KLF4 Regulates Abdominal Aortic Aneurysm Morphology and Deletion Attenuates Aneurysm Formation

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Background—KLF4 mediates inflammatory responses after vascular injury/disease; however, the role of KLF4 in abdominal aortic aneurysms (AAAs) remains unknown. The goals of the present study were to (1) determine the role of KLF4 in experimental AAA; and (2) determine the effect of KLF4 on smooth muscle (SM) cells in AAAs.

Methods and Results—KLF4 expression progressively increased at days 3, 7, and 14 after aortic elastase perfusion in C57BL/6 mice. Separately, loss of a KLF4 allele conferred AAA protection using ERTCre+ KLF4 flx/wt mice in the elastase AAA model. In a third set of experiments, SM-specific loss of 1 and 2 KLF4 alleles resulted in progressively greater protection using novel transgenic mice (MYHCre+ flx/flx, flx/wt, and wt/wt) in the elastase AAA model compared with control. Elastin degradation, MAC2, and cytokine production (MCP1, tumor necrosis factor-α, and interleukin-23) were significantly attenuated, whereas α-actin staining was increased in KLF4 knockout mice versus controls. Results were verified in global KLF4 and SM-specific knockout mice using an angiotensin II model of aneurysm formation. KLF4 inhibition with siRNA attenuated downregulation of SM gene expression in vitro, whereas in vivo studies demonstrated that KLF4 binds to promoters of SM genes by chromatin immunoprecipitation analysis. Finally, human aortic aneurysms demonstrated significantly higher KLF4 expression that was localized to SM cells.

Conclusions—KLF4 plays a critical role in aortic aneurysm formation via effects on SM cells. These results suggest that KLF4 regulates SM cell phenotypic switching and could be a potential therapeutic target for AAA disease. (Circulation. 2013;128[suppl 1]:S163-S174.)

Key Words: aneurysm ■ KLF4 ■ myocytes, smooth muscle ■ transcription factors ■ vascular biology

Abdominal aortic aneurysms (AAAs) comprise the tenth leading cause of death among men >65 and result in >15,000 surgical procedures annually in the United States.1,2 Although prevalence of aneurysms is on the rise, the mechanisms that regulate aortic aneurysm formation remain unclear.1,2 Currently, aneurysm formation is normally accompanied by destruction of the intimal layer and loss of aortic smooth muscle cell (SMC) contractile function.3,4 Furthermore, AAA formation is often accompanied by mural thrombus deposition, and infiltration by mesenchymal cells, processes influenced by immune regulation.5,6 Finally, collagen and elastin extracellular matrix of the abdominal aorta is thought to undergo degradation and contribute to AAA formation via a myriad of mechanisms modulated by inflammatory cells.5,6 The effects of SMC apoptosis accompanied by extracellular matrix degradation of elastin and collagen lead to aneurysm formation; however, the mechanism that regulates this process and the cell–cell interactions is unknown.

SMCs are remarkably plastic and transition from a quiescent contractile state to a proliferative-migratory state during a process known as phenotypic switching.7 This process is characterized by the coordinate downregulation of markers of differentiated SMCs, including SM22α, smooth muscle myosin heavy chain (SM-MHC), and SM α-actin genes required for SMC contraction.7 SMC phenotypic plasticity likely evolved for optimization of vascular repair after injury,8 although it is also widely accepted that SMC phenotypic switching plays a key role in development and progression of atherosclerotic lesions9 and regulation of plaque stability. Our laboratory was the first to demonstrate that SMC phenotypic switching was an early event in aortic aneurysm formation.1 However, there is currently little information to describe the mechanism of SMC phenotypic switching in the context of aneurysm formation.4

Previously, KLF4 has been shown to be a key factor required for SMC phenotypic switching after PDG-BB and POVPc treatments.10–13 KLF4 has also been shown to play a critical role in the maintenance of pluripotency in embryonic stem cells,14 then declines as embryonic stem cells differentiate into various cell types. KLF4 is transcriptionally induced in macrophages, SMCs, and other cell types with vascular
injury inflammation.\textsuperscript{5,16} KLF4 along with Oct4, Sox2, and c-myc is also capable of reprogramming somatic cells into induced pluripotent stem cells.\textsuperscript{17} In addition, global conditional KLF4 knockout mice demonstrated a delay in SMC phenotypic switching after carotid artery endothelial injury.\textsuperscript{18} Because SMC phenotypic switching occurs early in AAA formation, and this occurs, in part, through the effects of KLF4 on SM phenotype and aneurysm disease. In the present studies, we hypothesize that KLF4 deletion results in attenuated aneurysm formation and this occurs, in part, through phenotypic modulation of SMCs.

**Materials and Methods**

**Defining KLF4 in the Murine Elastase Aneurysm Model**

Two groups of 8- to 12-week-old wild-type (WT) C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) underwent the murine aortic aneurysm model with either elastase or saline perfusion (WT elastase, WT saline) as described previously.\textsuperscript{3,4,10-12} Infrarenal abdominal aortas from both groups were evaluated and harvested for tissue analysis at days 0, 3, 7, and 14.

**Murine Elastase Model**

Eight- to 12-week-old male mice were injected with intraperitoneal ketamine solution, perfused with elastase, and harvested as previously described, and the procedure was performed by Johnston et al.\textsuperscript{4,10-12} The aortas (or aneurysms, when present) were harvested, and either (1) snap-frozen in liquid nitrogen for analyses by real-time polymerase chain reaction (PCR) or protein extraction, or (2) incubated overnight for histology or immunohistochemistry. Animal care and use were in accordance with the Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the University of Virginia Institutional Animal Care and Use Committee (#3634) in compliance with the Office of Laboratory Animal Welfare.

**Creation of KLF4 Transgenic Mice**

ERTCre\textsuperscript{+} KLF4 flx/wt mice and ERTCre\textsuperscript{+} KLF4 flx/wt mice were created by breeding ERT2 tamoxifen Cre mice with KLF4 flx/flx mice.\textsuperscript{15,18,22} These mice are heterozygous knockouts of KLF4 in all cell types and are necessary to use as ERTCre\textsuperscript{+} KLF4 flx/flx mice are lethal injections as they develop epithelial and gastrointestinal defects.\textsuperscript{15,25} When performing experiments, ERTCre\textsuperscript{+} KLF4 flx/wt mice and their sibling age-matched controls were used when performing elastase perfusion. These mice underwent a series of 10 tamoxifen injections at 6 to 8 weeks during a 14-day period followed by 14 days of rest. These mice then underwent elastase perfusion followed by harvest 14 days later.

Mice were bred with ERTCre\textsuperscript{+} KLF4 flx/wt mice and MYHC\textsuperscript{+} KLF4 flx/wt, and MYHC\textsuperscript{+} KLF4 flx/wt mice were created by breeding MYHC\textsuperscript{+} KLF4 flx/wt mice with KLF4 flx/flx mice.\textsuperscript{15,18,22} These mice were then back-bred together to produce the experimental and control MYHC\textsuperscript{+} KLF4 flx/flx mice. The MYHC\textsuperscript{+} transgene has been used successfully in the past to demonstrate SM-specific knockout of various genes after tamoxifen injections.\textsuperscript{15,25} These mice underwent the same series of injections and injuries as mentioned for the ERTCre\textsuperscript{+} mice above.

ERTCre\textsuperscript{+} KLF4 flx/wt ApoE\textsuperscript{-/-} mice were created by breeding ERTCre\textsuperscript{+} KLF4 flx/flx onto an ApoE\textsuperscript{-/-} background. Once ERTCre\textsuperscript{+} KLF4 flx/flx ApoE\textsuperscript{-/-} mice were created, these mice were then bred with ApoE\textsuperscript{-/-} mice to produce the experimental ERTCre\textsuperscript{+} KLF4 flx/wt ApoE\textsuperscript{-/-} mice and their experimental controls. These mice underwent the same series of 10 tamoxifen injections at 6 to 8 weeks followed by 2 weeks of rest and then angiotensin II infusion via osmotic pump.

Mice were then back-bred together to create MYHC\textsuperscript{+} KLF4 flx/wt ApoE\textsuperscript{-/-} mice, which were then bred together to produce the experimental MYHC\textsuperscript{+} KLF4 flx/flx and flx/wt ApoE\textsuperscript{-/-} mice and their WT controls. These mice also underwent the same series of injections, 2 weeks of rest, and angiotensin II-infused osmotic pump insertion as mentioned previously.

**Angiotensin II Infusion**

Osmotic pumps (Alzet 2004, Durect Corp, Cupertino, CA) containing angiotensin II (1000 ng/kg per minute, Sigma Aldrich Inc, St. Louis, MO) were introduced into 10-week-old ERTCre\textsuperscript{+} KLF4 flx/wt ApoE\textsuperscript{-/-} and ERTCre\textsuperscript{+} KLF4 flx/wt ApoE\textsuperscript{-/-} male mice as previously described.\textsuperscript{26,27} Mice were housed and maintained at 70°F, at 50% humidity, and in 12-hour light-dark cycles per institutional animal protocols. All mice were fed ad libitum water and placed on high-fat diet (TD 88137, Harlan Teklad Inc, Indianapolis, IN) with no restrictions on movement. Aneurysm segments of the aortas (proximal to the renal arteries) were harvested after 28 days and processed for histology. At day 28, video micrometry measurements of the aortic wall diameter were performed in situ using a Q-Color3 Optical Camera (Olympus Corp, Center Valley, PA) using QCapture Pro Software version 6.0 (QImaging Inc, Surrey, Canada). MYHC\textsuperscript{+} KLF4 flx/flx, flx/wt, and wt/wt ApoE\textsuperscript{-/-} male mice underwent a similar procedure at 10 weeks as mentioned above. Kaplan–Meier curves and log-rank (Mantel–Cox) tests tracked the percentage survival of mice during the 28-day period.

In a second model of angiotensin II infusion, osmotic pumps containing angiotensin II were introduced into 10-week-old ERTCre\textsuperscript{+} KLF4 flx/wt and ERTCre\textsuperscript{−} KLF4 flx/wt male mice as previously described.\textsuperscript{26,27} After angiotensin II infusion, mice received biweekly injections of anti-transforming growth factor-β (TGFβ) antibody (R&D systems).\textsuperscript{29} After 28 days, aneurysmal segments were harvested as mentioned in the previous paragraph and percentage survival was tracked and log-rank (Mantel–Cox) tests were performed. The same procedure was performed for MYHC\textsuperscript{+} KLF4 flx/flx, flx/wt, and wt/wt mice at 10 weeks.

**mRNA and Protein Isolation**

mRNA and protein were extracted from frozen aortic tissue samples with TRIzol reagent (Invitrogen, Life Technologies, Grand Island, NY) as previously described.\textsuperscript{5,19,20}

**Real-Time Reverse Transcription PCR Analysis**

Using isolated mRNA from the mouse aortas and aortic SMCs, cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time reverse transcription PCR was performed using Sensifast SYBR Supermix (BioLine, Taunton, MA) with primers described previously.\textsuperscript{10,29-31} Target DNA was analyzed with Bio-Rad CFX Manager software (Bio-Rad, Hercules, CA) containing 18s rRNA, which served as a housekeeping reference. Levels of mRNA were standardised either with GAPDH or 18s mRNA, which served as a housekeeping gene for comparison. All experiments were run 3× in triplicate unless otherwise mentioned.

**Histology**

Murine aortas were harvested at euthanasia for histology analysis after undergoing left ventricular puncture and 4% paraformaldehyde antegrade perfusion at physiological pressure. Further fixation was achieved by overnight incubation in 4% paraformaldehyde at 4°C followed by paraffin embedding and sectioning at 5 µm. After microwave antigen retrieval, antibodies were bound and detected using VectaStain Elite Kit (Vector Laboratories Inc, Burlingame, CA). Antibodies for immunohistochemical staining were anti-rat Mac2 for macrophages (1:10000; Cedarlane Laboratories, Burlington, Canada), anti-mouse–anti-neutrophil (Ly 6B.2) for neutrophils (1:10000; AbD Serotec, Oxford, United Kingdom), anti-goat MMP2 for matrix metalloproteinase-2 (1:350; R&D Systems, Minneapolis, MN), anti-goat MMP9 for matrix metalloproteinase-9 (1:400; R&D Systems, Minneapolis, MN), and anti-mouse SMαA for SM α-actin.
α-actin (1:1000; Santa Cruz Biotechnology Inc, Santa Cruz, CA). Visualization color development was completed using diaminobenzidine (Dako Corporation, Carpinteria, CA) for SMα, Mac2, anti-neutrophil, CD3ε, and MMP9.

Confocal immunohistochemistry was performed using immunofluorescent staining for KLF4 (1:200 dilution, R&D); macrophages with mac-2 (1:1000, Cedarlane Laboratories), SMCs with SM α-actin (1:1000, Abcam), and nuclei with 4,6-diamidino-2-phenylindole (DAPI; 1:10000, Invitrogen). Appropriate controls verified staining procedures. Human confocal immunofluorescent staining was performed for KLF4 (1:1000) dilution followed by TSA amplification using a TSA kit (Invitrogen), SM α-actin for SMCs (1:1000, Cy3, Sigma Aldrich Corporation), and nuclei using DAPI (1:10000; Invitrogen). Images were acquired using Axiocam Software version 4.6 via ×10, ×40, and ×100 objectives and an Axiocam MRc camera (Carl Zeiss Inc, Thornwood, NY). Threshold-gated positive signal was detected within the AOI and quantified using ImagePro Plus version 7.0 (Media Cybernetics Inc, Bethesda, MD). Elastin degradation was quantified by counting the number of breaks per vessel and then averaged and graphed.

Cytokine Array

For the purpose of determining the effects of KLF4 total cell and SMC deletion on proinflammatory cytokines in the aortic wall, mouse cytokine arrays (R&D Systems) were performed using isolated protein from mouse aortas at day 14 according to the manufacturer’s instructions (n=3 mice per group). Protein samples from each group were pooled for analysis, and all samples were run in duplicate.

Mouse Aortic SMC Cultures

Mouse abdominal aortic SM was isolated and cultured as previously described. For siRNA transfections, cells at passages 8 to 10 were plated in all 6 wells of a 6-well plate at 1×10⁶ cells per well; 24 hours later, cells were transfected with either a single siRNA to mouse KLF4 or a nontargeting control. Twenty-four hours later, mouse aortic SMCs were stimulated by elastin degradation products, phorbol ester, TGFβ, retinoic acid or interleukin (IL)-1β (Upstate Biotechnology), or representative vehicle for 24 hours as described previously. Cells were harvested and RNA was extracted using the TriZol method, and reverse transcription PCR was performed as described previously.

Chromatin Immunoprecipitation Analysis

Quantitative chromatin immunoprecipitation assays were performed as described previously. For each in vivo experiment, 15 AAA samples were pooled into 3 sets of 5 samples each and chromatin immunoprecipitation analysis was performed. Experiments were performed in triplicate and 1 representative experiment was shown. Antibodies include polyclonal KLF4 (R&D Systems). Real-time PCR primers were designed as previously described for SM α-actin, SM22α, cFOS, and SM-MHC. For quantitative PCR, 2 μL of the 20-μL extracted DNA was used in 50 cycles of amplification in 3 steps: 95°C for 15 s, 55°C for 30 s, and 68°C for 45 s. At the end of the amplification cycles, dissociation curves were determined to rule out signal from primer dimers and other nonspecific dsDNA species. Data were normalized to IgG immunoprecipitated DNA levels. The size of the PCR products was confirmed on a 2% agarose gel stained with ethidium bromide.

Human Tissue Harvest

Normal abdominal aortas were obtained from transplant donors, and aneurysmal aortas were taken from patients undergoing elective open AAA repair. All aortic samples were harvested from patients without evidence of known collagen vascular disease. Aortic tissue specimens were explanted, placed on ice, and then immediately snap-frozen in liquid nitrogen. Collection of human tissue was approved by the patient’s written consent in compliance with the University of Virginia’s Human Subjects Review Committee (HSR #13178).

Statistical Methods

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Maximal aortic dilation (%) was calculated as [maximal aortic diameter−internal control diameter]/ internal control diameter] ×100%. The internal control was a small segment of normal abdominal aorta just distal to the renal arteries that was above the proximal ligation. This section was not perfused with elastase, but it was susceptible to blood pressure changes from volume loss during the harvest, as well as expected animal growth over time. Values are reported as mean±SE of the mean. Aortic dilation between groups was compared using Fisher exact test. Post hoc Tukey correction was applied to determine the significance of individual comparisons with α=0.05. When groups were compared, the Fisher exact test was used for significance. Pearson correlation coefficients (R) were used to determine strength of linear dependence and are reported as R value (95% confidence interval). Differences between groups for histological grading for macrophage infiltration, neutrophil infiltration, and elastin degradation were tested with the Fisher exact test. Angiotensin II experiment differences were measured using a log-rank (Mantel–Cox) test. All assays were performed in triplicate unless stated otherwise. Blinding to experimental groups was maintained as feasible. Data are presented as mean±SEM.

Results

KLF4 Is Elevated During Experimental Aneurysm Formation

KLF4 has previously been shown to be a key regulator of endothelial vascular injury and to be key in the downregulation of SM marker gene expression. Therefore, we sought to determine whether KLF4 expression increases during aneurysm formation. Aortic dilation in WT mice was significantly increased after elastase perfusion compared with saline perfusion. Although no differences in aortic dilation were present at day 3, aortic dilation was apparent at day 7 (61.8±4.0% with elastase versus 47.6±4.1% with saline; P<0.05) and most prominent 14 days after perfusion (90.1±3.7% with elastase versus 45.8±5.1% with saline; P<0.0001).

At baseline, murine aortas have low levels of KLF4 expression. Although there were no differences in KLF4 expression at 3 days, KLF4 expression at 7 and 14 days in elastase-perfused aortas increased significantly compared with saline-perfused aortas. Furthermore, by immunohistochemistry, KLF4 expression progressively increased after elastase perfusion as aortic diameter increased (Figure 1A; Figure 1B in the online-only Data Supplement). To determine which cells types specifically expressed KLF4, confocal fluorescence microscopy was performed to evaluate macrophages, SMCs, and KLF4 7 days after elastase exposure. KLF4 colocalized primarily to intramural SMCs and macrophages (Figure 1C; Figure 1B and IC in the online-only Data Supplement). Aortic samples from 14-day elastase-perfused aortas demonstrated decreased SM marker expression (data not shown), consistent with ours and others’ previous studies.

Heterozygous KLF4 Deletion Results in Attenuated Aneurysm Formation

With KLF4 levels upregulated in AAAs and known to alter vascular injury responses, we hypothesized that KLF4 plays a critical role in aneurysm formation. To test this, we required synthesis of unique transgenic mice because conventional KLF4 knockout mice are embryonic lethal.
Tamoxifen-inducible ERTCre+ KLF4 flx/wt mice and their controls were generated. These mice are total-cell tamoxifen-dependent KLF4 heterozygous knockouts and were necessary to use because inducible homozygous KLF4 knockouts are lethal because of epithelial skin and gastrointestinal defects. Heterozygous KLF4 knockout mice received 10 tamoxifen injections to induce KLF4 loss. After a 2-week recovery period, elastase perfusion was performed and mice were harvested at 14 days (Figure 2A). Heterozygous KLF4 knockout mice had significant reduction in maximal aortic dilation compared with WT mice (ERTCre+ KLF4 flx/wt: 50.19±17.19%, n=10 versus ERTCre− KLF4 flx/wt: 72.27±27.13%, n=17; \( P < 0.003 \); Figure 2B).

Next, given the significant difference in aneurysm formation between KLF4 flx/wt ERT Cre+ mice and their controls, we examined elastin degradation and expression of SM \( \alpha \)-actin and MAC2 into the medial cell layer at 14 days (Figure 2A). Heterozygous KLF4 knockout mice had significant reduction in maximal aortic dilation compared with WT mice (ERTCre+ KLF4 flx/wt: 50.19±17.19%, n=10 versus ERTCre− KLF4 flx/wt: 72.27±27.13%, n=17; \( P < 0.003 \); Figure 2B).

Figure 1. KLF4 is localized to smooth muscle (SM) cells and macrophages after elastase perfusion. A, C57Bl/6 mice underwent aortic perfusion with elastase or saline and animals were recovered. Aortas were harvested on days 0, 3, 7, and 14. Reverse transcription polymerase chain reaction for KLF4 gene expression relative to GAPDH from C57Bl/6 mice perfused with elastase or saline over time. n=6 mice per group. *\( P < 0.05 \) for wild-type (WT) elastase vs saline, whereas ++\( P < 0.005 \) for WT elastase vs saline via 2-way ANOVA. B, Aortic cross-sectional histology with KLF4 antibody at days 0, 3, 7, and 14 days after elastase perfusion (n=6 mice per group). C and D, Confocal immunohistochemistry performed on aortic tissue from a WT elastase-perfused mouse on day with macrophages (MAC2), SM cells (SM actin), KLF4, and cell nuclei (4′,6-diamidino-2-phenylindole [DAPI]). Confocal studies done at day 14 are not shown.

SM-Specific Deletion of KLF4 Attenuates Aneurysm Formation

As there are both SM and leukocyte sources of KLF4 (Figure 1C), we next sought to determine the contribution of SM-derived KLF4 in AAAs. To investigate this, SM-specific myosin heavy chain (MYH) Cre mice were bred with KLF4 flx/flx mice to produce novel MYHCre+ KLF4 Flx/Flx, MYHCre+ KLF4 Flx/wt, and MYHCre+ KLF4 WT/WT mice (Figure III in the online-only Data Supplement). Using a tamoxifen-elastase experimental design (Figure 3A), deletion of 1 or 2 alleles of SM-specific KLF4 showed significant step-wise protection from AAA formation compared with controls at 14 days (Figure 2C and 2D; Figure II in the online-only Data Supplement). The preceding data suggest that deletion of even 1 allele of KLF4 in the entire animal attenuates aneurysm formation and demonstrates that KLF4 is important for SMC marker gene expression, as well as macrophage and neutrophil recruitment into the aorta.
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85.89±24.45, n=11; Figure 3B). Elastin degradation was significantly less in heterozygous mice (MYHCre+ KLF4 flx/wt) and even greater protection in homozygous SM-deleted KLF4 mice (MYHCre+ KLF4 flx/flx; Figure 3C). Similarly, there was evidence of increasing protection from macrophage and neutrophil inflammation in the MYHCre+ KLF4 flx/wt and flx/flx animals compared with WT animals (Figure 3C; Figure IV in the online-only Data Supplement). SMC deletion of KLF4 resulted in significantly less MMP2 and MMP9 staining (Figure 3C and 3D; Figure IV in the online-only Data Supplement).

KLF4 Modulates Cytokine Expression and SM Marker Genes During Aneurysm Formation

To determine cytokines involved in AAA formation that may be modulated by KLF4, cytokine protein array analysis was performed on purified protein extracts from elastase-perfused aortic tissue harvested 14 days after perfusion. Aortic tissue from total-cell heterozygous deletion had significantly less MCP1, tumor necrosis factor-α, CCL12, and IL-23 (Figure 4). Conversely, there were increased levels of IL-10, a known anti-inflammatory cytokine (Figure 4). Interestingly, KLF4 did not modulate IL-1β CXCL12 or complement component 5A cytokine levels, which have been linked previously to experimental AAAs.20,37

Cytokine array analysis was also performed on aortic tissue harvested on day 14 from SM-specific homozygous KLF4-deleted mice (MYHCre+ KLF4 flx/flx) and WT control mice (MYHCre+ KLF4 wt/wt). Cytokine levels of MCP1, tumor necrosis factor-α, CXCL12, and IL-23 were decreased in KLF4-deleted mice (MYHCre+ KLF4 flx/flx, P<0.05 by Fisher exact test for all; Figure 4). Moreover, SM-specific knockout of KLF4 increased IL-10 levels. KLF4 knockout in SMCs did not modulate IL-1β CCL12 or complement component 5A cytokine levels (Figure 4). Of interest, CXCL12 was not modulated in global heterozygous KLF4 knockout mice (ERTCre+ KLF4 flx/wt), whereas SM-specific knockout of KLF4 modulated CXCL12. Conversely, CCL12 was modulated with total-cell heterozygous knockout of KLF4, whereas KLF4 was not modulated in the SM-specific knockout of KLF4.
To determine whether these protein cytokine levels are reflected in changes in staining patterns, we stained total-cell heterozygous and SM-specific KLF4 knockout mice with an antibody to MCP1, a cytokine we previously documented to be highly critical in experimental AAAs.3 Heterozygous and SM-specific knockout of KLF4 resulted in decreased MCP1 staining (Figure V in the online-only Data Supplement). Taken together, these data indicate that KLF4 deletion decreased protein cytokine levels of several relevant inflammatory cytokines in aortic aneurysms.

Figures 3A and 3B. Smooth muscle (SM)–specific knockout of KLF4 results in attenuated aneurysm formation. A, Model depicting the timeline of tamoxifen injections followed by recovery and subsequent elastase perfusion for the MYHCre+/−/KLF4 mice. B, Maximal percentage of aortic dilation is shown for the 3 groups of mice at day 14. Sample images are shown (MYHCre+/+ KLF4 Flx/FloxFlo: 57.97±18.64%, n=18 vs KLF4 Flx/FloxFlo: 64.83±16.84%, n=8 vs MYHCre+/− KLF4 wild-type [WT]/WT: 85.89±24.45%, n=11; P<0.05 by Fisher exact test). C, Elastin fibers shown with Verhoeff-Van Gieson (VVG) staining. SM cell staining shown by SM α-actin staining. Macrophage and matrix metalloproteinase-9 (MMP9) staining were visualized with MAC2 and anti-MMP9, respectively. D, Quantification of number of elastin breaks per vessel. *Significantly less elastin breaks per vessel with a P value of <0.05 compared with WT controls. Quantification of SM α-actin, MAC2, and MMP9 staining using imagePro. *Significantly less staining with a P value of <0.05 by Fisher exact test as compared with WT elastase-perfused controls.

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**siRNA to KLF4 Inhibits Downregulation of SMC Marker Genes In Vitro**

Previously, KLF4 has been shown to repress SMC marker gene expression in vivo and in cultured SMC through multiple mechanisms10,34,38 and most recently via directly binding to SM marker genes in vivo via the G/C repressor element to mediate transcriptional silencing after injury.13 Currently, there is no known role for KLF4 modulation of SM marker genes during aneurysm formation. To determine whether KLF4 could mediate SMC marker gene downregulation during aneurysm formation, we isolated primary mouse abdominal aortic SMCs. We next treated these cells with siRNAs to KLF4 or control siRNAs followed by novel treatment with elastin degradation products (10 ng/mL). We verified that KLF4 mRNA expression was downregulated via reverse transcription PCR with siRNA treatment (Figure 5A). Second, we examined the levels of SM α-actin, SM22α, and SM-MHC levels after elastin degradation product treatment (EDP; Figure 5B–5D). All 3 SM marker genes were downregulated with treatment of EDP for 24 hours, similar to what has been seen previously with PDGF-BB or POVPC treatments.10,12 Importantly, transfection of siRNAs to KLF4 resulted in a lack of downregulation of SM marker genes after EDP treatment (Figure 5B–5D). IL-1β did not demonstrate deregulation in response to siKLF4 (Figure 5E). These findings are consistent with our in vivo studies and suggest that KLF4 is a key modulator of SM gene expression in vitro after treatment with elastin degradation products.

In separate experiments, we demonstrated that KLF4 seems to be important for SM marker gene downregulation...
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in abdominal aortic SMCs after IL-1β treatment because we have shown previously that IL-1β and receptor-deleted mice are highly protected from aneurysm formation20 (Figure VI in the online-only Data Supplement). In additional subsequent experiments, we demonstrated that KLF4 overexpression alone seems to inhibit SM marker gene expression and that this effect is enhanced with cytokine treatment (Figure VII in the online-only Data Supplement). However, KLF4 did not negatively affect the expression of SM marker genes after retinoic acid, phorbol ester, or TGFβ treatment (Figures VIII–X in the online-only Data Supplement).

KLF4 Binds to Promoters of SMCs In Vitro and In Vivo During Aneurysm Formation

Previously, it has been demonstrated that KLF4 binds directly to SM marker genes after PDGF-BB and POVP treatment13; however, there is no evidence to date that KLF4 modulates SM marker genes during aneurysm formation. We treated abdominal aortic mouse SMCs with elastin degradation products for 24 hours after serum starvation and then performed chromatin immunoprecipitation analysis to determine whether KLF4 binds to SM marker genes after EDP treatment (Figure XI in the online-only Data Supplement). KLF4 significantly binds to the SM α-actin, SM22α, and SM-MHC promoters after EDP treatment (Figure XI in the online-only Data Supplement). These data indicate that KLF4 regulates SM marker gene downregulation in vitro after EDP treatment. Importantly, KLF4 binding to SMC marker genes has never been evaluated in vivo during aneurysm formation. Thus, chromatin immunoprecipitation assays were performed on aortas from WT mice 14 days after elastase perfusion. KLF4 was found to bind specifically to the promoters of SM marker genes SM α-actin, SM22α, and SM-MHC, whereas KLF4 did not bind the cFOS promoter either before or after elastase perfusion (Figure 6). Collectively, these data suggest a new model where KLF4 plays a role in the phenotypic modulation of SMCs and suggest that KLF4 plays a role in aneurysm formation via SMC modulation.

Figure 4. A to D, KLF4 deletion alters cytokines important to aneurysm formation after elastase perfusion. Protein array for ERTCre+ KLF4 flx/wt (global KLF4 heterozygous knockout), ERTCre− KLF4 flx/wt (global control), MYHCre+ wt/wt (smooth muscle [SM] control), and MYHCre+ KLF4 flx/flx (SM homozygous KLF4 knockout) elastase perfused and harvested at day 14 evaluating CXCL12, CCL12, complement component 5A, MCP1, tumor necrosis factor-α, interleukin (IL)-23, IL-1β, and IL-10. Three mouse aortas were pooled for each array. Results are the average of 2 independent experiments. *Significant downregulation of ERTCre+ KLF4 flx/wt mice in comparison with their wild-type (WT) controls with P value of <0.05 by Fisher exact test. **Significant downregulation of MYHCre+ KLF4 flx/flx mice in comparison with their WT controls with P value of <0.05 compared with WT control by Fisher exact test. **+Significant upregulation of ERTCre+ KLF4 flx/wt mice as compared with their WT controls with P value of <0.005 by Fisher exact test. +++Significant upregulation of MYHCre+ KLF4 flx/flx mice in comparison with their controls by Fisher exact test.

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Figure 5. Knockdown of KLF4 modulates smooth muscle (SM) marker genes after elastin degradation product treatment. A to E, Mouse abdominal aortas were isolated, the adventia and endothelial layer stripped, and the abdominal cells were placed in culture. Cells were cultured until passage 6 and SM marker genes were verified at passage 6 and then again at passage 11 for expression (data not shown). From these cultures, cells were plated at 1×10^6 cells per well and treated with siKLF4 or control, scrambled siRNA in serum-free media.25 Twenty-four hours after transfection, cells were treated with elastin degradation products for 24 hours and then harvested, RNA extracted, and reverse transcription polymerase chain reaction performed. Brackets, A significant response (P>0.05) to treatment with elastin degradation products. *Significant downregulation of vehicle-treated cells in response to siKLF4 expression as compared with control-treated siRNA by Fisher exact test. Results are the average of 3 independent experiments performed in triplicate.

KLF4 Deletion Attenuates Aneurysm Formation After Angiotensin II Treatment

Because the elastase perfusion model is criticized as an acute artificial (surgical) model of AAAs, we sought to determine whether KLF4 could also attenuate aneurysm formation in other established models of aneurysm formation. To determine whether KLF4 is protective of aneurysm formation, we used (1) ERTCre+ KLF4 flx/wt mice bred on an ApoE−/− background infused with angiotensin II for 28 days26 (Figure XIIA in the online-only Data Supplement); and (2) ERTCre+ KLF4 flx/wt mice infused with angiotensin II with a series of TGFβ injections (Figure 7A).28 Kaplan–Meier curves of ERTCre+ KLF4 flx/wt–deleted mice demonstrated that KLF4 protects from death because of aneurysm rupture in both angiotensin II models of aneurysm formation (Figure 7A and 7B; Figure VIA in the online-only Data Supplement; χ^2=4.276; P value=0.0387 by log-rank test).

To further study the role of KLF4 in SMCs specifically, we also used these 2 models of angiotensin II aneurysm formation in MYHCre+ KLF4 flx/flx, flx/wt, wt/wt. SM-specific

Figure 6. KLF4 binds smooth muscle (SM) cell marker genes in vivo during aneurysm formation. A to D, Fourteen-day elastase and saline-perfused C57/B6 mice underwent chromatim immunoprecipitation analysis for KLF4 binding in vivo during aneurysm formation.15 Aortas were ground and subsequently underwent formalin crosslinking and sonication. After sonication and preclearage, the homogenate underwent immunoprecipitation for either KLF4 or IgG. The precipitate was then washed and the crosslinking was reversed and proteins denatured. Reverse transcription polymerase chain reaction was then performed for SM α-actin, SM22α, SM-myosin heavy chain, and cFOS. Results are the average of 3 independent pooled experiments of 15 animals each performed in triplicate. *Significant binding over controls with a P value of <0.05 by Fisher exact test.
knockout of KLF4 was also protective of mouse survival during the 28-day course of aneurysm formation after angiotensin II pump insertion (Figure 7B; Figure XIIB in the online-only Data Supplement; \( \chi^2=4.886; P \text{ value}=0.035 \) by log-rank test). These data further demonstrate the critical importance of SM-derived KLF4 in 2 additional models of aneurysm formation enhancing our findings within the elastase model of aneurysm formation.

**Discussion**

Deletion of KLF4 results in decreased aortic aneurysm formation in both a heterozygous and a SM-specific knockout mouse elastase AAA model. KLF4 deletion also affected mouse survival from aneurysm rupture in 2 angiotensin II pump insertion models. Kaplan–Meier survival curves of ERTCre+/− KLF4 flx/wt mice after angiotensin II pump insertion with log-rank tests as indicated.28

Located in SMCs, a key unresolved question is the mechanism of activation of KLF4 expression in SMCs, as the cytokines that upregulate this gene are most likely key to the initiating progression of the aneurysm phenotype. Furthermore, KLF4 activation is of great interest given this gene is normally epigenetically silenced in virtually all differentiated somatic cells.39,40 The KLF4 promoter contains several conserved regulatory elements for AP-1, GATA-1, Sp1, nuclear factor-B, and HLH transcription factors22; however, few studies have directly assessed activation of the KLF4 promoter under different stimuli.30,41 There are several relevant unresolved issues regarding mechanisms responsible for activation of KLF4 during aneurysm formation. First, few studies have been published that have determined mechanisms that activate KLF4 on vascular injury or disease. These mechanisms may be key to blocking upregulation of KLF4, and their inhibition could represent a reasonable drug target against aneurysm formation. Second, the mechanisms responsible for controlling the different roles of KLF4 in various cell types during vascular injury and disease remain unclear and at times contradictory. It is possible that post-translational modifications, such as differential mRNA splicing, or chemical modifications, such as sumoylation or acetylation, regulate effects of KLF4 in different cell types. It has been shown that TGFβ-induced expression of SM α-actin in cultured SMCs is mediated, in part, through inactivation of KLF4 through protein sumoylation.42 Thus, selectively targeting these modifications of KLF4 to produce a net decline

**Figure 7.** KLF4 affects aneurysm formation after angiotensin II pump insertion. A, Model depicting the timeline of tamoxifen injections followed by recovery and subsequent angiotensin II pump insertion for ERTCre+/− KLF4 Flx/wt mice (global heterozygous KLF4-deleted mice). Kaplan–Meier survival curves of ERTCre+/− KLF4 Flx/wt mice after angiotensin II pump insertion with log-rank tests as indicated.28 B, Model for the MYHCre+ KLF4 flx/flx, flx/wt, and wt/wt mice and pump insertion. TGF indicates transforming growth factor.
in aneurysm formation may pose a challenge. Alternatively, targeting the down-stream effects of KLF4 activation could also be considered. Third, other Kruppel-like factors may be involved in regulating aneurysm formation. For example, KLF2 has been shown to have a profound role in atherosclerosis, and future investigation will merit studying both KLF2’s role during aneurysm formation and the interplay among multiple KLFs. In summary, further studies are needed to determine whether these factors regulate activation of KLF4 in SMCs in vivo and if post-translational modifications regulate KLF4 function.

These studies suggest that KLF4 could be a potential therapeutic target against aneurysm formation. However, there are several potential challenges associated with targeting a transcription factor as a potential drug target. Because KLF4 is a transcription factor and can have both positive and negative interactions on many different promoters in many different cell types, the challenge will be to specifically design a drug target that can modulate KLF4’s negative effects on aneurysm formation. Interestingly, our use of a KLF4 heterozygous tamoxifen-dependent total knockout of KLF4 (Figure 2) reflects decreases we would see with pharmacological inhibition of KLF4 and suggests that inhibition of KLF4 in all cell types would be overall beneficial for preventing aneurysm formation. However, it is unknown whether inhibition of KLF4 in humans would ultimately revert SMCs to a noninflammatory phenotype in AAAs. Another additional issue that should be considered is KLF4 is one of the transcription factors required for stem cell function. Our laboratory has demonstrated previously that stem cells seem to be proactive when added exogenously after aneurysm treatments; however, KLF4 expression during aneurysm formation seems to be detrimental for aneurysm formation. How are these issues resolved within the cellular context and what sort of modulated cell type does KLF4 cause SMCs to become? These issues have to be addressed and sufficiently understood in order for KLF4 to be used as a therapeutic target for aortic aneurysms.

In summary, the present studies provide the first direct evidence that KLF4 is an important regulator of aneurysm formation specifically in SMCs. Moreover, our results support a model whereby KLF4 mediates phenotypic switching of SMCs in vivo during aneurysm formation via binding to SM marker gene promoters. Although the present studies have focused on phenotypic modulation of SMCs and KLF4 during aneurysm formation, KLF4 also may have broad significance for the overall aneurysm phenotype given that KLF4 is already known to play key roles in mediating cellular function and proliferation in other cell types. However, further studies are needed to directly test whether KLF4 play similar roles in these other cell types during aneurysm formation.

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References


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Supplementary Material

**KLF4 Regulates Abdominal Aortic Aneurysm Morphology and Deletion Attenuates Aortic Aneurysm Formation**

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**Categories:** Vascular Biology, Basic Science Research, CV surgery: aortic and vascular disease
Supplemental Figures

Supplemental Figure 1. KLF4 protein quantification and z-stacking confocal. A) Quantitation of immunohistochemical staining of Figure 1B. B) Confocal immunofluorescence staining of WT elastase perfused aortas 7 days following injury. White boxes show regions of overlap between KLF4 and SM-actin while circle indicate regions of overlap between KLF4 and Mac2. C) Z-stacking confocal immunofluorescence staining of WT aortas as mentioned in Supplemental Figure 1B.

Supplemental Figure 2. Heterozygous deletion of KLF4 results in attenuated aneurysm formation A) Immunohistochemistry was performed on ERTCre+/- KLF4 flx/wt mice and sample images are shown. B) Staining for neutrophils is shown in brown using an anti-neutrophil antibody, MMP2 stain is shown in brown using an anti-MMP2 antibody. Quantitation of immunohistochemistry from part A. * indicates p-value <0.05.

Supplemental Figure 3. Staining for KLF4 in the smooth muscle specific knock-out mouse model. A-C) Immunohistochemical and confocal staining for KLF4 (red), SM a-actin(green) and DAPI(blue)

Supplemental Figure 4. Smooth muscle specific knock-out of KLF4 results in attenuated aneurysm formation. A) Pictoral examples of dilated aortas 14 days following elastase perfusion. B) Neutrophil staining was visualized using an anti-neutrophil antibody, MMP2 staining was visualized using an antibody to anti-MMP2. Quantitation of staining from part B. * indicates significant staining over controls.

Supplemental Figure 5: KLF4 deletion results in decreased MCP1 staining. A and B) Immunohistochemical staining of MCP-1 in ERTCre+ KLF4 flx/wt, ERTCre- KLF4 flx/wt, and MYHCre+ KLF4 flx/flx, flx/wt and wt/wt mice using an anti-MCP1 antibody.

Supplemental figure 6. Knock-down of KLF4 modulates smooth muscle marker genes following IL1β treatment. Mouse abdominal aortic smooth muscle cells were plated and. 24 hours following
transfection cells were treated with IL1β for 24 hours and then harvested and RNA extracted. * indicates significant expression over control treated siRNA. Results are the average of three independent experiments performed in triplicate.

**Supplemental Figure 7. Over-expression of KLF4 modulates smooth muscle marker genes with IL1β treatment.** Mouse abdominal aortic smooth muscle cells were plated and infected with control or KLF4 adenovirus. 24 hours following transfection cells were treated with IL1β for 24 hours and then harvested and RNA extracted. * indicates significant expression over control treated siRNA. Results are the average of three independent experiments performed in triplicate.

**Supplemental Figure 8. Smooth Muscle Marker gene expression following retinoic acid treatment.** Mouse abdominal aortic smooth muscle cells were plated and treated with siKLF4. 24 hours following transfection cells were treated with Retinoic Acid for 24 hours and then harvested and RNA extracted. * indicates significant expression over control treated siRNA. Results are the average of three independent experiments performed in triplicate.

**Supplemental Figure 9. Smooth Muscle Marker gene expression following TGFβ treatment.** Mouse abdominal aortic smooth muscle cells were plated and treated with siKLF4. 24 hours following transfection cells were treated with TGFbeta for 24 hours and then harvested and RNA extracted. * indicates significant expression over control treated siRNA. Results are the average of three independent experiments performed in triplicate.

**Supplemental Figure 10. Smooth Muscle Marker gene expression following phorbol ester treatment.** Mouse abdominal aortic smooth muscle cells were plated and treated with siKLF4. 24 hours following transfection cells were treated with phorbol ester for 24 hours and then harvested and RNA extracted. * indicates significant expression over control treated siRNA. Results are the average of three independent experiments performed in triplicate.
Supplement Figure 11: KLF4 bind smooth muscle cell marker genes in vitro following elastin degradation product treatment. A-C) Aortic smooth muscle cells were plated and treated as mentioned previously with elastin degradation productions for 24 hours following serum starvation. ChiP assays were performed for KLF4 and then qPCRs were run priming for SM actin, SM22, and SM-MHC promoter. * indicates significant binding over controls. Results were performed three times in triplicate.

Supplemental Figure 12. KLF4 is protective against angiotensin II aneurysm formation A) Model Depicting the process of tamoxifen injections followed by Angiotensin II treatment for the ERTCre +/- KLF4 Flx/wt ApoE/-/- mice. Kaplan-meier curves of ERTCre+/- KLF4 ApoE/-/- flx/wt mice following Angiotensin II treatment. P-values indicate significant survival over WT controls. B) Model depicting the process of tamoxifen injections and angiotensin II treatment of MYHCre+ KLF4. Kaplan-meier curves depicting percent survival free from aneurysm rupture following Angiotensin II treatment. P-values indicate significant survival over WT controls.
Supplementary Figure 1
A. ERT Cre KLF4 heterozygous knockouts

ERT Cre- KFL4 flx/wt (Control)
ERT Cre+ KFL4 flx/wt

B. ERTCre- KLF4 flx/wt
ERTCre+ KLF4 flx/wt

Histologic Grading

<table>
<thead>
<tr>
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<th>ERTCre- KFL4 flx/wt</th>
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<tr>
<td>Neutrophil</td>
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<td>MMP2</td>
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Supplementary Figure 2
Supplementary Figure 3
B. MyHCreKFL4 wt/wt  MyHCreKFL4 flx/flx

spleen

liver

colon

Supplementary Figure 3
A. MyHCre KLF4 knockouts

B. Neutrophil and MMP2 staining

C. MMP2 staining

Supplementary Figure 4
Supplementary Figure 5
Supplementary Figure 6
Supplementary Figure 7
Supplementary Figure 8
Supplementary Figure 9

A. 

- siControl
- siKLF4
- Vehicle
- TGFBeta (10 ng/mL)

SM-actin/18s mRNA

B. 

- siControl
- siKLF4
- Vehicle
- TGFBeta (10 ng/mL)

SM22a/18s mRNA

C. 

- siControl
- siKLF4
- Vehicle
- TGFBeta (10 ng/mL)

SM-MHC/18s mRNA

+ p-value <0.05
Supplementary Figure 10
A. KLF4/INPUT Fold Enrichment over the SM actin promoter

B. KLF4/INPUT Fold Enrichment over the SM22 promoter

C. KLF4/INPUT Fold Enrichment over the SM-MHC promoter

Supplementary Figure 11
Supplementary Figure 12

A.
- ERTCre+ KLF4 flx/wt ApoE-/-
  - Tamoxifen injections
  - AngII osmotic pump
  - Harvest Aorta
- ERTCre- KLF4 flx/wt ApoE-/-
- 6-8 weeks
- 10 weeks
- 14 weeks

B.
- MyHCre KLF4 wt/wt ApoE-/-
- MyHCre KLF4 flx/wt ApoE-/-
- MyHCre KLF4 flx/flx ApoE-/-

Same experimental design as above using:
- p=0.0245
- p=0.0343

Graphs showing survival free from aneurysm rupture following Ang II treatment.