Critical Role for GATA3 in Mediating Tie2 Expression and Function in Large Vessel Endothelial Cells

Endothelial phenotypes are highly regulated in space and time by both transcriptional and post-transcriptional mechanisms. There is increasing evidence that the GATA family of transcription factors function as signal transducers, coupling changes in the extracellular environment to changes in downstream target gene expression. Here we show that human primary endothelial cells derived from large blood vessels express GATA2, -3, and -6. Of these factors, GATA3 was expressed at the highest levels. In DNA microarrays of human umbilical vein endothelial cells (HUVEC), small interfering RNA-mediated knockdown of GATA3 resulted in reduced expression of genes associated with angiogenesis, including Tie2. At a functional level, GATA3 knockdown inhibited angiopoietin (Ang)-1-mediated but not Ang-2 is a functional antagonist of Tie2. At a functional level, GATA3 knockdown inhibited angiopoietin (Ang)-1-mediated but not angiogenesis, including Tie2. A functional level, GATA3 knockdown inhibited angiopoietin (Ang)-1-mediated but not vascular endothelial cell growth factor (VEGF)-mediated AKT signaling, cell migration, survival, and tube formation. In electrophoretic gel mobility shift assays and chromatin immunoprecipitation, GATA3 was shown to bind to regulatory regions within the 5′-untranslated region of the Tie2 gene. In co-immunoprecipitation and co-transfection assays, GATA3 and the Ets transcription factor, ELF1, physically interacted and synergized to transactivate the Tie2 promoter. GATA3 knockdown blocked the ability of Ang-1 to attenuate vascular endothelial cell growth factor stimulation of vascular cell adhesion molecule-1 expression and monocytic cell adhesion. Moreover, exposure of human umbilical vein endothelial cells to tumor necrosis factor-α resulted in marked down-regulation of GATA3 expression and reduction in Tie2 expression. Together, these findings suggest that GATA3 is indispensable for Ang-1-Tie2-mediated signaling in large vessel endothelial cells.

Endothelial cell phenotypes are differentially regulated in space and time. Phenotypic changes in the endothelium are mediated, at least in part, by differential activity of transcription factors. The GATA family of transcription factors consists of six members (GATA1 to -6). All GATA factors contain two highly conserved C2C2-type zinc fingers that can bind to DNA containing the consensus WGAATAR sequence (reviewed in Refs. 1, 2). The GATA family includes two subfamilies based on sequence homology and expression patterns. GATA1/2/3 are expressed primarily in hematopoietic cells, whereas GATA4/5/6 are expressed within various mesoderm- and endoderm-derived tissues such as the heart and gastrointestinal tract. Gene disruption studies have shown that all GATA proteins with the exception of GATA5 are required for development (3–8). In addition to their role in embryogenesis, there is increasing evidence that GATA proteins are important for cell type-specific gene expression and signal transduction in adult tissues.

Among the GATA factors expressed in endothelial cells, GATA2 has received the most attention. Indeed, GATA2 has been implicated in the regulation of a number of endothelium-specific genes, including prepro-endothelin (9), endothelial nitric-oxide synthase (10), von Willebrand factor (11), intercellular adhesion molecule-2 (12), Down syndrome critical region-1 (13), and vascular endothelial cell adhesion molecule (VCAM)3-1 (14). The functional role, if any, for other GATA factors in endothelial cells has remained largely elusive.

Tie2 is a protein-tyrosine kinase receptor that is specifically expressed in endothelial cells. Tie2 is activated by its ligand, angiopoietin (Ang)-1. Ang-2 is a functional antagonist of Ang-1. The binding of Ang-1 to Tie2 results in rapid receptor autophosphorylation, which in turn mediates endothelial cell survival, cell migration, angiogenesis, and barrier function (15–19). Targeted disruption of Ang-1 or Tie2 results in embryonic...
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lethality because of abnormal interactions between endothelial cells and mural cells and marked impairment of vascular integrity (20). Tie2 also plays an important role in the adult vasculature. For example, Ang-1-Tie2 signaling induces neoangiogenesis and promotes an anti-inflammatory phenotype (reviewed in Refs. 21, 22).

The mouse Tie2 promoter has been previously characterized (23–28). In standard transgenic mice, a promoter fragment spanning 2100 bp of 5′-flanking sequence, the first exon, and 10 kb of the first intron was shown to direct widespread endothelial cell-specific expression in transgenic mice (27). In vitro studies have demonstrated a functional role for Ets and GATA motifs in mediating Tie2 expression (25, 26). NERF2 and ELF1 have been shown to interact with the Ets motifs (26). However, the transcription factors that bind to the GATA motifs have yet to be identified.

In this study, we show that GATA2, -3, and -6 are expressed in cultured primary human endothelial cells. In human umbilical vein endothelial cells (HUVEC), GATA3 is expressed at higher levels compared with GATA2 and GATA6. Furthermore, we demonstrate that GATA3 is essential for mediating Tie2 expression and Ang-1-Tie2 signaling. These findings implicate an important role for GATA3 in endothelial cell biology.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Culture**—Human recombinant Ang-1 and VEGF were obtained from R & D Systems (Minneapolis, MN). Human recombinant tumor necrosis factor (TNF)-α was from PeproTech (Rocky Hill, NJ). Thrombin was from Calbiochem. HUVEC, human coronary artery endothelial cells (HCAEC), human dermal microvascular endothelial cells (HDMEC), human dermal lymphatic endothelial cells, and human pulmonary artery endothelial cells (HPAEC) were purchased from Clonetics (Clonetics, La Jolla, CA). Human afferent lymphoid lymphatic endothelial cells were obtained as described previously (29). All endothelial cells were cultured in EGM-2 MV complete medium (Clonetics). Human skin fibroblast (Clonetics) and COS-7 cells (ATCC CRL-1651) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human U937 cells (ICR-9021) were grown in RPMI 1640 medium plus 10% FBS.

**Plasmids, Transient Transfections, and Luciferase Assays**—The construction of Tie2-luc, Tie2 (GATA mut)-luc, Tie2 (Ets mut)-luc, and expression plasmids for human GATA3 and ELF1 was described previously (25, 26, 30). HUVEC and HEK-293 cells were transiently transfected with plasmid DNA using FuGENE HD reagent (Roche Applied Science), and luciferase activities were calculated using the Dual-Luciferase assay kit (Promega, Madison, MI) as described previously (31).

**siRNA against GATA3**—Control siRNA was obtained from Invitrogen. Two independent siRNA were generated against GATA-3 3′-untranslated region (oligo1, GCAGGGCCAUAGCAUGAACCCUAA, and oligo2, GCGGAAAGGAUGCAAGAAGUUUA). HUVEC were incubated with a mixture of 40 nM siRNA and Lipofectamine Max (Invitrogen) for 48 h at which time cells were processed for total RNA or for 72 h at which time cells were processed for protein.

**Generation of GATA3 Adenoviruses**—Human GATA3 was cloned by PCR with total RNA from HUVEC and GATA3-specific primers (sequences are shown in supplemental Table I. GATA3 cDNA was subcloned into pIRE2-EGFP (Clontech) and then transferred into the pShuttle and Adeno-X DNA (Clontech) using the Adeno-X adenoviral expression system (Clontech). All cloned and subcloned constructs were confirmed by restriction enzyme digestion and automated DNA sequencing.

**DNA Microarray Analysis**—RNA was harvested from si-control- or si-GATA3-treated HUVEC using TRIzol (Invitrogen). Preparation of cRNA and hybridization of probe arrays were performed according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA). File maker software was used to identify genes that demonstrated identical response patterns in the presence of two independent GATA3 siRNAs. Data were analyzed according to the MIAME rule. Annotation of the probe numbers and targeted sequences was based on data available on the Affymetrix web page.

**Quantitative Real Time PCR**—Two µg of TRIzol-extracted RNA was reverse-transcribed using SuperScript II enzyme and oligo(dT) primer as specified by Invitrogen. Real time PCR, including SYBR Green PCR reagent, was performed on an instrument according to instructions provided by the manufacturer (Applied Biosystems, Foster City, CA). To quantify the mRNA expression levels among GATA family, human GATA1–6 cDNAs were cloned. A titration curve was generated using successively diluted concentrations of cDNA for each GATA factor, normalized for cyclophilin A mRNA levels. The curve was then used to calculate the GATA levels in the test samples from endothelial cells. Primer pair DNA sequences are shown in supplemental Table I.

**Electrophoretic Gel Mobility Shift Assays (EMSA)**—Nuclear extraction and EMSA were carried out as described previously (31). To reduce nonspecific binding, 1 µg of poly(dI-dC) (Invitrogen) was added to the reaction mixture. Double-stranded oligonucleotides were labeled with [α-32P]dCTP and Klenow fragment and purified by spin column (Amersham Biosciences). Oligonucleotide sequences used for probes and competitors are shown in supplemental Table I. For supershift experiments, nuclear extracts were preincubated with monoclonal antibody against GATA3 (Santa Cruz Biotechnology, Santa Cruz, CA) or IgG for 30 min at room temperature. Recombinant GATA3 were generated using the TnT-coupled Wheat Germ Extract systems (Promega) with pcDNA3-GATA3 according to the manufacturer’s instructions.

**Chromatin Immunoprecipitation (ChIP) Analysis**—Endothelial cells were exposed to 1% formaldehyde (Wako Chemicals, Osaka, Japan) for 10 min at room temperature. The cells were washed twice with ice-cold PBS containing protease inhibitor mixture (Roche Applied Science), then scraped, and collected by centrifugation. The cell pellets were resuspended in SDS lysis buffer containing protease inhibitors (Upstate Biotechnology, Inc., Lake Placid, NY) and incubated for 10 min. The cross-linked chromatin was subjected to sonication using BioRuptor (Cosmobio, Tokyo, Japan) to obtain DNA fragments.
of ~500 bp. The chromatin complexes were collected by centrifugation at 4 °C and diluted 10-fold in ChIP dilution buffer containing protease inhibitors (Upstate Biotechnology, Inc.). For pre-clear experiments, 100 μl of salmon sperm DNA/Protein G were added (Upstate Biotechnology, Inc.). The samples were immunoprecipitated with 10 μg of anti-GATA3 or control IgG (Santa Cruz Biotechnology). Precipitated chromatin complexes were washed and eluted according to the manufacturer’s instructions (Upstate Biotechnology, Inc.). Quantitative real time PCRs were performed with the primer pairs as shown in supplemental Table I.

Western Blot Analysis—Endothelial cells were washed with ice-cold PBS, collected with a cell scraper, and lysed with RIPA buffer as described previously (30). The membrane was blocked with TBS-T containing 2% skim milk and incubated with primary antibodies against GATA3 (Santa Cruz Biotechnology), Tie2 (Santa Cruz Biotechnology), or β-actin (Sigma).

Immunoprecipitation—Endothelial cells were treated with si-control or si-GATA3 for 48 h, serum-starved (EBM2 containing 0.5% FBS) for 16 h, and then stimulated 500 ng/ml Ang-1 or 50 ng/ml VEGF for 15 min. Subsequently, cells were lysed with lysis buffer following the manufacturer’s protocol (Cell Signaling Technology, Beverly, MA) and then immunoprecipitated with anti-AKT (5G3) antibody (Cell Signaling Technology). Each sample was separated with 10% SDS-PAGE and immunoblotted with anti-phospho-AKT (Ser-473) or total AKT antibodies (Cell Signaling Technology, Beverly, MA) and then immunoprecipitated with anti-AKT (5G3) antibody (Cell Signaling Technology) overnight at 4 °C. The immunoprecipitated complexes were washed and eluted according to the manufacturer's instructions (Upstate Biotechnology, Inc.).

Monocyte Adhesion Assays—For fibroblast-endothelial cell co-culture, we employed a method previously described by Velazquez et al. (33). Briefly, HUVEC were treated with siRNA for 24 h and then harvested and fluorescent-labeled with PKH dye (Sigma). Migration assays were carried out with Biocat angiogenesis system (BD Biosciences). Fluorescently labeled cells were seeded on the upper chamber (1 × 10^5 cells/250 μl of EBM-2) and incubated with 750 μl of EBM-2 with or without Ang-1 in the lower chamber. After 24 h, migrated cells were visualized under the fluorescent microscope and quantified with fluorescence detect-cell image analyzer (Kurabo, Kyoto, Japan).

Cell Migration Assays—Endothelial cells were treated with siRNAs for 24 h and then serum-starved and labeled with PKH dye (Sigma). Migration assays were carried out with Biocat angiogenesis system (BD Biosciences). Fluorescently labeled cells were seeded on the upper chamber (1 × 10^5 cells/250 μl of EBM-2) and incubated with 750 μl of EBM-2 with or without Ang-1 in the lower chamber. After 24 h, migrated cells were visualized under the fluorescent microscope and quantified with fluorescence detect-cell image analyzer (Kurabo).

Cell Survival Studies—Endothelial cells were treated with siRNAs for 24 h and then incubated with EBM-2 basal media (Clonetics) containing 0.5% FBS for 18 h. Subsequently, cells were incubated with Dulbecco’s modified Eagle’s medium containing 0.5% FBS in the presence or absence of Ang-1 for 24 h. Cells were harvested and assayed for annexin V using flow cytometry (fluorescein isothiocyanate-conjugated annexin V, BD Biosciences). Alternatively, cells were fixed and processed for TUNEL staining using the VasoTACS in situ apoptosis detection kit (R & D Systems). Monocyte Adhesion Assays—Monocyte adhesion to HUVEC was assayed as described previously (34).

RESULTS

GATA3 Regulates Tie2 Expression and Function

GATA3 Is Expressed in Large Vessel Endothelial Cells—In DNA microarrays and real time PCR, large vessel endothelial cells (HUVEC, HCAEC, human afferent lymphoid lymphatic endothelial cells, and HPAEC) were shown to express GATA2, GATA3, and GATA6 but not GATA1, GATA4, or GATA5 (Fig. 1A and supplemental Fig. 1). To compare mRNA expression levels between the various GATA factors, we generated titration curves with known concentrations of GATA2, -3, and -6 cDNA. GATA3 was expressed at the highest levels, followed by GATA2 and GATA6 (Fig. 1B). In contrast to large vessel endothelial cells, human dermal microvascular endothelial...
A

| Number      | Name     | Large vessel | Microvascular |
|-------------|----------|--------------|---------------|
| NM_000459  | 206702_at| Tie-2        |               |
| NM_002049  | 210446_at| GATA1        |               |
| NM_032638  | 209710_at| GATA2        |               |
| NM_002051  | 209604_s_at| GATA3      |               |
| NM_002052  | 205517_at| GATA4        |               |
| NM_080473  | 238095_at| GATA5        |               |
| NM_005257  | 210002_at| GATA6        |               |
| NM_172373  | 212418_at| ELF1         |               |
| NM_201999  | 203822_s_at| NERF2     |               |
| BE561479   | 212581_x_at| GAPDH       |               |

B

mRNA expression in cells (x10 fmol/mg total RNA)

|    | GATA2 | GATA3 | GATA6 |
|----|-------|-------|-------|
| Mean | 0.1   | 0.3   |       |
| SD   | 0.01  | 0.1   |       |

* Significant difference

C

GATA3

PECAM1

Merge (GATA3/PECAM1/DAPI)
endothelium was clearly observed in the nuclei of PECAM1-
Although the background was higher, the GATA3 signal in
out immunohistochemical staining of the mouse aorta.
whether GATA3 is expressed in intact endothelium, we carried
obtained with HCAEC and HPAEC (not shown). To determine
alyzed primarily in the nucleus (Fig. 1
of GATA3 protein expression (Fig. 2
control. siRNA treatment also resulted in significant inhibition
action of HUVEC with two independent siRNAs (si-GATA3-a
and -6 mRNA levels in HUVEC. Data are expressed as mean
//H11021
0.001 compared with si-control.
\[\text{FIGURE 2. siRNA-mediated knockdown of GATA3 in primary human endothelial cells results in altered transcriptional profile.} \]
\( A \) quantitative real time PCR analyses of GATA3 mRNA in HUVEC transfected with
compared with GATA2 or GATA6 (supplemental Fig. II). In
immunohistochemical analyses of HUVEC, GATA3 was local-
ized primarily in the nucleus (Fig. 1C). Similar results were
obtained with HCAEC and HPAEC (not shown). To determine
whether GATA3 is expressed in intact endothelium, we carried
out immunohistochemical staining of the mouse aorta.
Although the background was higher, the GATA3 signal in
endothelium was clearly observed in the nuclei of PECAM1-
positive endothelial cell layer (arrows in supplemental Fig. III).

\[\text{GATA3 Knockdown in Cultured Human Primary Endothe-
lial Cells Results in an Altered Transcriptional Profile—To de-
termine the functional relevance of GATA3 in endothelial}
\] cells, we employed siRNA to knock down expression of the transcription factors bind to this region, we carried out EMSA
using a radiolabeled oligonucleotide probe that spans the con-
sensus GATA motifs (Fig. 3
\[\text{FIGURE 1. GATA3 is expressed in primary human endothelial cells.} \]
\( A \) heat map of selected genes in DNA microarrays of large vessel or microvascular endothelial cells. Expression values represent the average from two independent microarrays. Eisen cluster was performed with Gene Cluster software. Color intensity is relative to the median. \( B \), quantitative real time analyses of GATA2, -3, and -6 mRNA levels in HUVEC. Data are expressed as mean ± S.D. of 10 independent experiments. *, \( p < 0.01 \) compared with GATA3 expression in HUVEC. 
C, immunofluorescent staining of HUVEC for GATA3 (green) and PECAM1 (red). Nuclei were stained with 4',6-diamidino-2-phenylindole. Merged image is shown on the right. Bar, 50 \( \mu \)m.

\[\text{GATA3 Mediates Expression of Tie2 and Binds to the Proxi-
mal Promoter Region in Cultured Human Primary Endothe-
lial Cells—The DNA microarray data implicated GATA3 as a}
\] regulator of Tie2 expression. To validate these findings, we
employed real time PCR and Western blot analyses to quanti-
tate Tie2 expression in si-control- and si-GATA3-transfected
HUVEC. As shown in Fig. 3A, GATA3 knockdown in HUVEC
resulted in significant reduction in Tie2 mRNA and protein
levels (58.2 reduction and 93%, respectively). These effects were
rescued by Ad-mediated expression of siRNA-resistant
GATA3 (Fig. 3B).

We previously demonstrated that a 732-bp fragment of the
Tie2 promoter, including exon 1, contains information for
endothelium-specific expression in cultured cells and in Hprt-
targeted transgenic mice (25). The Tie2 promoter contains
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targeted transgenic mice (25). The Tie2 promoter contains
closely aligned clusters of three GATA sites and six Ets-binding
sites in the first exon (Fig. 3C). To determine whether GATA
transcription factors bind to this region, we carried out EMSA
using a radiolabeled oligonucleotide probe that spans the con-
sensus GATA motifs (Fig. 3C, hatched rectangle) and recombi-
nant GATA protein. As shown in Fig. 3D, incubation of the
DNA probe with recombinant GATA3 (but not control lysate)
yielded a specific DNA-protein complex (Fig. 3D, lanes 1 and 2,
arow). The DNA-protein complex was inhibited by the addi-
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C

Down regulated genes by si-GATA3 treatment

Up regulated genes by si-GATA3 treatment

D

Gene volume (n)

Functional category

24 Signaling/transcription
16 Metabolism
14 Angiogenesis and vascularization
11 Transporters and channels
6 Immune/inflammation and cytokines
4 Cell proliferation and apoptosis
3 Hemostasis and hematologic disorder
2 Extracellular matrix and cytoskeleton
2 Vasomotor/blood pressure
4 unknown

FIGURE 2—continued
GATA3 Regulates Tie2 Expression and Function

FIGURE 3. GATA3 binds to tandem GATA motifs in the Tie2 gene in primary human endothelial cells. A, quantitative real-time PCR analyses of Tie2 mRNA in HUVEC transfected with si-control and siRNAs against GATA3 (Oligo 1). Data are expressed as mean ± S.D. of expression levels relative to si-control values obtained in triplicate from at least three independent experiments. *, p < 0.0001 compared with si-control. B, Western blot analysis of HUVEC preinfected with either Ad-control or Ad-GATA3 (multiplicity of infection (moi) = 0.3) and transfected with si-control or GATA3 siRNA (Oligo 1). The membrane was immunoblotted with anti-Tie2 and anti-GATA3 antibodies. To control for loading, the membrane was stripped and re-probed with anti-β-actin antibody. The results are representative of three independent experiments.

Asterisks indicate homologous DNA sequences in human, mouse, and rat. GATA and Ets binding regions (as determined by previous DNase footprinting assays (25)) are underlined. The probe sequence used in EMSA is highlighted by a hatched bar. D, EMSA was performed with 32P-labeled probe incubated in the absence (lane 1) or presence (lanes 2–6) of recombinant GATA3. Binding reactions were performed in the presence of 100-fold molar excess of unlabeled wild-type probe without (lane 2) or with (lanes 3) or a mutation of the GATA motifs (lane 4). Alternatively, the reaction mixture was preincubated with anti-GATA3 antibody (lane 5) or control IgG (lane 6). The hatched bar and asterisk indicate specific DNA-protein and super-shifted complexes, respectively. The results are representative of at least three independent experiments. F, left, schematic representation of the Tie2 gene and real time PCR primer region for ChIP assay. Formalin-fixed chromatin was immunoprecipitated with antibodies against GATA3 or control IgG. Right, precipitated genomic DNA and preimmunoprecipitated chromatin (input) were quantified by real time PCR using the specific primer pairs as described in left and supplemental Table 1. Data are expressed as mean ± S.D. obtained from at least three time repeated assays. *, p < 0.001 compared with immunoprecipitated chromatin (primer A and B region) by anti-GATA3 antibody. N.S, nonsignificant.
GATA3 and ELF1 Physically Interact and Function Synergistically to Promote Tie2 Expression in Cultured Human Primary Endothelial Cells—Consistent with our previous observations, a mutation of the Ets cluster resulted in >90% reduction in Tie2-luc activity (Fig. 4A). A previous study demonstrated an important role for the Ets transcription factors, NERF2 and ELF1, in mediating endothelial expression of Tie2 (26). Indeed, our DNA microarrays confirmed that NERF2 and ELF1 are expressed in multiple types of primary human endothelial cells (see Fig. 1A). In co-transfection assays, ELF1 induced expression of the Tie2 promoter by 6.8-fold (Fig. 5A). Importantly, co-transfection with ELF1 and GATA3 resulted in synergistic
activation (15.5-fold) of the Tie2 promoter (Fig. 5A). Similar synergy was observed between NERF2 and GATA3 (not shown). To determine whether GATA3 and ELF1 directly interact, we carried out immunoprecipitation assays using COS-7 cells transiently transfected with expression plasmids for GATA3 (pcDNA3-GATA3), FLAG-tagged ELF1 (pFLAG-ELF1), and/or vector alone (pcDNA3 or pFLAG). As shown in Fig. 5B, GATA3 was detected in Western blots of immunoprecipitated ELF1. In contrast, no such interaction was observed in cells co-transfected with pcDNA3-GATA3 and pFLAG or pcDNA3 and pFLAG-ELF1 (Fig. 5B). To determine whether GATA3 and ELF1 physically interact in endothelial cells, nuclear extracts from HUVEC were prepared by nitrogen cavitation (30, 32) and processed for co-immunoprecipitation. Endogenous GATA3 was co-precipitated with anti-ELF1 antibody but not control IgG (Fig. 5C, lanes 2 and 3). Quantitation from two independent experiments revealed that ELF1 antibody immunoprecipitated 84% of total ELF1 in HUVEC and that ELF1 was physically associated with 7.6% of total cellular GATA3. Taken together, these findings suggest that GATA3 physically interacts and functionally synergizes with ELF1 to mediate Tie2 expression in endothelial cells.

GATA3 Knockdown in Cultured Human Primary Endothelial Cells Attenuates Ang-1-Tie2-mediated Signal Transduction, Cell Migration, Tube Formation, and Cell Survival—Previous studies have demonstrated that Ang-1-mediated phosphorylation of Tie2 results in activation of the phosphatidylinositol 3-kinase-AKT pathway (18, 36). To determine whether GATA3 knockdown is associated with functional defects in Ang-1-Tie2 signaling, we quantified Ang-1-induced phosphorylation of AKT in the presence or absence of siRNA against GATA3. In si-control-transfected cells, Ang-1 treatment resulted in a 6.9-fold increase in phospho-

**FIGURE 4.** GATA3 transactivates Tie2 promoter in primary human endothelial cells. A, HUVEC were transiently transfected with wild-type Tie2-luc or an identical construct containing a point mutation of the GATA motifs (Tie2 (GATAmut)-luc). Cells were co-transfected with pRL-CMV to normalize for transfection efficiency. *, \( p < 0.005 \) compared with the activity from wild-type Tie2-luc. B, HUVEC were treated either si-control or si-GATA3 and transiently transfected with wild-type Tie2-luc or Tie2 (GATAmut)-luc. Cells were co-transfected with pRL-CMV to normalize for transfection efficiency. *, \( p < 0.01 \) compared with the activity from wild-type Tie2-luc with si-control treatment. N.S., nonsignificant. C, COS-7 cells were transiently co-transfected with Tie2-luc or Tie2 (GATAmut)-luc and expression plasmids for human GATA3 (pcDNA3-GATA3) or control mock vector (pcDNA3). Data represent mean ± S.D. (relative to the control (pcDNA3)) obtained in triplicate from at least three independent experiments. *, \( p < 0.01 \) compared with control. N.S., nonsignificant.
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AKT levels (Fig. 6A, lanes 1–3). This effect was inhibited by 90% in si-GATA3-treated cells (Fig. 6A, lanes 2 and 4). Total AKT levels were unaffected by treatment with Ang-1 or transfection with siRNA. Thus, GATA3 deficiency attenuates Ang-1-Tie2-mediated activation of AKT.

Next, we wished to determine whether the effect of GATA3 on Tie2 expression and signaling influenced Ang-1-mediated endothelial cell function. First, we carried out cell migration assays using a modified Boyden chamber. Fluorescently labeled HUVEC transfected with control siRNA or siRNA against GATA3 were plated in the upper chamber. The addition of Ang-1 or VEGF in the lower chamber resulted in a 2.6- and 3.6-fold increase in migration of control siRNA-transfected cells, respectively (Fig. 6B). In contrast, Ang-1 (but not VEGF) failed to induce the migration of GATA3 siRNA-treated HUVEC (Fig. 6B). Thus, GATA3 knockdown blocks Ang-1-Tie2-mediated endothelial cell migration.

We performed sandwich tube formation assays of HUVEC co-cultured with fibroblasts. Fluorescently labeled HUVEC were incubated in a collagen gel, and the resulting mixture was overlaid with a fibroblast-containing collagen gel. As shown in Fig. 6C, Ang-1 treatment of HUVEC transfected with control

FIGURE 5. GATA3 and ELF1 physically interact and synergize to activate the Tie2 promoter in primary human endothelial cells. A, COS-7 cells were transiently co-transfected with Tie2-luc and expression plasmids for human GATA3 (pcDNA3-GATA3), human ELF1 (pCI-ELF1), and control vector (pCI) alone or in combination. Data represent mean ± S.D. (relative to the control (pcDNA3 plus pCI)) obtained in triplicate from at least three independent experiments. *, p < 0.0001; #, p < 0.01 compared with the activity from pcDNA3 plus pCl-transfected cells or pcDNA3 plus pCI-ELF1-transfected cells, respectively. B, expression vector in combinations of either pcDNA3, pcDNA3-GATA3, pFLAG, or pFLAG-ELF1 were co-transfected into COS-7 cells. Extracted proteins from the cells were untreated (input) or precipitated by anti-FLAG antibody and then separated by 10% SDS-PAGE. The transferred membrane was immunoblotted (IB) with anti-GATA3 (upper) or FLAG (lower) antibody. The arrows indicate GATA3 and FLAG-tagged ELF1.

C, nuclear extracts from HUVEC were immunoprecipitated (IP) by either anti-ELF1 antibody or control IgG. Supernatant (1:20 volume, lane 1) and immunoprecipitated fractions (lanes 2 and 3) were separated by 10% SDS-PAGE and immunoblotted with anti-GATA3 and anti-ELF1 antibodies. The arrows indicate GATA3 and ELF1.
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A

![Western Blot Image]

- **M.W. Marker (kDa):**
  - Minus: 1, 2, 3, 4
  - Plus Ang-1: 1, 2, 3, 4

- **Markers:**
  - P-AKT
  - Total AKT

B

- **Migration Level (pixels/10^5 area):**
  - SiRNA Control GATA3 Minus
  - SiRNA Control GATA3 Ang-1
  - Control GATA3 VEGF

- **Significance Levels:**
  - N.S.
  - *
  - #
siRNA promoted migration of endothelial cells toward the fibroblast layer, leading to the formation of capillary-like cords or tubes. Quantitation revealed a 3.1-fold increase in tube length in Ang-1-versus control-treated cells. siRNA-mediated knockdown of GATA3 resulted in abrogation of Ang-1-mediated tube formation (Fig. 6C). In contrast, GATA3 knockdown had no effect on VEGF-induced tube formation (Fig. 6C).

Ang-1-Tie2-AKT signaling has been shown to promote endothelial cell survival (37). To determine whether GATA3 plays a role in this pathway, HUVEC were transfected with si-control and si-GATA3, preincubated in serum starvation medium (0.5% FBS) for 18 h, serum-starved for an additional 24 h in the absence or presence of Ang-1, and then assayed for annexin V staining using fluorescence-activated cell sorter. As shown in Fig. 6D, 18 h of preincubation had little effect on si-control- and si-GATA3-transfected cells, whereas an additional 24 h of serum starvation resulted in marked apoptosis (93 and 88% annexin V-positive cells, respectively). Apoptosis of si-control- but not si-GATA3-transfected cells was significantly attenuated by Ang-1 (Fig. 6D). In addition, we performed TUNEL staining as a late apoptotic marker. Serum starvation of si-control- and si-GATA3-transfected endothelial cells resulted in 68 and 73% TUNEL positivity, respectively (supplemental Fig. VI). Ang-1 treatment inhibited the TUNEL-positive rate in si-control cells (97%) but not cells transfected with si-GATA3 (supplemental Fig. VI). Collectively, these findings suggest that GATA3 is a critical determinant of Ang-1-Tie2-mediated angiogenic signaling.

**GATA3 Mediates Anti-inflammatory Effect of Ang-1-Tie2 in Cultured Human Primary Endothelial Cells—** Consistent with previous studies (19, 34, 38), VEGF treatment induced VCAM-1 expression (Fig. 7A). Ang-1 significantly attenuated the VEGF stimulation of VCAM-1 in si-control-transfected cells (82%) but not si-GATA3-transfected cells (Fig. 7A). This effect of GATA3 knockdown was...
rescued by Ad-mediated expression of siRNA-resistant GATA3 protein (Fig. 7A). Incubation of HUVEC with VEGF promoted U937 monocyte adhesions (Fig. 7B). Ang-1 treatment effectively (74%) reduced VEGF-stimulated monocyte adhesion to control cells but had no effect on GATA3-deficient cells (Fig. 7B).

Compared with VEGF, thrombin and TNF-α induce higher levels of cell adhesion molecules in endothelial cells (34). Interestingly, TNF-α and thrombin have been shown to reduce the GATA3 expression in endothelial cells (39). We have observed similar results with TNF-α and thrombin in our DNA microarrays (data not shown). To confirm these findings, HUVEC were treated with 10 ng/ml TNF-α or 1.5 units/ml thrombin for 18 h and assayed for mRNA expression using real time PCR. As shown in Fig. 7C, both activation agonists resulted in a marked reduction in GATA3 mRNA levels. In keeping with the findings of this study, TNF-α and thrombin also reduced Tie2 expression (Fig. 7D). In contrast, neither agonist had an effect on NER2F or ELF1 levels.

FIGURE 6—continued

GATA3 Regulates Tie2 Expression and Function

GATA factors have been shown to play an important role in lineage determination. Indeed, gene disruption studies have shown that all GATA proteins with the exception of GATA5 are required for development (reviewed in Refs. 1, 2, 40). GATA2 is essential for hematopoiesis, GATA3 for central nervous development and fetal liver hematopoiesis, GATA4 for ventral body plan and formation of linear heart tube, and GATA6 for differentiation of visceral mesoderm and morphogenetic patterning of cardiac outflow tract and large arteries (1, 2, 40).

Previous studies have demonstrated that HUVEC express GATA2, GATA3, and GATA6 (41). In addition, GATA2 and GATA3 were identified in human coronary artery endothelial cells (42). Interestingly, GATA3 was reported to be expressed at higher levels in retinal endothelial cells compared with brain capillary endothelial cells (43). Our data indicate that GATA3 is preferentially expressed in endothelial cells derived from large vessels.

GATA2 has been implicated in the regulation of several genes in endothelial cells (9–14). However, with the possible exception of von Willebrand factor (44) and VCAM-1 (41), there is no information about GATA3 or GATA6 target genes in endothelial cells. This study is the first to comprehensively survey endothelial cells for GATA3-responsive transcripts. GATA3 knockdown resulted not only in the down-regulation of many genes but also in the induction of certain transcripts, suggesting that this transcription factor may act both as a positive and negative transacting factor in endothelial cells. In preliminary experiments using HUVEC, we have shown that the expression of some genes is affected by siRNA-mediated knockdown of any of the three GATA factors, whereas the expression of other genes is influenced only by combined knockdown of GATA factors (data not shown). These findings suggest that several GATA target genes were overlapped, but other targets were distinctly regulated by each GATA factor.

Tie2 expression is restricted to endothelial cells. Thus, an understanding of its transcriptional control mechanisms may provide important insights into lineage determination. We have shown that GATA3 binds to and activates the Tie2 promoter, induces expression of Tie2 protein and mRNA, and thus regulates Ang-1-Tie2 signaling. Our data point to a physical interaction and functional synergism between GATA3 and the Ets factor ELF. Both GATA3 and ELF are expressed in multiple cell types. In fact, no transcription factor is unique to the endo-
FIGURE 7. GATA3 and Tie2 regulate Ang-1-mediated anti-inflammation in HUVEC. A, quantitative real-time PCR analysis of VCAM-1 mRNA in HUVEC transfected with si-control or si-GATA3 or infected with Ad-control or Ad-GATA3 and treated in the absence or presence of VEGF or Ang-1. The results are expressed as mean ± S.D. from three independent experiments. *, p < 0.001 compared with VEGF treatment minus Ang-1 in each condition. N.S., nonsignificant. B, top, monocyte adhesion assay in HUVEC treated as shown in A. The data are representative from three independent optical images derived from two independent experiments. Adherent monocytes are shown in red. Bar, 100 μm. Bottom, quantification of the adherent monocytes. The mean ± S.D. values were calculated using cell image analyzer from six optical images. *, p < 0.01 compared with VEGF treatment minus Ang-1 in each condition. N.S., nonsignificant. C, quantitative real-time PCR analyses of GATA3, NERF2, and ELF1 mRNA levels in HUVEC treated for 18 h in the absence or presence of 10 ng/ml TNF-α or 1.5 units/ml thrombin. The results are representative of three independent experiments. *, p < 0.0001; **, p < 0.01; ***, p < 0.05 compared with minus stimulus. D, Western blot analysis of Tie2 protein levels in HUVEC treated for 18 h in the absence or presence of 10 ng/ml TNF-α or 1.5 units/ml thrombin. β-Actin is shown as loading control. The results are representative of three independent experiments. E, Western blot analysis of Tie2, GATA3, and β-actin in HUVEC infected with Ad-Control or Ad-GATA3 and then treated in the absence or presence of 10 ng/ml TNF-α for 18 h. The results are representative of three independent experiments.
GATA3 Regulates Tie2 Expression and Function

A prevailing view is that EC-specific gene expression is mediated by combinations of otherwise nonspecific transacting proteins. For example, De Val et al. (45) recently demonstrated a role for FoxC2 and the Ets factor, ETV2, in mediating cell type-specific expression in the endothelium. However, compared with ELF1, ETV2 is expressed at very low levels in HUVEC and HDMVEC (supplemental Fig. VII). Future studies will be required to determine whether GATA3-ELF1 plays a role in mediating lineage specificity of Tie2 in vivo.

Tie2 expression was observed in all endothelial cell types (see Fig. 1A). Compared with large vessel endothelial cells, dermal microvascular endothelial cells express low levels of GATA3 and high levels of GATA2. Based on these findings, we hypothesized that GATA2 rather than GATA3 may play a primary role in mediating Tie2 expression in microvascular endothelial cells. Indeed, GATA2 knockdown in HDMVEC and human dermal lymphatic endothelial cells resulted in >90% reduction in Tie2 mRNA levels (supplemental Fig. VIII shows HDMVEC). Moreover, GATA2 knockdown in HDMVEC inhibited Ang-1-mediated phosphorylation of AKT, cell migration, and tube formation (supplemental Fig. VIII). Together, these data raise the interesting possibility that Tie2 expression is regulated by different GATA factors in distinct blood vessel types. Definitive evidence for such a model will require generation and analysis of mice with conditional endothelial cell-specific knock-out of GATA2 and/or GATA3.

The GATA family of transcription factors has been implicated not only in the early differentiation of the endothelium (2) but also in the transduction of extracellular signals. For instance, insulin-like growth factor-1, low density lipoprotein, VEGF, TNF-α, and thrombin have each been shown to induce GATA2 activity at a post-transcriptional level without significantly changing GATA2 mRNA levels (14, 46, 47), whereas estrogen and transforming growth factor-β inhibit GATA2 binding (48, 49). In contrast to GATA2, we have shown that inflammatory agonists such as TNF-α and thrombin down-regulate GATA3 expression at an mRNA level. Together, these studies suggest that GATA transcription factors may behave like immediate early genes, serving to couple short changes in the extracellular environment to long term changes in downstream gene expression.

The Ang-Tie2 signaling axis plays a critical role in maintaining vascular integrity. Based on our results, it is interesting to speculate that in certain pathological states (e.g. sepsis, atherosclerosis or cancer), inflammatory mediators may result in GATA3-dependent inhibition of Ang-1-Tie2 signaling, thus contributing to endothelial cell dysfunction. It follows that therapies designed to maintain GATA3 activity might help to blunt the deleterious effects of inflammation on the endothelium.

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