The transcription factor KLF2 in vascular biology
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Endothelial KLF2 regulates mitogen activated protein kinase signaling through RhoA and the actin cytoskeleton

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Abstract

The shear stress-induced transcription factor Krüppel-like factor 2 (KLF2) confers anti-inflammatory properties to endothelial cells through inhibition of activator protein 1 (AP-1), presumably by interfering with upstream mitogen activated protein kinase (MAPK) cascades.

To gain further insight into the global effects of KLF2 on the endothelial phospho-proteome we used Kinex antibody arrays, followed by validation using conventional techniques. This showed two major modulatory roles for KLF2 on the MAPK network. First, KLF2 represses its own production by a MAPK-mediated negative feedback loop, through activation of RhoA and inhibition of externally regulated kinase 5 (ERK5) and myocyte enhancer factor 2 (MEF2). Both were previously implicated in the activating effects of both shear stress and HMG-CoA reductase inhibitors (statins) on anti-inflammatory KLF2 levels. Second, the phosphorylation of several actin cytoskeleton-associated proteins, like focal adhesion kinase, is markedly repressed by KLF2 explaining its effect on cellular architecture. Furthermore, we demonstrate that KLF2 inhibits the activation of all isoforms of Jun NH₂-terminal kinase (JNK) and its downstream targets ATF2 and c-Jun in a cytoskeleton-dependent manner. Finally, the delayed effects of KLF2 are related to its slow accumulation, associated with a similarly timed gradual formation of actin fibers, and increasing suppression of inflammatory gene expression, mimicking the effects of prolonged flow on endothelial morphology and gene expression.

Collectively, these findings provide mechanistic molecular explanations for the suppressive effects of shear-induced KLF2 on MAPK signaling that constitute an important part of the anti-inflammatory and auto-regulatory effects of KLF2 in vascular homeostasis and disease.
Introduction

Endothelial cells (ECs) in the arterial tree are chronically exposed to pulsatile blood flow and shear stress. But, at sites where shear stress is severely reduced or oscillatory, ECs show signs of dysfunction, which is generally regarded as the first event in the development of atherosclerosis. We and others have identified the endothelial transcription factor Krüppel-like factor 2 (KLF2) to be specifically induced by shear stress. Recently, 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins) were also found to induce KLF2 through inhibition of RhoA activity. Transcriptional induction of KLF2 by these stimuli was shown to involve a concerted mitogen activated protein kinase (MAPK) signaling pathway consisting of MAPK kinase 5 (MEK5), extracellular signal regulated kinase 5 (ERK5) and myocyte enhancer factor 2 (MEF2). Inhibition of transcription of the KLF2 gene by inflammatory cytokines was shown to depend on direct inhibition of MEF2 by histone deacetylase (HDAC) 4 and nuclear factor (NF)-κB. Overexpression of KLF2 was also shown to diminish endogenous KLF2 mRNA levels, suggesting that KLF2 interferes with the MAPK signaling pathway leading to transcription of the KLF2 gene.

MAPK signaling consists of basically four distinct signaling routes with complex interactions and has been implicated in numerous processes including inflammatory signal transduction in ECs. These four canonical routes comprise the ERK1/2, ERK5, Jun NH₂-terminal kinase (JNK) and p38 pathways. The anti-inflammatory and anti-atherosclerotic effects of shear stress on ECs have been attributed to MAPK signaling. Shear stress was shown to inhibit TNF-α-mediated MAPK signaling through the JNK pathway and simultaneously induce the MEK5/ERK5 axis, which prevents apoptosis. Cellular structures involved in endothelial MAPK signal propagation include the actin cytoskeleton and focal adhesions.

In recent years, it has been established that KLF2 acts as a central transcriptional regulator that establishes functional quiescent differentiation in endothelium. Most of these studies were performed using a functional genomics approach and therefore not designed to directly identify KLF2-mediated changes on the level of protein activity. Nonetheless, KLF2 was found to indirectly inhibit post-transcriptional activation of c-Jun and activating transcription factor 2 (ATF2), both members of the activator protein 1 (AP-1) complex. AP-1 is activated through phosphorylation by the MAPKs ERK1/2, JNK and p38. To elucidate how KLF2 regulates the complex MAPK network, we analyzed the phosphorylated proteome (kinome) in the absence and presence of exogenously expressed human KLF2 in human umbilical vein endothelial cells (HUVECs). We show that KLF2 inhibits the MEK5/ERK5/MEF2 MAPK pathway, activates RhoA and blocks the JNK MAPK pathway with the targets ATF2 and c-Jun in a cytoskeleton-dependent fashion.
Materials and methods

Cell culture and reagents
Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described. Confluent monolayers were grown from freshly isolated HUVECs and used before the fourth passage. Actinomycin D, Cytochalasin D, Lovastatin and Trichostatin A were purchased from Sigma (St. Louis, MO).

Lentiviral overexpression, Western blotting, microscopy and RT-PCR
Long-term lentiviral overexpression, microscopy and Western blotting were performed as previously described. The open reading frame of human MEF2B cDNA including stop codon was cloned into the pENTR-D-TOPO vector and shuttled to the pLenti4-V5-DEST vector according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). The P-ERK5 and RhoA antibodies was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the P-JNK, P-c-Jun and P-ATF2 antibodies were from Cell Signaling Technology (Danvers, MA). The KLF2 antibody was described previously. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described.

Luciferase reporter constructs and assay
KLF2 promoter fragments were obtained by PCR using KpnI and XhoI restriction sites-containing primers and human chromosomal DNA. Mutation of the transcription factor binding site was accomplished by PCR overlap mutagenesis, with which the core binding sequence for MEF2 (5’-TTTA-3’) was mutated into 5’-CGCG-3’. The core MZF1 site (5’-CCCC-3’) and the core TBP site (5’-TATA-3’) were mutated into 5’-TTTT-3’ and 5’-GGCC-3’, respectively. These constructs were cloned into the pGL3 basic Firefly luciferase reporter vector and transferred to the modified pRRL-cPPT-CMV-X2-PRE-SIN lentiviral vector, using the NotI and Sall restriction sites, according to our recently described procedure. A control thymidine kinase promoter driven Renilla luciferase construct was likewise transferred to the modified lentiviral vector. HUVECs were transduced at least 48h before luciferase activity was measured with the dual luciferase reporter assay system following the manufacturer’s protocol (Promega, Madison, WI) as recently described.

Kinex® protein array and microarray analysis
For protein array analysis, HUVECs from two independent isolates were lysed seven days after lentiviral transduction following the manufacturer’s protocol (Kinexus, Vancouver, Canada). These lysates were shipped and analyzed in duplicate by the manufacturer (Kinexus). Results were analyzed using a moderated t-test to correct for multiple testing. Microarray analysis was performed as described. Briefly, three independent HUVECs isolates were transduced with KLF2 lentivirus or empty control virus. Cells were lysed and RNA was isolated at 24h, 48h and 72h after transduction. For each timepoint and isolate, KLF2-transduced and control RNA were hybridized in duplo.

RhoA activity assay
Confluent monolayers of HUVEC were lysed in 50mM Tris-HCl, containing 1% NP-40, protease- and phosphatase-inhibitors. Active GTP-bound RhoA was isolated using GST-tagged Rhotekin (Tebu-bio, Heerhugowaard, the Netherlands) and glutathione-agarose beads (Sigma). RhoA levels were analyzed by Western blot.
Chapter 6

Statistical Analysis
Data were analyzed using unpaired student’s T-tests, when comparing two situations, or a one-way ANOVA with Bonferroni correction for multiple comparisons. Probability-values of less than 0.05 were considered significant. Protein array data were analyzed using a moderated T-test specifically designed for array analysis. Microarray data was analyzed as described.

Table 1. Summary of the effects of KLF2 on the endothelial (phospho-)proteome.
The Bayesian P-value and fold induction by KLF2 are indicated, with repressed proteins listed on the left and induced proteins on the right. Proteins highlighted in dark grey are implicated in MAPK signaling and proteins highlighted in light grey are implicated in actin cytoskeleton/integrin signaling.
Results

Delayed KLF2 protein accumulation and downstream effects after lentiviral overexpression

KLF2 confers a quiescent phenotype to ECs by inducing various transcriptional changes in both direct and indirect ways. One of the indirect effects of KLF2 is the inhibition of the AP-1 pathway, through inhibition of phosphorylation and nuclear localization of c-Jun and ATF2. Interestingly, inhibition of ATF2 by KLF2 was found to occur only marginally after 24 hours of shear stress, while complete abrogation of ATF2 activity was observed after long-term shear stress exposure (five days). To optimize lentiviral KLF2 overexpression to mimic the long-term effects of shear stress, we analyzed KLF2 protein levels in time after lentiviral transduction (Figure 1A). KLF2 protein levels gradually increase and correlate to the expression levels of the two best-known direct transcriptional targets of KLF2, endothelial nitric oxide synthase (eNOS) and thrombomodulin, as measured using real-time RT-PCR (Figure 1B). The optimal induction of these target genes is only reached after 72 hours of lentiviral KLF2 overexpression. Furthermore, the previously reported effect of exogenous overexpression of human KLF2 on greatly decreasing mRNA levels of endogenous KLF2 in HUVECs was subject to a similar timecourse (Figure 1B), thereby suggesting a direct negative feedback loop. This suppression seems a result of diminished synthesis as exogenous KLF2 did not affect stability of the endogenous KLF2 mRNA (Figure 1C).

KLF2 modulates the endothelial kinome

To understand how accumulated KLF2 completely inhibits MAPK signaling to AP-1, a global insight into the KLF2-mediated effects on the endothelial phospho-proteome (kinome), was analyzed on total protein lysates of mock- and KLF2-transduced HUVECs seven days after transduction using the Kinex® protein kinase antibody array, simultaneously probing changes in over 600 proteins (Table 1). Surprisingly, only a limited number (16/623 induced, 18/623 repressed) of phosphoproteins was significantly changed by KLF2. As positive control of the validity of the Kinex array, inhibition of phosphorylation of c-Jun by KLF2, shown previously by our group, could readily be confirmed by this array-based technique (Table 1). The most prominent KLF2-mediated effect on the endothelial kinome, however, is the inhibition of phosphorylation of focal adhesion kinase (FAK) (Table 1). FAK is known to be involved in actin cytoskeleton signaling as are other proteins regulated by KLF2, like crystallin αB and heat-shock protein 27 (HSP27). Another striking effect of KLF2 on the endothelial kinome, is the inhibition of phosphorylation of ERK5, which is known to be pivotal in the transcriptional activation of the KLF2 gene. These two pivotal observations were analyzed in more detail using conventional techniques.
Figure 1. KLF2 mRNA expression, protein levels and transcriptional activity after lentiviral transduction.

(A) HUVECs were transduced with mock (M) or KLF2 lentivirus (K), as previously described. After 24, 48 and 72 hours cells were lysed and KLF2 levels were analyzed by Western blot and densitometric quantification (N=3). α-Tubulin was used as a loading control. (B) HUVECs were transduced with mock- or KLF2-lentivirus for 24h, 48h and 72h and mRNA levels of the indicated genes were measured using real-time RT-PCR (N=5). Ratios of KLF2/Mock levels for each time point are expressed as fold change. (C) Mock- and KLF2-transduced HUVECs were treated with Actinomycin D (2 μg/ml) for 1, 2 or 4 hours or left untreated (set to 1 for each condition). Shown are average mRNA levels of endogenous KLF2 in three independent experiments, as quantified by real-time RT-PCR.
**KLF2 inhibits transcription of the KLF2 gene through inhibition of MEF2**

ERK5 is known to induce KLF2 transcription through activation of MEF2, which directly interacts with the KLF2 promoter. The observed downregulation of ERK5 phosphorylation by KLF2 (Table 1) could be quantitatively confirmed by Western blot analysis (Figure 2A). To further analyze the KLF2 promoter and the putative involvement of MEF2 in its auto-feedback regulation, we made use of our recently described lentiviral luciferase reporter assay, which enables stable integration of reporter constructs in the endothelial genome. A first analysis with constructs containing different lengths of the KLF2 promoter confirmed the validity of this approach. The 367bp minimal KLF2 promoter fragment immediately upstream of the translational start site contains the essential regulatory elements to study regulation of the KLF2 gene, as exemplified by its induction by an HMG-CoA reductase inhibitor (Supplemental Figure I). In depth analysis of this part of the promoter for the presence of evolutionary conserved putative transcription factor binding sites (TFBS) using Genomatix software (Munich, Germany), confirmed the presence of the reported MEF2 site, and revealed the presence of myeloid zinc finger 1 (MZF1) and TATA box-binding protein (TBP) binding sites within this region (Supplemental Figure II). We confirm that the MEF2 site is indeed functional in our reporter construct, as lentiviral overexpression of human MEF2B induces transcriptional activity of the wild-type minimal KLF2 promoter, but not of the promoter in which the core MEF2 site is mutated (Figure 2B). Next, using KLF2 promoter constructs containing mutations in these putative TFBS, we show that the magnitude of basal promoter activity is dependent on all analyzed TFBS, but the relieve of the reduction by KLF2 overexpression is only observed by mutation of the MEF2 site (Figure 2C). As specified by the numbers next to the bars, which indicate the fold induction by KLF2 overexpression, transcription through the promoter fragment where the MEF2 site is mutated is only 1.5-fold repressed by exogenous KLF2, in contrast to the three- to four-fold reduction in promoters containing the functional MEF2 site.

**KLF2 inhibits the MEK5/ERK5 pathway and activates RhoA**

To investigate the KLF2-mediated regulation of MEF2 activity, HUVEC transduced with the minimal KLF2 promoter reporter construct were stimulated with the class I and II HDAC inhibitor Trichostatin A (TSA), or Lovastatin, two known inducers of MEF2-dependent transcription (Figure 3A). TSA directly inhibits HDACs associated with MEF2 and Lovastatin inhibits the addition of geranylgeranyl pyrophosphate moieties to RhoA, which normally inhibits MEK5/ERK5 signaling to MEF2. While TSA induces KLF2 promoter activity in both mock- and KLF2-transduced HUVECs, Lovastatin greatly induced promoter activity in mock-transduced cells, but only to a minor extent in KLF2-transduced HUVECs, which is exemplified by the 6.3 fold repression by KLF2 in the presence of Lovastatin. Previously, it was shown that the MEK5/ERK5 pathway leading to KLF2 expression is sensitive to inhibition by RhoA, which can be alleviated by statin treatment. Our data confirm this mechanism, as substantiated by the finding that activity of the MEF2-mutated promoter
Figure 2. The KLF2-induced negative feedback loop inhibits KLF2 promoter activity through the conserved MEF2 site.

(A) Western blot analysis and quantification thereof showing levels of phosphorylated ERK5 (P-ERK5) in the presence (KLF2) or absence (Mock) of exogenous KLF2. (N=3). (B) Transduction with either MEF2B- (black bars) or mock-lentivirus (white bars) was performed at least five days before measurement and transduction with the -367 bp. to +1 bp. reporter lentivirus or a mutant thereof (-MEF2 site) was performed 48h before measurement. Average levels of luciferase activity of at least 3 experiments are shown. (C) Luciferase activity was measured after transduction with lentiviral reporter vectors containing parts of the wild-type KLF2 core promoter (-367 bp. to +1 bp. relative to ATG start-codon) or mutations thereof (MEF2, MZF1 and TATA box-binding protein sites mutated). Numbers next to the bars indicate fold change by KLF2 transduction vs. control (N=3). *P<0.05
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KLF2 regulates the phosphorylation of actin cytoskeleton associated proteins

KLF2 regulates the levels of many (phosphorylated) proteins that are associated with

Figure 3. KLF2 induces a negative feedback loop through induction of RhoA.

(A) Luciferase reporter assay, performed at least five days after transduction with either KLF2- (black bars) or mock-lentivirus (white bars) and 48h after transduction with the -367 bp. to +1 bp. reporter lentivirus or a mutant thereof (-MEF2 site). Trichostatin A (TSA) (1 μM) and Lovastatin (10 μM) were added 16 hours before luciferase measurement. Averages of at least 3 experiments are shown. Numbers next to the bars indicate fold induction by KLF2 transduction vs. control. (B) Active RhoA was isolated using GST-tagged Rhotekin and a representative subsequent Western blot for RhoA is shown. Three independent experiments were quantified and corrected for α-Tubulin. *P<0.05
actin cytoskeleton signaling (Table 1), which is consistent with the morphological changes seen in actin cytoskeleton architecture as well as with RhoA activation (Figure 3B), when KLF2 is overexpressed. Notably, KLF2 regulates phosphorylation of specific residues of proteins like crystallin αB, focal adhesion kinase (FAK) and heat-shock protein 27 (HSP27), without affecting their total protein levels (Table 1). FAK phosphorylations at tyrosine

![Figure 4. KLF2 inhibits JNK phosphorylation in an actin-dependent manner.](image)

(A and B) Summary of levels of the different post-translationally activated forms of FAK and Hsp27 in KLF2-transduced compared to mock-transduced cells, as analyzed using the Kinex® antibody micro-array. The category axis shows antibody specificity for the indicated phosphorylated amino acid residue. Fold induction by KLF2 is indicated on the Y-axis. (C) Western blot analysis of P-JNK1, 2 and 3 levels in mock- (white bar) and KLF2-transduced HUVECs (black bar) in the presence of DMSO (control) or Cytochalasin D (200 nM). α-Tubulin was used as a loading control. Quantifications are shown for four independent experiments. (D) Fluorescent photomicrographs of mock- and KLF2-transduced cells treated with DMSO or 200 nM Cytochalasin D, showing filamentous actin in red and nuclei in blue. The scale bar indicates 10 μm. *P<0.05, **P<0.01
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Residue 576, described to be induced by auto-activation,28 and at serine residue 732, induced by neuronal cyclin dependent kinase 5 (CDK5),29 are most significantly inhibited by KLF2 (Figure 4A). On the other hand, phosphorylation of FAK on tyrosine residue 397, known for its involvement in the activation of PI3K, is slightly induced by KLF2. Two different array antibodies specific for HSP27 phosphorylated at serine residue 82 show that KLF2 inhibits HSP27 activation (Figure 4B), normally a product of activated p38, protein kinase C and protein kinase D.30 Based on these results, we hypothesized that KLF2 might regulate MAPK signaling through actin cytoskeleton associated proteins, which we next set out to study.

KLF2 inhibits JNK and downstream targets c-Jun and ATF2 in an actin cytoskeleton-dependent manner

FAK has been implicated in the phosphorylation of JNK, the upstream kinase activating both AP-1 components c-Jun and ATF2. Actin-mediated activation of JNK via FAK only requires a small (140 amino acids) domain of FAK.31 KLF2 inhibits phosphorylation of serine residue 732 (Table 1), which lies in this domain, suggesting that KLF2 might interfere with JNK phosphorylation. Western blot analysis showed that phosphorylation of JNK1 and JNK2/3 is indeed inhibited by KLF2 (Figure 4C, left two lanes). To assess the role of the actin cytoskeleton in the KLF2-mediated inhibition of JNK activity, the actin cytoskeleton in mock- and KLF2-transduced HUVECs was disrupted with Cytochalasin D, which specifically binds actin dimers with very high affinity, thereby inhibiting actin polymerization.32 Cytochalasin D treatment prevented typical actin fiber formation in KLF2-transduced ECs, as assessed by fluorescence microscopy (Figure 4D). Strikingly, the mere disruption of the actin cytoskeleton completely blocked the inhibitory effect of KLF2 on phosphorylation of all JNK isoforms (Figure 4C).

The involvement of the actin cytoskeleton in the KLF2-mediated attenuation of JNK phosphorylation was tested for its involvement in downstream signaling to the JNK targets c-Jun and ATF2. To that end, mock- and KLF2-transduced HUVEC were treated with Cytochalasin D and we performed immunofluorescence analysis of phosphorylated c-Jun and phosphorylated ATF2 levels and localization. Using antibodies against two different phosphorylated forms of c-Jun (P-Ser63 and P-Ser73) and one antibody against phosphorylated ATF2 (P-Thr71), we show that disruption of the actin cytoskeleton can reverse the inhibitory effect of KLF2 on nuclear localization of both of these JNK-activated transcription factors (Figure 5).

Anti-inflammatory gene expression follows the KLF2-mediated changes in actin cytoskeleton architecture

Given the delayed effects of KLF2 on gene expression and autoinhibition (Figure 1), we next evaluated the effect on cytoskeleton rearrangements in more detail. As shown in figure
Figure 5. KLF2 inhibits nuclear localization of P-c-Jun and P-ATF2 in an actin-dependent manner.

Immuno-fluorescent photomicrographs and quantification thereof of mock- (A, E, I, M, Q, U, C, G, K, O, S and W) and KLF2-transduced (B, F, J, N, R, V) HUVECs treated with DMSO (A, B, E, F, I, J, M, N, Q, R, U and V) or Cytochalasin D (200 nM) (C, D, G, H, K, L, O, P, S, T, W and X). Phosphorylated c-Jun on serine residue 63 (A-H) or serine residue 73 (I-P) or phosphorylated ATF2 on threonine residue 71 (Q-X) are stained red and nuclei are stained blue with Hoechst 33342 (E-H, M-P and U-X). The scale bar indicates 10 μm. (Y) Average nuclear intensity was measured for the indicated fluorescent staining and condition (N=5). For each staining, student’s T-tests were performed comparing KLF2/DMSO vs. Mock/DMSO and KLF2/CytD vs Mock/CytD. ***P<0.001, N.S. P>0.05
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6A, the effects of KLF2 on actin stress fiber formation and maturation follow a similar timecourse as the slow accumulation of KLF2 (Figure 1). To test whether actin fiber formation is also required for the inhibitory effect of KLF2 on its own promoter, the actin cytoskeleton was disrupted with Cytochalasin D in mock- and KLF2-transduced cells. However, treatment with Cytochalasin D did not alter KLF2 promoter activity (Figure 6B).

Finally, effects on gene expression during this slow maturation were followed by genome-wide transcriptome analysis (Figure 6C and Supplemental table II). A modified Venn analysis of KLF2 induced genes during different stages of KLF2 overexpression indeed shows that of the 1281 genes that are regulated by prolonged KLF2 overexpression (7 days, 168h), only 16% (206 genes) is significantly regulated at 72h after KLF2 transduction. Of the 206 genes that were significantly regulated at 72h of KLF2 overexpression, only 72 genes (35%) are affected at 48h. At 24h after KLF2 transduction, only the transcript of KLF2 itself is induced, in accord with the results shown in figure 1. The earliest transcriptional targets of KLF2 comprise the known direct targets thrombomodulin (THBD) and eNOS, but also some of the most significant KLF2 downstream genes like aquaporin 1 (AQP1), cytochrome P450 1B1 (CYP1B1), hyaluronidase 2 (HYAL2) and the prostaglandin transporter SLCO2A1. The genes that constitute the previously reported anti-inflammatory effects of KLF2, however, are all regulated after full maturation of the actin fiber network (at 72h and 168h after transduction).
Figure 6. Actin cytoskeleton changes induced by KLF2 overexpression occur rapidly but are not involved in the negative feedback loop of KLF2.

(A) Mock- and KLF2-transduced HUVECs were fixed with paraformaldehyde at 24, 48 and 72 hours after transduction. F-actin is visualized in red, KLF2 in green and nuclei in blue.

(B) Mock- and KLF2-transduced HUVECs were transduced with the 367bp KLF2 promoter construct 48h before luciferase measurement. Cytochalasin D (200 nM) or DMSO (0.1%) was added 16h before luciferase measurement. Averages of three experiments are shown. Numbers indicate fold induction by KLF2.

(C) Modified Venn analysis of microarray expression data at 24h, 48h, 72h and 168h after lentiviral KLF2 transduction. Indicated are the numbers of genes per timepoint that meet the indicated criteria, but do not meet the criteria for an earlier timepoint. Inflammatory genes, TGF-β signaling genes and vasodilatation genes were previously published to be KLF2-dependent.\textsuperscript{10, 16-18} *P<0.05
Discussion

The shear stress-induced transcription factor KLF2 inhibits both its own production and the pro-inflammatory state of ECs cultured in the absence of flow. Using array-based phosphoproteome profiling we now show the concerted effects of KLF2 on the endothelial kinome. The two major MAPK routes affected are schematically represented in figure 7. First, MEF2-dependent transcription of the KLF2 gene itself is similarly repressed through RhoA-mediated inhibition of upstream MAPK signaling. Second, we show that KLF2 attenuates MAPK-mediated posttranslational activation of the AP-1 components c-Jun and ATF2 in an actin cytoskeleton-dependent manner and that anti-inflammatory gene expression is only displayed after KLF2-mediated changes in cytoskeleton architecture. In general, these results complete the basic architecture of the KLF2-dependent effects on MAPK signaling in ECs. Furthermore, this corroborates its role as a repressor of MAPK activation, as recently also demonstrated in relation to the proposed contribution of KLF2 to negative feedback effects that control EGF signaling in tumorigenesis.\textsuperscript{33}
Previous reports have established the essential contribution of the MEK5/ERK5/MEF2 axis to KLF2 transcription.\(^7,21\) We now show that the essential MEF2 site in the KLF2 promoter lies in an evolutionarily conserved region, as assessed using Genomatix software. In the same region, a putative MZF-1 binding site was identified, which also appears to be essential for normal transcription of the KLF2 gene in static ECs (Figure 2C). MZF-1 is known to be expressed in a myeloid-specific manner, which is in concurrence with the expression pattern for KLF2,\(^34\) offering a potential explanation for the cell type-specific expression of KLF2. Furthermore, MZF-1 is known to induce transcription of myeloid-specific genes in cells from the myeloid lineage, but to repress the same genes in non-myeloid cells,\(^35\) indicating that additional factors are required for lineage-specific transcription, such as MEF2. In accordance with this, ERK5, which activates KLF2 transcription via MEF2, has also been described to regulate tissue specific gene expression.\(^7\) Based on our phosphoproteome analysis (Table 1), we show that the signaling pathway leading to KLF2 expression is controlled by a negative feedback mechanism involving RhoA. Strikingly, this is precisely the molecule inhibited by HMG-CoA reductase inhibitors to induce KLF2 expression (Figure 7). This is achieved by inhibiting the synthesis of essential geranylgeranyl pyrophosphate moieties of RhoA, thereby increasing MEK5/ERK5/ERK5 activity, as was also shown for another RhoA inhibitor, exotoxin C3.\(^6\) Similarly, shear stress also induces KLF2 expression by activation of the MEK5/ERK5/MEF2 signaling pathway (Figure 7).\(^21\) Collectively, these data are consistent with a model in which the activating MEK5/ERK5/MEF2 signaling pathway is balanced by a concerted inhibition by KLF2-induced RhoA.

**Figure 7.** KLF2 attenuates the MEK5/ERK5/MEF2 cascade through RhoA activation and inhibits JNK and subsequent c-Jun and ATF2 activation in an actin-dependent manner.

Schematic representation of the indirect KLF2-mediated inhibition of the JNK MAPK pathway via the actin cytoskeleton and the RhoA dependent attenuation of the upstream MEK5/ERK5/MEF2 cascade.

![Diagram of KLF2 attenuation](image-url)
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Negative feedback mechanisms are essential to establish tightly regulated and controllable protein levels and the current model (Figure 7) has all the characteristics of a classic “negative feedback amplifier” so frequently described in MAPK signaling networks. In accordance with such model, KLF2 levels are indeed precisely regulated in a linear proportional relation to shear stress levels, for which we now postulate an underlying molecular mechanism. The details of this auto-inhibitory feedback loop, specifically how KLF2 might activate RhoA, need further detailed investigations, as no specific clues could be found in the current kinome data (Table 1) or expression data (Supplemental table I).

The second issue we addressed at the global kinome level, concerns the anti-inflammatory effects of KLF2, most notably through our previously reported effects on the AP-1 members ATF2 and c-Jun. The current data confirm our previous findings that KLF2 largely inhibits c-Jun phosphorylation but not its total protein levels, whereas ATF2 levels and phosphorylation are even slightly increased, but the active form (phosphorylated on Thr71) is excluded from the nucleus. Surprisingly, no gross changes in the MAPK network upstream of JNK could be detected (Table 1). Rather, a widespread effect of KLF2 on actin cytoskeleton related proteins and kinases was found. We now show that it is indeed the actin cytoskeleton itself that is responsible for the suppression of MAPK signaling, with all isoforms of JNK being affected (Figure 4C). Moreover, disruption of the actin cytoskeleton with cytochalasin D completely abolished the inhibitory effects of KLF2 on activation and nuclear localization of c-Jun and ATF2 (Figure 5) and suppression of inflammatory gene expression by KLF2 only occurs after full maturation of the actin cytoskeleton (Figure 6).

The actin fibers that are indirectly formed by KLF2 are highly similar in appearance to stress fibers formed by shear stress. Rho activation is known to underlie actin polymerization due to shear stress and this is in agreement with the finding that KLF2 induces RhoA (Figure 3B). In vivo, KLF2 expression levels are regulated by the fine balance between the shear stress-mediated activation through the MEKS/ERK5/MEF2 pathway and the inhibition through RhoA activation by KLF2. This could offer an explanation for the well-established atheroprotective effects of Rho Kinase inhibition, by Fasudil for instance, which would shift the balance towards induction of KLF2 and its downstream anti-inflammatory effects. Furthermore, it explains the reduced potency of statins versus shear stress, which we previously reported, as direct RhoA inhibition by statins will induce KLF2 expression in the absence of actin fiber formation. The importance of the actin cytoskeleton in the development of atherosclerosis, typically initiated due to absence of shear stress and hence KLF2, has recently been shown directly. Romeo and colleagues investigated the role of profilin-1 (Pfn1), an essential protein that dynamically regulates actin polymerization, proving that its diminished expression prevented pro-atherogenic MAPK signaling in endothelial cells and suppressed in vivo atherosclerosis in a mouse model. That study also showed that ATF2 activation was impaired in (atheroprotected) Pfn1+/− mice, which is in accordance with previous data and the data presented here that KLF2 inhibits activation of ATF2 in an actin dependent manner. Previously, we described that KLF2 inhibits the TGF-β-mediated induction of PAI-1, partly
through inhibition of MAPK signaling to c-Jun. In accordance with this finding, TGF-β-induced PAI-1 expression through MAPK signaling was also found to be actin cytoskeleton-dependent.

JNK has long been known as a pro-inflammatory mediator in many cell-types involved in atherosclerosis and insulin resistance. More recently, JNK was shown to be the central mediator of the inflammatory and atherogenic effects of advanced glycation endproducts in ECs. Our finding that KLF2 utilizes the actin cytoskeleton to indirectly inhibit JNK signaling suggests that the KLF2-mediated regulation of the actin cytoskeleton forms an essential part of the anti-inflammatory and anti-atherosclerotic effects of shear stress. Onset of shear stress induces remodeling of the actin cytoskeleton and the ECs align in the direction of the flow within 6 hours and the typical shear-induced actin stress fibers become visible approximately 24 hours after shear exposure. Furthermore, short-term shear stress exposure (for less than 6 hours) is known to activate JNK signaling. And even though KLF2 mRNA expression is induced in this short time-frame, its downstream targets like eNOS and thrombomodulin (which are indicative of KLF2 protein function) are not induced, while this is well established for prolonged shear stress (more than 24 hours). Similarly, KLF2 effects are evident only after prolonged overexpression, correlating with direct downstream target regulation as earliest events (Figures 1B and 6C) and actin fiber formation and inhibition of pro-inflammatory JNK and TGF-β signaling as delayed effects (Figures 5, 6A and 6C).

In conclusion, the current comprehensive analysis of KLF2 effects on endothelial MAPK signaling completes a general lay-out of the architecture of the suppressive effects of KLF2 on MAPK signaling that constitute an important part of the anti-inflammatory and autoregulatory effects of KLF2. A future, more detailed knowledge of this integrated signaling network might supply specific points of intervention that could mimic the protective effects of both shear stress and statins on endothelial function to prevent vascular disease.
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References


