Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Arterioscler Thromb Vasc Biol. 2013;33:2608-2617; originally published online August 22, 2013;
doi: 10.1161/ATVBAHA.113.302161
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Objective—Vascular endothelial cells (ECs) are continuously exposed to blood flow that contributes to the maintenance of vessel structure and function; however, the effect of hemodynamic forces on transforming growth factor-β (TGF-β) signaling in the endothelium is poorly described. We examined the potential role of TGF-β signaling in mediating the protective effects of shear stress on ECs.

Approach and Results—Human umbilical vein ECs (HUVECs) exposed to shear stress were compared with cells grown under static conditions. Signaling through the TGF-β receptor ALK5 was inhibited with SB525334. Cells were examined for morphological changes and harvested for analysis by real-time polymerase chain reaction, Western blot analysis, apoptosis, proliferation, and immunocytochemistry. Shear stress resulted in ALK5-dependent alignment of HUVECs as well as attenuation of apoptosis and proliferation compared with static controls. Shear stress led to an ALK5-dependent increase in TGF-β3 and Krüppel-like factor 2, phosphorylation of endothelial NO synthase, and NO release. Addition of the NO donor S-nitroso-N-acetylpenicillamine rescued the cells from apoptosis attributable to ALK5 inhibition under shear stress. Knockdown of TGF-β3, but not TGF-β1, disrupted the HUVEC monolayer and prevented the induction of Krüppel-like factor 2 by shear.

Conclusions—Shear stress of HUVECs induces TGF-β3 signaling and subsequent activation of Krüppel-like factor 2 and NO, and represents a novel role for TGF-β3 in the maintenance of HUVEC homeostasis in a hemodynamic environment. **(*Arterioscler Thromb Vasc Biol*. 2013;33:2608-2617.)**

Key Words: endothelium ■ hemodynamics ■ KLF2 protein, human ■ nitric oxide ■ transforming growth factor beta

Mechanical forces associated with blood flow play an important role in maintaining vessel structure and function. Endothelial cells (ECs) lining the vasculature are continuously exposed to shear stress as a result of fluid forces at the blood/EC interface. Shearing of ECs leads to reorganization of the cytoskeleton, morphological alterations, and production of a variety of substances that act on the ECs themselves and surrounding cells.[1–3]

Coordinated release of growth factors by cells of the vessel wall plays a critical role in development, stabilization, and function of the vasculature. Transforming growth factor-β (TGF-β) is a multifunctional growth factor that is a well-established modulator of vascular cells.[4] Of the 3 mammalian TGF-βs (TGF-β1, -β2, -β3), TGF-β1 and TGF-β3 are found in the vasculature. Structural homology for each isoform among species is ≈98% and ≈71% to 76% between the different isoforms, indicating specific roles for each isoform in vivo[5]; however, distinct effects of vascular-derived TGF-β1 and TGF-β3 have not been elucidated.

After its cleavage from an inactive precursor, TGF-β forms a dimer that binds the TGF-β receptor II on the endothelial surface. Subsequent recruitment of the TGF-β receptor I (ALK1 or ALK5 in ECs) into a tetrameric complex results in phosphorylation of the intracellular domain of TGF-β receptor I and the activation of smad transcription factors. Both TGF-β1 and TGF-β3 are detected in plasma in the absence of pathology, consistent with a role for these factors in vessel homeostasis,[6–8] as they play for epithelial cells.[9] In support of this notion, it has been shown that pre-eclampsia, which is characterized by systemic endothelial dysfunction, multiple end-organ ischemia, hypertension, proteinuria, and increased vascular permeability, is attributable, at least in part, to elevated levels of soluble endoglin (sEng), which neutralizes TGF-β1 and TGF-β3.[10] Similarly, experimental systemic inhibition of TGF-β1 and TGF-β3, achieved by overexpression of sEng, leads to vascular permeability and perfusion defects as well as apoptosis of both vascular and nonvascular tissues.[11] sEng overexpressing mice also demonstrate an essential role for TGF-β in maintaining the endothelium in a nonactivated state[12] and microvessel integrity and function in the retina and choroid plexus.[13,14] Previous reports demonstrate that TGF-β signaling between EC and mural cells participates in vessel stabilization in vivo and in vitro, and paracrine TGF-β signaling between ECs and surrounding mural cells and astrocytes is well documented.[11,15–17]

Received on: July 19, 2011; final version accepted on: August 5, 2013.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.113.302161/-/DC1.

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Nonstandard Abbreviations and Acronyms	
EC	endothelial cell
eNOS	endothelial NO synthase
HUVEC	human umbilical vein endothelial cell
KLF	Krüppel-like factor
sEng	soluble endoglin
TGF-β	transforming growth factor-β

Members of the Krüppel-like factor (KLF) family of transcription factors, in particular KLF2, act as central mediators of shear stress–induced changes in EC.[18–20] Shear stress–induced KLF2 regulates the expression of genes important in inflammation, thrombosis, and vessel tone, with an estimated ≈46% of flow-regulated genes downstream of KLF2 induction.[21–25] Among the KLF2-inducible factors regulating vessel tone is endothelial NO synthase (eNOS), the well-described shear stress–inducible enzyme that is responsible for formation of NO. NO, a gas, with a half-life of a few seconds, acts on underlying mural cells (pericytes and smooth muscle cells) to regulate vessel tone and on ECs to modulate inflammatory properties.[26–28] The precise mechanism by which the endothelium senses shear stress to increase both KLF2 and NO signaling is unclear.

Experimental inhibition of TGF-β by overexpression of sEng in vivo reveals an important role for TGF-β in maintaining vessel structure and function in the kidney, retina, mesentery, and choroid plexus[10,12–14]; however, the role of flow in modulating TGF-β signaling in ECs is poorly understood. We, therefore, investigated the effect of shear stress on TGF-β signaling in ECs and examined the relationship among flow-induced changes in TGF-β, KLF2, and NO signaling. Our results indicate that protective effect of shear stress on ECs is mediated via induction of TGF-β3 signaling and downstream KLF2 and NO signaling.

Materials and Methods

Detailed information on the materials and methods, including cell culture, RNA isolation and real-time polymerase chain reaction, Western blot analysis, and immunofluorescence are described in the online-only Supplement.

Results

Role of TGF-β Signaling in the Morphological Response of Human Umbilical Vein ECs to Shear Stress

Confluent monolayers of human umbilical vein ECs (HUVECs) were exposed to shear (10 dynes/cm^2) in serum-reduced medium, and cell morphology was examined every 24

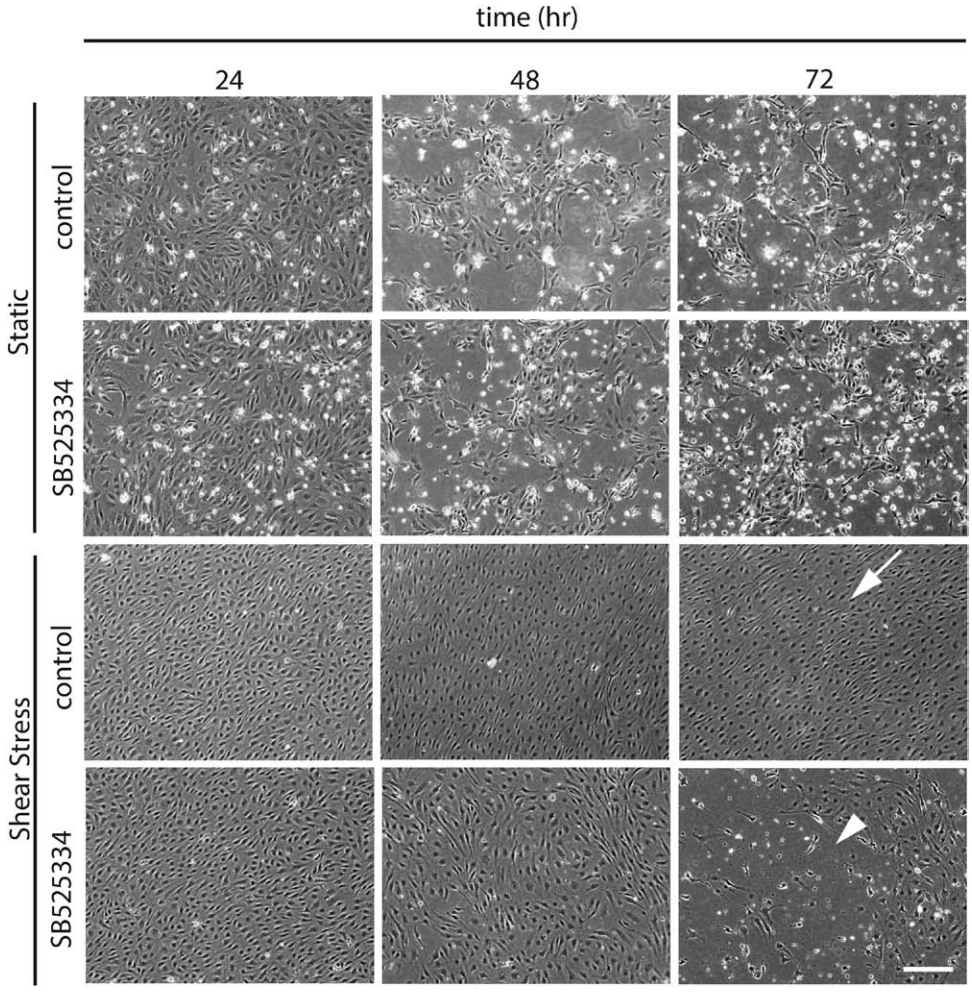

Figure 1. Effects of ALK5 inhibition on shear stress–induced changes in human umbilical vein endothelial cell (HUVEC) morphology. Shear stress led to HUVEC alignment along the flow path (arrow). Treatment of cells with the ALK5 inhibitor, SB525334, led to a disruption of the HUVEC monolayer after 72 hours (arrowhead).

hours for 3 days. Consistent with previous observations,[29] shear stress caused HUVECs to align in the direction of the flow path, with the cell alignment becoming more pronounced with time (Figure 1). In contrast, static cells detached from the plate so that after 3 days only ≈50% of the cells remained (Figure 1).

To examine the role of TGF-β receptor ALK5 signaling, HUVECs in a static and hemodynamic environment were exposed to 10 μmol/L SB525334 in the presence or absence of shear stress. The effect of SB525334 on static HUVECs was variable, with either no effect or a slight attenuation of cell loss compared with static controls (Figure 1). Addition of SB525334 to cells before exposure to shear stress led to a modest loss of cells at 24 or 48 hours and a dramatic loss of cells after 72 hours (Figures 1 and 2A). The exposure of cells to shear stress in the presence of SB525334 (Figure 1) resulted in ≈50% loss of cells, whereas throughout the well, the degree of cell loss per field of view varied from 0% to 100%. The inhibition of ALK4, ALK5, and ALK7 with 10 μmol/L SB431542 did not impair endothelial morphology (Figure I in the online-only Data Supplement). These results reveal that ALK5 is required to maintain the endothelial monolayer exposed to shear stress.

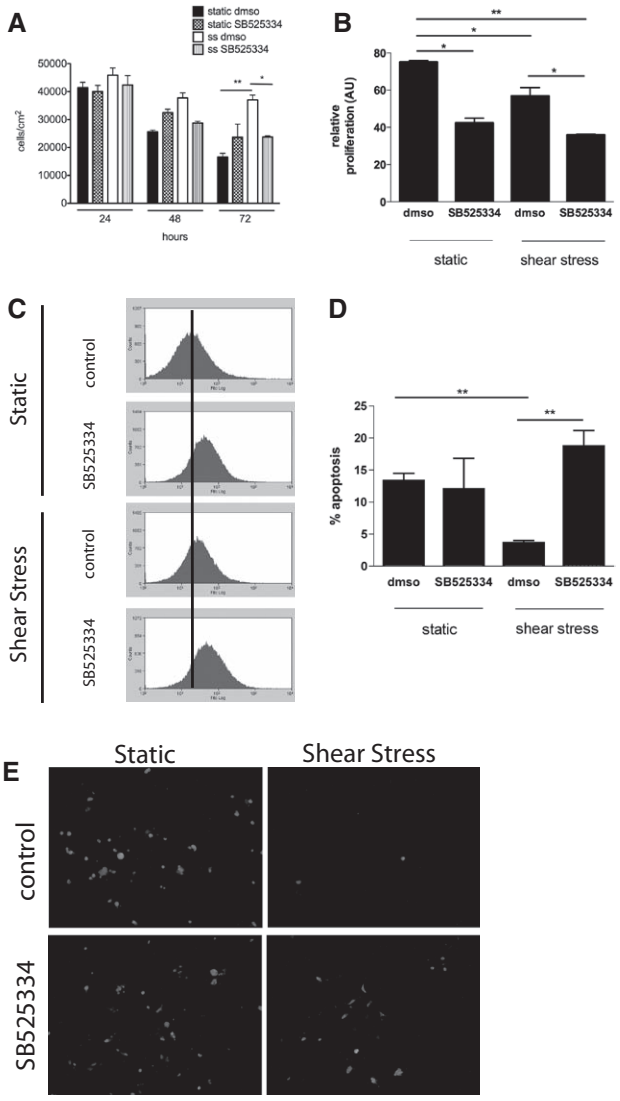

Figure 2. A, Shear stress of human umbilical vein endothelial cell (HUVEC) limited the decrease in cell numbers compared with cells grown under static conditions; SB525334 reduced this protective effect. **B** and **C**, Shear stress decreased HUVEC proliferation; SB525334 further decreased proliferation. **D**, Shear stress decreased HUVEC apoptosis and caspase-3 activation (**E**) and is dependent on ALK5. **P*<0.05; ***P*<0.01.

Effect of TGF-β Receptor Inhibition on HUVEC Proliferation and Apoptosis Under Shear Stress

Consistent with previous reports, exposure of HUVECs to shear stress reduced cell loss when compared with HUVECs under static conditions.[3,30,31] SB525334 blockade of ALK5 signaling in HUVEC exposed to shear stress led to an increase in cell loss (Figure 2A). As cell number may reflect changes in cell death and proliferation, HUVEC proliferation was determined using a cell tracer assay. Despite the increased cell number compared with static control, shear stress decreased HUVEC proliferation by ≈25%. Addition of SB525334 further decreased HUVEC proliferation under both static and shear stress conditions by ≈40% (Figure 2B and 2C).

Exposure of HUVEC to shear stress for 72 hours protected HUVEC from apoptosis, and inhibition of TGF-β signaling prevented this protection (Figure 2D). Whereas addition of SB525334 had no significant effect on the level of apoptosis in cells grown under static conditions, it led to an ≈5-fold increase in apoptosis compared with shear stress control cells. Immunofluorescent localization of cleaved caspase-3, a key component of caspase-dependent apoptosis, revealed that shear stress decreased the cleavage of caspase-3 in HUVECs (Figure 2E). These findings demonstrate that ALK5 limits endothelial apoptosis under shear stress conditions.

Effect of Shear Stress and TGF-β Receptor Inhibition on TGF-β Signaling in EC

To assess the effect of shear stress on TGF-β levels in HUVECs, conditioned media and cell lysates were collected after 72 hours and examined for active and total TGF-β1 and TGF-β3. Both latent and active TGF-β1 and TGF-β3 were detected in cell lysates, with active TGF-β3, but not TGF-β1, significantly increased by exposure to shear stress (Figure 3A). Examination of conditioned media revealed that neither total TGF-β1 nor total TGF-β3 was significantly increased under shear stress conditions; neither active TGF-β1 nor TGF-β3 was detected in media collected. Latent TGF-β1, but not TGF-β3 (latent or active), was detected in media, and the levels were not changed with shear stress (Figure 3A). Real-time polymerase chain reaction revealed that shear stress induced a ≈3-fold increase in TGF-β3; levels of TGF-β1 mRNA were unchanged. The induction of TGF-β3 by shear stress was blocked by the addition of SB525334 (Figure 3B). In contrast to increased TGF-β in EC exposed to shear stress, levels of ALK5 mRNA were unchanged (Figure II in the online-only Data Supplement).

To determine whether the shear stress–induced increases in the levels of TGF-β3 resulted in downstream signaling, intracellular smad2 proteins were examined after 24 hours of shear stress. Shear stress led to a 2.5-fold increase in the phosphorylation of smad2, in the absence of any significant changes in total smad2 levels (Figure 3C). Addition of SB525334 reduced the phosphorylation of smad2 under both static and

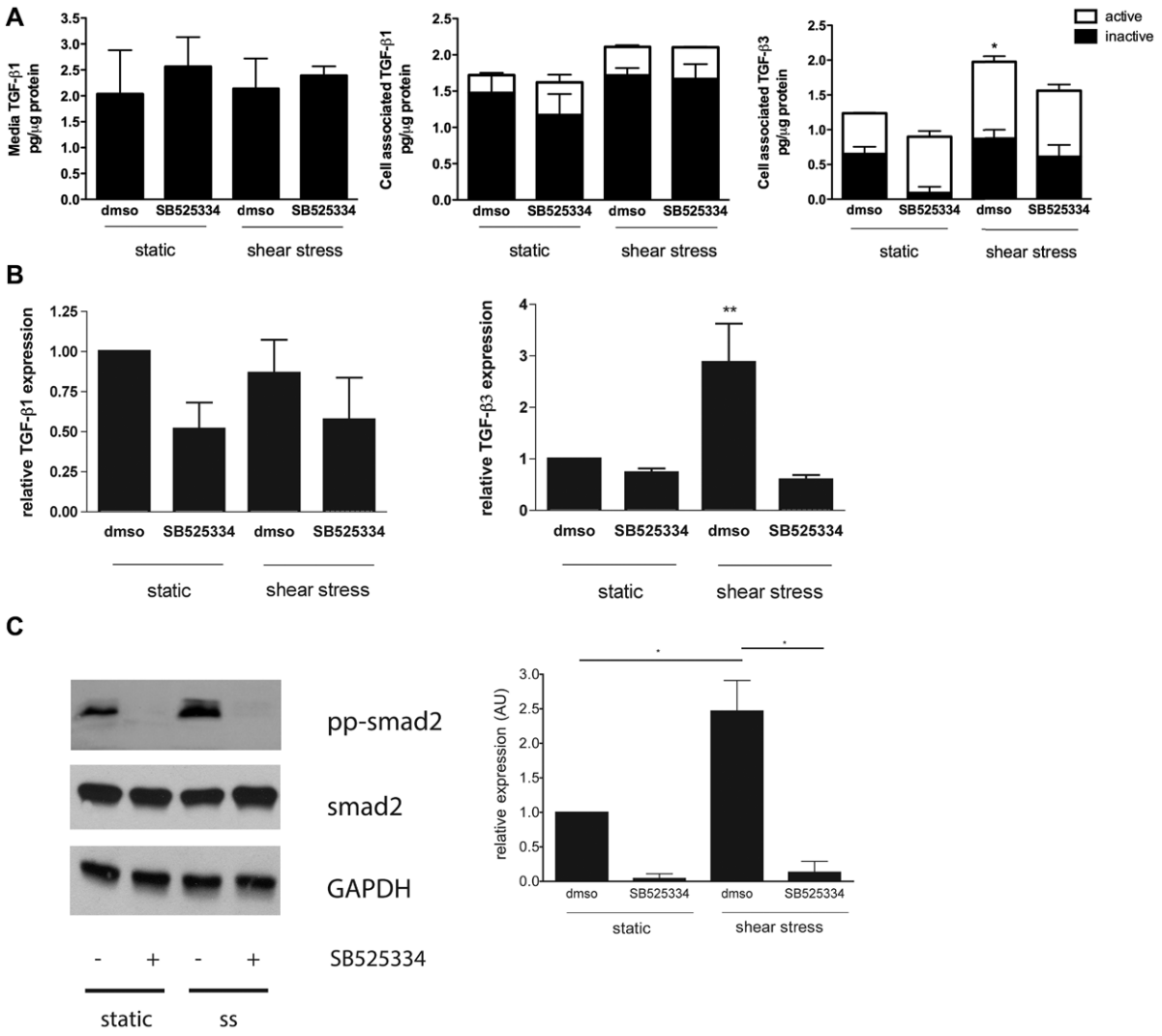

Figure 3. A, Transforming growth factor-β1 (TGF-β1) was detected in human umbilical vein endothelial cell (HUVEC) conditioned media. Shear stress increased active and latent cell–associated TGF-β3, but had no effect on levels of TGF-β1. **B**, Shear stress increased TGF-β3 mRNA and phosphorylation of smad2 (**C**). Addition of SB525334 inhibited the phosphorylation of smad2. *P<0.05; **P<0.01.

shear stress conditions without altering smad2 levels. These results reveal that shear stress of HUVECs increases TGF-β3 and activates downstream smad2 signaling.

Role of KLF2 and NO in Shear Stress–Induced TGF-β Signaling in HUVECs

KLF2 is a key transcriptional regulator of flow-induced changes in ECs.[20] To determine whether TGF-β signaling plays a role in flow-induced KLF2 signaling, HUVECs were treated with SB525334 under static and shear stress conditions and examined for changes in KLF2 mRNA and protein after 24 hours. Shear stress increased KLF2 mRNA (Figure 4A) and protein (Figure 4B and 4C), and the addition of SB525334 resulted in a significant (≈50%) decrease in both KLF2 mRNA and protein. Small interfering RNA (siRNA) targeting of ALK5 also led to a significant (≈30%) reduction of KLF2 mRNA (Figure III in the online-only Data Supplement). Addition of 0.1 ng/mL TGF-β3 induced KLF2 mRNA, which was partially reversible with siRNA targeting of ALK5 (Figure IV in the online-only Data Supplement).

NO signaling is the classic mediator of the vasoactive effects of shear stress in ECs. Exposure of HUVECs to shear stress increased eNOS protein phosphorylation (serine 1179) by ≈2.5-fold after 24 hours (Figure 4D). To determine whether TGF-β plays a role in the shear stress–induced formation and release of NO from HUVECs, the effect of TGF-β receptor inhibition was examined. Addition of SB525334 decreased eNOS phosphorylation in the absence of any changes in total eNOS protein (Figure 4D). Cell supernatants were examined for the stable NO breakdown product (NOx) using the Griess reaction. Shear stress of HUVECs resulted in an ≈2-fold increase in NOx release, which was significantly inhibited by the addition of SB525334 (Figure 4E).

To determine whether the morphological changes and increased apoptosis observed with the inhibition of TGF-β receptor in shear stressed HUVEC were mediated by decreased NO, the ability of exogenous NO to rescue the cells from these effects was assessed. Indeed, the addition of SNP (1 μmol/L) before the initiation of shear stress reversed the effects of TGF-β receptor inhibition on HUVEC morphology

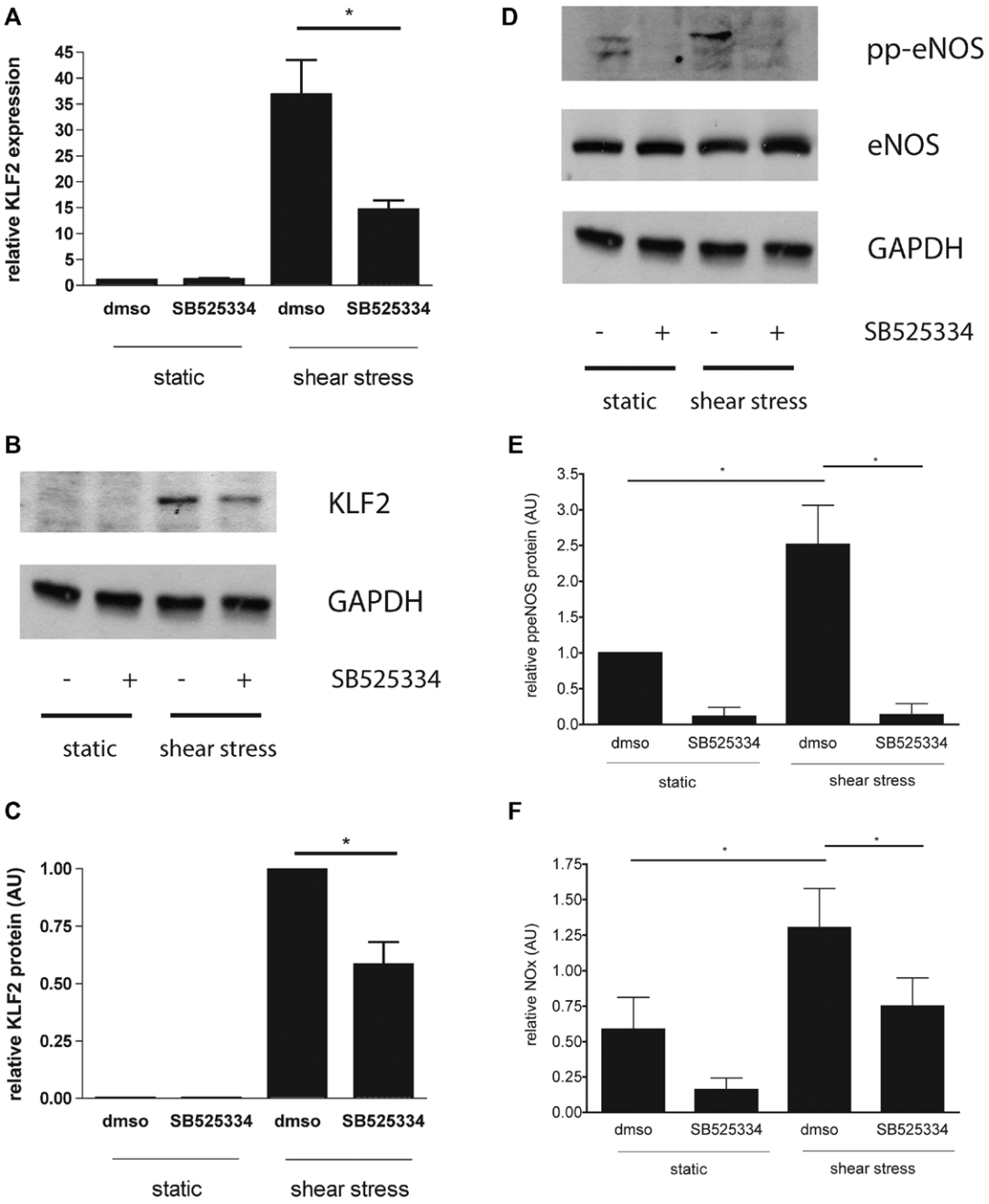

Figure 4. Shear stress increases human umbilical vein endothelial cell (HUVEC) Krüppel-like factor 2 (KLF2) mRNA (**A**) and protein (**B** and **C**); this effect was partially reversed by the inhibition of ALK5. Shear stress increased phosphorylation of endothelial NO synthase (pp-eNOS) protein (**D** and **E**) and release of NO breakdown product (NOx; **F**), which was blocked with inhibition of ALK5. *P<0.05.

and apoptosis (Figure 5A and 5B). Taken together, these results reveal that shear-induced TGF-β3 activation of ALK5 is upstream of KLF2-NO signaling.

Interaction Between KLF2 and TGF-β Signaling Under Shear Stress

To assess the interaction and signaling sequence between the TGF-β and KLF2 pathways, TGF-β1, TGF-β3, and KLF2 mRNA were knocked down using siRNA pools before exposure to shear stress for 24 hours. The specific siRNA pools decreased the levels of shear stress–induced TGF-β1, TGF-β3, and KLF2 mRNAs 5-, 3-, and 6-fold, respectively (Figure 6A–6C). Whereas reduction in the levels of TGF-β1 or KLF2 did not significantly alter TGF-β3 mRNA, knockdown of TGF-β3 led to a 4-fold decrease in KLF2 mRNA (Figure 6C).

To determine the relative contribution of TGF-β1, TGF-β3, and KLF2 to the protective effects of shear stress on HUVECs, each of these proteins was individually knocked down with siRNA and the cells were then exposed to shear stress for 72 hours. Reduction of TGF-β3 in HUVEC exposed to shear stress led to a disruption of the monolayer that was similar to that observed with the pharmacological inhibition of TGF-β receptor (compare Figures 6D and 5A), whereas the

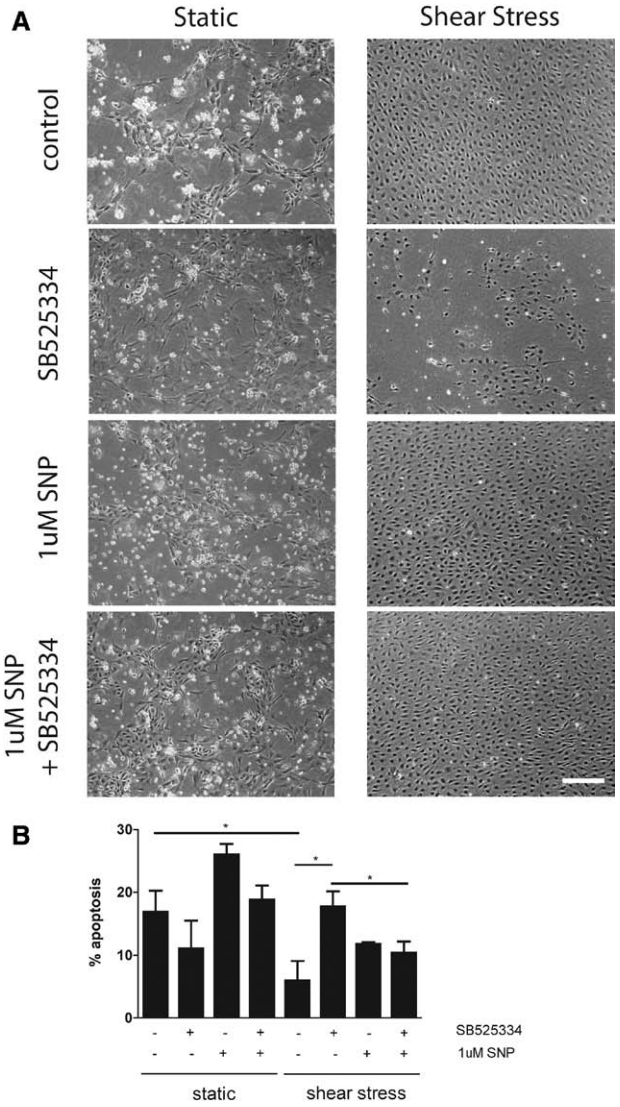

Figure 5. A, SB525334 prevented the protective effects of shear stress on human umbilical vein endothelial cells (HUVECs) at 72 hours, and addition of 1 μmol/L S-nitroso-N-acetylpenicillamine reversed this effect. **B**, Flow cytometry analysis of apoptotic HUVECs revealed that the addition of SNP prevented the increase in HUVEC apoptosis caused by the blockade of ALK5. *$P<0.05$.

knockdown of TGF-β1 had no effect. KLF2 knockdown also led to a notable disruption of the HUVEC monolayer under shear stress, although not as dramatically as TGF-β3 knockdown. Addition of SNP (1 μmol/L) completely reversed the effects of KLF2 knockdown, whereas the effect of TGF-β3 suppression was partially rescued with SNP. Although SNP limited HUVEC loss resulting from TGF-β3 inhibition, shear stress–induced HUVEC alignment was still significantly impaired. The suppression of TGF-β1 levels had no effect in the presence or absence of SNP (Figure 6D). Taken together, these results reveal that TGF-β3, not TGF-β1, is upstream of shear stress–induced KLF2.

Discussion

Physiological shear stress has been shown to be an important protective stimulus for the endothelium, limiting apoptosis and proliferation while maintaining endothelial quiescence and, in particular, suppressing the expression of proinflammatory proteins.[22,32] Our results are consistent with this function and expand this concept by demonstrating a critical role for TGF-β signaling in mediating the protective effects of physiological shear stress. Shear stress of ECs induces TGF-β3 expression and signal activation via the transmembrane receptor TGF-βRI (ALK5). Pharmacological inhibition of ALK5 before exposure to shear stress for 3 days led to a dramatic disruption of the endothelial monolayer and increased cell death. Specific knockdown of TGF-β3, but not TGF-β1, resulted in similar cell loss over the same time course. In line with these findings, TGF-β3, but not TGF-β1, was significantly upregulated under shear stress. These findings demonstrate a novel role for shear stress–induced TGF-β3.

Cyclic stretch has been reported to upregulate ALK1, but not ALK5 or TGF-βRII, in EC cocultured with smooth muscle cells, but not in EC in monoculture,[33] and other studies demonstrate that cyclic stretch induces autocrine TGF-β1 via activation of the ERK pathway.[34] Reports of bovine aortic ECs exposed to different types of shear stress revealed induction of TGF-β1 mRNA via K^+ channel activation,[35] with laminar and pulsatile flow having greater effects than oscillatory flow.[36] These findings suggest that TGF-β1 plays a role in atheroprotection because oscillatory flow strongly correlates with atheroprone regions of the vascular tree. Consistent with our observations, recent studies of mouse embryonic ECs reveal that shear stress induction of KLF2 is an ALK5-dependent process.[37]

Prior studies have reported the activation of TGF-β1 by contact of ECs with pericytes or astrocytes in vivo and induction of TGF-β1 in ECs exposed to shear stress.[35,38,39] However, in our study of EC monocultures exposed to shear stress, neither TGF-β1 nor ALK5 was regulated by shear stress. Rather, shear stress of HUVECs led to increased levels of TGF-β3 mRNA as well as both active and total protein. TGF-β3 protein was detected in association with cells but not in the conditioned media. Latent TGF-β1, but not active, was detectable in the conditioned media and was not changed in response to shear stress. These findings suggest autocrine/paracrine/juxtacrine roles for TGF-β1 and TGF-β3.

To determine whether there was a relationship between TGF-β signaling and flow-induced KLF2 and NO, we specifically targeted each component using siRNA. Knockdown of TGF-β3, but not TGF-β1, prevented the shear stress induction of KLF2. KLF transcription factors are well-documented mediators of the protective effects of shear stress, and these results indicate that the induction of TGF-β3 by shear stress is at least partially responsible for the activation of the KLF2 signaling cascade. TGF-β3 signaling did not mediate shear-induced KLF4 (Figure V in the online-only Data Supplement). KLF2 has been shown to enhance the anti-inflammatory properties of ECs,[40] and we have previously described that inhibition of TGF-β in vivo using sEng overexpression leads to a proinflammatory response of mesenteric ECs, further supporting the notion that these pathways modulate anti-inflammatory properties of the endothelium in vivo.[12]

Previous reports of overexpression of KLF2 in HUVECs under static conditions demonstrated activation of inhibitory smad signaling, a decrease in smad2 phosphorylation, and

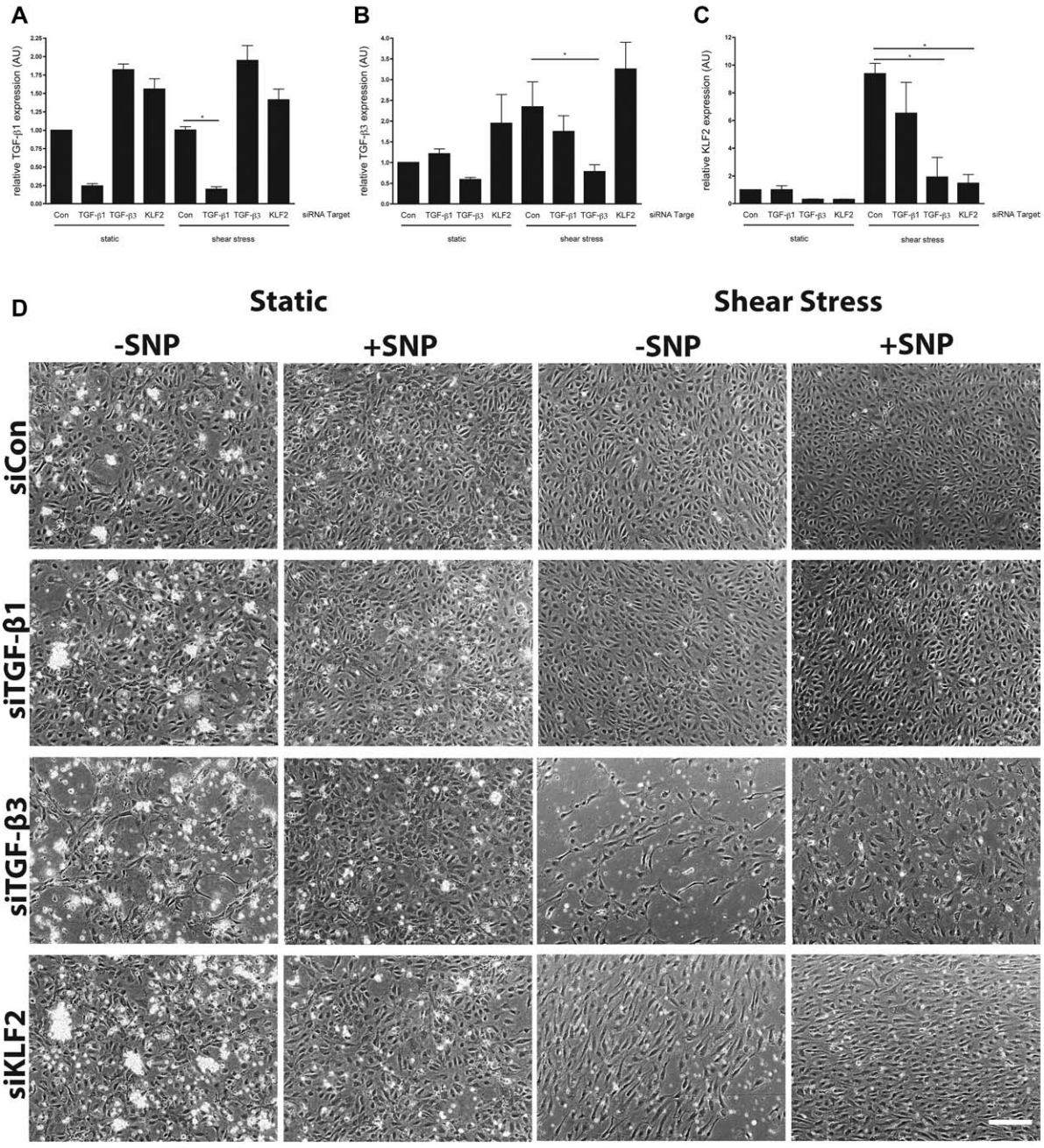

Figure 6. mRNA levels of (**A**) transforming growth factor-β1 (TGF-β1), (**B**) TGF-β3, and (**C**) Krüppel-like factor 2 (KLF2) after small interfering RNA (siRNA) treatment. **D**, siTGF-β3 significantly disrupted the human umbilical vein endothelial cell (HUVEC) monolayer. S-nitroso-N-acetylpenicillamine rescued this cell loss, but not loss of cell alignment. siKLF-2 partially impaired HUVECs morphology, which was fully rescued by the addition of exogenous S-nitroso-N-acetylpenicillamine. *P<0.05.

decreased TGF-β1 and TGF-β2, but not TGF-β3.[41] These results suggest a feedback mechanism that is consistent with our finding that TGF-β3, but not TGF-β1, induced expression of KLF2 under shear stress. Moreover, siRNA knockdown of KLF2 did not significantly alter the levels of TGF-β1 or TGF-β3, demonstrating that KLF2 is downstream of TGF-β signaling.

Despite their structural and biological similarities, it is clear that the TGF-β isoforms have distinct roles. For instance, TGF-β3, but not TGF-β1, protects keratinocytes against apoptosis in vitro and in vivo.[42] Gene transfer of TGF-β3, but not TGF-β1, inhibits constrictive remodeling and luminal loss after coronary angioplasty.[43] The molecular basis of these differences is unknown, though it may be attributable, at least in part, to the differences in binding to TGF-β modulating factors. For example, binding to cell surface and extracellular matrix heparan sulfate is isoform-specific and is thought to modulate their accessibility and activity.[44] TGF-β1, but not TGF-β3, binds heparan sulfate, potentiating its biological activity by limiting proteolytic degradation and inactivation by binding to α2-macroglobulin.[44] Similarly, TGF-β latency-associated protein, which is bound to TGF-β in its inactive

form, interacts more effectively with TGF-β3 than with the 2 other isoforms.[45] Recent studies reveal differences in binding affinity between the TGF-β isoforms and the TGF-β receptors. TGF-βRII binds TGF-β1 and TGF-β3 with similar affinity because of a conserved amino acid sequence; however, ALK5 binds TGF-β3 with much higher affinity than TGF-β1. The differential kinetics of ternary complex assembly persists with binary complexes, with 5-fold greater affinity of TGF-β3/TGF-βRII for ALK5, than TGF-β1/TGF-βRII.[46]

Developmental studies of large arteries revealed TGF-β3 localization in smooth muscle progenitor cells, but not in endothelium,[47] and TGF-β1, but not TGF-β3, mRNA in lung mesenchymal and ECs.[48] Although the role of TGF-β3 in angiogenic processes has not been elucidated, reports suggest elevated TGF-β3 may promote angiogenesis during development by increasing Flk-1 and CD31 expression,[49] whereas other reports demonstrate TGF-β1, -β2 and -β3 cooperate to facilitate tube formation.[50]

To assess the mechanism of shear stress–induced TGF-β signaling, we examined the effects of ALK5 inhibition on the well-documented shear-induced protective EC signaling components, NO and KLF2.[51–53] Shear stress of ECs activates eNOS enzyme activity proteoglycan via phosphorylation at serine 1179, leading to NO release, which acts on the underlying mural cells to regulate vessel tone.[53–55] In addition, NO has been shown to protect ECs from apoptosis.[3,56,57] Our data show that shear stress leads to increased phosphorylation of eNOS and NO release. These effects were blocked by the inhibition of ALK5 and rescued by the NO donor S-nitroso-N-acetylpenicillamine, demonstrating a clear role for the TGF-β signaling in mediating the shear stress induction of NO and suggesting that the protective effects of TGF-β3 are mediated largely by NO. Consistent with these findings, eNOS knockout mice display hypertension, impaired microvessel function, and leukocyte adhesion,[58–65] which also occur in sEng overexpressing mice (systemic TGF-β inhibition),[12–14] suggesting that the phenotype in mice in which TGF-β3 is impaired is at least partially a result of decreased NO.

siRNA knockdown studies revealed that TGF-β3, but not TGF-β1, was responsible for protecting ECs exposed to shear stress. Inhibition of ALK5 significantly blocked the induction of KLF2 mRNA and protein by shear stress, demonstrating that ALK5 is upstream of KLF2 in the signaling cascade. Inhibition of NO with L-NAME did not affect either TGF-β3 or KLF2 mRNA (data not shown), indicating that NO is downstream of both ALK5 and KLF2. Addition of exogenous NO fully reversed the effects of ALK5 inhibition; however, although NO prevented cell loss attributable to TGF-β3 knockdown, it did not prevent the loss of EC alignment. These results suggest that the action of TGF-β3 to align EC along the flow path is independent of NO signaling. Similarly, knockdown of KLF2 did not result in the same degree of cell loss as knockdown of TGF-β3 under shear stress, suggesting that KLF2 is not the only target for TGF-β3 signaling in ECs exposed to flow.

Further investigation of putative NO signaling activators induced by shear/TGF-β3 did not indicate a role for any of the previously described signaling molecules (pp-PLC-γ, PLC-γ, HSP90α, PI3k, pp-Akt, Akt, pp-ERK, ERK, pp-p38, p38, JNK, Mek5). Nonbiased proteomic analysis, however, did reveal a number of novel proteins regulated by shear stress induction of TGF-β3, but not TGF-β1. For example, the level of RhoA was decreased in sheared ECs, whereas siTGF-β3 treated sheared cells exhibited a significant increase in RhoA. Interestingly, RhoA has been previously shown to inhibit NO signaling via multiple pathways.[66] ALK1 signaling in ECs stimulates migration and proliferation, both of which are inhibited on ALK5/ALK1 complex formation. Proteomic analysis revealed the differential expression of 2 proteins that limit ALK1 signaling; shear stress increased EC PPP1CA (ALK1 inhibitor) and decreased CSNK2B (ALK1 enhancer), effects that were mediated by the activation of TGF-β3, but not TGF-β1.

We have described a novel effect of shear stress on ECs in which the induction of TGF-β3 expression leads to activation of KLF2 and NO signaling. Understanding the mechanisms underlying this effect sheds light on the potential homeostatic role for plasma TGF-β3 and should enable targeting of the TGF-β pathway in pathologies in which TGF-β3 signaling is perturbed such as pre-eclampsia and cancer.

Acknowledgments

We thank Drs Joseph Arboleda-Velasquez, Alisar Zahr, and Christine Bagley for critical review of the article.

Sources of Funding

This work was supported by EY05318 (P.A. D'Amore) and by the Core grant for Vision Research P30EY14104 (James Zieske) and Knights Templar Eye Foundation award (T.E. Walshe).

Disclosures

None.

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Significance

Vascular endothelial cells are continuously exposed to blood flow that contributes to the maintenance of vessel structure and function; however, the effect of hemodynamic forces on transforming growth factor-β (TGF-β) signaling in the endothelium is not well understood. We have described a novel effect of shear stress on endothelial cells, in which the induction of TGF-β3 expression leads to activation of Krüppel-like factor 2 and NO signaling, which are known to limit endothelial dysfunction and maintain endothelial homeostasis. The induction of TGF-β by shear stress was specific to TGF-β3, not TGF-β1, thus revealing their differential regulation. Each of the TGF-βs is strongly conserved across species, however their distinct roles are poorly described. Understanding the mechanisms underlying these changes sheds light on the potential homeostatic role of plasma TGF-β3 and should enable targeting the TGF-β pathway in pathologies in which TGF-β3 signaling is perturbed such as pre-eclampsia and cancer.

Supplemental Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) (provided by Ms. Kay Case, Center for Excellence in Vascular Biology, Brigham and Women's Hospital, Boston, MA), which are routinely employed to study ECs in vitro were cultured in EGM-2 medium (EBM-2, SingleQuots, Lonza), supplemented with 20% fetal bovine serum (Hyclone), L-glutamine, and penicillin/streptomycin. Cells were grown to confluence in 6-well plates prior to exposure to 10 dynes/cm^2 shear stress using an orbital shaker as previously described.[1,2] The TGF-β receptor inhibitor, SB-525334 (Tocris), which inhibits the kinase activity of ALK5, but not ALK1, ALK4 or ALK7, was added in Medium 199 (1:20 dilution of growth media) for 24 or 72 hr at 10 μM concentration to maximally inhibit ALK5 as previously described.[3] Equivalent volumes of DMSO were added to control wells. For inhibition of NO, 1 μM L-NAME was similarly applied.

siRNA knockdown

siRNA-pools (2 μM; Dharmacon) were prepared in 100 μl OPTI-MEM containing GlutaMAX for each well (6-well), mixed and incubated with transfection reagent (8 ul Oligofectamine in 92 μl OPTI-MEM/GlutaMAX). After incubation for 15 min, 200 μl aliquots of siRNA/Oligofectamine complexes were added to PBS-washed HUVECs at ~90% confluence with 800 μl OPTI-MEM/GlutaMAX. After 6 hr at 37°C, 1 ml HUVEC growth medium (without

gentamycin and pen-strep) was added per well for 12 hr before subjecting to shear stress.

FACS analysis of apoptosis and proliferation

Apoptotic cells were detected using the Vybrant® Apoptosis Assay Kit (Invitrogen, Carlsbad, CA) followed by FACS analysis using a FACSCAN flow cytometer. Cells were designated as viable (V), apoptotic (A) or necrotic (N). Cellular proliferation rates were determined using a Vybrant® CFDA SE (carboxy-fluorescein diacetate succinimidyl ester) Cell Tracer Kit. Loss of fluorescent tracer over time reflects cell proliferation, as the tracer concentration is halved with each cell division. Cell counts were performed using the Z1 Coulter Counter.

RNA isolation and reverse transcription-PCR analysis

RT-PCR was carried out using total RNA isolated from cells using TRIzol™ (Invitrogen), according to the manufacturers' specifications. Total RNA (1-2 μg) was reverse transcribed to cDNA using iSCRIPT, as per the manufacturer's specifications (BioRad). The gene-specific oligonucleotide sequences used were:

GAPDH: F- CAAATTCCATGGCACCGTCA; R- GGAGTGGGTGTCGCTGTTGA;

KLF2: F- GCACGCACACAGGTGAGAAG; R- ACCAGTCACAGTTTGGGAGGG;

TGF-β1: F- AAGTTGGCATGGTAGCCCTT; R- CCCTGGACACCAACTATTGC;

TGF-β3: F- CTGCTCGGAATAGGTTGGTT; R- AAATTCGACATGATCCAGGG.

For real-time PCR, reactions were performed on the LightCycler 480II (Roche) using 0.4 μM primers and the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions. Amplification of GAPDH was performed on each sample as a control for normalization.

SDS-PAGE and immunoblot analysis

Cell pellets were treated with lysis buffer and equal protein was fractionated by 10% (wt/vol) polyacrylamide resolving gels. After transfer to nitrocellulose membranes, non-specific protein binding was blocked by a 60-min incubation in phosphate-buffered saline, 0.1% Tween-20 (PBS-T) containing 5% (wt/vol) nonfat skim milk and 2.5% BSA. Membranes were then incubated overnight in PBS-T containing 2.5% BSA containing the following antibodies: pp-smad2 (1:500; Chemicon, Temecula, CA); smad2 (1:1000; Cell Signaling, Beverly, MA); KLF2 (1:500; Millipore, Bedford, MA); GAPDH (1:2000; Santa Cruz, Santa Cruz, CA); ppeNOS (1:500; Millipore, Bedford, MA); eNOS (1:500; Upstate, Lake Placid, NY). After two 10-min washes with PBS-T, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (Amersham; 1:5000) for 90 min at room temperature. After two further washes with PBS-T, immunoreactive proteins were identified by enhanced chemiluminescence (Pierce). Scanning densitometry was performed with image-analysis software (ImageJ).

Immunofluorescence

HUVECs were fixed and permeabilized in ice-cold methanol for 20 min, followed by three washes in ice-cold PBS and blocking in 3% Dky/goat serum plus 2.5% BSA in PBS-T (PBS containing 0.03% Triton X-100) overnight. Active caspase3 (1:100; Chemicon, Temecula, CA) was added for 1 hr before incubation with DyLight549 secondary antibody (1:100; Jackson ImmunoResearch, West Grove, PA).

Quantification of TGF-β

Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) were used to quantify TGF-β1 and TGF-β3 in cell lysates and in concentrated conditioned media (Ambicon Ultra-15 centrifugal filters, Millipore, Bedford, MA). Active TGF-β and total TGF-β after acid activation, following the manufacturer's instructions were analyzed.

Statistical analysis

For all experiments data are reported as mean ± SEM in at least three replicates per group unless otherwise indicated. Data were analyzed by student’s unpaired T-Test. P values <0.05 were taken to indicate statistical significance.

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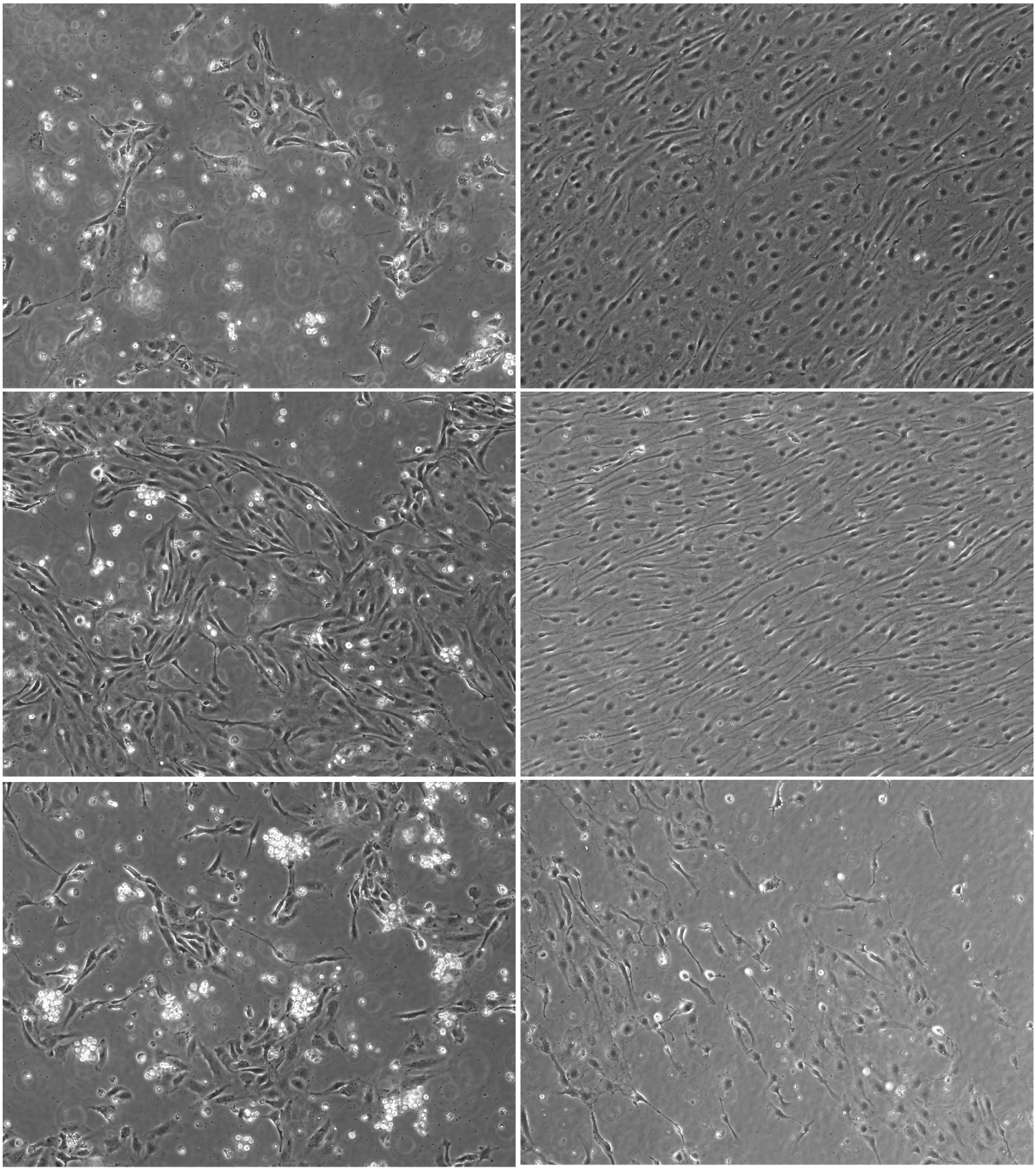
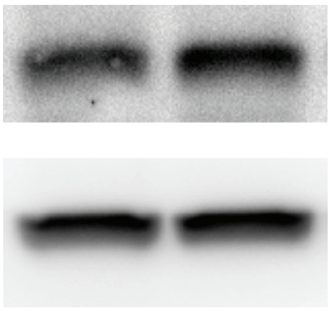
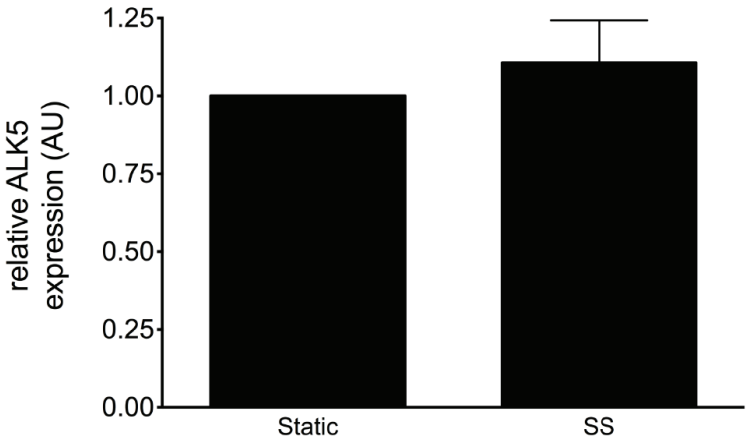
Supplemental Material



Supplemental Figure I

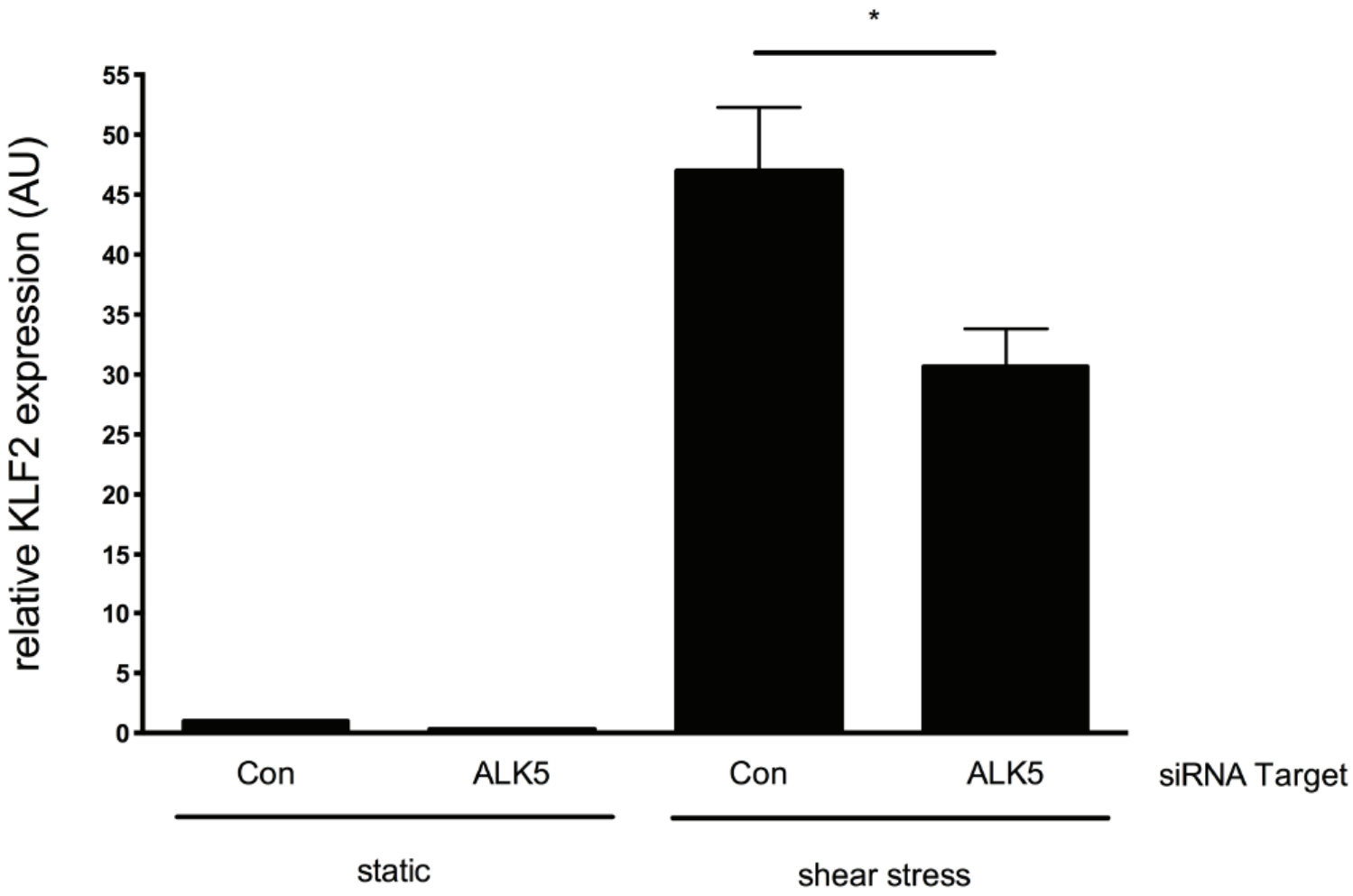
Supplemental Figure I. Effects of ALK5 inhibition on shear stress-induced changes in HUVEC morphology. HUVECs exposed to shear stress aligned along the flow path (arrow). Treatment of cells with SB525334, but not SB431542, led to a disruption of the HUVEC monolayer after 72 hr.





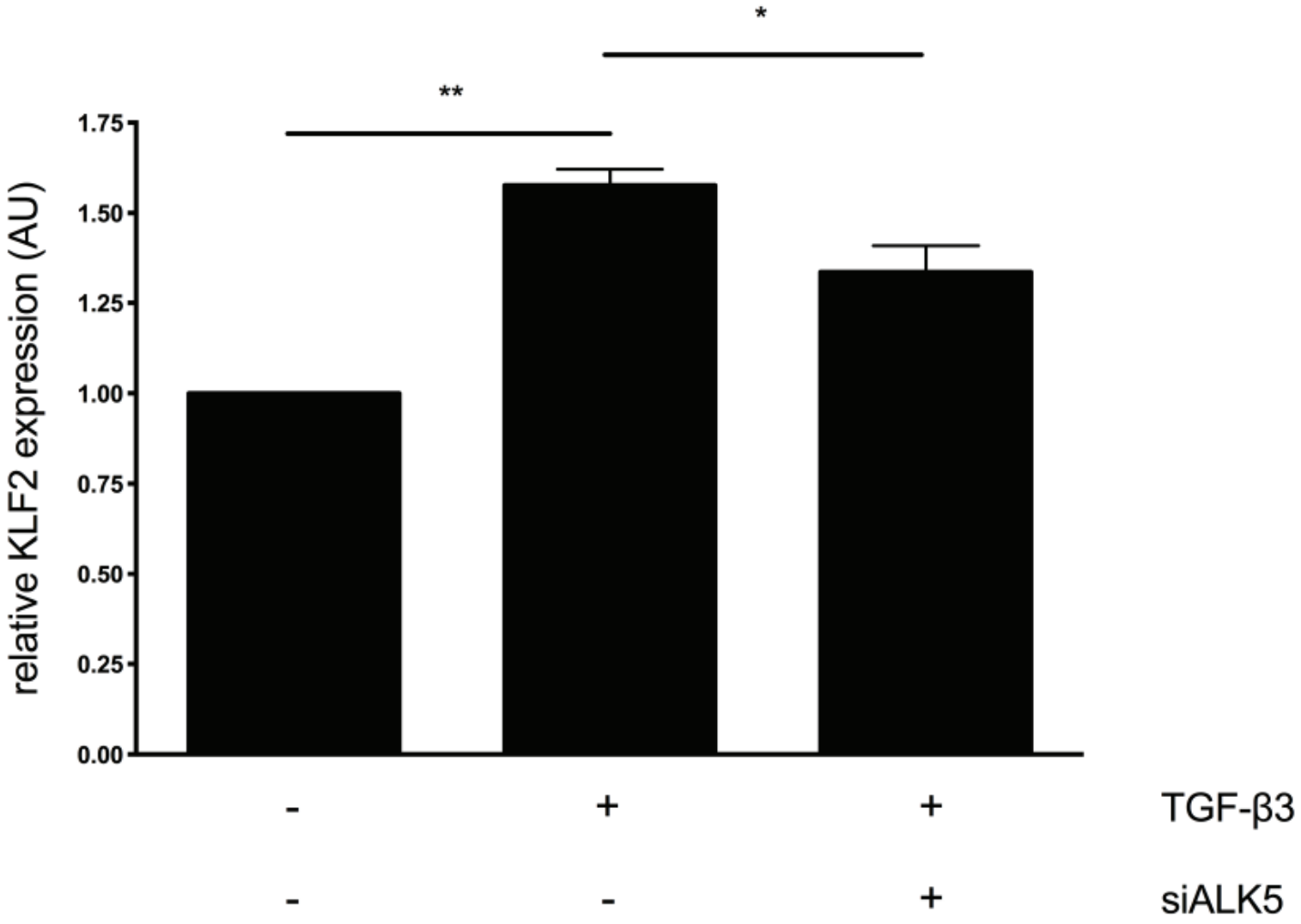
Supplemental Figure II

Supplemental Figure II. Effects of shear stress on endothelial ALK5 protein. Exposure of endothelial cells to shear stress for 72 hr did not alter ALK5 protein.



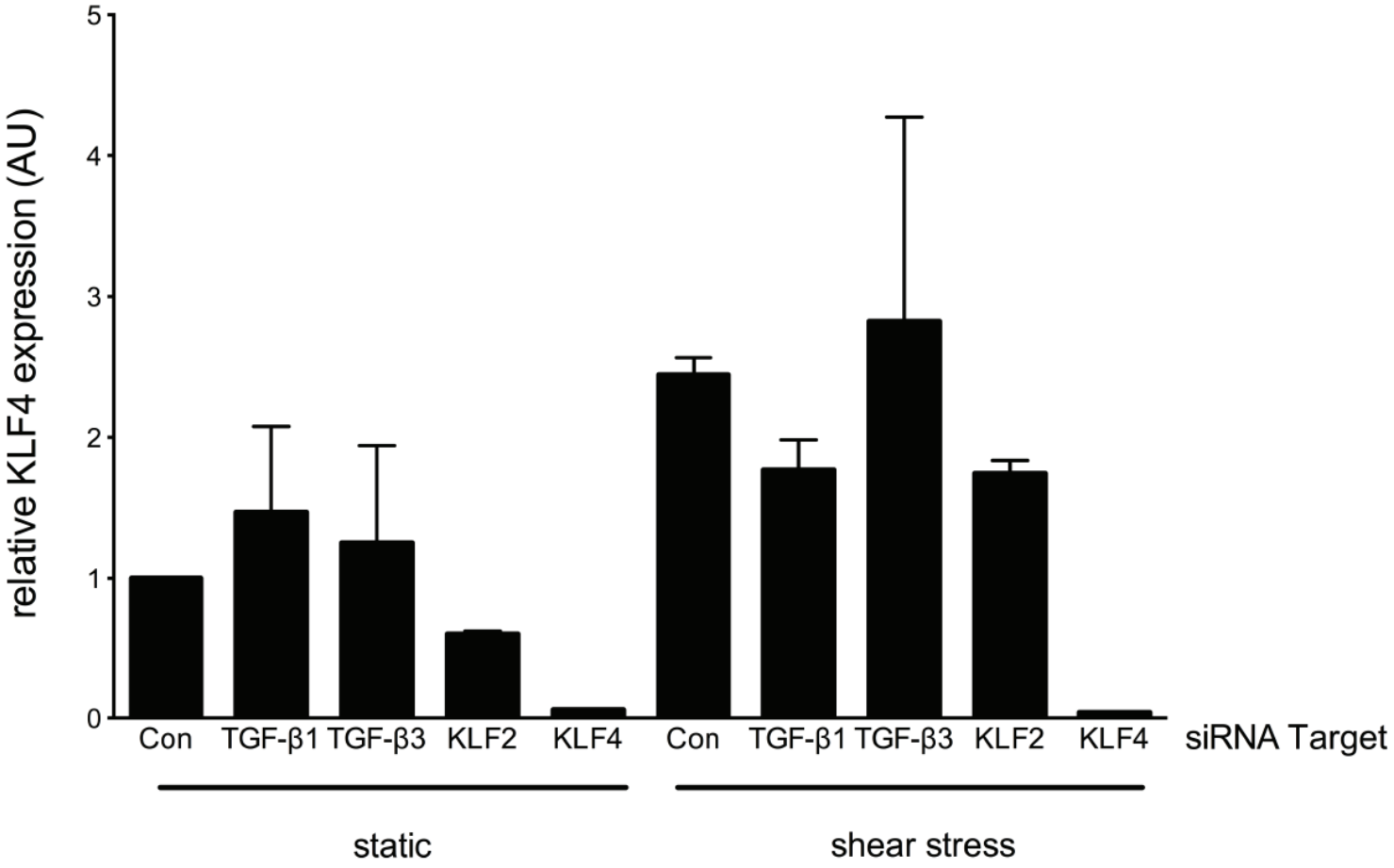
Supplemental Figure III

Supplemental Figure III. mRNA levels of KLF2 following siALK5 treatment. Exposure of endothelial cells to shear stress for 24 hr increased endothelial KLF2 mRNA and siALK5 significantly limited shear-induced KLF2.



Supplemental Figure IV

Supplemental Figure IV. Effects of TGF-β3 and ALK5 signaling on KLF2. Addition of 0.1 ng/ml TGF-β3 for 24 hr led to significantly increased levels of KLF2 mRNA were which were partially reversed by siRNA targetting of ALK5.



Supplemental Figure V

Supplemental Figure V. mRNA levels of KLF4 following siRNA treatment. siTGF-β1, siTGF-β3 or siKLF2 did not significantly alter induction of KLF4 after 24 hr of shear stress.