A Three-Kilobase Fragment of the Human Robo4 Promoter Directs Cell Type–Specific Expression in Endothelium

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Abstract—Robo4, a member of the roundabout family, is expressed exclusively in endothelial cells and has been implicated in endothelial cell migration and angiogenesis. Here we report the cloning and characterization of the human Robo4 promoter. The 3-kb 5′-flanking region directs endothelial cell–specific expression in vitro. Deletion and mutation analyses revealed the functional importance of two 12-bp palindromic DNA sequences at −2528 and −2941, 2 SP1 consensus motifs at −42 and −153, and an ETS consensus motif at −119. In electrophoretic mobility shift assays using supershifting antibodies, the SP1 motifs bound SP1 protein, whereas the ETS site bound a heterodimeric member of the ETS family, GA binding protein (GABP). These DNA–protein interactions were confirmed by chromatin immunoprecipitation assays. Transfection of primary human endothelial cells with small interfering RNA against GABP and SP1 resulted in a significant (∼50%) reduction in endogenous Robo4 mRNA expression. The 3-kb Robo4 promoter was coupled to LacZ, and the resulting cassette was introduced into the Hprt locus of mice by homologous recombination. Reporter gene activity was observed in the vasculature of adult organs (particularly in microvessels), tumor xenografts, and embryos, where it colocalized with the endothelial cell–specific marker CD31. LacZ mRNA levels in adult tissues and tumors correlated with mRNA levels for endogenous Robo4, CD31, and vascular endothelial cadherin. Moreover, the pattern of reporter gene expression was similar to that observed in mice in which LacZ was knocked into the endogenous Robo4 locus. Together, these data suggest that 3-kb upstream promoter of human Robo4 contains information for cell type–specific expression in the intact endothelium. (Circ Res. 2007;100:1712-1722.)

Key Words: endothelial cells ▪ Robo4 ▪ gene regulation ▪ transgenic mice

There is increasing evidence that migration and patterning of axons and blood vessels share similar guidance mechanisms. Among the guidance systems involved in axonal and vascular networks are the ephrin-Eph, netrin-unc5b, semaphorin-plexin, and slit-Robo molecules.1

Robo (roundabout) is a member of the neural cell adhesion molecule family. Robo was originally isolated from Drosophila melanogaster.2 The ligand for Robo, Slit, was first identified in Drosophila as an extracellular molecule involved in axonal branching and neural migration.3 In vertebrates, 3 Robo receptor family members (Robo1 to -3) and 3 Slit ligands (Slit1 to -3) have been implicated in guiding axon growth via repulsive signaling.2 A fourth Robo receptor family member, Robo4, was cloned and shown to be restricted in its tissue distribution to endothelial cells.4-5 Robo4 is expressed in areas of in vivo angiogenesis. For example, the receptor is present in the endothelial lining of blood vessels in the developing embryo,6 placenta,4 and tumors.4,7 Robo4 has also been detected in the endothelium of normal nonangiogenic tissues, including the heart and lung.6,7 Recent studies support a role for Robo4 in endothelial cell migration, proliferation, and angiogenesis.6,8-10 The goal of the present study was to dissect the mechanisms of cell type–specific expression of the Robo4 promoter.

Materials and Methods

Human umbilical vein endothelial cells (HUVECs), human coronary artery endothelial cells (HCAECs), human pulmonary artery endothelial cells (HPAECs), human microvascular endothelial cells (HMVECs), and human coronary artery smooth muscle cells (HCASmCs) were purchased from Cambrex (Walkersville, Md). Primary endothelial cells were cultured in endothelial growth medium-2-MV. HCASmCs were cultured in SmBM-2 medium. The human embryonic kidney cell line (HEK293) and HepG2, U937, LLC, and B16-F1 cells were grown in DMEM supplemented with 10% FCS (Hyclone, Logan, Utah). Plasmid constructions are detailed in the online data supplement at http://circres.ahajournals.org. Transient transfection of primary endothelial cells and HEK293 cells

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was performed using FuGENE 6 reagent (Roche Molecular Biochemicals, Mannheim, Germany). Nuclear extracts were prepared from HCAECs using Nuclear Extract Kit (Active Motif, Carlsbad, Calif) according to the instructions of the manufacturer. Chromatin immunoprecipitation (ChIP) assays were performed using a Chromatin Immunoprecipitation Assay Kit (Upstate, Lake Placid, NY) according to the instructions of the manufacturer. Immunoprecipitated genomic DNA fragments were quantified by real-time PCR using the primers to amplify the Robo4 proximal promoter region. Hprt-targeted mice were generated as described previously. The generation of the Robo4-lacZ knock-in mouse is detailed in the online data supplement. Expression level of Robo4, CD31, vascular endothelial (VE)-cadherin, and LacZ from mouse tissues and cultured cells was measured by real-time PCR. All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. An expanded Materials and Methods section is available in the online data supplement.

Results

Cloning and Sequence Analysis of the Human Robo4 Gene

Under in vitro conditions, endogenous Robo4 was expressed at high levels in primary human endothelial cells, but not HCAECs or HEK293 cells (please see Figure I in the online data supplement). Rapid amplification of cDNA ends revealed 2 common transcriptional start sites (supplemental Figure II). The most frequent transcriptional start site is designated as base pair number +1. The sequence of the upstream promoter region of the human Robo4 gene was determined (Figure 1). The promoter lacks a TATA box. The upstream region includes unique long direct repeat sequences and 12-bp and 26-bp palindromic sequences (Figure 1A). The sequence between 285 and +40 is 80% conserved between mouse and human and 76% conserved between rat and human (Figure 1B). Among the consensus binding sites that are conserved among all 3 species in the 300-bp proximal promoter are ETS and 2 SP1 motifs (discussed below).

Functional Analysis of the Human Robo4 Promoter in Cultured Cells

To compare the activity of the Robo4 promoter with that of other endothelial cell–specific promoters, HUVECs and/or HCAECs were transiently transfected with pGL3 containing the upstream promoter regions of intercellular adhesion molecule-2 (0.37 kb), E-selectin (3 kb), P-selectin (3 kb), ephrinB2 (2.8 kb), Flt-1 (1.4 kb), Robo4 (3 kb) (termed pGL3-Robo4), Tie1 (0.8 kb), or Tie2 (0.72 kb). The Robo4 promoter demonstrated higher activity compared with promoters for intercellular adhesion molecule-2, P-selectin, E-selectin, ephrinB2, Flt-1, and Tie2 and similar activity to the Tie1 promoter (Figure 2A shows HCAECs). To determine whether the Robo4 promoter contains information for endo-
thelial-specific expression in vitro, transient transfection assays were also performed in HEK293, HepG2 and U937 cells. As shown in Figure 2B, Robo4 promoter activity was significantly higher in HCAECs compared with HEK293 cells. As shown in Figure 2B, Robo4 promoter activity was significantly higher in HCAECs compared with HEK293 cells (5-fold), HepG2 cells (29-fold), and U937 cells (18-fold). Together, these findings suggest that the 3-kb upstream promoter of human Robo4 directs high-level cell type restricted expression in vitro.

To delineate the functional elements within the upstream promoter region, a series of deletion and mutant promoter fragments were fused to the luciferase reporter gene in pGL3, and the resulting constructs were transiently transfected into HCAECs. Sequential 5' deletions resulted in stepwise reduction of promoter activity, with Del1 (−2450) demonstrating 55%, Del2 (−1635) demonstrating 44%, Del3 (−1173) demonstrating 32%, Del4 (−930) demonstrating 25%, and Del5 (−329) demonstrating 15% of wild-type level (Figure 3A). Deletion of sequences between −329 and −228 (Del6) resulted in a slight increase in activity (29%), suggesting the presence of a repressor in that region.

To further delineate the enhancing region between −2450 and −3000, additional 5' deletions were generated and assayed for luciferase activity. As shown in Figure 3B, Del1–1 (−2867), Del1–2 (−2745), Del1–3 (−2644), and Del1–4 (−2550) demonstrated activity comparable to the full length 3-kb promoter. The Del1–5 promoter, containing a 2515 bp 5'-flanking sequence, resulted in a significant (70%) decrease in activity, whereas further deletions had no such effect. These data suggest that a 35-bp DNA region between −2550 and −2515 possesses enhancing activity. This region (which we term the Robo4 enhancer element 1 [REn1]) contains consensus binding sites for nuclear factor-κB, nuclear factor of activated T cells (NF-AT), SP1, glucocorticoid receptor, and activator protein 2y. However, single mutations of these motifs in the context of the 3-kb promoter failed to alter luciferase activity (Figure 3C). To further address the role of this region in mediating expression of Robo4, the REn1 was removed from the full-length promoter, inverted, or replaced with 2 different heterologous sequences. As shown in Figure 3D, none of these manipulations strongly affected promoter activity.

Based on these findings, we inferred that additional upstream DNA sequences between −3000 and −2551 must compensate for promoter activity in the absence of REn1. To test this hypothesis, 6 new internal–deletion constructs were generated in which the REn1 and progressive lengths of 5' sequence were removed from the full-length promoter. As shown in Figure 3E, there was little change in promoter activity with deletions of 100, 200, 300, and 400 bp. However, deletions of 435 bp (Del 435bp) or 465 bp (Del 465bp) resulted in 80% and 70% reduction in promoter activity, respectively, similar to that observed with Del1–5 (the same construct used in Figure 3B). These data support the existence of a second enhancer between −2950 and −2916 (which we term REn2) (Figure 3F).

To confirm the dual role for REn1 and REn2 in mediating Robo4 promoter activity, we generated new deletion constructs. Single deletions of REn1 or REn2 resulted in a 15% and 34% reduction in promoter activity, respectively, whereas a double deletion of REn1 and REn2 resulted in 42% decrease in activity (Figure 3G). Interestingly, REn1 and REn2 contain 12-bp palindromic DNA sequences (5’CAGAGCCCGAG in REn1; 5’TCTTGCTCTG in REn2) (Figure 3F). To determine whether these sequences were responsible for the enhancing activity of REn1 and REn2, we deleted the two 12-bp elements from the full-length promoter (Figure 3G). The resulting construct demonstrated a 56% reduction in promoter activity, similar to that observed with Del1–5. Taken together, these data support a role for the 12-bp palindromic sequences in mediating Robo4 promoter activity.

We next focused on the proximal region of the human Robo4 promoter because the sequence of the immediate upstream 300-bp region is highly conserved between species. As shown in Figure 3D, none of these manipulations strongly affected promoter activity. Based on the presence of a repressor in that region.
distinguish it from other ETS motifs in the promoter. The above sites were mutated alone or in combination (5 Mut and SP1[1,2]) in the context of the 3-kb Robo4 promoter, and the resulting mutants were assayed for activity in transient transfection assays. As shown in Figure 3H, a single mutation of the −119 ETS(1), −153 SP1(1), or −42 SP1(2) site resulted in a 90%, 40%, or 50% reduction in promoter activity, respectively, whereas mutations of the other sites had no significant effect. A double mutation of the SP1 sites (SP1[1,2]) resulted in a 65% reduction in promoter activity. These findings suggest that the ETS(1), SP1(1), and SP1(2) sites are critical determinants of Robo4 promoter activity.

**SP1 Binds to the Human Robo4 Promoter and Induces Promoter Activity**

To investigate whether SP1 binds to the SP1(1) and SP1(2) sites, electrophoretic mobility shift assay (EMSA) was performed. Incubation of a radiolabeled probe spanning the −153 SP1 site with nuclear extract from HCAECs resulted in a strong DNA–protein complex (Figure 4A, lane 2). The
complex was inhibited by addition of cold wild-type SP1 competitor, but not a mutant SP1 competitor (Figure 4A, lanes 3 to 6). Preincubation with anti-SP1 antibody resulted in a partial supershift of the specific DNA–protein complex, whereas control antibody had no such effect (Figure 4A, lanes 7 and 8). (The partial nature of the supershift may be explained by limiting amounts of antibody or the existence of a second DNA–protein complex that lacks SP1.) Finally, incubation of radiolabeled probe with in vitro–translated SP1 resulted in a DNA–protein complex of similar size to that obtained with nuclear extracts (Figure 4A, compare lanes 10 and 2). The latter complex was supershifted with anti-SP1 antibody (Figure 4A, lane 11). The same result was obtained in EMSA using a probe spanning the −42 SP1 site (Figure
To determine whether SP1 transactivates the Robo4 promoter through these 2 SP1 sites, cotransfection assays were performed in HEK293 cells using an SP1 expression vector. Overexpression of SP1 resulted in significant (8-fold) induction of Robo4 promoter activity (Figure 4C). A single mutation of SP1(1) or SP1(2) reduced the promoter activity to 6.5- or 5.5-fold, respectively. A double mutation of the SP1 motifs (SP1[1,2]) led to a further reduction in activity (4-fold). Taken together, these data suggest that SP1 regulates the Robo4 promoter activity through both the −42 and −153 SP1 sites.

**GABP Binds to the Human Robo4 Promoter and Induces Promoter Activity**

To identify the factor that binds to the −119 ETS(1) motif, EMSA was performed as described above using a probe that

![Figure 4. SP1 and GABP bind to the human Robo4 promoter and induce promoter activity. A and B, EMSA was performed with 32P-labeled SP1(1) or SP1(2) probe in the absence (lane 1) or presence of nuclear extract from HCAECs (lanes 2 to 8), in vitro–translated SP1 (lane 10 and 11), or negative control (lane 9). In competition assays, a 10- or 50-fold molar excess of unlabeled wild-type (lanes 3 and 4) or mutant (lanes 5 and 6) SP1 probe was added to the reaction mixture. In supershift assays, nuclear extract or in vitro–translated SP1 was incubated in the presence of anti-SP1 antibody (lanes 7 and 11, respectively) or control antibody (lane 8). The closed arrow indicates specific DNA–protein complex. The arrowhead indicates the supershifted complex. C, Cotransfection assay was performed in HEK293 cells using 0.3 µg of pcDNA3-SP1 expression vector (WT) or empty vector (−) and either wild-type pGL3-Robo4 (WT) or similar constructs containing mutations at either SP(1) or SP(1) site, or both of these sites, SP1(1,2). Luciferase light units are expressed as fold induction over the empty expression vector, pcDNA3. The data represent means ±SE of 6 replicates. *P<0.05 between WT with pcDNA3-SP1 and SP(2) or SP(1,2). D, EMSA was performed with 32P-labeled ETS probe, spanning the ETS(1) motif, in the absence (lane 1) or presence of nuclear extract from HCAECs (lanes 2 to 5). In competition assays, a 50-fold molar excess of unlabeled wild-type (WT) (lane 3) or mutant (Mut) (lane 4) ETS probe was added to the reaction mixture. The binding reaction was pre-incubated with a supershifting anti-ETS-1 antibody (lane 5). The bracket indicates specific DNA–protein complex. NS indicates nonspecific band. E, EMSA was performed using a classical ETS-1 consensus probe (lane 1 to 3) or ETS(1) probe (lane 4 to 5) in the absence (lanes 1 and 4) or presence of in vitro–translated ETS-1 protein (lanes 2, 3 and 5). As a negative control, in vitro–translated protein from an empty vector was added (lanes 1 and 4). Antibody to ETS-1 was added in lane 3. F, EMSA was performed with 32P-labeled ETS probe in the absence (lane 1) or presence of nuclear extract from HCAECs (lanes 2 to 7). In supershift assays, nuclear extracts were incubated in the presence of antibodies to GABPα (lane 3), GABPβ (lane 4), GABPβ/γ (lane 5), SP1 (lane 6), or control antibody (lane 7). The arrowhead indicates DNA-SP1 complex; the bracket indicates DNA-GABP complex. NS indicates nonspecific band. G, EMSA was performed using a classical ETS-1 consensus probe (lane 1 to 3) or ETS(1) probe (lane 4 to 5) in the absence (lanes 1 and 4) or presence of in vitro–translated ETS-1 protein (lanes 2, 3 and 5). As a negative control, in vitro–translated protein from an empty vector was added (lanes 1 and 4). Antibody to ETS-1 was added in lane 3. F, EMSA was performed with 32P-labeled ETS probe in the absence (lane 1) or presence of nuclear extract from HCAECs (lanes 2 to 7). In supershift assays, nuclear extracts were incubated in the presence of antibodies to GABPα (lane 3), GABPβ (lane 4), GABPβ/γ (lane 5), SP1 (lane 6), or control antibody (lane 7). The arrowhead indicates DNA-SP1 complex; the bracket indicates DNA-GABP complex. NS indicates nonspecific band. G, EMSA was performed with 32P-labeled ETS probe in the absence (lane 1) or presence of in vitro–translated GABP. Arrows indicate DNA–protein complexes derived from GABPα, GABPβ and -β, or GABPβ and -γ. H, Cotransfection assay was performed in HEK293 cells using 0.1 µg of each GABP expression vector (−) or empty vector (−) and either wild-type pGL3-Robo4 (WT) or a similar construct containing a mutation of the −119 ETS site (Mut). The data represent means ±SE of 6 replicates. *P<0.05 between control and GABPα, GABPβ/β, GABPβ/γ, GABPβ/β/γ or GABPα/β/γ with mutant promoter, respectively; #P<0.05 between Robo4 WT and mutant promoter.
contains this site. A strong DNA–protein complex was detected (Figure 4D, lane 2) and was inhibited by addition of wild-type, but not mutant cold ETS(1) competitor (Figure 4D, lanes 3 and 4). Supershift assays were performed with antibodies to ETS factors that have been previously implicated in endothelial cell gene regulation, including ETS-1, ETS-2, ELF-1, FLI-1, ERG, NERF, and PEA3. None of these antibodies resulted in a supershift or inhibited the specific DNA–protein complex (Figure 4D, lane 5 shows ETS-1). As a positive control for ETS-1 binding and supershifting activity of the ETS-1 antibody, a radiolabeled probe spanning the consensus ETS-1 binding motif was incubated with recombinant ETS-1. As shown in Figure 4E, ETS-1 protein bound to the classical ETS motif (but not ETS[1] from the Robo4 promoter), and the resulting DNA–protein complex was supershifted by ETS-1 antibody.

Based on the above results, we explored the potential role of another ETS factor that has not been previously described in endothelial cells, namely GA binding protein (GABP). GABP binds as a complex consisting of heterodimers of GABPα/H9251 and GABPα/H9252 or α/H9253. GABPα/H9253 is an alternative splice form of GABPα/H9252 (see review12). In supershift assays, preincubation with antibodies against GABPα or GABPβγ resulted in complete inhibition of the DNA–protein complex (Figure 4F, lanes 3 and 5). In contrast, anti-GABPβ antibody had minimal effect on DNA binding, and anti-SP1 antibody resulted in loss of a more slowly migrating DNA–protein complex (Figure 4F, lanes 4 and 6). In vitro translated protein consisting of GABPα, GABPα/β, or GABPα/γ resulted in specific DNA–protein complexes compatible with those observed with nuclear extracts (Figure 4G).

In cotransfection assays, overexpression of GABPα, alone or together with GABPβ or GABPγ, resulted in significant induction of Robo4 promoter activity, whereas GABPβ or GABPγ alone had no such effect (Figure 4H). Mutation of the ETS(1) site significantly attenuated GABP-mediated transactivation of the promoter (Figure 4H). Together, these data suggest that GABP plays an important role in mediating Robo4 expression.

**SP1 and GABP Bind to the Endogenous Human Robo4 Promoter in Primary Endothelial Cells**

To investigate whether SP1 and GABP bind to the Robo4 proximal region in endothelial cells, ChIP assay was performed. Formalin-fixed genomic DNA–protein complexes from HCAECs and HCASmCs were sheared by sonication (Figure 5A). Resulting small DNA–protein complexes were immunoprecipitated in the absence or presence of 8 μg of antibodies to SP1, GABPα, or Egr-1 (control IgG). Real-time PCR analysis was performed using the precipitated DNA fragments and primers for Robo4 proximal region, which included the SP1 and ETS(1) site. C, HCAECs were transfected with 100 pmol of siRNA against SP1, GABPα, or negative control siRNA using Lipofectamine 2000 reagent according to the instructions of the manufacturer. After 48 hours of incubation, total RNAs were prepared by RNeasy RNA extraction kit. Samples were assayed by real-time PCR for expression of SP1, GABPα, and Robo4.

**Small Interfering RNA–Mediated Knockdown of GABPα and SP1 Results in Significant Reduction of Endogenous Robo4 mRNA Expression**

To determine whether SP1 and GABPα play a role in mediating the endogenous expression of Robo4, small interfering RNA (siRNA) against these transcription factors were transfected into HCAECs. As shown in Figure 5C, siRNA
against SP1 resulted in a 5.3-fold reduction in SP1 and a 1.9-fold reduction of Robo4 expression, whereas siRNA against GABP resulted in 4.9-fold reduction in GABP and 2.2-fold decrease in Robo4.

The Three-Kilobase Human Robo4 Promoter Contains Information for Endothelial-Specific Expression in Mice

To determine whether the Robo4 promoter directs lineage-specific expression in vivo, the 3-kb promoter region was coupled to LacZ. A single copy of the transgenic cassette was targeted to the Hprt locus of mice using homologous recombination as previously described. High percentage chimeric males were bred to wild-type females. Resulting female agouti offspring were bred to generate stable lines and F2 males were assayed for LacZ expression.

Whole-mount staining of organs (brain, heart, lung, skeletal muscle, aorta, trachea, diaphragm, and esophagus) revealed widespread, although not uniform, -galactosidase activity in the vasculature (Figure 6). Tissue sections revealed LacZ staining in the endothelial lining of vessels in all organs examined (Figure 7B). Expression was greater in the microvessels compared with macrovessels. In the kidney, -galactosidase activity was highest in the glomeruli. In serial sections, LacZ colocalized with CD31. LacZ was not observed in any other cell type or lineage including peripheral blood cells and bone marrow (supplemental Figure III).

We compared expression of LacZ mRNA with that of endogenous Robo4 and 2 endothelial markers, CD31 and VE-cadherin, using real-time PCR of adult mouse tissues. As shown in Figure 7C, Robo4 transcripts were detected in all organs, according to the following rank order: lung>heart>kidney>skeletal muscle>liver>spleen=brain. Importantly, LacZ mRNA expression in Hprt-targeted mice followed a similar pattern. To control for vascular density, tissue samples were also assayed for CD31 and VE-cadherin. The pattern of LacZ and Robo4 expression mirrored that of VE-cadherin and CD31 (Figure 7C). (In the case of CD31, expression was relatively higher in spleen, presumably owing to positivity in hematopoietic cells.) These data suggest that expression of the transgene mimics that of the endogenous Robo4 gene, which in turn correlates with the degree of vascularization. LacZ expression was also detected in the endothelium of embryos and tumor xenografts (supplemental Figures IV and V).

As a second strategy for comparing the expression of the Hprt-targeted transgene and the endogenous gene, we knocked LacZ into the endogenous Robo4 locus (Figure 8A). Heterozygous F2 adult males demonstrated endothelial cell–specific expression of LacZ in the vasculature (Figure 8B and 8C). One exception was the brain, where mounts and tissue sections revealed a weak nonvascular distribution in the pia mater (data not shown). Compared with the Hprt locus–targeted mice, the LacZ knock-in animals demonstrated lower -galactosidase activity in the vasculature. Moreover, reporter gene expression in the aorta was more restricted to branch orifices and tributaries and was undetectable in the large arteries of the brain. Otherwise, the pattern of expression was similar between the two lines of mice, with predominant staining in the microvascular endothelium.

Discussion

We have cloned and characterized the human Robo4 promoter. Similar to the endogenous Robo4 gene, a 3-kb fragment of the upstream promoter directed high-level lineage-specific expression in cultured endothelial cells.
Deletion analyses revealed several positive regulatory regions. Using a series of mutational analyses, EMSA, ChIP, and siRNA-mediated knockdown experiments, we have demonstrated an important role for SP1 and GABP in governing basal expression of Robo4. In addition, we have identified 12-bp palindromic DNA sequences that are important for Robo4 promoter activity in endothelial cells. This DNA sequence does not conform to established cis-regulatory motifs and thus represents a potentially novel regulatory element. The mechanism by which the palindromic sequences mediate Robo4 expression is the focus of ongoing studies.

Members of the ETS family of transcription factors share an evolutionarily conserved DNA-binding domain of 85 aa with a winged-helix-turn-helix configuration. ETS factors bind to GGAA/T core sequences. Consensus ETS binding motifs have been identified within the promoters of several other endothelial cell genes, including Flt-1, Tie1, Tie2, and VE-cadherin. The functional relevance of ETS motifs in mediating endothelial cell gene expression has been demonstrated both in vitro and in vivo. Several ETS factors have been shown to mediate gene expression in endothelial cells, most notably ETS-1, ETS-2, ESE-1, NERF2, and ELF-1.

GABP (also known as nuclear respiratory factor [NRF]-2 and adenovirus E4 transcription factor [E4TF]-1) is unique among the ETS family of transcription factors in that it forms multimers, consisting of 2 structurally unrelated subunits: GABPα and GABPβ. GABPα contains the ETS DNA-binding region, whereas GABPβ is required for nuclear translocation and transactivation. GABPβ stabilizes the GABPα–DNA interaction more than 100-fold. Mammalian GABP is ubiquitously expressed in all tissues and has been implicated in several critical cellular processes including cellular respiration in mitochondria, differentiation, cell cycle, cell survival, and neuromuscular function. Mice that are null for GABPα are embryonic lethal and die before implantation. In addition to controlling the expression of housekeeping genes, GABP has been shown to regulate the expression of cell type–specific genes in several distinct lineages, including myeloid cells, lymphocytes, neuromuscular cells, hepatocytes, and mast cells. To our knowledge, this is the first study to demonstrate a role for GABP in promoting the expression of an endothelial cell–specific target gene.

Our data are consistent with the notion that SP1 and GABP are necessary for full basal expression of Robo4 in endothelial cells. They do not prove that these transcription factors mediate cell type–specific gene expression. Indeed, SP1 and GABP are expressed in other cell types, and ChIP assays in...
vascular smooth muscle cells revealed binding of both transcription factors to the Robo4 promoter in an otherwise nonexpressing cell type. Thus, other mechanisms must be responsible for cell type–specific gene expression. One possibility is that GABP interacts with cell type–specific transcription factors or coactivators to promote cell-specific responses.

Previous studies using standard transgenic mouse assays or Hprt locus targeting have demonstrated that the majority of endothelial-specific promoters direct expression to specific vascular beds (reviewed previously22). In the current study, the Hprt-targeted 3-kb Robo4 promoter directed expression in the embryonic and adult vasculature. Reporter gene expression was restricted to the endothelium. Expression was more prominent in microvessels compared with macrovessels. Even within microvessels, LacZ expression was nonuniform. In real-time PCR analyses, LacZ mRNA expression correlated with expression of endogenous Robo4. Moreover, the expression pattern was similar to that observed when the LacZ reporter gene was knocked into the Robo4 locus. Because the knock-in strategy involved deletion of Robo4 DNA sequences, including potential regulatory elements in the first 3 introns, it is formally possible that the LacZ expression does not precisely reflect the endogenous of the Robo4 gene. That caveat notwithstanding, our data suggest that the 3-kb human Robo4 promoter contains information for near-authentic expression in the endothelium.

Based on its exquisite cell type specificity, and its expression in the neovasculature, the Robo4 gene (and promoter) represents a powerful tool for dissecting the molecular basis of lineage determination and new blood vessel growth.

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Disclosures
None.

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The ETS transcription factor GABPalpha is essential for early embryo-

A Three-Kilobase Fragment of the Human Robo4 Promoter Directs Cell Type–Specific Expression in Endothelium

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SUPPLEMENTAL MATERIALS AND METHODS

Cell culture. HUVEC, HCAEC, HPAEC, HMVEC, and HCASmC were purchased from Cambrex (Walkersville, MD). Primary endothelial cells were cultured in endothelial growth medium-2-MV. HCASmC were cultured in SmBM-2 medium. The human embryonic kidney cell line (HEK293), HepG2, U937, LL/2 and B16-F1 cells were grown in DMEM supplemented with 10% fetal calf serum (Hyclone, Logan, UT). All cells were cultured at 37°C and 5% CO₂.

5' RACE. The 5' end of full length Robo4 cDNA was amplified by PCR using GeneRacer Kit with SuperScript™ III RT (Invitrogen, Carlsbad, CA) and 3 independent Robo4 gene-specific primers according to the manufacturer's instructions. Amplified fragments were cloned into the pCR4-TOPO vector. A total of 17 clones were randomly chosen for purification and DNA sequencing.

Plasmid constructions. To generate the luciferase constructs, the 5' flanking regions of the human ICAM-2 (0.37-kb), P-selectin (3-kb), E-selectin (3-kb), ephrinB2 (2.8-kb) and Robo4 (3-kb) genes were PCR amplified using the KOD DNA polymerase (Toyobo, Osaka, Japan), human genomic DNA, and forward and reverse primers containing restriction sites (Table 1). The amplified fragments of ICAM-2, P-selectin and E-selectin were digested with HindIII and cloned into the HindIII site of the pGL3 vector (Promega, Madison, WI). The amplified fragments of ephrinB2 and Robo4 were digested with NheI and cloned into the NheI site of the pGL3 vector. To generate pGL3-Flt-1 (1.4-kb),
pGL3-Flt-1 was digested with SpeI and HindIII and cloned into NheI (blunt ended) and HindIII sites of the pGL3 vector. The resulting construct was named pGL3-Flt-1(1kb). The upstream region of the Flt-1 promoter was PCR amplified using human genomic DNA and primers (Table 1), and cloned into the pGEM-T Easy vector (Promega). After verifying the sequence, the Flt-1 fragment was purified by digesting with EcoRI and ApaI, and cloned into MluI (blunt ended) and ApaI sites of pGL3-Flt-1(1kb). For the preparation of pGL3-mTie-1 plasmid, the 0.8-kb upstream region of the mouse mTie-1 promoter (described in 2) was subcloned into pGL3. pGL3-mTie-2 contains the 300-bp intronic enhancer coupled upstream to a 423-bp core promoter, as previously described 3.

The Robo4 mutants were prepared using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The Del3 construct (shown in Fig. 5A) was employed as template. Mutations (shown in Table 1) were based on data from previous reports 4-9. Once generated, the Del3 constructs containing the mutations were digested with StuI and XhoI. The mutant promoter fragments were purified and used to replace the StuI and XhoI fragment of the wild-type Robo4 promoter in pGL3.

To generate the Robo4 deletion constructs (Del1, 3, and 4), pGL3-Robo4 was digested with MluI and EcoRI (-2450), with MluI and ApaI (-1173), and with MluI and StuI (-930), respectively. The largest fragment was blunt-ended using T4 DNA polymerase, gel-purified and self-ligated. For generating Del2, 5 and 6, pGL3-Robo4 was digested with NheI and SmaI (-1635), NheI and blunted BamHI (-329), and NheI and blunted HindIII (-228), respectively, and the resulting fragment was cloned into MluI (blunt-ended) and
For the preparation of 5 deletion constructs (Del1-1, 1-2, 1-3, 1-4 and 1-5), Robo4 promoter upstream fragments were amplified by PCR. The promoter fragment for Del1-6 was made by annealing 2 oligonucleotides. These 6 fragments were digested with MluI and EcoRI, and cloned into MluI and EcoRI sites of pGL3-Robo4.

The Robo4 upstream mutants (NF-κB, NF-AT, SP1, GR and AP2γ) were prepared using the QuikChange II Site-Directed Mutagenesis Kit. The pGL3-Robo4 construct was employed as template. Primer sequences (shown in Table 1) were created by with or without modifying the sequence described previously. Once generated, the pGL3-Robo4 constructs containing the mutations were digested with KpnI and EcoRI. The mutant promoter fragments were purified and used to replace the KpnI and EcoRI fragment of the wild-type Robo4 promoter in pGL3.

For the preparation of Del REn1, Inverted, Replacement1, Replacement2, the intermediate plasmid, pGL3-ROBO4-BbsI, was prepared by PCR using 4 primers (shown in Table 1). pGL3-ROBO4-BbsI was digested with BbsI and blunted with T4 DNA polymerase. The resulting fragment was self-ligated for Del REn1. For the other constructs (Inverted, Replacement1, Replacement2), DNA fragments were prepared by annealing 2 oligonucleotides (shown in Table1), and inserted into the BbsI site of pGL3-ROBO4-BbsI.

For the preparation of Del 100bp, Del 200bp, Del 300 bp, Del 400bp, Del 435bp and Del 465bp, upstream promoter fragments were amplified by PCR with primers (shown in
Table1). The resulting fragments were digested with KpnI and MluI, and inserted into KpnI and MluI site in Del1-5.

For the preparation of the Del REn2 and Del REn1,2, DNA fragments were amplified by PCR using primers (shown in Table1) with pGL3-Robo4 or Del REn1 as a template. The resulting fragments were inserted into the MluI and EcoRI site in Del 435bp. For the preparation of Del REn1,2(12bp), an intermediate plasmid, REn1(12bp), was prepared. The REn1 DNA fragment without 12-bp was prepared by annealing 2 oligonucleotides (shown in Table1) and inserted into the BbsI site of the pGL3-Robo4-BbsI. By using the resulting REn1(12bp) and primers (shown in Table1), a DNA fragment with two 12-bp deletions from REn1 and REn2 was amplified by PCR. The resulting fragment was inserted between the MluI and EcoRI sites in Del 435bp.

For the preparation of expression vectors, the cDNAs for SP1, GABPs (GABPα, β and γ) were PCR amplified using HUVEC cDNA and primers containing restriction sites (Table 1). The resulting SP1 and GABP fragments were digested with EcoRI and XhoI, or KpnI and XbaI, respectively. The digested fragments were cloned into the pcDNA3 vector (Invitrogen). The DNA sequences of all constructs were verified by automated sequencing.

To generate the Hprt-targeting vector, pGL3-Robo4 vector was digested with HincII and NheI. The fragment containing the 3kb promoter was purified and cloned into the pSDK-lacZ vector containing LacZ cDNA to generate pRobo4-lacZ. pRobo4-lacZ was then
digested with *PmeI* and *NotI*. The resulting transgenic cassette was purified and cloned into the Hprt-targeting vector, pMP8II.

**Transient transfection assays.** Transient transfection of primary endothelial cells, HEK293, HepG2 and U937 cells was carried out using FuGENE 6 reagent (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, cells were seeded in 6-well plates (1 × 10^5 cells/well) 18-24 h prior to transfection. Cells were then incubated with a reaction mixture containing 1 μg of reporter gene construct, 100 ng of Renilla luciferase vector (pRL-CMV, Promega), and 3 μl of FuGENE 6 reagent. Cells were harvested 48 h later and assayed for luciferase activity by luminometer. Each assay was performed in triplicate at least 3 times. Co-transfection assays using HEK293 cells (1.5 x 10^5 cells/well) were carried out with 0.3 μg of reporter gene construct, 0.3 μg of expression vector, and 4 μl Lipofectamine (Invitrogen) as previously described \(^{15}\). The cells were harvested 20 h following transfection and assayed for luciferase activity.

**Nuclear extract, in vitro translation and EMSA.** Nuclear extracts were prepared from HCAEC using Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. In vitro translated SP1 and GABP was prepared using the TNT Quick Coupled transcription/translation System (Promega) and 1 μg of expression vector. To generate EMSA probes, oligonucleotides (sequences shown in Table 1) were annealed, labeled with T4 polynucleotide kinase and \([\gamma-P]^{32}\) ATP, and purified with G-50 micro columns (Amersham Pharmacia Biotech, Uppsala, Sweden). Binding reactions were carried out using 50 fmol of each probe, 3-5 μg of nuclear extract or 0.5-2 μl of in
vitro translated proteins in a SP1 buffer (10 mM Tris-HCl (pH7.5), 10 mM HEPES-NaOH (pH 7.9), 13% glycerol, 1 mM EDTA, 2 mM dithiothreitol (DTT), 50 mM KCl, 0.5 mM MgCl₂, 0.16 μg/μl poly dI-dC) or an ETS buffer (2 mM HEPES-NaOH (pH 7.9), 2% glycerol, 0.01 mM EDTA, 0.25 mM DTT, 5 mM KCl, 0.1 mg/ml bovine serum albumin, 5 ng/μl poly dI-dC) for 40 min at 4°. For competition and supershift assays, competitor oligonucleotides or antibodies were pre-incubated with nuclear extract or in vitro translated proteins for 10 min prior to addition of oligonucleotide probe. Antibodies to SP1 (PEP2), GABPα (H-180), GABPβ1 (C-14), GABPβ1/2 (H-265), and AML1 (N-20) used as a control antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody to GABPβ1 reacts only against GABPβ, whereas anti-GABPβ1/2 antibody reacts against both GABPβ and γ. Gel electrophoresis was carried out using 4% native polyacrylamide gel and 0.5xTBE buffer at 120V.

**Chromatin immunoprecipitation (ChIP) assay.** ChIP assays were performed using a Chromatin Immunoprecipitation Assay Kit (Upstate, Lake Placid, NY) according to the manufacturer's instructions. Briefly, crosslinked genomic DNA from HCAEC and HCASmC (1x10⁶ cells) was sheared by sonication (10 sec x 10 times at 30% power) using the Digital Sonifer Model 250 (Branson, Danbury, CT). The sheared DNA samples were analyzed by 1.7% agarose gel. Resulting DNA-protein complexes were immunoprecipitated in the absence or presence of 8 μg of antibodies to SP1, GABPα, or Egr-1 (as a negative control). Real-time PCR analysis was performed using the precipitated DNA fragments and primers for Robo4 proximal region, which included the SP1 and ETS(1) site (Table 1).
Generation and analysis of HPRT-targeted mice. Robo4-lacZ was targeted into the Hprt locus of the X chromosome of the BK4 ES cells (a generous gift from Sarah Bronson). The targeted ES clones were used for generating Robo4 promoter-lacZ chimeric mice as described previously. Chimeric males were bred to C57BL/6 females to obtain agouti offspring. Mouse lines generated from two independent ES clones were analyzed for β-galactosidase activity. LacZ staining was carried out as previously described.

Generation and analysis of Robo4-lacZ knock in mice. To generate the intermediate vector pMulti-DT, pMulti-DT 1.0 (a generous gift from Dr. J. Takeda) was digested with SpeI, and the resulting larger fragment was self-ligated. The 6.5kb of the mouse (129Sv/Tac) Robo4 gene (extending from -2560 into intron 8) was inserted into pMulti-DT by homologous recombination using two 300-bp Robo4 homology arms in E. coli, EL350. The resulting pMulti-Robo4 vector was targeted by homologous recombination in EL350 with a linear DNA fragment consisting of E. coli LacZ, an SV40 polyadenylation signal, and a neomycin/kanamycin resistance cassette, all flanked with Robo4 homology arms of ~300-bp. After the recombination, DNA sequence was verified to confirm that the resulting targeting vector contains the insertion of the LacZ and neomycin/kanamycin resistance cassettes between nucleotide +35 of Robo4 and nucleotide 105 of intron 3, with the attendant deletion of exon 1 coding sequence and all of exons 2 and 3. The linearized targeting vector was transfected into embryonic stem cells derived from 129/Sv (W4) by electroporation. The targeted ES clones were used for
generating Robo4-lacZ knock in chimeric mice. Chimeric males were bred to C57BL/6 females to obtain agouti offspring. Mouse lines generated from 2 independent ES clones were analyzed for β-galactosidase activity (as described for the Hprt-targeted mice).

**Quantitative real-time PCR.** Total RNA was prepared using the RNeasy RNA extraction kit with DNase I treatment following the manufacturer’s instructions (Qiagen). To generate cDNA, total RNA (100 ng) from each cell type, mouse organ or tumor xenograft was mixed and converted into cDNA using random primers and SuperscriptIII reverse transcriptase (Invitrogen). All cDNA samples were aliquoted and stored at –80°C. Primers were designed using the Primer Express oligo design software (Applied BioSystems, Foster City, CA) and synthesized by Genemed Synthesis (South San Francisco, CA). All primer sets were subjected to rigorous database searches to identify potential conflicting transcript matches to pseudogenes or homologous domains within related genes. Amplicons generated from the primer set were analyzed for melting point temperatures using the first derivative primer melting curve software supplied by Applied BioSystems. The sequences of all real-time PCR primers used in this paper are listed in Table 1. The SYBR Green I assay and the ABI Prism 7700 Sequence Detection System were used for detecting real-time PCR products from the reverse transcribed cDNA samples, as previously described 17. 18S rRNA was used as the normalizer. PCR reactions for each sample were performed in duplicate and copy numbers were measured as described previously 17. The level of target gene expression was normalized against 18S expression in each sample and the data presented as mRNA copies per 10^6 18S copies.
**Tumor xenografts.** Cultured LL/2 (Lewis lung carcinoma) and B16-F1 (melanoma) cells were trypsinized and harvested by brief centrifugation at room temperature. A total of $5 \times 10^6$ cells was suspended in 150 µL PBS, and the resulting suspension was injected subcutaneously into the right flank of a Robo4-lacZ F1 female or a negative control (F1 male) mouse. The tumors were allowed to grow for 12 days, at which time the mice were killed and tumor tissues were harvested for LacZ staining and real-time-PCR analyses.

**CD31 immunostaining.** 5-µm formaldehyde/glutaraldehyde-fixed sections of normal adult tissues, E10 embryos and tumor xenografts were carried out as previously described\(^1\), using rat anti-mouse PECAM-1 antibody (BD Biosciences Pharmingen, San Diego, CA), and goat anti-rat IgG biotinylated antibody (Dako, Carpinteria, CA).

**siRNA-mediated knockdown of SP1 and GABPα.** HCAEC (4 x 10^5 cells in a 6 cm plate) were transfected with 100 pmol siRNA for SP1 (Invitrogen), GABPα (Ambion) or negative control siRNA (Ambion) using Lipofectamine 2000 reagent according to the manufacturer’s instructions. After 48 h incubation, total RNAs were prepared by RNeasy RNA extraction kit described above. siRNA sequence or product name for SP1 or GABPα siRNAs are shown in Table 1.

**Statistical analyses.** Data were expressed as mean +/- standard error (SE). The statistical significance of differences of the means was determined by one-way analysis of variance and multiple comparisons by Tukey-Kramer multiple range test.
RESULTS

Cloning and sequence analysis of the human Robo4 gene

Under in vitro conditions, endogenous Robo4 was expressed at high levels in primary human endothelial cells, but not human coronary artery smooth muscle cells (HCASmC) or HEK293 cells (Fig. I). 5' RACE revealed two common transcriptional start sites (Fig. II). The most frequent transcriptional start site is designated as base pair number +1.

LacZ expression is not detectable in peripheral blood cells and bone marrow in Hprt-targeted mice

LacZ was not observed in any other cell type or lineage including peripheral blood cells and bone marrow (Fig. III).

The 3-kb human Robo4 promoter contains information for endothelial-specific expression in embryos and tumor xenografts

In whole mounts of day 10 (E10) embryos, LacZ expression was detected in the vasculature (Fig. IV shows staining in aorta and intersomitic vessels). In sections of the embryo, LacZ co-localized with CD31. Interestingly, some LacZ-positive cells were present in the lumen of the aorta, typically next to the endothelium (Fig. IV). It is unclear whether these represent hematopoietic cells (which are otherwise negative in adult
Peripheral blood and bone marrow in adults), endothelial precursor cells or endothelial
cells that have sloughed off the blood vessel wall.

Previous studies have demonstrated that Robo4 is expressed in tumor vasculature. To
determine whether the Robo4 promoter directs expression in tumor endothelium, Hprt-
targeted mice were injected subcutaneously with a suspension of LL/2 or B16-F1 cells.
Resulting tumors were allowed to grow to 0.25-0.5 cm³, at which time the tissue was
harvested for LacZ staining, immunohistochemistry, and in situ hybridization or real-time
PCR analyses. As shown in Fig. VA, the tumors contained many LacZ-positive
endothelial cells, which co-localized in serial sections with CD31. In situ hybridization
revealed a positive Robo4 signal in tumor endothelium (data not shown). Finally, in real-
time PCR analyses, LacZ and Robo4 transcripts were detected in tumors, in a similar
pattern to CD31 and VE-cadherin (Fig. VB).
REFERENCES


FIGURE LEGENDS

Supplemental Figure I. Robo4 expression in cultured cells. Shown are results of quantitative real-time PCR analyses (mRNA copy number per 10^6 copies 18S) of Robo4 (top) and Robo1 (bottom) in human umbilical vein endothelial cells (HUVEC), human coronary artery endothelial cells (HCAEC), human pulmonary endothelial cells (HPAEC), human microvascular endothelial cells (HMVEC), human coronary artery smooth muscle cells (HCASmC), HEK293 cells, HepG2 and U937 cells.

Supplemental Figure II. Determination of the transcription start site by the 5' RACE method. (A) The location of primers used for reverse transcription (RT primer) and PCR amplification (AS1, AS2 and AS3) are shown relative to the transcriptional start site of human Robo4. (B) Agarose gel analysis of the PCR-amplified fragments. (C) Shown is the 5' upstream sequence of human Robo4, 5' untranslated sequence, and the start codon (ATG). Circled nucleotides represent transcription start sites, with ratios indicating the number of clones (out of a total of 17) that revealed that start site. The arrows indicate the 2 major transcriptional start sites.

Supplemental Figure III. Absence of LacZ expression in blood cells. Peripheral blood and bone marrow cells were collected from Robo4-lacZ F1 mouse by brief centrifugation. These cells were washed with PBS, fixed, and stained with X-Gal overnight. Cells are counter-stained with nuclear fast red.
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**Supplemental Figure IV. LacZ expression in E10 embryos.** (A) Wild-type (WT) and transgenic embryos were harvested from F1 female Hprt-targeted mice carrying the Robo4-lacZ transgene gene and processed for whole mount staining with X-gal. (B) Sections of E10 embryos were double stained for CD31 and LacZ. Upper left shows co-localization in endothelial lining of aorta and intersomitic vessels. Upper right shows co-localization in heart. Lower panels are high power images of aorta.

**Supplemental Figure V. LacZ expression in tumor xenografts.** (A) Tumor xenografts were prepared by injecting LL/2 or B16-F1 cells to Robo4-lacZ F1 mouse. Tumor sections were stained using X-Gal (upper 2 panels) or both X-Gal and CD31 antibody (lower 4 panels). (B) Real-time PCR was used to measure Robo4, CD31, VE-cadherin or LacZ in tumors from Hprt-Robo4-lacZ mice or age-matched negative controls.
Supplemental Figure I

![Supplementary figure showing mRNA levels of Robo4 and Robo1 in different cell types.](image-url)
Supplemental Figure III

**Peripheral blood**

**Bone marrow**
Supplemental Figure IV
Supplemental Figure V