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

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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 down-regulation 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, down-regulation of GATA6 in endothelial cells led to increased expression of transforming growth factor (TGF) β1 and TGFβ2, whereas enhanced GATA6 expression, accordingly, suppressed Tgb1 promoter activity. High TGFβ1/β2 expression in GATA6-depleted endothelial cells increased the activation of the activin receptor-like kinase 5 (ALK5) 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.

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 interact with each other to form new capillaries (1–3). Although 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). To find effective treatment strategies, a detailed understanding of the regulatory signaling pathways that control activation and quiescence of endothelial cells is needed. Although 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 six 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 days 5.5 and 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 for 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

4 The abbreviations used are: VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cell; HUAEC, human umbilical arterial endothelial cell; HCMEC, human cardiac microvascular endothelial cell; 7AAD, 7-amino-actinomycin D; PAI-1, plasminogen activator inhibitor 1; ALKS, activin receptor-like kinase 5.
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 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, both of which act as angiogenesis inhibitory molecules.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—HUVECs, human umbilical arterial endothelial cells (HUAEcs), and human cardiac microvascular endothelial cells (HCMCs) were purchased from PromoCell. HUVECs and HUAECs were cultivated in endothelial cell growth medium (PromoCell) containing a growth factor mixture with sterile-filtered aqueous extract from mixed-sex bovine hypothalamic tissue 0.4%, fetal calf serum (FCS) 2%, epidermal growth factor (EGF) 0.1 ng/ml, hydrocortisone 1 µg/ml, 1 ng/ml basic FGF. HCMECs were cultured in Endothelial Cell Growth Medium MV for microvascular endothelial cells (PromoCell) containing a growth factor mixture with sterile-filtered aqueous extract from mixed-sex bovine hypothalamic tissue 0.4%, 5% FCS, 10 ng/ml EGF, and 1 µg/ml hydrocortisone. 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 Sigma 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 positive 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-conjugated secondary antibody (Invitrogen). was applied.

**Chromatin Immunoprecipitation (ChiP) Assay**—The ChiP assays were performed according to manufacturer’s directions (ChiP assay kit, Upstate Biotechnology). GATA6 antibodies (R&D Systems) and normal goat IgG (Santa Cruz) were used for immunoprecipitation. PCR was performed with the following primers: PECAM1 promoter, forward (5'-AGAACGCCAAGGCAAATGT-3') and reverse (5'-CTGGAAACCGGGAACATG-3'); EDN1 promoter, forward (5'-GGGCTCTGCTCTGGAATTTA-3') and reverse (5'-CCACCCCCAGAATGTTAT-3'); NOS3 promoter (forward, 5'-GGGCTCTGCTGACACCTGT-3') and reverse (5'-AGGGGGCTTCCAGTGT-3').

**siRNA Transfection**—HUVECs or HCMCs were transfected with either GATA6-specific siRNA duplex (SASL_Hs01_00123992, SASL_Hs01_00123992_AS; Sigma) or control siRNA (Ambion) using the GeneTrans II transfection reagent (MoBiTec) according to the manufacturer’s protocol. After 48 h of incubation in growth factor mixture 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 2 h either with an adenovirus that expresses GATA6 under the control of the constitutive CMV promoter (Ad.GATA6, 5 multiplicity of infection) or a control adenovirus, which expresses β-galactosidase also under the control of the CMV promoter (Ad.βgal, 5 multiplicity of infection) (22). The cells were then incubated for up to 48 h in growth factor mixture containing medium.

**Proliferation Assay**—DNA synthesis was measured as BrdU incorporation with a commercially available kit (Roche Applied Science). Cells were plated on gelatinized 96-well plates at 8000 cells/well in the presence of BrdU. After 24 h, 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 h, tube formation (number of closed circular structures/high-powered field at a magnification of 50×) was quantified.

**Migration Assay**—Endothelial cell migration was analyzed in 6.5-mm cell culture transwell inserts (8-µm pore size, Costar). 50,000 cells were added in the upper chamber in medium without growth factor mixture, whereas the lower chamber contained medium with added growth factor mixture. After 24 h, 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 mixture-containing medium for 5 h. The cells were then washed with PBS and incubated for 17 h 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 7-amino-actinomycin D (7AAD) with the use of a commercially available kit (BD Pharmingen). Thereafter, samples were analyzed by flow cytometry (FACS, BD Biosciences) for viable (annexin-V-negative and 7AAD-negative), early apoptotic (annexin-V-positive, 7AAD-negative), and late apoptosis (annexin-V-positive, 7AAD-positive) cells.
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 44k v2 kit from Agilent was used. 200 ng 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 Version 10.7.3.1. 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 up-regulated and down-regulated 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 the 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 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 of DNA/well of two different mouse Tgfβ1 promoter constructs, −406 bp and −1079 bp (kindly provided by Dr. Naoko Nakano, Tokyo, Japan) (24). To express GATA6, 1.5 μg of DNA of either GATA6 expressing construct or empty control vector were co-transfected with GeneTransII transfection reagent (MoBiTech). 48 h after transfection, cells were harvested, and luciferase activity was measured as previously described (25). A construct in which three 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 shuttle 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 the mean ± S.E. A one-way analysis of variance followed by Student-Newman-Keuls post hoc test was used to analyze differences between three or more groups; the unpaired Student’s t test was used to evaluate differences between two groups. A two-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) (Fig. 1A). Western blot analysis detected GATA6 protein in these cells, with levels paralleling the differences of the mRNA expression (Fig. 1B). 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. Im-
portantly, endothelial GATA6 expression was clearly detectable in the vasculature of different organs including the heart and kidney (Fig. 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 h; data not shown). However, exposure to hypoxia or a growth factor mixture for 4 h induced a strong nuclear translocation of GATA6 protein in HUVECs (Fig. 2A).

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 Fig. 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 three consecutive GATA motifs (GATA2, -3, and -6) and that either remained unstimulated (Unstim) or stimulated with growth factor mixture (GF) or hypoxia. Results from a representative 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 2. GATA6 activation in endothelial cells. A, immunofluorescence staining for GATA6 (red) in HUVECs that were unstimulated or stimulated for 4 h either with growth factor mixture or hypoxia. Samples were visualized using an Axiosvert 200M microscope (Carl Zeiss) with a 40 ×/0.75 lens (Carl Zeiss). The results are representative of three independent experiments. B, shown is a 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′ promoter region of the PECAM1, EDN1, or NOS3 genes, respectively. The results are representative of three independent experiments. C, shown is a 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, shown is 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 stimulated with growth factor mixture (GF) or hypoxia. Results from a representative experiment are shown (n = 3 per condition). The experiment was repeated twice with similar results.

A. Unstimulated Growth Factors Hypoxia

B. Promoters: PECAM1 EDN1 NOS3

C. Luciferase

D. GATA6 mRNA

E. siRNA Control GATA6

F. Luciferase (fold change)
GATA transcriptional activity under these circumstances was mainly due to GATA6 (Fig. 2F).

In summary, GATA6 translocated to the nucleus and activated transcription in endothelial cells that were exposed to either growth factors or hypoxia. Although 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. To assess the functional role of GATA6 in this program, we analyzed the ability of HUVECs and HCMCs to proliferate, migrate, and to form capillary/vessel-like structures on Matrigel after growth factor mixture stimulation. Down-regulation of GATA6 (by siRNA) in HUVECs and HCMCs led to a dramatically reduced proliferative response compared with control siRNA-treated cells (Fig. 3A). Moreover, the ability of the endothelial cells (HUVEC and HCMC) to form vessel-like structures on Matrigel was almost completely abrogated by the reduction in GATA6 (Fig. 3B and C). Finally, the capacity of the HUVECs and HCMCs to migrate toward a growth factor mixture gradient in a cell culture transwell system was significantly blunted in HUVECs and HCMCs with diminished GATA6 expression (Fig. 3D).

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. Fig. 3E shows that GATA6 protein levels in HUVECs with silenced endogenous GATA6

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**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 HCMCs transfected with a control siRNA or siRNA against GATA6 is shown. B, shown is quantification and representative images (C) of vessel-like structure formation on Matrigel in HUVECs and HCMCs when treated with a control siRNA or siRNA against GATA6. HUVECs were visualized using an Axiovert 200M microscope (Carl Zeiss) with a 5×/0.12 lens (Carl Zeiss). D, shown is a migration assay (assessed the amount of cells that successfully migrated along a growth factor gradient) with HUVECs or HCMCs transfected with a control siRNA or siRNA against GATA6. E, shown is a 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 a control. F, endothelial function was assessed by proliferation, vessel-like structure formation, and migration assays in cells that were treated like described under E. OD, optical density; HPF, high power field. For A, B, D, and 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.
expression could indeed be restored by adenoviral expression of mouse GATA6. As demonstrated in Fig. 3F and supplemental Fig. 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 (Fig. 4A and B). Although 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, Fig. 4C and 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 Fig. 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 7AAD and subsequent FACS analysis revealed similar levels 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 down-regulation (Fig. 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). To test this, we withdrew growth factor mixture from HUVECs for 17 h and subsequently quantified the total number of surviving cells. It is shown in Fig. 5C that far fewer cells survived growth factor withdrawal when GATA6 was down-regulated. To elucidate whether the endothelial cells with reduced GATA6 expression died either by apoptosis or necrosis, we stained HUVECs after 17 h of growth factor mixture 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 (Fig. 5D), whereas 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 down-regulation of GATA6 was indeed the reason for exaggerated cell death in the siRNA GATA6-treated cells (supplemental Fig. 2).

**Suppression of TGFβ1 and TGFβ2 Expression in Endothelial Cells by GATA6**—To obtain mechanistic insight into how GATA6 modulates endothelial function and survival, we performed transcriptome profiling in siRNA control and siRNA GATA6-treated HUVECs employing Agilent microarrays. Two microarrays from independent samples were analyzed per condition. As demonstrated in the heat map view, down-regulation of GATA6 by siRNA resulted in a significant at least 2-fold up- or down-regulation of 1145 genes versus siRNA control-treated cells (Fig. 6A). Genes from multiple
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A schematic representation of the promoter constructs is depicted above the panel in Fig. 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 toward ALK1**—Because we found highly elevated expression of TGFβ1 and TGFβ2 in the HUVECs with down-regulation of GATA6 and because it is known that high levels of TGFβ inhibit endothelial cell function and survival (29, 30), we hy-
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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, down-regulation; red, up-regulation 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. The expression level in cells transfected with a control siRNA was used as a reference and set to 1. The fold increase in the 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 is shown. As the Tgfb1 promoter construct, either the −1079 bp (Tgfb1−1079-Luc) or the −406 bp (Tgfb1−406-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 Tgfb1−1079-Luc.

Pothesized that the high TGFβ levels were critical for the impaired angiogenic function and survival in GATA6-depleted endothelial cells. 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 Fig. 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; although 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. Because ALK5 activation specifically leads to SMAD2 phosphorylation, we analyzed SMAD2 phosphorylation in GATA6-depleted HUVECs by Western blot with a phospho-SMAD2-specific antibody. As demonstrated in Fig. 7B, SMAD2 phosphorylation indeed specifically occurred in HUVECs with reduced GATA6 expression. Inhibition of ALK5 by the specific compound SB-431542 abolished SMAD2 phosphorylation in HUVECs treated with GATA6 siRNA (Fig. 7B). In turn, down-regulation 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 min (Fig. 7C). This confirmed TGFβ1 as a potent inducer of SMAD2 phosphorylation 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 (Fig. 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 (Fig. 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 hematopoietic 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. Although 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, whereas 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 toward a growth factor gradient, and 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 h 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, besides 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 down-regulation leads to suppression of PAI-1 expression, but down-regulation of GATA6 was reported to occur upon GATA3 reduction 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). Although 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 up-regulation of ID1), down-regulation 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. Although 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 to sustain endothelial cell function and survival (28). In summary, we identified a previously unknown critical proangiogenic role of the transcription factor GATA6 in endothelial cells that might render it an interesting target for angiogenesis related therapies in the future.

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