Arteriosclerosis, Thrombosis, and Vascular Biology

TRANSLATIONAL SCIENCES

ADAM (a Disintegrin and Metalloproteinase) 15 Deficiency Exacerbates Ang II (Angiotensin II)–Induced Aortic Remodeling Leading to Abdominal Aortic Aneurysm

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OBJECTIVE: ADAM (a disintegrin and metalloproteinase) 15—a membrane-bound metalloprotease from the ADAM (disintegrin and metalloproteinase) family—has been linked to endothelial permeability, inflammation, and metastasis. However, its function in aortic aneurysm has not been explored. We aimed to determine the function of ADAM15 in the pathogenesis of aortic remodeling and aneurysm formation.

APPROACH AND RESULTS: Male *Adam15*-deficient and WT (wild type) mice (10 weeks old), on standard laboratory diet, received Ang II (angiotensin II; 1.5 mg/kg per day) or saline (Alzet pump) for 2 or 4 weeks. Ang II increased ADAM15 in WT aorta, while *Adam15*-deficiency resulted in abdominal aortic aneurysm characterized by loss of medial smooth muscle cells (SMCs), elastin fragmentation, inflammation, but unaltered Ang II–mediated hypertension. In the abdominal aortic tissue and primary aortic SMCs culture, *Adam15* deficiency decreased SMC proliferation, increased apoptosis, and reduced contractile properties along with F-actin depolymerization to G-actin. Ang II triggered a markedly greater increase in THBS (thrombospondin) 1 in *Adam15*-deficient aorta, primarily the medial layer in vivo, and in aortic SMC in vitro; increased SSH1 (slingshot homolog 1) phosphatase activity and cofilin dephosphorylation that promoted F-actin depolymerization and G-actin accumulation. rhTHBS1 (recombinant THBS1) alone was sufficient to activate the cofilin pathway, increase G-actin, and induce apoptosis of aortic SMCs, confirming the key role of THBS1 in this process. Further, in human abdominal aortic aneurysm specimens, decreased ADAM15 was associated with increased THBS1 levels and loss of medial SMCs.

CONCLUSIONS: This study is the first to demonstrate a key role for ADAM15 in abdominal aortic aneurysm through regulating the SMC function, thereby placing ADAM15 in a critical position as a potential therapeutic target for abdominal aortic aneurysm.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: angiotensin II ■ disintegrins ■ elastin ■ myocytes, smooth muscle ■ thrombospondins

Aortic aneurysm—a permanent focal dilation of the aorta—is initially asymptomatic but potentially a devastating disease with no treatment other than corrective surgery. Aortic aneurysm results from a series of adverse structural and cellular events, such as degradation of the ECM (extracellular matrix), loss or dysfunction of the endothelial cells (ECs)[1] and smooth muscle cells (SMCs).[2,3] Abdominal aortic aneurysm (AAA) is more prevalent in old men (≈1%–2% of 65 year olds) than in women (≈0.5% of 70 year olds).[4–6] Although linked to atherosclerosis, AAA can also occur in the absence of and independent from atherosclerosis and metabolic complications.[7–9]

Aortic aneurysm is characterized by aberrant structural remodeling and cell loss, and as such, the role of

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Nonstandard Abbreviations and Acronyms

αSMA	alpha-smooth muscle actin
AAA	abdominal aortic aneurysm
ACE	angiotensin-converting enzyme
ADAM	a disintegrin and metalloproteinase
Adam15$^{-/-}$	Adam15 deficient
ADAMTS	a disintegrin and metalloproteinase with thrombospondin domain
Akt	protein kinase B
Ang II	angiotensin II
AoSMC	aortic smooth muscle cell
BrDU	5-bromo-2′-deoxyuridine
EC	endothelial cell
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
HAoEC	human aortic endothelial cell
MMP	matrix metalloproteinase
MT1-MMP	membrane type 1 matrix metalloproteinase
rhTHBS1	recombinant thrombospondin-1
SM-MHC11	smooth muscle myosin heavy chain
SM22	smooth muscle 22
SMC	smooth muscle cell
SSH1	slingshot homolog 1
TGFβ	transforming growth factor-beta
THBS	thrombospondin
TIMP	tissue inhibitor of metalloproteinase
VE-cadherin	vascular endothelial cadherin
WT	wild type
YAP	Yes-associated protein

proteases such as MMPs (matrix metalloproteinases), their inhibitors, TIMPs (tissue inhibitor of metalloproteinases), and cathepsins has been investigated in different models of aortic aneurysm.[3,10–12] However, the function of ADAMs (disintegrin and metalloproteinases)—a family of transmembrane proteases with diverse functions—in aortic aneurysm has been less explored. ADAM15 is a member of the ADAM family that can mediate proteolytic ectodomain shedding of various molecules, while the cytosolic domain of ADAM15 has recognition sites for a number of molecules that are important players in signal transductions; therefore, ADAM15 can have pleiotropic functions through different mechanisms.[13] Expression of ADAM15 was first detected in human ECs[14] and subsequently in animal vasculature.[15] The role of ADAM15 in endothelial permeability[16] and neovascularization[17] has been reported. ADAM15 has also been linked to impaired endothelial barrier function, transmigration of monocytes and inflammation in atherosclerosis,[18] and sepsis,[19] while ADAM15 has also been proposed to interrupt cell-cell adhesion[20] and block neovascularization.[15,21] However, function of ADAM15 in aortic aneurysm has not been studied.

Highlights

- Loss of ADAM15 (a disintegrin and metalloproteinase) increases susceptibility to abdominal aortic aneurysm following Ang II (angiotensin II) infusion without altering hypertensive response.
- In the absence of *Adam15*, Ang II infusion leads to decreased proliferation and contractility and increased apoptosis in aortic smooth muscle cells in vivo and in vitro.
- Abdominal aortic aneurysm in *Adam15*-deficient–Ang II mice is associated with elevated THBS (thrombospondin) 1 levels, cofilin activation (dephosphorylation), and F-actin depolymerization to G-actin.
- Consistent with our findings in this animal model, human abdominal aortic aneurysm specimens show reduced ADAM15, elevated THBS1, and loss of medial smooth muscle cell.

In this study, we aimed to determine the function of ADAM15 in arterial remodeling, hypertension, and aortic aneurysm. Ang (angiotensin II) is a hormone that is upregulated in most cardiovascular diseases, and as such, Ang II infusion has been widely used as a preclinical model of hypertension, vascular remodeling, and aortic aneurysm.[3,22] Using *Adam15*-deficient (*Adam15*$^{-/-}$) mice, primary cell culture of aortic ECs and aortic SMCs (AoSMCs), and AAA specimens from patients, we report a novel protective function for ADAM15 against AAA, such that its absence results in THBS (thrombospondin) 1 upregulation, dephosphorylation and activation of the cofilin pathway, and disruption of the actin filaments causing AoSMC dysfunction and loss in the aortic wall, leading to AAA formation.

MATERIALS AND METHODS

All data and materials supporting the findings of this study are available within this article and in the Data Supplement or from the corresponding authors upon reasonable request. The expanded Methods are available in the Data Supplement.

Human Aorta Specimens

Human AAA specimens were procured from patients undergoing open AAA surgery at the Northern Alberta Vascular Center/Grey Nuns Hospital (Edmonton, AB). Patients with aortic dissection or atherosclerosis were excluded. Control samples were nonaneurysmal healthy abdominal aortas obtained from donors through the Human Organ Procurement and Exchange Program at the University of Alberta Hospital. This study was approved by the Hearth Research Ethics Board and Covenant Health Research Center (Pro00089421). Clinical information regarding the human specimens is provided in Table I in the Data Supplement.

Experimental Animals and Alzet Pump Implantation

Male WT (wild type) and *Adam15*$^{-/-}$[15] mice received Ang II (1.5 mg/kg per day) or saline for 2 or 4 weeks by dorsal and subcutaneous implantation of Alzet micro-osmotic pumps (model 1002; Durect, Co) as before.[23] All experiments were performed according to the Animal Research: Reporting of In Vivo Experiments guidelines and in accordance with the guidelines of the University of Alberta Animal Care and Use Committee and the Canadian Council of Animal Care. Only male mice were used in this study since AAA is more prevalent in men.[12] However, it is critical to also understand the underlying mechanism of AAA in women as outlined in the scientific statement of the American Heart Association and the Arteriosclerosis, Thrombosis, and Vascular Biology Council,[24] but given the complexity that is added by the presence (or absence) of estrogen, a dedicated study to exploring the mechanism of AAA in women is warranted.

Telemetric Blood Pressure Measurement

The hypertensive effects of Ang II were measured in conscious and freely moving WT and *Adam15*$^{-/-}$ mice using telemetry as described.[23]

Ultrasound Aortic Imaging

Ultrasonic images of the abdominal aorta were obtained in mice anesthetized with 1.5% isoflurane, using the Vevo 3100 high-resolution imaging system (Visual Sonics, Toronto, Canada) as described previously.[25]

Macroscopic and Microscopic Imaging, Morphometric and Immunofluorescent Analyses of Mouse Aorta

At indicated time points, mice of either genotype were anesthetized and perfuse-fixed (formalin) as described.[26] The abdominal aorta was paraffin-embedded or frozen in optimal cutting temperature media. Verhoeff-Van Gieson, trichrome and Picrosirius red staining, and immunofluorescent staining for CD (cluster of differentiation) 68 (macrophage), Ly6G (lymphocyte antigen 6 complex locus G6D; neutrophil), αSMA (alpha-smooth muscle actin; SMC), 5-bromo-2′-deoxyuridine (BrDU) for proliferative cells, THBS1, and phosphorylated cofilin were performed as before.[27] F (filament)-actin and G (globular)-actin staining were performed on optimal cutting temperature–fixed sections as before.[28]

Ex Vivo Stress-Strain Assessment of the Abdominal Aorta

Aortic stress-strain relationship and aortic wall distensibility were assessed in the abdominal aorta of WT and *Adam15*$^{-/-}$ mice after 4 weeks of Ang II or saline infusion, using the DMT pin myography system (model P110; Danish Myo Technology, Denmark) according to the protocol provided by the manufacturing company (Danish Myo Technology).

Primary Human Aortic Endothelial Cell Culture, siRNA Transfection, and Permeability Assay

Primary human aortic ECs (HAoECs; PCS-100-011) were purchased from American Type Culture Collection and cultured as before.[27] HAoECs were used at passages 3 to 5 for the experiments described in this study. *Adam15* siRNA (Ambion; s13718, siRNA#16682) was used to knock down *Adam15* in HAoECs, and scrambled siRNA (Ambion; 4390844) was used as control. In vitro EC permeability assay was performed using a vascular permeability assay kit according to the manufacturer's instructions (EMD Millipore) as before.[27]

Primary Mouse AoSMC Culture

Primary AoSMCs were isolated from the abdominal aorta of 4-week-old male WT and *Adam15*$^{-/-}$ mice after removing the adventitia and the endothelial layer, as described previously.[27] For all experiments, AoSMCs were used at passages 3 to 5 to minimize phenotypic switching, which can occur with prolonged culture periods.

Protein and mRNA Extraction and Analyses

Flash-frozen abdominal aortas (below the diaphragm and above the iliac arteries) were freeze-crushed and suspended in tissue lysis buffer containing EDTA-free protease inhibitor cocktails (for protein extraction) or Trizol (for RNA extraction). Western blotting and gelatin zymography were performed as before.[25] β-actin or total protein (memcode-stained polyvinylidene fluoride membrane) were used as the loading control. The intensity of bands was quantified using the inbuilt ImageQuant TL software (version 7.0; GE Healthcare) and normalized to the corresponding loading control.

Total RNA was extracted from abdominal aorta of WT and *Adam15*$^{-/-}$ mice using TRIzol reagent (Invitrogen; 15596026) according to the manufacturer's protocol. Quantitative Taqman real-time polymerase chain reaction was performed using TaqMan primer-probe mix for each gene (Table II in the Data Supplement) and a LightCycler 480 II system (Roche) using the LightCycler 480 Probes Master kit (Roche; 04887301001). The reference gene 18S was used as the internal control.

Statistics

Statistical analyses were performed using IBM SPSS software (version 21). Normality of data distribution was determined by the Kolmogorov-Smirnov test. Comparisons between any 2 groups were performed with unpaired Student *t* test. Two-way ANOVA followed by LSD post hoc test was used to compare among multiple groups with 2 main factors (ADAM15 deficiency and Ang II infusion). Averaged values are presented as mean±SEM. For all in vitro experiments, the reported n value corresponds to the number of independent experiments. For in vivo data, n values refer to the number of mice used per experiment. Statistical significance was recognized at $P<0.05$.

RESULTS

Ang II Triggers AAA Formation in Mice Lacking ADAM15

Ang II is a well-known hormone that is upregulated in cardiovascular diseases. We investigated the role of ADAM15 in vascular pathogenesis in response to Ang II. After 4 weeks of Ang II infusion, *Adam15*$^{-/-}$ mice

developed AAA, whereas WT mice exhibited a uniform remodeling of the aorta with only 2.6% of mice (1 of 38) showing aortic aneurysm compared with 64.7% AAA incidents in *Adam15*$^{-/-}$ mice (22 of 34) including 5.8% rupture incidents, all of which occurred in the abdominal aorta (Figure 1Ai and 1Aii). Ultrasound analyses further confirmed a significantly greater increase in the systolic and diastolic diameter of the abdominal aorta, concomitant with a marked decrease in the aortic wall expansion index in *Adam15*$^{-/-}$-Ang II compared with WT-Ang II mice (Figure 1B and 1C). Consistent with the suppressed aortic expansion index detected in vivo, ex vivo compliance analysis of the abdominal aorta from WT and *Adam15*$^{-/-}$ mice revealed that 4 weeks of Ang II infusion markedly compromised aortic compliance in *Adam15*$^{-/-}$ compared with WT mice, as indicated by the upward shift in the stress-strain curve (Figure 1D). The difference between the saline-infused WT and *Adam15*$^{-/-}$ mice did not reach statistical significance (Figure 1D). Ang II infusion caused a significant rise in ADAM15 protein levels in the abdominal aorta of WT mice (Figure 1Ei and 1Eii), which could be an important compensatory mechanism that protected the WT mice from developing AAA, whereas mice lacking ADAM15 developed AAA. Hypertension can be a comorbidity for AAA. Daily and nightly blood pressure measurements in conscious mice over 4 weeks of Ang II infusion showed a significant but comparable hypertensive response in WT and *Adam15*$^{-/-}$ mice (Figure 1F; Figure I in the Data Supplement). Assessment of other ADAMs in the abdominal aorta showed that the Ang II–induced rise in *Adam9* was suppressed in *Adam15*$^{-/-}$ aortas, but the changes in *Adam10*, *Adam12*, and *Adam17* were comparable between WT and *Adam15*$^{-/-}$ mice (Figure IIA in the Data Supplement). Overall, *Adam15* deficiency increased susceptibility to AAA without altering the hypertensive response.

Adverse ECM Remodeling, Increased Inflammation, and Proteolytic Activities in Aneurysmal *Adam15*$^{-/-}$ Aorta

Histological assessment of the abdominal aorta in WT and *Adam15*$^{-/-}$ mice showed that consistent with the aneurysmal dilation, adverse remodeling of the aortic wall and the ECM was detected in *Adam15*$^{-/-}$-Ang II abdominal aorta. These included degradation of the elastin lamella (visualized by Verhoeff-Van Gieson staining), loss of medial SMCs, and excess adventitial collagen accumulation and outward remodeling of the aortic wall mainly due to excess collagen deposition (trichrome and Picrosirius red staining) in *Adam15*$^{-/-}$-Ang II compared with WT-Ang II mice (Figure 2A). As anticipated, Ang II increased mRNA expression of *Col Iα1* and *Col IIIα1* and *Eln* in the abdominal aorta of WT mice, whereas a greater increase in *Col Iα1* and markedly suppressed *Eln* levels were detected in *Adam15*$^{-/-}$-Ang II mice (Figure IIB in the Data Supplement).

One of the characteristics of aortic aneurysm is accumulation of inflammatory cells in the aortic wall.[3,27,29] Immunofluorescent staining revealed markedly greater accumulation of neutrophils and macrophages in the abdominal aorta of *Adam15*$^{-/-}$-Ang II compared with WT-Ang II aortas (Figure 2B). The increased aortic inflammation in *Adam15*$^{-/-}$-Ang II mice was also evident by the higher expression levels of inflammatory cytokines, *Tnfα*, *Il6*, and *Ccl2/Mcp1* in the abdominal aorta of these mice, while *Ilβ1* was elevated comparably in both genotypes following Ang II infusion (Figure 2C). Inflammatory cells such as macrophage and neutrophils can produce a number of MMPs, proteases that can mediate adverse aortic wall remodeling.[3,10,30] In vitro gelatin zymography showed a significantly greater increase in levels of MMP9 and cleaved MMP2 in the abdominal aorta from *Adam15*$^{-/-}$-Ang II compared with WT-Ang II mice (Figure 3A). In addition, MT1-MMP (membrane type 1 MMP)—a membrane-bound MMP and a potent collagenase—was significantly elevated in the abdominal aorta of *Adam15*$^{-/-}$-Ang II mice (Figure 3B). TIMPs are the only endogenous inhibitors of MMPs.[31] Our assessment of the 4 TIMPs (TIMP1–4) showed that TIMP1 was equally reduced in both genotypes following Ang II infusion, while TIMP2 was increased more in *Adam15*$^{-/-}$-Ang II, TIMP3 increased only in WT-Ang II, and TIMP4 remained unaltered (Figure 3C). TIMP2 and MT1-MMP collectively mediate the activation of pro-MMP2 to its cleaved form by forming a trimolecular complex.[31] As such, the rise in these two proteins could explain the higher levels of cleaved MMP2 in *Adam15*$^{-/-}$-Ang II abdominal aorta.

Cathepsins are a family of cysteine proteases produced by vascular SMCs and macrophages under inflammatory conditions that can mediate cell death and promote aneurysm development.[32,33] Given the heightened inflammation in *Adam15*$^{-/-}$-Ang II aortas, we assessed cathepsin levels as possible contributors to AAA in these mice. Ang II infusion increased cathepsin D and S and reduced cathepsin L and K to similar levels in the abdominal aorta of WT and *Adam15*$^{-/-}$ mice (Figure 3D). Therefore, the changes in these cathepsins cannot explain the more prevalent AAA incidents in *Adam15*$^{-/-}$ compared with WT mice, while the greater increases in key MMPs in *Adam15*$^{-/-}$-Ang II aortas could underlie the more severe adverse remodeling of the aortic ECM in this group.

ADAM15 Deficiency Preserves the Endothelial Barrier

Having found that inflammation is heightened in *Adam15*$^{-/-}$-Ang II aortas, we next examined whether this could be due to impaired EC barrier integrity in the

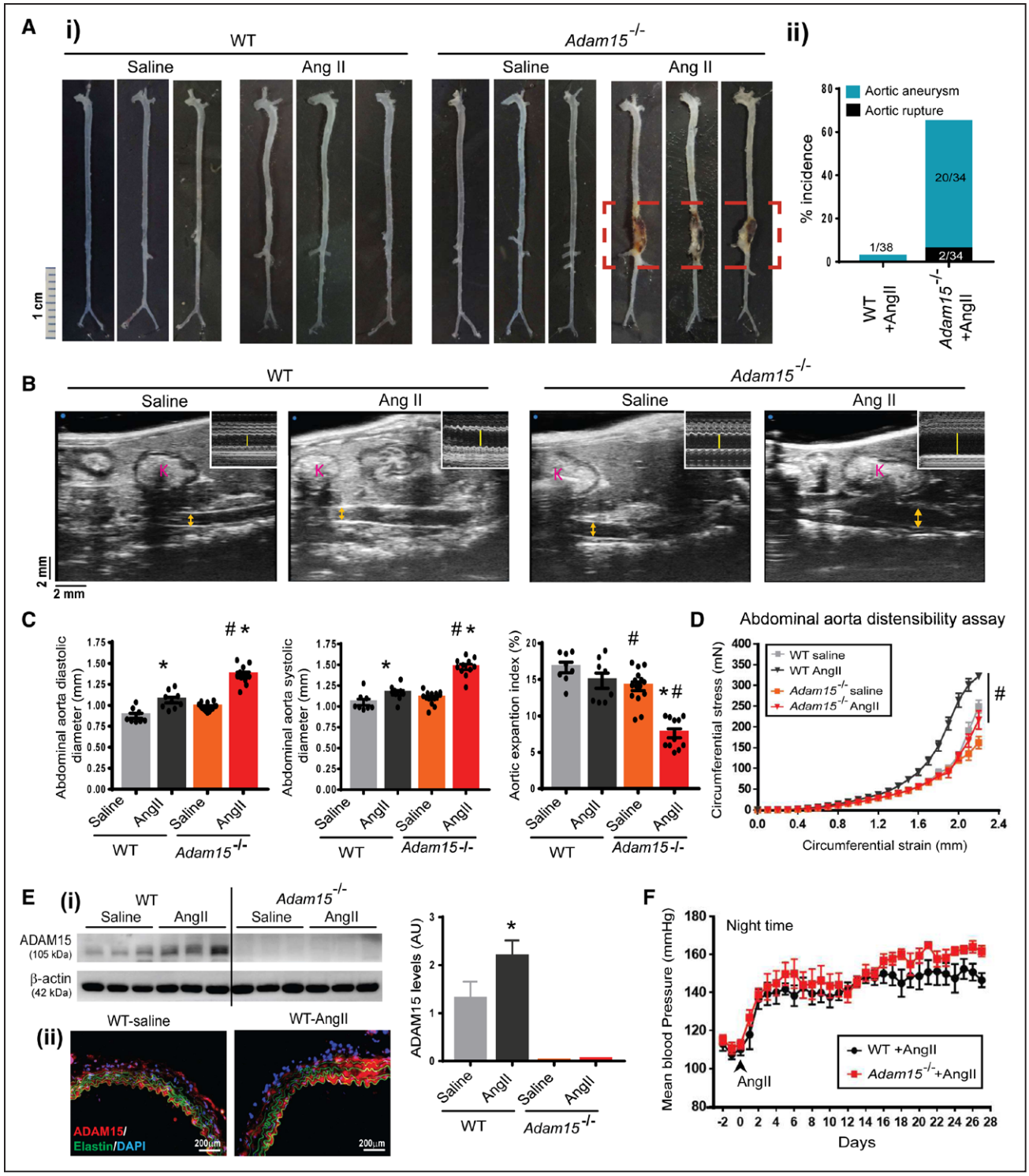

Figure 1. Loss of Adam15 triggers abdominal aortic aneurysm formation despite unaltered hypertensive response following 4 wk of Ang II (angiotensin II) infusion.

Ai, Representative pictures of whole aorta from WT (wild type) and *Adam15* knockout mice after 4 wk of Ang II or saline infusion. The red line box indicates the site of aneurysm. **Aii**, Demography of WT and *Adam15* knockout mice showing the frequency of abdominal aortic aneurysm formation and rupture in each genotype. **B**, Ultrasound images of the abdominal aorta (lumen is indicated in yellow line, K indicates kidney) in each genotype. Inset shows M-mode images of the lumen and the aortic wall. **C**, Average diastolic and systolic lumen diameters and expansion index of abdominal aorta in indicated groups (n=8–10/group). **D**, Aortic wall distensibility measured on abdominal aortic rings isolated from either genotype after 4 wk of Ang II or saline infusion (n=3–4/group). **Ei**, Representative immunoblot analysis for ADAM15 (a disintegrin and metalloproteinase 15) in the abdominal aorta from WT and Adam15 knockout mice after Ang II or saline infusion. **Eii**, Representative immunofluorescent images for ADAM15 staining (red), elastin autofluorescence (green), DAPI (4′,6-diamidino-2-phenylindole; blue) in abdominal aorta from WT mice. **F**, Mean arterial blood pressure recorded by telemetry before and after Ang II infusion. The arrow shows when Ang II Alzet pumps were implanted. Averaged values represent mean±SEM. **P*<0.05 vs corresponding saline; #*P*<0.05 vs corresponding WT group.

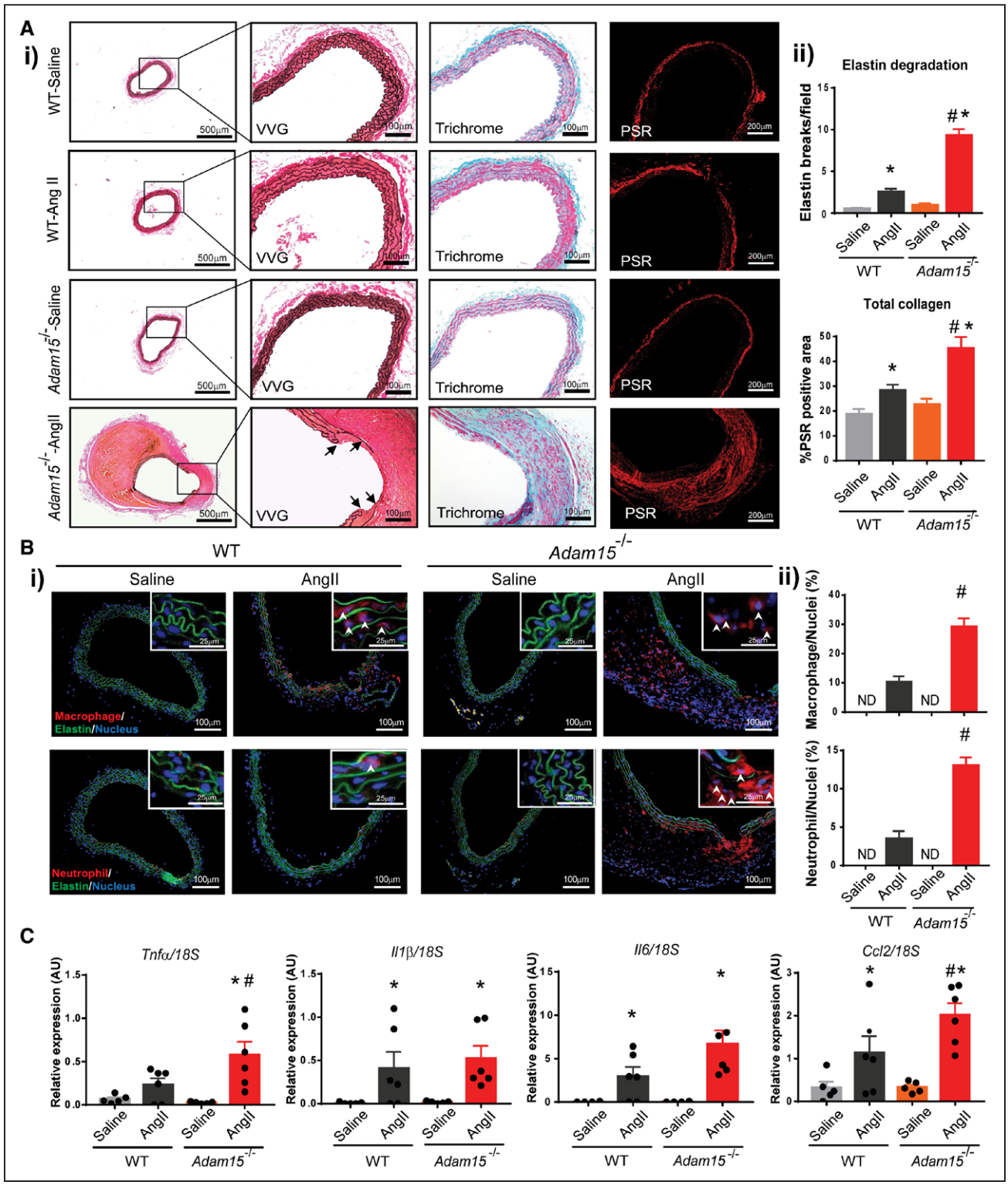

Figure 2. Abdominal aortic aneurysm in Adam15 knockout mice is associated with severe structural deterioration and inflammation in the aortic wall.

A, Representative full aortic cross section of abdominal aortas from either genotype after 4 wk of Ang II (angiotensin II) or saline infusion, stained for elastin (black; Verhoeff-Van Gieson [VVG]), collagen (blue) and smooth muscle cells (red/magenta; Gomori trichrome), and collagen (red; Picrosirius red [PSR]). Black arrows point to elastin breakage. **Aii**, Quantification of elastin breakage (VVG staining) and collagen content (PSR staining). **Bi**, Representative immunofluorescent staining for macrophage (CD [cluster of differentiation] 68^+ in red, **top**) and neutrophils ($Ly6G^+$ [lymphocyte antigen 6 complex locus G6D] in red, **bottom**) in the abdominal aorta after 2 wk of saline or Ang II infusion. Insets show higher magnification. **Bii**, Quantification of macrophage and neutrophil population in the aortic wall as a percentage of total cells in the area ($DAPI^+$; 4′,6-diamidino-2-phenylindole). **C**, Taqman mRNA expression for inflammatory cytokines in the abdominal aorta after 2 wk of Ang II infusion in the indicated groups. Values were normalized to internal loading control (18S). n=5 to 6/group. Averaged values represent mean±SEM. AU indicates arbitrary units; and ND, not detected. *P<0.05 vs corresponding saline group; #P<0.05 vs corresponding WT (wild type) group.

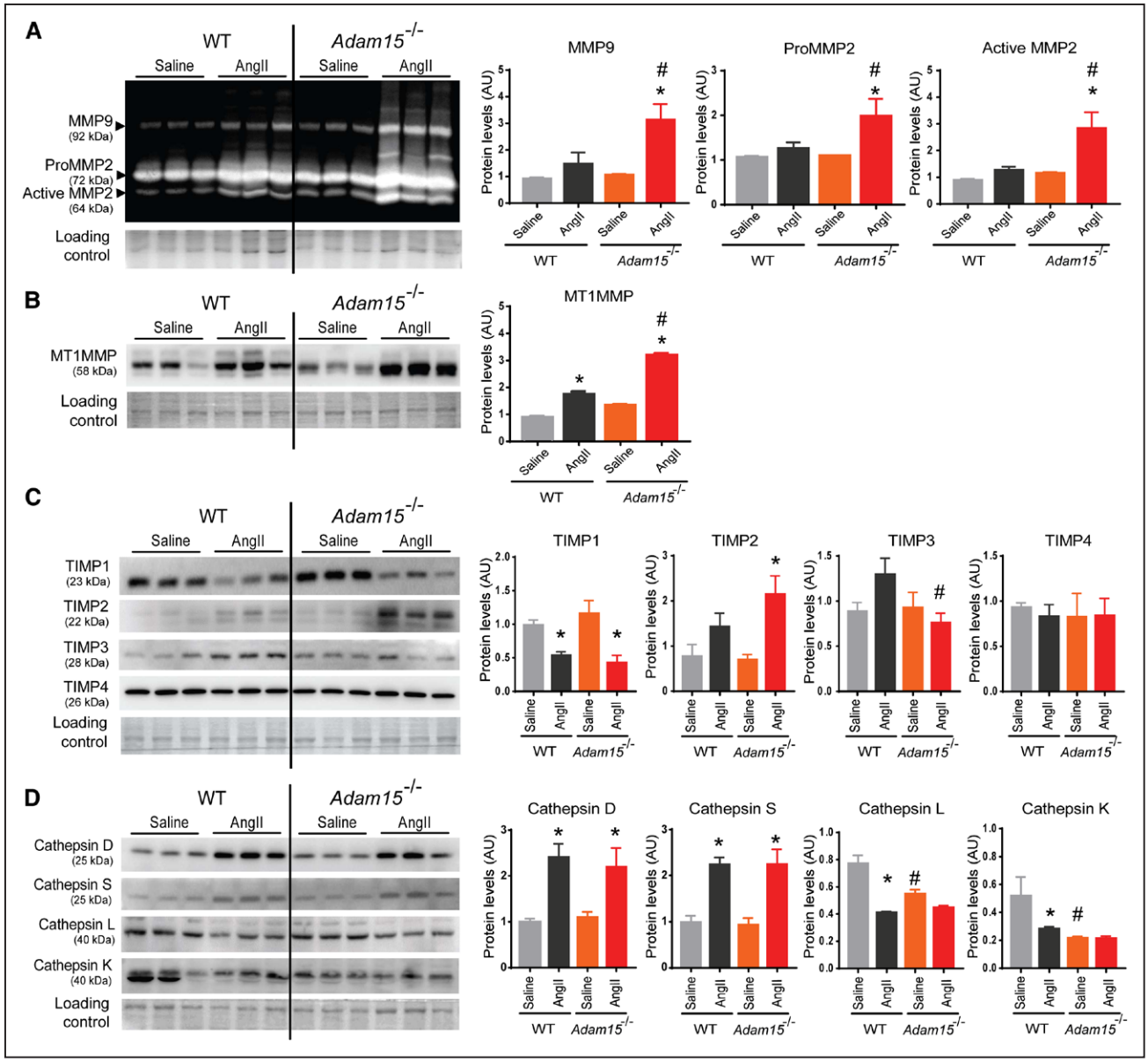

Figure 3. Ang II (angiotensin II) infusion triggered a greater increase in MMPs (matrix metalloproteinases), but not cathepsins, in the abdominal aorta of Adam15-deficient mice.

A, Representative in vitro gelatin zymography and averaged band intensities for MMP9, pro-MMP2, and MMP2 in indicated groups. Coomassie blue–stained gel was used as the protein loading control. n=6/group. **B**, Representative immunoblot and average protein levels for collagenase, MT1-MMP (membrane type 1 MMP; **C**), TIMPs (tissue inhibitor of metalloproteinases; TIMP1–4; **D**), and cathepsins D, S, L, and K. Values were normalized to loading control (Memcode-stained polyvinylidene fluoride membrane). Averaged values represent mean±SEM. AU indicates arbitrary unit. **P*<0.05 vs corresponding saline; #*P*<0.05 vs corresponding WT (wild type) group.

absence of *Adam15* facilitating infiltration of inflammatory cells into the subintimal tissue. We used HAoECs and siRNA (to knock down human *ADAM15*) to examine the impact of *ADAM15* loss in EC barrier integrity in response to Ang II. Immunoblotting confirmed that *ADAM15*-siRNA markedly reduced ADAM15 protein levels in HAoECs (Figure III in the Data Supplement). Immunofluorescent imaging showed that Ang II treatment (250 nM, 24 hours) reduced VE-cadherin (vascular endothelial cadherin)—a key adhesion molecule responsible for EC-EC connection—and increased F-actin stress fibers in *ADAM15*-intact HAoECs (+control siRNA). In contrast, *ADAM15* knockdown (+*ADAM15*-siRNA) preserved the presence of VE-cadherin in the cell membrane and at the EC-EC junctions with minimal stress fiber formation (Figure IIIA in the Data Supplement), also preserving another adhesion molecule, claudine 5 (Figure IIIB in the Data Supplement). To confirm that the observed changes in protein levels of adhesion molecules are reflected in endothelial permeability, we performed an in vitro permeability assay where HAoEC monolayers transfected with control siRNA or *ADAM15*-siRNA were treated with

Ang II. Ang II increased HAoEC permeability in *ADAM15*-intact (control) monolayer but to a significantly lower extent in *Adam15*$^{-/-}$ HAoEC monolayers (Figure IIIC in the Data Supplement). These data indicate that *Adam15* deficiency, in fact, preserves the endothelial barrier, and, therefore, increased endothelial permeability is not the mechanism responsible for the increased inflammation in *Adam15*$^{-/-}$-Ang II aortas.

Next, we examined whether *Adam15* deficiency impacts migration of HAoECs since this feature of ECs is critical for restoration of intimal integrity in a damaged artery. We used a 2-dimensional culture system to compare the rate of HAoEC migration to close a gap (scratch). Ang II increased the rate of EC migration in *Adam15*-intact HAoECs compared with the saline-treated group; however, *Adam15*$^{-/-}$ HAoECs showed a markedly reduced rate of migration significantly lower than the parallel *Adam15*-intact HAoECs group (Figure IIID in the Data Supplement). This impaired migratory ability corroborates a critical role of ADAM15 in EC migration, perhaps by weakening of the intercellular junctions to facilitate EC mobility. The suppressed EC mobility with *Adam15* deficiency could hinder the endothelial repair process subsequent to damage or cell death, which could lead to increased infiltration of inflammatory cells in *Adam15*$^{-/-}$-Ang II aortas.

SMC Dysfunction Underlies AAA in *Adam15*$^{-/-}$ Mice

Vascular SMCs play a critical role in the function and structural integrity of the aortic wall. SMC dysfunction promotes many vascular pathologies, while SMC damage or death can also serve as a proinflammatory signal. As such, we examined SMC survival and function in the aortic wall of WT and *Adam15*$^{-/-}$ mice. Immunostaining for SMC αSMA—the main SMC structural protein—showed a significant reduction in the abdominal aorta of *Adam15*$^{-/-}$-Ang II compared with WT-Ang II mice (Figure 4Ai and 4Aii). In addition, immunoblotting for SMC contractile proteins, SM-MHC11 (smooth muscle myosin heavy chain), SM22 (smooth muscle 22), and calponin, further showed that these proteins were markedly reduced in the abdominal aorta of *Adam15*$^{-/-}$-Ang II mice (Figure 4Bi and 4Bii).

Next, we investigated whether the reduced SMC density in the medial layer of the aorta is due to increased cell death or decreased proliferation. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining showed increased number of apoptotic cells in the abdominal aorta of *Adam15*$^{-/-}$-Ang II mice (Figure 4C), consistent with the elevated levels of cleaved caspase 3 (Figure 4Di and 4Dii) compared with WT-Ang II abdominal aorta. Autophagy marker LC3A/B was reduced in *Adam15*$^{-/-}$ aorta, therefore, eliminating autophagy as a cause of SMC loss in these mice (Figure 4D). SMC proliferation was assessed by staining for KI67—a protein that is expressed in proliferating but not in quiescent cells. Immunostaining for KI67 showed that Ang II significantly increased cell proliferation in WT but not in *Adam15*$^{-/-}$aortas (Figure 4E). Cell proliferation is associated with activation of a number of signaling factors including phosphorylation of ERK (extracellular signal-regulated kinase). Consistent with the KI67 staining data, the Ang II–induced rise in ERK1/2 phosphorylation was markedly greater in WT compared with *Adam15*$^{-/-}$ aortas (Figure 4F). Taken together, these data demonstrate that loss of *Adam15* leads to reduced SMC population in the aortic wall due to increased apoptosis and decreased proliferation, as well as their transformation to the less contractile phenotype.

THBS1 Is Upregulated in Aneurysmal *Adam15*$^{-/-}$ Aorta

To determine the underlying mechanism responsible for SMC loss and dysfunction in *Adam15*$^{-/-}$ -Ang II aorta, we investigated the contribution of signaling pathways that could target the actin filament remodeling in the AoSMCs. THBS1 is a key regulator of mechanotransduction in the aorta by regulating the actin filament dynamics in the SMCs.[34] THBSs are matricellular glycoproteins that can mediate a number of cellular functions.[35] Immunoblotting for the 4 THBSs showed that Ang II infusion increased THBS1 and THBS4 in the abdominal aorta from WT mice, while THBS1 (but not THBS4) levels increased to a markedly greater degree in *Adam15*$^{-/-}$-Ang II aorta (Figure 5A; Figure IVA in the Data Supplement). Although it has been reported that an increase in THBS1 in *Adam15*$^{-/-}$ mice was associated with increased TGFβ (transforming growth factor-beta) activity,[36] we found that activation of TGFβ (25-kDa band) and its downstream signaling pathway phospho-Smad2/3 was suppressed in Ang II–infused *Adam15*$^{-/-}$ mice (Figure IVB in the Data Supplement), which could underlie the decreased mRNA expression of elastin in the abdominal aorta in these mice (Figure IIB in the Data Supplement). Immunofluorescent staining for THBS1 showed that in WT-Ang II aorta, the increase in THBS1 was primarily localized to the intimal ECs, whereas in *Adam15*$^{-/-}$-Ang II aortas, high expression of THBS1 was found in the medial SMCs (Figure 5B), suggesting its contribution to SMC pathology in *Adam15*$^{-/-}$ aortas.

ADAMTSs (ADAMs with a THBS domain) are another family of extracellular proteases related to ADAMs but possess a THBS-binding site. Many functions of ADAMTSs have been linked to their THBS-binding ability,[37] while ADAMTS1 can also proteolytically process THBS1.[38] Ang II infusion reduced ADAMTS1 levels in aorta from both genotypes, increased ADAMTS4 only in WT, and did not alter ADAMTS2 or ADAMTS5 in either genotype (Figure

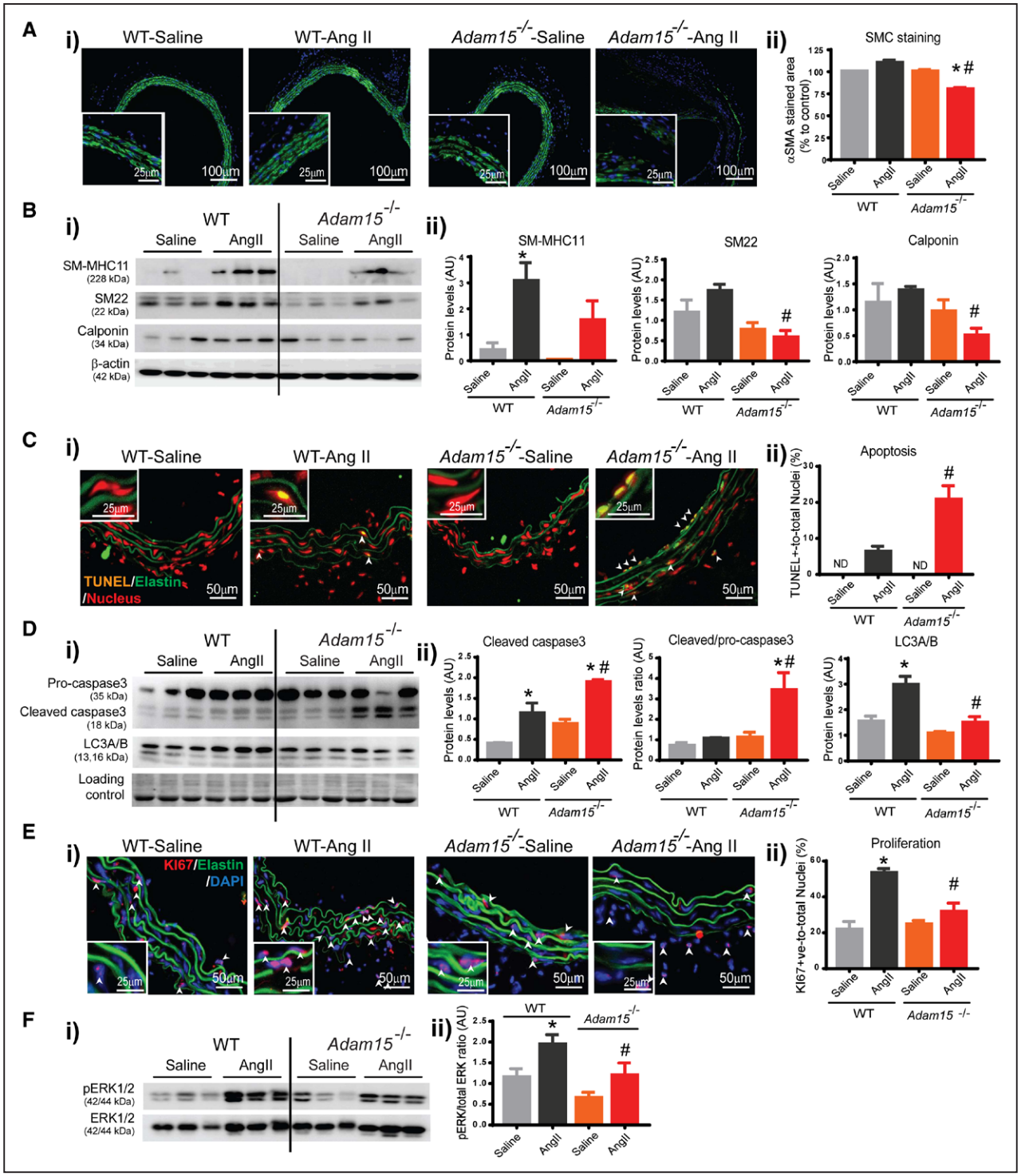

Figure 4. Absence of Adam15 promotes smooth muscle cell (SMC) apoptosis and suppresses proliferation in the abdominal aortic wall.

A, Representative immunofluorescent images (**Ai**) and quantification (**Aii**) of αSMA (alpha-smooth muscle actin; green) and DAPI (4′,6-diamidino-2-phenylindole) nuclear staining (blue) in WT (wild type) and *Adam15* knockout aorta. **B**, Representative immunoblots (**Bi**) and quantification (**Bii**) for smooth muscle cell markers, SM-MHC, SM22 (smooth muscle 22), and calponin. **C**, Representative fluorescent images (**Ci**) and quantification (**Cii**) of apoptotic cells in (TUNEL$^+$ cells, yellow, white arrows). Elastin autofluorescence appears green. **D**, Representative immunoblots (**Di**) and quantification (**Dii**) for molecular markers of apoptosis (pro- and cleaved caspase 3) and autophagy (autophagosome protein LC [light chain] 3A/B). **Ei**, Representative immunofluorescent staining for KI67 (red, marker of cell proliferation), and (**Eii**) quantification of percent KI67$^+$ nuclei (percentage of total nuclei). **F**, Representative immunoblots for total and phospho-ERK (extracellular signal-regulated kinase; **Fi**) and averaged phospho-to-total ratio (**Fii**). Insets, higher magnification. Immunoblot quantifications were normalized to loading control. Averaged values represent mean±SEM. AU indicates arbitrary units; and TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling. *P<0.05 vs corresponding saline; #P<0.05 vs corresponding WT.

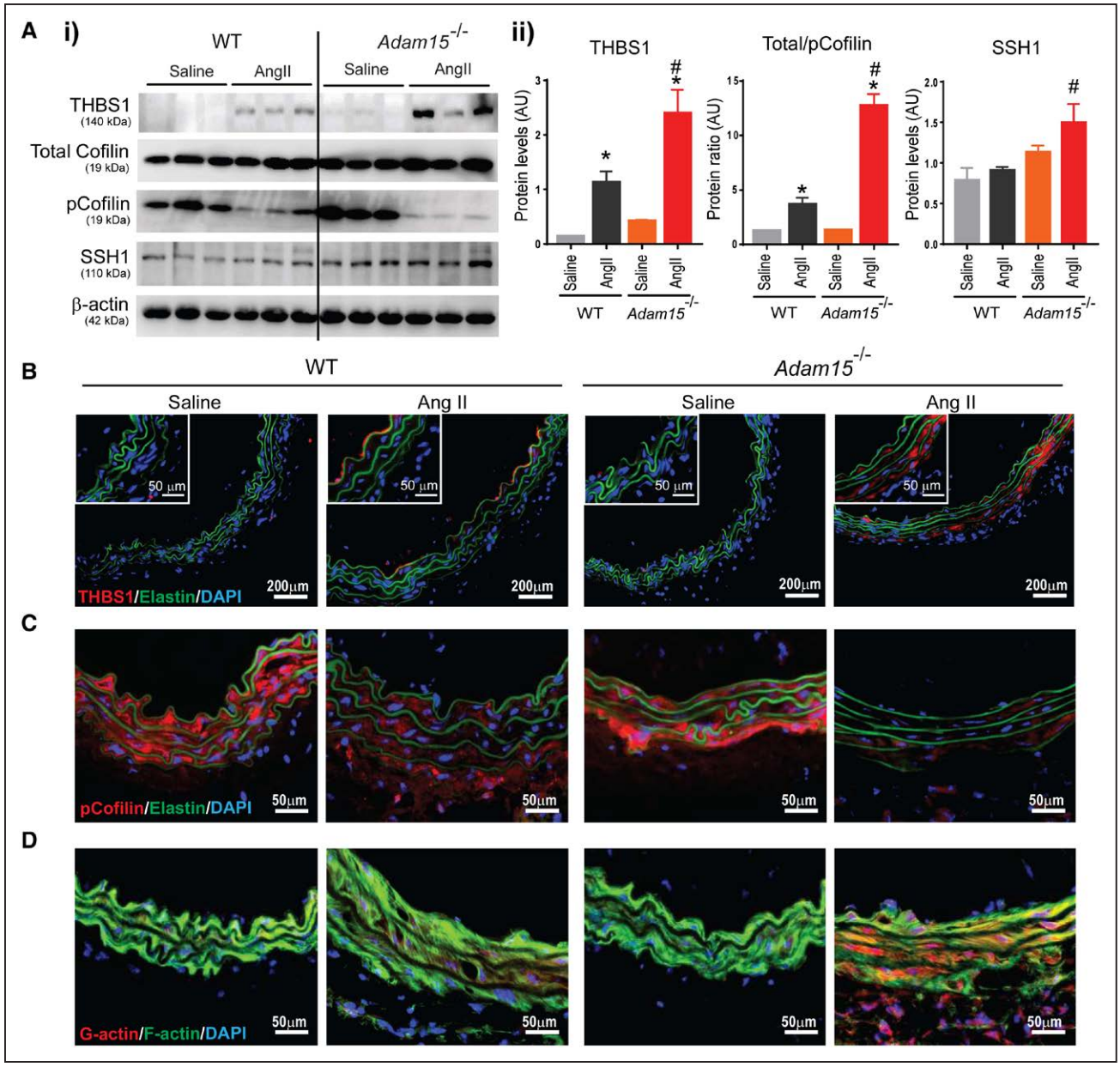

Figure 5. Ang II (angiotensin II) promotes thrombospondin-1 accumulation, cofilin dephosphorylation, and depolymerization of F-actin to G-actin in Adam15-deficient mice.

Ai, Representative immunoblots for THBS1 (thrombospondin-1), phosphorylated and total cofilin, and SSH1 (slingshot homolog 1 phosphatase) in abdominal aorta from WT (wild type) and *Adam15* knockout mice following 2 wk of Ang II or saline infusion. **Aii**, Averaged protein quantification, values normalized to β-actin. **B**, Representative immunofluorescent images for THBS1 staining (red), elastin autofluorescence (green). Insets, higher magnification. **C**, Representative immunofluorescent images for phosphorylated cofilin staining (red), elastin autofluorescence (green), DAPI (4′,6-diamidino-2-phenylindole) nuclear staining (blue). **D**, Representative immunofluorescent images for G-actin (actin monomer, red) and F-actin (fibrillar actin, phalloidin, green) in abdominal aorta from WT and *Adam15* knockout mice after 2 wk of Ang II or saline infusion. Averaged values represent mean±SEM. AU indicates arbitrary units. **P*<0.05 vs corresponding saline; #*P*<0.05 vs corresponding WT group.

IVB in the Data Supplement). ADAMTS4 levels are upregulated in human sporadic aneurysm,[39] and its deficiency was protective against aortic aneurysm induced by high fat and Ang II,[40] while ADAMTS1 and most ADAMTSs have been reported to be downregulated in AAA.[41] Therefore, the observed changes in ADAMTSs cannot explain the higher THBS1 levels, nor AAA formation in *Adam15*$^{-/-}$ mice.

Another factor that could contribute to SMC dysfunction is cofilin—an actin-binding protein that is activated upon dephosphorylation and plays an important role in actin polymerization.[42] We investigated whether cofilin could be involved in SMC dysfunction in *Adam15*$^{-/-}$ mice. The ratio of active-to-inactive cofilin (total-to-phosphorylated) increased following Ang II infusion in both genotypes; however, this increase was significantly greater

in *Adam15*$^{-/-}$-Ang II aortas (Figure 5Ai and 5Aii). This was consistent with a marked decrease in inactive cofilin (phosphorylated Cofilin) as detected by immunofluorescent staining of the abdominal aorta of *Adam15*$^{-/-}$-Ang II compared with WT-Ang II aortas (Figure 5C). Moreover, levels of SSH1 (slingshot homolog 1) protein, the cofilin phosphatase, were much greater in *Adam15*$^{-/-}$-Ang II aortas, which could mediate the higher dephosphorylation (activation) of cofilin in these aortas (Figure 5A). Cofilin, when active, promotes actin filament remodeling by depolymerizing F-actin filaments into G-actin monomers.[42–44] Immunofluorescent staining of the abdominal aortas for F-actin and G- actin showed a drastic increase in G-actin (red) compared with F-actin (green) levels in the SMCs of *Adam15*$^{-/-}$-Ang II compared with WT-Ang II aortas (Figure 5D). These data support the notion that increased cofilin activity in *Adam15*$^{-/-}$ aortas promotes adverse remodeling of the F-actin filaments to G-actin monomers, which could reduce contractile properties of the AoSMCs in these mice.

Adam15 Deficiency Suppresses Proliferation and Contractility of Primary AoSMC In Vitro

Having found that *Adam15*$^{-/-}$ mice exhibit reduced aortic compliance, impaired viability, and dysfunction of the medial SMCs in the aortic wall following Ang II infusion, we investigated the direct effects of *Adam15* loss on AoSMCs using a primary culture system in vitro. Treatment of WT and *Adam15*$^{-/-}$ AoSMCs with Ang II (or saline) showed that Ang II induced cell proliferation (assessed by BrdU incorporation) in WT but not in *Adam15*$^{-/-}$ AoSMCs (Figure 6Ai and 6Aii) but triggered apoptosis in *Adam15*$^{-/-}$ and not in WT AoSMCs (Figure 6Bi and 6Bii). Consistent with the reduced proliferation, phosphorylation of ERK1/2 and Akt (protein kinase B), important signaling pathways for cell growth and proliferation, was suppressed in *Adam15*$^{-/-}$- compared with WT-AoSMCs following Ang II treatment (Figure 6Ci and 6Cii). Despite the suppressed proliferation, *Adam15*$^{-/-}$ SMCs exhibited enhanced migration rate in the presence of Ang II (Figure 5A in the Data Supplement). The impact of *Adam15* deficiency on contractile properties of AoSMC was assessed by using an in vitro gel contraction assay. *Adam15*$^{-/-}$-AoSMCs exhibited reduced contractility at baseline and following Ang II treatment assessed as percentage decrease in gel surface area over 24 hours (Figure 6D). Consistent with this observation, levels of contractile SMC proteins SM22 and αSMC were significantly lower in *Adam15*$^{-/-}$-AoSMCs following Ang II treatment (Figure 6E). This decrease in contractile properties, however, was not associated with an increase in expression of the markers for SMC synthetic phenotypes, such as vimentin or fibroblast growth factor 2 (data not shown). Similar to our in vivo findings, Ang II treatment resulted in a marked rise in THBS1, total-to-phosphorylated cofilin, SSH1 levels (Figure 6E), and increased G-actin content (Figure VB in the Data Supplement) in *Adam15*$^{-/-}$ compared with parallel WT- AoSMCs. These in vitro data are in full agreement with our in vivo findings supporting the notion that *Adam15* deficiency impairs SMC proliferation, survival, and contractility.

THBS1 Is the Key Mediator for the Impaired AoSMC Survival and Function in the Absence of *Adam15*

Having found that Ang II triggers a greater increase in THBS1 levels in *Adam15*$^{-/-}$ abdominal aorta in vivo and in AoSMCs in vitro, and that this was associated with markedly impaired AoSMC viability and function, we asked whether THBS1 is the driving force for these adverse events in *Adam15*$^{-/-}$ AoSMCs. Primary culture of WT and *Adam15*$^{-/-}$ AoSMCs was treated with rhTHBS1 (recombinant THBS1; 2 μg/mL), and subsequently cell proliferation, apoptosis, and the relevant signaling pathways were examined. rhTHBS1 did not markedly impact AoSMC proliferation (Figure 7A) but increased apoptosis in WT and to a greater extent in *Adam15*$^{-/-}$ AoSMCs (Figure 7B) and decreased contraction more strongly in *Adam15*$^{-/-}$-AoSMCs (Figure VC in the Data Supplement), although the decrease in SM22 protein levels appears to be the result of *Adam15* deficiency (Figure 7C). The increased apoptosis could be through activation of the integrin-αvβ1-CD47 complex as we observed elevated integrin-αv and integrin-β1 levels in Ang II–infused *Adam15*$^{-/-}$ aorta (Figure VI in the Data Supplement), and this mechanism has been linked to THBS1-mediated SMC function and apoptosis.[45,46] Cofilin activity (total-to-phosphorylated ratio) was increased, whereas cell proliferation pathways (ERK1/2 and Akt) were suppressed in *Adam15*$^{-/-}$-AoSMCs following THBS1 treatment (Figure 7C). rhTHBS1 did not increase cofilin activity in WT-AoSMCs, suggesting that ADAM15 deficiency may be a requirement for THBS1-mediated cofilin activation and the subsequent depolymerization of F-actin to G-actin in AoSMCs.

AAA Specimens From Patients Show Reduced ADAM15 and Elevated THBS1 Levels

Abdominal aorta specimens from patients with AAA (male, nonatherosclerotic, nonhypertensive) were analyzed for ECM remodeling, medial SMC density, and expression of ADAM15 and THBS1. Human AAA specimens showed fragmented elastin fibers, reduced SMCs density (αSMA staining), reduced ADAM15 and increased THBS1 levels compared with nonaneurysmal control aortic specimens (male, nonatherosclerotic, nonhypertensive; Figure 8A). These observations are consistent with the changes we observed in our mouse model of AAA, supporting the link

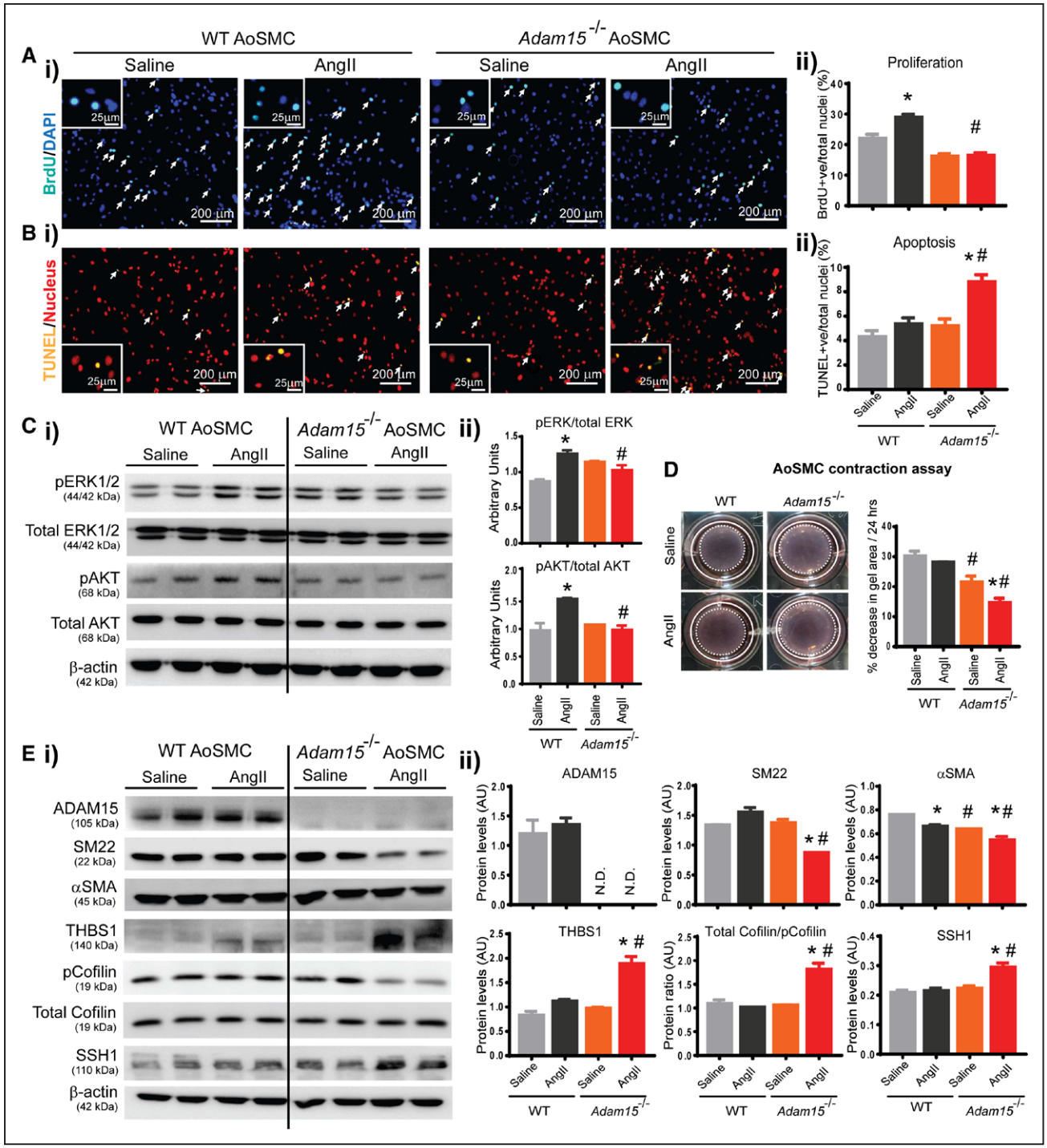

Figure 6. Absence of Adam15 reduces proliferation and contractile properties of primary aortic smooth muscle cells (AoSMCs). **Ai**, Representative immunofluorescent images for 5-bromo-2′-deoxyuridine (BrdU)-positive primary AoSMCs (green) following saline or Ang II (angiotensin II) treatment (250 nm/L, 24 h; DAPI [4′,6-diamidino-2-phenylindole] nuclear staining, blue). Inset, higher magnification. **Aii**, Averaged quantification of BrdU-positive cells as percentage of total cells. **Bi**, Representative terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining images of primary AoSMCs following saline or Ang II treatment (TUNEL$^+$, yellow). Insets, higher magnification. **Aii**, Averaged quantification of TUNEL-positive cells as percentage of total cells. **C**, Representative immunoblots (**Ci**) and quantification (**Cii**) for phosphorylated and total ERK (extracellular signal-regulated kinase), AKT (protein kinase B) from WT (wild type) and *Adam15*-deficient primary AoSMCs treated with saline or Ang II (250 nm/L, 24 h). **D**, Representative images of gel contraction assay for AoSMCs treated with saline or Ang II, and averaged percentage reduction in the gel area at 24 h (indicated with dotted line) as compared with zero hours (indicated with solid line). **E**, Representative immunoblots (**Ei**) and averaged quantification (**Eii**) for ADAM15 (a disintegrin and metalloproteinase 15), SM22α (smooth muscle 22α), αSMA (alpha-smooth muscle actin), THBS1 (thrombospondin-1), phosphorylated and total cofilin, and SSH1 (slingshot homolog 1). Values normalized to β-actin. n=3 to 4 mice/genotype/culture; at least 2 independent cultures. Average values represent mean±SEM. AU indicates arbitrary units; and ND, not detected. *P<0.05 vs saline; #P<0.05 vs corresponding WT.

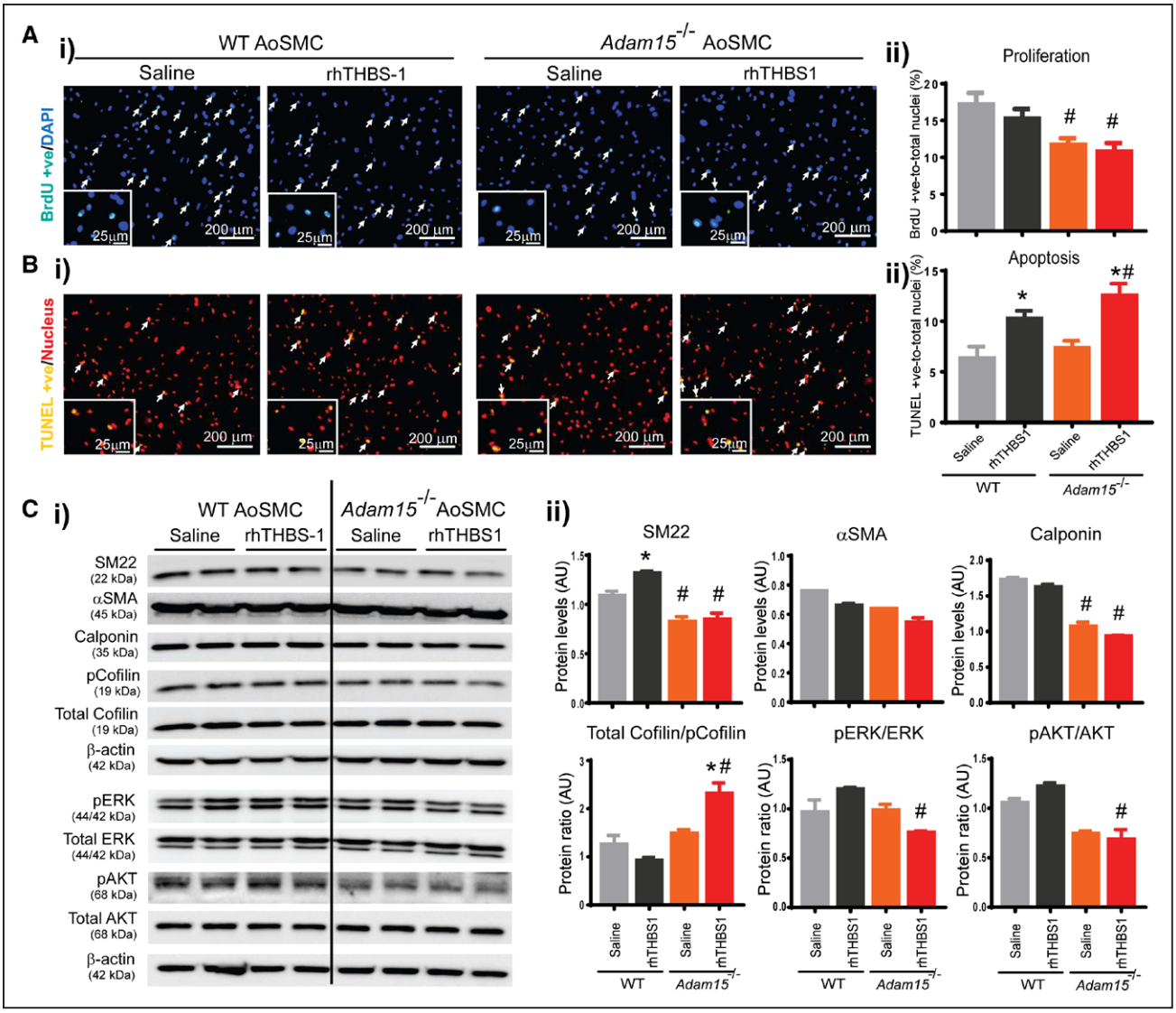

Figure 7. rhTHBS1 (recombinant human thrombospondin-1) reduces proliferation and suppresses contractile properties of primary aortic smooth muscle cells (AoSMC).

Ai, Representative 5-bromo-2′-deoxyuridine (BrDU) staining of primary mouse AoSMCs of either genotype treated with saline or rhTHBS1 (2 mg/mL, 24 h). Inset, higher magnification. **Aii**, Averaged quantification of BrdU-positive to total cells. **Bi**, Representative terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining images (TUNEL+, yellow) following treatment with saline or rhTHBS1. Inset, higher magnification. **Bii**, Averaged quantification of TUNEL-positive to total cells. **C**, Representative immunoblots (**Ci**) and averaged quantification (**Cii**) for SM22 (smooth muscle 22), αSMA (alpha-smooth muscle actin), calponin, phosphorylated and total cofilin, phospho- and total ERK (extracellular signal-regulated kinase), and phospho- and total AKT (protein kinase B). Values were normalized with to β-actin. n=3 to 4 mice per genotype per isolation and culture; at least 2 independent cultures per experiment. Averaged values represent mean±SEM. **P*<0.05 vs saline; #*P*<0.05 vs corresponding WT (wild type).

between ADAM15 loss and rise of THBS1 as a contributing mechanism to AAA formation.

DISCUSSION

AAA remains an important health risk as it is an asymptomatic disease, but if untreated, can lead to aortic rupture and death. Most importantly, there is no medical treatment available for AAA other than surgical repair. Medications that are commonly used for AAA patients (eg, β-blockers, ACE [angiotensin-converting enzyme] inhibitors, and statins) help reduce overall cardiovascular risks but do not reverse aneurysm development nor reduce its growth.[47,48] AAA is a multifactorial disease and involves a number of cellular and molecular events. Given the obvious structural destruction of the aortic wall, the role of proteases such as MMPs and cathepsins has been examined in different AAA models[11]; however, fewer studies have investigated the role of ADAMs in this disease. *Adam10* inhibition prevented AAA in a hyperlipidemia model in *Apoe*$^{-/-}$ mice.[49] Cell-specific loss of *Adam17* has been shown to be protective against TAA[27]

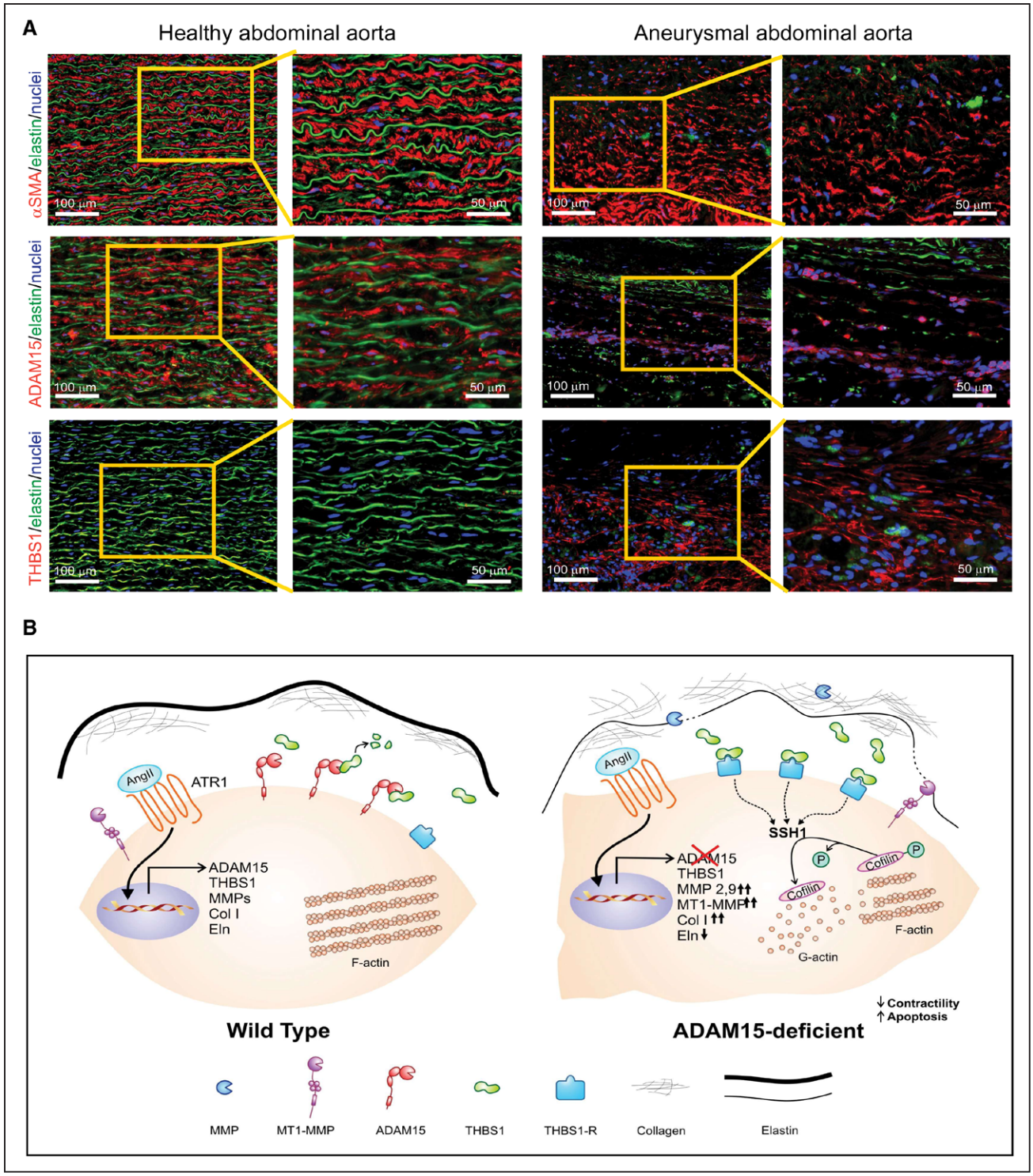

Figure 8. Human aneurysmal aortas show reduced ADAM15 (a disintegrin and metalloproteinase 15) and αSMA but increased levels of THBS1 (thrombospondin-1) compared with healthy human aortas.
A, Representative immunofluorescent images for αSMA (alpha-smooth muscle actin), ADAM15, and THBS1. Elastin appears as green autofluorescence. The boxed sections on the images are shown at higher magnification on the right. n=3 aortas (patients)/group. **B**, Schematic of how ADAM15 loss can lead to smooth muscle cell dysfunction and loss.

and AAA.[50] ADAM15 is highly expressed in the vasculature,[14,15] but its role in aortic aneurysm had not been explored. We found that in mice receiving regular chow diet, Ang II increases ADAM15 levels in the abdominal aorta. Mice lacking ADAM15 develop AAA following 4 weeks of Ang II infusion. Similarly, AAA specimens from patients exhibit a marked reduction in ADAM15 levels in the aortic wall. Therefore, ADAM15 could play

a protective role against AAA formation since its loss or decrease is associated with AAA. Through detailed in vivo and in vitro cellular and molecular analyses, we identified increased apoptosis, reduced proliferation, and reduced contractile properties of the AoSMCs as the primary mechanism underlying AAA in *Adam15*$^{-/-}$ mice, mediated mainly through upregulation of THBS1, activation (dephosphorylation) of cofilin, and depolymerization of F-actin filaments in SMCs (Figure 8B).

THBS1 is a matricellular protein that can regulate cell proliferation and apoptosis.[35,51,52] THBS1 levels are elevated in the plasma and aorta of patients with aortic dissection, along with markers of inflammation and SMC apoptosis.[53,54] Mice lacking THBS1 exhibit reduced AAA severity in different experimental models of AAA.[34,54] THBS1 can be proteolytically processed by ADAMTS1[38]; however, ADAMTS1 does not appear to be an important player in AAA,[41] and we found it to be decreased in the AAA tissues as also reported by others.[41] We found that Ang II upregulates THBS1, as also reported by others,[55,56] as well as ADAM15. ADAM15 could proteolytically process THBS1 or bind to THBS1, preventing it from activating its receptors, and as such, the absence of ADAM15 will result in highly elevated THBS1 levels. The marked rise in THBS1 levels in *Adam15*$^{-/-}$-Ang II mice was concomitant with increased inflammation, apoptosis, reduced proliferation, and contractile properties of AoSMCs in the abdominal aorta. Cofilin is an actin depolymerizing factor that has been reported to contribute to thoracic aortic aneurysm formation in *Fibulin 4-knockout mice.*[34] THBS1 can interact with about 12 receptors identified to date, including integrins, CD36 and CD47. In a mouse model of thoracic aortic aneurysm, CD36 and CD47 were found to not be the receptors for THBS1 function as deletion of these receptors did not reduce the severity of thoracic aortic aneurysm.[34] It is plausible that given the diversity of THBS1 receptors, the absence of one receptor is compensated for by other receptors. In *Adam15*$^{-/-}$ primary AoSMCs, rhTHBS1 (in the absence of Ang II) was sufficient to induce cofilin activation (dephosphorylation) and depolymerization of F-actin filaments, with suppressed contractility of these SMCs. THBS1 binds to integrin $\alpha v\beta 1$ to form focal adhesion, subsequently mediating nuclear shuttling of YAP (Yes-associated protein), thereby linking mechanotransduction to vascular remodeling.[45] THBS1-mediated apoptosis of SMCs could be through activation of the integrin complex, through association with CD47, as reported in fibroblasts,[46] or other membrane-associated proteins. AoSMCs are critical for renewal of the aortic ECM proteins and for the aortic contractile function. Therefore, impaired AoSMC contractility,[57,58] amplified by apoptotic loss, would be key contributor to aortic aneurysm formation. The observation that rhTHBS1 recapitulated the impairments in AoSMCs as observed in *Adam15*$^{-/-}$ aneurysmal aortas provides evidence for an important role of THBS1 in the absence of *Adam15* in causing AAA.

ADAM15 has been reported to mediate hyperpermeability of the endothelial barrier and that its loss could limit inflammation.[16,19] *Adam15*$^{-/-}$-Ang II mice exhibited increased aortic inflammation despite the preserved endothelial barrier in *Adam15*$^{-/-}$ ECs. We further found that *Adam15* deficiency impairs endothelial migratory ability that is essential in repairing the EC barrier. Moreover, THBS1 has been reported to trigger vascular inflammation by promoting adhesion and migration of mononuclear cells in murine models of AAA,[54] while apoptotic SMCs could serve as chemoattractant to signal migration of the inflammatory cells. Therefore, the increased THBS1, impaired EC migratory ability, and increased SMC apoptosis could collectively explain the heightened inflammation in *Adam15*$^{-/-}$ aorta.

In summary, our study provides strong in vivo and in vitro evidence that ADAM15 is a key player in AAA pathology. A decrease or loss of ADAM15 triggers impaired function and loss of SMCs leading to adverse aortic remodeling and AAA formation. These data provide novel insight into the critical role of ADAM15 in the molecular mechanism of aortic aneurysm—a potentially lethal disease that remains without effective medical therapy—and toward discovering effective therapeutic targets to prevent disease progression.

ARTICLE INFORMATION

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Disclosures

None.

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