Differential Regulation of Vascular Endothelial Growth Factor Receptors (VEGFR) Revealed by RNA Interference: Interactions of VEGFR-1 and VEGFR-2 in Endothelial Cell Signaling†

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ABSTRACT: Vascular endothelial growth factor (VEGF) plays a central role in vascular homeostasis. VEGF receptors (VEGFRs) include several subtypes that may have a differential role in endothelial signal transduction, but interactions among these receptors are incompletely understood. In these studies, we designed small interfering RNA (siRNA) duplexes that targeted specific VEGFR subtypes in bovine aortic endothelial cells (BAEC). siRNA-mediated downregulation of VEGFR-2 by its cognate siRNA resulted in a significant attenuation of VEGF-mediated signaling. Compared to control siRNA-treated cells, VEGFR-2 siRNA markedly inhibited VEGF-mediated activation of PI3K/Akt/GSK3-β as well as MAP kinase and PKC pathways. VEGFR-2 siRNA also blocked VEGF-stimulated phosphorylation and dephosphorylation of endothelial nitric oxide synthase (eNOS) at Ser^{1179} and Ser^{116}, respectively. VEGFR-2-specific siRNA had no effect on the abundance of VEGFR-1 protein. By contrast, VEGFR-1-specific siRNA markedly not only downregulated the abundance of VEGFR-1 but also significantly reduced VEGFR-2 protein and mRNA abundance. VEGFR-1 siRNA had no effect on the stability of VEGFR-2 protein or mRNA. However, VEGFR-1 siRNA significantly inhibited VEGFR-2 promoter activity, as determined in luciferase assays using VEGFR-2 promoter fusion constructs in transfected BAEC. Deletion of either the 5′ E box or the 3′ E box and the GATA element in the VEGFR-2 promoter completely abolished the inhibition of VEGFR-2 promoter activity elicited by VEGFR-1 siRNA. Taken together, our data suggest that VEGFR-1 receptor is a critical determinant of VEGFR-2 abundance, while VEGFR-2 is the key receptor directly responsible for endothelial cell signaling stimulated by VEGF.

Vascular endothelial growth factor (VEGF)[1] is a key regulator of endothelial function in physiological and pathological conditions (for reviews, see refs *1*−*5*). VEGF has been implicated in endothelial cell proliferation (*6*), migration (*7*), and differentiation (*8*), and also regulates vascular permeability (*9*) and angiogenesis (*8*). VEGF is a polypeptide that elicits its biological effects through binding to a family of cell surface receptor tyrosine kinases, of which the best characterized are VEGFR-1 (known also as flt-1) and VEGFR-2 (also known as KDR or flk-1) (*10*). Gene targeting of either of these receptors is lethal to mouse embryos (*11*, *12*). VEGFR-2^{null} embryos fail to develop blood islands and organized blood vessels (*12*), while the demise of VEGFR-1^{null} mice is related to excessive proliferation of angioblasts and disorganization of vascular channels (*11*, *13*). Analyses of the differential signaling pathways elicited by VEGFR-1 and VEGFR-2 have used antibody neutralization experiments (*14*) or have analyzed signaling pathways of chimeric receptors (*14*−*18*). VEGFR-1 was identified as a VEGF receptor a decade ago, yet the role of this molecule in endothelial signaling is incompletely understood. Indeed, it has been suggested (*19*) that VEGFR-1 might not be a primary receptor transmitting a mitogenic signal, but rather may serve as a "decoy receptor" that negatively regulates VEGF signaling by preventing binding of VEGF to VEGFR-2. In contrast, other studies (*14*, *17*, *18*, *20*) have used overexpression systems and analyses of chimeric VEGFR to suggest that VEGFR-1 plays a key role involving the activation of small G proteins and members of the MAP kinase family. It has also been reported that VEGFR-1 and VEGFR-2 can form a heterodimer (*21*, *22*), but the consequences of VEGFR heterodimerization have not been fully elucidated. Understanding the relationships between VEGFR-1 and VEGFR-2 has been hampered by the lack of suitable cellular systems that may permit studies of VEGFR

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[1] Abbreviations: VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor-1/flt-1; VEGFR-2, vascular endothelial growth factor receptor-2/flk-1/KDR; MAP kinase, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA; ERK, extracellular signal-regulated kinase 1/2; SAPK, stress-activated protein kinase; MEK, mitogen-activated ERK kinase 1/2; GSK3-β, glycogen synthase kinase 3-β; eNOS, endothelial isoform of nitric oxide synthase; FBS, fetal bovine serum; BAEC, bovine aortic endothelial cells; ANOVA, analysis of variance.

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loss of function on signaling pathways and cell responses. In these studies, we have developed and applied small interfering RNA (siRNA) "knockdown" methods to characterize the differential role of VEGFR subtypes in endothelial cells. Our data establish that VEGFR-2 is essential for VEGF-mediated signaling in cultured bovine aortic endothelial cells (BAEC), and identify a new role for VEGFR-1 in the maintenance of VEGFR-2 expression.

EXPERIMENTAL PROCEDURES

Materials. Fetal bovine serum (FBS) was from Hyclone (Logan, UT). All other cell culture reagents, media, LipofectAMINE 2000, and Trizol were from Invitrogen (Carlsbad, CA). Anti-VEGFR-1/flt polyclonal antibody (H-225) and anti-VEGFR-2/KDR monoclonal antibody (A-3) were from Santa Cruz Biotechnology (Santa Cruz, CA). VEGFR-2 antiserum, phosphotyrosine 4G10 monoclonal antibody, and phospho-eNOS (Ser^{116}) antibody were from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies directed against phospho-eNOS (Ser^{1177} in the human eNOS sequence, corresponding to Ser^{1179} in bovine eNOS), phospho-Akt (Ser^{473}), phospho-GSK3-β (Ser^{9}), phospho-ERK1/2 (Thr^{202}/Tyr^{204}), phospho-PKC (pan), phospho-p38 (Thr^{180}/Tyr^{182}), phospho-SAPK (Thr^{183}/Tyr^{185}), phospho-MEK ($Ser^{217/221}$), total Akt, and total ERK were from Cell Signaling Technologies (Beverly, MA). Monoclonal antibody directed against eNOS was from BD Pharmingen (Lexington, KY). Super Signal substrate for chemiluminescence detection and secondary antibodies conjugated with horseradish peroxidase were from Pierce (Rockford, IL). Determinations of protein abundance using immunoblot analyses were quantitated using a ChemiImager (AlphaInnotech, San Leandro, CA). Protein determinations were carried out with the Bio-Rad protein assay kit. Actinomycin D and recombinant $VEGF_{165}$ were from Calbiochem (San Diego, CA); VEGF was solubilized in Tris-buffered saline containing 0.1% bovine serum albumin and stored at −70 °C. Cycloheximide was from Biomol (Plymouth Meeting, PA). A VEGFR-2 cDNA probe and VEGFR-2−luciferase fusion promoter reporter constructs encoding −225 to +268, −169 to +268, −225 to +105, −225 to +5, and −95 to +268 promoter fragments were gifts from C. Patterson (University of North Carolina, Chapel Hill, NC) (*23*). All other reagents were from Sigma (St. Louis, MO).

siRNA Preparation. Small interfering RNA duplexes were designed on the basis of the sequences of bovine VEGFR-1 and VEGFR-2 published online [GenBank accession numbers X94263 and X94298 (*24*)]. The siRNA duplex for VEGFR-2 corresponds to bases 199−217 of the open reading frame of bovine VEGFR-2 mRNA: 5′-GGUCUCCGUUUAUUGCUUC-dTdT-3′ (prepared by Ambion, Inc., Austin, TX). We designed a total of three different siRNA duplexes for VEGFR-1, corresponding to bases 387−405, 38−56, and 213−231, respectively, from the open reading frame of the bovine VEGFR-1 mRNAs, noted VEGFR-1 siRNA 1, 2, and 3, respectively, in the text (where not indicated, VEGFR-1 siRNA 1 was used): 1, 5′-GGAAGAUAUUUCUGCUCCA-dTdT-3′; 2, 5′-AGAGCGACGUGUGGUCUUA-dTdT-3′; and 3, 5′-GCACAAAGACCCAAAAGAA-dTdT-3′. VEGFR-1 siRNA 1 was from Ambion, and VEGFR-1 siRNAs 2 and 3 were from Dharmacon, Inc. (Lafayette, CO). The RNA sequence used as a negative siRNA control was 5′-GCCCGCUUUGUAGCAUUCG-dTdT-3′ from Dharmacon Inc.

Cell Culture, Transfection, and Drug Treatment. Bovine aortic endothelial cells (BAEC) were obtained from Cell Applications, Inc. (San Diego, CA), and maintained in culture in Dulbecco's modified Eagle's medium supplemented with FBS (10%, v/v) as described previously (*25*). BAEC were plated onto gelatin-coated culture dishes and studied prior to cell confluence between passages 5 and 9. The day before transfection, the cells were split in a 1:5 ratio; BAEC were transfected with 30 nM siRNA using LipofectAMINE 2000 (0.15%, v/v) following the protocol provided by the manufacturer. LipofectAMINE 2000 was removed by placing the cells in fresh medium containing 10% FBS 5 h after transfection. Cells were analyzed 48 h following transfection, and were incubated in serum-free medium overnight prior to treatments with VEGF (10 ng/mL).

Immunoblots and Immunoprecipitation. Cell lysates were prepared using a cell lysis buffer containing Nonidet P-40; immunoblot analyses of protein expression and phosphorylation were assessed as we previously described in detail (*26*). For immunoprecipitation analysis, lysates from BAEC in 60 mm dishes were immunoprecipitated overnight with 4 μL/mL VEGFR-2 antiserum at 4 °C and for 1 h with protein A−Sepharose. After being washed three times with lysis buffer, immunoprecipitates were analyzed in immunoblots probed with phosphotyrosine antibody 4G10.

Protein Stability Analysis. Protein half-life was determined by Western blot analysis after cells had been treated with the protein synthesis inhibitor cycloheximide. Thirty-six hours following transfection with control or VEGFR-1 siRNA, cells were treated with 10 ng/mL cycloheximide, and cell lysates were subjected to immunoblot analysis as described above using specific antibodies. The intensities of bands corresponding to VEGFRs as well as kinase Akt were quantitated by densitometry.

Northern Blot Analysis. Northern blot analyses for VEGFR-2 transcripts in BAEC were performed as described previously (*23*, *27*). Briefly, endothelial cells in 10 mm dishes were rinsed twice with ice-cold PBS and scraped into 1 mL of Trizol reagent. Total RNA was isolated by ethanol precipitation following the manufacturer's protocol (Invitrogen). Typically, ∼30−50 μg of total RNA was obtained from cells in a 100 mm culture dish; 10 μg of total mRNA from each sample was resolved by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde, capillary-blotted onto a nylon membrane, and then UV-cross-linked. The VEGFR-2 probe was labeled to a high specific activity ($>10^6$ cpm/μg of DNA) by using a random primer labeling kit (Invitrogen). Membranes were then hybridized, extensively washed, and analyzed by autoradiography as previously reported (*23*, *27*). Typically, the membranes were exposed at −80 °C overnight. The intensities of labeled VEGFR-2 bands were quantitated by densitometry.

mRNA Stability Analysis. For the VEGFR-2 mRNA stability study, actinomycin D (5 μg/mL) was added to BAEC 36 h following siRNA transfection, and the cells were incubated for 1−6 h. Total RNA was isolated and analyzed in northern blots probed with the ^{32}P-labeled VEGFR-2 cDNA probe. Following autoradiography, intensities of the labeled VEGFR-2 bands were quantitated by densitometry.

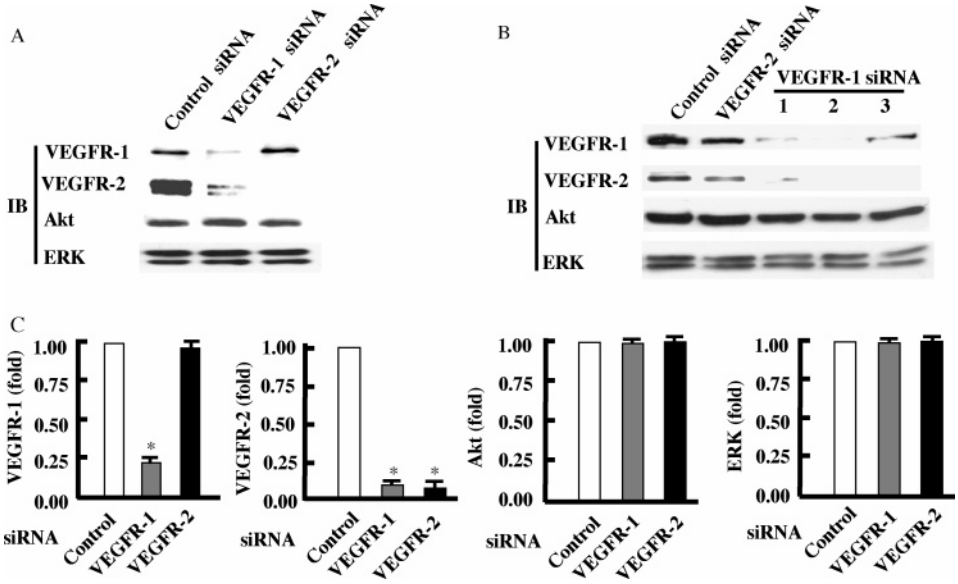

FIGURE 1: siRNA-mediated downregulation of VEGFRs in BAEC. (A) Shown here are immunoblots prepared from BAEC transfected with control siRNA, VEGFR-1 siRNA, or VEGFR-2 siRNA. Forty-eight hours after transfection, cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed separately with specific antibodies directed against VEGFR-1, VEGFR-2, kinase Akt, or ERK. The experiment shown is a representative of three independent experiments that yielded similar results. (B) Shown here is an immunoblot analyzed in BAEC cultures following transfection with control siRNA, VEGFR-2 siRNA, or three different VEGFR-1 siRNA duplex constructs (indicated as 1, 2, and 3). Forty-eight hours after transfection, cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed separately with specific antibodies directed against VEGFR-1, VEGFR-2, kinase Akt, or ERK. The experiment shown is representative of three independent experiments that yielded similar results. (C) Shown here are pooled data from three to six independent experiments quantitated by densitometry to determine the relative abundance of VEGFR-1, VEGFR-2, kinase Akt, and ERK1/2 following transfection with control (whire bars), VEGFR-1 (gray bars), or VEGFR-2 (black bars) siRNA constructs as indicated and then analyzed in immunoblots probed with specific antibodies directed against VEGFR-1, VEGFR-2, ERK, or Akt, as indicated. The asterisk indicates significant differences ($p < 0.01$) in protein abundance between VEGFR siRNA vs control siRNA-transfected BAEC as determined by ANOVA.

VEGFR-2 Promoter Activity Assay. For the promoter activity assay, BAEC were cultured in 12-well plates and cotransfected using LipofectAMINE 2000 containing 30 nM siRNA and 0.4 μg of plasmid DNA encoding a panel of VEGFR-2 promoter−luciferase fusion constructs (*23*). To correct for variations in transfection efficiency, 0.1 μg of pCMV-β-galactosidase was cotransfected in all experiments. Luciferase and β-galactosidase activities were determined 48 h after transfection (*23*). Data were normalized to β-galactosidase activity in each sample. Luciferase and β-galactosidase activities were performed following the manufacturer's protocols (Promega Co., Madison, WI).

Other Methods. All experiments were performed at least three times. Mean values for individual experiments were expressed as the mean ± the standard error. Statistical differences were assessed by ANOVA. A p value of less than 0.05 was considered statistically significant.

RESULTS

To explore the role of VEGFRs in endothelial cells, we designed siRNAs, targeting duplexes directed against bovine VEGFR-2 and VEGFR-1 (see the sequences in Experimental Procedures). After optimizing conditions for siRNA duplex transfection, we examined the ability of these siRNAs to "knockdown" expression of their cognate proteins. Transfection of BAEC with control siRNA had no effect on the abundance of a wide range of proteins as compared to untransfected cells (data not shown). As shown in Figure 1, transfection of BAEC with VEGFR-2 siRNA reduced VEGFR-2 protein abundance relative to that of control siRNA-transfected cells, without affecting the expression of VEGFR-1. Transfection with VEGFR-2 siRNA also had no effect on the expression of a variety of endothelial signaling proteins, including kinase Akt and MAP kinases ERK1/2. We next showed that transfection of BAEC with VEGFR-1 siRNA led to a reduction of VEGFR-1 protein abundance of 80 ± 5% (Figure 1C) without affecting the abundance of kinase Akt or ERK1/2 (Figure 1A,C). However, to our surprise, we found that transfection of BAEC with VEGFR-1 siRNA1 also markedly downregulated VEGFR-2 protein expression by 90 ± 3% ($p < 0.01$, $n = 6$) (Figure 1C). To exclude the possibility that the downregulation of VEGFR-2 protein expression by VEGFR-1 siRNA1 might be due to a nonspecific effect of this particular siRNA, we designed two other VEGFR-1-specific siRNA duplex constructs that target different sequences of the bovine VEGFR-1 transcript (siRNA VEGFR-1 2 and 3, as described in Experimental Procedures). We found that these two VEGFR-1 siRNAs were also able to specifically and dramatically downregulate VEGFR-2 protein expression without affecting kinase Akt or ERK1/2 protein abundance (Figure 1B).

We next studied the effects of siRNA-mediated VEGFR downregulation on VEGF-mediated signaling pathways in BAEC, focusing on protein kinase pathways that are activated by VEGF. We used phospho-specific antibodies to probe immunoblots prepared from siRNA-transfected BAEC treated with VEGF. As shown in Figure 2, in control siRNA-transfected cells, kinases Akt, GSK3-β, and ERK1/2 all underwent rapid and robust phosphorylation after the addition of VEGF, consistent with our previous observations in

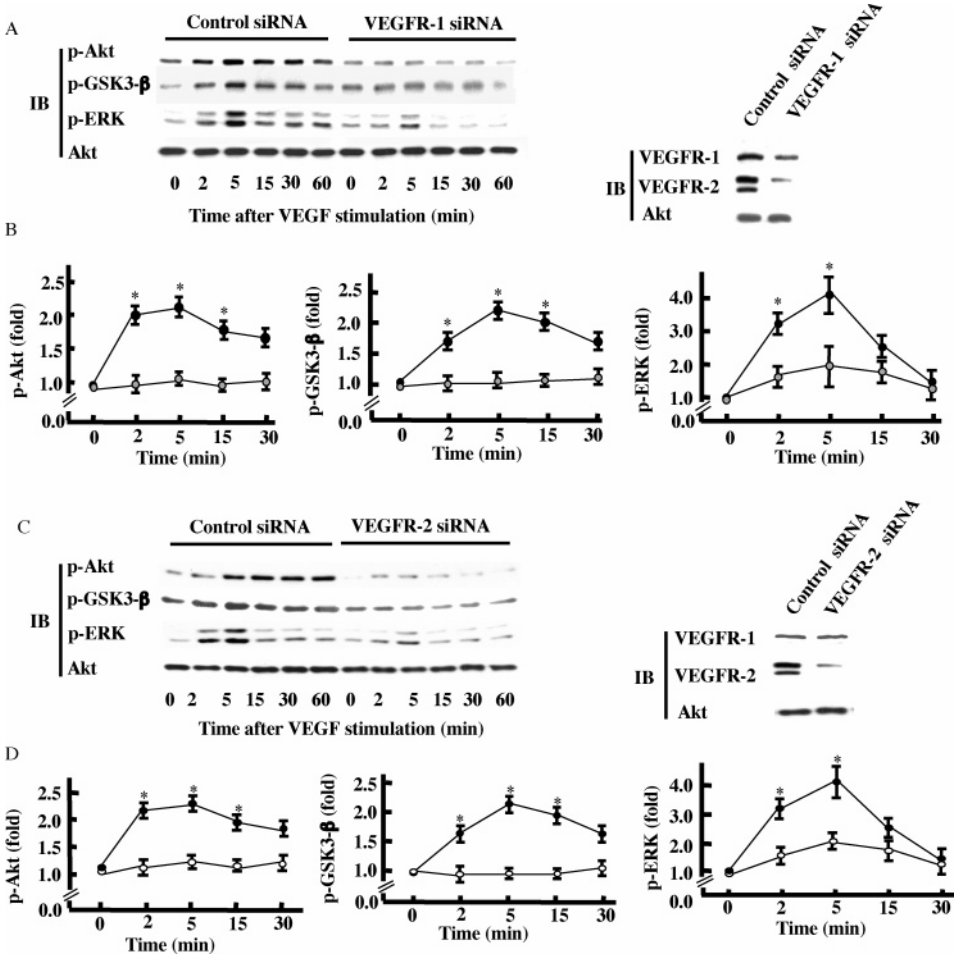

FIGURE 2: Effects of VEGFR-1 and VEGFR-2 siRNA transfection on VEGF-modulated protein kinase pathways. (A) Shown are results of immunoblots analyzed in BAEC cultures transfected with control siRNA or VEGFR-1 siRNA. Forty-eight hours after transfection, cells were treated with VEGF (10 ng/mL) for the times indicated. Cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed separately with specific antibodies directed against VEGFR-2, Ser^{473}-phospho-Akt, Ser^{9}-phospho-GSK3-β, phospho-ERK, and total Akt as shown. This experiment is representative of three independent experiments that yielded similar results. The inset shows the immunoblot results from transfected cells analyzed at time zero and probed with VEGFR-1, VEGFR-2, or total Akt antibodies to establish specific protein knockdown by the siRNA duplexes. (B) Shown here are pooled data from multiple experiments quantitating the relative abundances (normalized to the intensity of the total kinase Akt loading control) of Ser^{473}-phospho-Akt, Ser^{9}-phospho-GSK3-β, and phospho-ERK in BAEC transfected with either control (●) or VEGFR-1 (○) siRNA as indicated. The results were obtained from three to six independent experiments. The basal level of phosphorylation in control siRNA-transfected cells was defined as 1.0. The effects of VEGFR-1 siRNA on these phosphoprotein time course experiments were statistically significant at the $p < 0.01$ level (by ANOVA). (C) Shown are the results of an immunoblot analyzed in BAEC cultures transfected with control siRNA or VEGFR-2 siRNA and treated 48 h following transfection with 10 ng/mL VEGF for different times as indicated. Cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed separately with specific antibodies directed against VEGFR-2, Ser^{473}-phospho-Akt, Ser^{9}-phospho-GSK3-β, phospho-ERK, and total Akt. Shown is a representative of three independent experiments that yielded similar results. The inset shows the immunoblot results prepared from the same samples at time zero and probed with VEGFR-1, VEGFR-2, and total Akt antibodies. (D) Shown are the relative intensities (normalized with the loading control) of Ser^{473}-phospho-Akt, Ser^{9}-phospho-GSK3-β, and phospho-ERK analyzed by densitometry of immunoblots prepared from BAEC transfected with either control (●) or VEGFR-2 (○) siRNA as indicated. The results were obtained from three to six independent experiments. The basal level of phosphorylation in control siRNA-transfected cells was defined as 1.0. The effects of VEGFR-2 siRNA on these phosphoprotein time course experiments were statistically significant at the $p < 0.01$ level (by ANOVA).

nontransfected BAEC cultures (*28*−*30*). In VEGFR-1 siRNA-transfected cells, VEGF-promoted phosphorylation of kinases Akt, GSK3-β, and ERK1/2 was abrogated (Figure 2A,B). Transfection of BAEC with VEGFR-1 siRNA inhibited VEGFR-1 and VEGFR-2 expression significantly compared to that of the control siRNA-transfected cells (Figure 2A,B). These same immunoblots were probed with an antibody against total Akt, which documented equivalent protein loading under the different experimental treatments. In VEGFR-2 siRNA-transfected cells, VEGF-induced phosphorylation of kinases Akt, GSK3-β, and ERK1/2 was markedly attenuated; in these same cells, VEGFR-1 protein abundance was not affected while VEGFR-2 was down-regulated by 90% (Figure 2C,D).

We then tested tyrosine phosphorylation of VEGFR-2 following transfection with VEGFR-1 or VEGFR-2 siRNA. Following VEGF treatment of transfected cells, cell lysates were probed with VEGFR-2 monoclonal antibody to detect VEGFR-2 or were immunoprecipitated with VEGFR-2 antiserum and analyzed in immunoblots probed with phosphotyrosine antibody 4G10 to detect tyrosine-phosphorylated VEGFR-2 (Figure 3). As we had found in our previous experiments, VEGFR-1 as well as VEGFR-2 siRNA markedly knocked down VEGFR-2 protein abundance in BAEC.

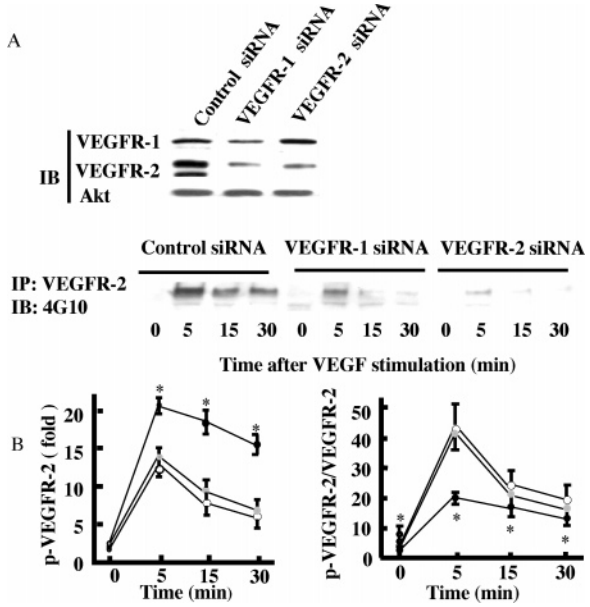

FIGURE 3: Effects of VEGFR-1 and VEGFR-2 siRNA transfection on VEGFR-2 tyrosine phosphorylation. (A) Shown are immunoblots analyzed in BAEC cultures following transfection with control siRNA, VEGFR-1 siRNA, or VEGFR-2 siRNA, as shown. Forty-eight hours after transfection, the cells were treated with 10 ng/mL VEGF for different times as indicated. Cell lysates were immunoprecipitated with VEGFR-2 antiserum, resolved by SDS−PAGE, and analyzed in immunoblots probed with anti-phosphotyrosine antibody as indicated. The experiment shown is representative of three independent experiments that yielded similar results. Cell lysates were also resolved by SDS−PAGE and analyzed in immunoblots probed separately with VEGFR-1, VEGFR-2, and total Akt antibodies. (B) Shown are the total and relative intensities of tyrosine phosphorylation of VEGFR-2 detected by densitometry of immunoblots prepared from BAEC transfected with either control (●), VEGFR1 (gray circles), or VEGFR-2 (○) siRNAs as indicated. The results were obtained from three independent experiments. The basal level of phosphorylation in control siRNA-transfected cells was defined as 1.0. The asterisk indicates a p of <0.05 for the effect of VEGFR-2 siRNA vs control siRNA.

However, the level of tyrosine phosphorylation of VEGFR-2 was less dramatically decreased such that the ratio of the level of VEGFR-2 tyrosine phosphorylation to the abundance of total VEGFR-2 significantly increased both in the basal state and following VEGF treatment. Nonetheless, there was still a significant $33 \pm 7\%$ decrease ($p < 0.05$, $n = 3$) (Figure 3B) in the overall level of tyrosine phosphorylation of VEGFR-2 following either VEGFR-1 or VEGFR-2 siRNA transfection.

Since these findings suggest that VEGFR-2 levels may be the principal determinant of VEGF responses, we explored the effects of VEGFR-2 siRNA on several additional kinase pathways. As shown in panels A and B of Figure 4, the kinases MEK, p38 MAP kinase, and SAPK underwent robust and rapid phosphorylation after the addition of VEGF in control siRNA-transfected cells. However, following transfection with VEGFR-2 siRNA, VEGF-induced phosphorylation of all three kinases was significantly attenuated. These same immunoblots were probed with an antibody against total ERK and documented equivalent protein loading under these different experimental treatments (Figure 4A,B). Other key VEGF-mediated responses were similarly abrogated by VEGFR-2 siRNA. As shown in panels C and D of Figure 4, phosphorylation of eNOS at Ser^{1179} was blocked in BAEC transfected with VEGFR-2 siRNA; VEGF-induced phosphorylation of PKC was also abrogated in these cells. Additionally, the VEGF-induced dephosphorylation of eNOS at Ser^{116} (*28*) was no longer seen following transfection with VEGFR-2 siRNA. These same immunoblots were probed with an antibody against total eNOS, and documented equivalent protein loading under these various conditions.

We next explored the mechanisms by which VEGFR-2 is downregulated by VEGFR-1 siRNA transfection. We first tested whether VEGFR-2 protein stability might be affected by VEGFR-1 siRNA transfection. We used cycloheximide to inhibit protein synthesis in control siRNA- or VEGFR-1 siRNA-transfected BAEC, and then performed immunoblot analysis using specific antibodies directed against VEGFR-2, VEGFR-1, and kinase Akt (Figure 5). We also performed pulse−chase experiments to explore the half-life of VEGFR-2 protein, which gave similar results (data not shown). Following transfection either with control siRNA or with VEGFR-1 siRNA, there was no change in the half-life of VEGFR-2 protein.

Since VEGFR-2 protein downregulation by VEGFR-1 siRNA is not due to decreased protein stability, we explored whether VEGFR-2 mRNA abundance or stability was affected by VEGFR-1 siRNA. We isolated total RNA from BAEC cultures transfected with either control or VEGFR-1 siRNA and analyzed northern blots probed with VEGFR-2 cDNA (*23*). We were intrigued to find that transfection of BAEC with VEGFR-1 siRNA strikingly reduced the abundance of VEGFR-2 transcripts ($80 \pm 9\%$ decrease relative to that of control siRNA-transfected cells, $n = 3$) (Figure 6). We next performed studies of RNA stability using actinomycin D to block transcription, and analyzed northern blots probed with the VEGFR-2 cDNA. We observed no change in VEGFR-2 mRNA half-life (~3 h) in BAEC cultures transfected with VEGFR-1 siRNA compared to control siRNA (Figure 7).

Since neither VEGFR-2 protein nor mRNA stability was affected by VEGFR-1 siRNA, we explored whether VEGFR-2 promoter activity might be affected by VEGFR-1 siRNA. We performed cotransfections in BAEC using a panel of VEGFR-2 promoter−luciferase fusion reporter constructs (as described in Experimental Procedures) with either VEGFR-1 siRNA or control siRNA. As shown in Figure 8, VEGFR-1 siRNA significantly decreased VEGFR-2 promoter activity by $46 \pm 11\%$ relative to that of control siRNA-treated cells ($p < 0.05$, $n = 3$). Deletion of the 5′ E box or the 3′ E box and the GATA element in the VEGFR-2 promoter decreased VEGFR-2 promoter activity in both control siRNA- and VEGFR-1 siRNA-transfected cells, and completely blocked the inhibitory effect of VEGFR-1 siRNA on VEGFR-2 promoter activity (Figure 8). Similarly, further deletions of VEGFR-2 promoter also decreased basal VEGFR-2 promoter activity, and eliminated the inhibitory effect of VEGFR-1 siRNA on VEGFR-2 promoter activity.

DISCUSSION

These studies have used siRNA methodologies to explore the interactions of VEGFR-1 and VEGFR-2 in endothelial cells. Transfection of BAEC with duplex siRNA constructs directed against VEGFR-2 attenuated VEGFR-2 expression by >90% without affecting VEGFR-1 protein abundance (Figure 1). We were surprised to note that VEGFR-1 siRNA

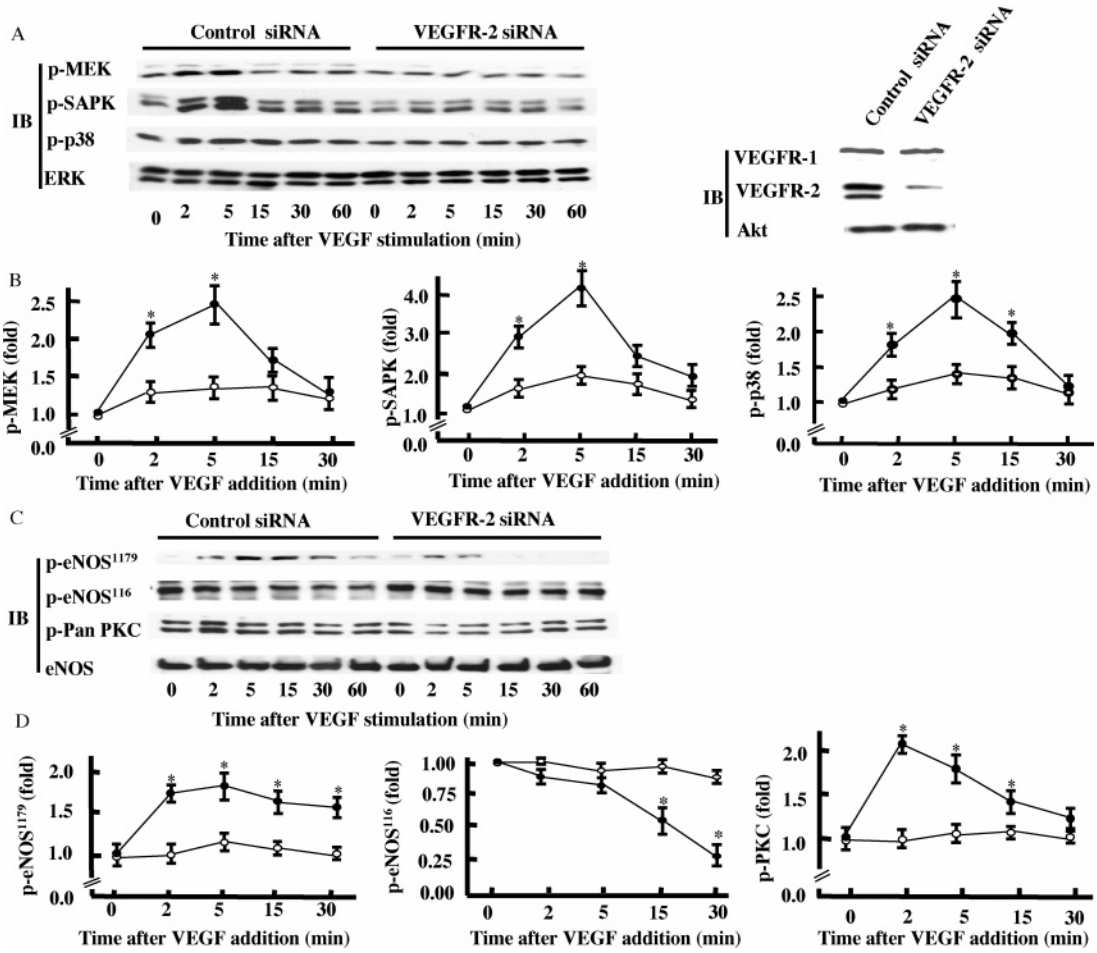

FIGURE 4: Effects of VEGFR-2 siRNA transfection on VEGF-induced signaling pathways in BAEC. (A) Shown are the results of immunoblots analyzed in BAEC cultures transfected with control siRNA or VEGFR-2 siRNA. Forty-eight hours after transfection, cells were treated with VEGF (10 ng/mL) for different times as indicated. Cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed separately with specific antibodies directed against phospho-MEK, phospho-SAPK, phospho-p38, and total ERK. The experiment shown is representative of three independent experiments that yielded similar results. The inset shows immunoblot results prepared from the same samples at the basal level and probed with VEGFR-1, VEGFR-2, and total Akt antibodies. (B) Shown are the relative intensities (normalized to the loading control) of phospho-MEK, phospho-p38, phospho-SAPK, and total ERK detected by immunoblot analyses in lysates obtained from BAEC 48 h after transfection with either control (●) or VEGFR-2 (○) siRNA as indicated following overnight starvation, and then treated with 10 ng/mL VEGF for indicated times. The results were obtained form three to six independent experiments. The basal level of phosphorylation in control siRNA-transfected cells was defined as 1.0. The asterisks indicate a significant effect of VEGFR-2 siRNA vs control siRNA at the $p < 0.01$ level. (C) Shown are immunoblots prepared from BAEC cultures transfected with control siRNA or VEGFR-2 siRNA and treated with VEGF for different times as indicated. Cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed separately with specific antibodies directed against Ser^{1179}-phospho-eNOS, Ser^{116}-phospho-eNOS, phospho-pan-PKC, or total eNOS. The experiment shown is representative of three independent experiments that yielded similar results. (D) Shown here are the results of pooled experiments identical in configuration to the experiment shown in panel C. Densitometry was used to establish the relative intensities (normalized to the total eNOS loading control) of Ser^{1179}-phospho-eNOS, Ser^{116}-phospho-eNOS, and phospho-pan-PKC detected in immunoblots from BAEC transfected with control (●) or VEGFR-2 (○) siRNA and treated with VEGF for the indicated times. These results represent pooled data from three to six independent experiments. The basal level of phosphorylation in control siRNA-transfected cells was defined as 1.0. The asterisk indicates a significant effect of VEGFR-2 siRNA vs control siRNA at the $p < 0.01$ level.

targeting duplexes had marked inhibitory effects on VEGFR-2 abundance (Figure 1). Transfection of BAEC with VEGFR-1 siRNA strikingly reduced VEGFR-2 protein (Figures 1−5) and transcript (Figure 6) abundance without affecting the stability of either VEGFR-2 protein (Figure 5) or mRNA (Figure 7). Knockdown of VEGFR-2 by siRNAs directed against either VEGFR-1 or VEGFR-2 led to the abrogation of VEGF-mediated signaling in these cells (Figures 2−4).

This study provides strong evidence for the central role of VEGFR-2 in endothelial signal transduction. This finding is consistent with prior reports (*15*, *31*) that used recombinant overexpression systems (with the limitations inherent to this experimental approach), as well as other previous studies (*17*, *18*) that analyzed chimeric proteins to make inferences about the roles of different VEGFR subtypes. Other reports (*32*) used neutralizing subtype-specific antibodies to explore the roles of VEGFR subtypes, or applied subtype-selective VEGF agonists (*19*, *33*−*36*), which may lead to its receptor phosphorylation on a site distinct from that phosphorylated by VEGF (*37*), have drawn similar conclusions about the key role of VEGFR-2. Our report presents novel findings about an important interaction between VEGFR-1 and VEGFR-2. Our data contrast sharply with a previous report (*38*), which found that antisense oligonucleotides directed against VEGFR-1 did not affect VEGFR-2 protein expression in cultured BAEC. The reasons for the discrepancy between our study and the previous report (*38*) are not entirely clear,

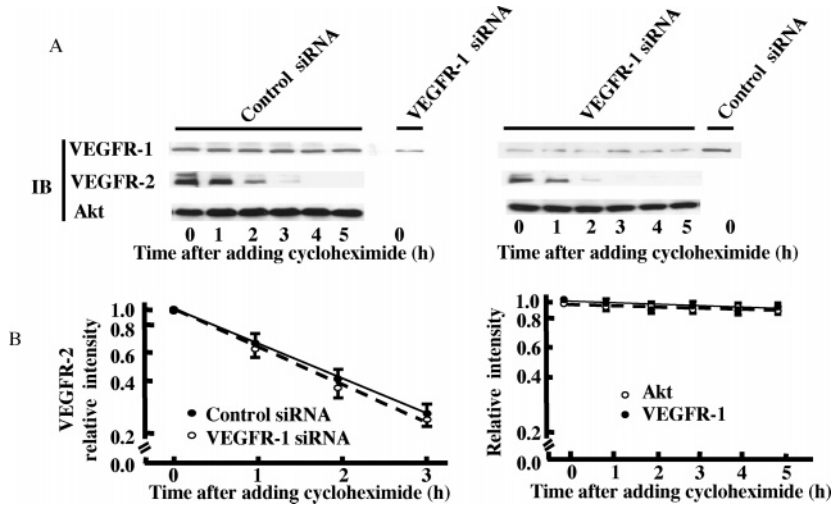

FIGURE 5: Effect of VEGFR-1 transfection on VEGFR-2 protein stability in BAEC. (A) Shown are the results of immunoblots analyzed in BAEC cultures transfected with control siRNA or VEGFR-1 siRNA. Transfected cells were treated with cycloheximide (10 ng/mL) for different times as indicated, and cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed separately with specific antibodies directed against VEGFR-1, VEGFR-2, and Akt. The data shown are representative of six independent experiments that yielded similar results. (B) Shown are densitometric data pooled from six similar independent experiments to determine the half-life of VEGFR-2 in BAEC cultures transfected with control (●) or VEGFR-1 (○) siRNA, treated with cycloheximide, and analyzed in immunoblots probed with antiserum directed against VEGFR-2. (C) Shown are densitometric data pooled from six similar independent experiments showing the relative intensity of Akt (●) and VEGFR-1 (○) in BAEC cultures transfected with control or VEGFR-1 siRNA and treated with cycloheximide and analyzed in immunoblots probed with specific antibody directed against Akt or VEGFR-1.

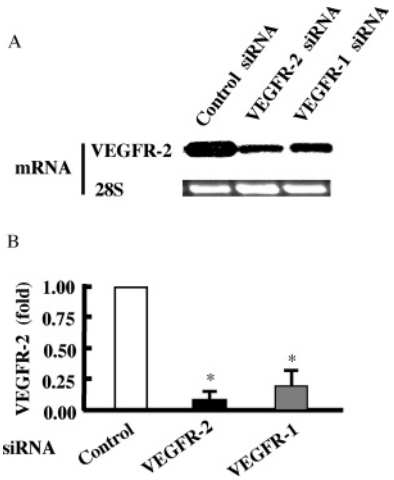

FIGURE 6: Effects of VEGFR-1 siRNA on VEGFR-2 mRNA abundance in BAEC. (A) Shown is a northern blot analyzing VEGFR-2 transcript abundance in BAEC transfected with control siRNA or with siRNA directed against VEGF-1 or VEGFR-2, as shown. This experiment was repeated three times with equivalent results. Shown below is the ethidium bromide-stained 28S RNA band, which serves as the loading control. (B) Shown is the densitometric analysis of VEFGR-2 transcript abundance pooled from three independent experiments in which northern blots were analyzed in BAEC transfected with control siRNA or with siRNA duplexes targeting VEGFR-1 or VEGFR-2, as shown. The asterisk indicates a significant decrease in VEGFR-2 transcript abundance for both VEGFR-1 and VEGFR-2 siRNA vs control, at the $p < 0.01$ level.

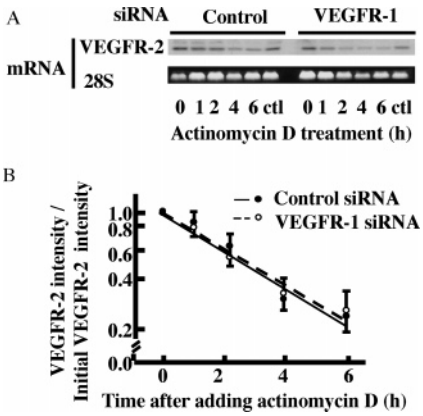

FIGURE 7: Effect of VEGFR-1 siRNA transfection on VEGFR-2 mRNA stability in BAEC. (A) Shown is an RNA stability experiment in which northern blots were analyzed at varying times following addition of actinomycin D to BAEC that had been transfected with control or VEGFR-1 siRNA. Total RNA was analyzed in northern blots probed with VEGFR-2 cDNA as indicated. Shown below are loading controls using 28S RNA. (B) Shown are densitometric data pooled from three experiments identical to the experiment shown in panel A. The abundance of the VEGFR-2 transcript was determined by northern blots analyzed at varying times following addition of actinomycin D to BAEC that had been transfected with control (●) or VEGFR-1 (○) siRNA.

but we note that the cell culture conditions in our report differ strikingly from those of the previous study. For example, in the prior report (*38*), BAEC were serum-starved for the duration of the antisense oligonucleotide treatment; by contrast, in our study, transfected cells were maintained in the presence of serum, and were serum-starved only prior to VEGF treatment. It is plausible that exposure of cells to serum following VEGFR-1 downregulation is required to activate the signaling pathways that suppress VEGFR-2 gene transcription. Other more subtle differences in experimental conditions may also explain these discordant observations.

There are several biologically plausible mechanisms whereby VEGFR-1 siRNA downregulates VEGFR-2 abundance. It seems less likely that VEGFR-1 siRNA somehow directly promotes VEGFR-2 mRNA degradation, since we found that three distinct VEGFR-1 siRNA targeting constructs each had similar effects leading to a decrease in VEGFR-2 transcript abundance. Furthermore, VEGFR-1 siRNA did not affect the half-life of VEGFR-2 transcript (Figure 7), as would have been expected if the VEGFR-1 siRNA promoted VEGFR-2 transcript degradation. Other

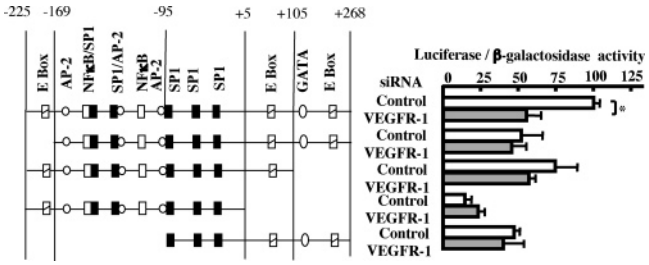

FIGURE 8: Effects of VEGFR-1 siRNA transfection on VEGFR-2 promoter activities in BAEC. Shown at the left are the schematic structures of a panel of VEGFR-2 promoter constructs (*23*), each of which is fused to luciferase and transfected into BAEC. At the right are shown the relative luciferase activities analyzed in BAEC that had been cotransfected with these promoter constructs along with control or VEGFR-1 siRNA duplexes, as indicated. All cells were also transfected with pCMV-gal plasmids, and the luciferase activity was normalized to β-galactosidase for each assay. The results shown are representative of three independent experiments, each conducted in duplicate, which yielded similar results. The asterisk indicates a significant ($p < 0.05$) effect on VEGFR-2 promoter activity of VEGFR-1 siRNA vs control siRNA.

nonspecific effects of VEGFR-1 siRNA that might lead to VEGFR-2 knockdown also seem unlikely because the siRNA targeting sequences were chosen following analyses to exclude constructs that have high levels of sequence identity with other known transcripts. We cannot definitively exclude the possibility that VEGFR-1 siRNA blocks the translation of VEGFR-2 mRNA. However, since we found that VEGFR-1 siRNA not only leads to a decrease in VEGFR-2 protein abundance (Figures 1−5) but also strikingly reduces VEGFR-2 mRNA abundance (Figure 6), we believe that an inhibitory effect of VEGFR-1 siRNA on VEGFR-2 translation is unlikely. The VEGFR-1 siRNA targeting constructs used in these studies would not be expected to affect expression of soluble VEGFR-1: the transcript for soluble VEGFR-1 terminates in intron 13, whereas the VEGFR-1 siRNA constructs used in these studies map to regions that are located 3′ to the terminus of the soluble VEGFR-1 transcript (*39*), corresponding to regions in the VEGFR-1 tyrosine kinase domain (exon 24) and in the receptor's C-terminal tail (exons 26 and 27). We feel that the most compelling mechanism for the suppression of VEGFR-2 by VEGFR-1 siRNA is via suppression of VEGFR-2 promoter activity: our observations are most consistent with an effect of VEGFR-1 knockdown on the abundance or functionality of transcription factors essential for VEGFR-2 gene induction (Figure 8).

These studies have shown that downregulation of VEGFR-2 by either VEGFR-1 or VEGFR-2 siRNA blocks the VEGF-induced signaling to eNOS, to PI3K/Akt/GSK3-β, and to MAP kinase signaling pathways in endothelial cells. Downregulation of VEGFR-2 alone is sufficient to attenuate VEGF-mediated signaling to eNOS, to protein kinase Akt, and to members of the MAP kinase family, which have been previously reported to be downstream effectors of VEGF signaling in these cells (*26*, *28*, *40*, *41*). Taken together, these findings suggest that VEGFR-2 is the key receptor mediating short-term responses to diverse cellular kinase pathways, with VEGFR-1 playing a central role in the induction of VEGFR-2 gene transcription.

Regulation of the VEGFR-2 promoter is complexly determined. In addition to diverse cis-regulatory sequences in the upstream promoter region, VEGFR-2 transcription is also modulated by control elements within its first intron that involve complex chromatin interactions (*42*). The VEGFR-2 promoter is further regulated by agonists such as TGF-β (*43*) that activate diverse transcriptional pathways. Our studies have shown that VEGFR-2 promoter activity is significantly attenuated following transfection of BAEC with VEGFR-1 siRNA (Figure 8). This inhibitory effect of VEGFR-1 siRNA is blocked by deletion of the 5′ E box in the VEGFR-2 promoter construct, and is also blocked by deletion of the 3′ E box and the GATA element. Although it is clear that the 5′ E box is important for regulation of VEGFR-2 promoter by VEGFR-1 siRNA, these studies do not resolve whether it is the 3′ E box or the GATA element that is more important for downregulation of VEGFR-2 promoter activity by VEGFR-1 siRNA. This specific GATA element in the VEGFR-2 promoter has been previously shown to play an important role in TGF-β-mediated downregulation of VEGFR-2 expression (*43*). Our finding that the E box is critical for regulation of VEGFR-2 promoter activity by VEGFR-1 siRNA is a particularly intriguing finding, in that previous studies have shown that members of the E box binding transcription factor family (basic helix−loop−helix proteins such as TAL1, HIF2α, EPAS1, and HERSR1), as well as other transcription factors such as Id, may play important roles in endothelial cell migration and in angiogenesis (*42*, *44*−*47*). It is plausible that coordinated functioning of VEGFR-1 and VEGFR-2 is essential for VEGF-mediated biological responses in vascular endothelium.

In conclusion, these studies have shown that siRNA-mediated knockdown of VEGFR-1 in cultured endothelial cells leads to the attenuation of VEGFR-2 promoter activity and to the loss of VEGF-mediated activation of diverse signaling pathways. VEGFR-1 and VEGFR-2 are key determinants of VEGF responsiveness in vascular endothelium, and interactions between these receptor tyrosine kinases may importantly influence the biological consequences of VEGF action in the vessel wall.

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