6/SMA human intimal hyperplasia
PCSK6/SMA
PCSK6/PCNA
Pcsk6/Sma mouse carotid artery
Pcsk6/Sma mouse intimal hyperplasia
Pcsk6/Sma
Pcna
Pcsk6/Sma rat carotid artery
Pcsk6/Sma rat intimal hyperplasia
Pcsk6/Sma
Pcna
100μm
50μm

Figure 4

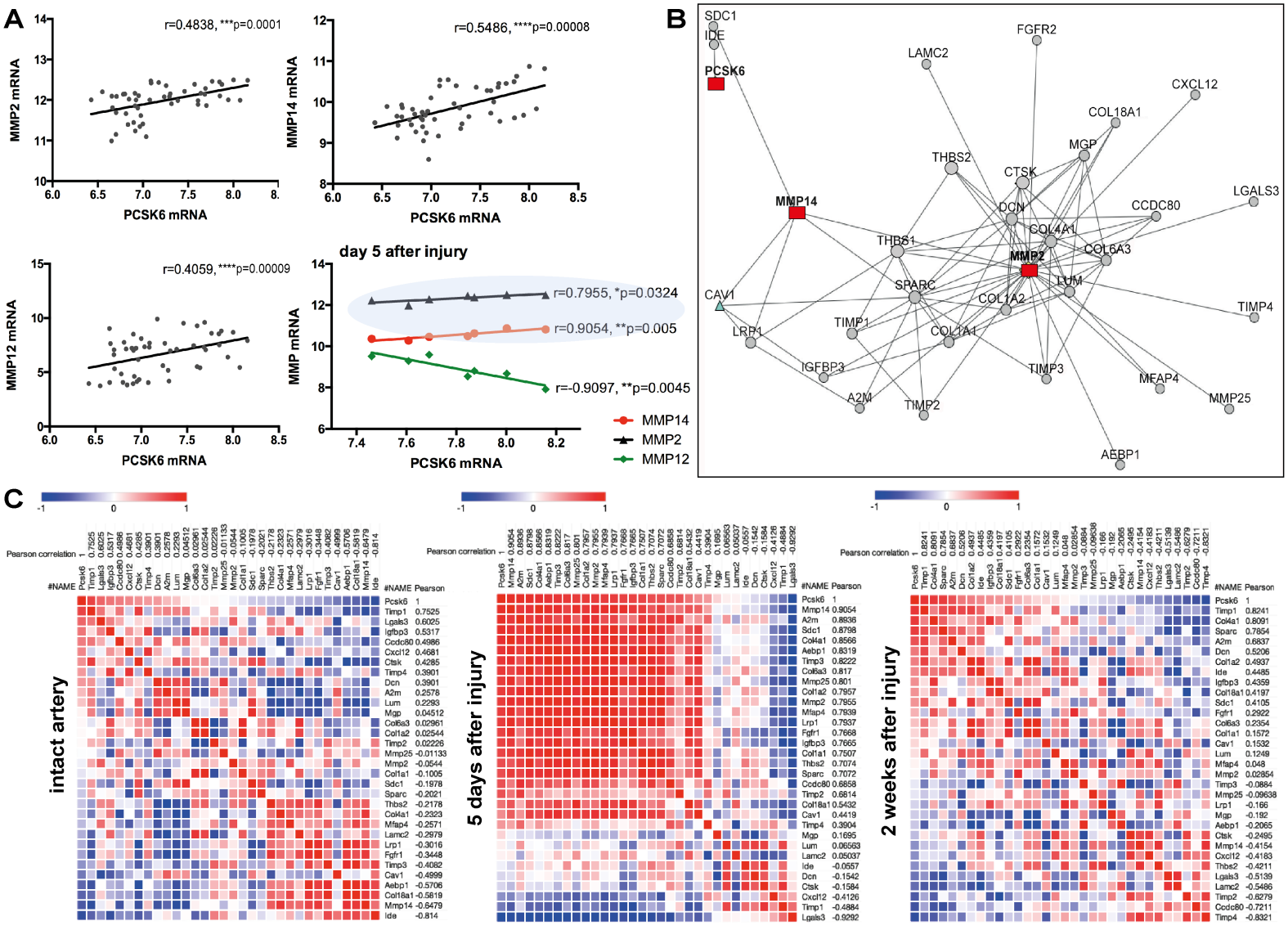

A
r=0.4838, ***p=0.0001
MMP2 mRNA
PCSK6 mRNA
r=0.5486, ****p=0.00008
MMP14 mRNA
PCSK6 mRNA
r=0.4059, ****p=0.00009
MMP12 mRNA
PCSK6 mRNA
day 5 after injury
r=0.7955, *p=0.0324
r=0.9054, **p=0.005
r=-0.9097, **p=0.0045
MMP mRNA
PCSK6 mRNA
MMP14
MMP2
MMP12
B
SDC1
IDE
PCSK6
LAMC2
FGFR2
CXCL12
COL18A1
MGP
THBS2
CTSK
LGALS3
MMP14
DCN
CCDC80
COL4A1
THBS1
COL6A3
MMP2
LUM
SPARC
CAV1
COL1A2
TIMP4
TIMP1
LRP1
COL1A1
IGFBP3
TIMP3
MFAP4
A2M
TIMP2
MMP25
AEBP1
C
Pearson correlation
#NAME
intact artery
5 days after injury
2 weeks after injury

Figure 5

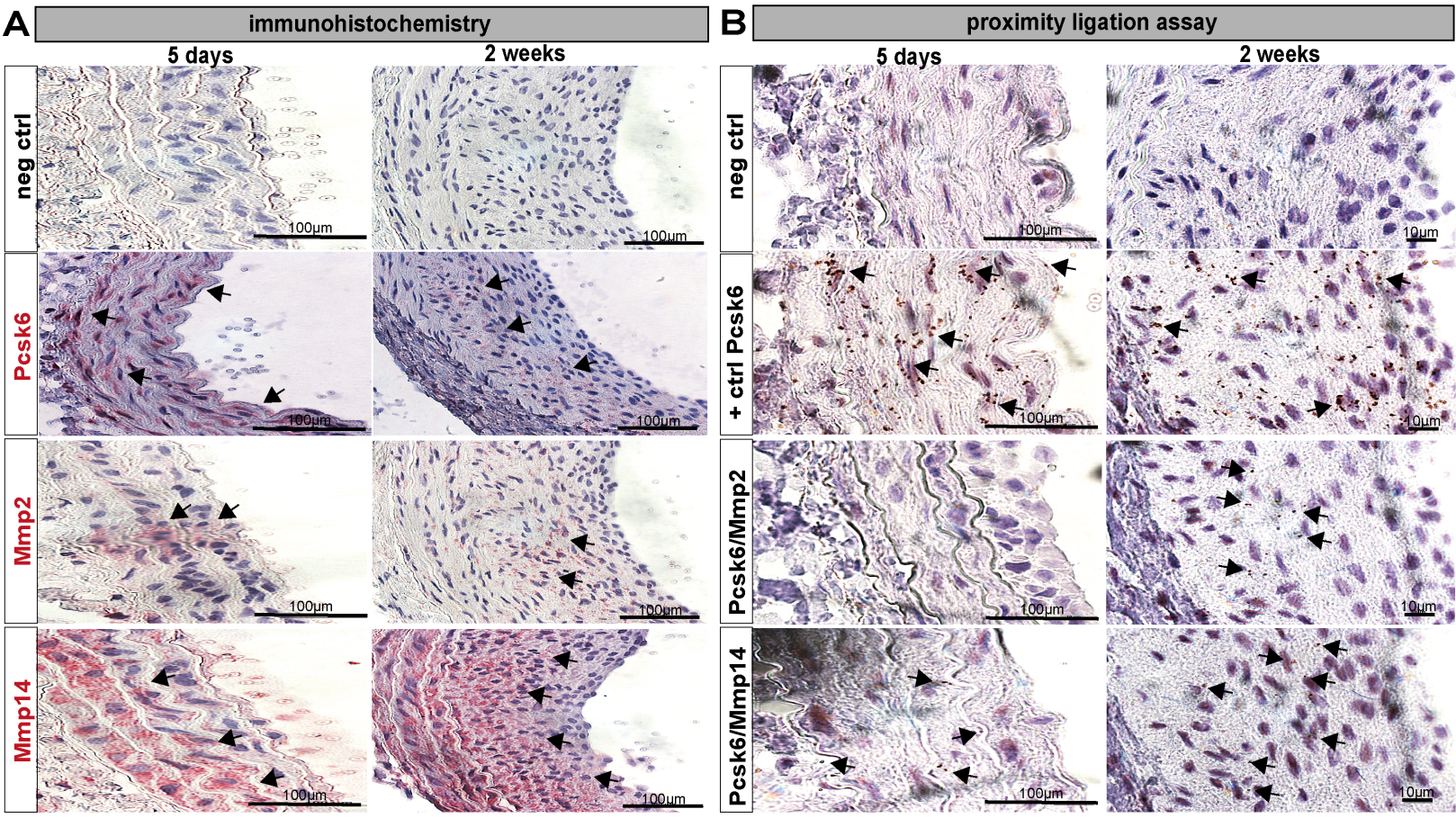

A
immunohistochemistry
5 days
2 weeks
neg ctrl
Pcsk6
Mmp2
Mmp14
100µm
B
proximity ligation assay
5 days
2 weeks
neg ctrl
+ ctrl Pcsk6
Pcsk6/Mmp2
Pcsk6/Mmp14
100µm
10µm

Figure 6

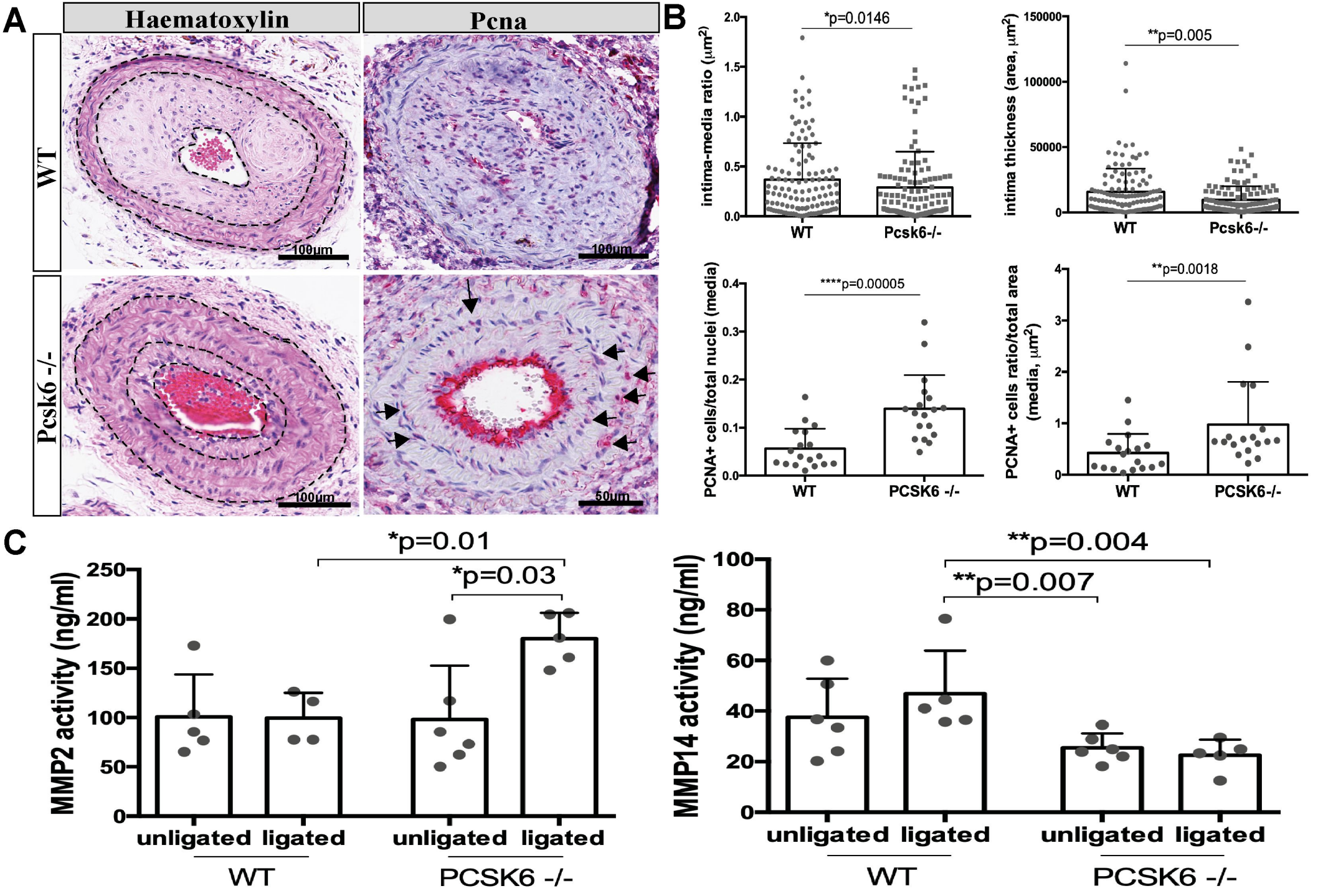
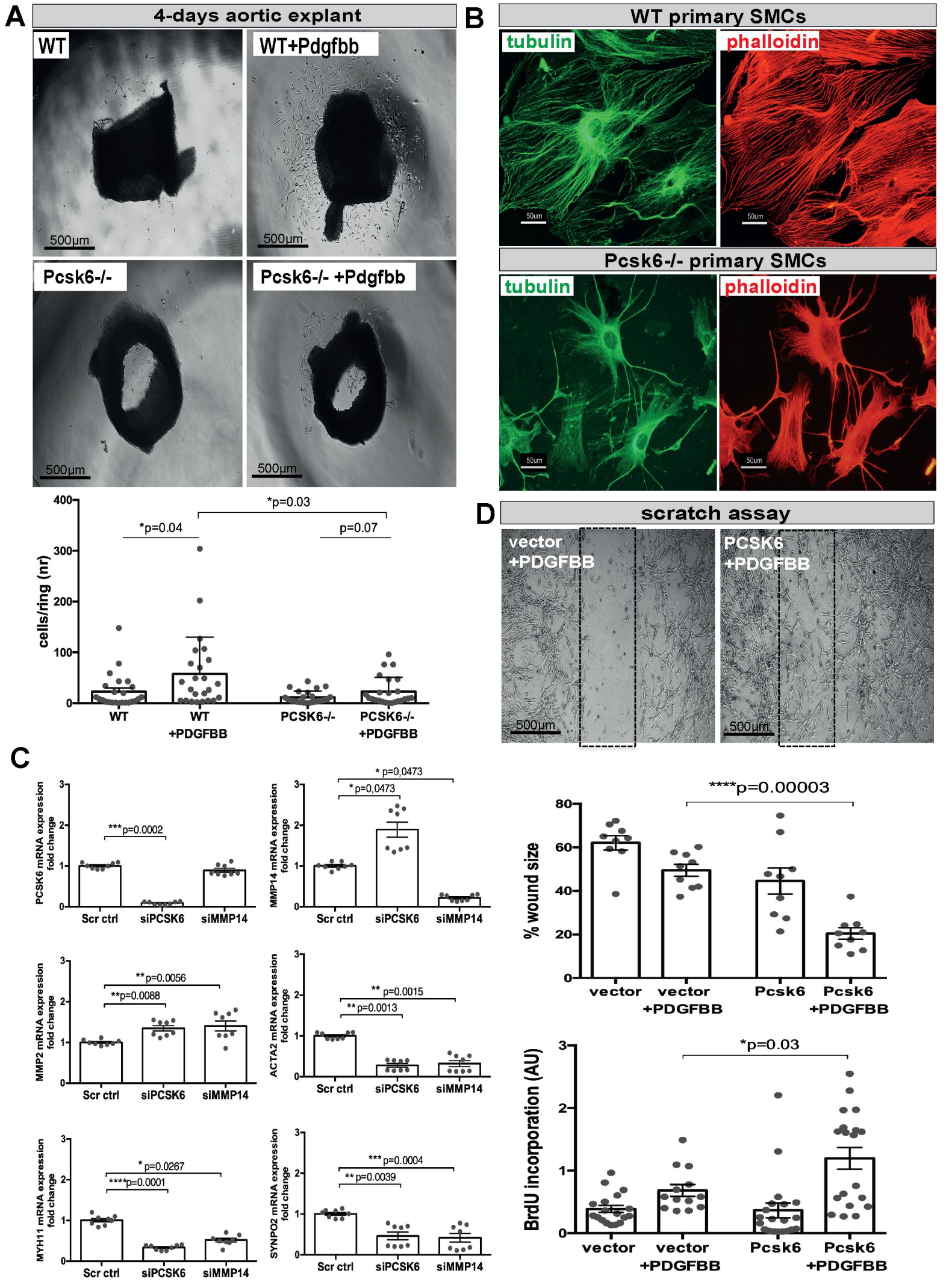
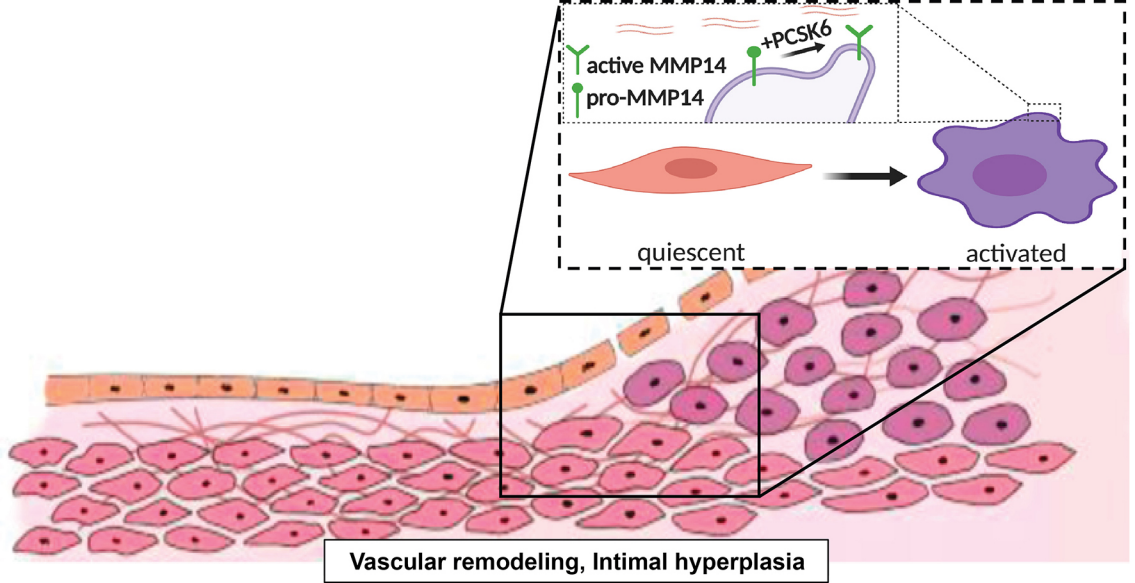

A
Haematoxylin
Pcna
WT
Pcsk6 -/-
100um
100um
100um
50um
B
intima-media ratio (μm²)
*p=0.0146
WT
Pcsk6-/-
intima thickness (area, μm²)
**p=0.005
WT
Pcsk6-/-
PCNA+ cells/total nuclei (media)
****p=0.00005
WT
PCSK6 -/-
PCNA+ cells ratio/total area (media, μm²)
**p=0.0018
WT
PCSK6-/-
C
MMP2 activity (ng/ml)
*p=0.01
*p=0.03
unligated
ligated
unligated
ligated
WT
PCSK6 -/-
MMP14 activity (ng/ml)
**p=0.004
**p=0.007
unligated
ligated
unligated
ligated
WT
PCSK6 -/-

Figure 7


A
4-days aortic explant
WT
WT+Pdgfbb
Pcsk6-/-
Pcsk6-/- +Pdgfbb
500µm
cells/ring (nr)
*p=0.03
*p=0.04
p=0.07
WT
WT +PDGFBB
PCSK6-/-
PCSK6-/- +PDGFBB
B
WT primary SMCs
tubulin
phalloidin
Pcsk6-/- primary SMCs
50um
D
scratch assay
vector +PDGFBB
PCSK6 +PDGFBB
C
PCSK6 mRNA expression fold change
***p=0.0002
MMP14 mRNA expression fold change
* p=0,0473
*p=0,0473
MMP2 mRNA expression fold change
**p=0.0056
**p=0.0088
ACTA2 mRNA expression fold change
** p=0.0015
**p=0.0013
MYH11 mRNA expression fold change
*p=0.0267
****p=0.0001
SYNPO2 mRNA expression fold change
***p=0.0004
**p=0.0039
Scr ctrl
siPCSK6
siMMP14
% wound size
****p=0.00003
vector
vector +PDGFBB
Pcsk6
Pcsk6 +PDGFBB
BrdU incorporation (AU)
*p=0.03


active MMP14
pro-MMP14
+PCSK6
quiescent
activated
Vascular remodeling, Intimal hyperplasia