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Vascular Injury Triggers Krüppel-Like Factor 6 (KLF6) Mobilization and Cooperation with Sp1 to Promote Endothelial Activation through Upregulation of the Activin Receptor-Like Kinase 1 (ALK1) Gene

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Abstract

Rationale—Activin receptor-Like Kinase-1 (ALK1) is an endothelial TGF-β receptor involved in angiogenesis. ALK1 expression is high in the embryo vasculature, becoming less detectable in the quiescent endothelium of adult stages. However, ALK1 expression becomes rapidly increased after angiogenic stimuli such as vascular injury.

Objective—To characterize the molecular mechanisms underlying the regulation of ALK1 upon vascular injury.

Methods and Results—Alk1 becomes strongly upregulated in endothelial (EC) and vascular smooth muscle cells (vSMC) of mouse femoral arteries after wire-induced endothelial denudation. In vitro, denudation of monolayers of Human Umbilical Vein Endothelial Cells (HUVEC) also leads to an increase in ALK1. Interestingly, a key factor in tissue remodeling, Krüppel-like factor 6 (KLF6), translocates to the cell nucleus during wound healing, concomitantly with an increase in the ALK1 gene transcriptional rate. KLF6 knock down in HUVECs promotes ALK1 mRNA downregulation. Moreover, *Klf6*$^{+/-}$ mice have lower levels of Alk1 in their vasculature compared with their wild type siblings. Chromatin immunoprecipitation assays show that KLF6 interacts with ALK1 promoter in ECs, and this interaction is enhanced during wound healing. We demonstrate that KLF6 is transactivating ALK1 gene, and this transactivation occurs by a synergistic cooperative mechanism with Sp1. Finally, Alk1 levels in vSMCs are not directly

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DISCLOSURES
None.

upregulated in response to damage, but in response to soluble factors, such as IL-6, released from ECs after injury.

Conclusions—ALK1 is upregulated in ECs during vascular injury by a synergistic cooperative mechanism between KLF6 and Sp1, and in vSMCs by an EC-vSMC paracrine communication during vascular remodeling.

Keywords

ALK1; KLF6; endothelial cell; vascular injury; smooth muscle cell; remodeling; cell culture; vascular biology

INTRODUCTION

Endothelial integrity is essential to regulate many aspects of vascular biology, including angiogenesis, inflammation, vasoconstriction, vasodilation, control of the blood pressure, blood clotting and barrier function. The consequences of endothelial injury have strengthened the concept of endothelium as an organ.[1,2] The impairment of the endothelial integrity leads to prothrombotic phenomena, aberrant angiogenesis, the loss of endothelial-selective permeability to leukocytes, and inflammatory processes.[3-6] All these consequences of endothelial dysfunction are associated with a range of diseases such as sepsis, haemolytic uremic syndrome, thrombotic thrombocytopenic purpura, diabetes and hypertension. Thus, the study of the regulatory mechanisms involved in vascular remodeling is a crucial step in the search of targets for therapy.

Upon vascular injury, a coordinated gene expression program is triggered among those genes coding for extracellular matrix proteins, growth factors, receptors and proteases.[7,8] One of these classes of proteins is the transforming growth factor-β (TGF-β) family, which includes TGF-β, activins and bone morphogenetic proteins (BMPs)[9]. Several lines of evidence support a pivotal role for TGF-β in response to injury: i) TGF-β expression is upregulated after injury;[8,10,11] ii) infusion of TGF-β polypeptide or transfection of TGF-β cDNA into injured arteries increases extracellular matrix production associated with the repair process;[12] iii) antibodies against TGF-β1 suppress intimal hyperplasia;[13] iv) radiation-mediated vascular injury causes a rapid and persistent increase in expression of TGF-β receptors and mediators;[14] v) BMP-9 is involved in postnatal retinal vascular remodeling[15]; and vi) TGF-β has a role in intimal thickening after vascular injury.[16,17] Moreover, increased TGF-β1 activity underlies the wound-healing response in liver,[18,19] kidney, lung[12] and vascular tissue.[20,21]

TGF-β family members exert their effects via type I (TβRI) and type II (TβRII) serine/threonine kinase receptors, helped by the co-receptors (TβRIII) and transduce the signal from the membrane to the nucleus through the intracellular Smad factors.[9] In endothelial cells (ECs), the TGF-β signaling acquires an important level of regulation due to the coexistence of two different type I receptors, the ubiquitous Activin receptor-like kinase 5 (ALK5), and ALK1.[22] ALK1 is highly expressed in the vascular structures of the embryo,[23-26] and it is essential during vascular development as demonstrated by the lethality of the *ALK1/Activin A receptor type II-like 1 (ACVRL1)* gene disruption. The *Alk1* knock out embryos die at E10.5, due to the absence of mature blood vessels in the yolk sac, showing aberrant hyperdilated vascular structures and clumps of blood cells.[27,28] Moreover, the heterozygous mutation of *ALK1* results in a vascular dysplasia called Hereditary Haemorrhagic Telangiectasia type 2 (HHT2), characterized by skin and mucosa telangiectases as well as liver and lung arteriovenous malformations (AVMs).[29,30] Despite of the essential role exerted by ALK1 in the vasculogenic process during embryonic

development, its expression is diminished in the quiescent endothelium during adult life[24]. The activation of the endothelial cell ALK1 expression is crucially upregulated in certain locations in response to several angiogenic stimuli.[24,31]

Krüppel-like factor 6 (KLF6) is a transcriptional regulator which mediates cellular differentiation and tissue development through its roles in growth-related signal transduction pathway, cell proliferation, apoptosis and angiogenesis.[32-34] KLF6 is considered as a damage-response factor that promotes tissue remodeling due to its ability of transactivating several target genes by direct binding to their promoters.[19,35] These genes comprise several members of the TGF-β signaling pathway such as *TGF-β1*, its receptors *TβRI* (ALK5) and *TβRII*,[36] the co-receptor *Endoglin (ENG)*,[37] *uPA* (urokinase-type plasminogen activator)[38] and *Col1A* (collagen 1A).[35] Furthermore, we have recently described a specific functional relationship between KLF6 and TGF-β pathway by the direct formation of a ternary Smad3-Sp1-KLF6 complex.[39] These effects suggest that KLF6 is a common regulatory factor for all the TGF-β functions related to injury, so KLF6 seems to orchestrate the repair mechanisms in order to return the endothelium to its regular state and to avoid the complications derived of its dysfunction.[40] In this article, we have explored the regulation of ALK1 expression under vascular injury. Our results demonstrate the transactivation of *ALK1* gene by KLF6 and as a consequence the ALK1 upregulation in the migrating ECs. These data provide new insights in the molecular mechanisms mediated by KLF6 for the coordination of the vascular remodeling process and provide additional evidences for a pivotal role of ALK1 in the activated state of the endothelial cell during the angiogenic response after vascular injury.

METHODS

Cell culture[37,41], expression vectors,[35,42,43] transfection and reporter assays,[41] stable infection of primary endothelial cell cultures,[44] real time PCR,[41] *in vitro* endothelial cell denudation,[37] immunofluorescence microscopy[37], flow cytometry,[37] immunohistochemistry, mechanical injury model in mouse femoral arteries,[45,46] laser microdissection (LMD) and chromatin immunoprecipitation[41] are described in an expanded manner in material and methods section of the supplementary data.

RESULTS

Alk1 expression is increased in vivo after endothelial injury

To assess the effect of vascular injury *in vivo* on Alk1 expression, we used a model of wire-induced endothelial injury in mouse. Mice were subjected to endothelial mechanical injury by using an angioplasty guidewire that removes the *tunica intima* (TI) of the hindlimb femoral artery. Then, the Alk1 expression levels post-injury were examined by immunohistochemistry after 4 weeks, when the proliferative response to arterial injury was prominent.[45,46] At day 28, a clear hyperplasia of the *neointima*, the new layer formed from *inner elastic lamina,* was detectable in the wounded area, as shown in Fig. 1. Alk1 expression was restricted to the endothelial single monolayer in uninjured femoral arteries. However, after injury, the hyperplasia was associated with a marked upregulation of Alk1 levels in the *neointima* (NI), and *tunica media* (TM), which is composed mainly by vascular smooth muscle cells (vSMC). These results suggest a potential active role for Alk1 during vascular remodeling after an acute injury, in concordance with previous findings of the involvement of TGF-β pathway in the formation of the *neointima*.[16]

KLF6 translocates to the nucleus prior to ALK1 upregulation in wounded endothelial cells

The wounding effect on Alk1 expression was next assessed using a model of endothelial injury *in vitro.* To this end, monolayers of Human Umbilical Vein Endothelial Cells

(HUVECs) were subjected to *in vitro* denudation and ALK1 levels were measured by flow cytometry. As expected, ALK1 levels were upregulated 2-3 hr after injury, reaching a 5 fold-increase after 24 hr (Fig. 2B). To unravel the molecular mechanism underlying the ALK1 upregulation after wounding, KLF6, an early damage response factor was studied. After 3 hours, KLF6 translocation to the nucleus was observed by fluorescence microscopy (Fig. 2A). The translocation is restricted to cells adjacent to the wound, within 300 μm, and decreases in more distant areas (Supplementary Fig. I). The nuclear localization of KLF6 is still evident after 8 hours, but at 24 hours, KLF6 has been shuttled back to the cytoplasm. In the same experiment, ALK1 surface levels underwent a time-dependent increase that peaked at 24 hours (5-fold upregulation), as measured by flow cytometry (Fig. 2B and 2C). Moreover, the expression of ALK1 and KLF6 transcripts was analyzed by real time PCR using total RNA from denuded HUVEC monolayers (Fig. 2D). KLF6 mRNA levels were markedly upregulated achieving a maximum of 6-fold induction after 2 hours and these levels were slowly decreased reaching basal levels after 24 hours. A similar profile was observed with ALK1, whose mRNA levels were upregulated after 2 hours, returning to basal levels after 10 hours. This kinetics of ALK1 mRNA induction is compatible with the subsequent increase in ALK1 protein levels shown at the cell surface (after 12-24 hours). As a positive control, KLF6-responsive endoglin[37] showed a similar upregulation. As negative control, the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were unaffected. Overall, these results demonstrate that upon *in vitro* denudation of endothelial monolayers, KLF6 induction precedes ALK1 upregulation in ECs, and this kinetics is compatible with the transcriptional regulation of *ALK1* by KLF6.

In vivo suppression of Klf6 leads to Alk1 downregulation

To assess the effect of Klf6 suppression in an *in vivo* model, Alk1 expression was studied in the liver of heterozygous $Klf6^{+/-}$ mice. This organ was selected because HHT2 patients present a high prevalence of hepatic AVMs.[30] Total liver RNA from either $Klf6^{+/+}$ or $Klf6^{+/-}$ mice was analyzed by real time RT-PCR (Fig. 3A). Levels of Klf6 mRNA in both 3-months and 6-months-old heterozygous mice were clearly diminished (~60% compared to control values) respect to their wild type siblings. Interestingly, basal levels of Alk1 mRNA in $Klf6^{+/-}$ heterozygous mice were expressed at lower levels than in wild type animals. As a positive control, endoglin levels were found to be decreased, although this reduction was more important in 6-months-old mice (Fig. 3A). Further support for the Klf6-dependent regulation of Alk1 was obtained from protein staining in the vessel walls of these mice. Liver sections from $Klf6^{+/-}$ heterozygous and their wild type siblings were paraffin-embedded and analyzed by immunohistochemistry (Fig. 3B). In wild type animals, Alk1 was clearly expressed in the endothelium of liver vessels. By contrast, Alk1 signal was much weaker in $Klf6^{+/-}$ heterozygous mice. Furthermore, Alk1 staining of quiescent endothelium from femoral arteries was found to be lower in $Klf6^{+/-}$ compared to $Klf6^{+/+}$ mice (Fig. 3C). Also, upon wire-induced endothelial injury, Alk1 protein was induced in both $Klf6^{+/+}$ and $Klf6^{+/-}$ mice (Fig. 3C), but laser microdissection studies showed that the upregulated Alk1 mRNA levels in $Klf6^{+/-}$ were lower than in $Klf6^{+/+}$ animals (Fig. 3D). In addition, a distinct vascular phenotype was observed (Fig. 3C,E). Thus, while the increase in the tunica media thickness was ~2-fold in WT mice, only a slight augmentation (1.2-fold) was observed in $Klf6^{+/-}$ mice. Remarkably, upon injury, the neointima of $Klf6^{+/+}$ mice was more than two-fold thicker than that of $Klf6^{+/-}$ animals. Taken together, these results agree with the crucial role of Alk1 in vascular remodeling and strongly support the involvement of Klf6 in the regulation of Alk1 gene expression.

ALK1 gene is a transcriptional target of KLF6

The experiments shown above suggest a transcriptional regulation of *ALK1* by KLF6. Supporting this view, various consensus motifs for KLF6 were identified in the −1,035/+210

fragment of the *ALK1* promoter, at positions −815, −739, −148, −109, −86 and +150 (Fig. 4A). The physical interaction between KLF6 and the *ALK1* promoter was examined by chromatin immunoprecipitation (ChIP). HUVEC monolayers were subjected to endothelial denudation and ChIP experiments were assayed using an anti-KLF6 antibody both in control situation and after 3 hours of endothelial denudation. KLF6-immunoprecipitated chromatin was subjected to PCR using two different couples of primers, encompassing the two clusters of KLF6 motifs present in the *ALK1* promoter sequence. As shown in Fig. 4A, KLF6 binding to *ALK1* promoter was detected in both amplified fragments (−872/−670 and −208/+38) under basal conditions. Moreover, upon endothelial wounding, the binding of KLF6 to *ALK1* promoter was enhanced, as shown in the densitometric analysis (Fig. 4A). This increase was especially evident in fragment −208/+38. These results indicate that, at least one KLF6 motif, within each cluster, is bound to KLF6 in vivo.

To assess the effect of this interaction, transcriptional experiments using *ALK1* promoter (p*ALK1*) constructs were performed. Transient transfections of the pGL2-p*ALK1*(−1,035/+210) reporter vector with increasing doses of KLF6 resulted in a marked activation (up to 13-fold) of *ALK1* promoter activity in HEK293T cells (Fig. 4B). Moreover, using a KLF6-free cellular model such as *Drosophila* Schneider S2 cell line (S2), a similar activation (up to 16-fold) of *ALK1* promoter activity was obtained. Overall, these results show KLF6 binding to, and transactivation of, the *ALK1* promoter. To assess the contribution of the different KLF6 motifs to *ALK1* transcription, a set of deletion constructs of the *ALK1* promoter were analyzed in HEK293T and S2 cells (Fig. 4C). In both cell lines, the highest KLF6-induced response was obtained with the two largest constructs (−1,035/+210 and −898/+50) containing 6 and 5 KLF6 binding motifs, respectively. The rest of the constructs (−587/+59, −422/+59 and −284/+59), all of them containing 3 KLF6 motifs, showed a proportional reduction in the KLF6-dependent transcriptional activity (~50%) respect to the largest constructs. These results demonstrate that KLF6 is able to interact with the −1,035/+210 fragment of *ALK1* promoter, stimulating its expression.

KLF6 upregulates ALK1 expression through a synergistic cooperation with Sp1

Based on our previous work, where we demonstrated that *ALK1* gene is Sp1-dependent for transcription initiation[41], the transactivator effect of KLF6 on the *ALK1* promoter segment −1,035/+210 was assessed in the absence or presence of Sp1 in HEK293T cells. As expected,[41] Sp1 overexpression induced (~4-fold) the transcriptional activity of the *ALK1* promoter construct (Fig. 5A). Similarly, overexpression of KLF6 achieved a 12-fold transactivating effect over the basal transcription rate of the *ALK1* promoter. Interestingly, simultaneous overexpression of KLF6 and Sp1 allowed a maximum transactivating effect of ~38-fold. Comparing the individual effects of Sp1 and KLF6 with the combined effect, it is obvious that the overexpression of both proteins leads to a clear synergistic cooperation, where the activating effect is much higher than the simple addition of each transcription factor independently. Next, the effect of both Sp1 and KLF6 overexpression on ALK1 protein levels was monitored by flow cytometry in ECs. As shown in Fig. 5B, overexpression of Sp1 and KLF6, independently upregulated ALK1 protein at the cell surface. Single transfections of these factors in HMECs increased ALK1 expression between 1.2- and 2-fold for Sp1 and KLF6, respectively, whereas cotransfection experiments showed a clear cooperation between Sp1 and KLF6 reaching an upregulation of 4.5-fold. Similarly, single nucleofections of Sp1 and KLF6 in HUVECs increased ALK1 expression 2.6- and 1.7-fold, respectively, although no synergistic effect was detected, probably due to a cytotoxic/apoptotic effect of the combined treatment for 48 hours in these primary cells (data not shown).

Because ALK1 is involved in endothelial cell migration,[47-51] a hallmark of activated ECs, we analyzed whether the KLF6-dependent upregulation of the ALK1 protein was associated

with a migratory phenotype. The human microvascular endothelial cell line-1 (HMEC-1) was transiently transfected with different combinations of Sp1 and KLF6 and then subjected to a wound healing assay. When both Sp1 and KLF6 are overexpressed in these ECs, their migration capacity was stimulated achieving the closure of the wound, after 24 hours (Fig. 5C). The same type of experiment was performed with primary cultures of HUVECs, previously electroporated with Sp1 and KLF6 expression vectors (Supplementary Figure II), yielding a similar wound healing kinetics (Fig. 5C). The toxicity derived from the Sp1/KLF6 combination in HUVECs was not observed in this case because the migration studies were carried out within a short time frame (12 hours). Taken together, these data demonstrate that *ALK1* is a KLF6 target gene and support the hypothesis that KLF6 acts cooperatively with Sp1 in order to promote endothelial activation of *ALK1* during vascular remodeling.

KLF6 and Sp1 knock down prevent ALK1 upregulation during wound healing

The expression of Sp1 and KLF6, independently or in combination, was silenced in HUVECs, during the wounding process by transfection of specific siRNAs (Supplementary Fig. III). Fig. 6A shows that silencing of Sp1, KLF6 or both resulted in the abolishment of the wound-mediated ALK1 induction as determined by flow cytometry analysis. When cellular migration was measured, as a function depending on ALK1 expression, in the presence of siRNA of Sp1, KLF6 or both, HUVECs were unable to close the disrupted monolayer (Fig. 6B) as compared to a correct healing when silencing was made with control scrambled siRNA. These changes are shown in Fig. 6C, where quantification of cell migration at each point time is expressed as number of migrated cells per field. These data corroborate that KLF6 in conjunction with Sp1, are essential for the upregulation of ALK1 during the angiogenic response to endothelial injury.

ALK5 is not implicated in ALK1 upregulation induced by vascular injury

In ECs there are two TGF-β type I receptors, the ubiquitous ALK5 and the endothelial ALK1. Because a cross-talk between ALK1 and ALK5 has been postulated,[47,50,52] the possible role of ALK5 in the wound-induced ALK1 expression was assessed by measuring changes in ALK5 levels and activity. As shown in Fig. 7A, ALK5 surface protein levels remained unchanged after wound healing and the same was true for the corresponding mRNA levels (Fig. 7B). To analyze the influence of ALK5 signal, ECs were treated with SB431542, a specific ALK5 kinase inhibitor. In spite of the suppression of ALK5 signaling, ALK1 expression was upregulated upon wound healing, although at a slightly lower extent than cells treated with vehicle (Fig. 7C,D). Upon scratching endothelial monolayers, the migration of cells is stimulated when the ALK5 pathway is inhibited (Fig. 7E), whereas the ALK5 inhibitor stimulated the promoter activity of the Id1 target gene in ECs (Fig. 7F). The efficiency of SB431542 treatment is demonstrated in HUVECs by the decrease in mRNA levels of *PAI-1*, a target gene of ALK5 (Supplementary Fig. IV). These results suggest that while wound-mediated upregulation of ALK1 is mostly independent of the ALK5 signaling pathway, inhibition of ALK5 activity favours signaling through ALK1, as shown by the stimulation of cell migration and activation of the Id1 target.

ALK1 is upregulated in smooth muscle cells after wounding through a paracrine communication with endotelial cells

As seen in Fig. 1, endothelial injury induces ALK1 upregulation in cells of the tunica media, suggesting the involvement of vSMC. As vSMCs are in close contact with ECs, we have explored the wounding effect in vSMCs. For this purpose, primary cultures of umbilical artery smooth muscle cells (UASMCs) were subjected to a scratching process and the surface levels of ALK1 were measured by flow cytometry. As observed in Fig 8A, UASMCs express high levels of ALK1 at their surface, but these levels remain unchanged along the wound healing process at variance with the increase of ALK1 in ECs after

wounding. Accordingly, levels of ALK1 mRNA were unaffected (Fig. 8B); the same was true for KLF6, as opposed to the increased levels of KLF6 observed in ECs after wound-healing (Fig. 2B,C,D). Staining of KLF6 in UASMC at different times after wound healing, did not reveal any nuclear translocation, either (Fig. 8C), at variance to ECs, where nuclear translocation is evident after 3h (Fig. 2A). Interestingly, when UASMCs were cultured with conditioned media from wounded-monolayers of ECs, a significant increase in ALK1 was evident at mRNA and protein levels, already after 2 hours (Fig. 8D,E). Furthermore, KLF6 mRNA levels were increased in parallel with ALK1 mRNA in UASMCs grown in conditioned media from ECs subjected to wound-healing. The increase in ALK1 protein was sustained up to 24 hours (Fig. 8E). Next, we assessed the contribution of the wound healing-dependent induction of KLF6 in ECs to the upregulated expression of ALK1 in UASMCs. KLF6 was overexpressed (using pCiNeo-KLF6) or suppressed (using pSuperKLF6) in HUVECs and the corresponding conditioned media were used to culture vSMCs. As shown in Fig 8F, culture media from KLF6 overexpressing HUVECs induced a modest, but significant increase (1.3-fold), in the expression levels of ALK1 of UASMCs. By contrast, culture media from HUVECs with a knocked down expression of KLF6 induced a marked reduction of ALK1 levels (0.6-fold) compared to mock treated cells. Of note, changes in ALK1 levels paralleled those of KLF6 in vSMCs (Fig. 8G). In summary, these results suggest that ALK1 is not directly upregulated in vSMCs in response to injury. Instead, endothelial injury triggers indirectly a similar KLF6-dependent stimulation of ALK1, in vSMCs. This response would constitute a paracrine mechanism operating from endothelium (intima) to smooth muscle cells (tunica media). In an attempt to analyse candidates responsible for this paracrine response, several cytokines and growth factors involved in angiogenesis were analyzed by ELISA in conditioned media from ECs following injury. The results showed a sustained increase of interleukin 6 (IL-6) in wounded cultures of HUVECs (from 6 to 24 h after wounding; Supplementary Fig. VI), suggesting the involvement of this cytokine in the upregulated expression of ALK1 in vSMCs after endothelial injury. To investigate the putative regulation of IL-6 by KLF6, immunohistochemistry staining of IL-6 in 4 weeks-injured femoral arteries from *Klf6*$^{+/-}$ mice in comparison with wild type littermates was carried out (Supplementary Fig. VIIA). In uninjured vessels from wild type and heterozygous animals the presence of IL-6 was almost undetectable. By contrast, IL-6 staining was clearly detected upon wire injury in different layers of the femoral artery. However, the increased signal of IL-6 in wild type mice was higher than that of *Klf6*$^{+/-}$ littermates, suggesting that KLF6 regulates IL-6 gene expression. Supporting this view, several putative consensus motifs for KLF6 were identified upon *in silico* analysis of both human and mouse IL-6 proximal promoter sequences (Supplementary Fig. VIII). Furthermore, ectopic expression of KLF6 stimulated more than 3-fold the promoter activity of a luciferase reporter construct driven by the human IL-6 promoter sequence (Supplementary Fig. VIIB). Taken together these experiments suggest that IL-6 is a target gene of KLF6. It can be speculated that KLF6 induction upon vascular injury modulates the expression of a set of target genes, including IL-6, which in turn upregulate *ALK1*. An in-depth analysis of these genes may shed light on the molecular mechanism triggered to achieve vascular repair.

DISCUSSION

ALK1 functions are closely related to vascular biology.[28,53,54] During embryogenesis, ALK1 is highly expressed in the vascular bed due to its critical requirement for a correct vasculogenic process, whereas in the adult life, the endothelium reaches quiescence and ALK1 expression levels drop[24]. In adults, the signaling network triggered by ALK1 is framed in the angiogenic process, when activated endothelial cell, proliferate and migrate, in order to develop new vessels from the preexistent ones, in response to certain stimuli.[23,50] After formation of the new sprouts, a resolution phase is needed, in which TGF-β-mediated

signaling blocks the proliferation and stabilizes the new vessel by the deposition of extracellular matrix.[47,49] Therefore, TGF-β signaling in the EC during angiogenesis is crucial, and needs to be highly regulated to control the balance between activating and resolving signals in each stage of the process. Having this in mind, a vascular damage, would require an immediate ALK1 upregulation for the correct coordination of the subsequent repair mechanisms, while ALK5-controlled pathway, should remain unchanged immediately after injury.

In Hereditary Hemorrhagic Telangiectasia, patients harboring mutations in ALK1 are able to develop normal vessels, and their vascular system is, overall, indistinguisible from that of a healthy subject. However, when angiogenesis is activated in areas exposed to different stimuli, such as inflammation, injury or hypoxia (second hit), ALK1 haploinsufficiency impairs the angiogenic process and may cause vascular lesions (telangiectases and Arteriovenous Malformations-AVMs),[55,56] as it is frequently the case of nasal mucosa, where vessels suffer from mechanical injury. Therefore, identification of molecular mediators implicated in the response to vascular injury may provide new insights for understanding the mechanism involved in the formation of telangiectases and AVMs in HHT patients. In addition, this information may be useful to elucidate the molecular basis of vascular embolotherapy, a common method used to treat pulmonary AVMs in HHT patients that involves vascular damage and remodeling induced with coils or balloons.[56]

Using both *in vitro* and *in vivo* models, we demonstrated that ALK1, but not ALK5 levels become strongly upregulated at the surface of ECs after vascular injury. We observed the existence of a temporal relationship between ALK1 upregulation and KLF6 translocation to the nucleus, in an *in vitro* endothelial wound healing model; a relationship that is compatible with a transcriptional involvement of KLF6 in *ALK1* gene expression regulation. Supporting this observation, ALK1 protein and mRNA levels are much lower in liver vasculature and in femoral arteries of *Klf6*$^{+/-}$ mice than those of wild type animals, and ectopic expression of KLF6 is able to markedly transactivate the *ALK1* promoter. Our recent studies on cloning and characterization of *ALK1* gene promoter[41] prompted us to explore the potential regulation by KLF6 of the *ALK1* gene. Based on the *in silico* analysis of the *ALK1* promoter sequence, six putative consensus binding sites for KLF6 were found along the −1,035/+210 p*ALK1* fragment. The motifs are located at positions −815, −739, −148, −109, −86 and +150. By chromatin immunoprecipitation we show that KLF6 indeed binds to the *ALK1* promoter and we demonstrate that, at least, one KLF6 motif is functional, within each one of the two KLF6 clusters located in −872/−670 and −208/+38, respectively. Of note, KLF6 binding to *ALK1* promoter is evident under basal conditions, being this interaction increased after vascular injury. Remarkably, three of the KLF6 motifs are surrounding the major transcriptional start site (TSS) (+1) driven by the transcription factor Sp1 through binding to the GC-rich regions in the TATA-less proximal promoter of *ALK1*[41]. These characteristics emphasize the importance of Sp1 in the basal mechanism of *ALK1* transcription and explain the strong synergistic cooperation observed between KLF6 and Sp1 potentiating *ALK1* transcriptional activity. Similarly, we have previously demonstrated the direct physical interaction and functional cooperation between Sp1 and KLF6 on the *ENG* promoter, in response to vascular injury [37]. There are also experimental evidences of similar regulatory mechanisms on the expression of other TGF-β family-related genes involved in vascular repair.[36,37] Among them are *TGF-β1, TβRI/ALK5, TβR-II*,[36] as well as other important key regulators of the vascular physiology like *Col1A*[35] and *uPA*.[38] Of note, the transcriptional activation of *uPA* by KLF6 activates latent TGF-β1 in vascular endothelial cells.[38] All these genes are intimately involved in endothelial homeostasis. Thus, even though KLF6 is ubiquitously expressed, following endothelium injury its endothelial expression is markedly increased, playing key roles in vascular biology.[34]

Recently, we described the TGF-β regulation of KLF6 and its splice variants and the cooperative transactivation effect on common target genes through a Smad3/Sp1/KLF6 interaction,[39] highlighting the tight relationship between KLF6 and the TGF-β pathway. ALK1 and Endoglin are involved in a common signaling pathway within the TGF-β system,[22,55,56] in agreement with the fact that *ALK1* and *ENG* gene mutations result in similar syndromic diseases, HHT2 and HHT1, respectively.[56] HHT is characterized by the presence of vascular lesions associated with fragile vessels and impaired vascular remodelling, likely a consequence of a deficient endothelial signaling in response to TGF-β. Indeed, experiments using heterozygous mouse models, $alk1^{+/-}$ or $eng^{+/-}$ have shown that vascular lesions develop upon an angiogenic stimulus, such as vascular injury, due to an inappropriate EC wound-healing response.[23,57] In this sense, endoglin cooperates with ALK1 in the proliferating responses of ECs and opposes to ALK5-promoted responses, including growth arrest, differentiation of ECs, recruitment of pericytes and production of extracellular matrix proteins.[50,52]

The signal(s) that triggers the KLF6 nuclear translocation remains to be elucidated. It can be postulated that the loss of intercellular contacts sustained by VE-cadherins and integrins might be a primary stimulus for KLF6 translocation in ECs affected by the wound. This nuclear translocation would promote an immediate stimulation of KLF6 target genes, including *Endoglin, ALK1,* and *KLF6* itself, to start the repair process. In the context of vascular homeostasis after endothelial damage, crucial players are the vSMCs in close contact with the EC layer, contributing to vessel stabilization and recovery. Alk1 is highly expressed in the vSMC layers surrounding the *tunica intima* as seen in Fig. 1, and it is especially conspicuous in the process of *neointima* formation after femoral denudation. At variance with ECs, neither ALK1 upregulation nor KLF6 translocation in cultured vSMCs was observed upon direct wounding. Thus, a cross-talk between endothelial and smooth muscle cell layers appears necessary for a correct homeostasis of the vessel wall. Indeed, *in vitro* experiments have shown that ALK1 is induced in vSMCs through a paracrine signal from the wounded endothelium. In this regard, the increase of IL-6 along the wounding process of ECs, suggests that this factor is a putative candidate to contribute to the paracrine effect on vSMCs surrounding the endothelium. Thus, the release of soluble factors, as IL-6, from the injured endothelium would serve to expand the repair signal by upregulating ALK1 from the inner to the more distant layers of the vessel. In addition, among the soluble factors secreted *in vivo*, it would be worth to analyze the levels of BMP-9, a specific ligand for ALK1 and endoglin.[58-60] BMP-9 is synthesized by the liver, circulates in human adult blood, is involved in vascular remodeling and induces vascular quiescence.[15,61] However, the in vitro effects of BMP-9 on endothelial proliferation and migration are controversial as some reports have described an inhibition, while others have described a stimulation of these functions.[58,59,62] Because upon vascular injury, EC proliferation and migration are increased, it will be of interest to find out whether the upregulation of ALK1 and endoglin in this setting is associated with changes in BMP-9 levels. Overall, the parallelism between ALK1 and endoglin in their pathophysiological functions as well as in their regulated gene expression in response to endothelial damage, support their involvement in the TGF-β-dependent events triggered by a vascular injury to recover the endothelial homeostasis. Because haploinsufficiency is the mechanism of pathogenicity in HHT1 and HHT2,[56,63] those stimuli able to upregulate the gene expression of *ALK1* and *ENG* constitute therapeutic targets to counteract the haploinsufficiency. In this regard, the characterization of KLF6 as a mediator of vascular injury in the induction of ALK1 and endoglin expression deserves an independent investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard Abbreviations

ACVRL1	Activin A receptor type II-like 1
ALK	Activin receptor-like kinase
ECs	Endothelial cells
ENG	Endoglin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HEK293T	Human embryonic kidney 293T cells)
HHT	Hereditary haemorrhagic telangiectasia
HMEC-1	Human microvascular endothelial cell line-1
HUVEC	Human umbilical vein endothelial cells
IL-6	interleukin 6
KLF6	Krüppel-like factor 6
p*ALK1*	*ALK1* promoter
S2	*Drosophila* Schneider embryonic cells
Sp1	Specificity protein 1
TGF-β	Transforming growth factor-β
UASMC	Umbilical artery smooth muscle cell
vSMC	vascular smooth muscle cells.

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Novelty and Significance

What Is Known?

- ALK1 is an endothelial TGF-β receptor involved in vascular remodeling and angiogenesis and whose expression is rapidly increased with angiogenic stimuli or upon vascular injury.
- The heterozygous deficiency of ALK1 gives rise to Hereditary Hemorrhagic Telangiectasia type 2 (HHT2), characterized by aberrant dilated vessels, and lack of capillary beds in certain areas.

What New Information Does This Article Contribute?

- Upon vascular injury, nuclear translocation of the transcription factor Kruppel-like factor 6 (KLF6) activates ALK1 gene transcription.
- The mechanism also involves a paracrine signal from endothelial cells that lead to the upregulation of ALK1 in smooth muscle cells.

Endothelial integrity is essential to regulate angiogenesis and vascular remodeling, but the repair mechanisms involved upon endothelial injury are poorly understood. ALK1 is an endothelial receptor whose expression is rapidly increased with angiogenic stimuli or upon vascular injury. In the present study we find that following endothelial injury, KLF6 translocates to the nucleus binding and activating the ALK1 gene promoter in synergy with Sp1 in endothelial cells. In addition, KLF6 translocation results in the release of soluble factors, including IL-6, which act on smooth muscle cells, increasing their ALK1 levels as well. This work demonstrates a key role of KLF6 in ALK1 upregulation after vascular damage both, in vitro and in vivo. These findings enhance our o understanding of the mechanism involved in vascular remodeling upon angiogenic stimuli or after endothelial denudation during embolotherapy of vascular lesions. Thus, KLF6 may be a therapeutic target to counteract ALK1 deficiency in HHT2.

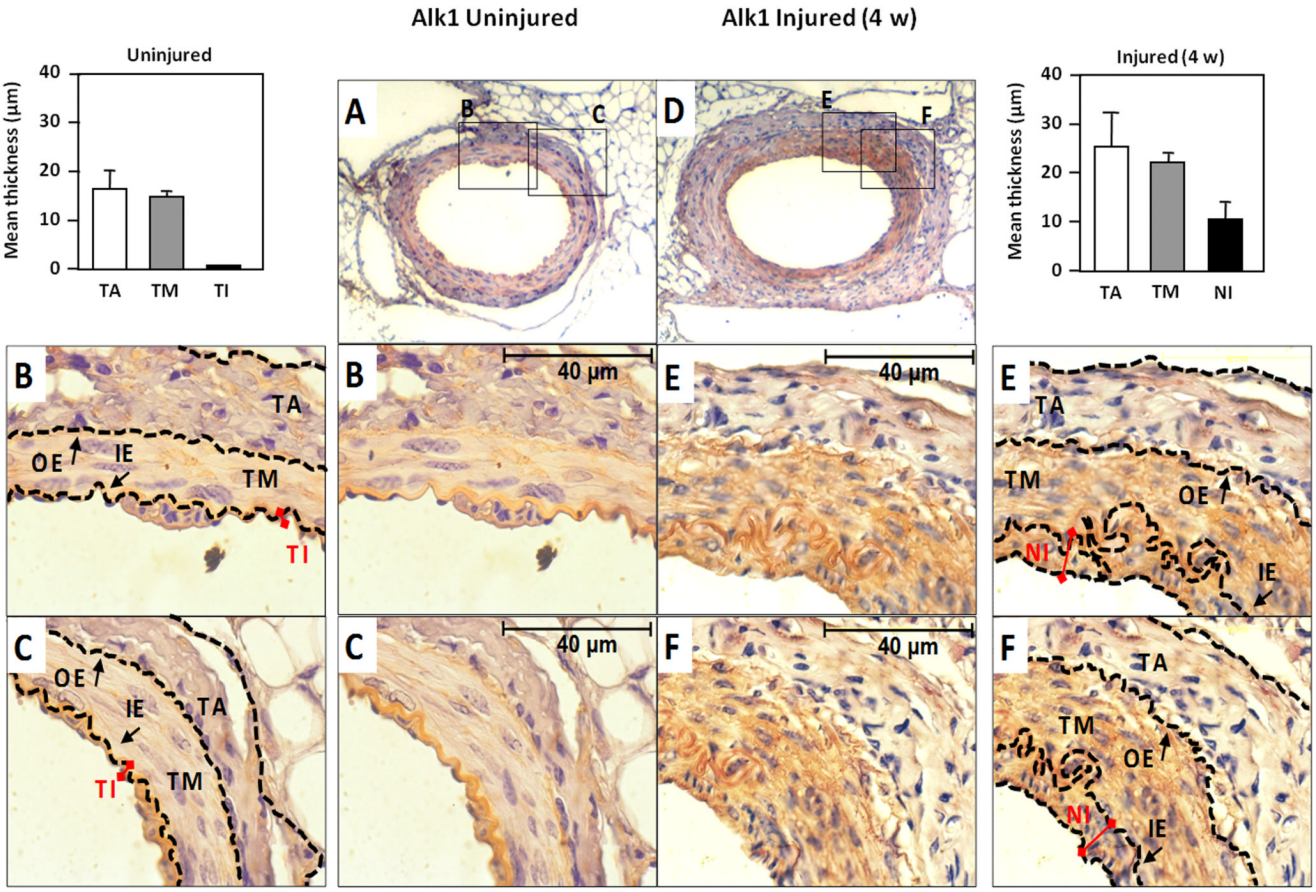

Figure 1. Alk1 expression is upregulated *in vivo* in neointima of mouse femoral artery after endothelial injury

A, D. Immunohistochemical staining of Alk1 in mouse femoral artery in (A) control, and (D) after endothelial injury (28 days). Pictures were taken at 25X magnification. **B, C, E and F.** Zoom (100X) of different areas of the vessel wall in each case. The outer (OE) and inner (IE) *elastica laminae*, divide the vessel wall into the three regions: *tunica adventitia* (TA), *tunica media* (TM) and *tunica intima* (TI) as indicated. In the injured vessel wall, the TI has been replaced for a hyperplasic neointima (NI), where Alk1 expression is highly increased. The thickness of both intima layers are indicated with red connectors. The mean thickness of each layer in both control and injured arteries was measured and the data are represented in the histograms.

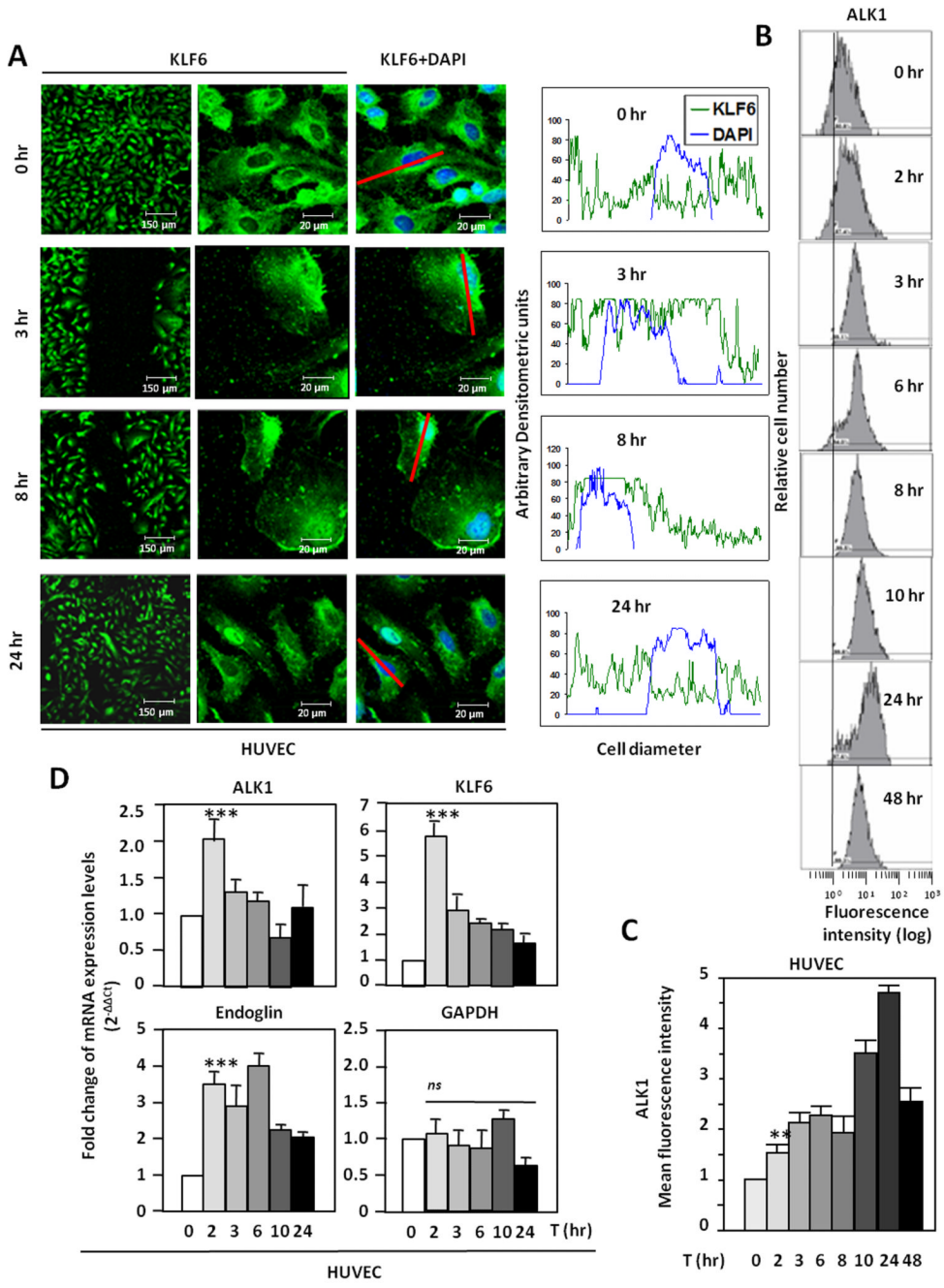

Figure 2. ALK1 expression is upregulated *in vitro* in endothelial cells after injury

A. Left, HUVECs were wounded *in vitro* and the intracellular location of KLF6 was tracked by immunofluorescence. Right, measurements of KLF6 (green) and DAPI nuclear staining (blue) along a longitudinal section of a representative cell (red line) of each condition. Fluorescence intensities were measured and represented in histograms using Image J™ software tool. Cellular distributions of both signals at distinct time points (0, 3, 8 and 24 hr) are shown. **B and C.** ALK1 protein levels in the surface of HUVECs from A were analyzed by flow cytometry. The time-dependent increase of the ALK1 mean fluorescence intensity is shown in C. **D**. Real time RT-PCR analysis of ALK1, KLF6, endoglin and GAPDH mRNA levels in HUVECs at different time points after the *in vitro* denudation.

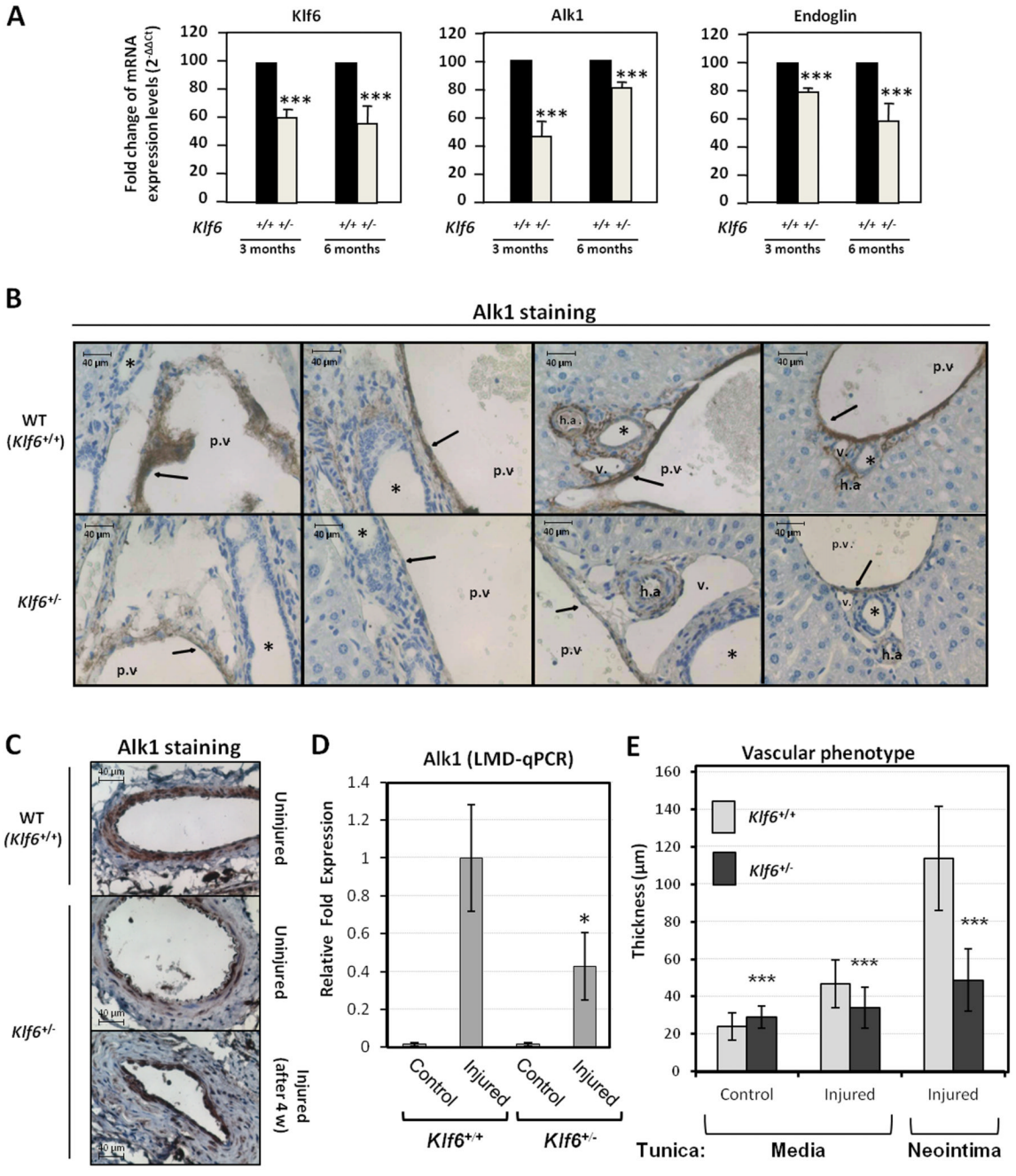

Figure 3. *Klf6*$^{+/-}$ heterozygous mice express lower levels of Alk1 in both basal condition and after endothelial injury

A. Real time RT-PCR of Alk1, Klf6 and endoglin levels from total liver mRNA of *Klf6*$^{+/-}$ heterozygous mice (3 and 6 months-old) compared to their wild type siblings. **B.** Immunohistochemistry of Alk1 in hepatic vasculature of *Klf6*$^{+/-}$ and *Klf6*$^{+/+}$ mice livers. Arrows highlight the Alk1 staining in ECs. The asterisks indicate the bile ducts. h.a, hepatic artery; p.v, portal vein; v, vein. **C.** Immunohistochemical staining of Alk1 protein in 4 weeks-injured femoral arteries from *Klf6*$^{+/-}$ mice in comparison with wild type littermates. **D.** Quantification of Ak1 mRNA by qPCR using laser microscopy microdissection (LMD)

from tissue sections of femoral arteries. **E.** Measurement of the tunica media and neointima 4 weeks post-injury. Each value represents the mean of at least 75 different measurements.

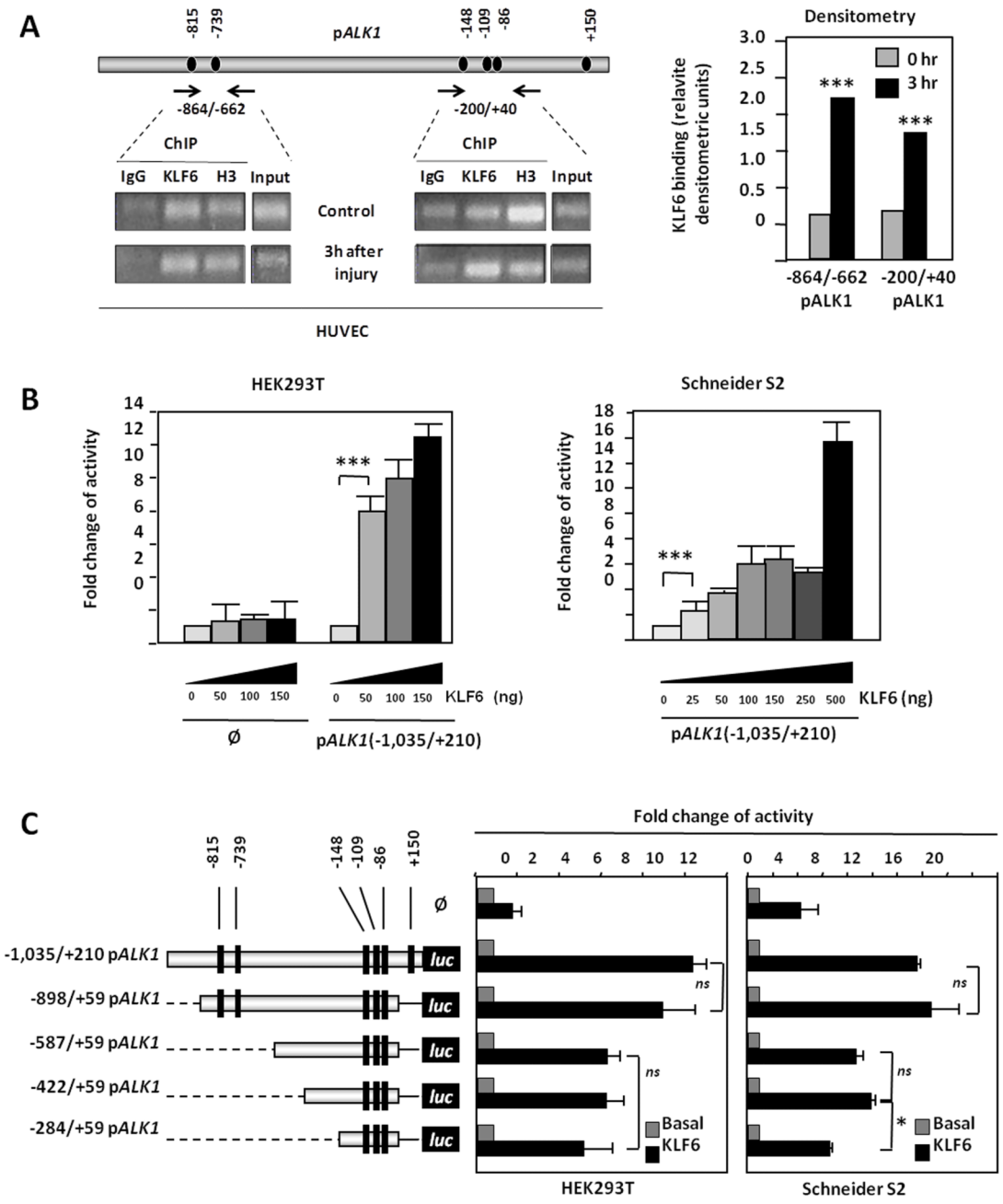

Figure 4. KLF6 transactivates *ALK1* promoter

A. KLF6 interacts with *ALK1* promoter in HUVECs and this interaction is increased after vascular injury. Left, chromatin immunoprecipitation (ChIP) of KLF6 over the two main KLF6-sites rich regions of the *ALK1* 5′-proximal promoter in HUVECs. Right, KLF6 binding was measured by densitometry of the individual bands and values of the (KLF6-IgG)/Input ratios were represented in both the control situation and 3 hr after the endothelial denudation **B.** Dose-response effect of KLF6 on the transcriptional activity of *ALK1* promoter in HEK293T cells (left) and *Drosophila* Schneider S2 cells (right). **C.** Effect of KLF6 on the transcriptional activity of 5′deleted constructs of *ALK1* promoter. Left, a

scheme of the different *ALK1* promoter constructs shows the distribution of the KLF6 consensus binding sites found along the *ALK1* promoter (black boxes). Right, transient co-transfection of KLF6 expression vector with different 5′ deleted constructs of *ALK1* promoter in both HEK293T and Schneider S2 cells.

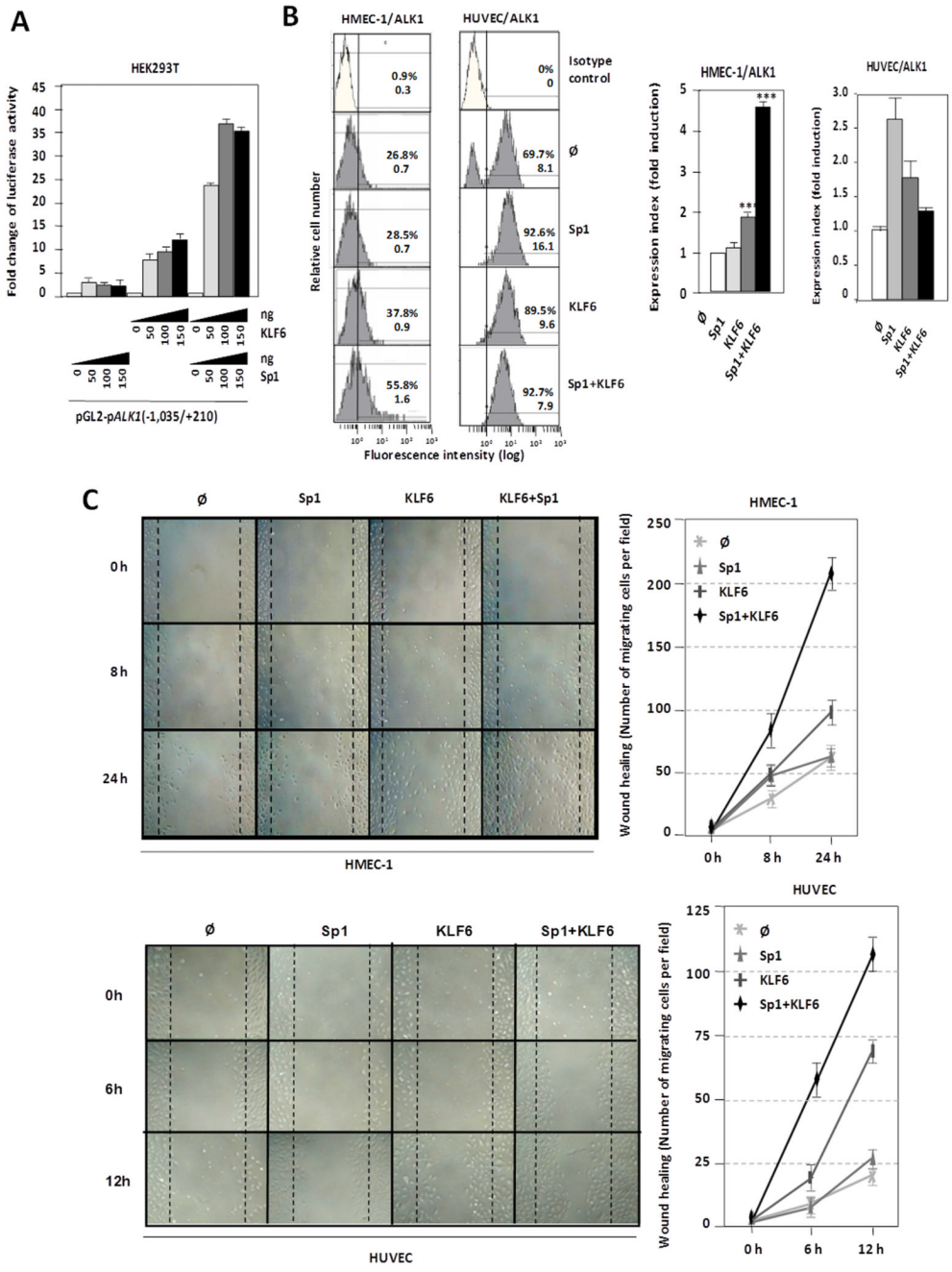

Figure 5. Functional cooperation between KLF6 and Sp1 in *ALK1* transactivation

A. Luciferase activity of the p*ALK1* (−1,035/+210) reporter in HEK293T cells transiently transfected with the indicated amounts of KLF6 and Sp1 expression vectors after 48hr. **B.** Left, flow cytometry of ALK1 protein levels at the surface of HMEC-1 and HUVEC cells 48hr after transfection with Sp1 and KLF6, as indicated. Right, quantification of expression index (fluorescence intensity normalized by number of positive cells) in each case is represented in the histograms. **C**. Wound healing experiments in HMEC-1 and HUVEC cells overexpressing Sp1 and KLF6, as indicated. After endothelial disruption, cells overexpressing both Sp1 and KLF6 close the wound much faster than control cells or those

overexpressing only Sp1 or KLF6. Right, quantification of the healing by measurement of the number of cells that have migrated to close the wound in each case.

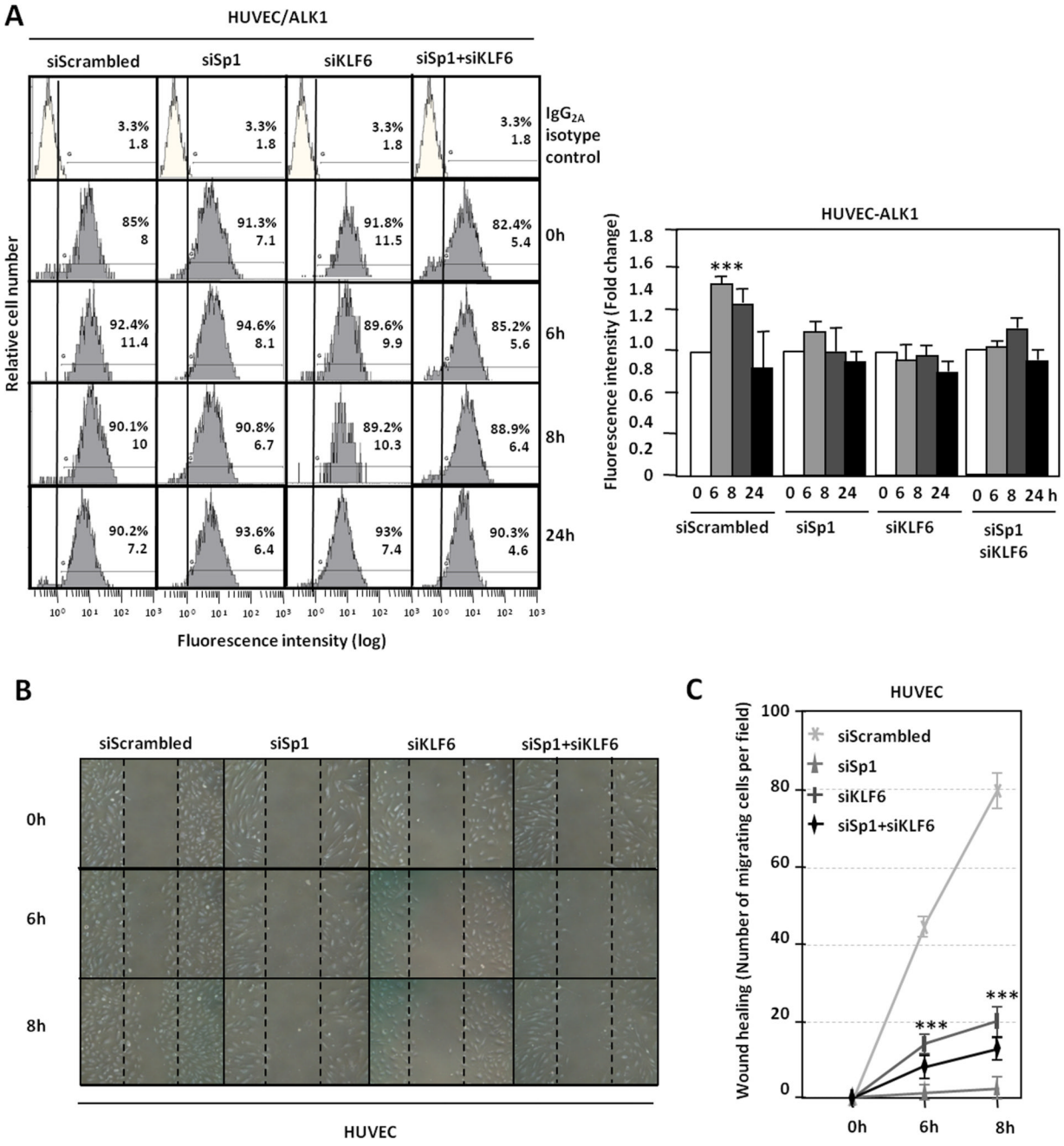

Figure 6. KLF6 and Sp1 knock down decrease ALK1 expression and inhibit ALK1 upregulation during wound healing

A. ALK1 levels on HUVEC surface 72h after transfection with siRNA Scrambled or siRNA specific for Sp1, KLF6 or both, at different time points of endothelial denudation *in vitro,* measured by flow cytometry. Right, representation of fold change in fluorescence intensity by bar histograms. **B.** Wound healing experiments in HUVEC cells after Sp1 and KLF6 knock down. C. quantification of the healing by measurement of the number of cells migrated to close the wound in each condition.

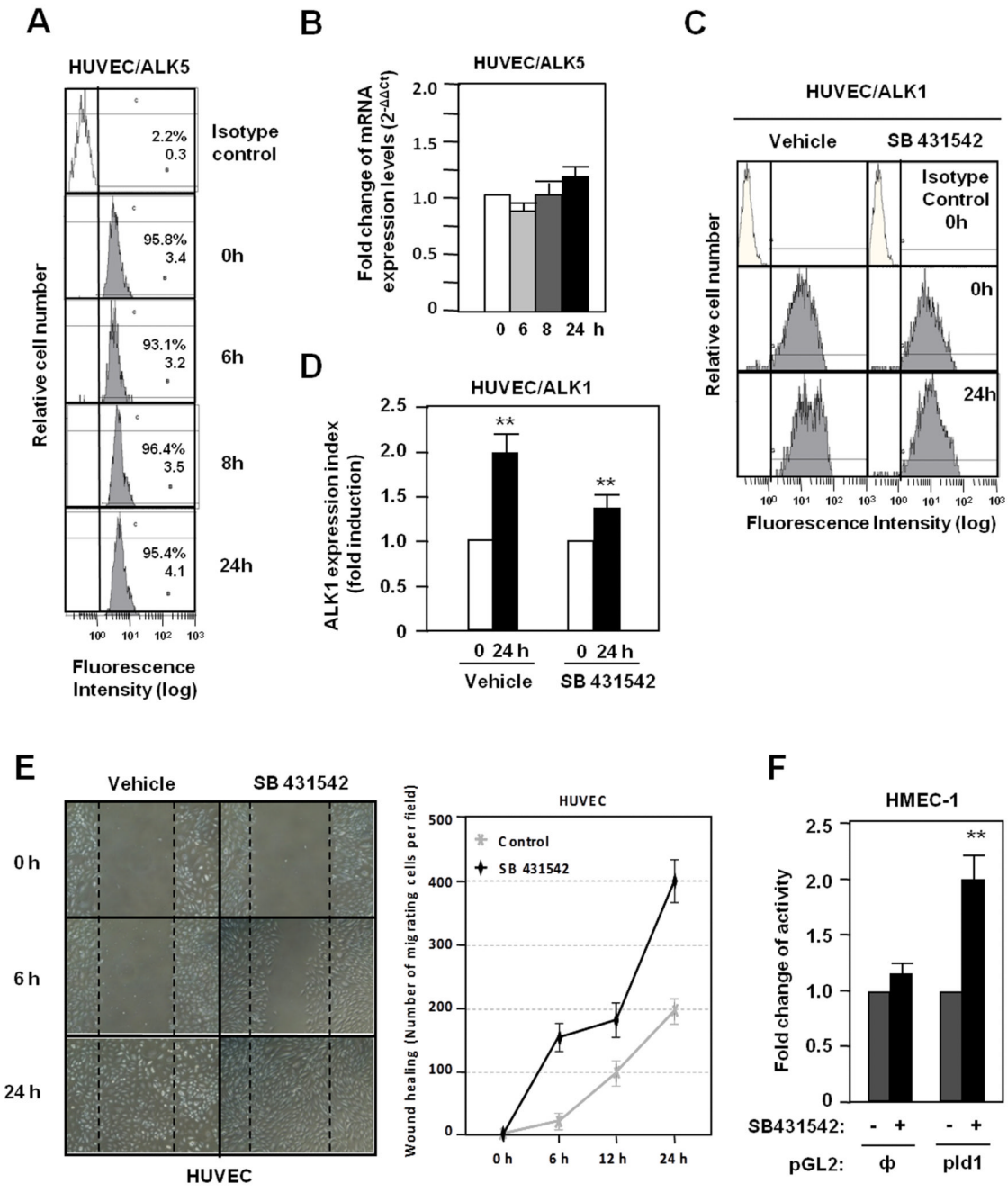

Figure 7. Role of ALK5 signaling in the wounding-induced ALK1 expression

A. ALK5 expression levels in HUVEC surface after wound healing measured by flow cytometry. **B**. ALK5 mRNA levels after wound healing measured by real time PCR. **C.** ALK1 expression in HUVEC surface after wound healing in absence or presence of the ALK5 kinase inhibitor SB431542, measured by flow cytometry. **D.** Histogram of ALK1 expression index from panel C. **E.** Wound healing assay of HUVECs treated or not with SB431542. Right, quantification of the endothelial migration expressed as number of migrating cells per field. **F.** Activity of Id1 promoter after treatment with SB431542 in

HMEC-1 cells previously transfected with the PGL2 reporter containing the Id1 promoter (pId1);, empty PGL2.

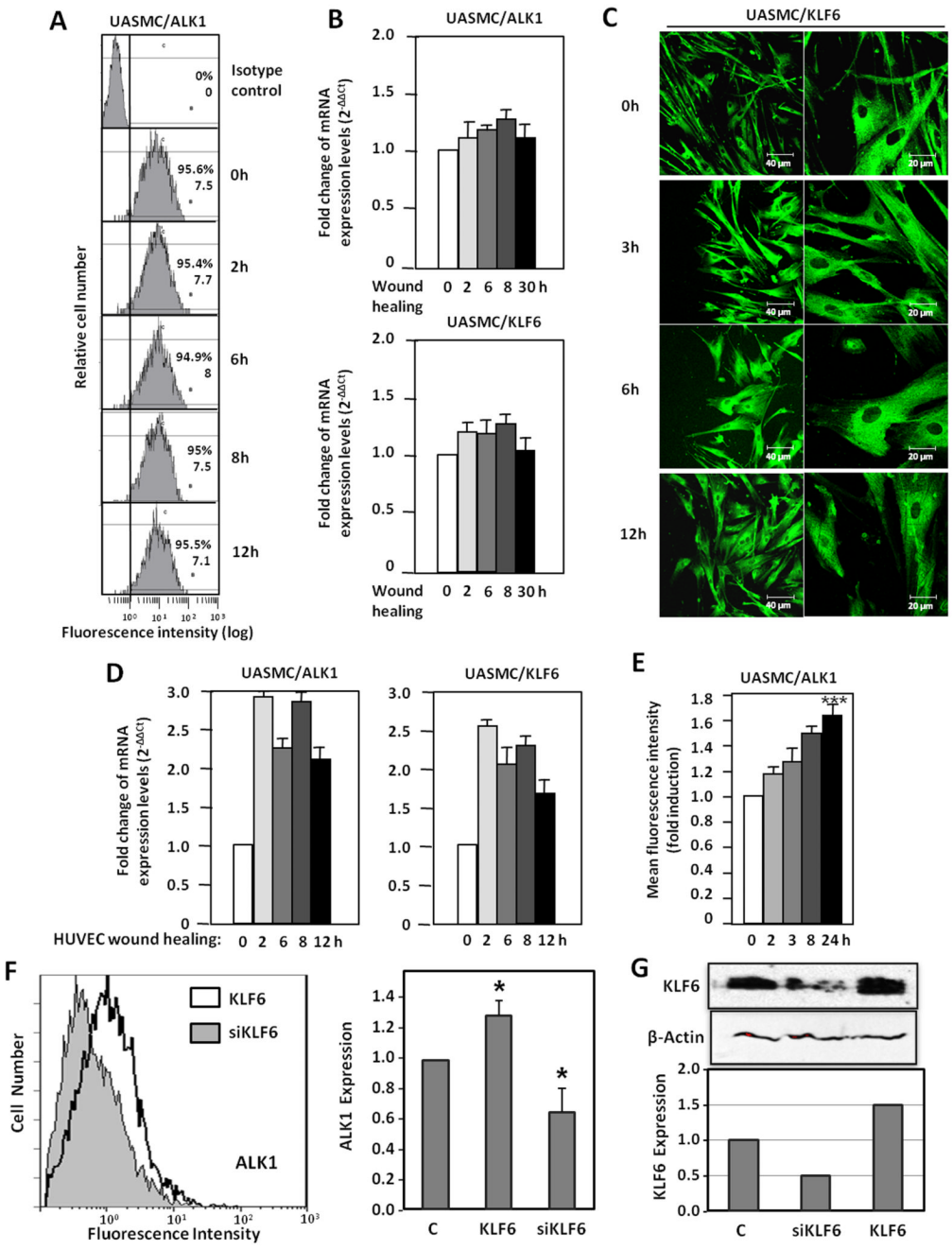

Figure 8. Paracrine effect of HUVEC denudation on ALK1 expression in vascular SMCs

A. ALK1 expression in the surface of vSMCs after denudation *in vitro*, measured by flow cytometry. **B**. Real time RT-PCR of ALK1 and KLF6 after vSMC denudation. **C**. Immunofluorescent staining of KLF6 in vSMCs during wound healing. **D,E**. Effect of conditioned media from HUVEC subjected to denudation at different time points on vSMC treated overnight. **D**. Real time RT-PCR of ALK1 and KLF6 transcripts. **E**. ALK1 expression measured by flow cytometry and represented as fold induction. **F,G**. Effect of conditioned media from HUVEC subjected to KLF6 overexpression (pCiNeoKLF6; KLF6), KLF6 suppression (pSuper-siKLF6; siKLF6) or mock transfection (C) on vSMC treated

overnight. **F**. ALK1 expression measured by flow cytometry (left) and represented as fold induction (right). **G**. Western blot analysis of KLF6 in total cell lysates. The intensity of the KLF6 band relative to β-actin intensity is represented in the histogram. UASMC, umbilical artery smooth muscle cells.