Molecular Medicine

Inhibition of KLF5–Myo9b–RhoA Pathway–Mediated Podosome Formation in Macrophages Ameliorates Abdominal Aortic Aneurysm

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Rationale: Abdominal aortic aneurysms (AAAs) are characterized by pathological remodeling of the aortic wall. Although both increased Krüppel-like factor 5 (KLF5) expression and macrophage infiltration have been implicated in vascular remodeling, the role of KLF5 in macrophage infiltration and AAA formation remains unclear.

Objective: To determine the role of KLF5 in AAA formation and macrophage infiltration into AAAs.

Methods and Results: KLF5 expression was significantly increased in human AAA tissues and in 2 mouse models of experimental AAA. Moreover, in myeloid-specific Klf5 knockout mice (myeKlf5−/− mice), macrophage infiltration, medial smooth muscle cell loss, elastin degradation, and AAA formation were markedly decreased. In cell migration and time-lapse imaging analyses, the migration of murine myeKlf5−/− macrophages was impaired, and in luciferase reporter assays, KLF5 activated Myo9b (myosin IXB) transcription by direct binding to the Myo9b promoter. In subsequent communostaining studies, Myo9b was colocalized with filamentous actin, cortactin, vinculin, and Tks5 in the podosomes of phorbol 12,13-dibutyrate–treated macrophages, indicating that Myo9b participates in podosome formation. Gain- and loss-of-function experiments showed that KLF5 promoted podosome formation in macrophages by upregulating Myo9b expression. Furthermore, RhoA-GTP levels increased after KLF5 knockdown in macrophages, suggesting that KLF5 lies upstream of RhoA signaling. Finally, Myo9b expression was increased in human AAA tissues, located in macrophages, and positively correlated with AAA size.

Conclusions: These data are the first to indicate that KLF5-dependent regulation of Myo9b/RhoA is required for podosome formation and macrophage migration during AAA formation, warranting consideration of the KLF5–Myo9b–RhoA pathway as a therapeutic target for AAA treatment. (Circ Res. 2017;120:799-815. DOI: 10.1161/CIRCRESAHA.116.310367.)

Key Words: aneurysm ■ KLF5 ■ macrophage ■ motility ■ Myo9b ■ myosin ■ podosome ■ RhoA

Abdominal aortic aneurysm (AAA) is a chronic inflammatory disease characterized by the remodeling of the aortic wall, and it frequently leads to high morbidity and mortality because of vascular dissection and rupture.1 Although AAA formation is a multifactorial process involving the infiltration of macrophages, release of proinflammatory cytokines and proteases, elastin breakdown, vascular smooth muscle cell (VSMC) apoptosis, and increased collagen turnover, macrophages are essential contributors to the pathogenesis of AAAs.2,3 Accordingly, monocytes/macrophages are reportedly activated by chronic inflammatory states, including atherosclerosis and oxidative stress, and by angiotensin II (Ang II) and inflammatory cytokines. Subsequently, macrophages infiltrate vessel walls, release proteases such as elastase, matrix metalloproteinase-12 (MMP-12) and metalloproteinases, and degrade extracellular matrix components such as collagen and elastin.4,5 Simultaneously, infiltrating macrophages secrete inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon-γ, interleukin-1β, and interleukin-6, into the media and adventitia of aneurysmal vessels, thereby, exacerbating inflammatory responses.6

Macrophage infiltration into vessel walls requires highly coordinated reorganization of actin cytoskeletal structures to create membrane protrusions called podosomes.7 Human and murine podosomes contain many of the structural components...
and signaling proteins that are commonly found in focal adhesions, including cortactin, five SH3 domains (Tks5), vinculin, paxillin, and talin. Podosomes appear as small dots (punctate staining) with a life span of 2 to 10 minutes. However, cytokine exposure promotes the accumulation of podosomes in ring-like structures called rosettes, which can last for hours, leading to sustained extracellular matrix digestion. In addition, podosomes contain several signaling proteins, includingSrc, FAK (focal adhesion kinase), PI(3)-kinase (phosphoinositide-3 kinase), GAP (p190Rho GTPase activating proteins), and Cdc42 (cell division cycle 42). Among these, Rho regulation has been shown to be a key intermediate in podosome formation. However, there is currently little understanding of the mechanism of macrophage migration in the context of podosome formation.

Myo9b (myosin IXB) is a member of the myosin class IX family and is a unique actin-based motor protein that contains a RhoGAP domain, which, like other RhoGAPs, is inhibitory to Rho signaling. Myo9b was reportedly localized with dynamic filamentous actin (F-actin) at the extending cell front and was shown to regulate Rho signaling and subsequent cell polarization and migration. Although Myo9b is expressed in osteoclasts and acts as a critical regulator of podosome patterning and osteoclast function, relationships between Myo9b and aneurysm development remain unknown.

Kruppel-like factor 5 (KLF5) is a DNA-binding transcriptional regulator that regulates several cellular processes, including development, differentiation, proliferation, and apoptosis. In the cardiovascular system, KLF5 is a target for Ang II signaling and is an essential regulator of cardiovascular remodeling. Although KLF5 is highly expressed in large and giant unruptured cerebral aneurysms, its specific contribution to podosome formation and macrophage infiltration during AAA formation has not been investigated.

In the present study, we determined whether KLF5 modulates macrophage infiltration during AAA formation by regulating podosome formation.

**Methods**

**Human Tissue Harvest**

AAA tissue specimens were obtained in the operating room from 11 male patients undergoing elective open AAA repair. Nonaneurysmal infrarenal aortic wall tissue specimens were also obtained from organ donors to serve as nonaneurysmal controls (n=7). Each of the surgical patients gave informed signed consent before donating tissue. All tissue specimens were taken after a protocol approved by the Human Tissue Research Committee of Hebei Medical University. One portion of each aortic wall specimen was fixed overnight in 10% neutral buffered formalin and processed for routine embedding in paraffin. An adjacent portion was snap-frozen in liquid nitrogen, stored at −80°C, and subsequently used for protein and nucleic acid extraction.

**Animal Experiments and Lesion Characterization**

All male mice were housed and handled according to the guidelines of the local Animal Care and Use Committee at Hebei Medical University. Myeloid-specific Klf5 knockout mice (myeKlf5−/− mice) were derived from breedings of floxed Klf5 mice, generously provided by Dr Huajing Wan, with heterozygous LysM-cre mice.
Data Supplement.

were evaluated in a minimum of 3 separate wells per experiment. The wild-type (WT) and myeKlf5 −/− macrophages were grown on cell culture inserts and incubated with 4% paraformaldehyde for 10 minutes. Proximity ligation assay was performed using the Rabbit PLUS and Mouse MINUS Duolink in situ proximity ligation assay kits with anti-Myo9b (mouse) and anti-cortactin (rabbit; Olink Bioscience, Uppsala, Sweden) according to the manufacturer’s protocol. Subsequently, slides were dehydrated, air-dried, and embedded in 4',6-diamidino-2-phenylindole–containing mounting medium.

Histology
Mice were anesthetized, euthanized, and perfused before the collection of aortas as described in Methods in the online-only Data Supplement, which also contains a detailed description of cell and tissue staining and visualization procedures.

Cell Culture
Bone marrow–derived macrophages and mouse VSMC, 293A cells, and mouse RAW 264.7 cells were cultured and in vitro CaPO4 treatment of smooth muscle cell (SMC) performed as detailed in Methods in the online-only Data Supplement.

Adenovirus Expression Vector and Plasmid Constructs
The expression plasmids of KLF5 were created by the placement of mouse KLF5 cDNAs into the pEGFP-C2 vector. The 5′ regulatory region of mouse Myo9b (~2000 to +1 bp) was amplified by PCR and cloned into the pGL3-Basic vector (Promega) to generate the Myo9b promoter–reporter pGL3-Myo9b-luc. Truncated Myo9b luciferase reporters were generated by cloning the −1164, −1059, −547, −382, and 287 to +1 regions of the Myo9b promoter into pGL3. Adenoviruses encoding Klf5 and control (Ad-null) wereentrusted to Invitrogen.

Small Interfering RNA Transfection
Small interfering RNAs (siRNAs) targeting mouse Klf5 and Myo9b were designed and synthesized by GenePharma (Shanghai, China). Nonspecific siRNA was purchased from Santa Cruz Biotechnology. Transfection was performed using Lipofectamine reagent (Invitrogen) following the manufacturer’s instructions. Twenty-hours after transfection, mouse RAW 264.7 cells were treated with TNF-α (10 ng/mL). Cells were then harvested and lysed for Western blotting.

Luciferase Assay for Myo9b Promoter Activity
Human embryonic kidney 293A cells were maintained as previously described. Cells were seeded into each well of a 24-well plate and grown for 24 hours prior to transfection with reporter plasmids and the control pTK-RL (Renilla luciferase reporter) plasmid. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Luciferase assays were performed after 24 hours using a dual luciferase assay kit (Promega). Specific promoter activity was expressed as the relative ratio of firefly luciferase activity to Renilla luciferase activity. All promoter constructs were evaluated in a minimum of 3 separate wells per experiment.

Cell Immunofluorescence, Isolation of RNA, and Quantitative Real-Time PCR, Western Blot Analysis, Oligonucleotide Pull-Down Assay, and Determination of the Amounts of Active Rho, Cdc42, and Rac Proteins
Assessment of macrophage morphology, expression level of mRNA and protein, and determination of the amounts of active Rho, Cdc42, and Rac proteins are detailed in Methods in the online-only Data Supplement.

Proximity Ligation Assay
The wild-type (WT) and myeKlf5−/− macrophages were grown on cell culture inserts and incubated with 4% paraformaldehyde for 10 minutes. Proximity ligation assay was performed using the Rabbit PLUS and Mouse MINUS Duolink in situ proximity ligation assay kits with anti-Myo9b (mouse) and anti-cortactin (rabbit; Olink Bioscience, Uppsala, Sweden) according to the manufacturer’s protocol. Subsequently, slides were dehydrated, air-dried, and embedded in 4',6-diamidino-2-phenylindole–containing mounting medium.

Fluorescence was detected using a confocal microscopy (DM6000 CFS, Leica).

Migration and Invasion Assays, Scanning Electron Microscopy, and Time-Lapse Experiments
Assessment of macrophage motility is detailed in Methods in the online-only Data Supplement.

Analysis of Microarray Data
For microarray analysis, total RNA was extracted by using TRIzol extraction method and a NucleoSpin RNA II kit (Macherey Nagel, Duren, Germany) from WT (n=3) and myeKlf5−/− (n=3) mouse aortas at day 14. Total RNA from each sample was quantified by the NanoDrop ND-1000, and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Total RNA of each sample was used for labeling and array hybridization as described in the following steps: (1) reverse transcription with Invitrogen Superscript ds-cDNA synthesis kit; (2) ds-cDNA labeling with NimbleGen one-color DNA labeling kit; (3) Array hybridization using the NimbleGen Hybridization System and followed by washing with the NimbleGen wash buffer kit, and (4) the arrays were scanned by the Agilent Scanner G2505C (KangChen Bio-tech, Shanghai, People’s Republic of China). Expression data were normalized through the Robust Multichip Average algorithm included in the NimbleScan software. All gene-level files were imported into Agilent GeneSpring GX software (version 12.1) for further analysis. Genes in 6 samples have values greater than or equal to lower cut-off: 100.0 (All Targets Value) were chosen for data analysis. Differentially expressed genes with statistical significance were identified through Volcano Plot filtering. Gene ontology analysis was applied to determine the roles of these differentially expressed genes played in these biological gene ontology terms.

Statistical Analysis of Experimental Data
Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Two-group comparisons were analyzed by the Welch Student’s t test to account for unequal vari-ances (except for higher-powered data sets with equivalent variance, where we opted for an unpaired t test). Two-sided P values <0.05 were considered significant and denoted with 1, 2, or 3 asterisks when lower than 0.05, 0.01, or 0.001, respectively.

Results
KLF5 Is Upregulated in Human and Experimental AAA
To investigate the roles of KLF5 in human aortic aneurysms, we initially determined KLF5 expression in AAA and normal aortic tissues. Demographic characteristics and representative axial computed tomography scans are presented with corresponding 3-dimensional volume-rendered images in Online Table I and Online Figure IA. KLF5 mRNA expression was significantly higher (P=0.0054) in human AAAs than in normal aortic tissues, as indicated by real-time PCR experiments (Figure 1A; Online Table II). Moreover, KLF5 immunostaining was markedly increased in human AAA tissues compared with that in control tissues (Figure 1B). Thus, to identify cell types that specifically express KLF5, we examined macrophages, VSMCs, and KLF5 in human AAA tissue using confocal fluorescence microscopy. In these experiments, KLF5 was primarily colocalized with intramural macrophages and...
Krüppel-like factor 5 (KLF5) is elevated in human abdominal aortic aneurysms (AAAs) and angiotensin II (Ang II)–induced AAA model. A, Quantitative real-time polymerase chain reaction (qRT-PCR) for KLF5 expression in human AAA tissues (n=11) and normal abdominal aorta (n=7). **P<0.01 vs control. B, Representative photographs of immunohistochemical staining for KLF5 in AAA tissue and control. Scale bars =50 μm. C, Confocal immunofluorescence performed on human AAA sections stained with macrophage marker (MAC-2), smooth muscle (SM) α-actin, KLF5, and 4',6-diamidino-2-phenylindole (DAPI). Arrows indicate KLF5+ MAC2-positive or KLF5+ SM α-actin–positive cells. Scale bars =50 μm. D and E, Statistics of KLF5+ MAC2-positive cells and KLF5+ SM α-actin–positive cells in human AAA tissue and control. *P<0.05 and **P<0.01 vs control. F, Representative photographs of hematoxylin and eosin (HE)–stained abdominal (Continued)
SMCs (Figure 1C; Online Figure 1B) in AAA lesions. Among all macrophage marker (MAC-2)-positive cells, the percentages of KLF5-positive macrophages were significantly higher in AAA than in controls (11.3±6.2% versus 2.8±1.6%; \( P<0.01 \) versus control; Figure 1D). Furthermore, the percentages of KLF5-positive SMCs were significantly higher in AAA than in controls (10.4±2.6% versus 2.3±1.1%; \( P<0.05 \) versus control; Figure 1E). These results suggest that both macrophages and VSMCs express KLF5 in human AAA.

We induced aneurysm formation in ApoE−/− mice by chronic infusion of Ang II. Infusion of Ang II for 4 weeks led to a significant increase in the aortic external diameter (1.63±0.11 mm; \( P<0.001 \)) and displayed an aeurysmal phenotype compared with saline-infused control (0.68±0.02 mm; Online Figure 1A and 1B). In addition, hematoxylin and eosin staining showed luminal dilation, aortic dissection, intraluminal thrombus, decreased smooth muscle layers, and wall thickening in Ang II–infused mouse aortas (Figure 1F).

Affected aortic tissues were harvested for RNA extraction and immunohistochemistry after the induction of the aneurysm. In these experiments, KLF5 expression was significantly increased by 3.37±0.79-fold (\( P<0.01, n=4 \)) in Ang II–infused aortic walls (Figure 1G). Furthermore, immunohistochemical KLF5 expression progressively increased with the aortic diameters after Ang II infusion (Figure 1H). Moreover, confocal immunofluorescence staining of KLF5, MAC2, and smooth muscle \( \alpha \)-actin, and DAPI. Scale bars =50 \( \mu \)m.

The role of macrophages in AAA formation, macrophage depletion was induced by injecting clodronate liposomes, and CaPO4-induced mouse AAA models were developed. As shown in Online Figure V, macrophage depletion markedly decreased infrarenal aortic expansion, with near absence of macrophage infiltration, suggesting that macrophages play an important role in AAA formation.

Correspondingly, coimmunostaining with anti-KLF5, anti-MAC2, anti-Ly6G and anti-smooth muscle \( \alpha \)-actin (SMA-\( \alpha \)) antibodies showed that KLF5-positive macrophages and neutrophils that had infiltrated the aortic wall were markedly fewer in myeKlf5−/− mice than in control mice (Figure 2E through 2G). A similar result was also obtained in CaPO4-induced AAA model (Online Figure IV through VII).

We further examined the influence of myeloid KLF5 expression on leucopoeisis by comparing total leucocyte counts between control and myeKlf5−/− mice. The results showed that the number of neutrophils and monocytes in peripheral blood was unaffected by KLF5 expression, and the total number of leucocytes and subpopulations were similar between control and myeKlf5−/− mice (Online Table III and Online Figure VIII and VIIIIB). Flow cytometry analysis showed that a significant increase in the fraction of SigF+ eosinophils was observed in bone marrow and spleen of myeKlf5−/− mice untreated with CaPO4. Ly6G+ neutrophils and CD11b+ monocytes did not change significantly in bone marrow, peripheral blood, or spleen of control and myeKlf5−/− mice, regardless of CaPO4 treatment. However, a significant decrease of CD11b+ monocytes in the spleen of CaPO4-treated myeKlf5−/− mice was detected (Online Figure VIIIIC through VIIIID).

To further validate whether myeKlf5−/− neutrophils affect aneurysm formation, we transiently depleted neutrophils in myeKlf5−/− and control mice by using rat anti-mouse Ly6G/Ly6C (Gr-1) monoclonal antibody and confirmed that neutrophils were depleted by flow cytometry (designated anti-Ly6G+myeKlf5−/− mice; Online Figure IXA and IXB). As shown in Online Figure IXC and IXD, anti-Ly6G+myeKlf5−/− mice also had reduction in maximal aortic dilation compared with control mice exposed to CaPO4 for 14 days (anti-Ly6G+myeKlf5−/−: 57.7±4.6% versus control: 87.1±6.3%; \( P<0.01 \)). Immunohistochemical staining showed that neutrophil depletion and myeloid Klf5 deficiency effectively protected against medial SMC loss induced by CaPO4 exposure and reduced the infiltration of neutrophils in myeKlf5−/− mice (Online Figure IXE through IXG). However, neutrophil depletion alone in WT mice could not alleviate the SMC loss induced by CaPO4 exposure and also did not reduce macrophage infiltration. Aortic dilation induced by exposure to CaPO4 showed no significant differences between WT and anti-Ly6G+ mice (Online Figure IXH through IXJ).

In addition, in the CaPO4-injured aortic tissues of WT mice, M1-type macrophages rather than M2-type macrophages infiltrated into AAA tissues, facilitating inflammation.
Figure 2. Myeloid-specific knockout of Krüppel-like factor 5 (KLF5) attenuates aneurysm formation. A, Representative photographs of abdominal aortic aneurysm (AAA) induced by angiotensin II (Ang II) infusion (dashed). Scale bar =2.5 mm. B, The maximal aortic external diameter for control (n=20) and ApoE−/−myeKlf5−/− mice (n=21) exposed to Ang II for 28 days. Data represent the mean±SEM. *P<0.05 vs control. C, Survival curve of Ang II-infused ApoE−/− (n=20) and ApoE−/−myeKlf5−/− mice (n=21). *P<0.05, log-rank test. D, Hematoxylin and eosin (HE)–stained representative sections of aortas from the Ang II-infused control (ApoE−/−) and ApoE−/−myeKlf5−/− mice. Scale bars =50 μm. E through G, Dual immunofluorescence staining of Krüppel-like factor 5 (KLF5; red), MAC2 (macrophage marker; green), Ly6G (green), and SMA-α (smooth muscle α-actin; green) in the injured aortas of control and ApoE−/−myeKlf5−/− mice exposed to Ang II for 28 days. Scale bars =50 μm.
and AAA formation; but in myeKlf5−/− mouse models, the infiltrating M1-type macrophages were reduced, whereas the infiltrating M2-type macrophages were increased. Moreover, the expression of M1 macrophage markers, including TNF-α, MCP-1 (monocyte chemotactic protein 1), IL-1β, and iNOS, was also downregulated, with increased expression of M2 markers, including Arg-1 (arginase 1), Mrc-1 (macrophage mannose receptor 1), PPARγ (peroxisome proliferator-activated receptor γ), and Retnal (Online Figure X).

Likewise, myeKlf5−/− macrophages derived from bone marrow of myeKlf5−/− mice exhibited the decreased expression of M1 markers, but the enforced expression of KLF5 in myeKlf5−/− macrophages rescued M1 marker expression induced by lipopolysaccharide treatment (Online Figure XI). Simultaneously, myeKlf5−/− macrophages showed less phagocytic activity than WT macrophages, as shown by zymosan phagocytosis assay (Online Figure XII). Together, KLF5 deletion in macrophages affects the proinflammatory response of macrophages and phagocytic function. We also confirmed that KLF5 deletion in macrophages did not significantly affect the proliferation of macrophages that had infiltrated the aortic wall, as evidenced by double-immunofluorescence staining of CaPO4-injured aortic sections with MAC2 and Ki67 (Online Figure XIII A), as well as by decreased proliferating cell nuclear antigen expression (Online Figure XIII B).

Defective In Vitro Migration of myeKlf5−/− Macrophages

To further investigate the roles of KLF5 in macrophage migration and infiltration, we prepared bone marrow–derived macrophages from control and myeKlf5−/− mice and compared macrophage migration using cell scratch migration assays. These experiments showed the diminished migration of myeKlf5−/− macrophages into cell-denuded gaps compared with WT macrophages (Figure 3A and 3B). In further experiments, Boyden chamber transwell migration assays were performed, with macrophages seeded in the upper chamber and Ang II–stimulated VSMCs in the lower chamber (Figure 3C), and the numbers of myeKlf5−/− and WT macrophages that migrated through the porous membrane were counted. These experiments showed that significantly fewer myeKlf5−/− macrophages than WT macrophages migrated across the membrane (Figure 3D and 3E). Accordingly, scanning electron microscopy showed that the pseudopodial projections formed by myeKlf5−/− macrophages when passing through membrane pores were fewer and shorter than those formed by WT macrophages (Figure 3F).

Moreover, reduced KLF5 expression was confirmed in myeKlf5−/− macrophages on the other side of the membrane using dual immunofluorescence staining (Figure 3G).

Taken together, the present observations indicate that myeKlf5−/− macrophages have impaired migration. However, the movement of macrophages in both directions across membranes may lead to the aberrant positioning and migration of Klf5-deficient macrophages at rate similar to that of WT macrophages. Thus, to validate these observations, time-lapse imaging experiments were performed, and the effects of KLF5 deficiency on forward and backward movement were investigated using chemotaxis assays in culture dishes. Because MCP-1 or TNF-α has been shown to contribute to mouse models of AAA,21,22 macrophages were seeded in culture dishes, and MCP-1 or TNF-α was added to one side of the culture dish to establish a spatial chemotactic gradient. Although WT macrophages exhibited marked time-dependent migration toward high concentrations of MCP-1 (Figure 3H and 3I), the movement of myeKlf5−/− macrophages was minimal (Figure 3H, 3J, and 3K). The results of TNF-α treatment were similar to those obtained with MCP-1 (Online Figure XV). Taken together, these data demonstrate that KLF5 is required for macrophage migration and infiltration during the formation of aneurysms.

KLF5 Mediates Myo9b Expression via Direct Binding to the Transforming Growth Factor-β Control Element Site 1 in the Myo9b Promoter

To identify genes that are regulated by KLF5 during macrophage migration and infiltration, we performed RNA expression profiling using microarray analyses of AAA and normal abdominal aortas from control and ApoE−/−myeKlf5−/− mice. The experiments showed that motility-related gene expression markedly differed between AAA and normal aortic tissues. Among identified genes, Myo9a (2.67-fold) and Myo9b (3.22-fold) were the most downregulated in experimental AAA tissues from Ang II–treated ApoE−/−myeKlf5−/− mice (Figure 4A). In subsequent gene ontology analyses, myosin filaments and complexes were the most enriched in gene ontologies targeted by the genetic deletion of KLF5 (Figure 4B). Furthermore, the quantitative real-time PCR analyses of motility-related gene expression after in vivo Ang II treatments demonstrated that Myo9a, Myo9b, and Macf1 were significantly downregulated in AAA tissues from ApoE−/−myeKlf5−/− mice compared with those from control mice (Figure 4C). Taken together, these data suggest that KLF5 is required for the expression of myosin genes.

To confirm that KLF5 directly regulates Myo9a and Myo9b gene expression, we generated KLF5 overexpressing and knockdown macrophages by infecting with adenoviruses encoding Klf5 or transfecting with siRNA against KLF5, respectively. In these experiments (Figure 4D and 4E), the overexpression of KLF5 significantly increased basal and TNF-α-induced Myo9a and Myo9b expression at transcription and translation levels. Conversely, the knockdown of KLF5 by siRNA against KLF5 blocked TNF-α-induced and basal Myo9a and Myo9b expression (Figure 4F and 4G). These results suggest that KLF5 plays a key role in the regulation of Myo9a and Myo9b expression, regardless of TNF-α stimulation.

The 2 class IX mammalian myosins Myo9a and Myo9b are actin-based motorized signaling molecules that negatively regulate RhoA signaling,23 and the impaired migration of Myo9b−/− peritoneal macrophages has been previously shown.22 Thus, to investigate the molecular mechanisms by which KLF5 promotes Myo9b gene expression, we used Transcription Element Search System string-based search (http://www.cbil.upenn.edu/tess/) programs and identified 5 putative binding sites of KLF5 (CACCC or its reverse sequence GGGTG; transforming growth factor-β control element [TCE] site) in the −1396 to +1 bp region of the Myo9b promoter (Figure 4H). Subsequently, to determine whether KLF5 regulates the expression of Myo9b gene expression via interactions with the Myo9b promoter, we performed luciferase reporter

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Figure 3. myeKlf5−/− macrophages are defective in their migration ability. A, Representative photomicrographs of a scratch assay performed with macrophages from ApoE−/− or ApoE−/−myeKlf5−/− mice immediately after scratching and 12 h later. B, Quantification of cells migrating into the scratch gap. The data are means±SEM from 3 independent experiments. *P<0.05 vs wild-type (WT). C, An in vitro coculture system is shown where macrophages were seeded in the top compartment and angiotensin II (Ang II)–stimulated smooth muscle cells (SMCs) in the bottom compartment. D, Boyden chamber assay showing WT and myeKlf5−/− macrophages traversed the filter to the other side and stained by hematoxylin and eosin (HE). E, Quantification of cells migrated to the lower side of the membrane. *P<0.05 vs WT. F, Scanning electron microscopy showing that myeKlf5−/− macrophages had shorter and fewer pseudopod projections. G, Dual immunofluorescence staining of Krüppel-like factor 5 (KLF5; red) and MAC2 (macrophage marker; green) in WT and myeKlf5−/− macrophages. Scale bars =50 μm. H, MCP-1 (monocyte chemotactic protein 1)–stimulated migration of WT and myeKlf5−/− macrophages was traced by using time-lapse imaging. I and J, Each line represents the movement of each macrophage from ApoE−/− and ApoE−/−myeKlf5−/− mice. K, Migration distances per hour were measured by Image J. n=21 cells, **P<0.001 vs WT. DAPI indicates 4′,6-diamidino-2-phenylindole; and VSMC, vascular smooth muscle cell.
Figure 4. Krüppel-like factor 5 (KLF5) mediates Myo9b (myosin IXB) expression via direct binding to the (transforming growth factor-β control element) TCE site 1 in the Myo9b promoter. A, A subset of the differentially expressed mRNAs detected in the injured aortas of control (ApoE<sup>−/−</sup>; n=3) and myeKlf5<sup>−/−</sup> (ApoE<sup>−/−</sup>myeKlf5<sup>−/−</sup>) mice (n=3) with microarray analysis was selected and summarized. B, The Gene Ontology enrichment analysis showing the significantly changed genes in the injured aortic tissues of myeKlf5<sup>−/−</sup> and control mice. C, Six changed genes were validated by quantitative real-time polymerase chain reaction (qRT-PCR) in the aortic tissues of control and myeKlf5<sup>−/−</sup> mice. *P<0.05 and **P<0.01 vs control. D and E, Macrophages were infected with Ad-null or adenoviruses encoding Klf5 (Ad-Klf5) for 24 h and then were treated or not with TNF-α (tumor necrosis factor-α; 10 ng/mL). The expression of KLF5, Myo9a, and Myo9b was analyzed by Western blotting (D) and qRT-PCR (E). *P<0.05 vs Ad-null, #P<0.05 vs Ad-null+TNF-α, **P<0.05 vs Ad-Klf5. F and G, Macrophages were transfected with nonspecific siRNA (si-NS) or siRNA against KLF5 (si-Klf5) for 24 h and then treated or not with TNF-α. The expression of KLF5, Myo9a, and Myo9b was examined as described earlier, *P<0.05 vs si-NS, **P<0.05 vs si-NS+TNF-α. H, A schematic map of the −1396 to +1 bp region of the Myo9b promoter showing the position of 5 KLF5-binding sites. I, A luciferase reporter controlled by the Myo9b promoter was transfected into 293A cells along with increasing amounts of KLF5 expression plasmid. (Continued)
assays in 293A cells using Myo9b promoter–luciferase reporter constructs containing murine Myo9b −1396 to +1 bp sequences. In these experiments, the overexpression of KLF5 concentration-dependently increased Myo9b promoter activity (Figure 4I). Subsequent truncation of the Myo9b promoter sequence from −1396 to −382 bp did not affect the activation of the promoter by KLF5. However, further deletion of −382 and −287 bp dramatically decreased the activation by KLF5 (Figure 4J), indicating that the TCE site 1 at −319 bp is critical for the KLF5-mediated transcriptional activation of Myo9b. Thus, to investigate whether KLF5 directly binds to TCE site 1 in macrophages, we conducted oligonucleotide pull-down assays using macrophage lysates and biotinylated oligonucleotide probes. As shown in Figure 4K, KLF5 bound only to the TCE site 1 of the Myo9b promoter, and mutation of the TCE site 1 abolished KLF5 binding. Furthermore, no KLF5 bands were detected when probes containing WT or mutant sites 2, 3, 4, or 5 were used. These results suggest that KLF5 activates Myo9b transcription by directly binding the TCE site 1 of the Myo9b promoter.

**KLF5 Deletion Reduces Podosome Rosette Formation in Macrophages by Downregulating Myo9b Expression**

Adhesion and migration of monocyte-derived cells are reportedly regulated by podosomes,7 and KLF5 was required for macrophage migration in the present study (Figure 3A through 3K). Thus, to investigate the relationships between the KLF5-regulated migration of macrophages and podosome formation, bone marrow–derived macrophages were prepared from control and ApoE−/−myeKlf5−/− mice and were treated with the inducer of podosome formation phorbol 12,13-dibutyrate (PDBu).24 Subsequently, podosome formation was visualized in macrophages according to the immunohistochemical colocalization of F-actin and cortactin (Figure 5A). Although podosomes can present as rosettes and as individual punctates,25 we hardly observed podosome rosettes in macrophages under normal culture conditions (data not shown), but stimulation with PDBu promoted podosome rosette formation (Figure 5A). However, no rosette structures were observed in myeKlf5−/− macrophages, regardless of the presence of PDBu. In addition, immunofluorescence staining with an anti-Myo9b antibody revealed the colocalization of Myo9b with F-actin and cortactin in podosome rosettes (Figure 5A), indicating that Myo9b participates in PDBu-induced podosome formation.

Because MCP-1 increased macrophage migration,26 we determined whether the numbers of cells carrying individual podosomes or podosome rosettes were altered in response to MCP-1 and whether the knockout of KLF5 affected MCP-1-induced podosome formation. These experiments showed significantly greater numbers of macrophages containing podosome rosettes among MCP-1-treated cells than among untreated cells. Moreover, the knockout of Klf5 significantly reduced the formation of podosome rosettes, regardless of MCP-1 treatment (Figure 5B). Conversely, the numbers of macrophages with individual podosomes did not differ between control and myeKlf5−/− macrophages in the presence and absence of MCP-1 or TNF-α (Figure 5C, Online Figure XVIC and XVII).

To further demonstrate Myo9b localization in podosome rosettes, coinmunofluorescence staining was performed using anti-vinculin, anti-Tks5 (both podosome marker proteins),27 and anti-Myo9b antibodies in PDBu-treated macrophages. In agreement with the above observations of Myo9b colocalization with F-actin and cortactin (Figure 5A; Online Figure XVIA), Myo9b colocalized with vinculin and Tks5 after PDBu and MCP-1 or TNF-α treatments (Figure 5D; Online Figure XVID). To confirm the interaction of Myo9b and cortactin, we performed an in situ proximity ligation assay. Because a proximity ligation assay signal can be detected when the proteins of interest are in close proximity, this technique enabled us to detect direct protein–protein (Myo9b and cortactin) interaction in macrophages. The interaction of Myo9b and cortactin in WT macrophages with PDBu and MCP-1 treatment was observed. The specificity of this interaction was confirmed by the absence of association between Myo9b with cortactin in myeKlf5−/− macrophages with same treatment (Figure 5E).

The specificity of anti-Myo9b antibody staining was confirmed by the absence of colocalization of Myo9a with cortactin and F-actin (Figure 5F), and defective podosome formation in myeKlf5−/− macrophages was rescued by the overexpression of KLF5, as indicated by the immunofluorescence staining of podosome markers (Figure 5G; Online Figure XVIE). Furthermore, although Myo9b immunostaining was barely detectable in myeKlf5−/− macrophages (Figure 5A and 5G), the enforced expression of KLF5 in myeKlf5−/− macrophages resulted in marked Myo9b staining (Figure 5G). Hence, KLF5-mediated Myo9b expression may be critical for the formation of podosome rosettes in MCP-1- or TNF-α-treated macrophages.

In further studies, we assessed podosome formation in abdominal aortas from mouse and human AAA using immunostaining. The staining of mouse and human AAA tissues for F-actin and cortactin showed the presence of podosome rosette structures that were remarkably similar to those observed in vitro (Figure 5H and 5I; Online Figure XVIF and XVIIH). Moreover, no staining of the podosome proteins cortactin and F-actin was observed in aortas from myeKlf5−/− littermates or human controls (Figure 5J and 5K; Online Figure XVIG and XVI I). These data suggest that podosomes form in vivo, their morphological characteristics are retained in tissues, and KLF5 facilitates podosome formation in vivo and in vitro.
Macrophage-Specific Knockdown of Myo9b Reduces Podosome Formation and Migration of Macrophages After Treatment With MCP-1

To further investigate the roles of Myo9b in podosome formation and macrophage migration, we knocked down endogenous Myo9b by transfecting macrophages with mouse Myo9b-specific siRNA or nonspecific siRNA. These experiments demonstrated the marked attenuation of Myo9b expression in siRNA targeting Myo9b–transfected macrophages compared with cells transfected with nonspecific siRNA after...
Figure 6. Myo9b (myosin IXB) knockdown reduces podosome formation and migration of macrophages induced by MCP-1 (monocyte chemotactic protein 1). A and B. Macrophages were transfected with siRNAs targeting Myo9b (si-Myo9b) or nonspecific siRNA (si-NS) for 24 h and then treated with MCP-1 for 24 h. Myo9b knockdown was determined by Western blotting with anti-Myo9b (A), podosomes were visualized by staining with rhodamine–phalloidin, anti-Myo9b, and anticortactin antibodies (B). Scale bars =10 μm. C. The percentage of podosome rosette–positive macrophages. *P<0.05 vs si-NS+MCP-1-untreated group; ***P<0.001 vs si-NS+MCP-1 group or si-NS+MCP-1-untreated group. D. The macrophage migration was determined by Boyden chamber assay. *P<0.05 vs (Continued)
treatment with MCP-1 (Figure 6A). Moreover, PDBu-induced formation of podosome rosettes was significantly decreased in siRNAs targeting Myo9b–transfected cells, regardless of MCP-1 stimulation (Figure 6B and 6C), suggesting that Myo9b is required for podosome formation in macrophages. Myo9b knockdown also led to significantly reduced macrophage migration compared with that in nonspecific siRNA–transfected cells, regardless of MCP-1 treatment (Figure 6D), again suggesting that podosome formation facilitates macrophage migration. Furthermore, we demonstrated that KLF5 overexpression promotes the formation of podosome rosettes in nonspecific siRNA–transfected macrophages, and this effect was abrogated after Myo9b knockdown using siRNA (Figure 6E and 6F). In addition, knockdown of Myo9b also reduced podosome formation and migration of macrophages after treatment with TNF-α (Online Figure XVIII). Taken together, these findings suggest that KLF5 promotes podosome formation in macrophages by regulating Myo9b expression.

RhoA-GTP Levels Increase After Knockdown or Deletion of KLF5 in Macrophages

Because the activation of small GTPases Cdc42, Rac1, and RhoA is required for cell migration,1 we investigated the roles of these molecules in TNF-α-induced podosome formation and cell migration. As shown in Figure 7A, the expression levels of GTP-bound Cdc42, Rac1, and RhoA were markedly increased within 15 minutes of TNF-α stimulation. In particular, GTP-bound RhoA expression was maximal at 30 minutes and subsequently decreased to basal levels by 6 hours (Figure 7A). Moreover, Myo9b expression progressively increased with decreases in active GTP-RhoA levels, suggesting a negative correlation between GTP-RhoA and Myo9b.

To confirm that KLF5-induced formation of podosomes is related to the activation of GTP-bound RhoA, Rac1, and Cdc42, we performed further experiments using KLF5-knockdown and -overexpressing macrophages. In these experiments, KLF5 overexpression clearly upregulated Myo9b and decreased GTP-bound RhoA expression, but did not affect the Rac1 and Cdc42 activation. In contrast, the knockdown of KLF5 markedly downregulated Myo9b expression and increased GTP-bound RhoA levels in macrophages (Figure 7B and 7C), indicating that KLF5-induced Myo9b negatively regulates RhoA signaling. In addition, the treatment of myeKlf5−/− macrophages with the RhoA-specific inhibitor Rhosin (30 μM) led to increased PDBu-induced podosome formation. However, the inhibition of RhoA signaling by Rhosin also increased podosome formation in WT macrophages, albeit slightly (Figure 7D and 7E). Accordingly, Boyden chamber assays showed significant decreases in the numbers of migrated myeKlf5−/− macrophages after treatment with Rhosin (Figure 7E). These data suggest that KLF5 and KLF5-regulated Myo9b lie upstream of the RhoA signaling, and KLF5, Myo9b, and RhoA coregulate macrophage podosome formation and migration.

Myo9b Is Upregulated and Localized to Macrophages in Human AAA

In the present study, KLF5 promoted podosome formation and macrophage infiltration by upregulating Myo9b. Thus, we performed further experiments to corroborate these data in human aortic aneurysms. Initially, we examined Myo9b expression in human AAA tissues and normal aortic tissues using confocal immunofluorescence staining for Myo9b (red) and showed markedly increased expression in human aneurysm tissues compared with normal tissues. Moreover, Myo9b localized in MAC2-positive macrophages, as indicated by the analyses of aortic tissue sections (Figure 8A). In addition, real-time PCR analyses showed significant increases in Myo9b mRNA expression in AAA samples compared with that in nonaneurysm tissues (Figure 8B), further suggesting that Myo9b is induced during human AAA formation.

Finally, we investigated the correlation between Myo9b expression and AAA expansion rates in patients with initial computed tomography diagnoses of AAAs. AAA expansion rates were calculated according to the changes in maximal anterior–posterior diameters of AAAs during the 1-year follow-up period. In the analysis, Myo9b mRNA expression in abdominal aneurysmal walls was positively correlated with AAA size. However, although a clear trend was observed, KLF5 expression was not significantly associated with AAA size, potentially reflecting more complicated functions (Figure 8C and 8D). These results suggest that Myo9b is a target of KLF5 and plays important roles in human AAA formation. Moreover, taken with the present in vitro and in vivo data, these observations suggest that Myo9b functions as an aneurysm-promoting factor during AAA formation and progression.

Discussion

Previous studies have shown that KLF5 is highly expressed in large and giant unruptured cerebral aneurysms.18 However, the mechanisms by which KLF5 participates in AAA formation remain unknown. Here we present in vitro and in vivo evidence of an important role of KLF5 during AAA formation. In particular, after the Ang II stimulation of abdominal aortas, KLF5 directly upregulated Myo9b expression and promoted macrophage migration by inducing the formation of podosome rosette structures and inhibiting RhoA signaling. Accordingly, (1) KLF5 knockdown attenuated macrophage migration in vitro and infiltration in vivo; (2) KLF5 directly bound the Myo9b promoter and induced Myo9b expression in macrophages; (3) KLF5 promoted podosome formation and migration in macrophages, partially through Myo9b and its downstream effector RhoA; (4) KLF5 and Myo9b were co-expressed in human AAA tissues and macrophages that had infiltrated vascular walls; and (5) the upregulation of KLF5 and Myo9b expression was correlated with the progressive dilation of human AAAs.
KLF5 has been identified as an oncogenic transcription factor that is highly expressed in tumor cell lines, and it reportedly promotes cell proliferation and migration in several cancer types, including breast, bladder, and intestinal cancers. In accordance, the present data show that KLF5 is substantially upregulated in human and experimental AAA...
tissues and is localized in SMCs and infiltrated macrophages. Although the proliferation-specific and antiapoptotic roles of KLF5 have been demonstrated in VSMCs, the corresponding roles in macrophages during aneurysm formation remain unclear.

In a previous study, AAA formation in global KLF4 and smooth muscle–specific KLF4 knockout mice was demonstrated using an Ang II model of aneurysm formation. Moreover, myeloid-specific conditional KLF6-deficient mice reportedly exhibited aortic dissection and the presence of intramural macrophages because of the reduced expression and secretion of granulocyte–macrophage colony-stimulating factor. Accordingly, myeloid-specific KLF5 knockout resulted in decreased aortic aneurysm formation and concomitantly reduced Myo9b expression in the present Ang II- or CaPO₄-induced mouse AAA models. Taken together, our results indicate that KLF5 acts as an important regulator of aneurysm formation by promoting macrophage infiltration and migration.

KLF5 was previously shown to promote the migration of basal keratinocytes by promoting the transcription of integrin-linked kinase and the consequent activation of Cdc42 and myosin light chain, which are critical for cell migration and motility. In the present study, several lines of evidence have implicated Myo9b as a direct target of KLF5. Specifically, KLF5 and Myo9b were coexpressed in human AAA tissues and macrophages, and the expression of Myo9b was decreased and increased after the knockdown and overexpression of KLF5, respectively. In addition, KLF5 directly bound the Myo9b promoter and promoted Myo9b expression in macrophages. Moreover, mutation of the KLF5-binding site in the Myo9b promoter abolished KLF5-stimulatory effects on Myo9b expression, and KLF5 and Myo9b promoted macrophage migration and podosome rosette formation.

KLF5 can be induced by Ang II or proinflammatory factors such as interleukin-1β, lipopolysaccharide, and TNF-α. Accordingly, KLF5 and Myo9b were induced after treatments with TNF-α, indicating that KLF5 and its downstream target gene Myo9b are responsive to various extracellular proinflammatory stimuli, including Ang II and CaPO₄. KLF5 has been shown to regulate target genes by interacting with nuclear factor-xB. However, it remains unclear whether KLF5 and nuclear factor-xB form a transcription complex at the Myo9b gene promoter.

Cell migration and invasion require the reorganization of actin cytoskeletal structures to produce membrane protrusions, such as podosomes and invadopodia. The formation of podosomes is necessary for cross-tissue migration during normal human development and is central to embryogenesis and angiogenesis, as well as atherosclerotic plaque formation and cancer cell metastasis. Podosomes are largely found in invasive cell types, including macrophages, osteoclasts, and dendritic cells, and podosome deficiency leads to the reduced immunity of monocyte-derived macrophages and the bone resorption of osteoclasts, potentially reflecting their functions in motility and matrix remodeling. Podosomes are also found in VSMCs and may mediate VSMC migration and contribute to intimal hyperplasia during vascular injury or atherosclerosis. Although phorbol esters and cytokines have been shown to induce podosome formation, the functional relationship of podosome formation in rosettes in human AAA and mouse experimental AAA tissues has not been previously characterized. However, the present data suggest that podosomes are critical for macrophage migration and infiltration.
The Rho family of GTPases play important roles in cell migration by regulating actin organization and microtubule dynamics, myosin activity, and cell–extracellular matrix and cell–cell interactions. Myo9b modulates lamellipodial protrusions and tail retractions by suppressing RhoA in migrating immune cells and contains a RhoGAP domain in its tail region. Similar to other RhoGAPs, this domain promotes the hydrolysis of Rho-bound GTP to switch Rho from the active GTP-bound conformation to the inactive GDP-bound state. Accordingly, as a motorized signaling molecule, Myo9b directly links Rho signaling with the actin cytoskeleton. Endothelial and SMC podosome rosettes have been found in tumor vasculature and in miR-143 (145) knockout mouse aortas and play important roles in cell migration. To our knowledge, Myo9b has not previously been shown to influence AAA formation, and the roles of podosomes in the actin-rich structures of AAA remain poorly understood. However, the present gain- and loss-of-function experiments show that KLF5 negatively regulates RhoA signaling by promoting Myo9b expression, which in turn affects macrophage migration in vitro and in vivo. Moreover, Myo9b was highly expressed in the macrophages of human AAA tissues, and its expression was positively correlated with the advanced dilation of AAAs in patients. Although RhoA mediates stress fiber formation and generates contractile forces that are required for the retraction of the trailing edge during cell migration, numerous studies show that the activation of RhoA products enhances the development of actin stress fibers and impairs the migration of cancer cells. Although these observations are consistent with our results, recent studies suggest tumor-suppressive roles of RhoA in human lung cancer and T cell lymphoma. In addition, the blockade of RhoA signaling significantly increased podosome formation in myekLF5−/− macrophages, suggesting KLF5-independent podosome formation. Indeed, Mark Schramp et al reported that ERK5 promotes Src-induced podosome formation in Src-transformed fibroblasts by limiting Rho activation. Pathways through which KLF5 modulates Rho activity, podosome formation, and cellular invasion are of considerable interest. Thus, the ensuing molecular mechanisms warrant further investigation.

In summary, the present data provide direct evidence that KLF5 is an important macrophage-borne stimulator of atherosclerosis, and it mediates Myo9b gene transcription by directly binding to the Myo9b gene promoter. Under these conditions, macrophage podosome formation and cell migration are promoted after the negative regulation of RhoA signaling. Taken together, these data show that the KLF5-dependent regulation of Myo9b/RhoA is required for podosome formation and cell migration in macrophages during AAA formation and warrant consideration of the KLF5–Myo9b–RhoA pathway as a therapeutic target for AAA treatment.

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Disclosures

None.

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Inhibition of KLF5–Myo9b–RhoA Pathway–Mediated Podosome Formation in Macrophages Ameliorates Abdominal Aortic Aneurysm
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SUPPLEMENTAL MATERIAL
Supplemental Material and Methods

Animal Experiments
To generate an Ang II-induced AAA model, eight-week-old ApoE<sup>−/−</sup> male mice were administered saline or Ang II (1000 ng/kg/min) via mini-osmotic pumps (Model 1004, Alzet, CA, USA) for 28 d to induce suprarenal aneurysms as described previously<sup>1</sup>. Briefly, mice were anesthetized with 1.5% isoflurane, and the pumps were placed into the subcutaneous space of the mice through a small incision in the back of the neck. A group of mice were infused with saline as controls. Animals were fed high-fat diet at the same time as Ang II infusion. After 28 d, all animals were anesthetized and perfused with cold saline. Digital photographs of the abdomens were taken to measure the maximum external diameters. The abdominal arteries were harvested for analysis of RNA, morphology and histology.

To induce abdominal aortic aneurysms, we performed mouse models of CaPO<sub>4</sub>-induced AAA as previously described.<sup>2</sup> Briefly, under the general anesthesia, midline incision was made and the infrarenal region of the abdominal aorta was isolated. A small piece of gauze soaked in 0.5 M CaCl<sub>2</sub> was applied perivascularly for 10 min. This gauze is then replaced with another piece of phosphate buffered saline (PBS)-soaked gauze for 5 min. Control mice received a single treatment of PBS soaked gauze for 15 min. After 14 d, all animals were anesthetized and perfused with cold 0.9% NaCl, and the abdominal arteries were harvested for analysis of RNA, morphology and histology.

Mouse aortic diameters were measured using a surgical microscope (OLYMPUS SZX7) equipped with a digital camera (Canon 600D). The digital images were processed using the Adobe Photoshop CS 5.1 software to calculate final maximal diameter of aorta from saline and Ang II infused mice or CaPO<sub>4</sub>-induced AAA mice. Maximal aortic dilation (%) was calculated as (maximal aortic diameter–internal control diameter) / internal control diameter×100%, with aortic diameters measured from the same photo while the mouse was alive. The internal control was a small segment of normal abdominal aorta just distal to the renal arteries that was above the proximal ligation and was not applied with CaPO<sub>4</sub>.

Histology
Murine aortas were harvested at euthanasia for histology analysis after undergoing left ventricular puncture and 4% paraformaldehyde perfusion at physiological pressure. Further fixation was achieved by overnight incubation in 4% paraformaldehyde at 4°C followed by paraffin embedding and sectioning at 4 µm. Ten consecutive sections were prepared for hematoxylin and eosin staining and EVG staining. Images were acquired using a Leica microscope (Leica DM6000B, LAS V.4.3, Switzerland). Morphometric analysis of the media thickness was performed in a blind manner. For each section, four random, noncontiguous microscopic fields were examined.

After microwave antigen retrieval, endogenous peroxidase activity was inhibited
by incubation with 3% H$_2$O$_2$. Sections were blocked with 5% goat serum in phosphate-buffered saline (PBS) and incubated overnight at 4°C with primary antibodies. After a PBS wash, the sections were incubated with secondary antibody at 37°C for 30 min. Immunohistochemical staining was visualized by use of a diaminobenzidine kit (Zhongshan Goldenbridge Biotechnology, Beijing, China) according to the manufacturer’s instructions. Sections were counterstained with hematoxylin to visualize nucleus. The primary antibodies included anti-mouse KLF5 Ab (1:200 dilution, GTX103289, GeneTex) and anti-mouse MAC2 Ab (1:200 dilution, 60207-1, Proteintech). Images were acquired using a Leica microscope (Leica DM6000B, Switzerland) and digitized with LAS V.4.4 (Leica).

Immunofluorescence staining was performed with 4 μm paraffin cross-sections from the abdominal artery. After deparaffinized with xylene and rehydrated, the slides were pre-incubated with 10% normal goat serum (710027, KPL, USA) and then incubated with primary antibodies anti-SMα-actin (1:50; sc-130616, santa), anti-F4/80 (1:50; sc-52664, santa), anti-KLF5 (1:100; GTX103289, GeneTex), anti-MAC2 (1:100; 60207-1, Proteintech). Secondary antibodies were fluorescein labeled antibody to rabbit IgG (1:200; 021516, KPL, USA) and rhodamine-labeled antibody to mouse IgG (1:200; 031806, KPL, USA), or fluorescein-labeled antibody to mouse IgG (1:200; 021815, KPL, USA), rhodamine-labeled antibody to rabbit IgG (1:200; 031506, KPL, USA). In each experiment, DAPI (157574, MB biomedical) was used for nuclear counter staining. Sections were stained simultaneously, using identical reagents and incubation times and negative controls were included. Images were captured by confocal microscopy (DM6000 CFS, Leica) and processed by LAS AF software.

**Hematological Analysis**

Peripheral blood was obtained by puncturing the cava vein. Blood was drawn into EDTA coated tubes (Monlab SL, Spain). Complete blood profiles and hemoglobin levels were obtained using an Abacus Junior Vet (Beckman) hematology analyzer. Values are shown as absolute counts and referenced against the normal range established for mice.

**Cytological Examination**

At the end point analysis, peripheral blood and bone marrow (tibia) smear were accomplished for each animal. All cytospins were stained with the Wright s-Giemsa’s stain method. Morphology was assessed by examination with a ×40 objective, and a 500-cell count was performed. Any cell which did not fit a definition exactly was counted with the category which it most closely resembled. All cells of granulocyte or monocyte lineage were included in the myeloid category for calculation of the M/E ratio.

**Macrophage and Neutrophil Depletion and Flow Cytometry Analysis**

Wild-type mice were injected intraperitoneally with 110 mg/kg of clodronate liposomes or equal volume of PBS liposomes 2 days before and 7 days after exposure to CaPO$_4$. The aortic tissues were rubbed through a stainless steel sieve (180 mesh) using a blunt instrument. and cell suspension was collected and washed with flow cytometry staining buffer. Murine Fc receptors were blocked using antibodies against murine CD16/32 antigens (eBioscience) for 15 min on ice, after which cells were washed and
then resuspended in 100 ml FACS buffer. Fluorochrome-conjugated antibodies (all from BioLegend) for PE-F4/80 and FITC-CD54 or FITC-CD206 were added for 30-45 min at room temperature. Corresponding isotype control antibodies were added to samples at the same concentrations as the antibodies of interest. After incubation, samples were washed three times and analyzed by FAC Sverse (FC500 MPL Beckman).

To determine whether neutrophil depletion encompasses the entire period of neutrophil infiltration, test mice were injected with 50 μl (0.025 mg) of rat anti-mouse Ly6G/Ly6C (Gr-1) antibody (BD Pharmingen, Franklin Lakes, NJ) 24 h before and after CaPO₄ application. Placebo mice were injected with 50 μl of rat serum. Neutrophil depletion was confirmed by flow cytometry. In brief, 0.5 to 1 ml of blood was collected by puncturing the cava vein under anesthesia with 1.5% isoflurane and kept at room temperature in Microvette 500 tubes coated with EDTA (Monlab SL, Spain). Erythrocytes were lysed in 100 volumes of hemolysis buffer containing 155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA, pH 7.3. The cells were then centrifuged at 500 g for 5 min at room temperature. The supernatant was discarded, and the pellet was suspended in 500 μl of cold PBS. The cells were again centrifuged at 500 g for 5 min at 4°C. The two previous steps were repeated, and the pellet was suspended in 1.5 ml of cold PBS containing 1% bovine serum albumin. To allow the detection of neutrophil, cells were incubated with 0.2 mg R-phycocerythrin-conjugated rat anti-mouse Ly6G monoclonal antibody (primary antibody, BD Pharmingen) and 0.2 mg R-phycocerythrin-conjugated rat anti-mouse IgG1, k immunoglobulin (isotype control, BD Pharmingen) for 1 h. Flow cytometry indicated that more than 95% of the neutrophils were depleted after 24 h by one injection of Ly6G/Ly6C compared with the controls.

**Cell Culture and Treatment**

Bone marrow-derived macrophages (BMMs) were obtained from femoral bone marrow suspensions of WT C57BL/6 mice and myeKlf5⁻/⁻ mice and plated at 3×10⁶ cells/mL and differentiated for 7 d in the presence of DMEM/10% FBS/10% murine G colony-stimulating factor (Pepro Tech, no. A2714)³. Mouse RAW 264.7 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin (100 U/100 mg) solution (Life Technologies, Grand Island, NY). Human embryonic kidney 293A cells were obtained from ATCC (Manassas, VA) and maintained in high glucose DMEM supplemented with 10% FBS. Cell cultures were maintained at 37°C in humidified incubators containing an atmosphere of 95% air, 5% CO₂.

Bone marrow–derived monocytes were grown in macrophage-inducing medium to a confluence of 80% to 100% before the cell monolayer was scratched using a sterile micropipette tip. After washes with PBS, fresh macrophage-specific medium was added. The images of the wounded area were captured immediately after the scratch and 12 h later to monitor macrophage migration into the wounded area. The migratory abilities were quantified by measuring the total number of cells in the scratched regions as well as by measuring the distance between cells in the scratch zone.
Mouse aortic smooth muscle cells (MASMCs) were isolated from thoracic aorta of WT C57BL/6 mice. The aortas were carefully dissected to remove excess connective tissue, then minced and digested in type III porcine pancreatic elastase (Sigma, no. E1250), type I collagenase (Sigma, no. C0130), and soybean trypsin inhibitor (Sigma, no. T6522) at 37°C for 1 h. The cells were plated in DMEM containing 10% FBS. Cells were maintained in 5% CO₂ at 37°C within a humidified atmosphere and determined to be SMCs by morphology and expression of SM α-actin.

In vitro CaPO₄ Treatments 10% FBS DMEM was replaced with 0.5% FBS DMEM 24 h prior to all cell treatment. Cells designated for CaPO₄ treatment were washed twice with 1x PBS before being treated with CaCl₂ (final concentration 0.05 M) diluted in 1xPBS. Treated cells were incubated at 37°C for 15 min, CaPO₄ solution was removed and cells were washed twice with 10%FBS DMEM and let rest 6 h at 37°C.

Cell Immunofluorescence (IF)
Immunofluorescence staining was performed on paraformaldehyde-fixed cells. Cells were permeabilized with 0.1% Triton X-100 in PBS and then blocked with 0.1% Triton X-100 and 5% BSA in PBS for 1 h, washed, and incubated overnight with the related primary antibodies anti-cortactin (1:100; 11381-1, Proteintech), anti-vinculin (1:100; 66305-1, Proteintech), anti-Tks5 (1:100; 18976-1, Proteintech), anti-Myo9a (1:50; sc-165026, Santa) and anti-Myo9b (1:50; sc-48246, Santa) at 4°C. Antibodies conjugated to Alexa Fluor 405, 488 and 568 were used as secondary antibodies. Actin was visualized with rhodamine-phalloidin, and nuclei were stained with DAPI. Coverslips were mounted with Mounting Medium for Fluorescence (Vectashield; Vector Laboratories). Images were captured by confocal microscopy (DM6000 CFS, Leica) and processed by LAS AF software. All images of primary cells were taken with 63×NA 0.75 objectives.

Isolation of RNA and Quantitative Real Time PCR
Total RNA was extracted from abdominal arteries, which were homogenized with gentle MACSTM Dissociator (Miltenyi Biotec), and cultured macrophages using the TRIzol (Invitrogen) according to the manufacturer’s instructions. The quality of the RNA was determined using a Biospectrometer (Eppendorf). 1 µg of RNA was subjected to reverse transcription using first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. qRT-PCR of mRNAs was performed using Platinum SYBR Green qPCR Super Mix UDG Kit (Invitrogen), and real-time PCR experiments were carried on an ABI 7500 FAST system (Life Technologies). Relative amount of transcripts was normalized with GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ formula as previously described⁴. All PCRs were performed in triplicate. The primer sequences were as Table 4.

Western Blot Analysis
Protein was isolated from cells as previously described⁵, electrophoresed on 10% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked with 5% dry milk in TTBS for 2 h at 37°C and incubated overnight at 4°C with the following primary antibodies: 1:1000 rabbit anti-KLF5 (GTX103289, GeneTex), 1:500 goat anti-Myo9a (sc-165026, santa), 1:500 goat anti-Myo9b (sc-48246, santa), 1:1000
mouse anti-Rac1 (66122-1-Ig, Proteintech), CDC42 (1:1000) and RhoA (1:1000) (rabbit antibodies obtained from Proteintech) and anti-β-actin antibody (1:2,000, Ambion). Membranes were washed and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Protein bands were detected by ECL plus (Thermo scientific) and β-actin served as an internal control for protein loading.

**Oligonucleotide Pull-down Assay**

Oligonucleotide pull-down assay was undertaken as described previously. Oligonucleotides containing the wild-type or mutant TCE site (site-1–5) sequence in the mouse Myo9b promoter with biotin added to their 5’-end were as follows:

**site-1 (wild-type):**
- biotin-5′-GTAGTGCTGGAAGCCGGAGGGTGCTCTT-3′ (forward)
- biotin-5′-AAGAGAGCACCCCTCCGGCTTCCAGCACTAC-3′ (reverse);

**mutant site-1:**
- biotin-5′-GTAGTGCTGGAAGCCGGAGGGAACTCTT-3′ (forward)
- biotin-5′-AAGAGAGGTCCCTCCGGCTTCCAGCACTAC-3′ (reverse)

**site-2 (wild-type):**
- biotin-5′-GATCCCAACTCACCTCGCTCCTCTCTCTT-3′ (forward)
- biotin-5′-TCAGAACGGGACGCAGGTTGGGAT-3′ (reverse)

**mutant site-2:**
- biotin-5′-GATCCCAATTTCCTGCTCCGGCTTCTCTTGA-3′ (forward)
- biotin-5′-TCAGAACGGGACGCAGGAAATTGGGAT-3′ (reverse)

**site-3 (wild-type):**
- biotin-5′-AAGCAAACACCCATGGGTGTAGTGAGTCAG-3′ (forward)
- biotin-5′-CTGACTCACTACACCACATGGGTGTTTGCTT-3′ (reverse)

**mutant site-3:**
- biotin-5′-AAGCAAATCCCATGGGAATAGTGAGTCAG-3′ (forward)
- biotin-5′-CTGACTCACTACATTCCCATGGGAATTTGGGAT-3′ (reverse)

**site-4 (wild-type):**
- biotin-5′-GAGCCTTTGTGTTTCAACCTGACCTCTTCAC-3′ (forward)
- biotin-5′-GTGAAGGTCAAGTGTTGAAACAAAGGCTC-3′ (reverse)

**mutant site-4:**
- biotin-5′-GAGCCTTTGTGTTTCAACCTGACCTCTTCAC-3′ (forward)
- biotin-5′-GTGAAGGTCAAGTGTTGAAACAAAGGCTC-3′ (reverse)

**site-5 (wild-type):**
- biotin-5′-ATCCCAGAGATGGCACCACCCACAAGGG-3′ (forward)
- biotin-5′-CCCTTTGTGGGTGGTCCATCTCTCTAGGAT-3′ (reverse)

**mutant site-5:**
- biotin-5′-ATCCCAGAGATGGCACCACCCACAAGGG-3′ (forward)
- biotin-5′-CCCTTTGTGGGTGGTCCATCTCTCTAGGAT-3′ (reverse).

Each pair of oligonucleotides was annealed following standard protocols. Macrophage lysates or purified recombinant proteins (GST and GST–KLF5) were precleared with Immunopure streptavidin–agarose beads (20 μl/sample, Pierce) for 1 h at 4°C. After centrifugation at 12000 g for 60 s at 4°C, the supernatant was incubated
with 100 pmol of biotinylated double-stranded oligonucleotides and 10 μg of poly (deoxyinosinic-deoxyctydilic) for 16 h at 4°C. DNA-bound proteins were collected with 30 μl of immobilized streptavidin–agarose beads followed by extensive washing. Bound proteins were separated by SDS-PAGE and subjected to Western blotting.

**Small Interfering RNA Transfection**
The siRNA sequences were as follows:

**KLF5 si-RNA:**
- Forward: 5'-GUUCCACAGACGUCAAUGATT-3'
- Reverse: 5'-UCAUUGACGUCUGUGGAACCTT-3'

**Myo9b si-RNA:**
- Forward: 5'-GGGAAGUUCAUCCAAGUCATT-3'
- Reverse: 5'-UGACUUGGAUGACUUCCCTT-3'

**Determination of the Amounts of Active Rho, Cdc42, and Rac Proteins**
The RhoA, Cdc42 and Rac1 activity assays were performed according to the manufacturer’s instructions (Upstate Cell Signaling Solutions, Lake Placid, NY). Active Rho (Rho-GTP) was isolated with GST–rhotekin RBD (GST fusion protein containing the RhoA binding domain of rhotekin) and active Cdc42/Rac1 with GST-PAK-Crib (GST fusion protein containing the Rac/Cdc42-binding domain of p21-activated kinase). Cells were washed with ice-cold PBS and lysed on ice in cell lysis buffer. After determining the protein concentrations by the method of Bradford, equal amounts of total protein per sample were incubated either with GST–rhotekin RBD or GST-PAK-Crib immobilized on glutathione-Sepharose 4B beads (GE Healthcare) at 4°C for 60 or 30 min, respectively. The beads were pelleted and washed three times with lysis buffer. Bound Rho proteins (GTP-RhoA, GTP-Rac1, or GTP-Cdc42) were eluted from the beads with SDS gel sample buffer, boiled for 5 min, and separated by 12% SDS-PAGE. Active Rho proteins were detected by immunoblotting using the following antibodies: anti-RhoA (ProteinTech), anti-Rac1 (ProteinTech), or anti-Cdc42 (ProteinTech). Signals were quantified using Image J.

**Migration and Invasion Assays**
Bone marrow–derived monocytes were grown in macrophage-inducing medium to a confluence of 80% to 100% before the cell monolayer was scratched using a sterile micropipette tip. After washes with PBS, fresh macrophage-specific medium was added. The images of the wounded area were captured immediately after the scratch 0 and 24 h later to monitor macrophage migration into the wounded area. The migratory abilities were quantified by measuring the total number of cells in the scratched regions.

Invasion assays were performed using a Boyden Chamber CytoSelect Cell Migration Assay kit with polycarbonate membrane inserts (8 μm pore size; Cell Biolabs Inc.). Briefly, bone marrow–derived macrophages (10^5 cells) were added to the upper wells at 2×10^4 cells/well. A coverslip pre-seeded with 10^5 CaPO_4-instimulated VSMC were placed at the bottom of the lower. After 6 h at 37°C in 5% CO_2, non-migratory cells on the upper membrane surface were removed, and the cells that traversed and spread on the lower membrane surface were fixed with methanol and stained with HE stain (Modified solution, Sigma). By utilizing a
microscope with a 40× objective, the number of migratory cells per membrane was enumerated. Four random fields in each filter were examined. Each experiment was performed in triplicate, and migration was expressed as the mean ± SEM of total cells counted per field.

**Scanning Electron Microscopy**

Scanning electron microscopy was used to examine the morphological characteristics of macrophages in migration assays. Cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3). After washing in buffer, cells were dehydrated in a graded series of ethanol solutions. After critical-point drying (Bal-Tec CPD 030), the samples were sputtered with gold (Denton Desk II sputter coater), and the probes were examined by scanning electron microscopy (FEI XL30 ESEM; Philips). Macrophages from 3 myeKlf5−/− mice and 3 WT mice were analyzed.

**Time-Lapse Experiments**

Macrophages from femoral bone marrow suspensions of WT mice and myeKlf5−/− mice were cultured in vitro. Time-lapse images were acquired on a Leica AF6000 inverted microscope equipped with an on-stage incubation chamber that maintained the temperature at 37°C and the CO2 concentration at 5% at all times. Macrophages were imaged via a 40× objective, and repetitive imaging was performed every 15 min for up to 12 h. Quantification of cell shapes and determination of the location were performed on an image analysis by using NIH Image J software. The cells were connected to generate the trace for the grouping of directions and calculation of Mean velocity of migrating cells. All cells counted were separated by at least 300 μm from all other cells at all times. We counted 20-35 cells for each type of pattern.

**Phagocytosis Assay**

The phagocytic ability of WT and myeKlf5−/− macrophages was detected using the CytoSelect™ 96-well phagocytosis assay kit (Cell Biolabs Inc., San Diego, CA, USA), following the manufacturer’s instructions. Macrophages (1×10^4 cell/well) were plated in 96-well plates and incubated overnight at 37°C to allow adherence to the plate. Subsequently, non-opsonized zymosan particles were added incubated at 37°C for 2 h. The amount of engulfed zymosan particles was determined using a colorimetric assay at an absorbance of 405 nm.

**Supplemental References**


Online Figure I

A

Representative axial CT and its corresponding 3D volume-rendered imaging of abdomen from a clinical normal-appearing abdominal aorta (a, c) and an AAA (b, d).

B

Rabbit IgG + Mouse IgG (primary antibodies)

488 Donkey 546 Donkey

Anti-Mouse IgG Anti-Rabbit IgG Merged

Online Figure I

Analysis of human aneurysmal and normal aortic tissues. (A) Representative axial CT and its corresponding 3D volume-rendered imaging of abdomen from a clinical normal-appearing abdominal aorta (a, c) and an AAA (b, d). (B) Representative images of immunohistochemical quality controls. Human aortic cross-sections were incubated with the indicated IgG antibodies followed by incubation with the indicated secondary antibodies. Scale bar=50 μm.
Online Figure II

**A**

Saline  |  Ang II

**B**

![Boxplot showing maximal aortic external diameter](image)

**C**

KLF5  |  MAC2  |  Merge

**D**

KLF5  |  SMA-α  |  Merge

**E**

Rabbit IgG + Mouse IgG (primary antibodies)

488 Donkey  |  546 Donkey

Anti-Mouse IgG  |  Anti-Rabbit IgG  |  Merged
Online Figure Ⅱ

Ang II-induced abdominal aortic aneurysm in ApoE−/− mice. (A) Representative photographs of aorta from ApoE−/− mice infused with Ang II (1000 ng/kg/min) or saline. (B) The external diameter of the aorta (0.68 ± 0.02 mm with saline vs. 1.63 ± 0.77 mm with Ang II), n=20 per group. ***P<0.001 vs. saline. (C and D) Representative photographs of aortic cross-sections from ApoE−/− mice infused with Ang II for 28 d. Sections were stained with anti-KLF5 (red) and anti-MAC2 (green) (C) or anti-KLF5 (red) and smooth muscle α-actin (SMA-α, green) (D). (E) Representative images of immunohistochemical quality controls. Sections from ApoE−/− mice infused with Ang II for 28 d were stained with an isotype control antibody. Scale bars=50 μm.
Online Figure Ⅲ

A

B

Days after CaPO₄ treatment

% Abdominal aortic dilation (maximum/control)

Days after CaPO₄ treatment

C

D

E

F

G
Online Figure III

KLF5 is elevated in CaPO₄-induced AAA model. (A) Representative photographs of male C57BL/6 mouse abdominal aortic dilation at different days after application of CaPO₄. (B) Maximal percentage of aortic dilation is shown for CaPO₄-treated mice and PBS-treated mice (control). *P < 0.05 and **P < 0.01 vs. control. (C) Representative hematoxylin and eosin (HE)-stained sections of mouse aortas exposed to CaPO₄ for 0, 3, 7 and 14 d. Scale bar=200 μm. (D) KLF5 mRNA expression in the injured aortas of C57BL/6 mice treated with CaPO₄ or PBS was determined by qRT-PCR. Data represent the mean±SEM. *P<0.05 vs. PBS. n=4 in each group. (E) Representative photographs of aortic cross-sections stained with KLF5 antibody (brown) on days 0, 3, 7, and 14 after CaPO₄ exposure. Scale bars=50 μm. n=4 in each group. (F and G) Confocal immunofluorescence performed on CaPO₄-treated mouse aortic sections stained with F4/80, SMα-actin, KLF5, and DAPI. Scale bars=50 μm.
Online Figure IV

A

[Images of tissue sections, Control vs. myeKlf5−/−]

B

 [% Abdominal aortic dilation (maximum/control)]

C

 [Images of HE and EVG staining, Control vs. myeKlf5−/−]

D

 [Graph showing Media thickness (μm), Control vs. myeKlf5−/−]

E

 [Images of DAPI, MAC2, and KLF5 staining, Control vs. myeKlf5−/−]
Online Figure IV

Macrophage-specific knockout of KLF5 attenuates aneurysm formation. (A) Representative photographs of abdominal aortas from WT (control) and myeKlf5−/− mice exposed to CaPO₄ for 14 d. Scale bars=2.5 mm. (B) The maximal percentage of aortic dilation for control and myeKlf5−/− mice exposed to CaPO₄ for 14 d. Data represent the mean±SEM, n=10 in each group. *P < 0.05 vs. control. (C) Representative photographs of HE-stained sections of aortas from the CaPO₄-induced control and myeKlf5−/− mice and Elastica Van Gieson (EVG)-stained elastin fibers. Scale bars=200 μm. (D) Quantification of media thickness in aortas of control and myeKlf5−/− mice exposed to CaPO₄ for 14 d. *P<0.05 vs. control. (E) Dual immunofluorescence staining of KLF5 (red) and MAC2 (green) in the injured aortas of control and myeKlf5−/− mice exposed to CaPO₄ for 14 d. Scale bars=50 μm.

Online Figure V

The effects of macrophage depletion on CaPO₄-induced AAA formation. (A) Representative photographs of abdominal aortas from clodronate liposome- or PBS liposome-administered mice (n=5) exposed to CaPO₄ for 14 d. Upper panel: H&E staining; Lower panel: F4/80 staining. Scale bars=200 μm and 50 μm. (B) Quantification of infrarenal aortic diameters from clodronate liposome- or PBS liposome-administered mice (*P<0.05, Student’s t-test, n=5 for each group). Results are from three independent experiments. Data are presented as means ± SEM.
KLF5 expression is determined by immunofluorescence staining in endothelial and neutrophils in CaPO₄-induced AAA model. (A) Immunofluorescence staining of KLF5 and CD31 (endothelial cell) on abdominal aortas from control and myeKlf5⁻/⁻ mice exposed to CaPO₄ for 14 d. Scale bars=50 µm. (B) Immunofluorescence staining of KLF5 and Ly6G (neutrophil) on abdominal aortas from control and myeKlf5⁻/⁻ mice exposed to CaPO₄ for 14 d. Scale bars=100 µm.
Knockout of KLF5 reduces infiltration of neutrophils and T-lymphocytes in CaPO₄-induced AAA model. Representative images of infiltration of neutrophils (positive staining for Ly6G) and T-lymphocytes (positive staining for CD3e) into abdominal aorta from CaPO₄-induced control and myeKlf5⁻/⁻ mice. Scale bars= 50 μm.
Online Figure VII

A

WT

myeKlf5−/−

B

WT

myeKlf5−/−

C

WT

myeKlf5−/−

D

WT

myeKlf5−/−

E

WT

myeKlf5−/−

F

Bone Marrow

Ly6G+ neutrophils population (%) Control CaPO4 WT myeKlf5−/−

CD11b+ monocytes population (%) Control CaPO4 WT myeKlf5−/−

SigF+ Eosinophils population (%) Control CaPO4 WT myeKlf5−/−
Online Figure VIII
Characterization of the bone marrow, blood and spleen myeloid compartment using flow cytometry. (A) Bone marrow cytospins from WT and myeKlf5⁻/⁻ mice. Scale bars=20 μm. (B) Representative Wright's-Giemsa's staining images of peripheral blood smear from WT and myeKlf5⁻/⁻ mice. Scale bars=20 μm. Indicate: Lymphocytes (up arrows), neutrophils (down arrows) and monocytes (right arrows). (C-H) Population and quantification of Ly6G⁺ neutrophils (C), CD11b⁺ monocytes (D) and SigF⁺ eosinophils (E) in bone marrow (F), peripheral blood (G) and spleen (H) in WT and myeKlf5⁻/⁻ mice exposed to CaPO₄ or PBS for 14 d. Results are from three independent experiments. Data are presented as means ± SEM. *P<0.05 vs. WT mice.
Online Figure IX

The effect of neutrophil depletion on CaPO₄-induced AAA formation. (A and B) Charts representing the quantification of circulating neutrophils by flow cytometry in mice treated with R-phycoerythrin-conjugated rat anti-mouse Ly6G monoclonal antibody for 5 d. (A) Neutrophils labeled with fluorescent dye exhibited strong fluorescence and high cell counts in control mice treated with PBS (see peak A). (B) Second peak (peak A) was almost nonexistent in mice treated twice with the anti-neutrophil antibody, indicating significant neutrophil depletion. (C) Representative
photographs of abdominal aortas from the CaPO₄-induced control (WT) and anti-Ly6G⁺myeKlf5⁻/⁻ mice exposed to CaPO₄ for 14 d. Scale bars=2.5 mm. (D) The maximal percentage of aortic dilation for control and anti-Ly6G⁺myeKlf5⁻/⁻ mice exposed to CaPO₄ for 14 d. Data represent the mean±SEM, n=10 in each group. **P <0.05 vs. control. (E) Representative images of neutrophil (positive staining for Ly6G) infiltration into the abdominal aorta of control and anti-Ly6G⁺myeKlf5⁻/⁻ mice exposed to CaPO₄ for 14 d. (F and G) Immunolocalization of macrophages (positive staining for MAC2) (F) and vascular smooth muscle cells (positive staining for SMA-α) (G) in abdominal aorta from control and anti-Ly6G⁺ myeKlf5⁻/⁻ mice exposed to CaPO₄ for 14 d. Areas of abdominal aortic cross-sections (E, F, G) represented in boxes are shown at higher magnification. Scale bars= 50 μm. (H) Representative photographs of abdominal aortas from the CaPO₄-induced WT and anti-Ly6G⁺ mice exposed to CaPO₄ for 14 d. Scale bars=2.5 mm. (I) The maximal percentage of aortic dilation for WT and anti-Ly6G⁺ mice exposed to CaPO₄ for 14 d. Data represent the mean±SEM, n=8 in each group. (J) Immunolocalization of neutrophils (positive staining for Ly6G), macrophages (positive staining for MAC2) and vascular smooth muscle cells (positive staining for SMA-α) in abdominal aorta from WT and anti-Ly6G⁺ mice exposed to CaPO₄ for 14 d. Areas of abdominal aortic cross-sections represented in boxes are shown at higher magnification. Scale bars= 50 μm.

Online Figure X

A

Control

myeKlf5⁻/⁻
Online Figure X

Myeloid KLF5 deficiency results in attenuated M1 and enhanced M2 macrophage markers in CaPO₄-injured aortic vessels. (A) Representative flow cytometry plots of macrophages in aortic tissues from control and myeKlf5⁻/⁻ mice exposed to CaPO₄ for 14 d. Phenotype of macrophages was analyzed by F4/80 vs CD54 (M1-type macrophages are defined as F4/80⁺CD54⁻) and F4/80 vs CD206 (M2-type macrophages are defined as F4/80⁺CD206⁻) fluorescence intensity. (B) M1 (TNF-α, iNOS, MCP-1 and IL-1β) and M2 (Arg-1, Mrc1, PPARγ, and Retnla) macrophage markers were analyzed by qRT-PCR in CaPO₄-induced aortic samples (n=3). *P<0.05 and **P<0.01 vs. WT.
**Online Figure XI**

**KLF5 deficiency inhibits macrophage M1 polarization.** (A) The expression of M1 markers, including TNF-α, MCP-1, IL-1β and iNOS, was decreased in KLF5-deficient macrophages stimulated by LPS for 24 h. *P<0.05 vs. WT. n=3 in each group. (B) The expression of M1 marker genes increased in KLF5-overexpressing macrophages. *P<0.05 vs. Ad-null. n=3 in each group. (C and D) Western blot analysis of iNOS expression. KLF5-deficiency (C) reduced, while overexpression of KLF5 (D) increased iNOS expression in LPS-treated macrophages. n=3 in each group.

**Online Figure XII**

**KLF5 deletion in macrophages slightly impairs phagocytosis.** Phagocytic activity was determined using a phagocytosis assay. Data normalized to WT macrophage were presented as the mean ± SEM and analyzed versus respective controls using a Student’s t test (n=3). *P<0.05 vs. WT.
Online Figure XIII

KLF5 promotes macrophage recruitment in AAA but does not affect proliferation. (A) Macrophages (green) recruited into CaPO₄-injured abdominal aortic tissues showed Ki67 (red) negative-staining by immunofluorescence. Scale bars= 50 μm. (B) Western blot analysis of PCNA in WT and KLF5-deficient macrophages. *P< 0.05 vs. WT. n=3 in each group.

Online Figure XIV
Online Figure XIV
Morphology of WT and myeKlf5−/− macrophages observed by scanning electron microscopy. WT and myeKlf5−/− macrophages traversed the filter to the other side of Boyden chamber were observed by scanning electron microscopy.

Online Figure XV
myeKlf5−/− macrophages are defective in their migration ability. (A) TNF-α-stimulated migration of WT and myeKlf5−/− macrophages was traced by using time-lapse imaging. (B and C) Each line represents the movement of each macrophage from WT and myeKlf5−/− mice. (D) Migration distances per hour were measured by Image J. n=21 cells, ***P<0.001 vs. WT.
Online Figure XVI

**KLF5 deletion reduces podosome-rosette formation in macrophages through down-regulating Myo9b expression.** (A) The WT and myeKlf5−/− macrophages were treated or not with TNF-α for 24 h, and then were stimulated for 30 min with PDBu. Fluorescence staining of cortactin (anti-cortactin) and F-actin (rhodamine-phalloidin) was used to visualize podosomes. Scale bars=10 μm. (B and C) The percentage of individual podosome- and podosome-rosette-positive macrophages. *P<0.05 vs. untreated or WT group. (D) Colocalization of Myo9b with F-actin or vinculin and Tks5 in macrophages treated with PDBu and TNF-α. Scale bars=10 μm. (E) myeKlf5−/− macrophages were infected with Ad-Klf5 or Ad-null for 24 h and then treated with TNF-α and PDBu. Podosomes were visualized as described above. Scale bars=10 μm. (F and H) Fluorescence staining of cortactin (anti-cortactin) and F-actin (rhodamine-phalloidin) on AAA tissue sections of the CaPO₄-induced WT mice (F) and human AAA (H). Scale bars=20 μm. (G and I) The percentage of podosome-rosette-positive macrophages in AAA tissues of the CaPO₄-induced WT mice and human AAA. The data represent mean±SEM of 3 independent experiments in which 400 cells were analyzed.
Online Figure XVII

The effect of TNF-α on podosome formation in WT and myeKlf5⁻/⁻ macrophages. Fluorescence staining with anti-cortactin, anti-Myo9b antibodies or rhodamine-phalloidin was used to visualize podosomes in WT and myeKlf5⁻/⁻ macrophages treated or not with TNF-α. Scale bars=10 μm.
Online Figure XVIII

Myo9b knockdown reduces podosome formation and migration of macrophages induced by TNF-α. (A and B) Macrophages were transfected with si-Myo9b or si-NS for 24 h and then treated or not with TNF-α for 24 h. Myo9b knockdown was determined by Western blotting with anti-Myo9b (A). Podosomes were visualized by staining with rhodamine-phalloidin, anti-Myo9b and anti-cortactin antibodies. Scale bars=10 μm (B). (C) The percentage of podosome-rosette-positive macrophages. *P < 0.05 and **P < 0.01 vs. si-NS+TNF-α-untreated group or si-NS+TNF-α group. (D) The macrophage migration was determined by Boyden chamber assay. *P < 0.05 vs. si-NS+TNF-α-untreated group; ***P < 0.001 vs. si-NS+TNF-α-untreated group or si-NS+TNF-α group.
### Online Table I

**Table 1 Clinical information**

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<th>AAA (N = 11)</th>
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<td><strong>Age (yr)</strong></td>
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<tr>
<td><strong>Aorta size (mm)</strong></td>
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<tr>
<td><strong>WBC (10⁹/L)</strong></td>
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<tr>
<td><strong>RBC (10¹²/L)</strong></td>
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<tr>
<td><strong>PLT (10⁹/L)</strong></td>
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<td><strong>NEUT (10⁹/L)</strong></td>
</tr>
<tr>
<td><strong>LYMph (10⁹/L)</strong></td>
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<tr>
<td><strong>MONO (10⁹/L)</strong></td>
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<tr>
<td><strong>Blood pressure (mm Hg)</strong></td>
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<tr>
<td><strong>Systolic</strong></td>
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<tr>
<td><strong>Diastolic</strong></td>
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<tr>
<td><strong>Serum creatinine (μmol/L)</strong></td>
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<td>Diabetes mellitus</td>
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<td>Smoker (%)</td>
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Plus–minus values are means ±SD.
Online Table 2

Table 2 KLF5, Myo9b mRNA level and expansion diameter in AAA patients

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<th>KLF5 mRNA</th>
<th>Myo9b mRNA</th>
<th>Expansion Diameter</th>
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<tbody>
<tr>
<td>1</td>
<td>2.31±0.75</td>
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<td>3</td>
<td>2.32±0.55</td>
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<tr>
<td>4</td>
<td>1.78±0.64</td>
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<tr>
<td>5</td>
<td>1.45±0.42</td>
<td>2.02±0.37</td>
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<tr>
<td>6</td>
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<td>7</td>
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<td>11</td>
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Plus–minus values are means ±S.D.
Table 3 Hematologic analysis of WT and KLF5<sup>-/-</sup> mice

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<th>Parameter</th>
<th>WT</th>
<th>KLF5&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Normal range</th>
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<td>White blood cell count (x10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>5.99 ± 0.26</td>
<td>5.47 ± 0.19</td>
<td>[5–15]</td>
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<td>Lymphocytes, (x10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>4.23 ± 0.21</td>
<td>4.18 ± 0.12</td>
<td>[3.4–7.44]</td>
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<tr>
<td>Monocytes, (x10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>0.38 ± 0.03</td>
<td>0.35 ± 0.04</td>
<td>[0.0–0.6]</td>
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<tr>
<td>Granulocytes, (x10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>0.91 ± 0.07</td>
<td>0.87 ± 0.03</td>
<td>[0.5–3.8]</td>
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Data are expressed as mean±s.e.m. Peripheral blood differentials are shown as absolute counts.
## Online Table 4

### The primers for real-time PCR

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<th>Primer</th>
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<th>Primer</th>
<th>Sequence (5' to 3')</th>
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<td>GAPDH</td>
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<tr>
<td></td>
<td>reverse</td>
<td>TGGGAGTAGACAAGTACACCCC</td>
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<tr>
<td>IL-1β</td>
<td>forward</td>
<td>CAACCCACAAAGTATATTTCCTCGATG</td>
<td>KLF5</td>
<td>forward</td>
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<tr>
<td></td>
<td>reverse</td>
<td>GATTCCACACTCTCCAGCTGCA</td>
<td>(human)</td>
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<tr>
<td>MCP-1</td>
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<td>Myo9b</td>
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<td>ATGTCTGGACCCATTTCCTC</td>
<td>(human)</td>
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