THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 42, pp. 29109–29124, October 16, 2009

Critical Role for GATA3 in Mediating Tie2 Expression and Function in Large Vessel Endothelial Cells*[S]

Received for publication, July 4, 2009, and in revised form, August 10, 2009 Published, JBC Papers in Press, August 12, 2009, DOI 10.1074/jbc.M109.041145

Haihua Song[‡], Jun-ichi Suehiro[‡], Yasuharu Kanki[‡], Yoshiko Kawai[§], Kenji Inoue[¶], Hiroyuki Daida[¶], Kiichiro Yano[‖], Toshio Ohhashi[§], Peter Oettgen[**], William C. Aird[‖1], Tatsuhiko Kodama[‡ ‡‡], and Takashi Minami[‡ ‡‡2]

*From the [‡]Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo 153-8904, Japan, [‡‡]Translational Systems Biology and Medicine Initiative, The University of Tokyo, Tokyo 113-8655, Japan, the [‖]Department of Molecular and Vascular Medicine, Beth Israel Deaconess Medical Center/Harvard Medical School, Boston, Massachusetts 02215, the [§]Department of Physiology, School of Medicine, Shinshu University, Matsumoto 390-8621, Japan, the [¶]Department of Cardiovascular Medicine for Diseases of Old Ages, Juntendo University School of Medicine, Tokyo 113-8421, Japan, and the **Division of Cardiology, Beth Israel Deaconess Medical Center/Harvard Institutes of Medicine, Boston, Massachusetts 02215*

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 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 WGATAR 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

* This work was supported, in whole or in part, by National Institutes of Health Grants PO1 HL76540 (to W. C. A. and P. O.) and R01HL082927 (to W. C. A.) from NHLBI. This work was also supported by the fund for Science and Technology from the Ministry of Education, Culture, Sports, Science, and Technology in Japan and in part by Takeda Science Foundation in Japan (to T. M.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. I–VIII and Table I.

[1] To whom correspondence may be addressed: Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, MA 02215. E-mail: waird@bidmc.harvard.edu.

[2] To whom correspondence may be addressed: The Research Center for Advanced Science and Technology, the University of Tokyo, 4-6-1 Komaba, Meguro Tokyo 153-8904, Japan. Tel./Fax: 81-3-5452-5403; E-mail: minami@med.rcast.u-tokyo.ac.jp.

[3] The abbreviations used are: VCAM, vascular cell adhesion molecule; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial cell growth factor; Ang, angiopoietin; TNF, tumor necrosis factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyltransferase-dUTP nick end labeling; siRNA, small interfering RNA; EMSA, electrophoretic gel mobility shift assay; HCAEC, human coronary artery endothelial cell; HDMVEC, human dermal microvascular endothelial cell; HPAEC, human pulmonary artery endothelial cell; ChIP, chromatin immunoprecipitation; Ad, adenovirus.

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 (HDMVEC), 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 (JCRB-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, GCAGGGCAGUAUCAUGAAGCCUAA, and oligo2, GGCAUGAAGGAUGCCAAGAAGUUUA). 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 pIRES2-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 digestions 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-^{32}P]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). For co-immunoprecipitation assays, COS-7 cells were co-transfected with pFLAG-ELF1 and pcDNA3-GATA3 expression plasmids using the FuGENE-HD reagent (Roche Applied Science) as instructed by the manufacturer. Two days later, the transfected cells were lysed, and extracts were prepared as described previously (30). The extracts were incubated with anti-FLAG antibody (Sigma) overnight at 4 °C. The resulting mixture was incubated with protein G-Sepharose (Amersham Biosciences), washed, and separated on 10% SDS-polyacrylamide gel. The gel was transferred to the membrane, which was incubated either with anti-FLAG antibody or anti-GATA3 antibody (R & D Systems). Alternatively, confluent HUVEC (2.5×10^7 cells) were harvested for nuclear extract according to the nitrogen cavitation method to preserve protein-protein associations (30, 32). Total nuclear extract was incubated with 30 μg of anti-ELF1 antibody (Santa Cruz Biotechnology) or rabbit control IgG (Santa Cruz Biotechnology) overnight at 4 °C. The immunoprecipitated samples were separated with 10% SDS-PAGE and immunoblotted with an anti-GATA3 antibody (R & D Systems) and anti-ELF1 antibody (Santa Cruz Biotechnology).

Sandwich Tube Formation 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 PKH2 (Sigma). Cells were re-seeded in 24-well plates (1×10^5 cells/well) containing type I collagen gel (Koken, Tokyo, Japan) and EBM-2 basal media. The resulting mixture was overlaid with a second layer of collagen gel containing 5×10^4 fibroblasts per well. The double-layered sandwich gel was then incubated with 500 μl of EBM-2 in the presence or absence of 500 ng/ml Ang-1 or 50 ng/ml VEGF. Two days later, a branched capillary network was visualized under a fluorescent microscope. Images from at least three different areas in each well were captured and measured by using 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 Biocoat 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).

Immunofluorescent Studies—HUVEC or frozen tissue sections (10 μm) obtained from 8-week-old female mice (C57/BL6j) were fixed and incubated with a rat monoclonal anti-PECAM1 antibody (1:50 dilution) (Pharmingen, San Diego, CA) or a goat polyclonal anti-GATA3 antibody (8 μg/ml) (R & D Systems) overnight at 4 °C. Sections were washed three times in PBS and incubated with secondary antibody labeled with Alexa-Fluor 647 (for GATA3) or Alexa-Fluor 488 (for PECAM1) (1:50 dilution) (Invitrogen) for 1 h at room temperature. The slides were then washed in PBS, mounted in ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen), and examined by fluorescence microscopy.

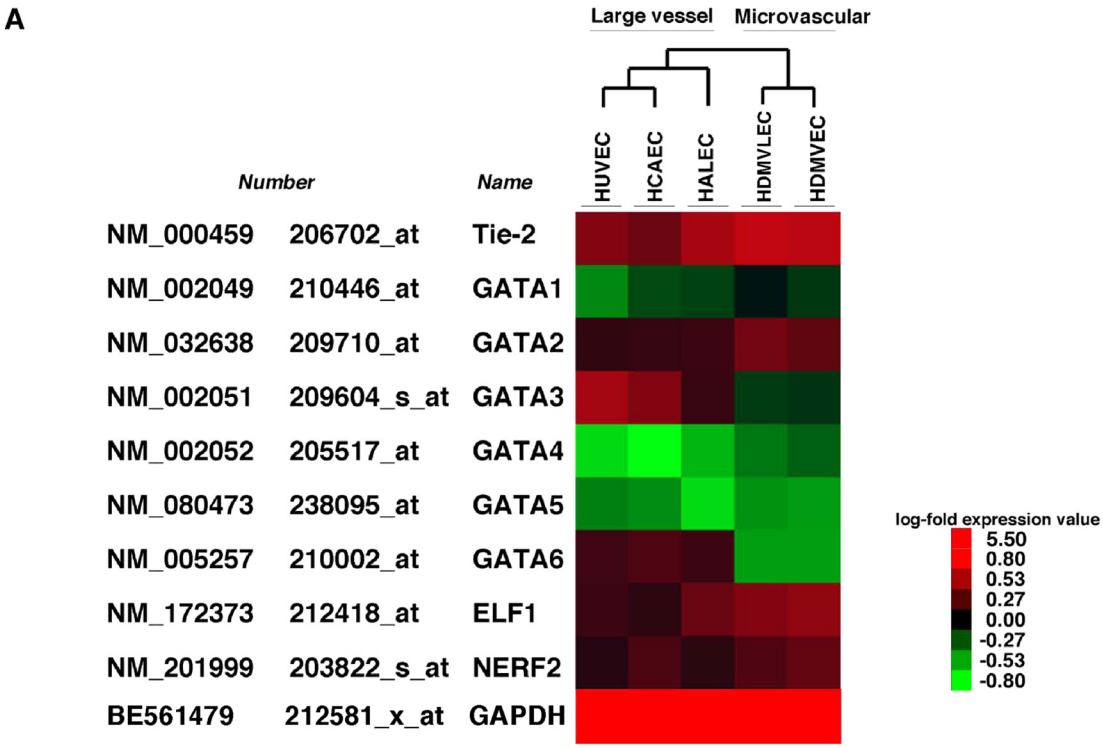
Cell Survival Studies—Endothelial cells were treated with siRNAs for 24 h and then incubated with EBM-2 basal media (Clonetics) containing 0.5% FBS for 18 h. Subsequently, cells were incubated with Dulbecco's modified Eagle's medium containing 0.5% FBS in the presence or absence of Ang-1 for 24 h. Cells 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). In brief, confluent HUVEC were pretreated with or without 500 ng/ml Ang-1 and then treated with 50 ng/ml VEGF for 6 h. PKH-26 (Sigma)-labeled U937 cells were added to HUVEC-seeded plate. 90 min later, cells were washed and examined by fluorescent microscopy.

Statistics—Data are shown as mean ± S.D. p values were calculated using two-tailed unpaired Student's t test. $p < 0.05$ was considered significant.

RESULTS

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. 1*A* and supplemental Fig. I). 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. 1*B*). In contrast to large vessel endothelial cells, human dermal microvascular endothelial


A
Large vessel
Microvascular
HUVEC
HCAEC
HALEC
HDMVLEC
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Number
Name
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0.00
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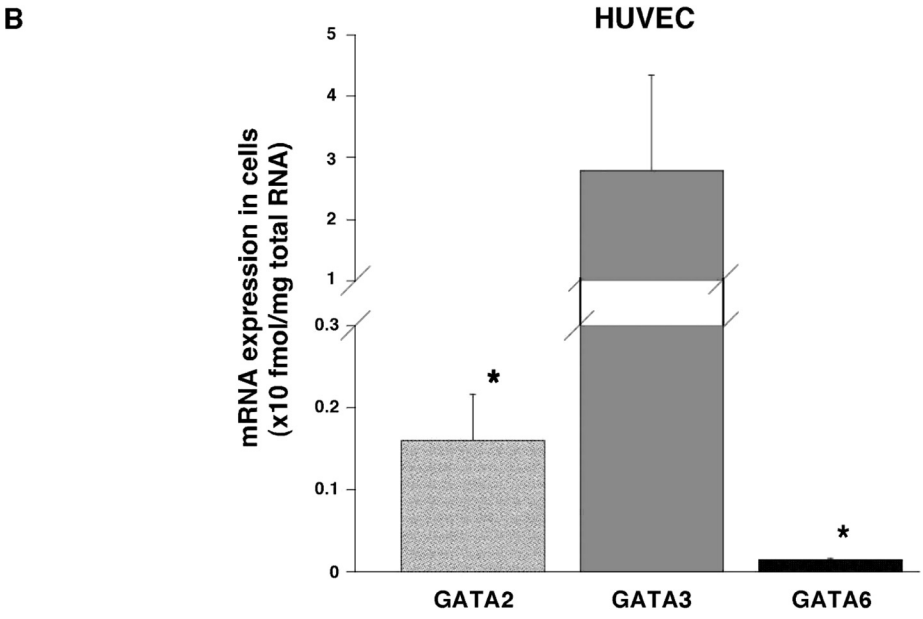
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HUVEC
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(x10 fmol/mg total RNA)
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3
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1
0.3
0.2
0.1
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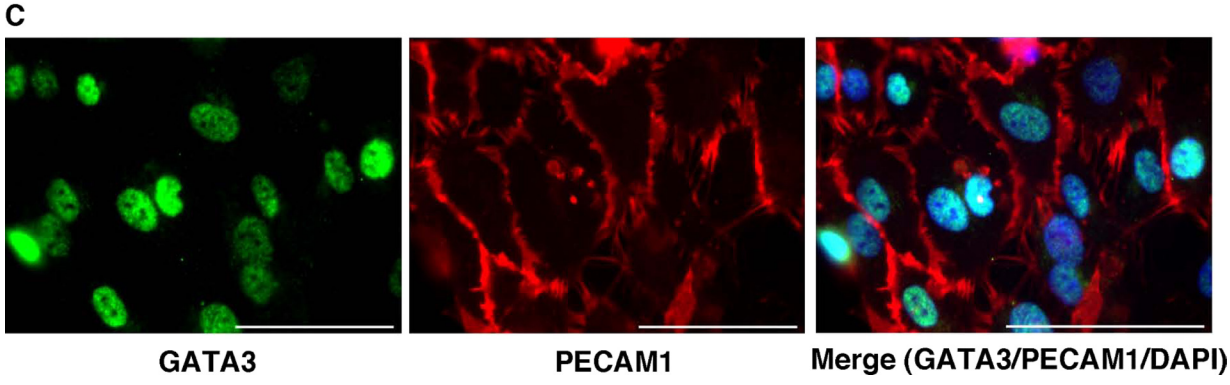
*
GATA2
GATA3
GATA6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GATA3
PECAM1
Merge (GATA3/PECAM1/DAPI)

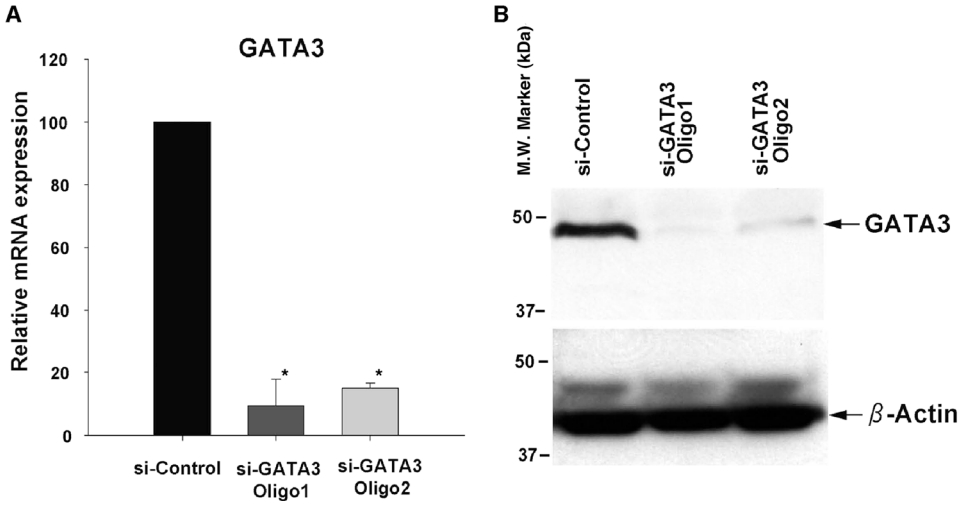


FIGURE 2. **siRNA-mediated knockdown of GATA3 in primary human endothelial cells results in altered transcriptional profile.** *A,* quantitative real time PCR analyses of GATA3 mRNA in HUVEC transfected with si-control and two independent siRNAs against GATA3 (*Oligo1* and *Oligo2*). Data are expressed as mean ± S.D. of expression levels relative to cyclophilin A obtained in triplicate from at least three independent experiments. *, $p < 0.001$ compared with si-control. *B,* Western blot analysis of HUVEC transfected with si-control and two independent siRNAs against GATA3 (*Oligo1* and *Oligo2*). The membrane was immunoblotted with anti-GATA3 antibody. To control for loading, the membrane was stripped and re-probed with anti-β-actin antibody. The results are representative of four independent experiments. *C,* heat map from DNA microarrays of HUVEC transfected 2 days earlier with si-control or one of two independent siRNAs against GATA3 (Oligo1 and Oligo2). The results show the up-regulated and down-regulated gene clusters using the Eisen cluster software. Color intensity is relative to the median (*black*). *D,* functional annotation of GATA3-regulated genes in HUVEC. The data were filtered for genes demonstrating >2.5-fold up-regulation or down-regulation in si-GATA3-transfected cells and subjected to Gene Ontology analysis.

cells demonstrated low levels of GATA3 mRNA (supplemental Fig. I). Consistent with the mRNA data, Western blot analyses of HUVEC revealed higher levels of GATA3 protein (≈10-fold) compared with GATA2 or GATA6 (supplemental Fig. II). In immunohistochemical analyses of HUVEC, GATA3 was localized primarily in the nucleus (Fig. 1*C*). 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).

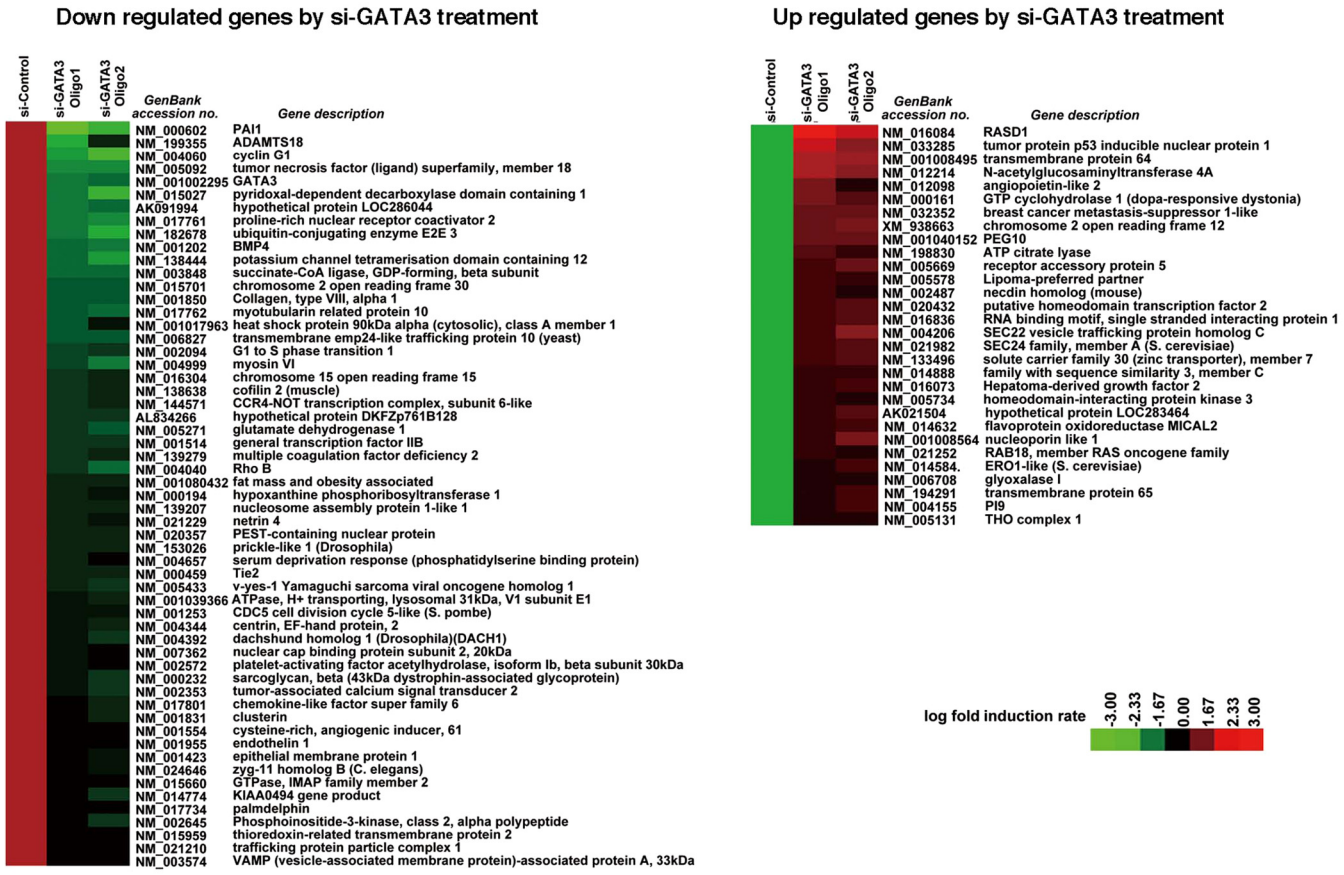
GATA3 Knockdown in Cultured Human Primary Endothelial Cells Results in an Altered Transcriptional Profile—To determine the functional relevance of GATA3 in endothelial cells, we employed siRNA to knock down expression of the transcription factor in HUVEC. As shown in Fig. 2*A*, transfection of HUVEC with two independent siRNAs (si-GATA3-a and si-GATA3-b) against GATA3 resulted in 90 and 82% reduction of GATA3 mRNA, respectively, compared with si-control. siRNA treatment also resulted in significant inhibition of GATA3 protein expression (Fig. 2*B*). To identify potential GATA3 target genes, we carried out DNA microarrays with HUVEC transfected with si-control, si-GATA3-*oligo1*, or si-GATA3-*oligo2*. The data were filtered for those genes that were >2.5-fold up-regulated or down-regulated in response to the two independent GATA3 siRNAs, compared with control siRNA. A total of 57 genes were down-regulated and 20 genes up-regulated in GATA3-deficient HUVEC (Fig. 2*C*). Functional annotation, using Gene Ontology (35), revealed three predominant gene clusters as follows: signaling/transcription, metabolism, and angiogenesis (Fig. 2*D*). For example, siRNA-mediated knockdown of GATA3 resulted in increased mRNA expression of Ang-like (Angptl)-2 and Ras-derived (RASD)-1 and decreased expression of plasminogen activator inhibitor-1, bone morphogenetic protein-4, endothelin-1, and Tie2. These changes were validated using quantitative real time PCR (supplemental Fig. IV and discussed below). Thus, GATA3 is involved in mediating the expression of functionally relevant target genes in endothelial cells.

GATA3 Mediates Expression of Tie2 and Binds to the Proximal Promoter Region in Cultured Human Primary Endothelial 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 quantitate Tie2 expression in si-control- and si-GATA3-transfected HUVEC. As shown in Fig. 3*A*, 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. 3*B*).

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 closely aligned clusters of three GATA sites and six Ets-binding sites in the first exon (Fig. 3*C*). To determine whether GATA transcription factors bind to this region, we carried out EMSA using a radiolabeled oligonucleotide probe that spans the consensus GATA motifs (Fig. 3*C*, *hatched rectangle*) and recombinant GATA protein. As shown in Fig. 3*D*, incubation of the DNA probe with recombinant GATA3 (but not control lysate) yielded a specific DNA-protein complex (Fig. 3*D, lanes 1* and *2*, *arrow*). The DNA-protein complex was inhibited by the addi-

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 (*black*). *HDMLVEC*, human dermal microvascular lymphatic endothelial cells. *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 μm.

C



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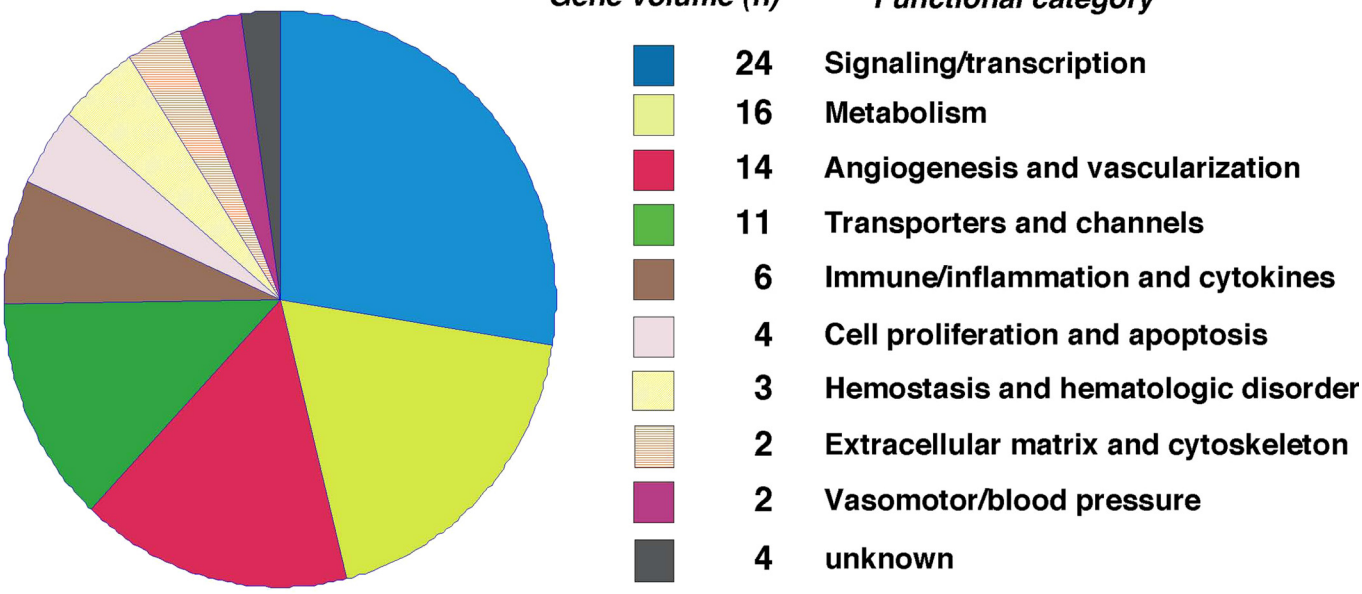


Up-and down-regulated genes cluster

FIGURE 2—*continued*

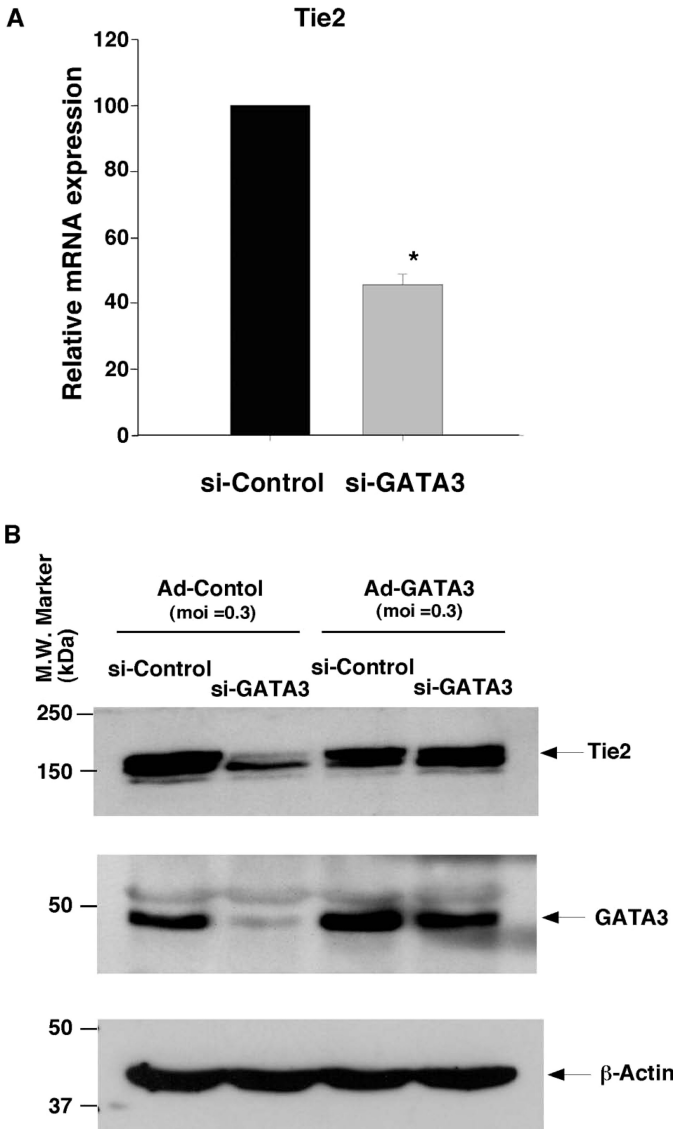


FIGURE 3. **GATA3 binds to tandem GATA motifs in the *Tie2* gene in primary human endothelial cells.** *A,* quantitative real time PCR analyses of *Tie2* mRNA in HUVEC transfected with si-control and siRNAs against GATA3 (Oligo1). Data are expressed as mean ± S.D. of expression levels relative to si-control 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 (Oligo1). 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. *C,* schematic representation of the highly conversed GATA- and Ets-binding motifs in the first exon of the *Tie2* gene. *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 ^{32}P-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 with (*lane 4*) or without (*lane 3*) a mutation of the GATA motifs. Alternatively, the reaction mixture was preincubated with antibodies against GATA3 (*lane 5*), or control IgG (*lane 6*). The *arrow* indicates specific DNA-protein complex. The *asterisk* indicates super-shifted complex. The results are representative of at least three independent experiments. *E,* EMSA was performed with nuclear extracts from HUVEC. Binding reactions were performed in the presence of 100-fold molar excess of unlabeled wild-type probe without (*lane 2*) or with (*lane 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 *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 I. 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.

tion of 100-fold molar excess of unlabeled self-competitor but not by a similar concentration of self-competitor containing point mutations of the three GATA motifs (Fig. 3*D*, *lanes 3* and *4*). Moreover, the DNA-protein complex was super-shifted in the presence of anti-GATA3 antibody but not isotype-matched control IgG (Fig. 3*D*, *lanes 5* and *6*). To determine which of the three GATA sites contributed to GATA binding, 100-fold molar excess of cold competitor containing a single or double mutation of the GATA motifs was added to the reaction mixture. Each combination of GATA mutations inhibited the specific DNA-protein complex (supplemental Fig. V). Thus, all three GATA sites are essential for stable GATA-DNA interaction.

Incubation of the GATA site-containing *Tie2* probe with nuclear extracts from HUVEC also resulted in slowly migrating DNA-protein complexes (Fig. 3*E, lane 2, hatched rectangle*). The highest of these bands demonstrated a similar motility to that obtained with *in vitro* translated recombinant GATA3. The DNA-protein complexes were inhibited in the presence of 100-fold molar excess of self-competitor but not mutant competitor (Fig. 3*E, lanes 3* and *4*). Preincubation with anti-GATA3 antibody, but not control IgG, resulted in the appearance of a pronounced super-shifted complex (Fig. 3*E, lanes 5* and *6, asterisk*). To provide further evidence for an interaction between GATA3 and the Tie2 promoter, we carried out ChIP assays. In HUVEC, a region spanning the GATA motif in exon 1 of *Tie2* was immunoprecipitated using anti-GATA3 antibody (Fig. 3*F*). As a negative control, the GATA3 antibody failed to immunoprecipitate a region in intron 1 that lacks a GATA consensus element (Fig. 3*F*). Together, these data suggest that GATA3 binds to the *Tie2* promoter in HUVEC.

GATA3 Transactivates the Tie2 Promoter in Cultured Human Primary Endothelial Cells—To verify a functional role for GATA DNA-protein interactions in mediating Tie2 expression, we carried out transient transfection assays in HUVEC using wild-type *Tie2* promoter sequence coupled to luciferase (Tie2-luc) or an identical construct containing a point mutation of one or more of the GATA motifs. Compared with the wild-type promoter, the triple GATA mutant (Tie2 (GATAmut)-luc) demonstrated a 60% reduction in reporter gene expression (Fig. 4*A*). In contrast, single mutations of the three GATA sites failed to alter promoter activity in HUVEC (data not shown). Consistent with these results, siRNA-mediated knockdown of GATA3 resulted in a significant reduction of wild type but not GATA mutant *Tie2* promoter activity (Fig. 4*B*). In co-transfection assays using COS-7 cells, GATA3 significantly induced *Tie2* promoter activity (3.5-fold) but failed to transactivate the GATA mutant promoter (Fig. 4*C*).

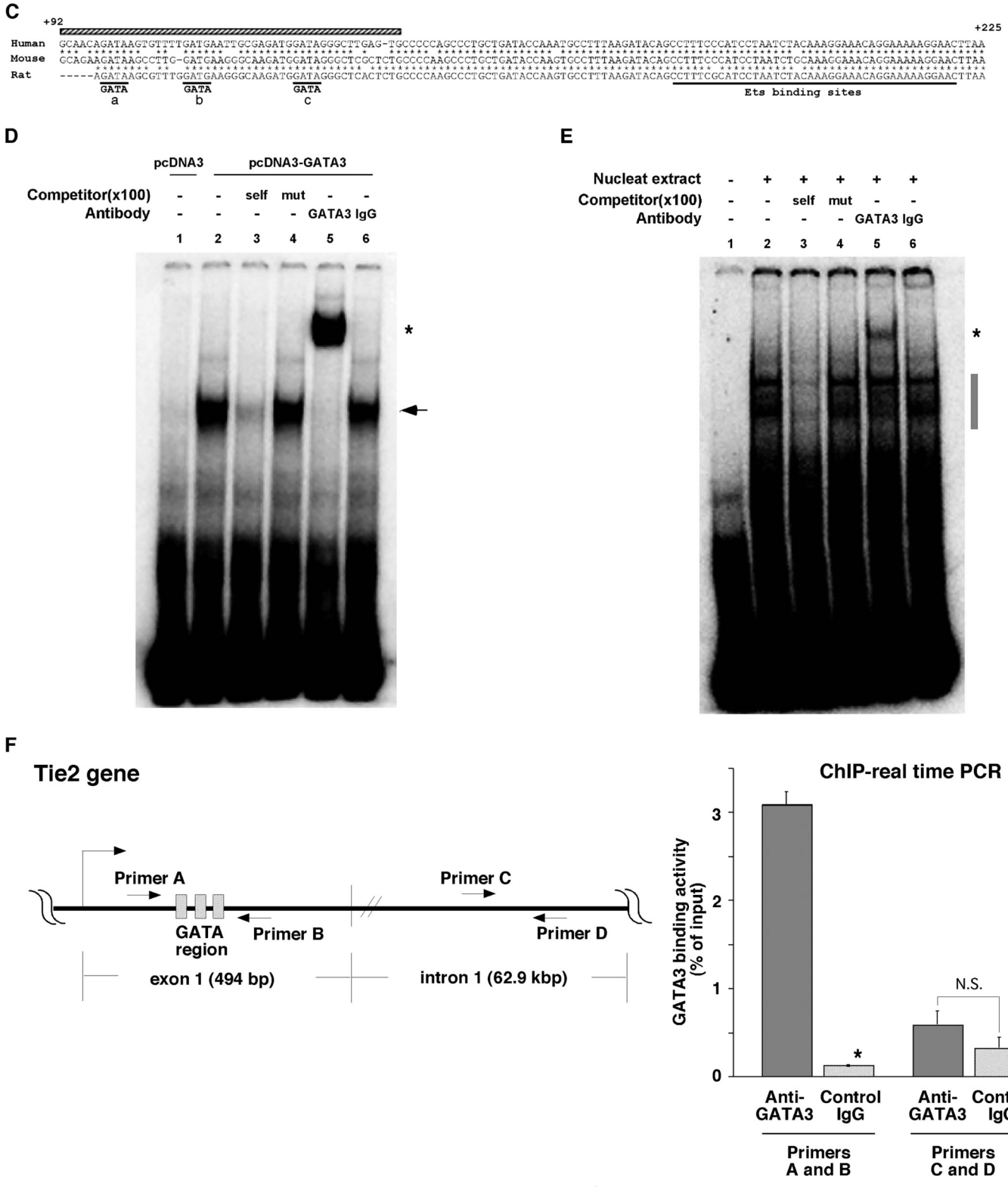


FIGURE 3—*continued*

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. 4*A*). 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. 1*A*). In co-transfection assays, ELF1 induced expression of the *Tie2* promoter by 6.8-fold (Fig. 5*A*). Importantly, co-transfection with ELF1 and GATA3 resulted in synergistic

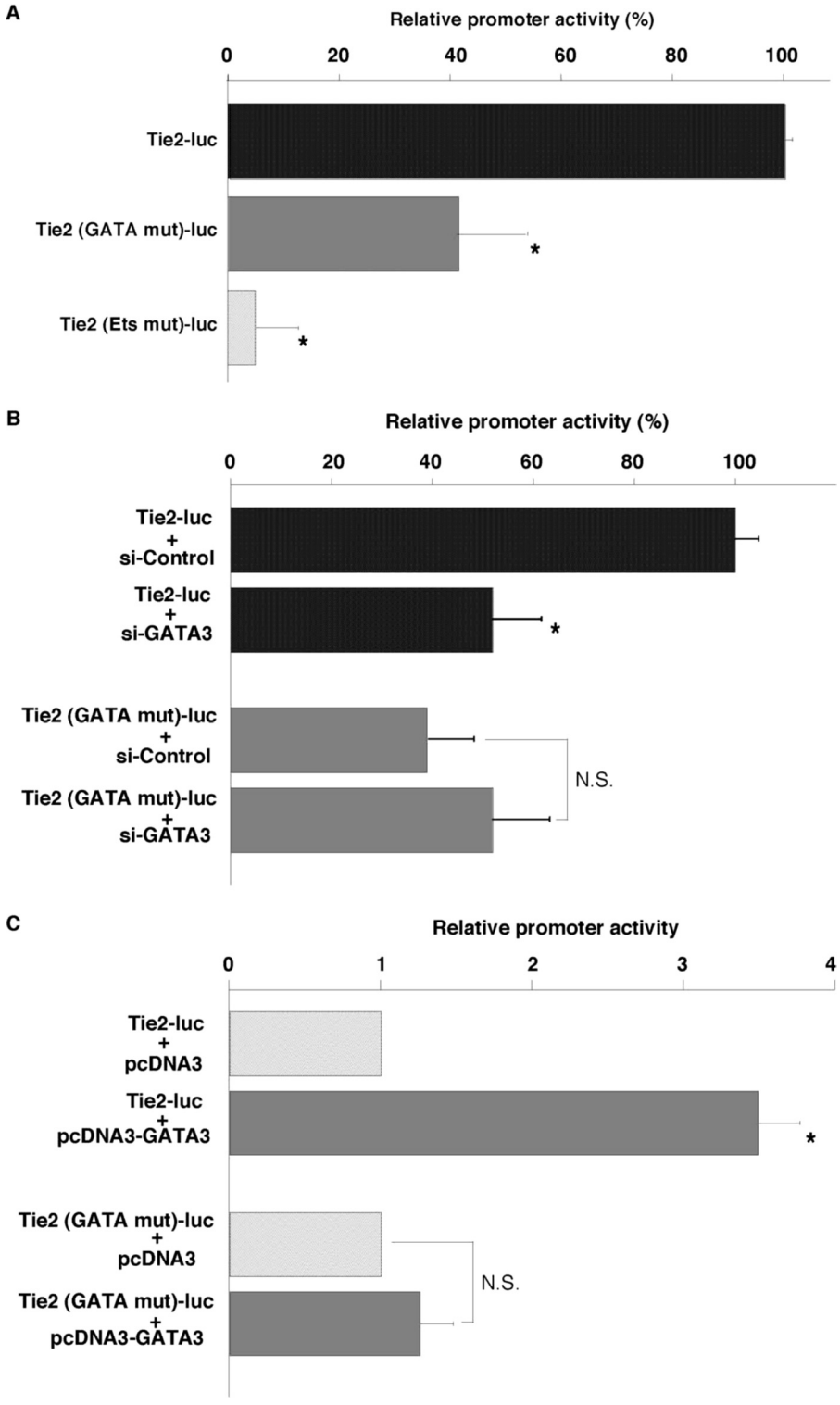


FIGURE 4. **GATA3 transactivates *Tie2* promoter in primary human endothelial cells.** *A,* HUVEC were transiently transfected with wild-type Tie2-luc or an identical construct containing a point mutation of the GATA motifs (Tie2 (GATAmut)-luc). Cells were co-transfected with pRL-CMV to normalize for 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.

activation (15.5-fold) of the *Tie2* promoter (Fig. 5*A*). 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. 5*B*, 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. 5*B*). 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. 5*C*, *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-

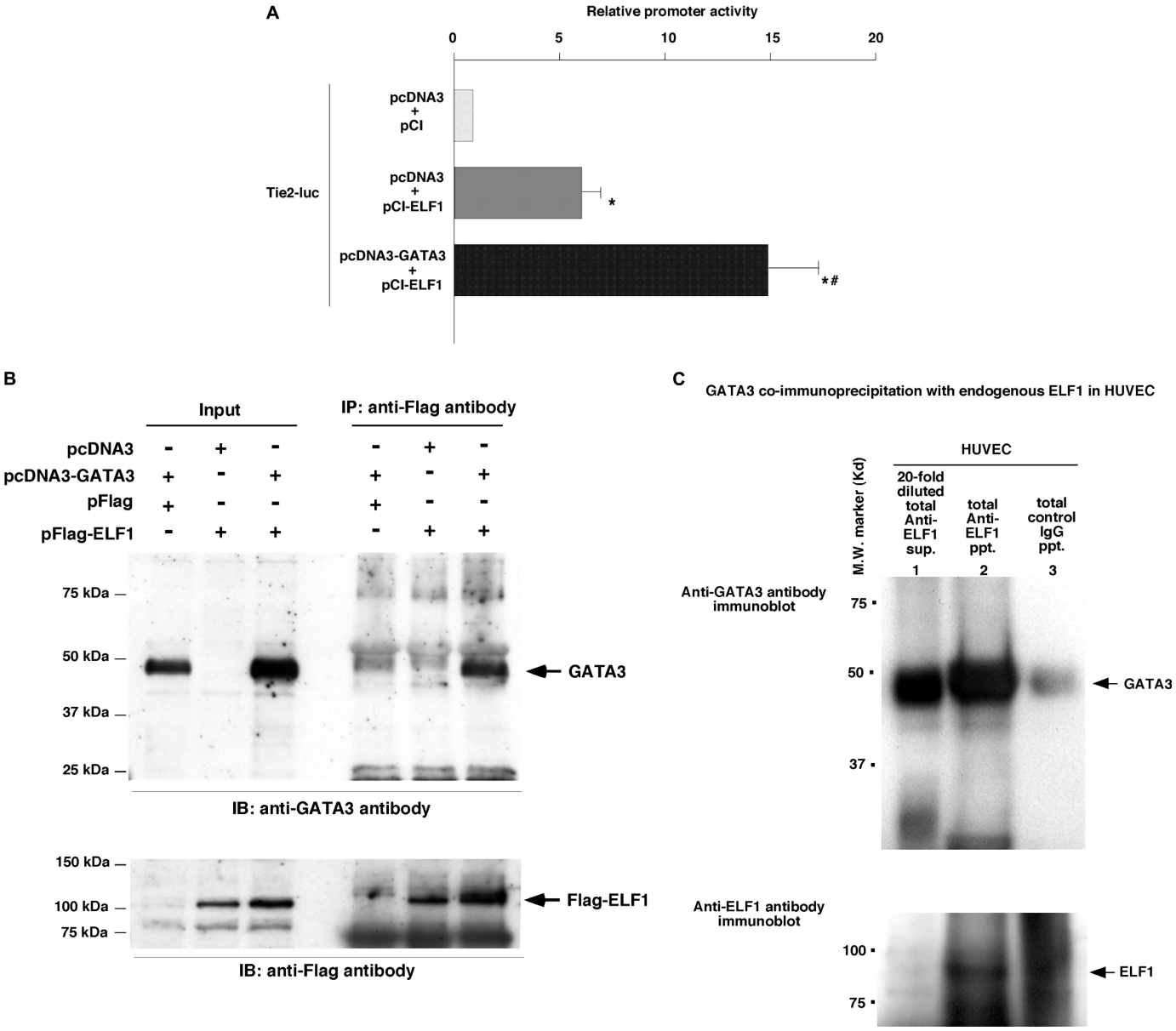
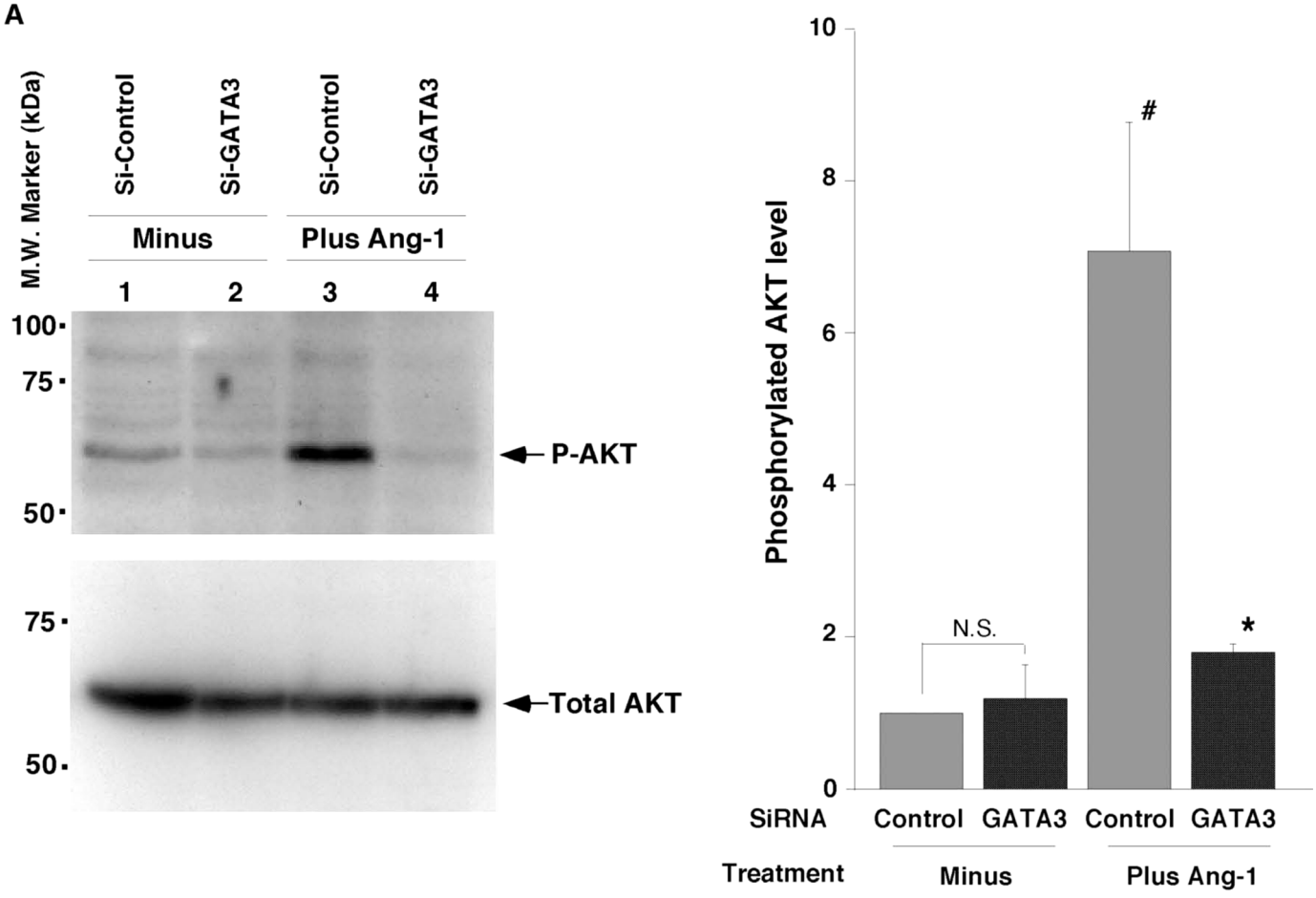


FIGURE 5. **GATA3 and ELF1 physically interact and synergize to activate the *Tie2* promoter in primary human endothelial cells.** *A*, COS-7 cells were transiently co-transfected with Tie2-luc and expression plasmids for human GATA3 (*pcDNA3-GATA3*), human ELF1 (*pCI-ELF1*), and control vector (*pCI*) 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 pCI-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.

AKT levels (Fig. 6*A*, *lanes 1–3*). This effect was inhibited by 90% in si-GATA3-treated cells (Fig. 6*A*, *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. 6*B*). In contrast, Ang-1 (but not VEGF) failed to induce the migration of GATA3 siRNA-treated HUVEC (Fig. 6*B*). 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. 6*C*, Ang-1 treatment of HUVEC transfected with control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M.W. Marker (kDa)
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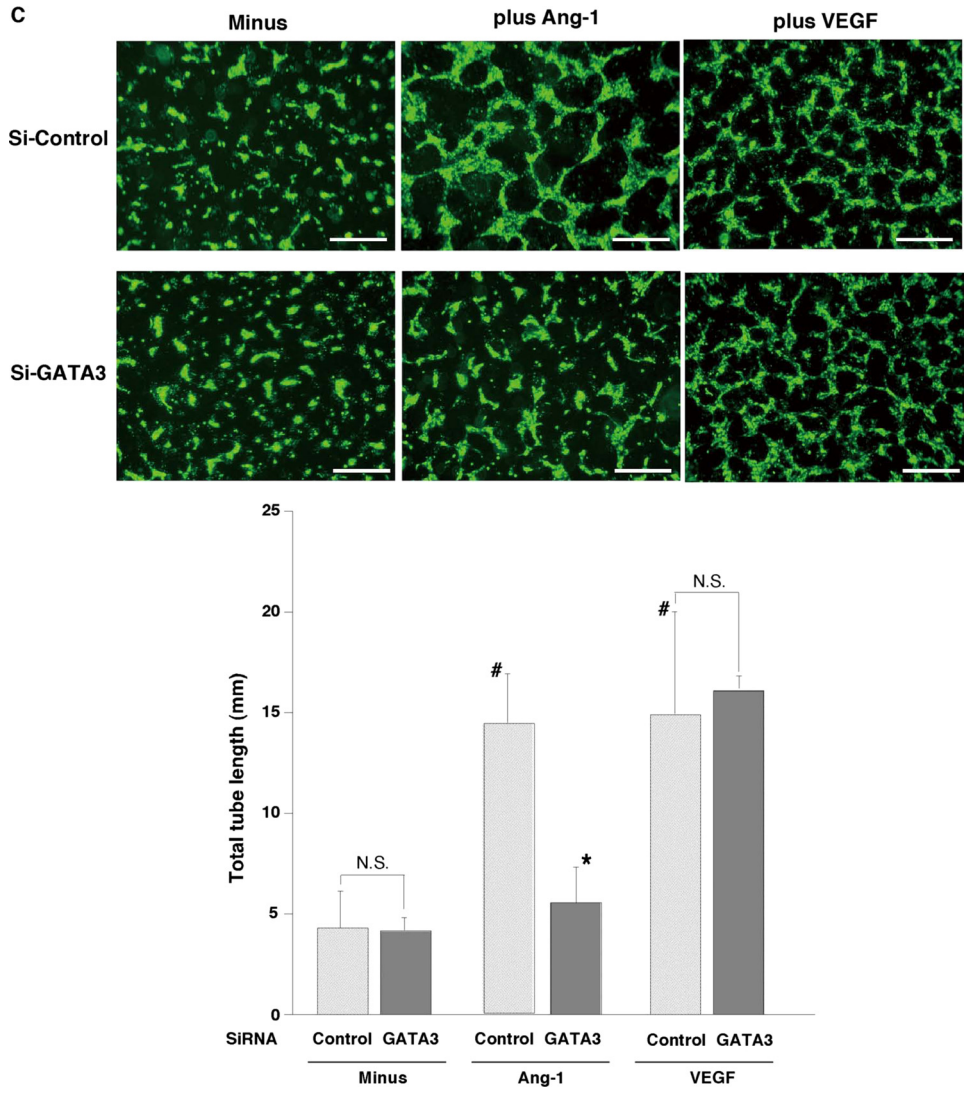

FIGURE 6—*continued*

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. 6*C*). In contrast, GATA3 knockdown had no effect on VEGF-induced tube formation (Fig. 6*C*).

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. 6*D*, 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. 6*D*). 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. 7*A*). Ang-1 significantly attenuated the VEGF stimulation of VCAM-1 in si-control-transfected cells (82%) but not si-GATA3-transfected cells (Fig. 7*A*). This effect of GATA3 knockdown was

FIGURE 6. **siRNA-mediated knockdown of GATA3 attenuates Ang-1-induced AKT phosphorylation, migration, tube formation, and cell survival in primary human endothelial cells.** *A, left,* HUVEC were transfected with si-control (*lanes 1* and *3*) or si-GATA3 (*lanes 2* and *4*) for 2 days, serum-starved for 18 h, and treated in the absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of Ang-1 for 10 min. Samples were immunoprecipitated with anti-AKT antibody and immunoblotted with phospho-specific AKT antibody. The membrane was stripped and re-probed for total AKT. *Right,* quantitation of phospho-AKT levels relative to si-control-transfected cells treated in the absence or presence of Ang-1. Data are expressed as mean ± S.D. from three independent experiments. #, $p < 0.0001$; *, $p < 0.0001$ compared with without Ang-1 control and control plus Ang-1, respectively *N.S.,* nonsignificant. *B,* modified Boyden chamber assay of HUVEC transfected with si-control (*Control*) or si-GATA3 (*GATA3*) and treated in the presence or absence of Ang-1 or VEGF. Shown are the mean ± S.D. of migrated cells quantified by cell image analyzer in three independent experiments. #, $p < 0.01$; *, $p < 0.001$ compared with minus control and control plus Ang-1, respectively. *N.S.,* nonsignificant. *C,* tube formation assay of si-control- or si-GATA3-transfected HUVEC plated on collagen gel and overlaid with fibroblast-containing collagen with or without Ang-1. Capillary-like tube morphology was observed under the fluorescent microscopy. *Bar,* 500 μm. Quantification of the tube length (*bar graph*) was calculated using the cell image analyzer from four arbitral optical images per experiment. Data are expressed as mean ± S.D. from three independent experiments. #, $p < 0.005$; *, $p < 0.001$ compared with without Ang-1 control and control plus Ang-1, respectively. *N.S.,* nonsignificant. *D,* HUVEC were incubated with EBM-2 + 0.5% FBS for 18 h and then further incubated with Dulbecco's modified Eagle's medium + 0.5% FBS (*Starvation*) for 24 h in the presence or absence of Ang-1. Cells were harvested and stained with annexin V-fluorescein isothiocyanate. Count of annexin V-positive cells were performed by flow cytometry. Data are presented as mean ± S.D. from six independent experiments. *, $p < 0.0001$ compared with starvation alone. *N.S.*, nonsignificant.

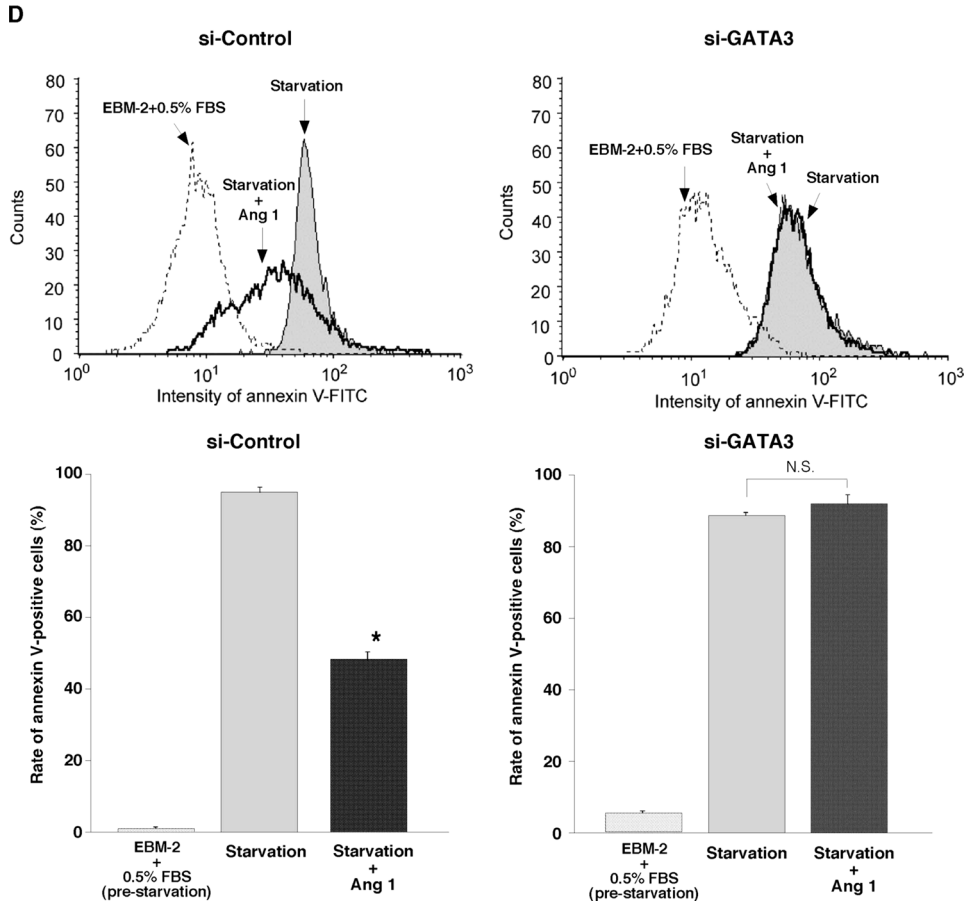

FIGURE 6—*continued*

rescued by Ad-mediated expression of siRNA-resistant GATA3 protein (Fig. 7*A*). Incubation of HUVEC with VEGF promoted U937 monocyte adhesions (Fig. 7*B*). Ang-1 treatment effectively (74%) reduced VEGF-stimulated monocyte adhesion to control cells but had no effect on GATA3-deficient cells (Fig. 7*B*).

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 data, 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. 7*C*, 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. 7*D*). In contrast, neither agonist had an effect on NERF2 or ELF1 levels. Infection of HUVEC with adenovirus overexpressing GATA3, but not control adenovirus, reversed agonist-mediated repression of Tie2 (Fig. 7*E*). Taken together, these findings suggest that inflammation negatively influences Ang-1-Tie2 signaling through a GATA3-dependent mechanism. Indeed, changes in GATA3 expression may be a critical determinant of Tie2-mediated endothelial cell function under the physiological or pathological conditions.

DISCUSSION

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-

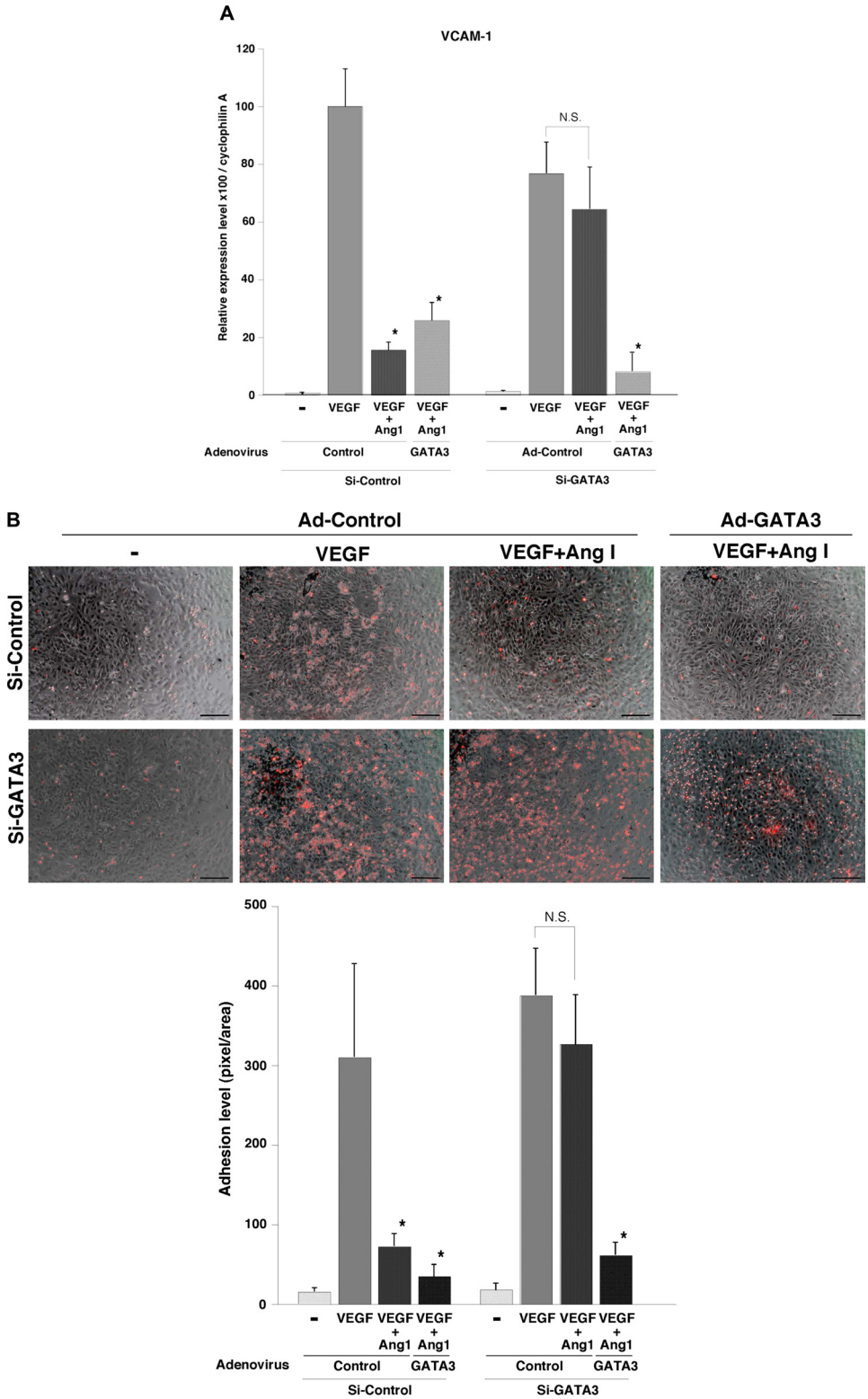

FIGURE 7. **GATA3 and Tie2 regulate Ang-1-mediated anti-inflammation in HUVEC.** *A,* quantitative real time PCR analysis of VCAM-1 mRNA in HUVEC transfected with si-control or si-GATA3 or infected with Ad-control or Ad-GATA3 and treated in the absence or presence of VEGF ± Ang-1. The results are expressed 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 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.

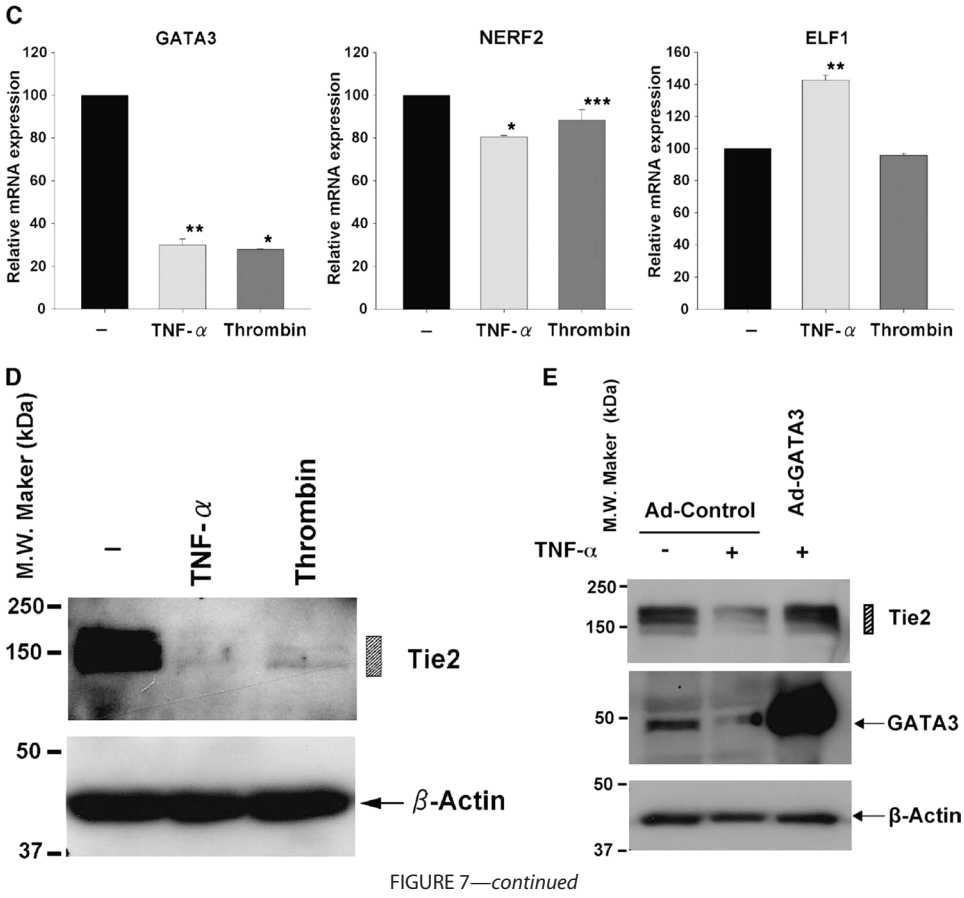

FIGURE 7—*continued*

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.

thelium. 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. 1*A*). 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)

Acknowledgment—We thank to Dr. Nobuyuki Takakura for helpful comments about migration assay.

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