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Novel Roles of GATA1 in Regulation of Angiogenic Factor AGGF1 and Endothelial Cell Function*[S]

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AGGF1 is an angiogenic factor, and its deregulation is associated with a vascular malformation consistent with Klippel-Trenaunay syndrome (KTS). This study defines the molecular mechanism for transcriptional regulation of *AGGF1* expression. Transcription of *AGGF1* starts at two nearby sites, −367 and −364 bp upstream of the translation start site. Analyses of 5′- and 3′-serial promoter deletions defined the core promoter/regulatory elements, including two repressor sites (from −1971 to −3990 and from −7521 to −8391, respectively) and two activator sites (a *GATA1* consensus binding site from −295 to −300 and a second activator site from −129 to −159). Both the *GATA1* site and the second activator site are essential for AGGF1 expression. A similar expression profile was found for GATA1 and AGGF1 in cells (including various endothelial cells) and tissues. Electrophoretic mobility shift assay and chromatin immunoprecipitation assays demonstrated that GATA1 was able to bind to the *AGGF1* DNA *in vitro* and *in vivo*. Overexpression of GATA1 increased expression of AGGF1. We identified one rare polymorphism −294C>T in a sporadic KTS patient, which is located in the GATA1 site, disrupts binding of GATA1 to DNA, and abolishes the GATA1 stimulatory effect on transcription of *AGGF1*. Knockdown of GATA1 expression by siRNA reduced expression of AGGF1, and resulted in endothelial cell apoptosis and inhibition of endothelial capillary vessel formation and cell migration, which was rescued by purified recombinant human AGGF1 protein. These results demonstrate that GATA1 regulates expression of *AGGF1* and reveal a novel role for GATA1 in endothelial cell biology and angiogenesis.

The *AGGF1* gene, previously known as *VG5Q*, encodes an angiogenic factor with 714 amino acid residues (1). *AGGF1* was identified through genetic analysis of Klippel-Trenaunay syndrome (KTS, MIM #149000),[2] which is a congenital vascular disorder composed of capillary malformations, venous malformations or varicose veins, and hypertrophy of the affected tissues (2–5). KTS is a congenital disorder, but most cases are sporadic. The genetic basis of KTS is complex and may involve multiple genes, environmental factors, and their interactions (6). To date, identification of susceptibility genes associated with KTS has relied upon gross cytogenetic defects reported in KTS patients. Three chromosomal abnormalities have been identified in three separate KTS patients: two balanced translocations t(5.11)(q13.3;p15.1) and t(8,14)(q22.3;q13), and an extra supernumerary ring chromosome 18 (7–9). Chromosomal breakpoints involved in KTS translocation t(5;11)(q13.3; p15.1) have been fully characterized. No gene has been identified within a 100-kb region flanking the chromosome 11p15.1 translocation breakpoint. In contrast, the chromosome 5p13.3 breakpoint is located in the promoter/regulatory region of the *AGGF1* gene and leads to increased transcriptional activation of *AGGF1* by 3-fold (1). The results suggest that deregulation of *AGGF1* is associated with KTS. However, the molecular mechanism for the deregulation is not known. In this study, we defined the promoter of *AGGF1* and important *cis*-acting DNA elements or *trans*-acting nuclear factors in the regulation of the *AGGF1* gene. We show that translocation t(5:11) increases transcription of *AGGF1* by removing *cis*-acting DNA elements that repress expression of *AGGF1*.

AGGF1 protein contains a N-terminal coiled-coil motif, an OCRE motif, a forkhead-associated domain, and a C-terminal G-patch domain (1, 10). Purified human recombinant AGGF1 protein promotes angiogenesis as potently as VEGF (1). AGGF1 protein is released outside endothelial cells when angiogenesis starts (1). It binds strongly to endothelia cell surface, and may act in an autocrine fashion (1). Strong expression of *AGGF* mRNA was detected in cells relevant to KTS, including endothelial cells, vascular smooth muscle cells, and MG-63 osteoblasts (1). Tissue immunostaining studies with an anti-AGGF1 antibody identified strong AGGF1 protein expression in blood

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table 1.

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[2] The abbreviations used are: KTS, Klippel-Trenaunay syndrome; VEGF, vascular endothelial growth factor; SNP, single nucleotide polymorphism; HUVEC, human umbilical vein endothelial cell; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; siRNA, small interference RNA; TSS, transcription start site; HBMEC, human brain microvascular endothelial cell.

vessels embedded in various tissues including the heart, kidney, tail, and limb and co-localized with an endothelial specific marker CD31 as well as a vascular smooth muscle cell-specific marker, smooth muscle cell α-actin (1). In a small case control study, the frequency of a single nucleotide polymorphism (SNP) in AGGF1, E133K, was found to be greater in cases (3.8%) than in controls (1), but later studies found that E133K showed a frequency of 2.2–3.3% in other general control populations (6, 11, 12). These results argue that SNP E133K is unlikely to confer a risk of KTS. On the other hand, a recent large scale case control study employing a STRUCTURE program demonstrates that two common SNP*s* in *AGGF1*, exonic SNP rs7704267 and intronic SNP rs13155212, are significantly associated with susceptibility of KTS even after adjustment of population structural parameters of the cases and controls (6). Therefore, *AGGF1* remains a strong candidate gene associated with risk of KTS.

GATA factors are important transcription factors that mediate cell-specific gene expression. There are six members in the GATA family of transcription factors. GATA1 is a key transcription factor that is central to the differentiation, proliferation, and/or apoptosis of erythroid (13), megakaryocytes (14), eosinophilic cells (15), and mast cells (16). However, the potential role of GATA1 in endothelial cells has not been studied. In this study, we uncovered a novel role of GATA1 in endothelial cells through the promoter analysis of *AGGF1*. We identified an *AGGF1* promoter SNP, −294C>T, that affects a *cis*-acting DNA element at this location that preferentially interacts with GATA1. SNP −294C>T disrupted GATA1 binding to DNA in endothelial cells and markedly reduced transcription activation of *AGGF1*. Furthermore, siRNA against *GATA1* effectively knocked down expression of *GATA1*, reduced expression of *AGGF1*, resulted in cell apoptosis, and subsequently inhibited endothelial cell vessel formation and cell migration. The effects by *GATA1* siRNA were rescued by recombinant human AGGF1 protein. Together, these results suggest that GATA1 regulates expression of AGGF1 in endothelial cells and is involved in AGGF1-mediated angiogenesis and other endothelial cell phenotypes.

EXPERIMENTAL PROCEDURES

Study Subjects—185 KTS patients were enrolled in North America for this study. The diagnosis of KTS was based on published reports (2–4). This study has been approved by the Cleveland Clinic Foundation and Mayo Clinic Institutional Review Boards on Human Subject Research. Informed consent was obtained from all participants according to the standards established by the local Institutional Review Boards.

Identification of SNPs in AGGF1—SNP identification was carried out using direct DNA-sequencing analysis. The 2-kb promoter/regulatory region/5′-untranslated region of *AGGF1* was PCR-amplified using two pairs of primers (supplemental Table 1) and sequenced.

TaqMan SNP Assays—The frequency of SNP −294C>T variant in the *AGGF1* promoter/regulatory region was analyzed in 1764 non-KTS control samples using the TaqMan 5′-allelic discrimination assay as described previously (17–20). The assay probes (supplemental Table 1) were ordered using the Assay-By-Design service from the Applied Biosystems.

Construction of AGGF1 Promoter-luciferase Reporter Genes with Various Deletions and SNP −294C>T—Previously we reported an *AGGF1* luciferase reporter gene (8.4kb-AGGF1p-luc) for assaying transcriptional activity of the *AGGF1* promoter by fusing an 8.4-kb DNA fragment containing the promoter/regulatory region of *AGGF1* to the luciferase gene in pGL3-Basic vector (Promega, Madison, WI) (1). The 8.4kb-AGGF1p-luc construct was digested with NheI and re-ligated, resulting in the 7kb-AGGF1p-luc reporter gene (Fig. 1). The 7kb-AGGF1p-luc construct was digested with NheI/EcoRI, NheI/NdeI, NheI/EcoRV, and NheI/ApaI, respectively, blunt-ended, and re-ligated, which resulted in 7.5kb-, 5.7kb-, 5kb-, 4kb-, 1.9kb-AGGF1p-luc, and 1.1kb-AGGF1p-luc reporter genes.

Further deletions were created based on the 1.1kb-AGGF1p-luc reporter gene using a PCR-based method. For the 5′-deletion series, the forward primers were designed and contained a unique NheI site. The reverse primer was designed based on the vector sequences after the unique XhoI site. Each PCR fragment was cut with NheI and XhoI and cloned into the NheI/XhoI-cut 1.1-kb AGGF1p-luc plasmid. For the 3′-deletion series, the forward primer was designed based on the sequence spanning the NheI site. The reverse primers were designed to contain a unique XhoI site. Each PCR fragment was cut with NheI and XhoI and cloned into the NheI/XhoI-cut 1.1kb-AGGF1p-luc plasmid.

SNP—294C>T in the *AGGF1* promoter/regulatory region was introduced into the core −536-bp-AGGF1p-luc reporter gene or −8.4kb-AGGF1p-luc reporter gene by site-directed mutagenesis using the mega-primer PCR-based method (21). The deletions involving the two activator sites were created by PCR and subcloning. The PCR primers used for creating deletions and SNP −294C>T are shown in supplemental Table 1. All mutant constructs were verified by DNA sequencing analysis.

Cells Culture and Transfection—Human umbilical vein endothelial cells (HUVECs) and other cells were cultured as described (1) and transfected by electroporation using a Nucleofector device and HUVEC kits (Amaxa, Inc., Cologne, Germany). Transfection of HEK293 cells was carried out using Lipofectamine 2000 (Invitrogen) as described previously (22, 23).

Transcriptional Assays—The transcription activation activity was measured by the luciferase assay as described (24, 25).

RT-PCR and Western Blot Analyses, and Immunofluorescence Staining—Total RNA was isolated from HUVECs and other cells using the TRIzol reagent (Invitrogen), treated with DNase I (Roche Applied Science, Indianapolis, IN), and used for RT-PCR analysis as described (1). Western blot analysis and immunostaining studies were performed from various cells and mouse tissues as described previously (1).

Primer-extension Analysis—The exact transcription start sites of *AGGF1* were determined by primer extension analysis using the Primer Extension System AMV Reverse Transcriptase Kit (Promega) and as described before (26). The primer extension products were run in parallel with a DNA sequence ladder obtained by cycle sequencing using the same [γ-^{32}P]ATP-labeled primer extension primer with the

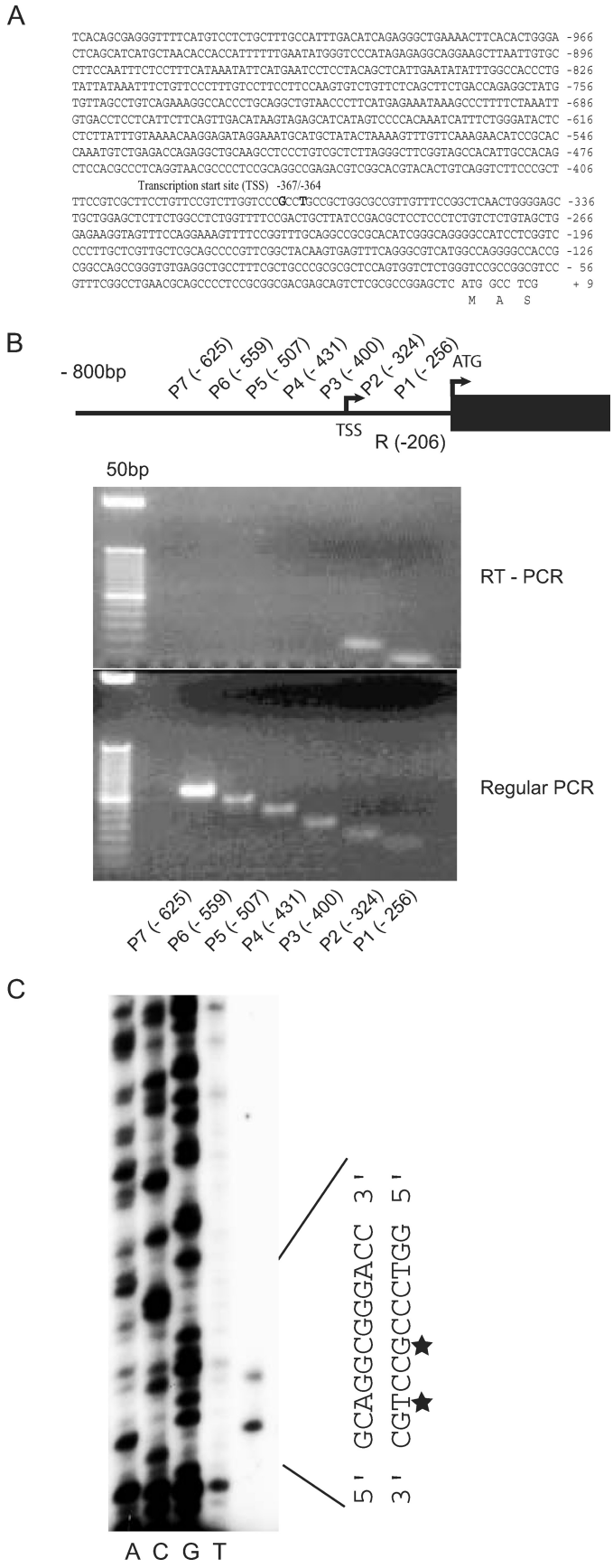


FIGURE 1. **Mapping of the TSSs of the *AGGF1* gene.** *A*, the nucleotide sequence of the human *AGGF1* promoter/regulatory region is shown. Only the region from −1045 bp to +9 bp from the translation start site is shown. The A residue of the start codon ATG is designated as "+1." *B*, identification of the TSSs of *AGGF1* by RT-PCR analysis. RT-PCR was performed using total RNA isolated from HUVECs. *R*, reverse primer; P1 to P7, a series of forward primers with locations indicated by the number of bp from the translation start site; *top panel*, results from RT-PCR; *bottom panel*, positive control for PCR primers (regular PCR with genomic DNA). *C*, precise mapping of the TSSs for human *AGGF1* gene using primer extension analysis. Primer extension reactions were performed with total RNA samples isolated from HUVECs. The extended products were analyzed with a 6% denatured urea polyacrylamide gel together with a sequencing ladder generated using the same primer and plasmid DNA samples. The nucleotide sequence readout is shown on the *right*. The TSSs are shown with *stars*.

AGGF1p-luc plasmid DNA as the template as described before (27).

Preparation of Nuclear Lysates and EMSAs—Nuclear extracts for HUVECs or transfected HEK 293 cells were prepared using NE-PER Nuclear and Cytoplasmic Extract kits (Pierce). The probes for EMSA were designed based on the sequences from the *AGGF1* promoter/regulatory region (supplemental Table 1). Positive control probes for *GATA1* binding and *TFII-I* binding sites were described (28, 29) and are shown in supplemental Table 1. EMSA was carried out as described (30, 31).

For supershift EMSA, a rat anti-GATA1 antibody (sc-266) and goat anti-GATA2, GATA3, GATA4, and GATA6 antibodies (1 μg, Santa Cruz Biotechnologies, Santa Cruz, CA) were added to the reaction mixture, and the mixture was incubated on ice for 15 min before addition of the probe. For competition EMSA experiments, excessive unlabeled probes were added to the binding reaction mixture before addition of the labeled probes.

Chromatin Immunoprecipitation Assays—ChIP assays were carried out with solutions prepared following the protocol from Upstate Biotechnology (Lake Placid, NY). Chromatin was sheared by sonication for 15 min to short fragments of ~200 to 1000 bp in a water bath with generation of high power ultrasound (15 cycles of 30 s on and 30 s off, 1 cycle/min) at the maximum power. To reduce nonspecific background, protein A-agarose (Pierce) was presaturated with herring-sperm DNA (Sigma). Immunoprecipitation was performed with 1 μg of a rat anti-GATA1 antibody (Santa Cruz Biotechnology). The normal anti-rat IgG was used as a negative control. After immunoprecipitation, the mixture was extracted with phenol/chloroform and precipitated with ethanol. Immunoprecipitated DNA was analyzed by PCR. ChIP assays were replicated three times. The PCR primers used for ChIP assays were designed based on the core promoter/regulatory region of *AGGF1* and are shown in supplemental Table 1.

Matrigel Endothelial Vessel Formation Assays—The property of HUVECs to spontaneously form capillary vessels in Matrigel basement membrane matrix (BD Biosciences, Oxford, UK) was assessed as described previously (1, 32). For the AGGF1 rescue experiments, purified recombinant human AGGF1 protein was mixed with Matrigel at 4 °C, which was placed back to an incubator for 30 min, resulting in solid Matrigel ready for Matrigel vessel formation assays.

Analysis of Cell Migration by a Scratch Assay—HUVECs treated with or without siRNA were plated on polylysine-coated two-well chamber slides (BD Bioscience) at 5×10^4 cells/well in endothelial basal medium supplemented with EGM-2 SingleQuots (IGF1, epidermal growth factor, and VEGF) (Lonza, Walkersville, MD),

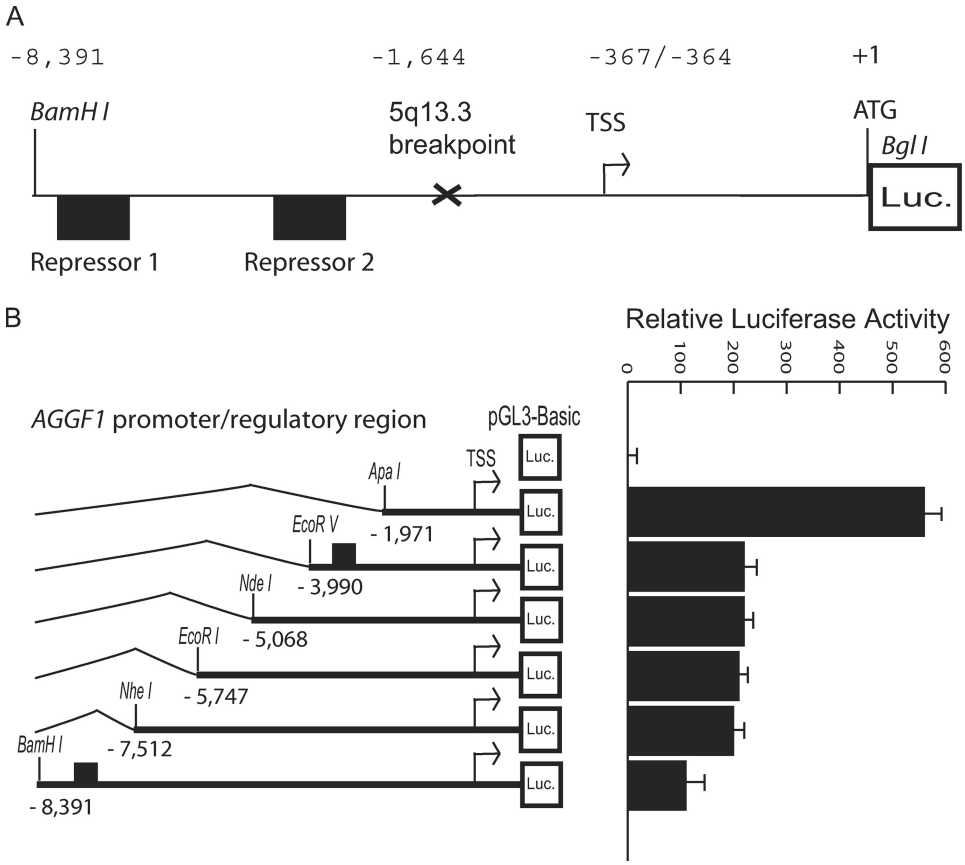


FIGURE 2. **5′-deletion analysis of the human *AGGF1* promoter.** *A*, schematic representation of the original *AGGF1* promoter luciferase reporter, *8.4kb-AGGF1p-luc* (1). An 8.4-kb genomic DNA upstream of the *AGGF1* TSS between BamHI and BglI restriction sites was cloned into luciferase reporter vector pGL3-Basic. The chromosome 5p13.3 breakpoint site is located at the position of −1644 bp from the translation start site. The locations of two repressor sites (rectangle) are indicated. TSS, transcription start site. *B*, a series of promoter deletions were created based on reporter 8.4kb-*AGGF1p-luc*. The luciferase activity of each deletion mutant in HUVECs is shown on the *right*. Data shown represent three independent experiments with the luciferase activity of each deletion mutant measured in triplicate. The luciferase activity of each deletion was normalized to the activity of the pGL3-Basic vector.

which was changed every 24 h. Two days after plating, a scratch was applied using a 20-μl pipette tip. Chambers were washed with endothelial growth media and replaced with endothelial basal medium supplemented with EGM-2 SingleQuots (Lonza). 16 h after the scratch, cells were photographed. For the AGGF1 rescue experiments, purified recombinant human AGGF1 protein or bovine serum albumin control was coated on slides, followed by the scratch assay as described above.

Apoptosis Assays—HUVECs were treated with GATA siRNA or control scramble siRNA. After 24 h, cells were harvested, and apoptosis was analyzed using flow cytometry that detects DNA breaks labeled by a fluorescein anti-bromodeoxyuridine antibody and total cellular DNA labeled by propidium iodide (APO-BRDUTM Kit, BD Pharmingen).

Statistical Analysis—Data are shown as mean ± S.E. Statistical analysis was performed using Student's *t* test for comparing two groups and analysis of variance for comparisons among groups. A *p* value of 0.05 was considered to be significant.

RESULTS

Identification of the Transcription Start Sites of the AGGF1 Gene in HUVEC—BLAST searches of public databases, including the NCBI data base, identified five cDNA or expressed sequence tags clones that match the *AGGF1* genomic sequence, including HSU84971, AI939311, AA311507, BX426365, and BX442568. The 5′-start sites of these five clones are 289, 327, 334, 338, 349, or 360 bp from the translation start site, respectively. The data suggest that the transcription start site (TSS) of *AGGF1* is at least 360 bp from the translation start site ATG (we designate the position of the A residue of codon ATG as +1 throughout the text) (Fig. 1*A*).

RT-PCR analysis was used to experimentally map the TSS of *AGGF1*. We designed a reverse primer (R) located at the position of −206 and a series of forward primers at positions of −625 (P7), −559 (P6), −507 (P5), −431 (P4), −400 (P3), −324 (P2), and −256 (P1), respectively (Fig. 1*B*). RT-PCR analysis of total RNA isolated from HUVECs with primer combinations P1/R and P2/R yielded positive signals, whereas other combinations did not produce any PCR signal. The data suggests that the TSS of *AGGF1* is located between positions −400 and −324.

To map the TSS of *AGGF1* more precisely, primer extension analysis was carried out. As shown in Fig. 1*C*, transcription of *AGGF1* starts at two sites that are 2 bp apart. The first TSS is at the position of −367 and the second one is at −364 (Fig. 1, *A* and *C*). The second TSS appears to be used more frequently than the more upstream one (Fig. 1*C*).

Structural Characteristics of the Core Promoter of AGGF1—DNA sequences for a region of 1045 bp upstream from the *AGGF1* translation start site are shown in supplemental Fig. S1. Notably, the core promoter/regulatory region of *AGGF1* is highly GC-rich (62%). There are more than 50 CpG dinucleotide repeats, including 5 HpaII/MspII restriction sites (CCGG) (supplemental Fig. S1).

Identification of Two Upstream Cis-acting DNA Elements That Repress the Transcription of AGGF1—A series of six 5′-deletions were created for the *AGGF1* promoter in 8.4kb-AGGF1p-luc (Fig. 2). The deletions were transiently transfected into HUVEC, and transcriptional activity was measured. Compared with the promoter-less reporter, the −1971 AGGF1p-luc exhibited a 600-fold increase of transcriptional activity.

Removal of sequences from −8391 to −7521 increased transcription activity of *AGGF1* by 2-fold, suggesting that there is a *cis*-acting DNA element at the region that represses

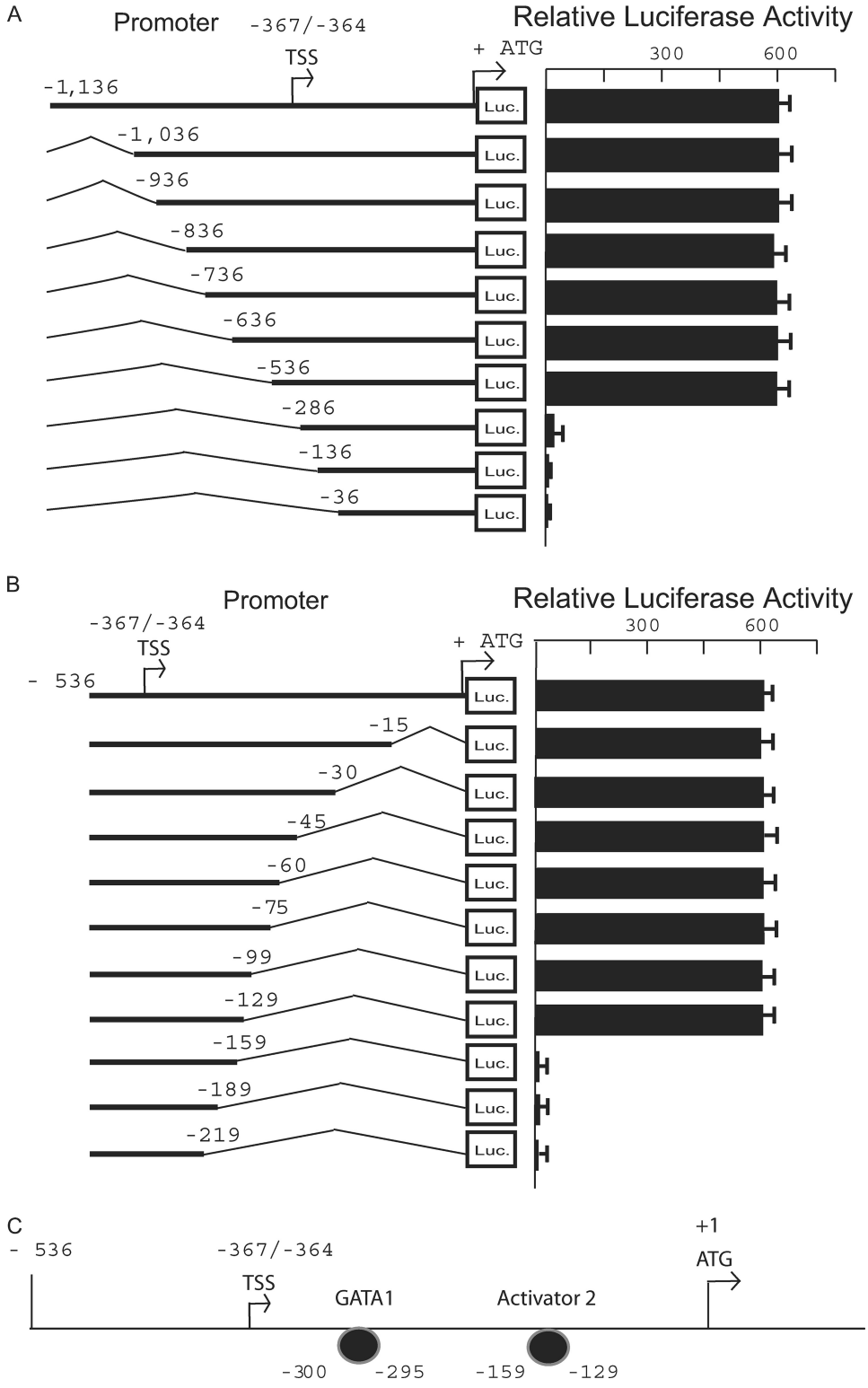


FIGURE 3. **Fine mapping of the core promoter of *AGGF1*.** *A*, 5′-deletions and their luciferase activities in HUVECs. *B*, 3′-deletions and their luciferase activities in HUVECs. *C*, schematic diagram showing the locations of two *cis*-acting activator elements, the GATA1 site, and the second activator site. *TSS*, transcriptionstart site.

expression of *AGGF1*. Removal of sequences from −3990 to −1971 increased *AGGF1* transcription activity by an additional 3-fold, indicating the presence of the second repressor element within the promoter/regulatory region of *AGGF1* (Fig. 2).

Identification of a Cis-acting DNA Element from −129 to −159 That Increases Transcription of AGGF1—To map the region responsible for the basal promoter activity, additional nine 5′-serial promoter truncations, including −1036, −936, −836, −736, −636, −536, −286, −236, and −36 deletions, were created and analyzed (Fig. 3*A*). Removal of sequences between −536 and −286 drastically reduced transcription of the *AGGF1* promoter. These data suggest that the basal promoter of *AGGF1* is located between −536 and −286 from the translation start site, which is consistent with earlier results that the transcription start sites (−367 and −364) of *AGGF1* are located in this region. A deletion of the region from −286 to −136 reduced the AGGF1 expression by another 2-fold, which may implicate a weak promoter in the region. The luciferase activities for the −136 and −36 deletions were low (5.5 ± 0.7 and 5.0 ± 0.3, respectively), but still 5-fold higher than the calibration value of 1 for the empty vector, which may reflect nonspecific activation or implicate another weak promoter in the region.

Analyses of additional ten 3′-serial promoter deletions revealed an interesting *cis*-acting DNA element that is essential for the expression of *AGGF1* (Fig. 3*B*). The *cis*-acting DNA element is located between −129 to −159 from the translation start site (Fig. 3*B*, 3*C*).

Identification of an SNP, −294C>T, at a GATA1 Binding Site in the Promoter/Regulatory Region of AGGF1 in a Patient with KTS—One novel SNP, which changes C to T at the −294, was identified in the promoter region of *AGGF1* in a sporadic male KTS patient (Fig. 4*A*). The patient was an adopted child who was affected with a capillary malformation of the abdomen, a large capillary malformation of the right leg, hypertrophy of the right leg, and important venous malformations. The patient underwent several operations and hospitalizations because of symptoms of KTS. SNP −294C>T was not present in 1764 control individuals, suggesting that it is a rare SNP. SNP −294C>T occurs at a

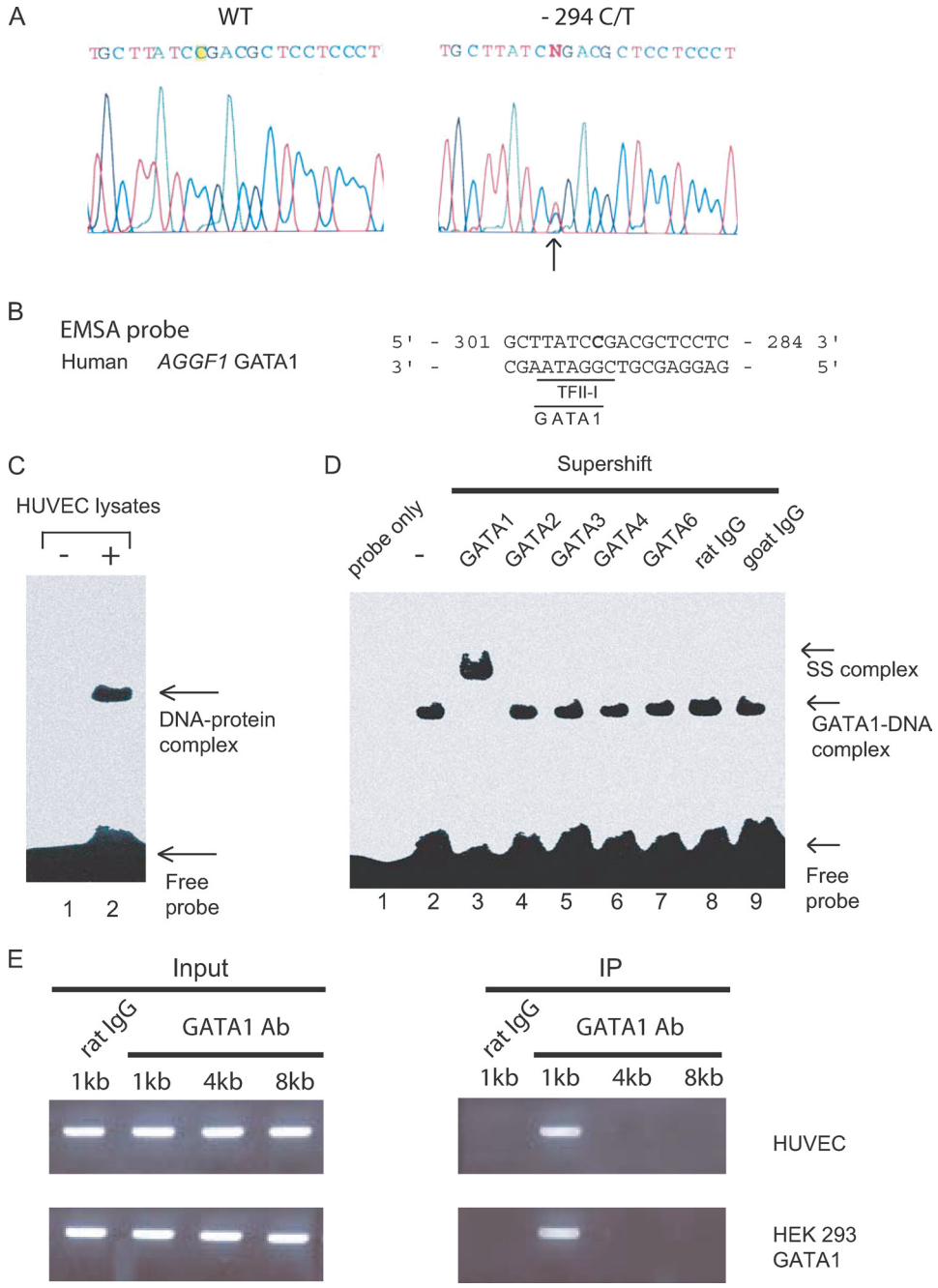


FIGURE 4. **Binding of GATA1 to the *AGGF1* promoter/regulatory region and identification of a novel SNP −294C>T at the *GATA1* DNA binding site in a patient with KTS.** *A*, identification of *AGGF1* promoter SNP −294C>T in a patient with KTS. The sequences for the wild-type allele and the rare variant allele are shown. The SNP occurs at the position of −294 bp from the translation start site. *B*, sequence of a double strand oligonucleotide probe used for EMSA. *C*, EMSA studies detected a DNA-protein complex in HUVECs with the EMSA probe. *D*, supershift EMSA with (*lanes 2–12*) or without (*lane 1*) nuclear extracts from HEK293 cells transfected with GATA1. *Lanes 3–7*, EMSA with preincubation of a rat anti-GATA1 or goat anti-GATA2, -GATA3, -GATA4, and -GATA6 antibodies, respectively. *Lanes 8* and *9*, negative controls. *SS complex*: supershifted complex. Similar results were obtained with nuclear extracts from HUVECs (data not shown). *E*, ChIP analysis detected binding of the GATA1 protein from HUVEC or HEK293 cells with transient expression of GATA1 to the *AGGF1* promoter *in vivo*. Primers that can amplify the *AGGF1* promoter were used for the PCR analysis. *GATA1 Ab*, presence of an anti-GATA1 antibody. *1kb*, ChIP with PCR primers covering the *AGGF1* promoter fragment with the GATA1 binding site (located within a 1-kb region upstream from the translation start site); *4kb* and *8kb*, ChIPs with PCR primers covering other *AGGF1* promoter fragments without the GATA1 binding site (located 4 kb and 8 kb upstream from the translation start site, respectively).

consensus binding site for GATA1 (−295 to −300) or TFII-1 (−293 to −298), but later studies indicate that it is a GATA1 binding site (Fig. 3*C*).

GATA1 Interacts Directly with the AGGF1 Promoter in Endothelial Cells—An EMSA was performed using a double-stranded oligonucleotide encompassing the region from −301 to −284 (EMSA1, Fig. 4*B*) and HUVEC nuclear extracts. As shown in Fig. 4*C*, incubation of nuclear extracts from HUVECs with the ^{32}P-labeled EMSA1 resulted in the formation of a DNA-protein complex. The DNA-protein interaction appeared to be specific because it was eliminated by addition of 50-fold excess of unlabeled EMSA1 but not affected by addition of 50-fold excess of three nonspecific control double-stranded oligonucleotides (NS1, NS2, and NS3) (supplemental Fig. S2). A 50-fold excess of an unlabeled *GATA1* oligonucleotide from the human *β-globin* promoter (EMSA2) (28) eliminated the DNA-protein complex, but even a 300-fold excess of a consensus *TFII-I* oligonucleotide from the c-*fos* promoter (EMSA3) (29) did not have any effect (supplemental Fig. S2). These data suggest that the DNA-protein complex is a complex with GATA1. This conclusion is further confirmed using supershift EMSA studies. The specific DNA-protein complex was shifted in EMSA by using nuclear extracts preincubated with an anti-GATA1 antibody, but not with antibodies against other GATA factors expressed in endothelial cells, including GATA2, GATA3, GATA4, GATA6, and negative control IgG (Fig. 4*D*). Together, these data suggest that the interval from −301 to −284 of the *AGGF1* promoter/regulatory region can interact preferably with GATA1.

To verify that the endogenous, native GATA1 protein binds to the *AGGF1* promoter *in vivo*, a conventional ChIP assay was performed using a specific monoclonal GATA1 antibody and specific primers for the *AGGF1* promoter region (for primer sequences, see supplemental Table 1). In ChIP assays using HUVEC extracts, GATA1 specifically binds to the *AGGF1* promoter (Fig. 4*E*). The same results were obtained

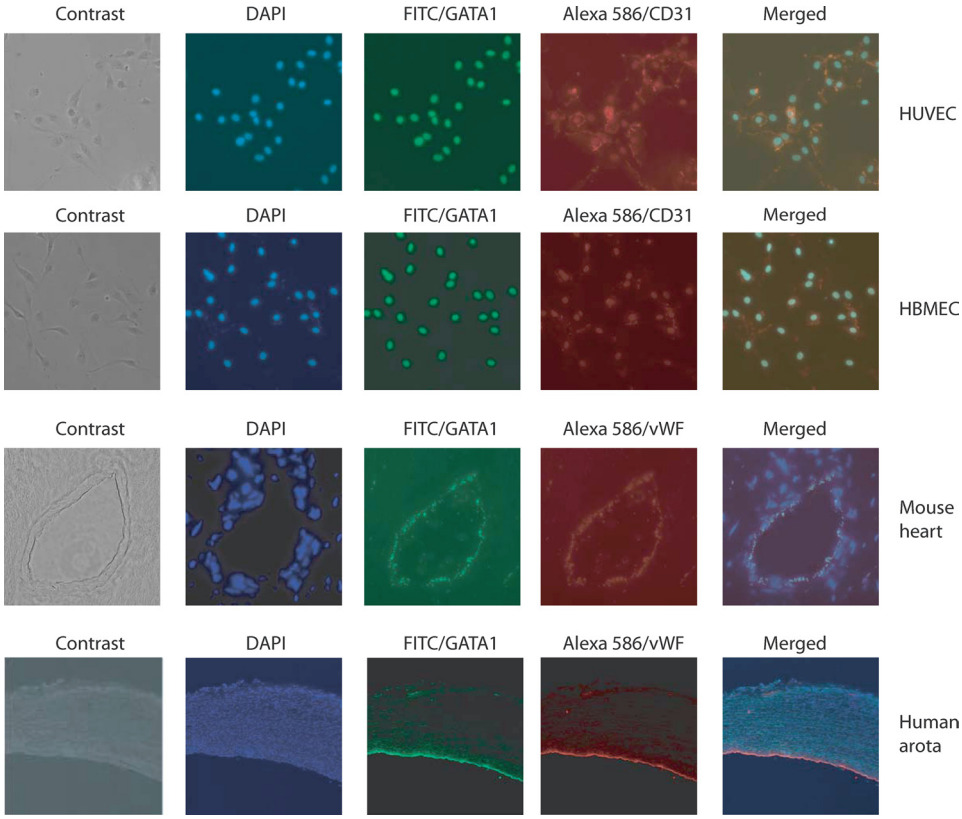


FIGURE 5. **Strong expression of GATA1 in endothelial cells.** Immunofluorescence staining with an anti-GATA1 antibody was used to detect the expression of GATA1 (*green*) in the nuclei (*blue*) of HUVECs and HBMECs. Both HUVECs and HBMECs expressed endothelial cell marker CD31 (tagged with Alexa 586). GATA1 co-localized with another endothelial cell marker vWF (tagged with Alex 586) in the endothelial layer (endothelium) of a large vessel in the mouse heart and a human aorta. 4′,6-Diamidino-2-phenylindole (*DAPI*) was used to stain nuclei.

using protein extracts from HEK293 cells transiently transfected with a GATA1 expression plasmid (Fig. 4*E*). These data further indicate that GATA1 can interact with a *cis*-DNA element in the *AGGF1* promoter/regulatory region.

GATA1 mRNA and Protein Are Expressed in Endothelial Cells—Immunostaining studies showed that GATA1 was expressed strongly in the nuclei of some representative endothelial cells, including HUVECs and HBMECs (human brain microvascular endothelial cells) (Fig. 5). Similarly, GATA1 was strongly co-localized with von Willebrand factor in the endothelium of large vessels in the mouse heart and human aortas (Fig. 5).

Semi-quantitative RT-PCR and Western blot analyses showed that *GATA1* was expressed in various tissues including the heart, liver, brain, lung, kidney, aorta, and bone marrow, and its expression was higher in the aorta than in other organs tested (Fig. 6, *A* and *B*). *GATA1* expression was detected in three cell lines (HEL, THP1, and U937) and three types of endothelial cells (HUVECs, HBMECs, and human coronary artery endothelial cells (Fig. 6, *A* and *B*)). The expression levels in HEL cells were higher than that in endothelial cells.

Western blot analysis revealed that expression of the GATA1 protein was restricted to the liver in neonatal mice at the age of P2 (Fig. 6*C*) and P3 (data not shown), consistent with the results by Northern blot analysis (32). Starting at P5, GATA1 expression is detected in other tissues/organs (Fig. 6*C*). Similar expression patterns were observed for AGGF1 except that a low level of AGGF1 expression was detected in the heart, brain, and kidney at the age of P2 (Fig. 6*C*).

GATA1 Trans-activates the AGGF1 Promoter—To assess the effect of GATA1 on the *AGGF1* promoter activity, co-transfection experiments were performed using the core −536-AGGF1p-luc construct (containing nucleotides from −536 to −129 of the *AGGF1* promoter/regulatory region) and an expression construct containing the *GATA1* cDNA or an empty expression vector as control. Co-transfection of the *GATA1* expression construct into HUVECs resulted in markedly increased transcription activation of the *AGGF1* promoter compared with the empty expression vector (Fig. 7*A*). No luciferase activity was detected with the parental pGL3-basic luciferase vector together with the *GATA1* expression vector, indicating that the transactivation of the *AGGF1* promoter requires the presence of the DNA binding site for GATA1. Similar results were obtained with a longer version of the AGGF1p-luc reporter gene, the −8.4kb-AGGF1p-luc luciferase reporter (Fig. 7*A*, *right panel*).

GATA2 Does Not Regulate Expression of AGGF1—GATA2 is highly expressed in endothelial cells and control expression of VEGFR2 (33, 34), thus we determined whether GATA2 could regulate expression of AGGF1. Knockdown of *GATA2* by siRNA does not affect expression of AGGF1 in HUVEC by both RT-PCR and Western blot analyses (supplemental Fig. S3*A*). HUVECs with overexpression of GATA2 did not increase the transcriptional activity of *AGGF1* promoter (supplemental Fig. S3*B*). These data indicate that GATA2 does not affect expression of AGGF1.

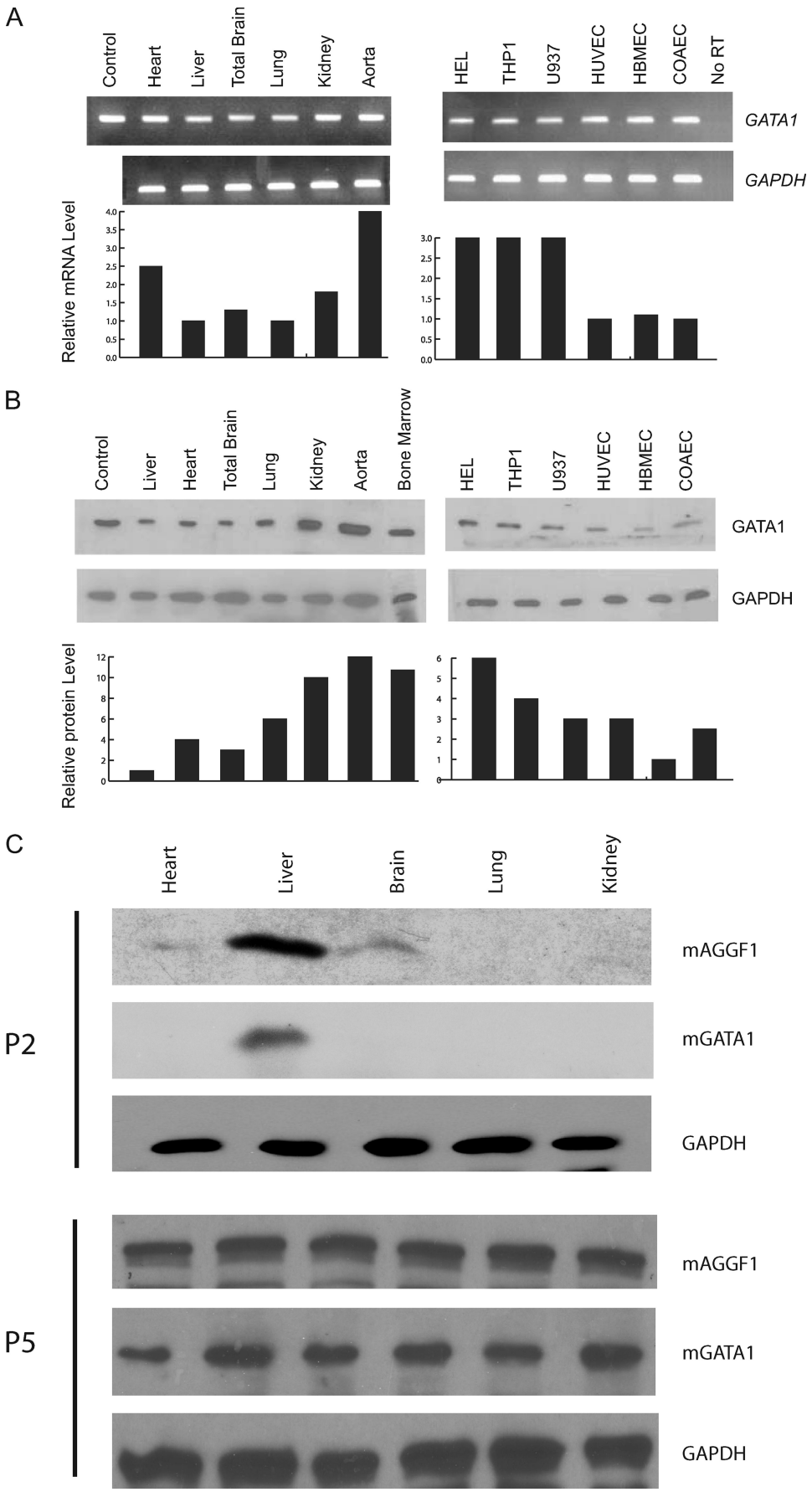
SNP −294C>T Weakens the GATA1-DNA Complex—To determine whether the formation of GATA1-DNA complex is affected by the *AGGF1* promoter SNP −294C>T identified in a KTS patient, we carried out EMSA experiments using a mutant EMSA1 probe containing the SNP (Fig. 7*B*). As shown in Fig. 7*B*, more GATA1-DNA complex was formed with the wild-type probe with the C allele than the mutant probe with the T allele.

SNP −294C>T Reduces Expression from the AGGF1 Promoter—Transcriptional activity of the *AGGF1* promoter was markedly reduced by 75.2% after introduction of the −294T allele (Fig. 7*A*). The AGGF1p-luc reporter was not responsive to increased expression of GATA1 (Fig. 7*A*). These data suggest that *AGGF1* promoter SNP −294C>T reduces *AGGF1* expression.

Both the GATA1 Site and the Second Activator Site from −129 to −159 Are Essential for Expression of AGGF1—A deletion of the GATA1 site reduced expression of *AGGF1* by more than 4-fold, and so does the deletion of the second activator site from −129 to −159 (Fig. 7*C*). A deletion of both sites abolished the transcriptional activity of the *AGGF1* promoter to almost the basal level (Fig. 7*C*). These data suggest that the GATA1 site and the second activator site are essential for expression of *AGGF1*.

Knockdown of Endogenous Expression of GATA1 by siRNA Impairs Endothelial Vessel Formation and Endothelial Cell Migration and Induces Apoptosis—To determine the cellular roles of GATA1-mediated *AGGF1* expression, we knocked down expression of *GATA1* in HUVECs and studied its potential roles in endothelial tube formation, migration, and apoptosis. A specific siRNA targeted to GATA1 or a scramble siRNA was transfected into HUVECs. Semi-quantitative RT-PCR analysis showed that, compared with the control scramble siRNA, *GATA1* siRNA reduced expression of *GATA1* by 80%. The expression level of *AGGF1* was also decreased by 70%, but expression of control genes, including *VEGF*, *GATA2*, and *GAPDH*, was not affected by *GATA1* siRNA (Fig. 8*A*). A similar level of inhibition of GATA1 and AGGF1 expression by the *GATA1* siRNA was achieved at the protein level (Fig. 8*B*).

It has been reported previously that an antisense oligonucleotide and siRNAs targeted *AGGF1* inhibited endothelial vessel formation in an *in vitro* Matrigel angiogenesis assay (1). Because GATA1 regulates the expression of *AGGF1*, we examined the role of GATA1 siRNA in endothelial vessel formation. 4 μM GATA1 siRNA was electroporated into HUVECs and plated on Matrigel-coated plates to allow capillary vessel formation. The formation of mature, well connected vessels was counted under an inverted phase-contrast microscope (40× magnifi-



cation) in 12 randomly selected fields in 3 wells. GATA1 siRNA inhibited endothelial vessel formation (Fig. 8*C*).

GATA1 siRNA was transfected into HUVECs, and its effects on endothelial cell migration and apoptosis were also examined. GATA1 siRNA reduced HUVEC migration (Fig. 8*D*) and increased apoptosis (Fig. 8*E*). The control scramble siRNA did not affect HUVEC migration or apoptosis (Fig. 8, *D* and *E*).

Purified Recombinant Human AGGF1 Rescues Effects by GATA1 siRNA—The AGGF1 protein is released outside of HUVECs when angiogenesis starts, and purified human AGGF1 protein can promote strong angiogenesis in a chicken embryo angiogenesis assay (1). Here we assessed whether purified recombinant human AGGF1 protein can rescue the effects of GATA1 siRNA (Fig. 9*A*). HUVECs were transfected with GATA1 siRNA and used for endothelial vessel formation, migration, and apoptosis in the presence of 6.4 μg of recombinant human AGGF1 protein or control bovine serum albumin. Interestingly, more mature endothelial vessels were formed with AGGF1/Matrigel mixture than with control bovine serum albumin/Matrigel (Fig. 9*B*), suggesting that inhibition of endothelial vessel formation by GATA1 siRNA can be rescued by recombinant human AGGF1 protein applied externally. In the scratch cell migration assay, recombinant human AGGF1 protein rescued the inhibition of HUVEC migration mediated by knockdown of GATA1 expression by siRNA (Fig. 9*C*). In the endothelial cell apoptosis assay, purified human AGGF1 protein was able to rescue GATA1 siRNA-medicated HUVEC apoptosis (Fig. 9*D*).

DISCUSSION

AGGF1 plays a role in angiogenesis and altered expression of AGGF1 is associated with vascular malformations consistent with KTS. Here, we demonstrate that GATA1 is involved in transcriptional activation of the *AGGF1* gene. We identified a consensus DNA binding site for GATA1 centered at −295 bp to −298 bp from the translation start site. Both EMSA and ChIP studies demonstrated that GATA1 interacted specifically with the GATA1 binding site (Fig. 4). Overexpression of GATA1, but not GATA2, increased transactivation of the *AGGF1* promoter (Fig. 7 and supplemental Fig. S3). Knockdown of *GATA1* expression, but not GATA2 expression, by siRNA decreased expression of *AGGF1* (Fig. 8 and supplemental Fig. S3). These data indicate that GATA1 is an important regulator of the *AGGF1* gene.

Previous studies showed that purified human AGGF1 promoted angiogenesis as potently as VEGF and knockdown of *AGGF1* expression inhibited endothelial vessel formation (1). Similarly, knockdown of *GATA1* expression reduced expression of *AGGF1* and resulted in inhibition of endothelial vessel formation in an *in vitro* Matrigel angiogenesis assay (Fig. 8). The current study shows that knockdown of GATA1 expression inhibited endothelial cell migration and induced endothelial cell apoptosis (Fig. 8). The effects of *GATA1* siRNA on endothelial vessel formation, endothelial cell migration, and apoptosis can be rescued by purified recombinant human AGGF1 protein (Fig. 9). These data suggest that the effects of GATA1 on endothelial cell phenotypes may be through regulation of the expression of AGGF1. Significantly, our results suggest that the function of GATA1 is not necessarily restricted to the hematopoietic linkage cells, and on the contrary, this study uncovers a novel role of GATA1 in endothelial cell biology and angiogenesis.

The *AGGF1* promoter/regulatory region have several interesting features. First, it lacks the TATA box and has two transcription start sites located −367 bp and −364 bp from the translation start site. Thus, the *AGGF1* promoter joins a growing list of vascular genes that use a TATA-less promoter and possess multiple, closely spaced transcription initiation sites, including the promoters for *GPIIb1*, α_2 and α_5 integrins, platelet *GPIX*, *CD11a*, and *CD11b* (35–40). Second, the sequences flanking the TSS and translation start site of the *AGGF1* gene are highly C/G-rich and contain more than 50 CpG islands. The CpG islands can become the target of DNA methylation. Also, they differ from other chromatin in several other respects, including a reduction in histone H1 and a general absence of nucleosomes in the region (41). Methylation may be one mechanism by which the expression of the *AGGF1* gene is regulated at the "pre-transcriptional" level. Third, the *AGGF1* gene uses a functionally analogous initiator element (first described for the terminal deoxytransferase gene promoter (42) to direct transcription initiation.

Functional results in this study demonstrate that, in addition to GATA1, several other putative *trans*-acting factors may regulate expression of *AGGF1*. It is possible that the *AGGF1* promoter is regulated both positively and negatively by other *trans*-acting factors. First, there are two *cis*-acting DNA elements that repress expression of *AGGF1*, one located from −8391 bp to −7512 bp (repressor 1) from the translation start site, and the other located from −3990 bp to −1971 bp (repressor 2) (Fig. 2*B*). Future studies are needed to precisely define the minimum sequences for the two repressors and to identify potential proteins that bind to the two sites. Second, the 536-bp DNA fragment from *AGGF1* translation start site is capable of driving the highest expression of the *AGGF1* gene (Fig. 3). The full expression of *AGGF1* requires GATA1 that binds to a consensus GATA1 DNA binding site centered from −295 bp to −300 bp, and a *cis*-acting element located from −159 to −129 bp from the translation start site (Fig. 3). Both the GATA1 site and the second activator site are essential for expression of *AGGF1* (Fig. 7*C*). The *AGGF1* −295 to −300 GATA site consists of GGATAA, a deviated version of the canonical GATA consen-

FIGURE 6. **Detection of GATA1 expression in a variety of tissues and cells.** *A*, RT-PCR analysis. *Control*, a mammalian expression plasmid for GATA1; *No RT*, negative control with RNA but without reverse transcriptase in the reaction. *B*, Western blot analysis. *Control*, nuclear extracts from HEK293 cells transfected with a mammalian expression plasmid for GATA1. The tissues samples, including the heart, liver, brain, lung, kidney, aorta, and bone marrow were from mice. Note that the *lanes* for the heart and liver are reversed in *B* compared with *A* and *C*. *HEL*, *THP1*, and *U937* are different types of human cells. *HUVEC*, human umbilical vein endothelial cells; *HBMEC*, human brain microvascular endothelial cells; *COAEC*, human coronary artery endothelial cells. Housingkeeping gene GAPDH was used as a loading control in both RT-PCR and Western blot analyses. The bands from RT-PCR and Western blot analyses were scanned, quantified, and plotted after calibration with GAPDH bands. *C*, expression profile of GATA1 and AGGF1 in neonatal mice at the age of P2 and P5 by Western blot analysis.

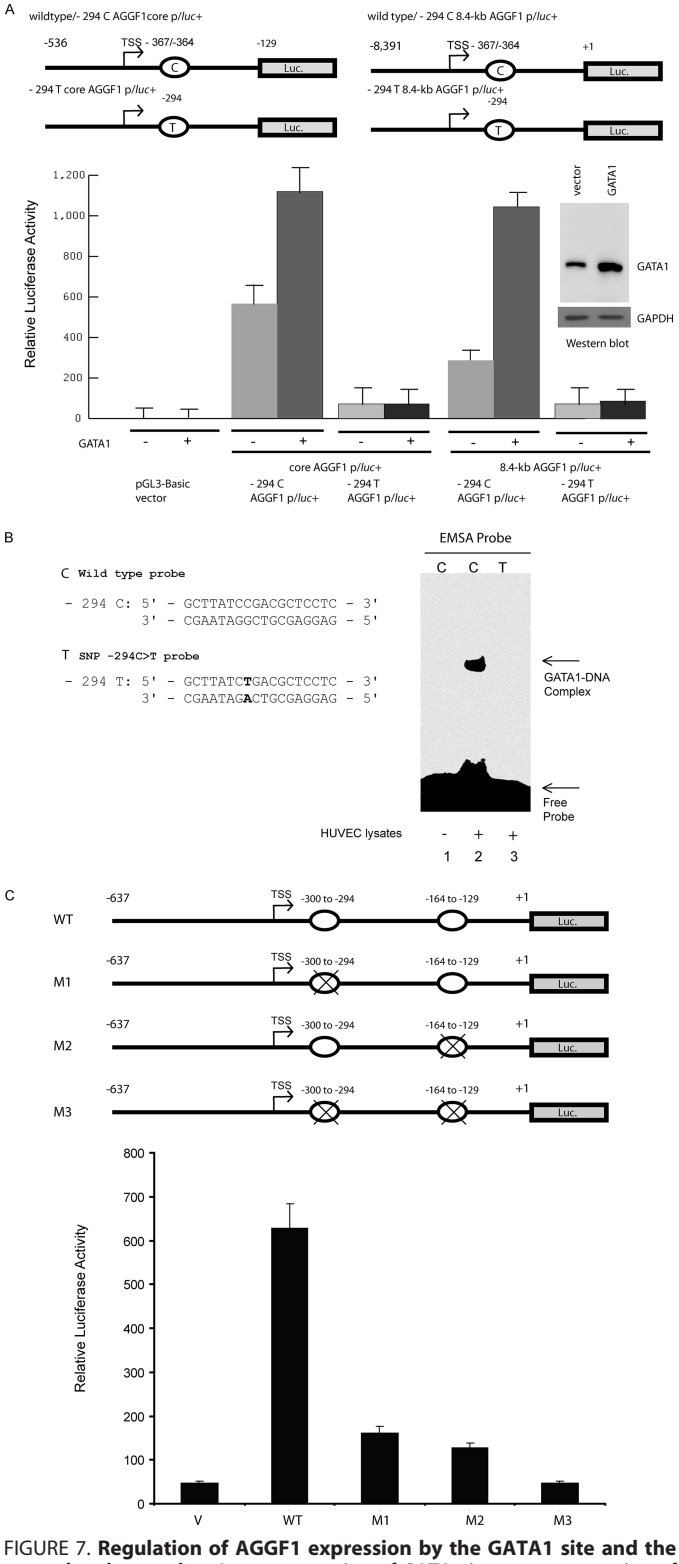
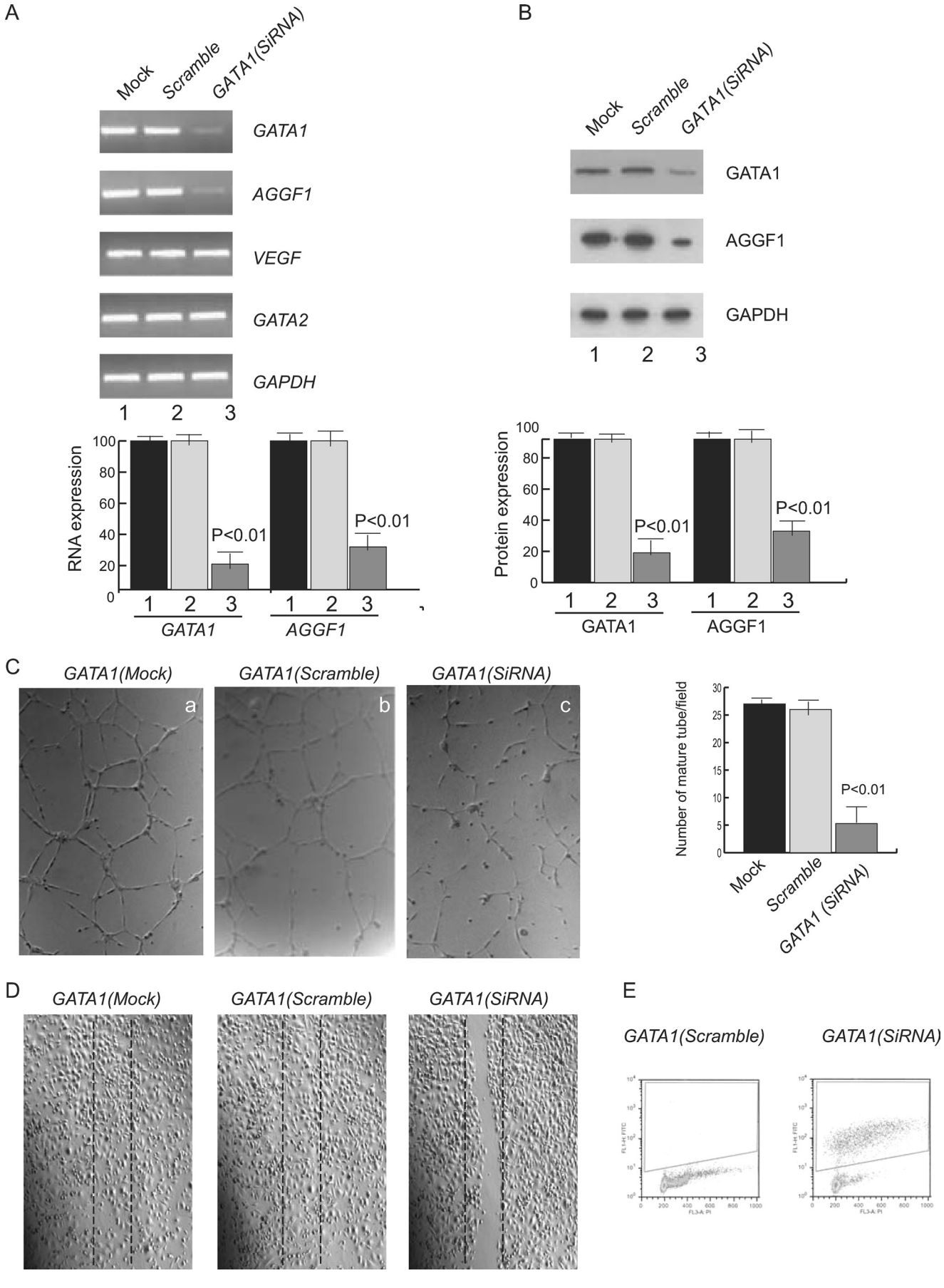


FIGURE 7. **Regulation of AGGF1 expression by the GATA1 site and the second activator site.** *A*, overexpression of GATA1 increases expression of AGGF1 and SNP −294C>T reduces transcription activation of the *AGGF1* promoter. Transcriptional activity for wild-type −294C and mutant −294T −536-*AGGF1p-luc* reporter genes (*left panel*) or longer 8.4kb-AGGF1p-luc reporter genes (*right panel*) are shown. HUVECs were co-transfected with a reporter genes and pcDNA3-GATA1 mammalian expression plasmid. Transcriptional activities are shown as relative luciferase activities on *y* axis. GATA1 strongly activates transcription of the wild-type core *AGGF1* promoter, but not the mutant promoter. Western blot analysis showed an increased expression level of GATA1 in HUVECs transfected with a GATA1 expression construct compared with HUVECs transfected with the expression vector. GAPDH was used for loading control. *B*, SNP −294C>T affects binding of GATA1 to DNA. Sequences of EMSA probes for wild-type (*C*) or mutant GATA1 binding sites (*left*) and results of EMSA (*right*) are shown. *Lane 1*, ^{32}P-labeled C probe alone; *lane 2*, EMSA for ^{32}P-labeled C probe and HUVEC nuclear extracts; *lane 3*, EMSA for ^{32}P-labeled mutant T probe and HUVEC nuclear extracts. *C*, transcriptional activity assays for mutant *AGGF1* promoters with a deletion of the GATA1 site (*M1*), the second activator site (*M2*), or both (*M3*).

sus sequence, (A/T)GATA(G/A). Interestingly, such deviation appeared to abrogate the binding of GATA2, -3, -4, and -6 but not GATA1, as demonstrated by the supershift assay with GATA antibodies (Fig. 4*D*). Thus, unlike other members of the GATA family, GATA1 possesses the unique binding specificity to GGATAA in the *AGGF1* promoter. It is such a unique binding affinity of GATA1 to the *AGGF1* promoter that defines GATA1 as the specific regulator of AGGF1 expression. However, this could not explain why the KTS promoter polymorphism reduced GATA1 binding, because the KTS polymorphism resulted in the reversal of the deviated sequence back to the canonical GATA consensus sequence AGATAA. It would be interesting for future studies to determine whether the flanking sequence is involved in regulating GATA1 binding to *AGGF1* promoter. It is unknown how the second *cis*-acting activator element regulates the expression of *AGGF1*, but it is notable that it contains three direct CA(G/T)GG repeats (5-GTGAGTTT**CAGGG**CGT**CATGG**C**CAGGG**G-CCA-3′). This *cis*-acting element may increase the expression of AGGF1 by binding a transcription factor or by its unique structure feature. Third, the core *AGGF1* promoter showed potential binding sites for a number of well known transcription factors, including GR, HIF-1, C/EBPb/a, NF-κB, Elk-1/c-Ets, MZF1, Th1/E4, STATX, and NKX2. Whether these factors regulate expression of *AGGF1* or not is a question that needs to be addressed in the future.

The genetic basis of KTS largely is unknown. Molecular characterization of a translocation t(5;11) associated with KTS has led to the molecular cloning of *AGGF1*. We previously reported that translocation t(5:11) increased the transcription activity of *AGGF1* by 3-fold. The chromosome 5p13.3 translocation breakpoint is located −1644 from the translation start site (Fig. 2*A*). Translocation t(5;11) is expected to remove the two repressor sites (−8391 to −7512; −3990 to −1971), resulting in increased *AGGF1* expression. The present study identifies a −294C>T polymorphism in the *AGGF1* promoter/regulatory region in a severe KTS case (large capillary malformations, important venous malformations, hypertrophy of the right leg, multiple surgeries, and hospitalizations). The −294C>T polymorphism reduces binding of GATA1 to the *AGGF1* promoter and dramatically decreases transactivation of *AGGF1*, indicating that it is a functional variant. The −294C>T polymorphism was identified in one of 185 KTS patients, but in none of 1764 non-KTS controls. Fisher's exact test revealed a trend, but not significant association with KTS (two-tailed $p = 0.095$). The KTS patient was an adopted child, and studies on family members are not possible. One final note is that −294C>T acts by a loss-of-function mechanism, whereas translocation t(5:11) acts by a gain-of-function mechanism. One interpretation of the data is that −294C>T is merely a rare polymorphism that is not associated with KTS. The alternative interpretation is that both


A
Mock
Scramble
GATA1(SiRNA)
GATA1
AGGF1
VEGF
GATA2
GAPDH
1 2 3
RNA expression
100
80
60
40
20
0
P<0.01
P<0.01
1 2 3
1 2 3
GATA1
AGGF1
B
Mock
Scramble
GATA1(SiRNA)
GATA1
AGGF1
GAPDH
1 2 3
Protein expression
100
80
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20
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1 2 3
GATA1
AGGF1
C
GATA1(Mock)
GATA1(Scramble)
GATA1(SiRNA)
a
b
c
Number of mature tube/field
30
25
20
15
10
5
0
P<0.01
Mock
Scramble
GATA1 (SiRNA)
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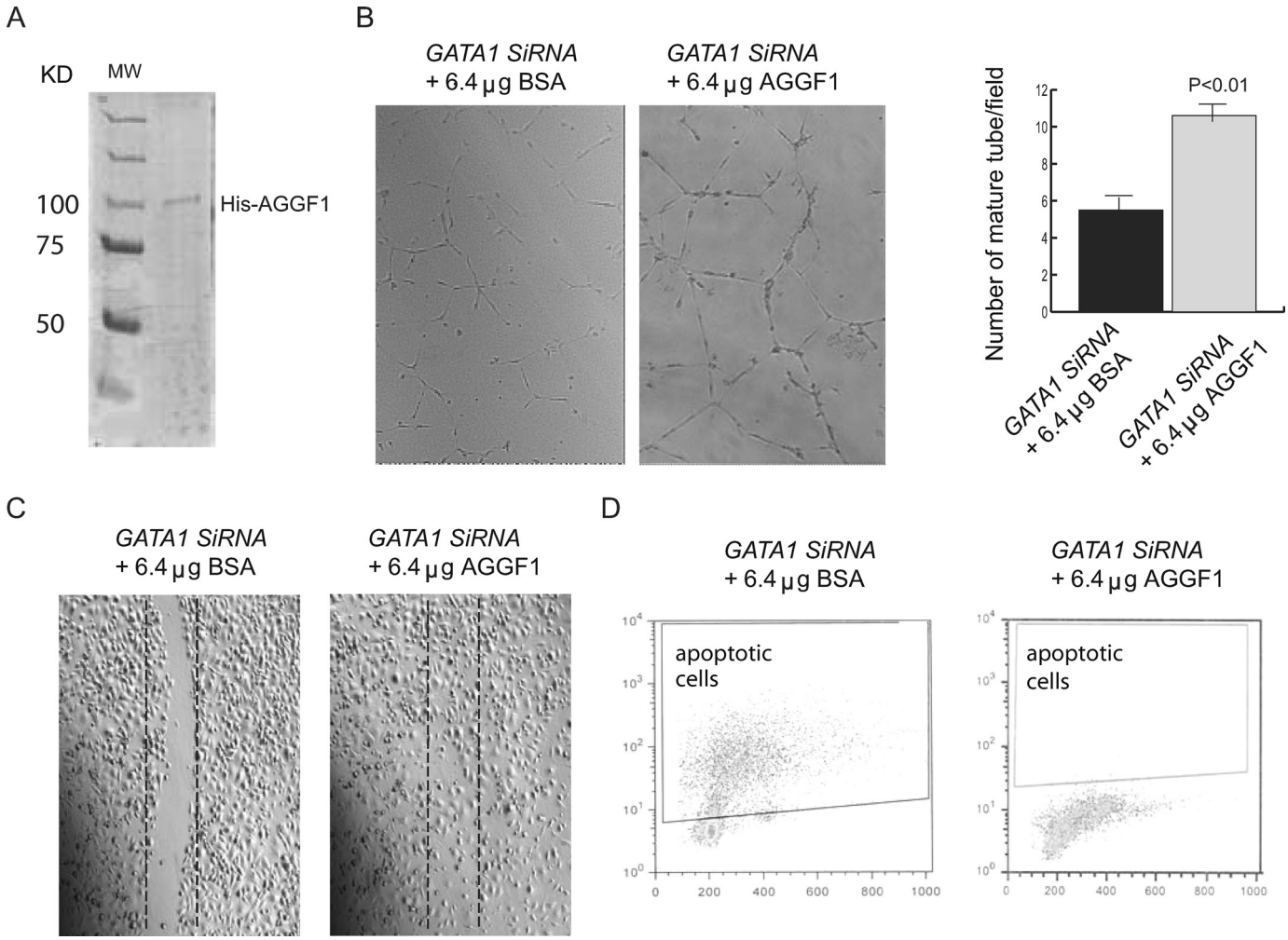

FIGURE 9. **AGGF1 rescues the effects of *GATA1* siRNA.** *A*, SDS-PAGE showing the quality of bacterially purified human AGGF1 protein. *B*, *in vitro* Matrigel endothelial tube formation assay. Quantification was from three independent experiments ($n = 3$; $p < 0.01$). *C*, HUVEC migration by a scratch assay ($n = 4$). *D*, HUVEC apoptosis assay by TUNEL ($n = 4$).

loss-of-function and gain-of-function mechanisms of AGGF1 are associated with risk of KTS. There are precedents that both loss-of-function and gain-of-function mutations in the same gene cause one disease. For example, both loss-of-function and gain-of-function mutations in *TBX5* cause Holt-Oram syndrome (24, 25, 43). Expression of a key angiogenic factor like AGGF1 is under delicate regulation in cells, and either up-regulation or down-regulation of AGGF1 can have a deleterious effect and increased risk of KTS. Heterozygous VEGF knockout mice died during embryogenesis (E9.5), and 2- to 3-fold overexpression of VEGF also led to embryonic lethality (E12.5-E14) (44). It should be noted that the *in vivo* effect of SNP −294C>T in human tissue could not be assessed due to lack of human samples from the SNP carrier. Future studies are needed to test whether the association between SNP −294C>T and KTS can be further established.

In conclusion, the present study defines molecular mechanisms for the transcriptional regulation of the expression of the *AGGF1* gene. We defined the precise TSSs and mapped the regulatory motifs within the *AGGF1* promoter/regulatory region. We identified one rare SNP in the *AGGF1* promoter, −294C>T, associated with one sporadic KTS patient. Functional analysis of the SNP led to the finding that GATA1 is a key regulator of the *AGGF1* gene. Further studies revealed a novel role of GATA1 in endothelial cells. Our results indicate that GATA1 plays important roles in endothelial vessel formation, endothelial cell migration, and apoptosis, likely by regulation of expression of AGGF1 as exogenous recombinant human

FIGURE 8. **Identification of new cellular roles of GATA1 in endothelial cells.** *A*, RT-PCR analysis was used to determine the expression levels of GATA1, AGGF1, VEGF, GATA2, and GAPDH with treatments of HUVEC with phosphate-buffered saline buffer (mock), scramble siRNA, and *GATA1* siRNA. Quantification was based on three independent experiments ($n = 3$). *B*, Western blot analysis showed that the *GATA1* siRNA reduced expression of GATA1 and AGGF1. GAPDH serves as a loading control. Quantification was based on four independent experiments ($n = 4$). *C*, *in vitro* Matrigel endothelial vessel formation assay. HUVECs from a confluent monolayer were induced to form sprouts on the Matrigel (capillary vessel morphogenesis). HUVECs were transfected with electroporation buffer (Mock) (*a*), scramble siRNA (*b*), and *GATA1* siRNA (*c*). The number of well connected tubes was counted and is shown in the *right graph*. *D*, HUVEC migration by a scratch assay. *E*, HUVEC apoptosis assay by TUNEL.

AGGF1 protein can rescue the defects caused by *GATA1* siRNA.

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