KLF11 (Krüppel-Like Factor 11) Inhibits Arterial Thrombosis via Suppression of Tissue Factor in the Vascular Wall

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Objective—Mutations in Krüppel like factor-11 (*KLF11*), a gene also known as maturity-onset diabetes mellitus of the young type 7, contribute to the development of diabetes mellitus. KLF11 has anti-inflammatory effects in endothelial cells and beneficial effects on stroke. However, the function of KLF11 in the cardiovascular system is not fully unraveled. In this study, we investigated the role of KLF11 in vascular smooth muscle cell biology and arterial thrombosis.

Approach and Results—Using a ferric chloride–induced thrombosis model, we found that the occlusion time was significantly reduced in conventional *Klf11* knockout mice, whereas bone marrow transplantation could not rescue this phenotype, suggesting that vascular KLF11 is critical for inhibition of arterial thrombosis. We further demonstrated that vascular smooth muscle cell–specific *Klf11* knockout mice also exhibited significantly reduced occlusion time. The expression of tissue factor (encoded by the *F3* gene), a main initiator of the coagulation cascade, was increased in the artery of *Klf11* knockout mice, as determined by real-time quantitative polymerase chain reaction and immunofluorescence. Furthermore, vascular smooth muscle cells isolated from *Klf11* knockout mouse aortas showed increased tissue factor expression, which was rescued by KLF11 overexpression. In human aortic smooth muscle cells, small interfering RNA–mediated knockdown of KLF11 increased tissue factor expression. Consistent results were observed on adenovirus-mediated overexpression of KLF11. Mechanistically, KLF11 downregulates *F3* at the transcriptional level as determined by reporter and chromatin immunoprecipitation assays.

Conclusions—Our data demonstrate that KLF11 is a novel transcriptional suppressor of *F3* in vascular smooth muscle cells, constituting a potential molecular target for inhibition of arterial thrombosis.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2019;39:402-412. DOI: 10.1161/ATVBAHA.118.311612.)**

Key Words: diabetes mellitus ■ gene ■ Krüppel-like factors ■ thrombosis ■ tissue factor ■ vascular disease

Thrombosis is a common pathology underlying many cardiovascular diseases: myocardial infarction, stroke, and venous thromboembolism, which collectively cause more than one-fourth of deaths worldwide.[1] The primary pathogenic process in arterial thrombosis is the rupture of the atherosclerotic plaque, which promotes platelet recruitment, adhesion, aggregation and activation, and results in thrombus growth.[2] Given the importance of the vascular smooth muscle cells (VSMCs) in vessel homeostasis and pathogenesis of vascular diseases,[3] it is of great interest to identify molecular signaling pathways that mediate the effects of both physiological and pathophysiological stimuli in VSMCs.

See accompanying editorial on page 309

The KLF (Krüppel-like factor) family is a subclass of Cys2/His2 zinc-finger DNA-binding proteins.[4] The KLF family plays critical roles in the maintenance of vascular homeostasis and further affects multiple vascular diseases.[5,6] In fact, emerging data from population genetics studies suggest that *KLF11* gene polymorphisms are significantly associated with type 2 diabetes mellitus.[7] Maturity-onset diabetes mellitus of the young type 7, an early-onset type 2 diabetes mellitus, is caused by mutations in the *KLF11* gene.[7] In pancreatic β cells, KLF11 regulates insulin transcription by directly binding or via increasing the expression of another maturity-onset diabetes mellitus of the young gene, pancreatic-duodenal homeobox-1 *(PDX-1)*.[8,9] Moreover, KLF11 reduces hepatic triglyceride levels by increasing fatty acid oxidation.[10] Apart from the metabolic disorder, diabetes mellitus accelerates vascular pathology and enhances thrombotic risk,[11,12] which

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Nonstandard Abbreviations and Acronyms

α-SMA	α-smooth muscle actin
AP-1	activator protein-1
BMT	bone marrow transplantation
$FeCl_3$	ferric chloride
HASMC	human aortic smooth muscle cell
IL	interleukin
KLF	Krüppel-like factor
KLF11 KO	KLF11 knockout mice
MAPK	mitogen-activated protein kinase
MASMC	mouse aortic smooth muscle cell
MCP-1	monocyte chemoattractant protein-1
PAR	protease-activated receptors
PKB	protein kinase B
PKC	protein kinase C
TAT	thrombin-antithrombin
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TIEG	transforming growth factor-β-inducible early gene
VSMC	vascular smooth muscle cell
WT	wild type

account for the major causes of morbidity and mortality in diabetic patients.[13]

As a transcription factor, KLF11 is expressed ubiquitously and is abundant in adipose tissue, testis, breast, artery, and lung (GTEx database). RNA sequencing data show that the mRNA level of KLF11 is modest in human endothelium and moderate in human aortic smooth muscle cells (HASMCs).[6] Prior studies from others and our group demonstrated that KLF11 plays an important role in maintaining vascular homeostasis.[14,15] Specifically, KLF11 inhibits endothelial activation in the presence of inflammatory stimuli and functions as a peroxisome proliferator-activated receptor-γ coregulator to attenuate middle cerebral artery occlusion–induced stroke in mice.[16] These in vitro and in vivo observations form the basis of the current view that KLF11 is a vessel protective factor. However, the role of KLF11 in VSMC biology and thrombosis has not been explored.

In the present study, we focused on VSMC KLF11 and, using in vivo VSMC-specific loss-of-function approaches, demonstrated that this factor is an inhibitor of experimental arterial thrombosis through transcriptional repression of tissue factor (TF, encoded by the *F3* gene) in VSMCs.

Materials and Methods

The data that support the findings of this study are available from the corresponding author on reasonable request. An extended version of this section is available as Extended Materials and Methods. in the online-only Data Supplement

Animals

Conventional *Klf11* knockout mice (*Klf11* KO) and wild-type (WT) mice in the C57BL/6J background were previously described[14] and the knockout was confirmed (Figure I in the online-only Data Supplement). The floxed-*Klf11* (*Klf11*$^{fl/fl}$) mice were generated by injecting blastocysts developed from *Klf11* targeted embryonic stem cells (*Klf11*$^{tm2a(KOMP)Wtsi}$, UCDavis, Knockout Mouse Project) into C57BL/6J mice. Exon 3 of the *Klf11* gene was flanked by *LoxP* elements. The genotype was determined from mouse tail clippings and a pair of polymerase chain reaction primers flanking the downstream *LoxP* region (Figure IIIA in the online-only Data Supplement). The inducible smooth muscle cell–specific *Klf11* KO (*Sm-Klf11* KO) mice were generated by cross-breeding *Klf11*$^{fl/fl}$ mice with *Myh11-CreER*^{T2} mice (019079, Jackson Laboratory).[17] The primer sequences for mouse genotyping are shown (Table I in the online-only Data Supplement). The *Myh11-CreER*^{T2}*-(+)/Klf11*$^{fl/fl}$ mice (*Sm-Cre/Klf11*$^{fl/fl}$ +TAM) and *Myh11-CreER*^{T2}*-(−)/Klf11*$^{fl/fl}$ mice (*Klf11*$^{fl/fl}$+TAM) were treated with tamoxifen (TAM; intraperitoneal injections, 80 mg/kg per day for 5 consecutive days). *Myh11-CreER*^{T2}*-(+)/Klf11*$^{fl/fl}$ mice treated with the same volume of vehicle corn oil (*Sm-Cre/Klf11*$^{fl/fl}$+Oil) were used as the control group. Fourteen days after tamoxifen or corn oil treatment, the reduction of KLF11 expression in the aorta was confirmed by real-time quantitative polymerase chain reaction and Western blot (Figure IIIB–IIIC in the online-only Data Supplement). Because the *Myh11-Cre/ER*^{T2} transgene is inserted on the Y chromosome,[18] only male *Sm-Cre/Klf11*$^{fl/fl}$ mice can be generated using this approach. All animal care and experimental procedures were approved by the University of Michigan Animal Care and Use Committee.

Ferric Chloride–Induced Carotid Artery Thrombosis Model

Ferric chloride ($FeCl_3$)-induced carotid artery thrombosis was conducted as previously described[19] and detailed in the Extended Materials and Methods in the online-only Data Supplement.

Bone Marrow Transplantation

The protocol for syngeneic bone marrow transplantation (BMT) was previously described[20,21] and detailed in the Extended Materials and Methods in the online-only Data Supplement.

Prothrombin Time and Activated Partial Thromboplastin Time Measurements

Platelet-poor-plasma was collected as previously described[22] and detailed in the Extended Materials and Methods in the online-only Data Supplement.

Tail-Bleeding Assay

Conventional *Klf11* KO mice and WT mice (8–10-week-old male) were used in this experiment. As previously described,[23] a transverse incision at the 5 mm distal end of the tail was performed and the tail was immersed in saline at 37°C. Bleeding time was recorded as the time to cessation of bleeding.

Reagents and Antibodies

Reagents and Antibodies are listed in the Extended Materials and Methods in the online-only Data Supplement.

Cell Culture

HASMCs (CC-2571, Lonza) were cultured in smooth muscle growth medium-2 containing 5% FBS (CC-3182, Lonza) and used within 10 passages. Before thrombin stimulation, the HASMCs were made quiescent with DMEM/F12 with 0.5% FBS for 48 hours. A7r5 cells (CRL-1444, ATCC) were cultured in DMEM/F12 supplemented with 10% FBS) and 50 mg/mL of a penicillin/streptomycin mix. Mouse aortic smooth muscle cells (MASMCs) were isolated from the conventional *Klf11* KO mice and WT mice (3–4-week-old male) as previously described.[17,24] Details are described in the Extended Materials and Methods in the online-only Data Supplement. The purity of MASMCs was validated by immunostaining for α-SMA (α-smooth muscle actin). All cells were cultured in a 5% CO_2 humidified incubator at 37°C.

Preparation of Washed Murine Platelets and Platelet Aggregation Assay

The collection of washed murine platelets and platelet aggregation assay were performed as previously described[23] and detailed in the Extended Materials and Methods in the online-only Data Supplement.

Protein Extracts and Western Blot

All protein extractions and Western blots were performed as previously described.[25]

Adenoviral Constructs

Adenoviral vectors overexpressing human KLF11 or LacZ control were generated as previously described.[26] The procedures are detailed in the Extended Materials and Methods in the online-only Data Supplement.

Statistical Analysis

All quantitative data are presented as mean±SEM. Statistical analysis was performed using the GraphPad Prism 7. All data were first subjected to Shapiro-Wilk normality test and *F* test to evaluate homogeneity of variances. For normally distributed data with similar variances among groups, unpaired Student *t* test with Welch correction was used for 2-group comparisons and 1-way ANOVA followed by Tukey test was used for >2 groups comparisons. Two-way ANOVA followed by Bonferroni test was applied for comparisons of grouped data under different conditions. Nonparametric Mann-Whitney test was used for data not normally distributed. All results were representative from at least 4 independent experiments.

Results

KLF11 Deficiency Aggravates Arterial Thrombosis In Vivo

To assess the role of KLF11 in arterial thrombosis, we used the conventional *Klf11* KO mice previously reported[14] and applied a $FeCl_3$-induced thrombosis model for studying arterial thrombosis.[19] The occlusion time in the *Klf11* KO male mice was significantly reduced to an average of 62% of that in WT C57BL/6J male mice (Figure 1A). A similar prothrombotic phenotype was also observed in *Klf11* KO female mice, with the occlusion time reducing to 56% of that in the WT C57BL/6J female mice (Figure 1B). To exclude the effects of *Klf11* KO in blood cells (such as platelets, neutrophils, and macrophages) in this prothrombotic phenotype, we performed BMT in *Klf11* KO and WT mice. The reduced occlusion time in the *Klf11* KO group transplanted with WT bone marrow was not rescued when compared with WT mice transplanted with WT bone marrow (Figure 1C).

The hemostatic status in the *Klf11* KO male mice was evaluated by measuring the function of coagulation factors and platelets. Our results showed that KLF11 did not alter the prothrombin time and activated partial thromboplastin time, which reflect the function of extrinsic/common or intrinsic coagulation pathways, respectively (Figure 1D–1E). Bleeding time and thrombin-antithrombin (TAT) complexes were also unchanged in the *Klf11* KO mice, indicating that the general hemostatic status was normal in the conventional KLF11-deficient mice (Figure 1F–1G). To evaluate whether the deficiency of KLF11 in megakaryocytes can affect the platelet function, we isolated washed platelets and performed thrombin-induced platelet aggregation assay. The maximum platelet aggregation induced by thrombin did not show significant differences between WT and conventional *Klf11* KO mice (Figure II in the online-only Data Supplement).

Smooth Muscle Cell–Specific KLF11 Deficiency Aggravates $FeCl_3$-Induced Arterial Thrombosis

Taking into account that the hemostatic status in the circulation was not affected and the BMT cannot rescue the occlusion time in the conventional *Klf11* KO mice, the phenotype in *Klf11* KO mice might result from molecular changes specific to the vascular wall. To further identify the role of VSMC KLF11 in thrombosis, we generated tamoxifen-inducible *Sm-Klf11* KO (*Myh11-CreER^{T2}/Klf11$^{fl/fl}$*+TAM) mice (Figure IIIA in the online-only Data Supplement). *Klf11$^{fl/fl}$* mice treated with tamoxifen (*Klf11$^{fl/fl}$*+TAM) and *Myh11-CreER^{T2}/Klf11$^{fl/fl}$* mice treated with corn oil (*Sm-Cre/Klf11$^{fl/fl}$*+Oil) were used as controls. KLF11 deficiency in the aortic media of *Sm-Klf11* KO mice was confirmed at the mRNA and protein levels (Figure IIIB–IIIC in the online-only Data Supplement). The specificity of the Myh11-CreER^{T2} in the aorta had been confirmed previously, as no expression was detected in the cells from blood and bone marrow.[27] In the $FeCl_3$ thrombosis model, *Sm-Klf11* KO mice exhibited a similar prothrombotic phenotype as that observed in the conventional *Klf11* KO mice. The occlusion time in the *Sm-Klf11* KO mice was significantly reduced to an average of 67% of that in the *Sm-Cre/Klf11$^{fl/fl}$*+Oil control mice (Figure 1H). Our data suggest that VSMC KLF11 protects against arterial thrombosis.

TF Expression Is Increased in the Vascular Wall of Conventional *Klf11* KO Mice

TF is an important initiator of the coagulation cascade, which can generate insoluble fibrins and form a thrombus. The vascular wall TF contributes to arterial thrombosis in cardiovascular diseases, such as atherosclerotic plaque rupture and myocardial infarction.[28] VSMC TF is critical for arterial thrombus formation in the mouse $FeCl_3$ thrombosis model.[19] To determine whether TF is an effector mediating the enhanced thrombosis resulting from KLF11 deficiency, we measured the expression of TF in the aorta of conventional *Klf11* KO mice and WT mice in basal conditions. First, the activity of circulating microvesicle-associated TF showed no significant differences between *Klf11* KO and WT mice (Figure 2A). We applied anti-mouse TF 1H1 antibody[29] or rat IgG to validate the specificity of this assay. Next, we observed increased *F3* mRNA level and TF activity in the isolated carotid artery of conventional *Klf11* KO mice (Figure 2B and 2C). The elevated aortic TF activity can also be blocked by TF 1H1 antibody. Furthermore, the immunofluorescence staining data showed that the TF protein was consistently upregulated in the vascular wall, and colocalized with α-SMA, a specific marker of VSMCs. Moreover, the *Klf11* KO mice also showed increased TF expression under lipopolysaccharide-induced inflammatory conditions (Figure 2D). The relative TF intensity in the vascular wall was quantified and statistically analyzed. Collectively, our data suggest that KLF11 negatively regulates TF levels in VSMCs in vivo. Similar to the data in Figure 1G, no significant difference in TAT complexes was

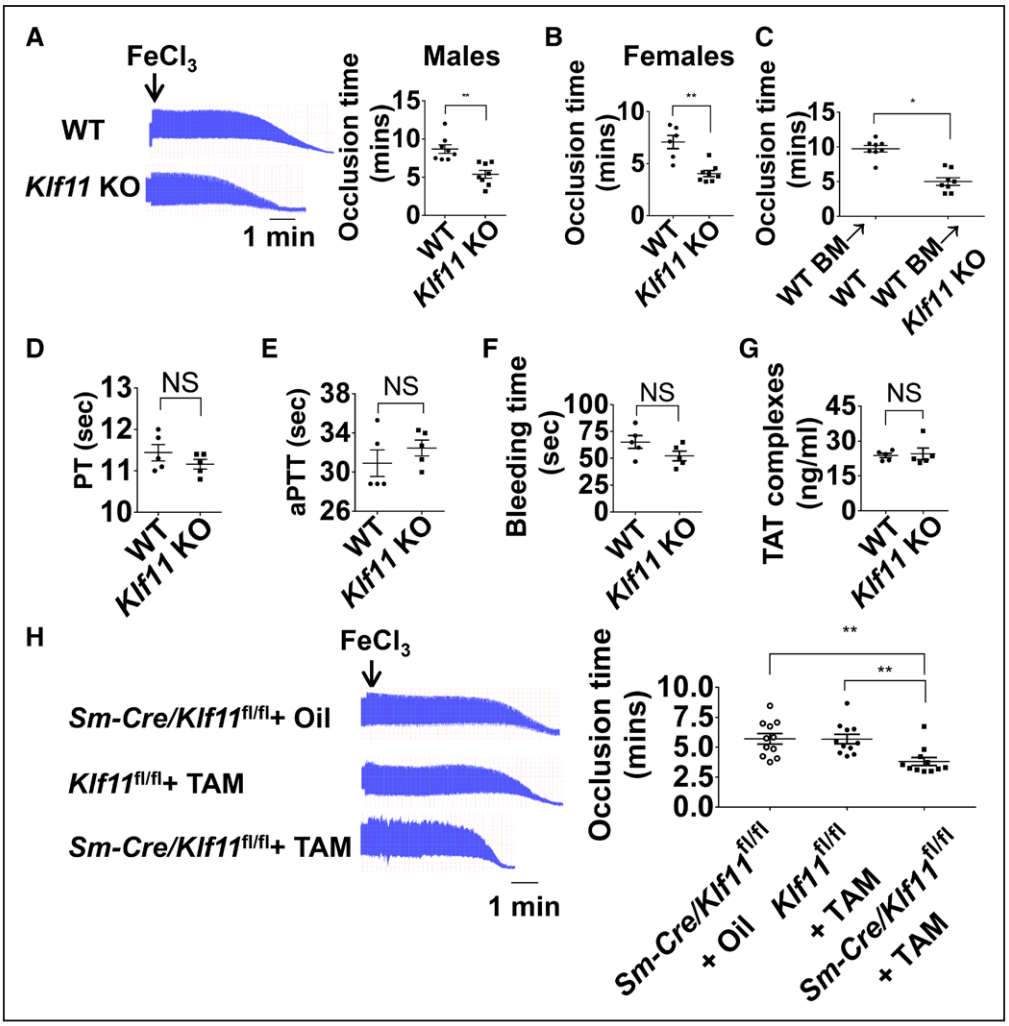

Figure 1. KLF11 (Krüppel-like factor 11) deficiency aggravates arterial thrombosis. **A**–**C**, The left carotid arteries of WT (wild type) and conventional *Klf11* KO (knockout) mice were subjected to 10% $FeCl_3$ to induce arterial thrombosis. **A**, Representative images of blood flow detected by ultrasound are shown with each division representing 8 seconds (**left**) and the corresponding occlusion time (**right**) determined in WT and *Klf11* KO male mice (n=8/group). **B**, Occlusion time in WT and *Klf11* KO female mice (n=6–8/group). **C**, WT male mice transplanted with WT bone marrow (BM) were designated as WT BM→WT, *Klf11* KO male mice transplanted with WT bone marrow were designated as WT BM→*Klf11* KO. The carotid artery occlusion time after bone marrow transplantation was recorded as in **A** (n=8/group). ***P*<0.01 or **P*<0.05 using unpaired Student *t test*. **D**–**G**, Prothrombin time (PT), activated partial thromboplastin time (aPTT), bleeding time and thrombin-antithrombin (TAT) complexes were measured from WT and *Klf11* KO male mice (n=5/group). NS, no significance using unpaired Student *t* test (**D**, **F**, **G**) or nonparametric Mann-Whitney test (**E**). **H**, The left carotid arteries of *Sm-Cre/Klf11*$^{fl/fl}$+TAM (*Myh11-CreER*^{T2}/*Klf11*$^{fl/fl}$+tamoxifen [TAM]) mice and controls: *Sm-Cre/Klf11*$^{fl/fl}$+ Oil (*Myh11-CreER*^{T2}/*Klf11*$^{fl/fl}$+ corn oil) and *Klf11*$^{fl/fl}$+TAM (*Klf11*$^{fl/fl}$+ tamoxifen) mice, were subjected to 10% $FeCl_3$ to induce thrombosis. Representative images of blood flow detected by ultrasound are shown and the occlusion time in control and *Sm-Klf11* KO mice was recorded (n=11/group). ***P*<0.01 using one-way ANOVA followed by Tukey test.

observed between WT and *Klf11* KO mice in basal conditions. However, there were higher TAT complexes in *Klf11* KO mice after lipopolysaccharide treatment (Figure IV in the online-only Data Supplement).

KLF11 Overexpression Rescues the TF Upregulation in KLF11-Deficient MASMCs

As a complementary and necessary approach, MASMCs were isolated from male and female conventional *Klf11* KO mice and WT mice. The isolated MASMCs were characterized by immunostaining for α-SMA (Figure V in the online-only Data Supplement). The *Klf11* deficiency was confirmed by real-time quantitative polymerase chain reaction (Figure 3A and 3E). A higher TF expression was observed in the MASMCs from both male and female *Klf11* KO mice compared with WT mice (Figure 3B–3D and 3F–3H, Ad-LacZ [adenovirus-mediated overexpression of LacZ]). In both sexes, restoration of KLF11 can rescue the phenotype in *Klf11* KO MASMCs. As expected, the upregulated TF expression was significantly alleviated after KLF11 overexpression, at both mRNA and protein levels (Figure 3B–3D and 3F–3H, Ad-KLF11 [adenovirus-mediated overexpression of KLF11]). These results indicate that endogenous KLF11 is required to prevent excessive TF upregulation, a hallmark of VSMC involvement in thrombosis.[19]

KLF11 Inhibits TF Expression in HASMCs

To determine whether KLF11 is essential to regulate TF in VSMCs, we measured TF expression upon KLF11 knockdown in HASMCs. The efficiency of small interfering RNA–mediated KLF11 knockdown was confirmed at the mRNA

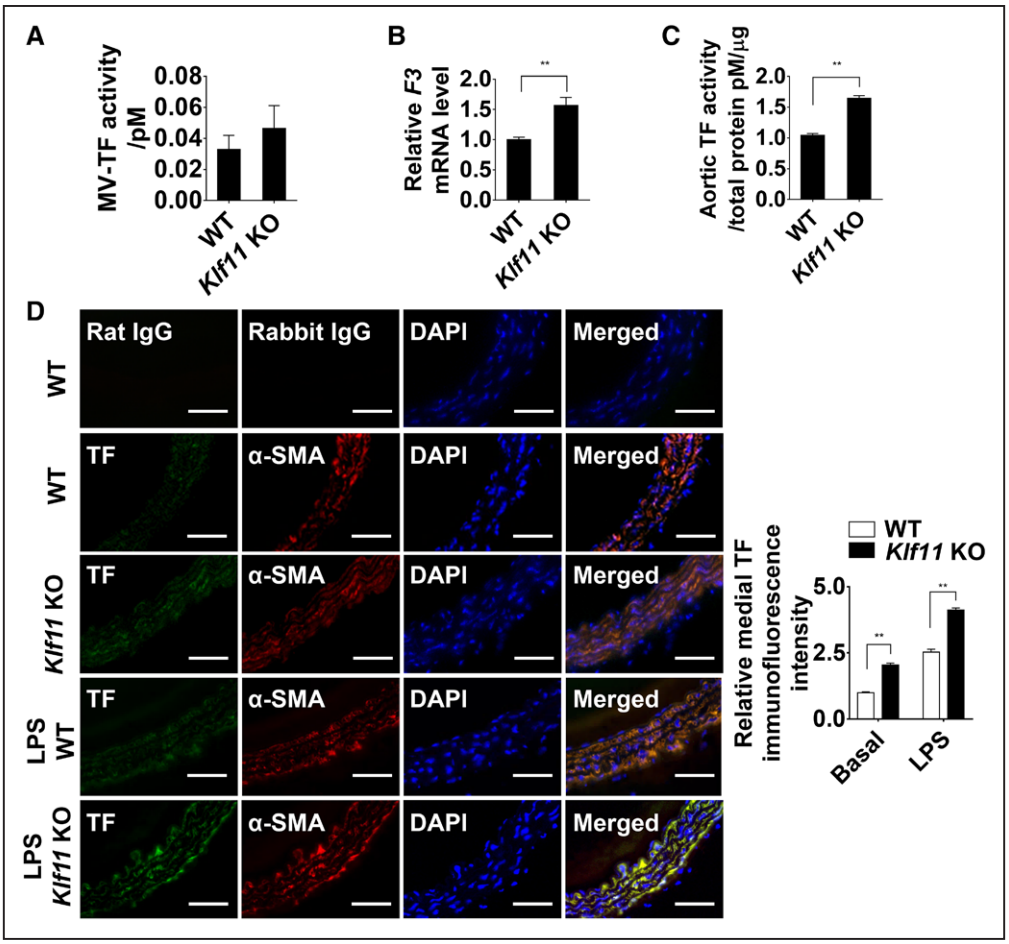

Figure 2. KLF11 (Krüppel-like Factor 11) deficiency induces tissue factor (TF) expression in arterial wall. **A**, Activity of microvesicles-associated tissue factor (MV-TF) in the plasma after preincubation with IgG or TF 1H1 antibody (n=6/group). Data are presented by subtracting the amount of FXa (coagulation factor Xa) generated in the presence of TF 1H1 antibody from the amount of total FXa generated in the presence of IgG. **B**, *F3* mRNA level in carotid arteries from wild-type (WT) and *Klf11* knockout (KO) mice. The mRNA level was normalized by *18S* and is presented relative to the WT group set as 1 (n=4/group). **C**, The aortic TF activity was measured and presented as in **A**, after preincubation with IgG or TF 1H1 antibody and normalized to the total protein quantity (n=6/group). ***P*<0.01 using unpaired Student *t* test (**A**–**C**). **D**, Expression of TF (Alexa 647, displayed in green) and α-SMA (α-smooth muscle actin, Alexa 568, displayed in red) in mouse aorta at basal level or 4 h after lipopolysaccharide (LPS; 30 μg/kg) tail vein injection was visualized by immunofluorescence staining. Respective IgG staining was used as negative control. Scale bars=50 μm. Quantification was performed from 4 mice, randomly selecting 3 different medial regions from each specimen and dividing the TF immunofluorescence intensity by medial area (indicated by α-SMA positive cells). Data are presented relative to the basal level of the WT group set as 1. ***P*<0.01 using 2-way ANOVA followed by Bonferroni test. DAPI indicates 4',6-diamidino-2-phenylindole.

and protein levels (Figure 4A). The KLF11 knockdown in HASMCs increased both the basal and thrombin-induced expression of TF at the mRNA and protein levels (Figure 4B–4D). Similar effects were observed in HASMCs stimulated with 10% FBS-containing culture medium (Figure 4E–4G).

Apart from TF, other factors can also affect the formation of arterial thrombus. In HASMCs, the expression of other thrombosis related factors, such as tissue factor pathway inhibitor (*TFPI*) and protease-activated receptor-1 (*PAR-1*), and inflammatory genes, such as monocyte chemoattractant protein-1 (*MCP-1*) and interleukin 1 beta (*IL-1*β) were not significantly changed in the KLF11-deficient HASMCs (Figure VI in the online-only Data Supplement).

Furthermore, we upregulated KLF11 in primary HASMCs to determine whether KLF11 regulates TF in vitro. In HASMCs, adenovirus-mediated overexpression of KLF11 (Figure 5A) significantly inhibited the thrombin-induced TF expression at both mRNA and protein levels (Figure 5B–5D). Similarly, KLF11 also suppressed TF expression in HASMCs stimulated with 10% FBS-containing culture medium (Figure 5E–5G). Thus, our data suggest that KLF11 potently inhibits TF in human VSMCs under either thrombin stimulation or normal serum conditions.

Interestingly, KLF11 overexpression also decreased TNF-α (tumor necrosis factor alpha)-induced TF expression in human umbilical vein endothelial cells (ECs) at both mRNA and protein levels (Figure VII in the online-only Data Supplement), suggesting that endothelial KLF11 also may have an important role in thrombosis.

KLF11 Inhibits *F3* Transcription

Next, we determined the mechanism that mediates the regulation of *F3* by KLF11. *KLF11* was originally identified as the transforming growth factor-β–inducible early gene 2 (*TIEG2*), with a preference to bind at GC-rich sequences (GGGTG).[30] Transcription factor binding site analysis of the human *F3* gene (Genomatix) revealed a relatively conserved TIEG binding site (–177 to –161 bp) upstream of

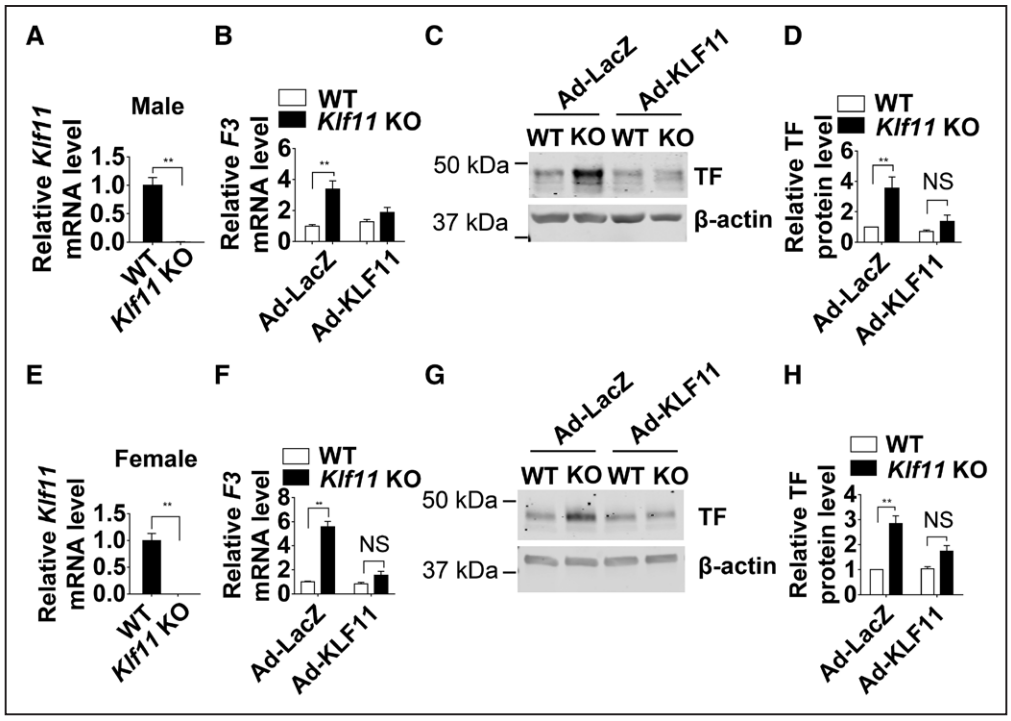

Figure 3. KLF11 (Krüppel-like Factor 11) overexpression rescues the tissue factor (TF) upregulation in KLF11-deficient MASMCs (mouse aortic smooth muscle cells). MASMCs were isolated from male (**A**–**D**) and female (**E**–**H**) wild-type (WT) or *Klf11* knockout (KO) mice. **A** and **E**, *Klf11* mRNA level of MASMCs from WT and *Klf11* KO mice. The mRNA level was normalized by *18S* and is presented relative to the WT group set as 1 (n=4/group). ***P*<0.01 using unpaired Student *t* test. **B**–**D** and **F**–**H**, MASMCs isolated from WT or *Klf11* KO mice were infected with Ad-LacZ or Ad-KLF11 (50 MOI). **B** and **F**, *F3* mRNA level of MASMCs was normalized by *18S* and is presented relative to the WT infected with Ad-LacZ group set as 1 (n=4/group). **C** and **G**, Representative Western blot of TF protein levels. **D** and **H**, Band density from 4 independent Western blots was quantitatively analyzed and normalized against β-actin. The WT infected with Ad-LacZ group was set as 1. ***P*<0.01 or NS, no significance using 2-way ANOVA followed by Bonferroni test (**B**, **D**, **F**, **H**). Ad-KLF11 indicates adenovirus overexpressing KLF11; Ad-LacZ, adenovirus overexpressing LacZ; and MOI, multiplicity of infection.

the *F3* transcription start site (Figure 6A). The TIEG binding site is conserved among human, mouse, rat, and rabbit (Figure 6B). To determine whether this TIEG binding site is a functional KLF11 binding region, we performed chromatin immunoprecipitation assay in the HASMCs infected with Ad-flag-KLF11 (adenovirus overexpressing flag-tagged KLF11) or Ad-LacZ. Our data suggest that KLF11 can bind to the region containing this TIEG binding site (Figure 6C). To determine whether KLF11 regulates *F3* at the transcriptional level, we generated luciferase reporter constructs, which were under the control of different lengths (−906/+162 and −556/+162) of the human *F3* promoter. In A7R5 cells, a rat aortic smooth muscle cell line, transfected with different reporter constructs, KLF11 overexpression significantly reduced the luciferase activity (Figure 6D). Next, we deleted the TIEG bind site (−177 to −161) in the *F3* promoter–driven luciferase construct. As expected, the deletion of the TIEG binding site significantly attenuated the KLF11 inhibition of *F3* promoter-driven luciferase activity (Figure 6E) from the reporter plasmid. In conclusion, we identified that KLF11 inhibits *F3* expression at the transcription level through direct binding to the *F3* promoter (Figure 6F).

Discussion

In the current study, we observed an increase of arterial thrombosis in both genetically engineered conventional *Klf11* KO mice and VSMC-specific *Klf11* KO mice. In cultured HASMCs, we demonstrated that KLF11 inhibits TF expression. Mechanistically, KLF11 directly binds to the *F3* promoter region and thereby suppresses the transcription of *F3*. This study demonstrated a potential role for VSMC KLF11 in arterial thrombosis.

The KLF family modulates cardiovascular activity through regulation of metabolism and inflammation in the cardiovascular system.[4–6] Endothelial KLF2 and KLF4 have been reported to inhibit thrombus formation by inhibiting the transcription of prothrombotic factors (such as plasminogen activator inhibitor 1 and TF) and increasing the expression of antithrombotic factors (such as thrombomodulin) under inflammatory conditions.[6,31–33]

We previously identified that KLF11 inhibits endothelial activation[14] and attenuates endothelial dysfunction in the mouse middle brain artery occlusion–induced stroke model.[16] Our current finding that VSMC KLF11 inhibits arterial thrombosis advances the understanding of the protective role of KLF11 in vascular diseases. Population genetics studies identified that mutations in the *KLF11* gene are positively associated with type 2 diabetes mellitus.[7] Cardiovascular events are the major causes of death in diabetes mellitus.[34] Our study points to a potentially beneficial effect of KLF11 on cardiovascular complications in diabetic patients. Follow-up studies are warranted to determine the role of VSMC KLF11 in diabetes mellitus associated cardiovascular diseases, such as atherosclerosis, thrombosis, and angiogenesis.

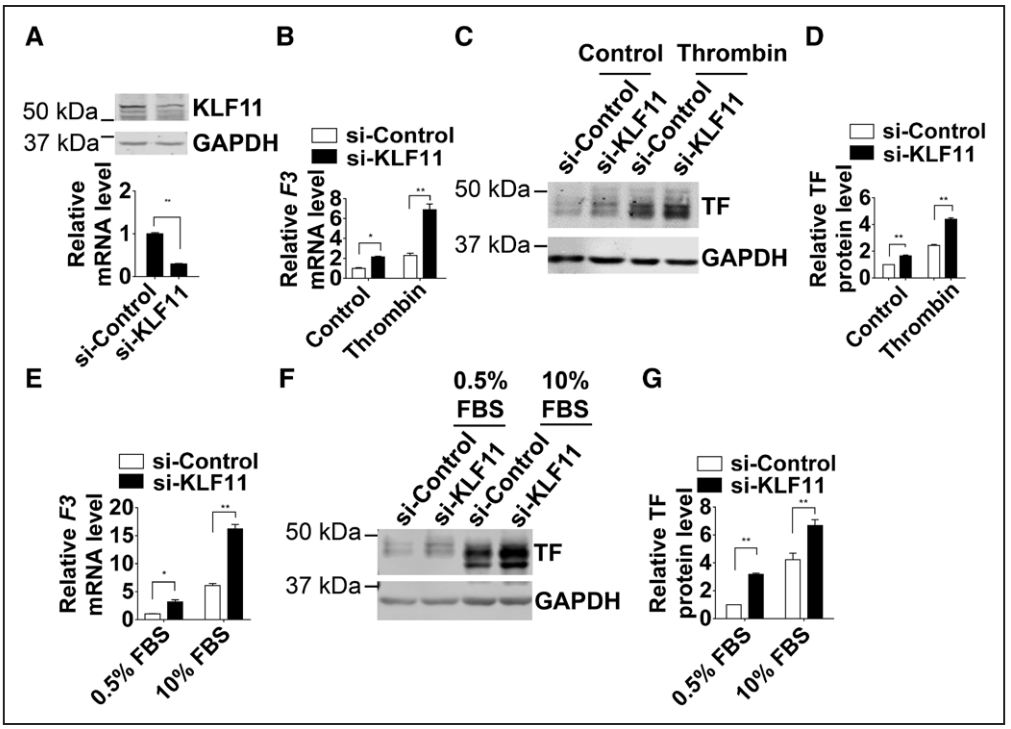

Figure 4. Tissue factor (TF) expression is increased upon KLF11 (Krüppel-like Factor 11) knockdown in HASMCs (human aortic smooth muscle cells). HASMCs were transfected with si-Control or si-KLF11 (40 nmol/L) and 24 h later serum starved with 0.5% FBS for 48 h. Three days after transfection, HASMCs were exposed to thrombin (3.24 μg/mL; **B**–**D**) or 10% FBS (**E**–**G**) for 4 h. **A**, The knockdown efficiency of KLF11 was determined by real-time quantitative polymerase chain reaction and Western blot. The mRNA level was normalized by *GAPDH* and is presented relative to HASMCs transfected with si-Control group set as 1 (n=4/group). ***P*<0.01 using unpaired Student *t*-test. **B** and **E**, *F3* mRNA level of HASMCs was normalized by *GAPDH* and is presented relative to HASMCs transfected with si-Control group set as 1 (n=4/group). **C** and **F**, Representative Western blot showing the protein level of TF. **D** and **G**, Band density from 4 independent Western blots was quantitatively analyzed and normalized against GAPDH. HASMCs transfected with si-Control group was set as 1. **P*<0.05, ***P*<0.01 using two-way ANOVA followed by Bonferroni test (**B**, **D**, **E**, **G**). si-Control indicates nontargeting small interfering RNA control; and si-KLF11, small interfering RNA targeting KLF11.

In this study, we used the $FeCl_3$ thrombosis model, a widely used mouse arterial thrombosis model,[35] to study the role of KLF11 in the vascular wall in vivo. The penetration of $FeCl_3$ from the adventitia triggers thrombosis.[36] In this study, we found that conventional *Klf11* KO mice were more prothrombotic. The BMT study and the measurement of microvesicle-associated TF activity excluded a potential involvement of KLF11 from blood cells and circulation-derived TF. Similar prothrombotic phenotype was observed in the *Sm-Klf11* KO mice, which further demonstrated the antithrombotic effects of KLF11 in the vascular wall under basal conditions.

TF is critical in maintaining the balance between hemostasis and thrombosis.[37] VSMCs in human atherosclerotic plaques express high levels of TF.[38–40] Interestingly, a previous study using low-TF mice demonstrated that the vascular wall derived TF, rather than leukocytes derived TF, is responsible for the macrovascular thrombosis.[41] Therefore, the lower expression of TF in the vascular wall, especially in the VSMCs, can limit the initiation of the TF-dependent coagulation cascade and thus be a potentially protective mechanism from the prolonged occlusion time in vivo. Compared with ECs, VSMCs have a higher constitutive expression of TF and are considered as the primary source of TF in the arterial wall.[42] The expression of TF can be rapidly induced in VSMCs after artery injury[43] and contributes to thrombosis events after plaque rupture.[38,44] The *SM22*-driven VSMC-specific TF-deficient mice showed an increase in occlusion time in $FeCl_3$-induced arterial thrombosis, indicating a key role of VSMC-derived TF in arterial thrombosis.[19] Although TF is not the only key factor in thrombosis formation, inhibition of TF activity by a monoclonal antibody[45] or administration of its counterpart recombinant TFPI[46] showed beneficial effects, which make inhibition of TF a potential pharmaceutical target for thrombosis. In addition, it has been reported that the deficiency of TFPI in smooth muscle cells can reduce the occlusion time in $FeCl_3$ model.[47] However, in this study, we found that *TFPI* expression was not significantly changed in the KLF11-deficient VSMCs. Therefore, the increased TF can at least partially account for the prothrombotic phenotype in the KLF11-deficient VSMCs.

It is well known that thrombin causes positive feedback effects on the coagulation cascade, including promoting the contact activation pathway.[48] However, numerous studies indicated that thrombin can also induce TF expression in VSMCs.[49–52] Thrombin can bind to PAR (protease-activated receptors) on human aortic SMCs and activate PKB (protein kinase B), PKC (protein kinase C), and MAPK (mitogen-activated protein kinase) pathways, which could induce TF expression.[53–55] Our data demonstrated that KLF11 directly binds to the *F3* promoter and inhibits its activity in VSMCs, which account for the KLF11-dependent decreased *F3* transcription under thrombin stimulation. The effect of KLF11 on

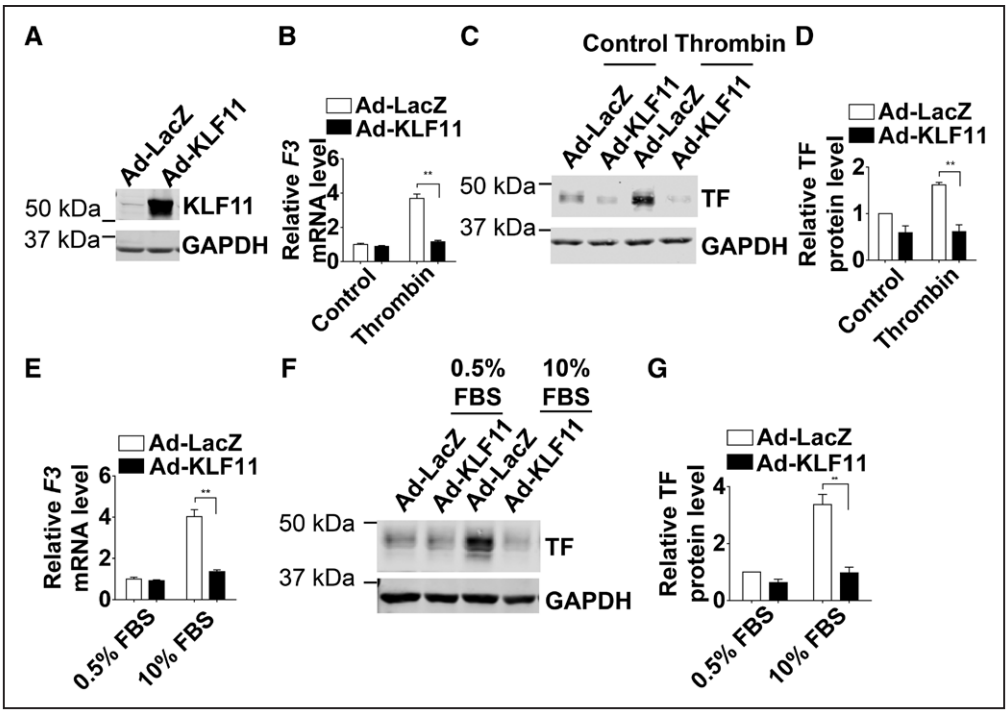

Figure 5. Tissue factor (TF) expression is decreased upon KLF11 (Krüppel-like factor 11) overexpression in HASMCs (human aortic smooth muscle cells). HASMCs were infected with Ad-LacZ or Ad-KLF11 (50 MOI). Twelve hours after infection, HASMCs were serum starved with 0.5% FBS for 48 h and then stimulated by thrombin (3.24 μg/mL; **B**–**D**) or 10% FBS (**E**–**G**) for 4 h. **A**, The overexpression of KLF11 was determined by Western blot. **B** and **E**, *F3* mRNA level of HASMCs from each group was normalized by *GAPDH* and is presented relative to HASMCs infected with Ad-LacZ group set as 1 (n=4/group). **C** and **F**, Representative Western blot showing the protein levels of TF. **D** and **G**, Band density from 4 independent Western blots was quantitatively analyzed and normalized against GAPDH. HASMCs infected with Ad-LacZ group was set as 1. ***P*<0.01 using 2-way ANOVA followed by Bonferroni test (**B**, **D**, **E**, **G**). Ad-KLF11 indicates adenovirus overexpressing KLF1; Ad-LacZ, adenovirus overexpressing LacZ; and MOI, multiplicity of infection.

thrombin-activated signaling pathways (such as PARs, PKB, PKC, and MAPK) warrants future investigation.

In the present study, we demonstrated that KLF11 binds to the *F3* promoter to inhibit its transcription (Figure 6). In basal conditions (serum starvation), TF is expressed at low level in VSMCs, and endogenous KLF11 is abundant enough to maintain the low level of TF by direct binding to the *F3* promoter. Thus, overexpression of KLF11 did not further inhibit *F3* transcription (Figure 5). The chromatin immunoprecipitation (Figure 6C) and luciferase (Figure 6D–6E) experiments were performed in normal growth conditions (10% FBS). Under these conditions, we observed the inhibitory effect of KLF11 on *F3* transcription (Figure 5). On thrombin or serum stimulation, overexpressed KLF11 may compete with other transcription factors, such as AP-1 (activator protein-1),[56] whose binding region (−172 to 160 bp) at the *F3* promoter overlaps with the KLF11 binding region (−177 to 161 bp). However, KLF11 deficiency may vacate the KLF11 binding site and abolish the repressive effect of KLF11 on the *F3* promoter activity. Therefore, KLF11 deficiency can increase *F3* transcription even under basal conditions (Figure 4).

The $FeCl_3$ model is a commonly used experimental model to study arterial thrombosis.[57] However, whether the endothelium is denudated and the internal elastic lamina is intact in this model are still controversial.[58,59] The mechanisms mediating the VSMC-derived effect on arterial thrombosis in the $FeCl_3$ model remain to be investigated. Noteworthy, despite an intact internal elastic lamina upon $FeCl_3$ infiltration,[58,59] VSMC-specific TF knockout inhibited the thrombus formation in the $FeCl_3$ model.[19] In the present study, we found that smooth muscle cell–specific *Klf11* KO mice had a prothrombotic phenotype in association with increased TF, indicating that VSMC KLF11 can maintain the antithrombotic state through transcriptional control of *F3*. We also found that KLF11 overexpression potently inhibited TNF-α–induced TF expression in human umbilical vein ECs at both the mRNA and protein levels (Figure VII in the online-only Data Supplement), although it should be highlighted that endogenous levels of KLF11 in ECs are low.[6] Because the contribution of endothelium is unclear in the $FeCl_3$ model,[58,59] the function of EC KLF11 in thrombosis warrants further investigation independently in other appropriate thrombosis models.

Interestingly, although there was no significant difference in TAT complexes between WT and *Klf11* KO mice in basal conditions, there were higher TAT complexes in *Klf11* KO mice after lipopolysaccharide treatment (Figure 1G and Figure IV in the online-only Data Supplement). In basal conditions, TF is constitutively expressed in the vascular wall and expressed at a lower level in circulation.[60,61] After lipopolysaccharide stimulation, TF expression is significantly induced not only in vascular wall but also monocytes.[62,63] We have demonstrated that KLF11 inhibits TF expression in VSMCs and ECs (Figures 3–5 and Figure VII in the online-only Data Supplement). Whether KLF11 also has inhibitory effects on TF expression in monocytes and thereby inhibits the prothrombotic status under lipopolysaccharide treatment warrants future investigation.

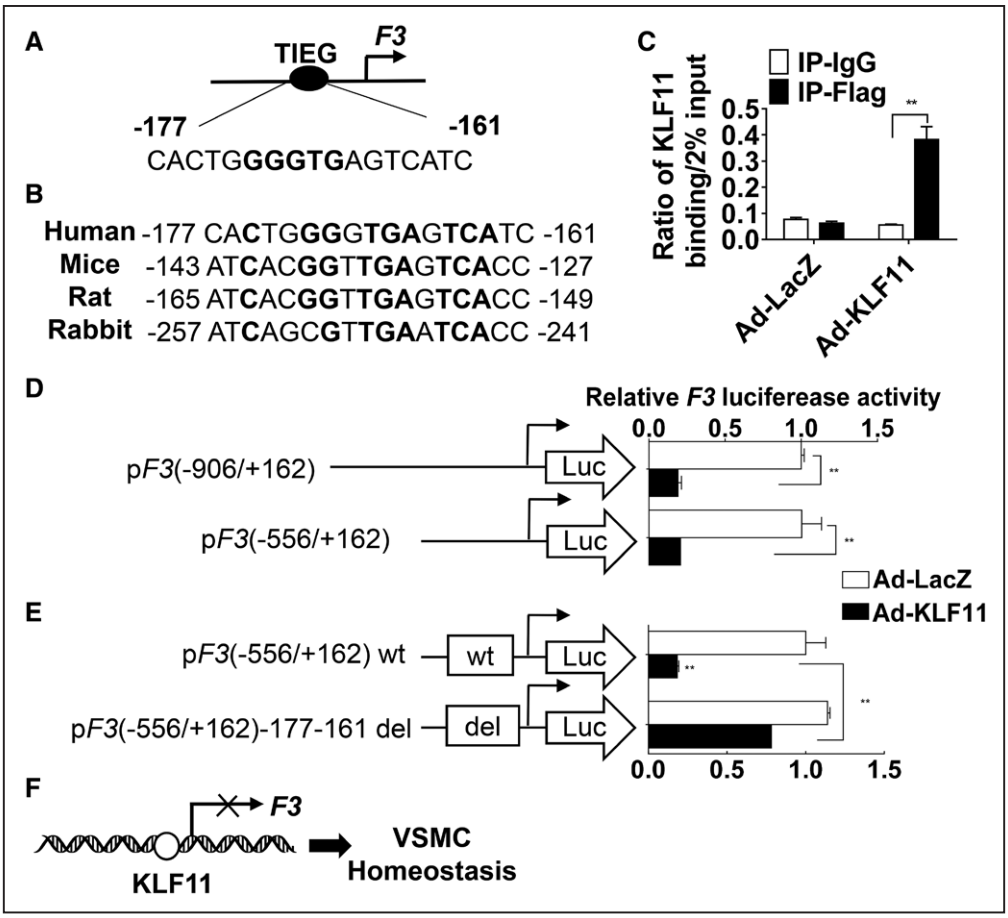

Figure 6. KLF11 (Krüppel-like Factor 11) inhibits *F3* (coagulation factor III) transcription. **A**, A diagram showing the simplified structure of the human *F3* promoter region with an illustration of the TIEG (transforming growth factor-β–inducible early gene) binding site. KLF binding region is shown in bold. **B**, The bold bases indicate the conservation of the TIEG binding site among species. **C**, Human aortic smooth muscle cells were infected with Ad-LacZ or Ad-Flag KLF11. Forty-eight hours after infection, the binding of KLF11 to the *F3* promoter was determined by chromatin immunoprecipitation assays using an antibody against Flag (n=4/group). **D**–**E**, A7r5 cells were transfected with Luc (luciferase) reporter driven by 2 different length (**D**), or wt (wild type) or del (region deleted; **E**) of the *F3* promoter and then infected with Ad-LacZ or Ad-KLF11 (50 MOI). Two days later, the luciferase activity was measured and normalized by *Renilla* activity. The results are presented relative to A7r5 cells transfected with p*F3* (*F3* promoter region; −906/+162; **D**) or wt (**E**) and infected with Ad-LacZ group set as 1 (n=4/group). ***P*<0.01 using 2-way ANOVA followed by Bonferroni test (**C**, **D**, **E**). **F**, Schematic summary: KLF11 inhibits *F3* transcription by directly binding to the *F3* promoter region. Ad-KLF11 indicates adenovirus overexpressing KLF11; Ad-LacZ, adenovirus overexpressing LacZ; IP, immunoprecipitation; MOI, multiplicity of infection; and VSMC, vascular smooth muscle cell.

Moreover, the conversion of prothrombin to thrombin is the common pathway in the coagulation cascade.[61] Under lipopolysaccharide treatment, apart from the increased TF expression, the abnormality of other coagulation factors may also contribute to the elevated TAT complexes in the *Klf11* KO mice.

In summary, utilizing gain- and loss-of-function strategies, we demonstrated an important homeostatic role of KLF11 as an antithrombotic factor in the vascular wall. We uncovered KLF11-dependent transcriptional inhibition of *F3* in VSMCs as the potential mechanism underlying this antithrombotic effect. Our findings extend the current understanding of the roles of KLF11 in the vascular system. Manipulation of this novel molecular target could contribute to therapeutic strategies aimed at controlling thrombosis under pathological conditions and diabetic vascular pathologies at large.

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Disclosures

None.

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Highlights

- Vascular smooth muscle cell–specific KLF11 (Krüppel-like Factor 11) protects against arterial thrombosis.
- Tissue factor is increased in the KLF11-deficient vascular smooth muscle cells.
- KLF11 inhibits *F3* at the transcriptional level.