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GATA6 Promotes Angiogenic Function and Survival in Endothelial Cells by Suppression of Autocrine Transforming Growth Factor Beta/Activin Receptor-Like Kinase 5 Signaling

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ABSTRACT

Understanding the transcriptional regulation of angiogenesis could lead to the identification of novel therapeutic targets. We showed here that the transcription factor GATA6 is expressed in different human primary endothelial cells, as well as in vascular endothelial cells of mice *in vivo*. Activation of endothelial cells was associated with GATA6 nuclear translocation, chromatin binding and enhanced GATA6 dependent transcriptional activation. siRNA mediated downregulation of GATA6 after growth factor stimulation led to a dramatically reduced capacity of macro- and microvascular endothelial cells to proliferate, migrate or form capillary-like structures on Matrigel. Adenoviral overexpression of GATA6, in turn, enhanced angiogenic function, especially in cardiac endothelial microvascular cells. Furthermore, GATA6 protected endothelial cells from undergoing apoptosis during growth factor deprivation.

Mechanistically, downregulation of GATA6 in endothelial cells led to increased expression of transforming growth factor (TGF) β1 and TGFβ2, while enhanced GATA6 expression, accordingly, suppressed *Tgfb1* promoter activity. High TGFβ1/β2 expression in GATA6 depleted endothelial cells increased the activation of the activin receptor-like kinase (ALK)5 and SMAD2, and suppression of this signaling axis by TGFβ neutralizing antibody or ALK5 inhibition restored angiogenic function and survival in endothelial cells with reduced GATA6 expression. Together, these findings indicate that GATA6 plays a crucial role for endothelial cell function and survival, at least in part, by suppressing autocrine TGFβ expression and ALK5 dependent signaling.

INTRODUCTION

Angiogenesis describes the sprouting of new capillaries from the endothelial cells of post-capillary venules (1,2). It is one of the central processes that controls and enables embryonic and fetal development and that is required to maintain functional and structural integrity of the organism in postnatal life. There are many circumstances in the adult organism (for example organ ischemia or tumor growth), under which the normally quiescent endothelial cells become activated by growth factors (like VEGF-A, basic fibroblast growth factor, epidermal growth factor) or hypoxia to proliferate, migrate and to interact with each other to form new capillaries (1-3). While on the one hand research efforts are under way to therapeutically enhance angiogenesis during organ ischemia, on the other hand angiogenesis inhibitors are being developed for cancer therapy (4,5). In order to find effective treatment strategies, a detailed understanding of the regulatory signaling pathways that control activation and quiescence of endothelial cells is needed. While a lot of different growth factors and their receptors have been identified that either facilitate or block angiogenesis, its transcriptional regulation is far less well studied (6). Here we examined the role of the transcription factor GATA6 for angiogenic function and survival in endothelial cells. GATA6 belongs to the family of GATA proteins, of which 6 GATA factors have been identified. All GATA factors share a conserved DNA binding domain consisting of two zinc finger motifs that mediate binding to the consensus DNA site (A/T)GATA(A/G) (7). During mouse embryonic and fetal development GATA6 is widely expressed in different cells and tissues (7). *Gata6* null mice die between embryonic day 5.5-7.5 due to defects in visceral endoderm function and extraembryonic development (8,9). When these defects were overcome by either tetraploid embryo complementation or by cell specific elimination of GATA6 only in smooth muscle/neural crest cells, important functions of GATA6 *in vivo* were demonstrated for liver differentiation and growth, or morphogenetic patterning of the cardiac outflow tract, respectively (10,11). In the adult mouse, GATA6 expression is detectable in many organs, including the heart, aorta, stomach, and in vascular smooth muscle cells (12). Functionally, GATA6 is critically involved in lung epithelial regeneration and the regulation of vascular smooth muscle cell proliferation and function in the adult mouse (13-15). Interestingly, primary human umbilical vein endothelial cells (HUVECs) express high levels of GATA6, which regulates the expression of the vascular cell adhesion molecule-1 (VCAM-1) in these cells (16). Other important endothelial cell genes like *NOS3* (encoding for the endothelial nitric oxide synthase), *PECAM1* (encoding for the PECAM1 protein) and *EDN1* (encoding for endothelin-1) are also targets of GATA transcription factors (17-20). However, the functional importance of GATA6 for angiogenic function and survival of endothelial cells is currently unknown.

In this study we demonstrated that GATA6 is crucial for the promotion of endothelial cell

function and survival, at least in part, by suppressing autocrine release of TGFβ1 and TGFβ2 that both act as angiogenesis inhibitory molecules.

Experimental Procedures

Cell culture and reagents- Human umbilical vein endothelial cells (HUVECs), human umbilical arterial endothelial cells (HUAECs) and human cardiac microvascular endothelial cells (HCMECs) were purchased from PromoCell. HUVECs and HUAECs were cultured in Endothelial Cell Growth Medium (PromoCell) containing a growth factor cocktail with ECGS/H 0.4% (sterile-filtered, aqueous extract from mixed-sex bovine hypothalamic tissue), FCS (fetal calf serum) 2%, EGF (epidermal growth factor) 0.1 ng/ml, hydrocortisone 1 µg/ml, bFGF (basic fibroblast growth factor) 1 ng/ml. HCMECs were cultured in Endothelial Cell Growth Medium MV for microvascular endothelial cells (PromoCell) containing a growth factor cocktail with ECGS/H 0.4%, FCS 5%, EGF 10 ng/ml, hydrocortisone 1 µg/ml. Endothelial cells were used for experiments at passage 2. The rat heart endothelial cell line (RHE-A) was cultured in DMEM containing 10% FCS.

The pan-specific TGFβ blocking antibody (AB-100-NA) was purchased from R&D systems, recombinant human TGFβ1 was purchased from Cell Signaling and SB-431542 (ALK5 inhibitor) was purchased from Sigma.

RNA in situ hybridization- This analysis was performed on 10 µm paraffin sections following a standard procedure with digoxigenin-labeled antisense riboprobes against GATA6 and Semaphorin 7A (Genbank: NM_011352, as negative control, because no expression could be detected in heart or kidney with this method) (21).

Western blotting and immunostaining- Western blots were performed using the following antibodies: GATA2 (Santa Cruz), GATA3 (ProteinTech), GATA6 (R&D Systems), ACTIN (Sigma), phospho-SMAD1/5, phospho-SMAD2, SMAD2 and SMAD1/5 (Cell Signaling). Immunostaining for GATA6 was performed after fixation of HUVECs with 100% ethanol by overnight incubation with GATA6 antibody (R&D Systems) in 5% BSA-PBS, before an ALEXA 568 coupled secondary antibody (Invitrogen) was applied.

ChIP assay- The chromatin-immunoprecipitation (ChIP) assays were performed according to manufacturer's directions (ChIP Assay kit, Upstate). GATA6 antibodies (R&D Systems) and normal goat IgG (Santa Cruz) were used for immunoprecipitation. PCR was performed with the following primers: for *PECAM1* promoter, forward, 5'-AGAACGCCAAGGCAAATGT-3', reverse, 5'-CTGGAAACCGGGAACAATG-3'; for *EDN1* promoter, forward, 5'-GGCGTCTGCCTCTGAAGTTA-3', reverse, 5'-CCAGCCCCAGACAATGTTAT-3'; and for *NOS3* promoter, forward, 5'-GGCTCTGCTGGACACCTG-3', reverse, 5'-AGGGGGCTCTCCAGTGCT-3'.

siRNA transfection- HUVECs or HCMECs were transfected with either GATA6-specific siRNA duplex (SASI_Hs01_00123992, SASI_Hs01_00123992_AS, Sigma) or control siRNA (Ambion) using the GeneTrans II transfection reagent (MoBiTec) according to the manufacturer's protocol. After 48 hours of incubation in growth factor cocktail containing medium, cells were used either for functional analysis or RNA/protein preparation.

Adenoviral infection- To overexpress GATA6, endothelial cells were infected at 37°C for two hours either with an adenovirus that expresses GATA6 under the control of the constitutive CMV promoter (Ad.GATA6, 5 MOI) or a control adenovirus, which expresses β-galactosidase also under the control of the CMV promoter (Ad.βgal, 5 MOI) (22). The cells were then incubated for up to 48 hours in growth factor cocktail containing medium.

Proliferation assay- DNA synthesis was measured as BrdU incorporation with a commercially available kit (Roche). Cells were plated on gelatinized 96-well plates at 8000 cells/well in the presence of BrdU. After 24 hours, BrdU incorporation was determined by ELISA using an anti-BrdU antibody.

In vitro angiogenesis assay- The formation of vessel (or capillary)-like structures was assessed on growth factor-reduced Matrigel (BD Biosciences) in 96-well plates. For this procedure, endothelial cells were plated on Matrigel at a density of 10,000 cells per well. After 24 hours, tube formation (number of closed circular structures/high-powered field at a magnification of 50x) was quantified.

Migration assay- Endothelial cell migration was analyzed in 6.5mm cell culture transwell inserts (8µm pore size, Costar). 50,000 cells were added in the upper chamber in medium without growth factor cocktail, while the lower chamber contained medium with added growth factor cocktail. After 24 hours, the migrated cells that attached at the bottom of the lower chamber were counted. The data are expressed as the mean number of migrated cells per high power field.

Cell death assays- Cell death was induced by growth factor deprivation. Cells were seeded onto gelatinized 6-well-plates at a density of 150,000 cells/well and cultured in growth factor cocktail containing medium for 5 hours. The cells were then washed with PBS and incubated for 17 hours in medium without serum or growth factors. The number of surviving cells was determined microscopically. Furthermore, the adherent cells were collected and stained with FITC-conjugated annexin-V and 7AAD (7-amino-actinomycinD) with the use of a commercially available kit (BD Pharmingen). Thereafter, samples were analysed by flow cytometry (FACS, Becton Dickinson) for viable (annexin-V-negative and 7AAD -negative), early apoptotic (annexin-V-positive, 7AAD -negative), and late apoptotic/necrotic (annexin-V-positive and 7AAD -positive) cells as described previously by others (23).

Agilent Microarray- For gene expression profiling, the Human Gene expression 4x 44k v2 kit from Agilent was used. 200ng of total RNA was transcribed into cDNA, amplified using T7 RNA polymerase while incorporating cyanine 3-labeled CTP and then hybridized according to the manufacturer´s protocol (Quick Amp, Agilent). Signal intensities were extracted from scan images using Feature Extraction Software v10.7.3.1. GeneSpring GX11 (Agilent) was used for further statistical analysis. Genes with significant expression differences between siRNA control versus siRNA GATA6 treatment were selected based on the assumption of equally distributed upregulated and downregulated genes using unpaired student´s t-test with a P value cut-off of 0.05 and an average fold change ≥ 2.

Real-time PCR- Total RNA was isolated from cells using NucleoSpin RNA Kit (Macherey-Nagel). cDNA was then synthesized from 2 µg of total RNA with random hexamer priming and Super Script III (Invitrogen) at 50°C for 50 min and forwarded to amplification with a specific primer sets using SYBR-green technology (Invitrogen) and the Stratagene Mx3005P PCR cycler. The sequences of the primers used in real-time PCR in this study are listed in Supplemental Table 1.

Luciferase assay- Cells were transiently transfected with 1.5 µg DNA/well of two different mouse *Tgfb1* promoter constructs -406 bp and -1079 bp (kindly provided by Dr. Naoko Nakano, Tokyo, Japan) (24). To express GATA6, 1.5 µg DNA of either GATA6 expressing construct or empty control vector were co-transfected with GeneTransII transfection reagent (MoBiTech). 48 hours after transfection, cells were harvested and luciferase activity was measured as previously described (25). A construct, in which 3 consecutive GATA binding sites were fused to a luciferase expression cassette downstream of a minimal metallothionein promoter (this construct was kindly provided by Licio Collavin and Claudio Santoro) (26), was cloned into the pshuttle vector, and an adenovirus (Ad.GATA-Luc) was generated with the Adeasy adenoviral system kit (Stratagene) according to the manufacturer´s protocol. HUVECs infected with this adenovirus were either treated with control siRNA or GATA6 siRNA or were exposed to growth factor containing medium or hypoxia as indicated, before luciferase activity was determined.

Statistical Analysis- All values are presented as mean ± SEM. 1-way ANOVA followed by Student-Newman-Keuls post-hoc test was used to analyze differences between 3 or more groups; the unpaired student´s t-test was used to evaluate differences between 2 groups. A 2-tailed P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

GATA6 expression in vascular endothelial cells in vitro and in vivo. We started our analysis by determining the expression level of GATA6 mRNA and protein in different kinds of primary human endothelial cells. Using quantitative RT-PCR, we detected GATA6 mRNA expression in venous (HUVECs), arterial (HUAECs) and also cardiac microvascular endothelial cells (HCMECs) (Figure 1A). Western blot analysis detected GATA6 protein in these cells, with levels paralleling the differences of the mRNA expression (Figure 1B). In order to

investigate whether GATA6 is also expressed in vascular endothelial cells in the whole organism *in vivo*, we analyzed mouse tissue for GATA6 mRNA by *in situ* hybridization. Importantly, endothelial GATA6 expression was clearly detectable in the vasculature of different organs including the heart and kidney (Figure 1C).

GATA6 activation by growth factors and hypoxia in endothelial cells. Because angiogenic activity in endothelial cells is induced by a combination of growth factors and also by hypoxia, we tested whether GATA6 could be activated by these stimuli in HUVECs. GATA6 mRNA and protein expression levels were not influenced by hypoxia or growth factor stimulation (stimulation for 6 or 24 hours, data not shown). However, exposure to hypoxia or a growth factor cocktail for 4 hours induced a strong nuclear translocation of GATA6 protein in HUVECs (Figure 2A).

In order to analyze whether this enhanced nuclear translocation was associated with increased chromatin binding by GATA6, we immunoprecipitated fragmented chromatin of stimulated or unstimulated HUVECs with anti-GATA6 IgG or control IgG (ChIP assay) and subsequently examined the immunoprecipitates for the presence of well characterized GATA binding sites in promoter regions of known endothelial GATA target genes (*NOS3*, *PECAM1* and *EDN1*). As depicted in Figure 2B, GATA site promoter DNA was selectively detected in growth factor stimulated endothelial cells (but not in unstimulated cells) after chromatin immunoprecipitation with anti-GATA6 IgG, but not with unspecific IgG. Interestingly, hypoxia did not induce increased GATA6 DNA binding, at least not to the examined promoter DNA of the *NOS3*, *PECAM1* and *EDN1* gene (data not shown).

We next wanted to analyze whether the capability of endothelial GATA6 to activate transcription is also modulated by growth factors or hypoxia. For this purpose, we generated an adenoviral vector encoding an artificial promoter, in which luciferase expression is driven by 3 consecutive GATA binding sites (Ad.GATA-Luc, Figure 2C) (26). In order to be able to dissect GATA6 dependent GATA factor activity from that of the other endothelial cell GATA factors (mostly GATA2 and GATA3, data not shown) we sought to specifically downregulate GATA6 in HUVECs by an siRNA approach. As demonstrated in Figure 2D, GATA6 mRNA expression was reduced by about 80% in HUVECs treated with the GATA6 siRNA compared to HUVECs treated with control siRNA. Western blot analysis revealed a similar downregulation of GATA6 protein in GATA6 siRNA treated HUVECs (Figure 2E). Importantly, the expression of the other endothelial GATA factors (GATA2 and GATA3) was not affected by the GATA6 specific siRNA oligonucleotide (Figure 2E). As shown in Figure 2F, GATA dependent luciferase activity in HUVECs treated with control siRNA was dramatically enhanced by growth factor cocktail stimulation and also mildly increased by hypoxia. Downregulation of GATA6 by siRNA led to a markedly reduced GATA dependent luciferase activity after growth factor or hypoxia stimulation, indicating that the increased GATA transcriptional activity under these circumstances was mainly due to GATA6 (Figure 2F).

In summary, GATA6 translocated to the nucleus and activated transcription in endothelial cells that were exposed to either growth factors or hypoxia. While growth factor stimulation also induced direct DNA binding of GATA6, this was not evident after hypoxia stimulation.

GATA6 is necessary for angiogenic function in endothelial cells. Because GATA6 was activated in response to growth factor stimulation in endothelial cells, we wanted to assess the role of GATA6 in the growth factor response in these cells. During angiogenesis, endothelial cells are stimulated to proliferate, migrate and to form capillary-like tube structures. In order to assess the functional role of GATA6 in this program, we analyzed the ability of HUVECs and HCMECs to proliferate, migrate and to form capillary/vessel-like structures on Matrigel after growth factor cocktail stimulation. Downregulation of GATA6 (by siRNA) in HUVECs and HCMECs led to a dramatically reduced proliferative response compared to control siRNA treated cells (Figure 3A). Moreover, the ability of the endothelial cells (HUVEC and HCMECs) to form vessel-like structures on Matrigel was almost completely abrogated by the reduction in GATA6 (Figure 3B and 3C). Finally, the capacity of the HUVECs and HCMECs to migrate towards a growth factor cocktail gradient in a cell culture transwell system was significantly blunted in HUVECs and HCMECs with diminished GATA6 expression (Figure 3D).

In order to test whether the functional defects observed in the siRNA GATA6 treated endothelial cells were indeed the consequence of

reduced GATA6 expression, we infected siRNA GATA6 treated HUVECs with an adenovirus expressing mouse GATA6, which could not be targeted by the siRNA directed against human GATA6. Figure 3E shows that GATA6 protein levels in HUVECs with silenced endogenous GATA6 expression could indeed be restored by adenoviral expression of mouse GATA6. As demonstrated in Figure 3F and Supplemental Figure 1, the functional defects in GATA6 siRNA treated cells (proliferation, vessel-like structure formation and migration) could be rescued by the restoration of GATA6 expression through Ad.GATA6, but not by a control adenovirus expressing β-galactosidase (Ad.βgal).

Enhancement of angiogenic function in cardiac microvascular endothelial cells by GATA6 overexpression. We next wished to analyze whether augmented GATA6 expression could promote the angiogenic program in endothelial cells. For this purpose, we infected HUVECs and HCMECs with either a GATA6 overexpressing adenovirus (Ad.GATA6) or a β-galactosidase (Ad.βgal) expressing control adenovirus. Western blot and immunofluorescence staining revealed GATA6 nuclear overexpression in the Ad.GATA6 treated cells (Figure 4A and B). While enhanced GATA6 expression only modestly enhanced cell migration without affecting vessel-like structure formation in HUVECs, the angiogenic program (vessel-like structure formation and cell migration) was robustly augmented in cardiac microvascular endothelial cells (HCMECs, Figure 4C, D).

GATA6 is necessary for the maintenance of endothelial cell viability. We then wanted to examine whether GATA6 is also necessary for endothelial cell survival. As demonstrated in Figure 5A, cell survival was not impaired in HUVECs with reduced GATA6 expression when the cells were cultured in the presence of growth factors. In agreement with these results, direct staining of these endothelial cells with annexin-V and 7-amino-actinomycinD (7AAD) and subsequent FACS analysis revealed similar level of early apoptotic (annexin-V positive, 7AAD negative, lower right field) and late apoptotic/necrotic cells (annexin-V positive, 7AAD positive, upper right field) in HUVECs with or without GATA6 downregulation (Figure 5B).

We next asked whether reduced GATA6 expression might predispose HUVECs to cell death during the exposure of external stress (like for example growth factor withdrawal). In order to test this, we withdrew growth factor cocktail from HUVECs for 17 hours and subsequently quantified the total number of surviving cells. It is shown in Figure 5C that far fewer cells survived growth factor withdrawal when GATA6 was downregulated. In order to elucidate whether the endothelial cells with reduced GATA6 expression died either by apoptosis or necrosis, we stained HUVECs after 17 hours of growth factor cocktail withdrawal with annexin-V and 7AAD and conducted a FACS analysis. We found that the amount of early apoptotic cells was more than doubled in the siRNA GATA6 treated cells (Figure 5D), while there was only a mild increase in late apoptotic/necrotic cells. This enhanced cell death upon growth factor withdrawal and treatment with GATA6 siRNA could be overcome by adenoviral expression of mouse GATA6, indicating that downregulation of GATA6 was indeed the reason for exaggerated cell death in the siRNA GATA6 treated cells (Supplemental Figure 2).

Suppression of TGFβ1 and TGFβ2 expression in endothelial cells by GATA6. In order to obtain mechanistic insight into how GATA6 modulates endothelial function and survival, we performed transcriptome profiling in siRNA control (Co) and siRNA GATA6 (G6) treated HUVECs employing Agilent microarrays. Two microarrays from independent samples were analyzed per condition. As demonstrated in the heat map view, downregulation of GATA6 by siRNA resulted in a significant at least twofold up- or downregulation of 1145 genes versus siRNA control treated cells (Figure 6A). Genes from multiple different functional categories (for example genes related to cellular metabolism, communication, but also cell biogenesis, proliferation, cell cycle, gene regulation and cell death) were affected by reduced GATA6 expression (data not shown). The entirety of regulated genes (at least twofold) is listed in a Supplemental Microsoft Excel Table. The expression of 20 genes that were found regulated at least by twofold in the array, known angiogenesis regulators (*VEGFA*, *ANGPT2* [angiopoietin-2], *HIF1A, FGFR1* and *AKT1*), or known endothelial GATA factor target genes (*NOS3*, *PECAM1* and *EDN1*) was also measured by quantitative real-time PCR (Figure 6B). Although angiogenic function was inhibited in HUVECs that were treated with GATA6 specific siRNA, the expression of some

angiogenesis promoting genes (*PECAM1*, *EDN1*, *IL11*, *PDGFA* and *HIF3A*) was significantly upregulated in these cells, while others were not significantly altered in their expression (*VEGFA*, *ANGPT2, NOS3, ID1* [inhibitor of DNA binding-1], *GHR* [growth hormone receptor], *FGFR1*, *AKT1* and *HIF1A*). Expression of the receptor of the angiopoietins, *TIE2*, was slightly, but significantly downregulated (by 40%) in HUVECs with silenced GATA6 expression. Besides these changes in the expression levels of proangiogenic genes, an unexpected pattern of gene regulation emerged related to the TGFβ signaling pathway: Both *TGFB1* (3-fold) and *TGFB2* (5-fold) were strongly upregulated in HUVECs with reduced GATA6 levels, as was *SMAD2* (1.6-fold) (an intracellular signaling protein downstream of the TGFβ receptor) and genes that are known to be induced upon TGFβ stimulation of cells (*TFPI2* [tissue factor pathway inhibitor 2], *FN1* [fibronectin-1] and *SERPINE1* [PAI-1, plasminogen activator inhibitor 1]) (27,28). Interestingly, it has previously been demonstrated that high levels of TGFβ inhibit angiogenic function and survival in endothelial cells, similarly to what we observed here upon downregulation of GATA6 (29). We hypothesized therefore that expression of TGFβ1 and TGFβ2 is normally suppressed by GATA6 to promote a pro-angiogenic state. In order to analyze whether GATA6 could be able to directly influence the activity of the *Tgfb1* gene promoter, we cotransfected a luciferase coupled mouse *Tgfb1* promoter construct together with a GATA6 over-expressing plasmid or empty plasmid into rat heart endothelial cells. As demonstrated in Figure 6C, the *Tgfb1* promoter (-1079 bp fragment containing a GATA binding site at -821 bp) was markedly suppressed by concomitant GATA6 expression. In contrast, a short *Tgfb1* promoter fragment (-406bp), not containing a GATA site, was not suppressed by GATA6 expression (Figure 6C). A schematic representation of the promoter constructs is depicted above the panel in Figure 6C.

In summary, GATA6 suppressed TGFβ expression in endothelial cells through direct effects on the gene promoter.

GATA6 enhances endothelial cell function and survival by shifting the balance between ALK5 and ALK1 activation towards ALK1. Because we found highly elevated expression of TGFβ1 and TGFβ2 in the HUVECs with downregulation of GATA6 and because it is known that high levels of TGFβ inhibit endothelial cell function and survival (29,30), we hypothesized that the high TGFβ levels were critical for the impaired angiogenic function and survival in GATA6 depleted endothelial cells. In order to test this hypothesis, we cultured HUVECs that were treated with either control or GATA6 specific siRNA in the presence of a TGFβ neutralizing antibody (targeting both TGFβ1 and TGFβ2) or control IgG. As shown in Figure 7A, the TGFβ neutralizing antibody indeed partially rescued the deleterious effects of GATA6 deficiency on endothelial cell function (proliferation and vessel-like structure formation), indicating the functional importance of the high TGFβ levels under these circumstances. It has been reported that the dose dependent effects of TGFβ on endothelial cells reflect the activation of different type I receptors: While low doses of TGFβ1 induce activation of ALK1, which subsequently phosphorylates SMAD1/5 proteins and *activates* angiogenic function and survival, high TGFβ doses activate ALK5, which phosphorylates SMAD2 and *inhibits* angiogenic function and survival of endothelial cells (28,30). Therefore, we hypothesized that ALK5 might be activated in the GATA6 depleted HUVECs. Since ALK5 activation specifically leads to SMAD2 phophorylation, we analyzed SMAD2 phosphorylation in GATA6 depleted HUVECs by Western blot with a phospho-SMAD2 specific antibody. As demonstrated in Figure 7B, SMAD2 phosphorylation indeed specifically occurred in HUVECs with reduced GATA6 expression. Inhibition of ALK5 by the specific compound SB-431542 abolished SMAD2 phophorylation in HUVECs treated with GATA6 siRNA (Figure 7B). In turn, downregulation of GATA6 or treatment with the ALK5 inhibitor did not influence SMAD1/5 phosphorylation (as a marker for ALK1 activation). A similar pattern with increased SMAD2 phosphorylation, but without an effect on SMAD1/5 phosphorylation was observed when recombinant TGFβ1 (at 0.05 ng/ml; no effects were observed with 0.005 ng/ml) was added to HUVECs for 90 minutes (Figure 7C). This confirmed TGFβ1 as a pontent inducer of SMAD2 phophorylation in endothelial cells.

Interestingly, ALK5 inhibition by SB-431542 also rescued the functional defects (cell proliferation, vessel-like structure formation, cell migration) and enhanced cell survival in HUVECs with reduced abundance of GATA6 (Figure 7D). Accordingly, our data demonstrate that GATA6 usually suppresses the expression of TGFβ1 and TGFβ2, consequently leading to inhibition of ALK5

and SMAD2 activation and thereby enhancing angiogenic function and survival in endothelial cells (Figure 7E).

DISCUSSION

In this study we identified GATA6 as an important transcriptional regulator in vascular endothelial cells that enables angiogenic function and endothelial cell survival.

GATA6 was shown to be first induced during embryonic endothelial cell development in the hemangioblast, which is the common progenitor of endothelial and hematopoetic cells (31). Our analysis revealed GATA6 mRNA and protein expression in various fully differentiated primary human endothelial cells of venous, arterial or cardiac microvascular origin. Importantly, we were also able to demonstrate endothelial GATA6 expression in the mouse vasculature *in vivo*. While that had never been shown before, GATA6 expression in HUVECs was previously reported. In relation to the other endothelial expressed GATA factors GATA2 and GATA3, one study showed GATA6 to be the highest expressed among the three, while the other found it as the lowest in HUVECs (16,32). We detected GATA6, GATA3 and GATA2 at comparable levels in HUVECs, HUAECs and HCMECs (data not shown). Interestingly, only GATA2 and GATA6, but not GATA3 was previously demonstrated to be induced in the hemangioblast (31).

Endothelial cells in the adult organism are quiescent, but become activated in response to growth factors that are released from adjacent cells or by hypoxia that emerges within a tissue for example after blockage of a major artery. Activated endothelial cells engage in angiogenesis by proliferating, migrating towards a growth factor gradient and by forming an immature capillary network (1-3). Because we hypothesized that GATA6 might functionally play a role during the early activation phase of angiogenesis, we assessed GATA6 activation after stimulation with growth factors or hypoxia. Indeed, only 4 hours of stimulation led to dramatically enhanced GATA6 nuclear translocation and DNA binding (the latter only in the case of growth factor stimulation) and was associated with increased transcriptional activity of GATA factors as measured by a GATA-dependent luciferase reporter assay. It should be noted, that total cellular GATA activity was measured in this assay, including beside GATA6, the activity of GATA3 and especially GATA2, for which enhanced nuclear translocation after growth factor stimulation had also been demonstrated in endothelial cells (33). Still, when GATA6 expression was reduced by a specific siRNA, the GATA dependent transcriptional activity was diminished by about 70% after stimulation, indicating that GATA6 is a major contributor to GATA activity in HUVECs under these circumstances.

In line with its rapid activation, we found that GATA6 plays an essential role for angiogenic function: HUVECs or HCMECs with reduced GATA6 abundance were substantially impaired in their ability to proliferate, form capillary/vessel-like structures or to migrate upon growth factor exposure. GATA6 overexpression, in turn, promoted endothelial cell function especially in cardiac microvascular endothelial cells. In addition, the presence of GATA6 protected against endothelial cell apoptosis after withdrawal of growth factors. Interestingly, similar functions, at least for capillary/vessel-like structure formation have also been described for GATA2 and GATA3 in endothelial cells, indicating overlapping function of these GATA factors (32,33). It should be noted, however, that there appears to be a major difference concerning their specific target genes (32,33). Although GATA6 downregulation also slightly reduced TIE2 expression, most of the transcriptional changes reported to occur upon GATA3 reduction were not found after GATA6 downregulation (in the case of PAI-1 [encoded by the *SERPINE1* gene], for example, regulation even goes in opposite directions: GATA3 downregulation leads to suppression of PAI-1 expression, but downregulation of GATA6 in our study led to a strong upregulation of PAI-1) (32). While VEGFR2 was reported as a major target of GATA2 in endothelial cells, neither GATA3 nor GATA6 appear to regulate it (33). Of note, unlike what we and others have previously reported for GATA4 in cardiac myocytes, GATA6 did not influence the expression of VEGF-A (25,34). In contrast, we found that GATA6 suppresses the expression of TGFβ1 and TGFβ2, which were not at all found as targets of GATA3 (32). It is thought that different protein interaction partners or the DNA sequence flanking the core GATA binding element could determine selectivity for specific promoter binding sites among the different GATA proteins, when multiple GATA factors are expressed in a certain cell type (7,35). We demonstrated here that GATA6 can directly suppress the *Tgfb1* promoter in

endothelial cells, and accordingly, suppression of this promoter had been previously shown to be mediated by the GATA binding site at -821bp in antigen stimulated T-cells (24).

TGFβ is generally regarded as an inhibitor of angiogenic function and survival in endothelial cells (29); however, low doses of this cytokine – in contrast – can even stimulate angiogenesis (36,37). These dose dependent effects of TGFβ are the consequences of specialized intracellular signaling in endothelial cells: TGFβ binds to a heterotrimeric complex of type I (also known as activin receptor-like kinase, ALK) and type II serine/threonine kinase receptors at the cell membrane (30). While in most cells, the TGFβ signal is propagated within the cell by ALK5, endothelial cells also express ALK1 (30). ALK1 appears to enhance the endothelial cell function in response to low TGFβ concentrations through activation of SMAD1/5 and induction of proangiogenic target genes like *ID1* (28,38). ALK5, in contrast, becomes activated within endothelial cells by high TGFβ doses and inhibits endothelial cell function and survival via SMAD2 activation and induction of anti-angiogenic target genes like *SERPINE1* (28,38). While regardless of the presence or absence of GATA6, ALK1 appears to be similarly activated in HUVECs (as reflected by SMAD1/5 activation and mild, but insignificant upregulation of *ID1*), downregulation of GATA6 expression dramatically increased ALK5 activation as evident by induction of SMAD2 phosphorylation and increased expression of the *SERPINE1* gene. Because inhibition of TGFβ or ALK5 rescued endothelial cell function and survival in HUVECs with reduced GATA6, we conclude that GATA6 directs endothelial cell function and survival, at least in part, by modulation of the intricate balance between ALK5 and ALK1 dependent signaling. While previously the dose of *exogenous* TGFβ was implicated as a regulator of ALK5 or ALK1 predominance, we identified an *endogenous* GATA6 dependent pathway that allows endothelial cells to independently regulate autocrine TGFβ release and fine tuning of the ALK5/ALK1 balance in order to sustain endothelial cell function and survival (28).

In summary, we identified a previously unknown critical pro-angiogenic role of the transcription factor GATA6 in endothelial cells, which might render it an interesting target for angiogenesis related therapies in the future.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. GATA6 expression in endothelial cells. A. Quantitative real-time PCR analysis of GATA6 mRNA in human umbilical vein endothelial cells (HUVECs), human umbilical arterial endothelial cells (HUAECs) and human cardiac microvascular endothelial cells (HCMECs). The results are representative of three independent experiments (each with n=3 per cell type). **B.** Representative Western blot for GATA6 from HUVEC, HUAEC and HCMEC total cell lysates. ACTIN was used as a loading control. **C.** In situ hybridization of mouse heart and kidney sections stained for GATA6 mRNA (blue). Specific expression of GATA6 in endothelial cells is indicated by arrow. GATA6 expression was also detected in the adjacent myocardium and in smooth muscle cells. Staining for *Semaphorin 7A* was used as a negative control. Samples were visualized using an Axiovert 200M microscope (Carl Zeiss) with a 40x/ 0.75 lense (Carl Zeiss).

Figure 2. GATA6 activation in endothelial cells. A. Immunofluorescence staining for GATA6 (red) in HUVECs that were unstimulated or stimulated for 4 hours either with growth factor cocktail or hypoxia. Samples were visualized using an Axiovert 200M microscope (Carl Zeiss) with a 40x/ 0.75 lense (Carl Zeiss). The results are representative of three independent experiments. **B.** Chromatin immunoprecipitation (ChIP) assay in unstimulated or growth factor stimulated HUVECs. The immunoprecipitation (IP) was performed with GATA6 antibodies or a nonspecific IgG; recovered DNA as well as total input DNA (Input) were analyzed by PCR using primers that bind in the 5´prime promoter region of the *PECAM1*, *EDN1* or *NOS3* genes, respectively. The results are representative of three independent experiments. **C.** Schematic illustration of the GATA-luciferase (Ad.GATA-Luc) construct that was adenovirally delivered for reporter assays to measure total GATA dependent transcriptional activity. **D.** Real-time PCR analysis of GATA6 mRNA in HUVECs transfected with a control siRNA or siRNA against GATA6. Expression in cells transfected with a control siRNA was used as reference and set to 1. The results are representative of three independent experiments (with n=2-3 per condition in each). **E.** Representative Western blot analysis of GATA2, 3 and 6 protein abundance in HUVECs transfected with a control siRNA or siRNA against GATA6. ACTIN was used as a loading control. **F.** Luciferase activity in HUVECs that were infected with Ad.GATA-Luc and transfected either with a control (Co) siRNA or a siRNA against GATA6 (G6) and that either remained unstimulated (Unstim) or were stimulated with growth factor cocktail (GF) or hypoxia. Results from a respresentative experiment are shown (n=3 per condition). The experiment was repeated twice with similar results.*$p<0.01$ versus siRNA control, unstimulated; #$p<0.01$ versus siRNA control with growth factor stimulation; §$p<0.05$ versus siRNA control with hypoxia stimulation.

Figure 3. GATA6 is essential for endothelial cell function. A. Endothelial cell proliferation (assessed by BrdU incorporation with a spectrophotometric absorbance assay) in HUVECs or HCMECs transfected with a control siRNA or siRNA against GATA6. **B.** Quantification and representative images (**C**) of vessel-like structure formation on Matrigel in HUVECs and HCMECs when treated with a control siRNA or siRNA against GATA6. HUVECs were visualized using an Axiovert 200M microscope (Carl Zeiss) with a 5x/ 0.12 lense (Carl Zeiss). **D.** Migration assay (assessed the amount of cells that successfully migrated along a growth factor gradient) with HUVECs or HCMECs transfected with a control siRNA or siRNA against GATA6. **E.** Western blot for GATA6 and ACTIN (Loading Control) in HUVECs, in which GATA6 protein in GATA6 siRNA treated cells was restored using adenoviral GATA6 (Ad.GATA6) expression. The β-galactosidase expressing adenovirus (Ad.βgal) was used as control. **F.** Endothelial function was assessed by proliferation, vessel-like structure formation and migration assays in cells that were treated like described under **E**. For **A, B, D, F:** Data from a representative experiment are shown (n=3-5 per condition). The experiment was repeated twice with similar results. *$p<0.05$ versus siRNA Control; #$p<0.05$ versus siRNA-GATA6 + Ad.GATA6.

Figure 4. GATA6 overexpression enhances endothelial cell function. A. Representative Western blot analysis of GATA6 expression in HUVECs infected with an adenovirus expressing GATA6 (Ad.GATA6) or β-galactosidase (Ad.βgal). ACTIN was used as a loading control. **B.** Immunofluorescence staining for GATA6 (red) in HUVECs infected with an adenovirus expressing GATA6 (Ad.GATA6) or β-galactosidase (Ad.βgal). Samples were visualized using an Axiovert 200M microscope (Carl Zeiss) with a 40x/ 0.75 lense (Carl Zeiss). **C-D.** Quantification of vessel-like structure formation **(C)** and migration **(D)** in HUVECs and HCMECs 48 h after infection with Ad.βgal or Ad.GATA6. Data from a representative experiment are shown (n=3 per condition). The experiment was repeated twice with similar results.*p<0.05 versus Ad.βgal.

Figure 5. GATA6 is necessary to maintain viability in endothelial cells. A. Quantification of viable (surviving) HUVECs 48 hours after treatment with a control siRNA or siRNA against GATA6. The cells were kept in growth factor cocktail containing medium. Data from a representative experiment are shown (n=2-3 per condition). The experiment was repeated twice with similar results. **B.** Flow cytometric analysis of HUVECs treated as described under **A.** and stained with annexin-V and 7AAD. The numbers in the lower-right quadrant of the plots indicate the number of annexin-V positive cells (early apoptotic cells), whereas in the upper-right quadrant the number of cells staining positive for both annexin-V and 7AAD (late apoptotic/necrotic cells) is indicated. Data from a representative experiment are shown; the experiment was repeated twice with similar results. **C.** Quantification of viable (surviving) HUVECs 17 hours after growth factor withdrawal was started and when treated with a control siRNA or siRNA against GATA6. Data from a representative experiment are shown (n=3 per condition). The experiment was repeated twice with similar results. *p<0.01 versus siRNA control. **D.** Flow cytometric analysis of HUVECs stained with annexin-V and 7AAD 17 hours after growth factor withdrawal was started. Data from a representative experiment are shown; the experiment was repeated twice with similar results.

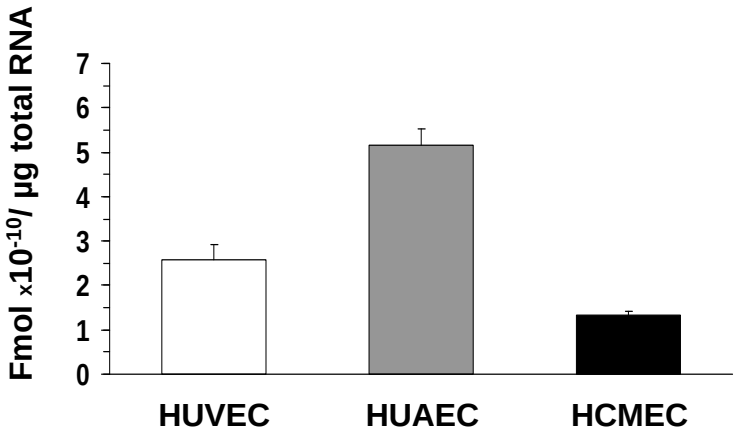
Figure 6. GATA6 suppresses TGFβ in endothelial cells. A. RNA isolated from HUVECs treated with a control (Co) siRNA (columns 1 and 2) or siRNA against GATA6 (G6, columns 3 and 4) was subjected to gene expression array analysis. The heat map plots represent the expression level of genes: green – downregulation, red – upregulation relative to the control (black). **B.** Quantitative real-time PCR analysis of the indicated genes in HUVECs that were regulated as indicated in the cells treated with GATA6 siRNA versus control siRNA. Expression level in cells transfected with a control siRNA was used as reference and set to 1. The fold increase in mRNA level is indicated. Data from at least two independent experiments (each with n=2-3 per condition) are shown. *p<0.01 versus sicontrol; #p<0.05 versus sicontrol. **C.** GATA6 directly regulates the *Tgfb1* promoter. Relative luciferase activity from rat heart endothelial (RHE-A) cells transfected with a *Tgfb1* promoter luciferase construct and co-transfected with a GATA6- expressing or a control plasmid. As *Tgfb1* promoter construct, either the -1079 bp (TGFβ1-1079-Luc) or the -406 bp (TGFβ1-406-Luc) construct was used. A schematic representation of the *Tgfb1* promoter constructs is shown above the bar graph**.** Data from a representative experiment are shown (n=3); the experiment was repeated twice with similar results. *p<0.01 versus TGFβ1-1079-Luc.

Figure 7. GATA6 facilitates endothelial cell function by suppressing ALK5/SMAD2 signaling. A. Proliferation and vessel structure formation assays when HUVECs transfected with control (Co) siRNA or GATA6 (G6) siRNA were additionally treated with a TGFβ neutralizing antibody or control IgG. Data from a representative experiment are shown (n=3 per condition). The experiment was repeated twice with similar results. *p<0.05 versus other groups **B.** Representative Western blot analysis of phospho (P) - or total SMAD2 and SMAD1/5 proteins from HUVECs transfected with a control siRNA or GATA6 siRNA and additionally treated with a specific ALK5 inhibitor (SB-431542, 10μM) as indicated. Each condition is represented in dublicates. ACTIN was used as a loading control. **C.** Representative Western blot analysis of phospho (P) - or total SMAD2 and SMAD1/5 proteins from HUVECs treated with recombinant TGFβ1 protein for 90 minutes as indicated. Each condition is represented in dublicates. ACTIN was used as a loading control. **D.** Proliferation, vessel-like structure formation, cell migration and cell death assays when HUVECs transfected with a control siRNA or GATA6 siRNA were additionally treated with the ALK5 inhibitor (SB-431542, 10μM) as indicated. Data from a representative experiment are shown (n=3 per

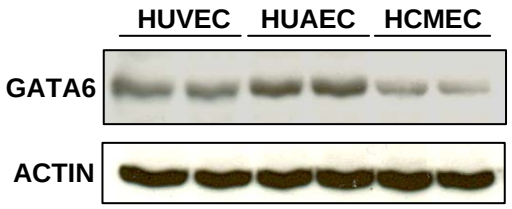
condition). The experiment was repeated twice with similar results.*$p<0.05$ versus other groups; #$p<0.05$ vs. siRNA control (Co) **E.** Schematic representation of the functional role of GATA6 in endothelial cells. Left side: GATA6 suppresses TGFβ1/β2 expression and thereby keeps ALK5-SMAD2 activation at a low level. ALK1-SMAD1/5 signaling was previously shown to enhance angiogenic function and survival in endothelial cells (28,38). Right side: Downregulation of GATA6 results in increased production of TGFβ, enhanced activation of the ALK5-SMAD2 cascade and inhibition of endothelial function and survival. Activation of ALK1-SMAD1/5 is not influenced by GATA6.

Figure 1.

A.


Fmol x10-10/ µg total RNA
7
6
5
4
3
2
1
0
HUVEC
HUAEC
HCMEC

B.


HUVEC
HUAEC
HCMEC
GATA6
ACTIN

C.

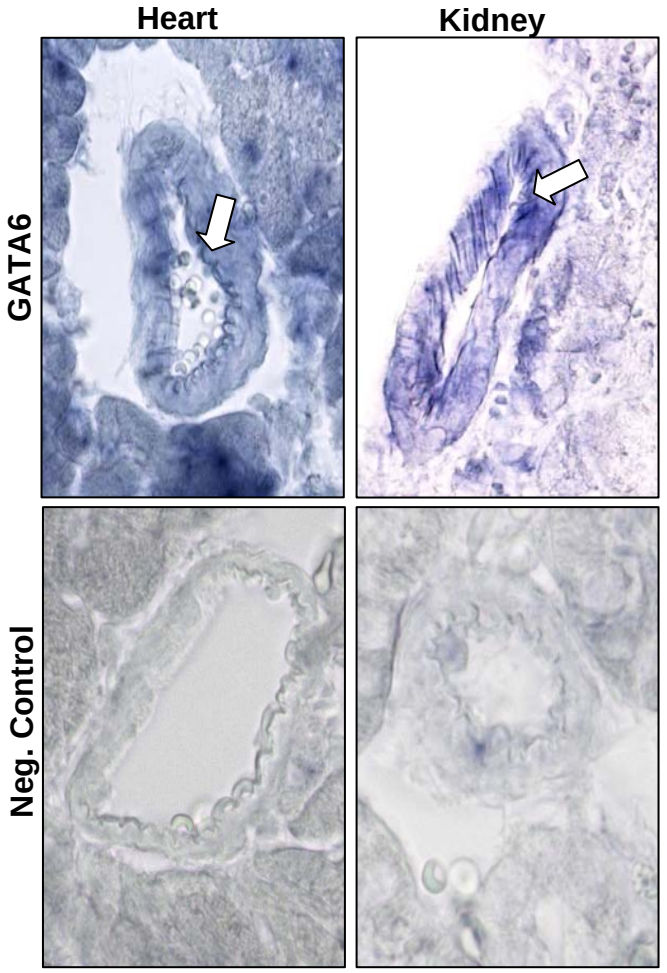
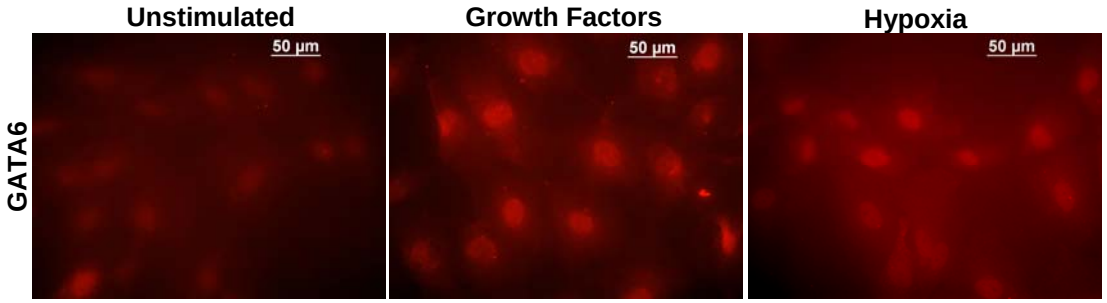
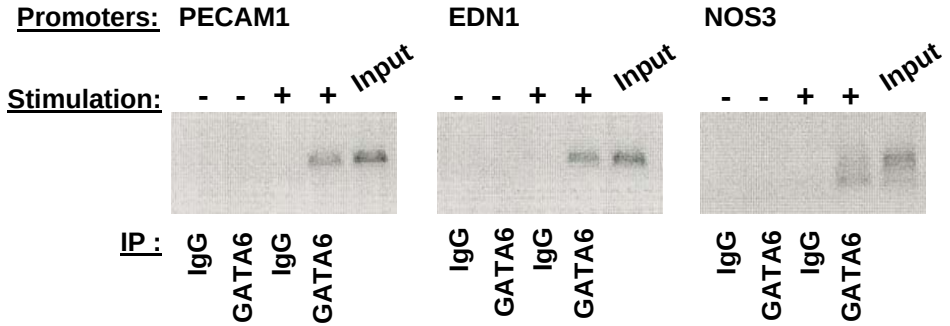

Heart
Kidney
GATA6
Neg. Control

Figure 2.

A.



B.



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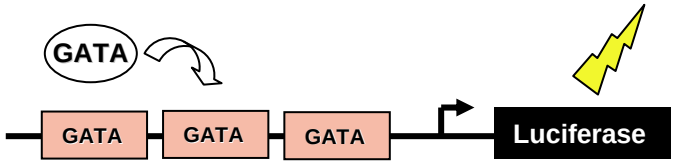

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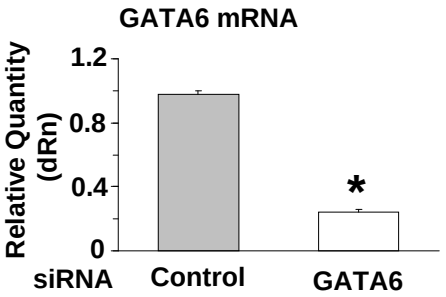

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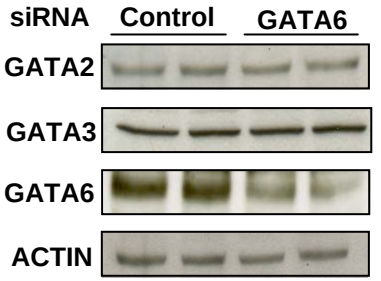

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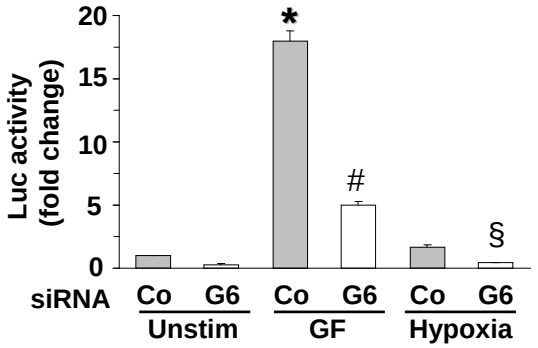


Figure 3.

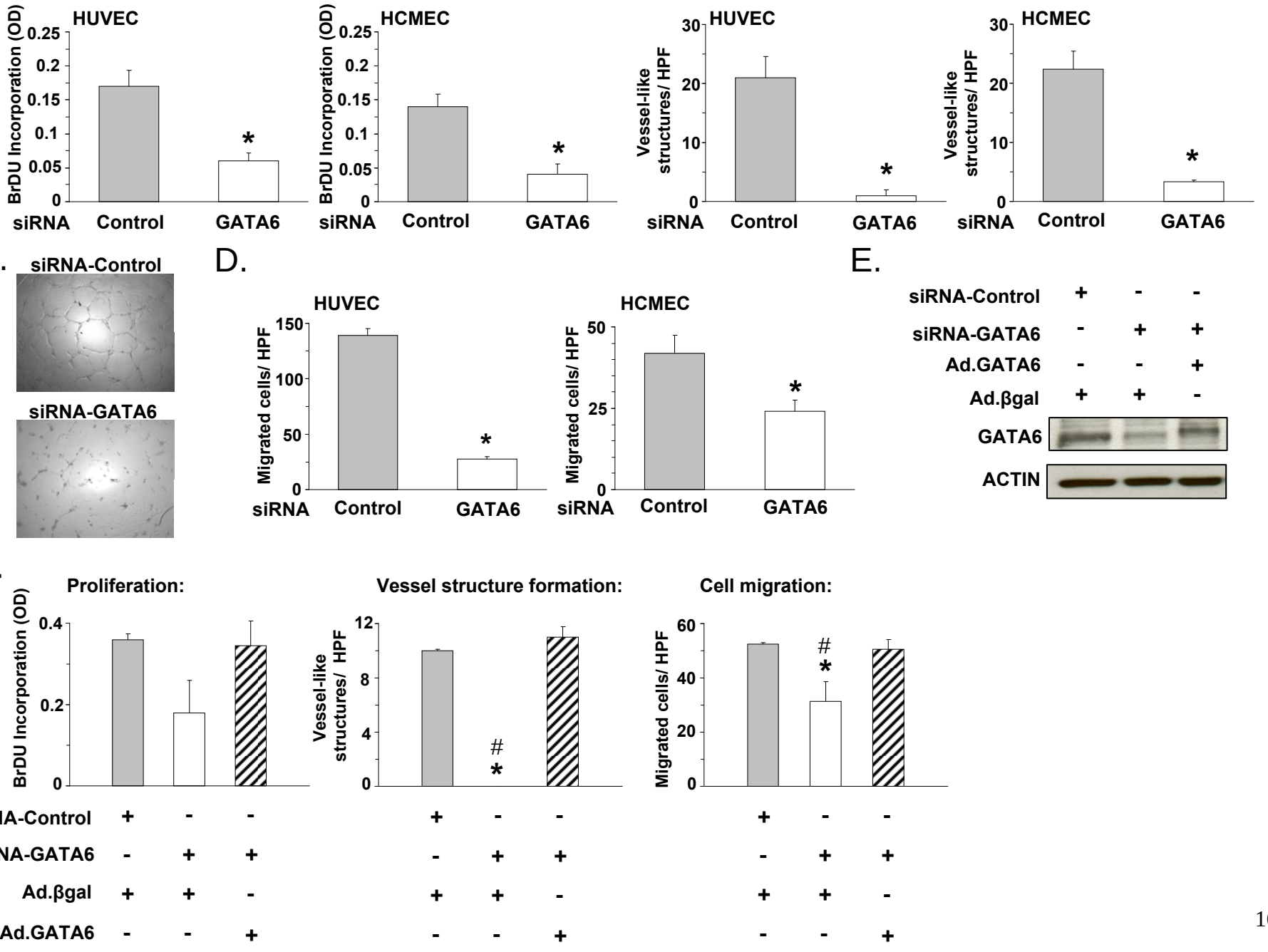
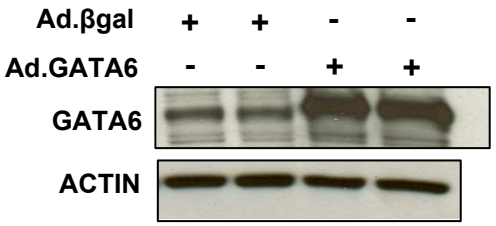

A.
HUVEC
BrDU Incorporation (OD)
0.25
0.2
0.15
0.1
0.05
0
*
siRNA
Control
GATA6
HCMEC
BrDU Incorporation (OD)
0.25
0.2
0.15
0.1
0.05
0
*
siRNA
Control
GATA6
B.
HUVEC
Vessel-like structures/ HPF
30
20
10
0
*
siRNA
Control
GATA6
HCMEC
Vessel-like structures/ HPF
30
20
10
0
*
siRNA
Control
GATA6
C.
siRNA-Control
siRNA-GATA6
D.
HUVEC
Migrated cells/ HPF
150
100
50
0
*
siRNA
Control
GATA6
HCMEC
Migrated cells/ HPF
50
25
0
*
siRNA
Control
GATA6
E.
siRNA-Control + - -
siRNA-GATA6 - + +
Ad.GATA6 - - +
Ad.βgal + + -
GATA6
ACTIN
F.
Proliferation:
BrDU Incorporation (OD)
0.4
0.2
0
Vessel structure formation:
Vessel-like structures/ HPF
12
8
4
0
#
*
Cell migration:
Migrated cells/ HPF
60
40
20
0
#
*
siRNA-Control + - -
siRNA-GATA6 - + +
Ad.βgal + + -
Ad.GATA6 - - +

Figure 4.

A.



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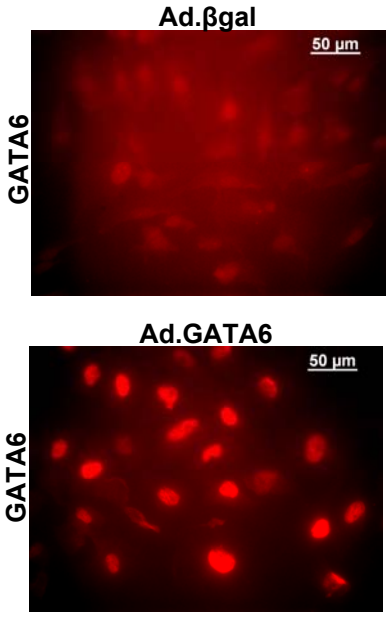

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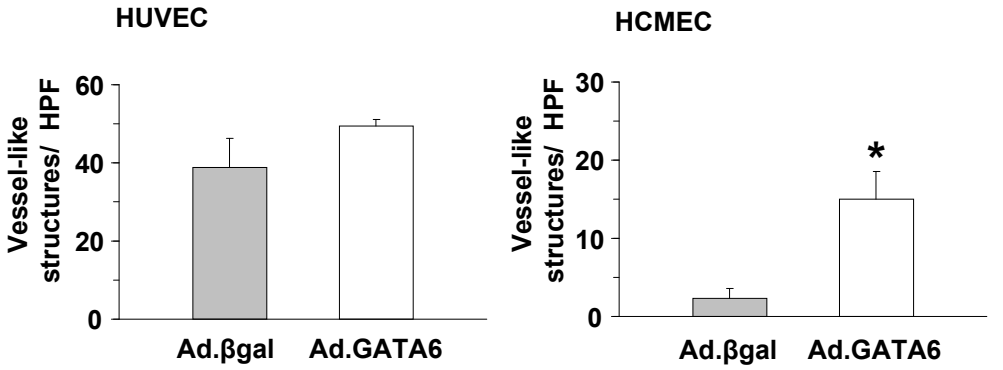


D.

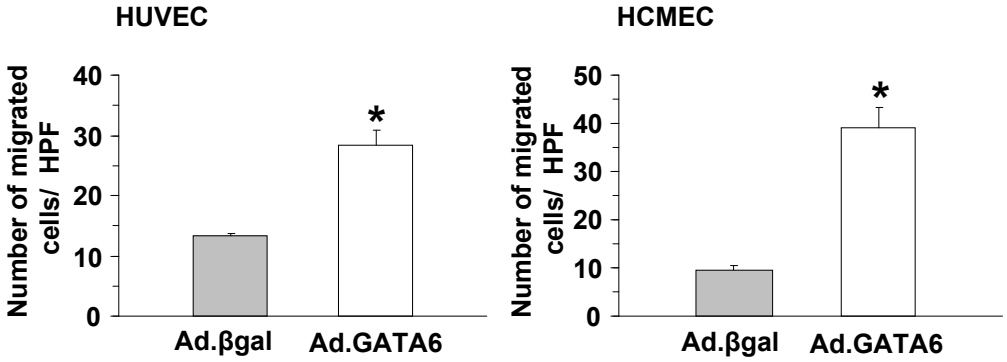
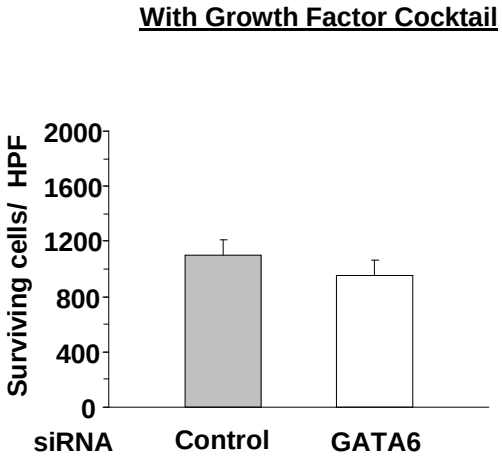
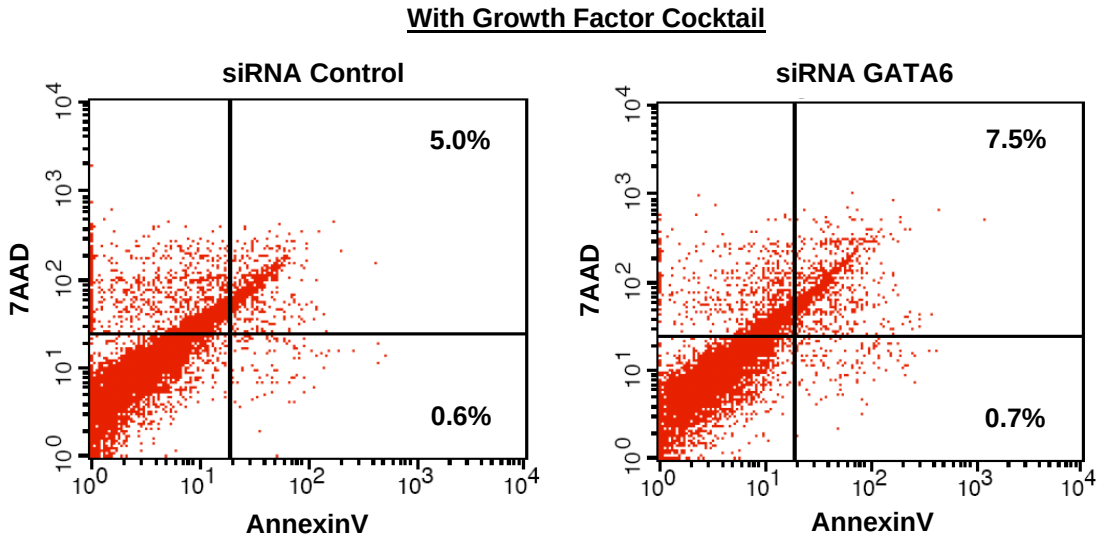


Figure 5.

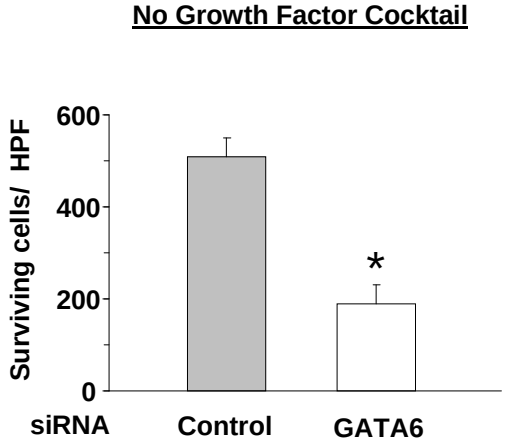
A.



B.



C.



D.

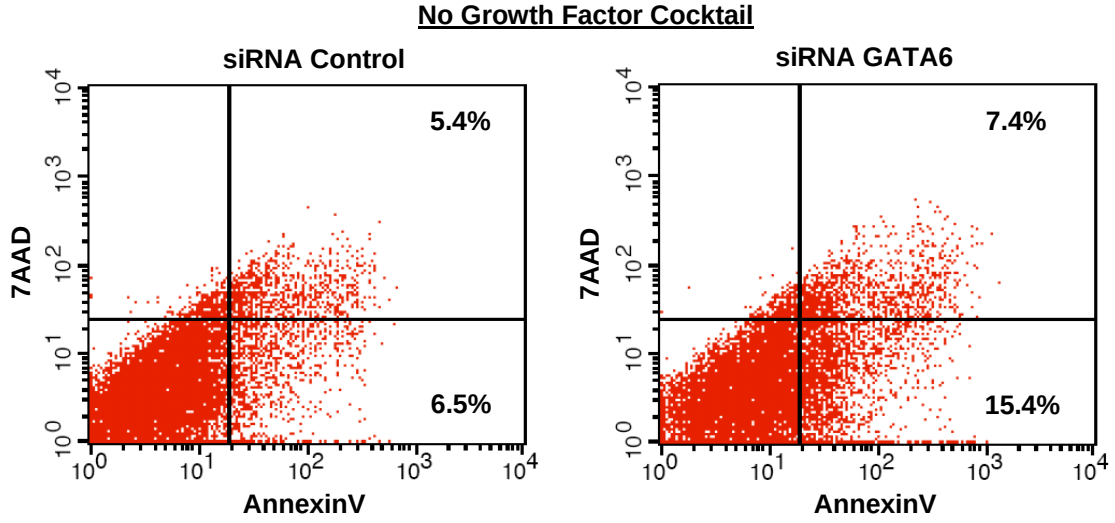
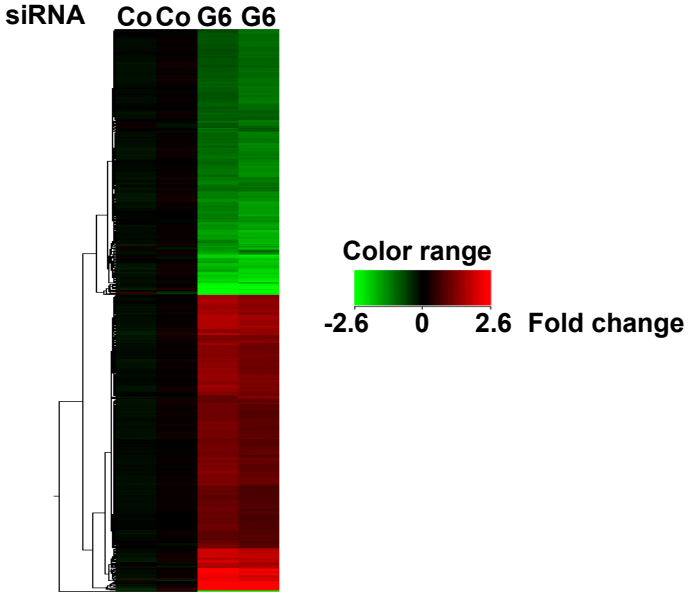
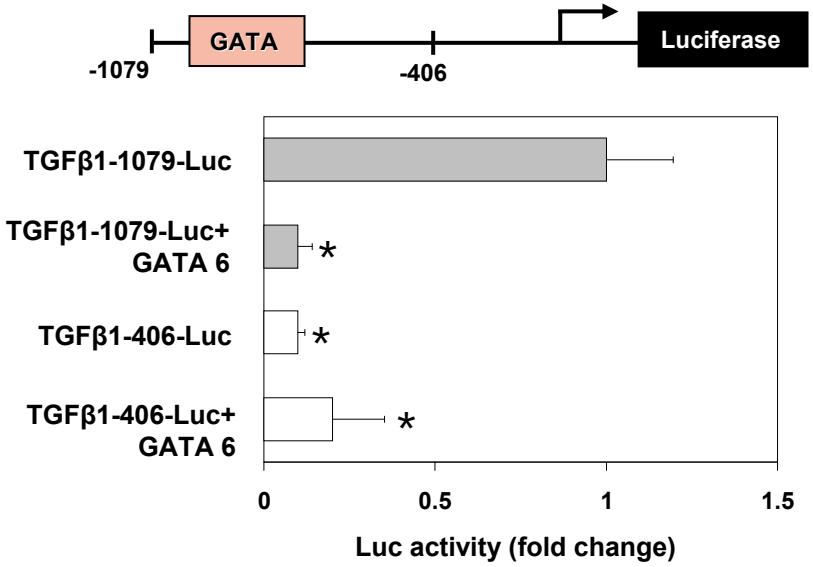


Figure 6.

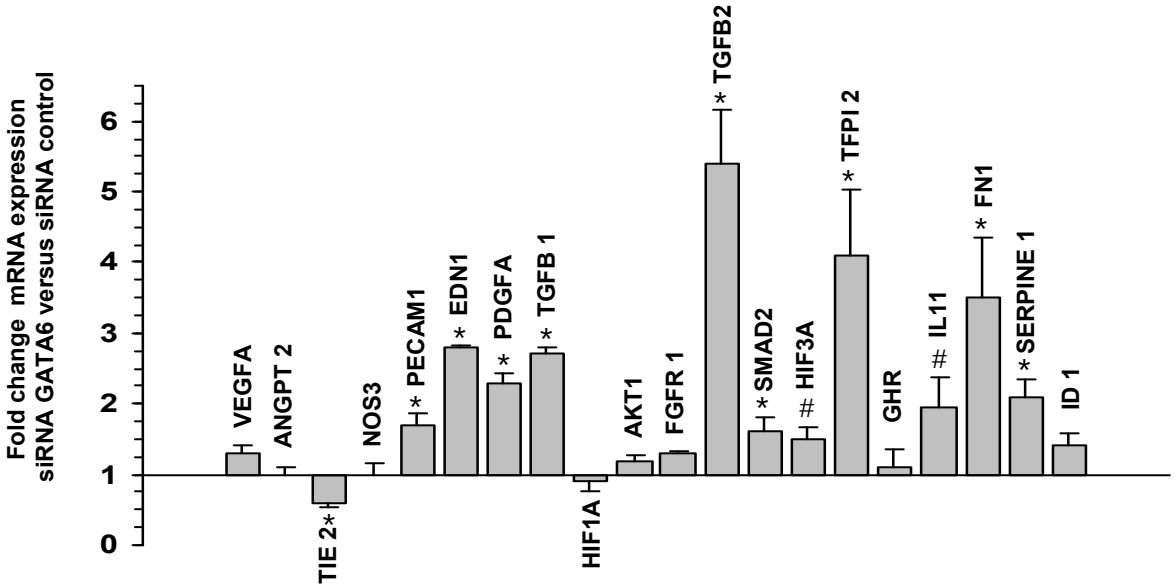
A.

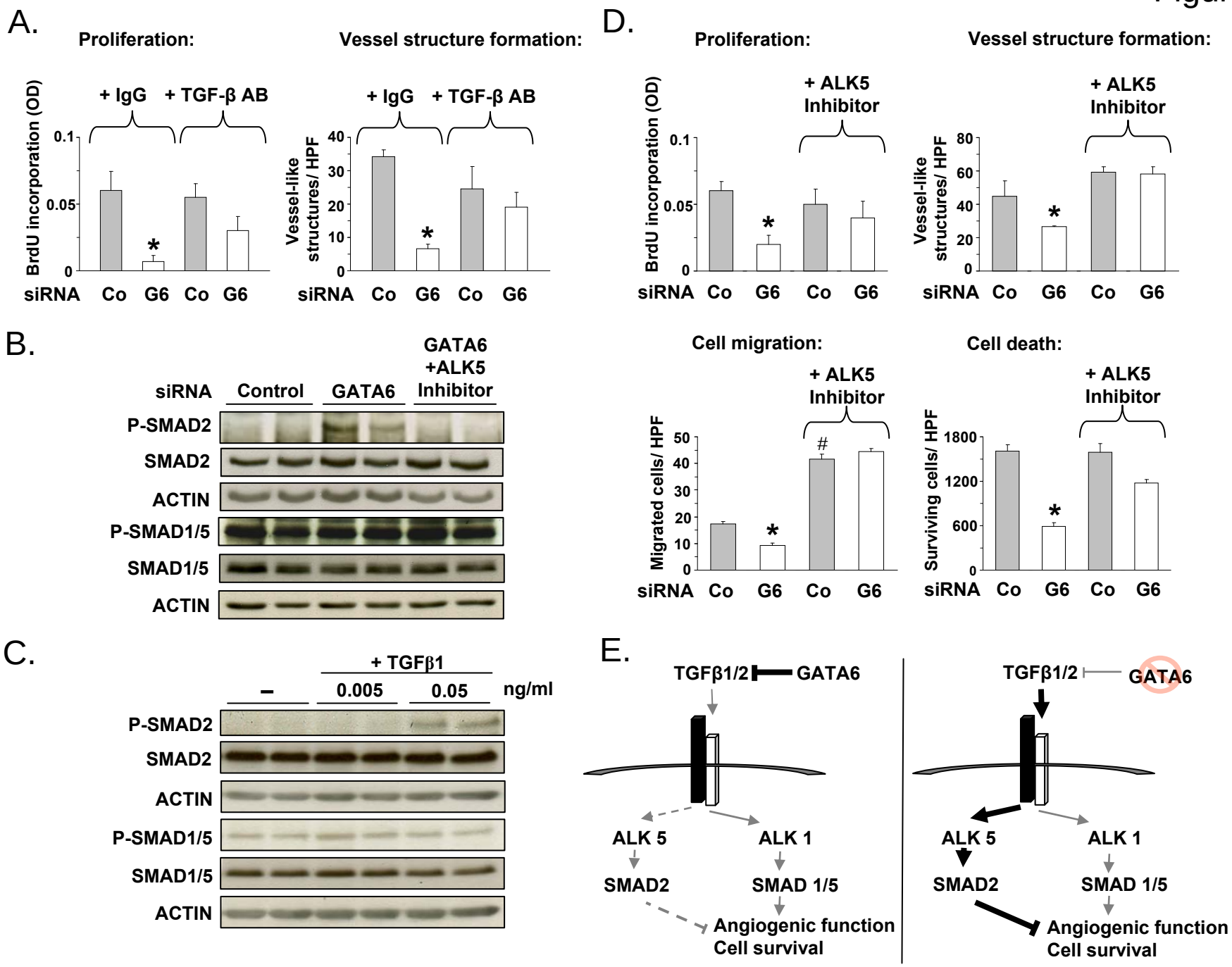

siRNA
Co Co G6 G6
Color range
-2.6 0 2.6 Fold change

C.


GATA
Luciferase
-1079
-406
TGFβ1-1079-Luc
TGFβ1-1079-Luc+ GATA 6
TGFβ1-406-Luc
TGFβ1-406-Luc+ GATA 6
*
0
0.5
1
1.5
Luc activity (fold change)

B.


Fold change mRNA expression siRNA GATA6 versus siRNA control
0
1
2
3
4
5
6
VEGFA
ANGPT 2
TIE 2*
NOS3
* PECAM1
* EDN1
* PDGFA
* TGFB 1
HIF1A
AKT1
FGFR 1
* TGFB2
* SMAD2
# HIF3A
* TFPI 2
GHR
IL11
* FN1
* SERPINE 1
ID 1


A.
Proliferation:
Vessel structure formation:
+ IgG
+ TGF-β AB
BrdU incorporation (OD)
Vessel-like structures/ HPF
siRNA Co G6 Co G6
B.
GATA6 +ALK5 Inhibitor
siRNA Control GATA6
P-SMAD2
SMAD2
ACTIN
P-SMAD1/5
SMAD1/5
ACTIN
C.
+ TGFβ1
ng/ml
P-SMAD2
SMAD2
ACTIN
P-SMAD1/5
SMAD1/5
ACTIN
D.
Proliferation:
Vessel structure formation:
+ ALK5 Inhibitor
Cell migration:
Cell death:
Migrated cells/ HPF
Surviving cells/ HPF
E.
TGFβ1/2
GATA6
ALK 5
ALK 1
SMAD2
SMAD 1/5
Angiogenic function
Cell survival