TGF-β regulates the expression of transcription factor KLF6 and its splice variants and promotes co-operative transactivation of common target genes through a Smad3–Sp1–KLF6 interaction

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INTRODUCTION

KLF6 (Krüppel-like factor 6) is a transcription factor and tumour suppressor with a growing range of biological activities and transcriptional targets. Among these, KLF6 suppresses growth through transactivation of TGF-β1 (transforming growth factor-β1). KLF6 can be alternatively spliced, generating lower-molecular-mass isoforms that antagonize the full-length WT (wild-type) protein and promote growth. A key target gene of full-length KLF6 is endoglin, which is induced in vascular injury. Endoglin, a homodimeric cell membrane glycoprotein and TGF-β auxiliary receptor, has a pro-angiogenic role in endothelial cells and is also involved in malignant progression. The aim of the present work was to explore the effect of TGF-β on KLF6 expression and splicing, and to define the contribution of TGF-β on promoters regulated by co-operation between KLF6 and Sp1 (specificity protein 1). Using co-transfection, co-immunoprecipitation and fluorescence resonance energy transfer, our data demonstrate that KLF6 co-operates with Sp1 in transcriptionally regulating KLF6-responsive genes and that this co-operation is further enhanced by TGF-β1 through at least two mechanisms. First, in specific cell types, TGF-β1 may decrease KLF6 alternative splicing, resulting in a net increase in full-length growth-suppressive KLF6 activity. Secondly, KLF6–Sp1 co-operation is further enhanced by the TGF-β–Smad (similar to mothers against decapentaplegic) pathway via the likely formation of a tripartite KLF6–Sp1–Smad3 complex in which KLF6 interacts indirectly with Smad3 through Sp1, which may serve as a bridging molecule to co-ordinate this interaction. These findings unveil a finely tuned network of interactions between KLF6, Sp1 and TGF-β to regulate target genes.

Key words: alternative splicing, endoglin, growth regulation, similar to mothers against decapentaplegic 3–specificity protein 1–Krüppel-like factor 6 interaction (Smad3–Sp1–KLF6 interaction), transactivation, transforming growth factor-β (TGF-β).

Abbreviations used: C-ter, C-terminus; DBD, DNA-binding domain; DMEM, Dulbecco’s modified Eagle’s medium; (E)CFP, (enhanced) cyan fluorescent protein; (E)YFP, (enhanced) yellow fluorescent protein; FCS, fetal-calf serum; FRET, fluorescence resonance energy transfer; GAL4, yeast transcription factor Gal4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione transferase; GTF, general transcription-factor; HEK-293T, human embryonic kidney-293T; HUVEC, human umbilical-vein endothelial cell; KLF6, Krüppel-like factor 6; LUC, luciferase; MH1, MAD homology 1 domain; siRNA, small interfering RNA; Smad3, similar to mothers against decapentaplegic 3; Sp1, specificity protein 1; Sv, splice variant; TGF-β, transforming growth factor-β; Tj/F, TGF-β receptor; uPA, urokinase plasminogen activator; WH, wound healing; WT, wild-type; ZAD, zinc-finger-associated domain.

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function through an unknown mechanism [8]. Expression of these Svs (splice variants) is increased in some cancers, is associated with a poor prognosis [18,19] and may contribute to functional inactivation of KLF6, even in tumours in which there is no loss of heterozygosity or inactivating mutation. However, the role of the KLF6 Svs in regulating full-length KLF6 activity in tissue remodelling is unclear.

TGF-β is induced through a KLF6–Sp1 interaction during vascular injury and inflammation [4], whereas it increases endoglin transcription through a Smad3 (similar to mothers against decapentaplegic 3)–Sp1 interaction [20]. Smads are signal-transducing and transcription factors mediating TGF-β signals [21]. Once TGF-β1 binds to the serine/threonine kinase receptor type II, it forms a tetra-complex with another serine/threonine kinase receptor type I and phosphorylates it, which in turn phosphorylates Smads 2 and/or 3, depending on the cell type [21]. Phosphorylated Smads 2 and 3 bind to Smad 4, which together translocate into the nucleus, where they bind to their cognate binding motifs on the promoters of target genes and activate them by co-operating with the general transcription-factor machinery [21]. Therefore the co-operation of KLF6–Sp1 in responsive genes might be further enhanced by TGF-β1 through a Smad3–Sp1 interaction, but this has not yet been established. Moreover, it is uncertain whether TGF-β directly affects KLF6 expression or splicing. If so, this would represent a potentially appealing way to enhance growth suppression through increased KLF6 activity.

In the present study we explored the effect of TGF-β on KLF6 biology at three different levels: (1) KLF6 expression; (2) KLF6 alternative splicing; and (3) promoter transactivation regulated by co-operation between KLF6 and Sp1.

**MATERIALS AND METHODS**

**Materials**

Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN, U.S.A.)

**Cells**

HUVECs (human umbilical-vein endothelial cells) were grown in medium 199 containing 20% (v/v) FCS (fetal-calf serum) and 50 µg/ml bovine brain extracts on 0.5%- (w/v)-gelatin-coated dishes. COS-7 monkey kidney cells, HeLa human epithelial cells from a cervical carcinoma, HepG2 human hepatic cells and human-derived renal epithelial HEK-293T (human embryonic kidney-293T) cells were grown in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% FCS. U-937 and THP-1 human monocytic cell lines were grown in RPMI-1640 medium supplemented with 10% FCS. Drosophila Schneider SL-2 cells were grown in Shield and Sang DES (Drosophila-enriched Schneider) insect medium (Sigma–Aldrich, St Louis, MO, U.S.A.) supplemented with 10% FCS.

Where indicated, cells were treated with TGF-β (10 ng/ml) (R&D systems) for progressive incubation times. Wound-healing treatments were applied by disrupting confluent monolayers of HUVECs with micropipette tips so that ~75% of the surface was denuded.

**Flow cytometry**

For semi-quantitative analysis of the amount of KLF6 expression, U-937, THP-1, HepG2 and HUVECs were fixed in 3.5% (v/v) formaldehyde and were permeabilized with 100 µg/ml lysophosphatidylcholine before incubation with the primary antibody to KLF6 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 1 h at 4°C. Cells were then incubated with FITC-labelled rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) for 30 min at 4°C and washed with cold PBS. The fluorescence intensity of each cell type was estimated with an EPICS® XL™ flow cytometer (Coulter, Hialeah, FL, U.S.A.) using logarithmic amplifiers. The amount of KLF6 expression is expressed as the Expression Index, obtained by multiplying the percentage of KLF6 positive cells by the mean fluorescence intensity of the total population of cells. A minimum of 10 000 cells was counted for each experimental time-point.

**RNA analysis by real-time PCR**

Total RNA was isolated from HUVECs, THP-1 and HepG2 cells using the RNeasy kit (Qiagen, Hilden, Germany) and was reverse-transcribed using avian-myeloblastosis-virus reverse transcriptase. The resulting cDNA was used as a template for PCR performed with a combination of specific oligonucleotide primers: WT (wild-type) human KLF6, forward: 5′-CGGACGCACACA-GGAAAAA-3′; reverse: 5′-CGGTCGTCCTTCGGAGGAGT-3′; total human KLF6, forward: 5′-CTGCGGCTCTGAGGAGT-3′; reverse: 5′-TCCACAGATCTTCTGCCTGTC-3′; human GAPDH (glyceraldehyde-3-phosphate dehydrogenase), forward: 5′-CAATGTACCCCTTCATTGACC-3′; reverse: 5′-GA-TCTCCTCCTGGAAGATG-3′; collagen type I, forward: 5′-CTGCGTGGTCTCTAAAGGTGAG-3′; reverse: 5′-TAGCACCATCATTGCAGA-3′; endoglin, forward: 5′-GCCTCGAGAGGTGCTTCT-3′; reverse: 5′-TGCAAGGAAGACACTGCTGT-AC-3′.

All experiments were performed in triplicate and the amounts of both WT and total KLF6 were normalized to GAPDH expression. To calculate the fold change in KLF6 alternative splicing, the fold change in total KLF6 mRNA levels (WT KLF6 plus alternatively spliced KLF6 transcripts) was divided by the fold change in WT KLF6 alone.

For Sp1 knockdown expression in HUVECs, confluent cells were infected with supernatants of HEK-293T cells transfected with the lentiviral system pSHI H1-Puro small-hairpin RNA of Sp1 (targeting site for Sp1 knockdown: TGATCGGTGGCCCTGAA) or a random sequence [non-target siRNA (small interfering RNA)] as a negative control, following the protocol recommended by the manufacturer [SBi (System Biosciences), Mountain View, CA, U.S.A. (http://www.systembio.com/downloads/Manual_pCDF_Vector_v5.pdf)].

**Endoglin and collagen promoter constructs and transactivation assay**

LUC (luciferase) reporters for the endoglin promoter pCD105 (~350/+50)-pXP2 and the collagen type I(1) promoter, pGL-Col1, have been described elsewhere [4]. Transient transfection was performed using the Superfect transfection reagent (Qiagen) in serum-free medium containing 1 µg each of either pCD105 (~350/+50)-pXP2 or pGL-Col1, with or without indicated amounts of expression vectors for Sp1, wild-type KLF6, KLF6 Svs KLF6-Sv1 and Sv2, Smad3, and TGF-β type 1 receptor. Sv1 and Sv2 alternatively spliced products arise from the use of native cryptic splice sites within exon 2 [8]. KLF6 Sv1 contains a novel 21-amino-acid carboxy domain resulting from out-of-frame splicing of its terminal exon. KLF6 Sv2 has lost a zinc-finger-binding domain and part of the C-terminal transactivation domain. Whereas WT KLF6 contains 283 amino acids, with three zinc fingers as its binding domain, Sv1 has 195 amino acids and...
no zinc fingers, and Sv2 has 241 amino acids and two zinc fingers. The expression vectors for Sp1 and KLF6 were in plasmids pAC for transfections into Drosophila cells or in pCIneo for expression in mammalian cells. All transfections contained the same amount of total DNA (2 μg), with the balance composed of the corresponding empty expression vectors. The KLF6-GAL4 (KLF–yeast transcription factor protein Gal4) and GAL4-LUC reporter were used as previously described [2]. LUC activity was determined in each cell lysate using a TD20/20 luminometer (Promega, Madison, WI, U.S.A.). Correction for transfection efficiency was made by co-transfection with pCMV-

β

RI and was separated by SDS/8%-w/v)-PAGE under reducing conditions. Proteins were transferred to Hybond-C extra nitrocellulose membranes [Amersham Biosciences (now GE Healthcare), Hillerod, Denmark] and probed with the antibodies indicated. Signals were developed using the Super Signal reagent (Pierce, Rockford, IL, U.S.A.) for enhanced chemiluminescence. Experiments were repeated at least three times independently with similar results, and representative results are shown in the corresponding figures. The expression vector pCMV5-TβRI (pCMV5-TGF-β receptor 1) (ALK-5), which encodes for the constitutively active form of this receptor and the GST-fusion protein GST–KLF6 was described previously [7].

For sequential immunoprecipitation experiments, 75%-confluent 100-mm-diameter plates of HEK-293T cells were either doubly transfected with 3 μg each of KLF6 and Smad3 expression vectors (pcDNA3.KLF6 and pcDNA3.Smad3.Flag respectively) or triply transfected with these two vectors and with, additionally, 3 μg of pcDNA3.Sp1. First, immunoprecipitation was performed with anti-KLF6 (Santa Cruz Biotechnology). After washing, the precipitates were subjected to a second immunoprecipitation with anti-Flag. Total protein extracts and the first and second immunoprecipitates were subjected to SDS/10%-PAGE and Western blotting. Western blots were incubated with anti-Flag, anti-KLF6 and anti-Smad3 antibodies respectively (Santa Cruz Biotechnology).

**Plasmid constructions for FRET (fluorescence resonance energy transfer)**

KLF6-WT-EYFP vector was constructed by recloning a BamHI/EcoRI fragment from the KLF6 expression vector into the pEYFP vector (Clontech Laboratories Inc., Mountain View, CA, U.S.A.) as described in [22] (where EYFP is enhanced cyan fluorescent protein). Sp1 Cter (C-terminus) (amino acids 556–763)-EYFP (Cter-enhanced yellow fluorescent protein) and Smad3-EYFP or -ECFP vectors were made by recloning both a NotI/XbaI fragment from pEYFP or pECFP vector (Clontech) plus an EcoRI/EcoRI fragment from the Sp1 and Smad3 expression vectors respectively into the pcDNA3 vector (Invitrogen Co., Carlsbad, CA, U.S.A.).

**Analysis of FRET**

HEK-293T cells were transfected with a combination of KLF6-EYFP and each of Sp1 Cter-EYFP, Smad3-EYFP and control EYFP, or a combination of Smad3-EYFP and either Sp1 Cter-EYFP or control EYFP, using Lipofectamine Plus® reagent (Invitrogen). Cells were incubated in DMEM containing 10% FCS for 48 h, and then treated with 2.5 ng/ml TGF-β in the same serum containing medium for 6 h, or scratched by pipette tip as described in [22], and the incubation was continued in the same serum-containing medium for 6 h. Cells were fixed with 10% formaldehyde, and FRET was determined using a model LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, NY, U.S.A.), an excitation wavelength of 458 nm and an emission wavelength of 523 nm. For the photobleach method, pre-photobleach CFP (cyan fluorescent protein) (donor) images were first acquired using an excitation wavelength of 458 nm and an emission wavelength of 480 nm. Cells were then exposed to 514-nm laser for 20 s at maximum power to eliminate the yellow color for YFP (yellow fluorescent protein) (acceptor photobleaching). Thereafter, cells were re-exposed to the 458-nm laser again. The intensity of the emission signal at 480 nm, recovered by acceptor bleaching, was detected. Fluorescence intensities at 480 nm obtained before and after the bleaching were compared in each sample.

**RESULTS**

Cell type-specific regulation of KLF6 expression by TGF-β1

KLF6 up-regulates several genes involved in the TGF-β signalling pathway, including TGF-β1, its type I and II receptors (TβRI and TβRII) and uPA [4,7]. However, it is not known whether there is a reciprocal induction of KLF6 by TGF-β1. To address this question, we measured the effect of TGF-β1 on the expression of KLF6 in several cell lines using flow cytometry. As seen in Figure 1 (top panel), KLF6 expression in both monocytic U-937 cells and promonocytic THP-1 cells was up-regulated in a time-dependent manner up to 8-fold after 24 h (P < 0.001). A similar effect was also observed in primary HUVEC cultures, although the increase was only sustained from 0 to 12 h of TGF-β1 treatment accompanying an increment of only 50% at 12 h (P < 0.01), followed by a decrease between 12 and 24 h to the basal level (Figure 1, middle panel). By contrast, the expression of KLF6 in HepG2 and HeLa cells was down-regulated after TGF-β1 treatment and decreased to 30 and 80% of basal levels respectively after 24 h (P < 0.001) (Figure 1, bottom panel), suggesting that KLF6 responsiveness to TGF-β1 was cell-type-specific.

KLF6 alternative splice products functionally antagonize full-length KLF6 in regulating wound-repair genes

Because it was unclear whether alternative Svs antagonize KLF6’s transactivation of genes involved in tissue repair as they do in cancer, we explored the impact of KLF6 Sv1 and Sv2 on two target genes, namely those coding for endoglin and collagen type 1, whose promoters are transactivated by full-length KLF6 (Figures 2A and 2B respectively). Whereas overexpression of WT (=full-length) KLF6 transactivated both endoglin- and collagen-type 1 promoters (black bar), overexpression of Sv1 and Sv2 down-regulated them even below basal levels (bars 3 and 4 respectively). Compared with Sv1, Sv2 had a more prominent suppressive effect. Treatment with TGF-β1 (10 ng/ml) increased the transactivation of the endoglin gene in the presence of
WT KLF6, but not when variants Sv1 and Sv2 of KLF6 were transfected (hatched bars 1–4), indicating a dominant-negative effect of these variants. Furthermore, the combination of WT KLF6 with Sv1 and Sv2 attenuated the effect of exogenously overexpressed WT KLF6 (bars 5 and 6 respectively) and suppressed transactivation activity down to the levels obtained with KLF6 siRNA (white bar 2), suggesting that KLF6 Svs could functionally antagonize full-length KLF6 in tissue repair.

We next examined whether TGF-β1 might affect KLF6 alternative splicing (Figure 2C). Using real-time PCR with a primer set capable of specifically detecting only WT KLF6 and a primer set that detects both WT and all Svs, we quantified full-length and total (full length + alternatively spliced forms) KLF6 mRNAs and calculated the amount of Sv mRNA. To calculate the fold change in KLF6 alternative splicing, the fold change in the mRNA levels of total KLF6 (wild-type KLF6 + alternatively spliced KLF6 transcripts) was divided by the fold change in wild-type KLF6 alone. This method for determining KLF6 Sv expression has been validated extensively [8,31]. TGF-β1 transiently increased KLF6 alternative splicing in THP-1 cells after 4 and 6 h treatment, followed by more sustained and highly significant decreases after 8 h and longer treatment (Figure 2C). In HUVECs, after a transient increase at 1 h, there was a decrease in KLF6 splicing starting after 2 h treatment (Figure 2D). By contrast, in HepG2 cells, TGF-β1 had no significant or sustained effect on alternative splicing (Figure 2E).

**TGF-β1 enhances Sp1–KLF6 co-operation through Smads**

We previously reported that KLF6 and Sp1 may functionally and physically interact to regulate target gene expression [4]. On the basis of these previous findings, we next explored whether TGF-β1 might regulate KLF6-target-gene expression by modulating KLF6–Sp1 interactions through Smads.

To address this question, we utilized reporter constructs containing the proximal upstream regions of the endoglin- and collagen-type-I-gene promoters, both of which are TGF-β-responsive through Smad3–Sp1 interactions [4,23]. The results of these experiments are shown in Figure 3. In HeLa cells, which express high levels of endogenous Sp1, transfection of either KLF6 (sample 2) or Smad3 (sample 3) alone led to a 5- and 2.5-fold stimulation in endoglin (Figure 3i, panel A) and collagen type I (Figure 3i, panel B) promoters respectively, whereas co-transfection of both KLF6 and Smad3 (sample 4) led to synergistic increases up to 18- and 10-fold for endoglin (Figure 3i, panel A) and collagen-type-I (Figure 3i, panel 3B) gene transactivation respectively, suggesting that the TGFβ1/Smad3 pathway further enhances Sp1–KLF6 transcripctional co-operation in mammalian cells. The overexpression of Smad3 in these experiments by co-transfection mimics the effect of exogenous TGF-β treatment, since Smad3 is the effector of the TGF-β pathway. However, the strongest transactivation was obtained when using both TGF-β (10 ng/ml) and transfecting Smad3 (Figure 3i, panel A, samples 1–4).

Because these findings did not directly establish a functional requirement for Sp1 in mediating KLF6–Smad3 synergy and involvement of Sp1 as an adaptor, we examined whether KLF6 and Smad3 could still functionally co-operate in the transcriptional regulation of the endoglin gene when Sp1 expression was knocked down by transient transfection with siRNA (Figure 3i, panel C). In HEK-293T cells, which express very low levels of KLF6, simultaneous overexpression of Smad3 and KLF6 by transient co-transfection transactivated the endoglin-gene promoter 5-fold (black bar for sample 4). However, this apparent co-operation of KLF6 and Smad3 (compare the black bars for samples 2 and 3 with sample 4), was abrogated by transfecting siRNA to Sp1, which decreased the transcriptional activity to the levels resulting from KLF6 transfection alone (2-fold) (white bar for sample 4). Control siRNA to random sequences had no effect (hatched bar for sample 4).

The preceding result was further supported by transfection of endoglin- or collagen-type-I-gene promoters in Drosophila Schneider cells (Figures 3i, panels D and E), which lack endogenous Sp1 and KLF6 [2]. In these cells lacking Sp1, transfected KLF6 and Smad3 showed no functional co-operation, but rather a slight competition (compare samples 2 and 3 with the sample 4 combination), whereas Sp1 and Smad3 showed an additive effect (sample 5). Similarly, Sp1 and KLF6 showed a cooperative effect (sample 6) in the transactivation of the endoglin (Figure 3i, panel D) and collagen-I-gene (Figure 3i, panel E) promoters, as reported previously [4,20,23]. Smad3 additionally enhanced KLF6/Sp1 synergy from 28- and 22-fold to 32- and 26-fold respectively (sample 8) in transactivating the endoglin- and collagen-I-gene promoters. These results suggested that KLF6...
Effect of TGF-β on KLF6 and target genes

Figure 2 Changes in KLF6 alternative splicing after TGF-β1 treatment in different cells

(A and B) HEK-293T cells were co-transfected with pCD105 (−50/+350)-pXP2 (pEndoglin, A) or pGL-Col1 (pCollagen, B) and 1 μg of either WT KLF6 (KLF6wt) (black bar 2), KLF6 Sv1 (Sv1) (black bar 3), KLF6 Sv2 (Sv2) (black bar 4) or a mixture of 0.5 μg of WT and 0.5 μg of Sv1/Sv2 (black bar 5). For the first two bars in (A), endogenous KLF6 was knocked down with KLF6 siRNA (white bar). In addition, the effect of TGF-β treatment (10 ng/ml) was assessed in hatched bars 1–4 in (A). The results are given in arbitrary units of LUC activity. Representative results obtained from three different experiments with replicable results are shown. *Statistical significance at least P < 0.05 between control (pEndoglin, pCollagen) and KLF6 co-transfected. In case of the white bar (siRNA), the asterisk (*) means that the value was statistically significant (P < 0.05) compared with the corresponding black bar 2. (C–E) After THP-1, HUVEC and HepG2, cells were treated with 10 ng/ml TGF-β1 for the various times indicated and cell lysates were prepared. Total RNA was extracted from each cell lysate, and levels of WT KLF6 and its Svs in each sample were measured using real-time PCR. To calculate the fold change in mRNA levels of KLF6 alternative splicing, the fold change in mRNA levels of total KLF6 (WT KLF6 plus alternatively spliced KLF6 transcripts) was divided by the fold change in wild-type KLF6 alone. *Statistical significance at least P < 0.05.

and Smad3 co-operate functionally in an indirect manner via their common partner Sp1 as an adaptor. These findings also suggested a novel mechanism in KLF6 target gene regulation involving the formation of KLF6–Sp1–Smad complexes on target promoters.

Next, the endogenous functional outcome of this co-operation between KLF6, Sp1, TGF-β and Smad was assessed by treating HUVECs with TGF-β (10 ng/ml) and by in vitro disruption of HUVEC monolayer [WH (wound healing)] at different times to induce KLF6. As shown in Figure 3i, panel A, WH treatment significantly induced the expression of KLF6 mRNA between 2- and 5-fold, on the basis of quantitative real-time PCR. Having validated that WH induced endogenous KLF6, levels of endoglin and collagen mRNA were measured either in untreated cells, after TGF-β treatment or after KLF6 induction (by WH) or a combination of both stimuli. As seen in Figure 3i, panels B and C, there was a synergistic effect of KLF6 and TGF-β, which significantly increased endoglin mRNA from 3 to 25 h, with a peak of 22.5-fold induction. In the case of type I collagen (Figure 3i, panel C), the co-operation between TGF-β and KLF6 (WH) was significant after 3 h of combined treatment, whereas the effect of WH was predominant after 12 and 25 h of combined treatment.

Together, these data indicate a synergistic effect between KLF6 and TGF-β in endothelial cells, measured by an increase in endoglin and collagen type I mRNAs.

KLF6 and Smad3 form a multimeric complex with Sp1 as a bridging molecule

The potential complexing of KLF6, Sp1 and Smad3 was examined by GST pull-down experiments using total extracts from COS-7 cells with or without stimulation of the TGF-β1/Smad3 signalling pathway (Figure 4A). When GST–KLF6 was precipitated with glutathione–Sepharose beads, both endogenous Sp1 and transfected Smad3 were pulled-down together (Figure 4A, lanes 1 and 2). The overexpression of Smads (Smad3/4 here) is sufficient to trigger TGF-β signalling (Smad3 phosphorylation), even in the absence of ligand stimulation (TGF-β) [24]; we additionally examined whether there was further stimulation following co-transfection of the constitutively active receptor TβRI [24]. The activation of Smad3/4 by co-transfection of constitutively active TβRI yielded only a small increase in the amount of Sp1 recovered (lane 1). When the transfection lacked Smad3, only Sp1 could be recruited by KLF6 (lane 3). The control pull-down with GST–Sepharose beads alone did not give any bands (results not shown).

The co-immunoprecipitation of Sp1, Smad3/4 and KLF6 was further demonstrated in an endogenous system using HUVECs grown under control conditions or 24 h after either WH and TGF-β treatment, as described previously. Under both conditions, Sp1 immunoprecipitates also pulled down KLF6 and Smad 3/4 (Figure 4B). When human IgG was used as a control antibody, no...
Figure 3 Effect of Sp1, KLF6 and Smad3 on transactivation of ENG and COL promoters in mammalian and insect cells

(i) Panels A and B: the transactivation effect of different expression vectors for KLF6, Smad3, and a combination of both, on the endoglin (ENG, A) and collagen (COL, B) promoter constructs, respectively in HeLa cells. In each transfection, 1 μg of reporters, 0.5 μg of KLF6 and 250 ng of Smad3 expression vectors were used. At 24 h after transfection, cell lysates were prepared and the LUC activity in each lysate was determined as described in the Materials and methods section. The results are expressed as fold induction, comparing with values obtained with vacant vector 1. Lane 1, reporter (endoglin or collagen-1); lane 2, reporter + KLF-6; lane 3, reporter + Smad3; lane 4, reporter + KLF6 + Smad3. Black bars represent the untreated samples, whereas hatched bars represent samples that were additionally treated with TGF-β (10 ng/ml). Panel C: the same experiment was carried out using HEK-293T cells. The transactivation effect of different expression vectors for KLF6 and Smad3 and in combination with the endoglin promoter construct. In each transfection, 1 μg of reporters, 0.5 μg of KLF6 and 250 ng of Smad3 expression vectors were used. At 24 h after transfection, cell lysates were prepared and the LUC activity in each lysate was determined as described in the Materials and methods section. The results are expressed as fold induction, comparing with values obtained with vacant vector (sample 1). Lane 1, reporter (endoglin); lane 2, reporter + KLF6; lane 3, reporter + Smad3; lane 4, reporter + KLF6 + Smad3. Moreover, the co-operative effect between KLF6 and Smad3 was also assessed by silencing Sp1 using transfection with 0.5 μg of siRNA (siSp1; white bar) and using as a control a random siRNA sequence (siRandom, hatched bar). Panels D and E: the same type of experiment as in panels A–C was performed, but instead Schneider Drosophila cells were used, which express neither Sp1 nor KLF6. The transactivation effect of different expression vectors for Sp1, KLF6 and Smad3, and a combination of them, on both on the endoglin (ENG, D) and collagen (Col, E) promoter constructs respectively was assayed. In each transfection, 1 μg of reporters, 0.5 μg of KLF6 and Sp1, and 250 ng of Smad3 expression vectors, were used. At 48 h after transfection, cell lysates were prepared and the LUC activity in each lysate was determined as described in the Materials and methods section. The results are expressed as fold induction, comparing with values obtained with vacant vector (sample 1). In the eight samples shown, ‘R’ means the reporter. The experiments were repeated five times, and each experiment had two or three replicate points. The experiments shown are representative of the results obtained. (ii) Panel A: RNA from KLF6 was quantitated after 0, 3, 12 and 25 h of WH in HUVECs by real-time PCR. The increase in RNA is expressed as fold induction. Panels B and C: RNA from endoglin and type I collagen was quantified after 0, 3, 12 and 25 h in HUVECs either, untreated or subjected to TGF-β treatment (10 ng/ml), WH or the combination of both treatments by real-time PCR. The increase in RNA is expressed as fold induction. Asterisks show that the differences are significant with P at least <0.05. Experiments were repeated at least twice, using triplicates, and referred to GAPDH RNA as endogenous control.
band of immunoprecipitation was detected. Further, the amount of KLF6 recovered was higher after treatment with either TGF-β or WH (lane T, Figure 4).

To reinforce this conclusion, Drosophila Schneider cells, which lack endogenous KLF6 and Sp1, were co-transfected with a combination of expression vectors for either Sp1 or KLF6 and/or Flag-Smad3. At 48 h after transfection, total extracts from these cells were subjected to immunoprecipitation using an anti-Flag antibody to identify Flag-Smad3 (Figure 4C, right-hand panel). Using this approach, only Sp1 could be coinmunoprecipitated with Smad3, and KLF6 was not recovered in the immunoprecipitates with Smad3 when Sp1 was not present. These data suggest that Sp1 is necessary for the formation of the complex, since there is not direct physical interaction between KLF6 and Smad3.

Altogether, the formation of two-component endogenous complexes (Sp1–KLF6 and Sp1–Smad3) has been demonstrated (Figure 4B). Figure 4(C) shows, in addition, that Sp1 is needed for Smad3–KLF6 complex formation in Schneider S2 cells. The functional data support co-operation between KLF6, Smad3 and Sp1. However, at this point it would still be an overstatement to argue that a ternary complex is really formed. To claim for the existence of such a complex we performed sequential double co-immunoprecipitation in HEK-293T cells, either doubly or triply transfected with expression vectors for KLF6 and Smad3, or KLF6, Smad3 and Sp1. The performance of a triple-transfection experiment in S2 and double-sequential co-immunoprecipitation was not viable, owing to the lower transfection efficiency in these cells. The results of the experiment in HEK-293T cells are shown in Figure 4(D). First we immunoprecipitated using an antibody against the first component, KLF6. Secondly, we eluted and made a subsequent immunoprecipitation using an antibody against the second component, Smad3, using the Flag tag, and in this way we were able of detect Sp1, the third component of the ternary complex. There is a substantial enrichment of the ternary complex after the second immunoprecipitation (the enrichment detected in Smad3 being relevant), which is dependent on the DBD or ZAD (zinc-finger-associated domain) KLF6 activation domain [19] after transient transfection of COS-7 cells and then immunoprecipitation with anti-Flag antibodies from cell extracts. Co-immunoprecipitates were Western-blotted with an Sp1-specific antibody. As seen in Figure 5(A), Sp1 was co-immunoprecipitated only when cells were transfected with the KLF6 transactivation domain (lane 3 in ‘IP Flag’). Combined with our previous finding [4], this result suggests that the Sp1–KLF6 interaction requires the DBD of Sp1 and the transactivation domain of KLF6. The specificity of the Flag immunoprecipitation was documented as shown in Figure 5(B), where expression of both KLF6 DBD and ZAD domains can be observed in the total extracts (lanes 1 and 3), but not when the Flag cDNA construct was eliminated when the acceptor is bleached, thereby yielding an increase in donor fluorescence. Supplementary Figure S2(B) shows the fluorescence intensity of emission at 480 nm before and after bleaching. Recovery of emission at 480 nm was seen in the cells co-transfected with combinations of KLF6–ECFP and Sp1 Cter–EYFP (panel a) and KLF6–ECFP and Sp1 Cter–EYFP (panel d), but not in the cells co-transfected with combinations of KLF6–ECFP and Smad3–YFP (panel f), KLF6–EYFP and EYFP (panel h) or Smad3–ECFP and EYFP (panel j). The average numbers in the recovery rate in each sample are presented under each of corresponding photographs. In conclusion, FRET confirmed a direct interaction between KLF6 and Sp1 Cter and between Smad3 and Sp1 Cter in living cells, but not between KLF6 and Smad3.

Sp1 interacts with the transactivation domain of KLF6

We previously demonstrated that Sp1 interacts with KLF6 through its C-terminal DBD (DNA-binding domain) [4]. However, the domain of KLF6 interacting with Sp1 has not been identified. We addressed this question by expressing a Flag-tagged KLF6 DBD or ZAD (zinc-finger-associated domain) KLF6 activation domain [19] after transient transfection of COS-7 cells and then immunoprecipitation with anti-Flag antibodies from cell extracts. Co-immunoprecipitates were Western-blotted with an Sp1-specific antibody. As seen in Figure 5(A), Sp1 was co-immunoprecipitated only when cells were transfected with the KLF6 transactivation domain (lane 3 in ‘IP Flag’). Combined with our previous finding [4], this result suggests that the Sp1–KLF6 interaction requires the DBD of Sp1 and the transactivation domain of KLF6. The specificity of the Flag immunoprecipitation was documented as shown in Figure 5(B), where expression of both KLF6 DBD and ZAD domains can be observed in the total extracts (lanes 1 and 3), but not when the Flag cDNA construct was eliminated from the transfection (lane 2).

We also examined whether KLF6 S1v and S2v interacted with Sp1 by separately transfecting each Flag-tagged KLF6 Sv, using the Flag-tagged WT KLF6 as a positive control and the empty expression vector as a negative control. Flag immunoprecipitates were then subjected to an Sp1 Western blot. As shown in Figure 5(C), both S1v and S2v interacted with Sp1, similarly to WT KLF6, although S2v appeared to do so more efficiently. These data are consistent with binding studies using WT KLF6, since both S1v and S2v retain all or part of the transactivation domain. Moreover, it localizes the binding site between KLF6 and Sp1 to amino acids 1–201, since this is the region shared by both the KLF6 Svs and WT KLF6.

DISCUSSION

Combined, these findings provide insight into the regulation of the endoglin gene, a key target of KLF6 after vascular injury, and define a precise molecular arrangement that involves Sp1 and
Figure 4  For legend see facing page
HEK-293T cells. HEK-293T cells were doubly or triply transfected with KLF6 and Smad3, or KLF6, Smad3 and Sp1 expression vectors respectively. Lane 1 shows the results obtained with double transfection of KLF6 and Smad3, whereas lane 2 displays the triple-transfection results. On the left-hand side, Western blots of total extracts are shown. On the right-hand side the results for the first immunoprecipitation using KLF6 antibody and the second immunoprecipitation with the anti-Flag tag are shown. There is a substantial enrichment of Smad3 in the ternary complex after the second immunoprecipitation, which is dependent on the transfection of Sp1.

Smad3. The results also contribute to our understanding of tumorigenesis, since there is an emerging role of endoglin in neoplasia. Endoglin (CD105) is a homodimeric cell membrane glycoprotein functioning as a TGF-β auxiliary receptor [5]. It plays an important role in vascular development, remodelling and neo-angiogenesis. Endoglin is highly expressed in endothelial cells of the tumour vasculature and at much lower levels in tumour cells [25,26]. There is also evidence supporting its involvement in malignant progression by its direct effect on tumour cells themselves. Thus loss of endoglin expression in cultured human prostate-cancer cells enhances cell migration and invasiveness [27], and endoglin-heterozygous (Eng<sup>+</sup>) mice exhibit accelerated malignant progression during chemically induced skin carcinogenesis in vivo [28]. The latter results suggested that endoglin could act as a suppressor of malignancy in carcinogenesis.

The present results and those obtained previously [4] suggest that KLF6 co-operates with Sp1 in transcriptionally regulating KLF6-responsive genes and that this co-operation is further enhanced by TGF-β1 by at least two mechanisms (Figure 6A). First, in specific cell types, TGF-β1 may decrease KLF6 alternative splicing such that there is a net increase in full-length growth-suppressive KLF6 activity. TGF-β1 may indirectly increase the relative expression of KLF6-regulated genes by favouring the expression of full-length, relative to alternatively spliced, forms of KLF6, the net effect of which might be to inhibit growth. Secondly, KLF6–Sp1 co-operation is further enhanced by the TGF-β1/Smad pathway via the likely formation of a tripartite complex of KLF6, Smad3 and KLF6 in which KLF6 interacts indirectly with Smad3 through Sp1, as uncovered using sequential immunoprecipitation, albeit following triple transfection rather than assessing endogenous protein interactions. Sp1 appears to serve as a bridging molecule in this complex. The physical interaction between Sp1 and KLF6 requires the N-terminal transactivating domain of KLF6 and the C-terminal DBD of Sp1 [4]. On the other hand, Smad3 and Smad3 reportedly make physical contact between the two glutamine-rich transactivating domains of Sp1 and MH1 (MAD homology 1 domain)-binding domain of Smad3 [29].

On the basis of accumulated data, a model of basal and injury-associated (or inflammatory-stimulus-mediated) endoglin gene regulation is proposed, which defines the sequence of events involving KLF6, Sp1 and Smad3 (Figure 6B). Figure 6B depicts the basal transcription of the endoglin gene [20]. It must be pointed out that, in the absence of Sp1 expression, when knocking down Sp1 by infection of endothelial cells with a small interfering RNA lentiviral vector, endoglin transcription is abolished (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/419/bj4190485add.htm).

Upon TGF-β1 stimulation associated with injury, this cytokine signals through its cognate receptors to Smad3/4 (Figure 6C), which then translocates to the nucleus, where they physically interact with Sp1. Smad3/4 also interact with the GTF (general transcription factor) machinery, which is further reinforced through interaction with Sp1, thereby synergizing transcription [20]. Endothelial injury or inflammatory events stimulate de novo synthesis of KLF6 and its translocation to the nucleus (as an early event). In the nucleus, KLF6 interacts with Sp1, enhancing endoglin-gene transcription (Figure 6D) and other KLF6-responsive promoters involved in the TGF-β1 system.
Figure 6  Model of basal and injury-associated endoglin-gene regulation

This model defines the sequence of events involving KLF6, Sp1 and Smad3. (A) As TGF-β increases, the alternative splicing of KLF6 is inhibited, leading to more net unopposed KLF6-mediated growth suppression. Binding of Sp1 and Sp2 to Sp1 suggests a potential mechanism whereby these splice forms may antagonize endoglin-gene expression through cytoplasmic sequestration of this bridging factor, which is critical to maximal endoglin-gene transactivation. (B) Basal transcription of the endoglin gene. On TGF-β stimulation associated with injury and/or inflammation, this cytokine signals through its cognate receptors to Smad3/4 (C), which translocate to the nucleus where they physically interact with Sp1. Smad3/4 also interact with the GTF machinery, thereby synergizing transcription. Endothelial injury or inflammatory events stimulates de novo synthesis of KLF6 and its translocation to the nucleus (as an early event). In the nucleus, KLF6 interacts with Sp1, the binding domain (BD) or C-terminal part of Sp1 with the transactivating domain (AD) of KLF6, enhancing endoglin transcription (D) and other KLF6-responsive promoters involved in the TGF-β system, thereby increasing expression and activation of TGF-β. This may eventually culminate in the formation of the Smad3–Sp1–KLF6 multimeric complex (E) (as a late event), where Sp1 would be the bridge between the KLF6 transactivating domain and the MH1 domain of Smad3.

The present model is not only relevant to injury or inflammatory events, but also in carcinogenesis. Whereas endoglin is a co-receptor of the TGF-β signalling system, and its role in carcinogenesis is the modulation of the TGF-β signal, KLF6 regulates the promoters of endoglin and of TGF-β receptors including TGF-β itself, and is a tumour suppressor by activating cell-cycle regulators, including p21, arresting cell division in a p53-independent manner. The modulation of KLF6 splicing by TGF-β might suggest an additional pathway whereby TGF-β is tumour-suppressive through down-regulation of growth-promoting KLF6 splice forms. On the other hand, TGF-β’s synergistic action on the KLF6–Sp1 co-operation through Smads is also relevant to tumorigenesis, since the endoglin and KLF6 genes are tumour suppressors, and TGF-β is a known modulator of the first stages of carcinogenesis [30]. Taken together, the findings further refine our understanding of the intersecting pathways underlying growth regulation and injury responses by TGF-β, KLF6 and Sp1.

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SUPPLEMENTARY ONLINE DATA

TGF-β regulates the expression of transcription factor KLF6 and its splice variants and promotes co-operative transactivation of common target genes through a Smad3–Sp1–KLF6 interaction

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Figure S1 One-hybrid analysis of Gal4–KLF6 after transient transfections with Sp1 and Smad3 expression vectors

HEK-293T cells were transiently transfected with 0.5 μg of the reporter Gal4 LUC vector and 0.5 μg of each of the expression vectors Gal4–KLF6, Smad3, Sp1, alone or in combinations Gal4–KLF6–Smad3 and Gal4–KLF6–Sp1. The results are expressed as fold induction relative to the LUC activity of the reporter vector alone. The experiments were repeated three times, with three replicates for each transfection. The result shown is representative.

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Figure S2 Interaction between KLF6, Sp1 and Smad3 as measured by FRET

(A) Intensity images (FRET), obtained at an excitation wavelength of 458 nm and an emission wavelength of 523 nm, are shown for the cells co-transfected with combinations of KLF6–ECFP and Sp1 Cter–EYFP (panel a), Smad3–ECFP and Sp1 Cter–EYFP (panel c), KLF6–ECFP and Smad3–EYFP (panel e), KLF6–ECFP and the control EYFP (panel g) and Smad3–ECFP and the control EYFP (panel i). Corresponding bright-field images are also shown (panels b, d, f, h and j). The scale bars represent 3 μm. Regions surrounded by the dotted line indicate the nucleus. (B) Intensity image (donor), obtained at an excitation wavelength of 458 nm and an emission wavelength of 480 nm before (panels a, c, e, g and i) and after (panels b, d, f, h and j) acceptor bleaching are shown for the cells co-transfected with combinations of KLF6–ECFP and Sp1 Cter–EYFP (panels a and b), Smad3–ECFP and Sp1 Cter–EYFP (panels c and d), and KLF6–ECFP and Smad3–EYFP (panels e and f), KLF6–ECFP and the control EYFP (panels g and h) and Smad3–ECFP and the control EYFP (panels i and j). Relative changes obtained by comparisons between before and after acceptor photobleach in each combination were calculated and are presented as percentages under each micrograph. The scale bars represent 3 μm.

Figure S3 Endoglin-gene expression is absolutely dependent on Sp1 expression

Confluent cultures of HUVECs were infected with supernatant containing lentivirus expressing either an Sp1 siRNA (si Sp1) or a random silencer sequence (si Random). RNA was collected from cells after 24 and 48 h infection and the levels of endoglin RNA (RNA ENG after infection, fold reduction versus si Random) were measured by real-time PCR using 18 S RNA as endogenous control.

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