Knockout of Adamts7, a Novel Coronary Artery Disease Locus in Humans, Reduces Atherosclerosis in Mice

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Background—Genome-wide association studies have established ADAMTS7 as a locus for coronary artery disease in humans. However, these studies fail to provide directionality for the association between ADAMTS7 and coronary artery disease. Previous reports have implicated ADAMTS7 in the regulation of vascular smooth muscle cell migration, but a role for and the direction of impact of this gene in atherogenesis have not been shown in relevant model systems.

Methods and Results—We bred an Adamts7 whole-body knockout mouse onto both the Ldlr and Apoe knockout hyperlipidemic mouse models. Adamts7+/Ldlr−/− and Adamts7+/Apoe−/− mice displayed significant reductions in lesion formation in aortas and aortic roots compared with controls. Adamts7 knockout mice also showed reduced neointimal formation after femoral wire injury. Adamts7 expression was induced in response to injury and hyperlipidemia but was absent at later time points, and primary Adamts7 knockout vascular smooth muscle cells showed reduced migration in the setting of tumor necrosis factor-α stimulation. ADAMTS7 localized to cells positive for smooth muscle cell markers in human coronary artery disease lesions, and subcellular localization studies in cultured vascular smooth muscle cells placed ADAMTS7 at the cytoplasm and cell membrane, where it colocalized with markers of podosomes.

Conclusions—These data represent the first in vivo experimental validation of the association of Adamts7 with atherogenesis, likely through modulation of vascular cell migration and matrix in atherosclerotic lesions. These results demonstrate that Adamts7 is proatherogenic, lending directionality to the original genetic association and supporting the concept that pharmacological inhibition of ADAMTS7 should be atheroprotective in humans, making it an attractive target for novel therapeutic interventions. (Circulation. 2015;131:1202-1213. DOI: 10.1161/CIRCULATIONAHA.114.012669.)

Key Words: atherosclerosis | coronary artery disease | genetics | genome-wide association study | metalloproteases | mice, knockout

Coronary artery disease (CAD) is a heritable disease and a leading cause of death and morbidity. Recent large-scale genome-wide association studies (GWASs) have discovered and replicated >40 loci for CAD and myocardial infarction (MI). One of these loci on chromosome 15 contains the gene ADAMTS7 (a disintegrin and metalloproteinase with thrombospondin motifs-7), and was initially identified by our group through GWASs of angiographic CAD. This finding has since been replicated multiple times for both CAD and MI in further meta-analyses, suggesting that ADAMTS7 is a novel regulator of atherogenesis in humans. To date, however, the lack of human expression quantitative trait loci (eQTL) data in relevant tissues creates uncertainty about whether ADAMTS7 is the causal gene at the locus and prevents elucidation of the directionality between the actions of ADAMTS7 and disease pathogenesis.

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ADAMTS7 is a member of the ADAMTS family of secreted zinc metalloproteases with characteristic protein domain composition, including at least 1 thrombospondin type I repeat. These proteins have highly homologous N-terminal protein domain structure and organization, but the C-terminal halves vary significantly, which is believed to confer substrate
specificity. Previous research on ADAMTS7 has centered mainly on its role in bone and cartilage growth because cartilage oligomeric matrix protein (COMP) has been identified as a substrate. ADAMTS7 can regulate endochondral bone formation through interactions with COMP, but COMP is also expressed in vascular smooth muscle cells (VSMCs) and vasculature. Additional studies with virus-mediated overexpression and knockdown in vivo and in vitro suggest that ADAMTS7 might modulate VSMC phenotype switching and migration.

Here, we present the first report of an Adamts7 knockout (KO; Adamts7−/−) mouse and demonstrate that Adamts7 deficiency in both the Ldlr−/− and Apoe−/− hyperlipidemic mouse models markedly attenuates the formation of atherosclerotic lesions; furthermore, wire-injury experiments in the Adamts7−/− mouse show reduced neointima formation. We demonstrate that Adamts7 gene expression is induced transiently in the mouse vasculature in response to stress both in the wire injury model and in the atherosclerosis experiments, that tumor necrosis factor-α (TNFα) induces Adamts7 expression in primary VSMCs, and that VSMCs of Adamts7−/− mice show reduced TNFα-induced migration. Finally, immunostaining in human diseased coronary arteries reveals colocalization of ADAMTS7 with cells positive for VSMC markers, and immunofluorescence in human aortic SMCs shows subcellular localization with leading edges of migrating VSMCs.

**Methods**

Expanded methods, including methods for generation of Adamts7 mouse strains, plasma lipid measurements, X-gal staining, vascular injury studies, primary aortic VSMC studies, aortic SMC immunofluorescence, and immunohistochemistry, are available in the online-only Data Supplement.

**Atherosclerosis Studies**

Adamts7−/− mice were crossed onto the Ldlr and Apoe hyperlipidemic KO mouse backgrounds, and single-KO (Ldlr or Apoe alone) and double-KO (dKO) mice were obtained. Experimental dKO mice 8 to 12 weeks of age and control littermates were fed a Western diet (D12079Bi, Research Diets) for 16 weeks (Adamts7+/Ldlr−) or 10 weeks (Adamts7+/Apoe−), and then the mice were euthanized. Aortas were collected from the base of ascending aorta to the iliac bifurcation for en face lesion measurement, whereas aortic roots and brachiocephalic arteries (BCAs) were either X-gal stained as described above or paraffin embedded and used for lesion area quantification and immunohistochemistry.

For atherosclerosis lesion quantification, whole aortas were stained with Oil Red O as previously described, and then the positively stained lesion area was measured. Data are reported as a percentage of entire aortic area. For aortic roots, lesion area was measured on hematoxylin and eosin–stained sections from paraffin-embedded hearts. We quantified lesion area in 5 serial sections (80 μm between sections). For selection of the 5 sections, we defined a zero point, and immunofluorescence in human aortic SMCs shows subcellular localization with leading edges of migrating VSMCs.
which is the first section that captures all 3 leaflets of the aortic valve moving from the aortic arch toward the ventricles. Then, the 2 serial sections proximal (+1 and +2) and distal (−1 and −2) to the zero point were quantified. For each mouse, the data were represented as either the average lesion area (average area across the 5 points) or the area under the curve produced by integration of the total areas across all 5 sections (ie, the integrated area under the curve of root lesions).

Statistical Analysis
Data are presented as means, and all error bars represent standard errors. Statistical significance was tested with unpaired Student t tests, and a value of \( P \leq 0.05 \) was considered significant. For atherosclerosis studies, unpaired \( t \) tests were used to compare percent lesion area of aorta (in face) or actual lesion area (aortic roots) of \( \text{Adamts7}^{-/+} \) animals with control \( \text{Adamts7}^{+/+} \) animals for each sex separately. For vascular injury studies, unpaired \( t \) tests were used to compare neointimal area, neointima-to-media ratio, and percent stenosis of \( \text{Adamts7}^{-/-} \) animals with control \( \text{Adamts7}^{+/+} \) animals. For quantitative polymerase chain reaction studies, results from untreated \( \text{Adamts7}^{+/+} \) cells/tissue were set to 1, and all other groups were compared with that group via an unpaired \( t \) test.

Results
Characterization of \( \text{Adamts7} \) Expression in Wild-Type and KO Mice
We examined \( \text{Adamts7} \) mRNA expression in a variety of murine tissues harvested from female C57BL/6 wild-type (WT) mice. \( \text{Adamts7} \) expression varied greatly between tissues (Figure 1A), with the highest levels in heart, brain, lung, intestine, and adrenal gland. In contrast, low but detectable levels were observed in liver, spleen, kidney, brown fat, thyroid, and skeletal muscle. We commissioned the generation of an \( \text{Adamts7} \) KO mouse (\( \text{Adamts7}^{-/-} \)) in which the gene was ablated by use of a gene trap strategy with \( \beta \)-galactosidase gene (\( \text{lacZ} \)) insertion into intron 4 of the \( \text{Adamts7} \) locus (Figure 1B). By design, splicing from exon 4 to the gene trap cassette results in a severely truncated protein containing only the signal peptide, the prodomain, and a small portion of the metalloprotease domain. The trapping cassette contains a \( \text{LacZ} \) reporter gene allowing X-gal staining as readout of active \( \text{Adamts7} \) expression. \( \text{Adamts7} \) gene KO was verified by real-time quantitative polymerase chain reaction with the use of 2 probe sets, 1 set that spans the exon 5 to 6 junction and 1 set that spans the exon 23 to 24 junction (Figure 1C). \( \text{Adamts7}^{-/-} \) animals exhibited >95% reduction of the message in heart and lung (Figure 1C). X-gal staining of multiple tissues from \( \text{Adamts7}^{-/-} \) mice was consistent with the mRNA expression data from C57BL/6 mice and confirmed that the reporter was working. High levels of \( \beta \)-galactosidase activity were observed in the heart and pulmonary vasculature but not other tissues such as the liver and spleen (Figure 1D). Immunohistochemistry analysis for markers of cardiomyocytes (\( \alpha \)-actinin), endothelial cells (platelet endothelial cell adhesion molecule-1), and epithelial cells (E-cadherin) localized the positive X-gal staining and thus \( \text{Adamts7} \) expression to the myocardium of the heart and tunica media of...
the pulmonary vasculature (Figure I in the online-only Data Supplement). Adamts7−/− mice display no outward phenotypes, reproduce normally, and appear healthy under chow-fed conditions.

**Adamts7 KO Decreases Atherosclerosis in Hyperlipidemic Mouse Models**

Atherosclerosis was quantified in Adamts7/Ldlr and Adamts7/Apoe dKO mice and control littermates. Mice were fed a Western diet for 16 weeks (Ldlr) or 10 weeks (Apoe). Aortas and aortic roots were harvested for lesion area quantification, whereas root and BCA sections were used for lesion characterization by immunohistochemistry. On a Western diet, no differences in plasma lipid levels were observed between experimental groups (Table I in the online-only Data Supplement).

Adamts7/Ldlr dKO males (n=31) and females (n=34) displayed significant reductions in aortic lesion area as measured by en face (37% \( P=0.001 \) and 52% \( P=0.0001 \), respectively; Figure 2A and 2B). Similarly, aortic roots from Adamts7/Ldlr dKO males and females showed decreased lesion area (32% \( P=0.001 \) and 25% \( P=0.0001 \), respectively) compared with control Ldlr−/− male (n=21) and female (n=28) littermates (Figure 2C and 2D; and Figure IIA–IID in the online-only Data Supplement).

Marked reductions in atherosclerosis were seen also on the Apoe−/− background; male (n=16) and female (n=13) Adamts7/Apoe dKO mice displayed significantly reduced lesion formation by en face (62% \( P<0.0001 \) and 54% \( P<0.0001 \), respectively; Figure 3A and 3B) with a similar pattern in aortic roots (18% \( P=0.07 \) and 22% \( P=0.04 \), respectively) compared with control Apoe−/− male (n=9) and female (n=10) mice (Figure 3C and 3D and Figure IIE–IIH in the online-only Data Supplement).

We characterized the cellular and collagen composition of aortic root and BCA lesions from the Adamts7/Ldlr dKO animals and their control Ldlr−/− littermates (n=8 per group, average of 3 serial sections per animal) by immunohistochemistry or Masson trichrome staining of lesions with subsequent quantification. No significant difference in SMC content (smooth muscle-22–positive signal) or intensity of contractile marker staining (eg, smooth muscle–myosin heavy chain) was observed in BCA lesions, but trends toward reduced macrophage and increased collagen content were observed in BCAs (Figure 4E–4H). A similar pattern of increased collagen in aortic roots of atherosclerotic mice was observed (Figure III in the online-only Data Supplement).

**Adamts7 Deficiency Reduces the Neointimal Response to Vascular Injury and TNFα-Stimulated Migration of SMCs**

Previous reports have implicated ADAMTS7 in SMC phenotype modulation in response to vascular injury. To examine the role of Adamts7 in the response to vascular injury, we performed femoral artery wire injury and sham surgeries on Adamts7−/− and WT littermates. Compared with WT littermates...
at 28 days after injury, *Adams7*-null animals displayed a 64% reduction \((P=0.05)\) in neointima formation (Figure 5A and 5B), a 47% reduction in intima-to-media ratio (Figure 5C), and a 61% decrease in percent stenosis \((P=0.003;\) Figure 5D). Coincident with this reduction in neointima, expression of contractile markers within neointima, including smooth muscle–myosin heavy chain, smooth muscle α-actin, and smooth muscle-22α, was significantly greater in *Adams7*\(^{-/-}\) mice after vascular injury compared with control mice (Figure IV in the online-only Data Supplement). Thus, after acute mechanical vascular injury, ablation of the *Adams7* gene reduced neointimal formation and appeared to maintain VSMC phenotype.

In light of vascular injury findings, we tested whether primary aortic SMCs from *Adams7*\(^{-/-}\) mice had reduced migration compared with those from control WT littermates. In initial studies, WT and KO VSMCs plated on collagen I, fibronectin, laminin, or uncoated plates displayed no differences in migratory ability. In prior studies of rat primary VSMCs, TNFα induced *Adams7* expression, \(^{13}\) so we examined TNFα effects in WT VSMCs and found a similar upregulation \((\approx 6\text{-}fold; P=0.0007)\) of *Adams7* (Figure 5E). Migration studies performed after 24 hours of TNFα treatment revealed a reduction in migration of TNFα-stimulated *Adams7*\(^{-/-}\) VSMCs compared with WT cells in dishes coated with collagen or laminin (Figure 5F and 5G), whereas unstimulated cells showed no difference (Figure V in the online-only Data Supplement). This supports the concept that, in vitro, protective VSMC phenotypes in *Adams7*\(^{-/-}\) are revealed in the setting of inflammatory stress, consistent with the observed vascular protection during in vivo physical and hyperlipidemic vascular insults.

**Adams7 Expression Is Temporal and Transient During Vascular Injury and in Atherosclerosis**

Our data suggest that *Adams7* modulates VSMC phenotype and migration during inflammatory stress and mechanical injury and that *Adams7* deficiency markedly reduces atherosclerotic lesions in hyperlipidemic mice. However, the atherosclerosis data did not yield clear differences in relative matrix or SMC composition of lesions at the time of sacrifice (after 16 and 10 weeks of Western diet feeding in *Ldlr*\(^{-/-}\) and *Apoe*\(^{-/-}\) models, respectively). Thus, we posited that murine *Adams7* might function predominantly in early lesion formation and that the imprint of *Adams7* deficiency is less visible on specific morphologies in more mature murine lesions. The β-galactosidase reporter in the exon-trapping cassette of the *Adams7*\(^{-/-}\) mouse (Figure 1B) permits X-gal staining in harvested tissues, allowing identification of cells that actively transcribe the *Adams7* gene in animals carrying a KO allele. In light of the lack of working antibodies toward mouse *Adams7*, we used this approach to examine the time course of vascular expression of *Adams7* in our vascular wire injury model and in atherosclerosis models.

First, we studied various time points after femoral wire injury \((n=4\text{ per time point})\). At day 1 after injury (Figure 6A) and day 28 after injury (time of harvest, data not shown), we saw no evidence of X-gal staining. However, at day 7 after injury (Figure 6B), blue staining indicative of β-galactosidase expression was apparent in injured vessels, localized predominantly to the media and adventitia, and this staining was almost completely absent at day 14 (Figure 6C). When considered in context of the reduction in lesions at day 28 after injury in *Adams7*\(^{-/-}\) mice, this suggests that *Adams7* expression is

**Figure 4.** Smooth muscle cell, macrophage, and collagen staining of brachiocephalic artery (BCA) lesions from *Adams7*\(^{-/-}\);*Ldlr*\(^{-/-}\) animals compared with controls. Representative immunohistochemistry staining of smooth muscle-22 (SM22; A), smooth muscle–myosin heavy chain (SM-MyHC; C), Mac-3 (E), and Masson trichrome (G) from the BCAs of *Adams7*\(^{-/-}\);*Ldlr*\(^{-/-}\) animals and control littermates \((n=9\text{ per group})\) is shown. Quantification of the positive signal inside the lesion (B, D, and F) or the blue staining in the trichrome (H) is shown on the right of the images and presented as percent of lesion area or mean pixel intensity.
induced early and transiently in response to the acute injury and affects remodeling at day 28 through its actions during the early pathophysiological response. Thus, the effects of Adamts7 deletion during injury may be confined to the early phase when VSMC migration plays a critical role.16

In this context, we analyzed X-gal staining patterns in BCA and aortic roots from Adamts7/Apoe dKO mice fed a Western diet for varying lengths of time (n=3 per time point). BCAs from mice fed a Western diet for 1 week showed no atherosclerotic lesion formation and no positive X-gal staining (Figure 6D). At 4 weeks on a Western diet, however, a subset of Adamts7−/−, Apoe−/− mice had developed lesions in the BCA, and they displayed strong positive X-gal staining in the both media and lesion neointima (Figure 6E). Immunohistochemistry analysis of these lesions showed overlap with cells positive for smooth muscle α-actin but not smooth muscle-22 (Figure VI in the online-only Data Supplement). In support of a transient induction of Adamts7 during the development of atherosclerosis, X-gal staining was not observed in BCA lesions at later time points (eg, 10 weeks), even though mature atherosclerotic plaques were present (Figure 6F). A similar pattern was observed in aortic roots of these mice; the only positive X-gal

Figure 5. Neointimal formation after femoral artery wire injury is attenuated by ablation of mouse Adamts7. A, Wire injury was performed on femoral arteries of Adamts7−/− and wild-type (WT) animals (n=5 and 4, respectively), vessels were harvested at 28 days, and neointima was assessed by hematoxylin and eosin staining. Representative sections from WT and knockout (KO) femoral arteries are shown. There was reduced (B) neointima (64%), (C) intima-to-media ratio (47%), and (D) percent stenosis (61%) in Adamts7−/− compared with WT mice. E, Adamts7 expression measured by TaqMan real-time polymerase chain reaction in primary vascular smooth muscle cells (VSMCs) from Adamts7 WT or KO mice after treatment with 25 ng/mL tumor necrosis factor-α (TNFα) for 24 hours (n=3). F and G, Migration of TNFα-stimulated Adamts7 KO primary VSMCs on collagen- or laminin-coated plates (n=3) is reduced compared with WT cells.
staining was observed in roots from mice fed a Western diet for 4 weeks (data not shown). X-gal staining of cultured primary VSMCs of Adamts7−/− mice revealed modest positive staining under resting conditions, but this X-gal staining was markedly increased after treatment with TNFα (Figure 6G). These observations suggest that Adamts7 is induced transiently and expressed only during earlier stages of atherogenesis in mouse, likely via inflammatory (eg, TNFα) and hyperlipidemic triggers. This is consistent with its induction in the early phase of femoral artery injury and suggests that Adamts7 may be active at early phases of both mechanical vascular injury and Western diet/hyperlipidemia–induced vascular stress and atherosclerosis.

ADAMTS7 Is Expressed in Human Atherosclerosis and Localizes to the Migrating Edge of VSMCs

Our rodent data suggest a role for Adamts7 in promoting VSMC phenotypic transition and migration during arterial injury and hyperlipidemic atherogenic stress. To place these rodent observations in a human context, we obtained sections of diseased human coronary arteries (n=12) and performed immunohistochemical studies (Figure 7 and Figures VII and VIII in the online-only Data Supplement). ADAMTS7 consistently colocalized in the vascular media with the VSMC markers smooth muscle-22 and smooth muscle α-actin in all lesions examined (Figure 7A–7D). In fibrotic atherosclerotic lesions, ADAMTS7 also colocalized with smooth muscle-22–positive signal in the neointimal region of the lesion (Figure 7B). Interestingly, in more advanced lesions, smooth muscle-22 and smooth muscle α-actin consistently stained the fibrous cap, but limited ADAMTS7 staining was observed in those regions (Figure 7B and 7C). Conversely, in these advanced lesions, ADAMTS7 staining was observed in the lesion neointima in regions sometimes negative for VSMC markers (Figure 7B and 7C). In contrast, ADAMTS7 staining did not colocalize with the macrophage marker CD68 in any lesions examined (eg, Figure 7E). These data show that ADAMTS7 is expressed in human arteries and lesions in medial VSMCs and...
suggest that ADAMTS7 stains neointimal cell populations, only a subset of which express classic VSMC markers.

To determine the subcellular localization of ADAMTS7 in human VSMCs, we also performed immunofluorescence in primary human aortic SMCs. Anti-ADAMTS7 staining demonstrated abundant protein expression in cytoplasmic granules and at the cell membrane (Figure 8A). ADAMTS7 was also found at the leading edge of cells and colocalized with cytoskeletal filaments and Na+/K+ ATPase, a cell surface–expressed protein (Figure 8B and 8C). Furthermore, ADAMTS7 colocalized at the cell membrane with cortactin (Figure 8D), a marker of focal adhesions and podosomal complexes that orchestrates VSMC migration through vascular matrix.17

Discussion

Through GWASs, we identified ADAMTS7 as a novel locus for CAD in humans.2 The association with this chromosome 15 locus has been reproduced in independent large GWAS meta-analyses.3–5 The ADAMTS7 locus, however, has no relationship to traditional risk factors; the direction of its action in atherosclerosis is unknown; and the precise mechanisms of action in atherosclerosis remain to be determined. Indeed, whether ADAMTS7 is definitely the causal gene at the locus remains uncertain. Here, we present the first report of Adamts7−/− mice and show that Adamts7 deficiency causes marked protection from Western diet–induced atherosclerosis in multiple hyperlipidemic mouse models, reduced neointimal formation after arterial injury, and decreased VSMC migration in vitro. These data not only strongly implicate ADAMTS7 as the causal gene at this human CAD locus but also provide evidence for a proatherogenic role of the gene in vivo and conversely an antiatherogenic effect of its genetic inhibition. Overall, our findings in 2 well-validated rodent model systems suggest that ADAMTS7 promotes atherosclerosis and that targeting its function could be a novel therapeutic strategy for atherosclerosis, CAD, and MI in humans.

Recent GWASs have identified >40 loci for CAD and MI. Unlike ADAMTS7, few novel loci have the combination of a clear candidate at the locus and plausible biology for the candidate gene. We discovered ADAMTS7 as a locus for coronary atherosclerosis using angiographic CAD as the outcome, and subsequent studies have shown that ADAMTS7 also relates to MI. However, its association is most robust for angiographic CAD, a marker of coronary atherosclerotic burden, suggesting that ADAMTS7 is likely to relate to clinical events through
Circulation
March 31, 2015

the development and progression of atherosclerosis. Our present findings in mice are consistent with such an action in the clinical setting.

The family of ADAMTS proteases degrades extracellular matrix and has grown to 19 members. ADAMTS family members are associated with vascular diseases, including thrombotic thrombocytopenic purpura,18 Weill-Marchesani syndrome,19 and atherosclerosis.20 Like all ADAMTS proteins, ADAMTS7 includes a signal peptide, a prodomain, a catalytic domain, a disintegrin-like domain, a central thrombospondin type I-like repeat, a cysteine-rich domain, a spacer region, and 7 C-terminal thrombospondin type I repeats.15 Unlike other metalloproteinases, ADAMTSs demonstrate narrow substrate specificity as a result of their C-terminal exosites. ADAMTS7 has been implicated in bone development and in inflammatory arthritis through degradation of COMP, a pentameric glycoprotein involved in inherited human chondrodysplasias.10 Previously published data suggest that the ADAMTS7-COMP interaction may extend to the vasculature. VSMC phenotypic switching and matrix remodeling play important roles in vascular disease and contribute to the development and progression of atherosclerosis. Studies in rats suggest that COMP may maintain VSMCs in a contractile phenotype.21 Adenoviral overexpression of ADAMTS7 increased COMP cleavage and neo-intima in injured arteries, whereas overexpression of COMP blocked ADAMTS7 effects.13 Whether acute effects using transient overexpression extend to vascular pathophysiology and atherosclerosis during germline modulation has not been studied.

Most domains in human and mouse Adams7 are highly conserved, rendering the mouse as a useful model for actions in human disease. We obtained whole-body KO mice, bred them onto both the Ldlr−/− and Apoe−/− KO hyperlipidemic mouse models, and performed atherosclerosis studies in both males and females of each strain. Loss of Adams7 clearly and significantly reduced atherosclerotic burden in the aortas and aortic roots of these mice in both sexes and both strains. Femoral artery wire injury is an established model in mice for studying VSMC phenotype transitions and matrix remodeling during vascular injuries of relevance to clinical vascular pathophysiology, for example, restenosis during coronary angioplasty and stent implantation. At 28 days after femoral injury or sham surgery, Adams7−/− mice had reduced neo-intima formation and intima-to-media ratio compared with WT mice. Primary aortic VSMCs from Adams7−/− mice displayed decreased migration on collagen- and laminin-coated plates, but only in the setting of TNFα stimulation. TNFα, a proinflammatory cytokine, and, more broadly, activation of innate immune proinflammatory signals have been implicated in atherosclerosis in both rodent hyperlipidemic models22 and

Figure 8. ADAMTS7 localizes to the leading edge and podosome-like structures in primary human aortic smooth muscle cells (hAoSMCs). A through C, Immunofluorescent staining for ADAMTS7 and the cell surface marker Na+/K+ ion channel in primary hAoSMCs. Each row is imaging of a different cell in the same experiment. D, Costaining for ADAMTS7 and cortactin, a marker of podosomes, in primary hAoSMCs. Arrowheads identify regions of strong overlap in focal adhesion-like structures.
human studies, and in the modulation of VSMC migration. It is plausible that ADAMTS7 plays a key role in mediating VSMC responses to inflammatory stresses in atherosclerosis and facilitates VSMC phenotype transition and localized matrix remodeling.

We observed a lack of Adams7 expression in mouse aortas under resting conditions. In combination with our data in mouse atherosclerosis and femoral injury, this suggests, at least in these mouse models, that induction of Adams7 may be important in VSMC phenotype transition during injury response and development of atherosclerosis but not in murine vascular homeostasis. The lack of any vascular developmental defects in germine Adams7 deletion also supports this concept. X-gal staining of mouse vasculature at various time points after femoral wire injury or Western diet feeding confirms that Adams7 vascular expression is transient and temporal, peaking early after both mechanical and hyperlipidemic vascular insults. Stimulation of primary VSMCs with TNFα also dramatically increased positive X-gal staining of these cells, suggesting that TNFα (and other proinflammatory cytokines) may play a key role in Adams7 induction in response to injury.

Understanding the relationship between CAD risk alleles in the ADAMTS7 genomic region and expression levels of ADAMTS7 in disease-relevant cells could facilitate translation of our rodent findings into human therapeutics. Interestingly, in available eQTL data sets with large sample sizes (eg, LCL eQTL from the Multiple Tissue Human Expression Resource [MuTHER] study; n=850), the lead single-nucleotide polymorphisms from the PennCath (rs1994016), Coronary Artery Disease Genome-Wide Replication and Meta-Analysis (CARDIoGRAM; rs3825807), and Coronary Artery Disease Genetics Consortium (C4D; rs4380028) GWAS studies display a significant association with ADAMTS7 expression (eQTL: P<0.0011, P<0.00097, and P<0.0028, respectively), matching the directionality and causality of our in vivo data; that is, the CAD risk alleles are associated with higher ADAMTS7 expression. However, there currently are no large eQTL or genomic allelic imbalance data that provide adequate power to determine eQTL Directionality in the most pertinent human cells and vascular tissues (ie, coronary lesions, coronary or aortic SMCs) to more definitively support that ADAMTS7 functions to promotes human atherosclerosis and CAD. Work by our group and consortia such as Genotype-Tissue Expression Project (GTEx) will soon produce data sets of our rodent findings into human therapeutics. Interestingly, ADAMTS1, 4, and 5 also have previously been localized to podosome-like structures in response to extracellular stimuli and mediate migration through cell-tissue matrixes.

Conclusions
ADAMTS7 is a genetic locus for CAD and MI in human, and our data are the first to show in vivo that ADAMTS7 is proatherosclerotic and may promote progression and complications of the disease by modulating VSMC phenotype. Because ADAMTS7 has a narrow substrate specificity, it has promise as a potentially safe drug target. Our findings provide support for the development of ADAMTS7 inhibitors in the prevention and treatment of human coronary atherosclerotic diseases.

Acknowledgments
We acknowledge Weili Yan for help with performing the wire injury experiments and Andrea Stout for imaging assistance. We also thank the heart failure and transplantation nurses, cardiothoracic surgeons, and operating room staff at the University of Pennsylvania and the Gift-of-Life donor program (Philadelphia, PA) for their assistance with heart tissue procurement.

Sources of Funding
These studies were funded by National Institutes of Health grant HL111694 (to Dr Reilly) and Transatlantic Network of Excellence Grant 10CVD03 from the Fondation Leducq (to Dr Rader). Dr Reilly is also supported by National Institutes of Health grants DK090505, HL-113147, HL107643, and HL108636. Dr Bauer is also supported by American Heart Association Postdoctoral Fellowship 12040456.

Disclosures
None.

References
1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie...
Through genome-wide association studies, we recently identified the metalloproteinase ADAMTS7 as a locus for coronary artery disease. Other than low-density lipoprotein cholesterol–related genes, few of the >40 validated loci for coronary artery disease identified through genome-wide association studies have known mechanisms of action in atherosclerotic heart diseases. Indeed, for many loci, the gene(s) at the locus is (are) unknown or uncertain. ADAMTS7 is relatively unique as a genome-wide association study locus in having a single strong candidate gene at the locus, plausible biological mechanisms, and potential for therapeutic translation. Here, we present the first report of the Adamts7-deficient (Adamts7−/−) mouse, which, once crossed onto a hyperlipidemic background, displays reduced atherosclerotic lesion formation after prolonged feeding of a Western diet. We also show that Adamts7 expression is induced in response to both mechanical wire injury and hyperlipidemia and that primary smooth muscle cells from Adamts7−/− mice display reduced migration when stimulated by tumor necrosis factor-α. Our findings are the first in vivo data to provide directionality to the genetic association between ADAMTS7 and coronary artery disease in humans. These data clearly demonstrate that ADAMTS7 is proatherogenic, likely through modulation of vascular smooth muscle cell phenotype in response to injury and inflammation. Importantly, these data provide early promise that inhibition of ADAMTS7, which is tractable given its narrow substrate specificity, might represent a novel mechanism for prevention and treatment of clinical cardiovascular diseases. Thus, it is one of the very few novel nonlipid findings from genome-wide association studies of coronary artery disease that have the potential to be a bona fide novel therapeutic target.
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Circulation. 2015;131:1202-1213; originally published online February 20, 2015;
doi: 10.1161/CIRCULATIONAHA.114.012669

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Supplemental Methods:

*Generation of Adamts7 mouse strains:* Adamts7$^{-/-}$ mice were generated by request at the Knockout Mouse Project (KOMP, University of California, Irvine). More information about the construction of these mice can be found on the KOMP website (https://www.komp.org/geneinfo.php?geneid=18338). Vascular injury studies were performed in female mice, and wild-type (WT) littermates were used as controls. All mice were generated on pure C57B/6 backgrounds, and all animal experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania.

The expression of Adamts7 mRNA was analyzed in tissues of 12 week old WT and Adamts7$^{-/-}$ mice by RT-PCR using multiple TaqMan probe-sets (Mm01239070_g1 and Mm01239067_m1, Life Tech, Grand Island, NY) and levels expressed relative to β-Actin expression. For determining Adamts7 gene expression in mouse tissues, RNA was prepared from the indicated tissue from three female C57BL/6 mice. TaqMan expression analysis was carried out using probe Mm01239067_m1 and normalized to β-Actin. The tissue with the lowest level of expression was set to one (ovaries in this instance), and all tissue expression values are reported as relative fold-difference. Tissue distribution of Adamts7 was examined by X-gal staining for the β-galactosidase reporter as positioned in the exon-trapping cassette in the Adamts7 locus of the Adamts7$^{-/-}$ mouse.

*X-gal Staining:* Tissues were dissected and fixed in 2% paraformaldehyde (PFA) for 1 hour. After six 30 minute PBS washes, tissues were incubated at 37°C overnight in X-gal stain (PBS containing 2mM MgCl2, 5mM Potassium Hexacyanoferrate (iii), 5mM
Potassium Hexacyanoferrate (ii) trihydrate, 0.01% NP40, 0.1% Deoxycholate, and 1.0 mg/ml X-Gal in dimethylformamide). Tissues were then washed six more times in PBS, and then imaged.

Vascular Injury Studies: 8-week old Adams7⁻⁄⁻ and WT littermates were subjected to femoral artery wire-injury or sham surgery as previously described, with slight modification¹. Briefly, one side of the femoral artery was exposed by blunt dissection while mice were under anesthesia a flexible angioplasty wire (0.35-mm diameter; Cook Inc, Bloomington, IN) was inserted into the femoral artery toward the iliac artery for 3 minutes to denude and dilate the artery. The femoral artery on the other side was sham-operated as a control. Mice (N=5 and 4, for Adams7⁻⁄⁻ and WT respectively) were euthanized 28 days post-surgery and perfused with 4% PFA. Femoral arteries were harvested and paraffin embedded for morphometric and histological analysis. Morphometric analysis was performed on 4 serial cross sections with a customized program (Phase 3 Imaging Systems, Glen Mills, PA) of Image Pro. The area of the lumen, the area inside the internal elastic lamina, and the area inside the external elastic lamina were estimated. Percentage stenosis was calculated as the ratio of the intimal area to the area inside the original internal elastic lamina, and the ratio of intima to media was determined.

Primary Aortic VSMC studies: Primary aortic smooth muscle cells were isolated as previously described ² from Adams7⁺⁄⁺ and Adams7⁻⁄⁻ mice aged 8-10 weeks. Migration assays were performed using the Radius™ Cell Migration Assay (Cell Biolabs, Inc, San Diego, CA) as per manufacturers instructions. Cells were treated for 24 hours prior to gel spot removal or X-gal staining with 25ng/ml recombinant TNFα (Life Tech, Grand Island, NY) or water.
Aortic SMC Immunofluorescence: hAoSMCs were obtained commercially (Lonza, Switzerland) and maintained in Smooth Muscle Growth Medium-2 (SmGM-2, Clonetics, Walkersville, MD) per manufacturer protocol. For immunofluorescence, 1.5x10^5 hAoSMCs were seeded on chamber slides. Twenty-four hours later, cells were fixed in 4% PFA for 15 minutes, permeabilized in 0.2% Triton X-100 for 5 minutes, washed and incubated in 1% BSA/PBS for 30 minutes at room temperature. Cells were treated with antibodies for ADAMTS7 (H00011173-A01, Novus Biologicals, Littleton, CO), Na+/K+ ATPase (sc-28800, Santa Cruz Biotechnology, Santa Cruz, CA), or Cortactin (ab11066, Abcam, Cambridge, MA), and then incubated with Donkey anti-mouse Alexa Fluor 488 and Goat anti-Rabbit Alexa Fluor 647 secondary antibodies (1:1000; Life Tech, Grand Island, NY) in 0.3%BSA/PBS. Purified rabbit and mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) served as controls (Supplemental Figure 8). Deconvolution microscopy images were acquired using the Deltavision Core Deconvolution Microscope system (Applied Precision Inc Issaquah, WA), using an Olympus IX70 microscope and softWoRx acquisition and analysis software (Applied Precision Inc Issaquah, WA).

Immunohistochemistry: All IHC, including that for sections of mouse aortic root, mouse BCA, mouse femoral artery, and human coronary arteries was performed in the UPenn Cardiovascular Institute Histology Core, and all relevant protocols are available at http://www.pennmedicine.org/heart/research-clinical-trials/core-facilities/histology-gene-expression/. All tissue sections were deparaffinized and pretreated using heat antigen retrieval with Bull's Eye Decloaker (BioCare Medical, Concord, CA) or 1.5% Proteinase K. After washing with 0.1% PBST and blocking with normal serum for 30-60 minutes at 25°C, sections were incubated with primary antibody in 0.1% PBST overnight at 4°C. For all work with mouse tissues, antibodies used included mouse anti-Fibronectin (1:50; Santa Cruz, Santa Cruz, CA), mouse anti-Collagen IV (1:100; Millipore, Billerica, MA),
goat anti-Sm22a (1:100; ab10135, Abcam, Cambridge, MA), mouse anti-SM-α-actin (1:200; A5228, Sigma, St. Louis, MO), mouse anti-SM-MyHC (1:100; Biomedical Technologies Inc., Stoughton, MA), and rat anti-Mac-3 (1:100; #550292, BD BioSciences, San Jose, CA). For staining of human coronary artery sections, we used anti-ADAMTS7 (1:100; ab45044, Abcam, Cambridge, MA), anti-CD68 (1:100; ab955, Abcam, Cambridge, MA) and the same anti-Sm22α and anti-SM-α-actin listed above. After washing with 0.1% PBST, sections were incubated with Alexa Fluor 488 or 568 Goat anti-mouse or Donkey anti-goat IgG (1:250; Life Tech, Grand Island, NY) for 1 hour at 25°C, or were subjected to ImmPRESS TSA detection for 60 minutes at room temperature. Secondary antibodies alone served as controls (Supplemental Figure 8). All slides then washed with 0.1% PBST and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

*Plasma Lipid Measurements:* Whole blood (200 µL) was collected via retro-orbital sinus plexus from mice after 4 hours of fasting for plasma lipid analyses. Plasma was obtained after centrifugation for 7 minutes at 10,000 rpm in a microcentrifuge and stored in aliquots at −80°C.
Supplemental Table 1: Plasma lipid levels in both the Ldlr and Apoe hyperlipidemic KO mouse models are unchanged in the setting of Adamts7 deficiency. Plasma lipid values for total cholesterol (TC), HDL, Non-HDL, and triglycerides (TG) are shown for all atherosclerosis experimental groups. The units for all measurements are mg/dL. The value in parentheses is the standard deviation of the group. * denotes a p-value < 0.05 as compared to control group by Student’s t-test.

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Supplemental Figure 1: *Adams7* is expressed in myocardium and pulmonary vasculature as evidenced by Xgal staining. A) Xgal staining of whole heart reveals
ubiquitous expression of Adamts7 in cardiomyocytes throughout the heart. B) 200x zoom of the boxed region from panel A showing that while myocardium expression of Adamts7 is high, in the resting state there is little to no positive staining in the wall of the aortic root. C) X-gal staining in heart does not colocalize with markers (PECAM-1) for endothelial cells, but D) does localize with markers (α-Actinin) of cardiomyocytes. E-H) Staining of Xgal-stained lung sections with antibodies directed at E-cadherin and PECAM-1 to differentiate between trachea and pulmonary vasculature. Adamts7 expression is localized to the media of the vasculature, as positive X-gal staining colocalizes with positive PECAM-1 staining for the endothelial layer and not positive E-Cadherin staining for bronchial epithelial cells. Panels F and H provide 200x enlargement of boxed regions in E and G.
Supplemental Figure 2: Integrated AUC analysis of aortic root lesion area in both Ats7/Ldlr and Ats7/ApoE atherosclerotic experimental models reveals decreased lesion area. For each animal, a “Zero Point” was established at the first section where all 3 leaflets of the aortic valve were visible. Lesion area was quantified at this section and the two sections above (+1 and +2) and below (-1 and -2). The average lesion area of these 5 sections is shown (A, C, E, G) for all experimental groups, and the area under the resultant curve is also shown (B, D, F, H). * denotes p-value < 0.05, ** denotes p-value < 0.01.
Supplemental Figure 3: Trend towards increased collagen content of aortic root lesions in Adamts7-deficient atherosclerotic mice. A-C) Representative images of Collagen I IHC in aortic root sections from hyperlipidemic Adamts7 WT (N=6) and KO mice (N=10), and quantitation of lesion Collagen I content represented as percent lesion area.
Supplemental Figure 4: Vascular ECM deposition is reduced and SMC phenotype
markers preserved in *Adams*7−/− animals following femoral artery injury. (A) Trichrome staining, as well as collagen-IV and fibronectin immunohistochemistry revealed greatly reduced ECM deposition in injured vessels of *Adams*7−/− compared to WT. (B) IHC for contractile VSMC markers (SM22α, SM-α-actin, and SM-MyHC) revealed preserved expression in the injured vessels of *Adams*7−/− mice compared to WT (N=5 and 4, respectively). Far right column is quantification of signal from wire-injury lesions, as measured as mean pixel intensity in neointimal region. * denotes *p*-value < 0.05, ** denotes *p*-value < 0.01.
Supplemental Figure 5: Primary $\textit{Adams}7^{+/+}$ and $\textit{Adams}7^{-/-}$ VSMCs display no difference in migration on extracellular matrices under resting (non-TNF\textalpha) stimulated conditions. A-B) Representative images from Radius\textsuperscript{TM} 24-Well Cell Migration Assay used for quantitation at 1-hour and 6-hours post-gel spot removal. C-F) Migration of $\textit{Adams}7$ WT and KO primary aortic VSMCs (N=3/timepoint) for 12 hours on uncoated tissue culture plates (C), plates coated with fibronectin (D), collagen I (E), and laminin (F).
Supplemental Figure 6: Positive X-gal staining in BCA lesions of Ats7/Apoe dKO mice after 4-weeks of western diet feeding co-localize with SM-α-actin positive cells. IHC analysis of X-gal stained BCA atherosclerotic lesions from Ats7/Apoe dKO mice after 4-weeks of western diet feeding. Positive X-gal staining overlaps with SM-α-actin staining (A) but not that of SM22 (B).
Supplemental Figure 7: Additional IHC staining of atherosclerotic human coronary arteries (HCAs) for ADAMST7 and markers of SMCs and macrophage.

Representative IHC staining from 4 HCA samples is shown for ADAMTS7 and A) SM22, B) SM-a-actin, and C) CD68, with merge of ADAMTS7 and cell marker staining in right column with the lumen demarcated by “L”.

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Supplemental Figure 8: Negative controls for immunostaining. A) Negative controls shown for each type of staining done in human coronary arteries. Top row is example of positive IHC staining with indicated antibody, and bottom row is 2nd antibody only in the same region of sample. B) Immunofluorescence of hAoSMCs incubated with mouse or rabbit IgG and Alexa Fluor 488 or 647. All panels are of the same view indicated in the DAPI panel.
Supplemental Material References
