Site-directed Mutagenesis in the B-Neuropilin-2 Domain Selectively Enhances Its Affinity to VEGF₁₆₅, but Not to Semaphorin 3F*

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Neuropilins (NRPs) are 130-kDa receptors that bind and respond to the class 3 semaphorin family of axon guidance molecules (SEMAS) and to members of the vascular endothelial growth factor (VEGF) family of angiogenic factors. Two NRPs have been reported so far, NRP1 and NRP2. Unlike NRP1, little is known about NRP2 interactions with its ligands, VEGF₁₆₅ and SEMA3F. Cell binding studies reveal that VEGF₁₆₅ and SEMA3F bind NRP2 with similar affinities, 5.2 and 3.9 nM, respectively, and are competitive NRP2 ligands. Immunoprecipitation studies show that the B (b₁b₂) extracellular domain of NRP2 is sufficient for VEGF₁₆₅ binding, whereas SEMA3F requires both the A (a₁a₂) and B domains. To identify residues of B-NRP2 involved in VEGF₁₆₅ binding, point mutations were introduced by site-directed mutagenesis. VEGF₁₆₅ is a basic protein. Reduction of the electronegative potential of B-NRP2 by exchanging acidic residues for uncharged alanine (B-NRP2 E284A,E291A) in the 280–290 b₁-NRP2 loop resulted in a 2-fold reduction in VEGF₁₆₅ affinity. Conversely, enhancing the electronegative potential (B-NRP2 R287E,N290D and R287E,N290S) significantly increased VEGF₁₆₅ affinity for B-NRP2 by 8- and 6.6-fold, respectively. The mutagenesis did not affect SEMA3F/B-NRP2 interactions. These results demonstrate that it is possible to alter VEGF₁₆₅ affinity for NRP2 without affecting SEMA3F affinity. They also identify NRP2 residues involved in VEGF₁₆₅ binding and suggest that modifications of B-NRP2 could lead to potentially high affinity selective inhibitors of VEGF₁₆₅/NRP2 interactions.

Neuropilins (NRP1 and NRP2)³ are transmembrane receptors for members of the class 3 semaphorin family of neuronal guidance mediators (SEMA3A-G) and for the vascular endothelial growth factor (VEGF) family of angiogenic factors. NRPs play a prominent role in neuronal wiring, normal blood vessel development, and tumor angiogenesis (1–5). NRP1 and NRP2 are often differentially expressed. In the neuronal system, NRP1 is expressed on sympathetic neurons. Binding of SEMA3s to NRPs and complexing with plexins results in axon repulsion and growth cone collapse (6–8). There is a specificity in SEMA3s binding to NRPs. For example, SEMA3A acts via NRP1, whereas SEMA3F is functional via NRP2 (9–11). Recently, it has been demonstrated that SEMA3E acts independently of NRPs and is instead a direct ligand for plexin D1 (12).

In the vascular system, NRP1 is confined to the arterial compartment, whereas NRP2 is expressed by venous and lymphatic endothelial cells (EC) (13–16). It is apparent that NRP1 promotes angiogenesis in vivo. NRP1 and NRP1/NRP2 mouse knockouts have major defects in yolk sac and embryonic vasculature (17, 18). NRP2 mutant mice show abnormal development of small lymphatic vessels and capillaries, suggesting a selective role for NRP2 in lymphatic vessel development (13). In zebrafish embryos, NRP1 knockdown with morpholinos results in loss of the angiogenic intersegmental vessels (19, 20).

Tumor cells also express NRPs (21, 22) which bind VEGF₁₆₅ and SEMA3A/F (21, 23). In tumors, overexpression of NRP1 results in markedly increased tumor progression and angiogenesis (24, 25). Anti-NRP1 antibodies have an additive effect with anti-VEGF therapy in reducing tumor growth, suggesting that NRP1 is a co-receptor for VEGFR-2 (26). Clinically, it appears that high expression levels of NRP1 and NRP2 correlate with poor outcomes (27–30).

Interestingly, SEMA3s not only mediate axon guidance but angiogenesis as well, suggesting cross-talk between these 2 processes (2, 3, 31, 32). Whereas VEGF₁₆₅ promotes angiogenesis, SEMA3s inhibit angiogenesis. For example, SEMA3A and SEMA3F inhibit steps in angiogenesis such as adhesion, migration, and capillary sprouting (23, 33). Overexpression of SEMA3F in melanoma cells inhibits tumor angiogenesis and metastasis (34).

The ability of two structurally disparate proteins, VEGF₁₆₅ and SEMA3s, to bind to one single receptor (NRP) has engendered considerable interest in NRP structure. That VEGF₁₆₅ and SEMA3A (Collapsin-1) are competitive inhibitors in EC binding, EC migration, and dorsal root ganglia collapse assays suggests possible overlapping binding sites on NRP1 (23).
NRP1 and NRP2 have about 40% amino acid identity but share similar domain structure (1) composed of a large extracellular domain (~840 amino acids), a very short transmembrane region (25 amino acids) and a short cytoplasmic sequence (42 amino acids) in which no signaling consensus sequences have been identified so far. The NRP extracellular domain contains: (i) an A domain consisting of two a-domain repeats (a1a2), (ii) a B domain consisting of two b-domains repeats (b1b2), and (iii) a C domain, most likely involved in NRP oligomerization (35–39). Our previous studies using pull-down methods indicated that VEGF165 binds solely to the B domain of NRP1 (40). Domain deletion studies have also demonstrated that the B-NRP1 domain is essential for VEGF165 binding (41). In addition, both A- and B-NRP1 domains are needed for SEMA3A binding (37, 41).

Compared with NRP1, the structural features of NRP2 necessary for ligand binding have not been reported yet. In this report, we show that SEMA3F competes with VEGF165 for NRP2 binding and that VEGF165 needs only the B domain for optimal binding, whereas SEMA3F needs both the A and B domains. Moreover, point mutations were made by site-directed mutagenesis to pinpoint VEGF165 binding sites based on information provided by the crystal structure of the b1-NRP1 domain (42) and by the over 50% identity of the NRP1 and NRP2 b1 domains. VEGF165 is a basic protein. Mutation of 2 electronegative glutamates to neutral alanines in the 280–290 loop of b1-NRP2 decreased VEGF165 affinity by 2-fold. On the other hand, enhancing the electronegative potential of the same b1 loop increased VEGF165 affinity by 8-fold. The point mutations do not affect SEMA3F binding. These studies identify specific NRP2 residues that bind VEGF165. Furthermore, because B-NRP2 can inhibit VEGF165 binding to EC, further modifications of this NRP2 region to increase its affinity for VEGF165 could lead to more potent VEGF165 antagonists to target angiogenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—PfuTurbo™ High-Fidelity DNA polymerase and the QuickChange® XL Site-directed Mutagenesis Kit were from Stratagene (La Jolla, CA). The vectors pCR®III-TOPO, pcDNA3.1, and pSecTag2B were from Invitrogen. The DNA Ligation Kit was from TAKARA Bio Inc. The restriction enzymes HindIII and EcoRV were from New England Biolabs, Inc. (Beverly, MA). FuGENE® 6 transfection reagent was from Roche Diagnostics. HEPES, NaCl, Na2HPO4, NiSO4, imidazole, bovine serum albumin, and heparin sodium salt from Porcine Intestinal Mucosa were from Sigma. SDS sample buffer was from Boston BioProducts, Inc. (Boston, MA). Carrier-free VEGF165 was from R&D Systems (Minneapolis, MN); VEGF165 was provided by the National Cancer Institute. 125I-Sodium was from PerkinElmer Life Sciences, Inc. and the IODO-BEADS® were from Pierce. NAP 5 columns were from GE Healthcare. Mouse anti-Myc antibody clone 9E10 was from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-V5 antibody was from Invitrogen; ECL™ anti-mouse horseradish peroxidase-linked whole antibody (from sheep) was from Amersham Biosciences. Protein G-Sepharose™ 4 Fast Flow was from GE Healthcare. Western Lightning™ was from PerkinElmer Life Sciences. HiTrap™ Chelating column and HiTrap™ Desalting column were from Amersham Biosciences. SYPRO® Ruby protein gel stain and Quick Start™ Bradford Dye Reagent were from Bio-Rad. Fetal bovine serum, Dulbecco’s modified Eagle’s medium, l-glutamine/penicillin G/streptomycin sulfate mixture, CD 293 chemically defined medium, and Dulbecco’s phosphate-buffered saline (PBS) were from Invitrogen; Ham’s F-12 medium was from Cellgro Mediatech, Inc. (Herndon, VA). Endothelial basal media-2 and endothelial growth media-2 SingleQuots were from Cambrex BioScience Inc. (Walkersville, MD).

**Cell Culture**—Parental porcine aortic endothelial cells (PAEC) were kindly provided by Dr. Lena Claesson-Welsh (University of Uppsala, Uppsala, Sweden) (43). PAEC NRP1 and NRP2 were established as previously described (21, 34). PAEC were grown in Ham’s F-12 medium containing 10% fetal bovine serum and 1% l-glutamine/penicillin G/streptomycin sulfate. Human umbilical vein endothelial cells (HUVEC) were from Clonetics/BioWhittaker (Walkersville, MD) and were cultured in endothelial basal media-2 supplemented with endothelial growth media-2 SingleQuots. HEK293 cells were from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% l-glutamine/penicillin G/streptomycin sulfate.

**Cloning and Mutagenesis**—The A-, B-, and AB-NRP2 domains were PCR amplified using the human NRP2 construct in the pcDNA3.1(+) vector as a template. The sites of HindIII and EcoRV were added to the forward and reverse primers, respectively, for consequent enzymatic digestion. PfuTurbo™ High Fidelity DNA polymerase was used for the PCR (95 °C for 2 min, 30 cycles: 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; 72 °C for 10 min) together with the following primer pairs: A-NRP2, forward, 5'-CCTACCAAGCCTGCGAGGAGGTGCTTTGTAATC and reverse, 5'-TACAGATACTTGGACCAACGTGATAACGCC; B-NRP2, forward, 5'-CCTACCAAGCCTTTGCGAGGAGGTGCTTTGTAATC and reverse, 5'-TACAGATATCTTGGACCAACGTGATAACGCC; AB-NRP2, forward, 5'-CCTACCAAGCCCTTTGCGAGGAGGTGCTTTGTAATC and reverse, 5'-TACAGATATCTTGGACCAACGTGATAACGCC.
The mutations in the B-NRP2 domain were introduced into the QuikChange XL Site-directed Mutagenesis Kit according to the manufacturer’s protocol using the pSecTag2B B-NRP2 plasmid as a template. The mutagenesis primers are as follows: B-NRP2 R287E,N290D, forward, 5‘-GGAGTCTGGCAGATGGATGGAACAGATCGTGC and reverse, 5’-GGACCTGATCTGGATCTCAGCAATCTGGCCAGATCCCTC; B-NRP2 R287E,N290D, forward, 5’-GGAGTCTGGCAGATGGATGGAACAGATCGTGC and reverse, 5’-GGACCTGATCTGGATCTCAGCAATCTGGCCAGATCCCTC; B-NRP2 E284A,E291A, forward: 5’-GGCACATGTCATGCATAGCTAGCACAGTGCC and reverse, 5’-GATCTGTGACTAGCAATCCGCGAAGCCAGACCGATTCG. The constructs were sequenced to prove sequence integrity.

**Expression and Purification of NRP2 Extracellular Domains and Semaphorins**—The domain constructs were transiently transfected overnight into HEK293 cells using the transfection reagent FuGENE 6. The following day the culture medium was replaced by serum-free CD 293 medium and the conditioned media were collected after 48 h. The conditioned medium was centrifuged at 3500 × g for 20 min, adjusted to 30 mM NaH2PO4, pH 7.6, 150 mM NaCl, filtered on a 0.22-μm filter, and applied onto a 1-ml HiTrapTM chelating column loaded with NiSO4 and attached to a fast protein liquid chromatography system. After washing with 30 mM NaH2PO4, pH 7.6, 150 mM NaCl, 5 mM imidazole, the proteins were eluted with 30 mM Na2HPO4, pH 7.6, 150 mM NaCl, 250 mM imidazole. The fractions were analyzed by SDS-PAGE followed by ImageJ. The dissociation constants (Kd values) of the different domains and B-NRP2 mutants for VEGF165 and SEMA3F were determined using the Quick StartTM Bradford dye reagent, an aliquot of the samples was measured in a counter.

**Iodination of VEGF165 and SEMA3F and Binding to PAEC NRP2—VEGF165 and SEMA3F were iodinated using IODO-BEADS® as previously described by Soker et al. (21). Specific activities of ~60,000 and ~10,000 cpn/ng were obtained for the two proteins, respectively.

To determine the dissociation constants (Kd values) of 125I-VEGF165 and 125I-SEMA3F for PAEC NRP2, the cells were incubated in 96- or 48-well plates with increasing concentrations of the radioligand for 1 h at 4 °C in binding buffer (Ham’s F-12, 20 mM HEPES, pH 7.5, 0.5% bovine serum albumin, 1 μg/ml heparin sodium salt). The supernatant was aspirated, the cells were thoroughly washed with cold PBS, lysed with 0.2 N NaOH, and the cell-associated radioactivity was measured in a γ-counter. The values at each concentration point were corrected for the nonspecific binding obtained on the parental PAEC. The dissociation constants (Kd values) were calculated from average values of at least two experiments using the Software GraphPad nonlinear regression fitting parameters for one-site binding.

For the competition-binding experiments, PAEC NRP1 and NRP2 were incubated in 48-well plates with 125I-VEGF165 (5 ng/ml) in binding buffer at increasing concentrations of cold SEMA3F (0 to 250 nM) for 1 h at 4 °C or PAEC NRP2 were incubated with 125I-SEMA3F (25 ng/ml) in binding buffer at increasing concentrations of cold VEGF165 (0 to 200 nM). Afterward, the cells were washed with cold PBS, lysed with 0.2 N NaOH, and cell-associated radioactivity was measured in a γ-counter. The experiment was repeated in duplicates two to three times.

**RESULTS**

**VEGF165 and SEMA3F Are Competitive NRP2 Ligands**—Porcine aortic endothelial cells (PAEC) do not express VEGF165 and SEMA3F.
B-NRP2 Mutants with Selective Enhanced Affinity for VEGF₁₆₅

The B-domain of NRP2 Is Essential and Sufficient for Optimal Interaction with VEGF₁₆₅. Whereas SEMA3F Requires Both A- and B-NRP2—Previous studies have implicated the B domain of NRP1 as the VEGF₁₆₅ binding site (40, 41). However, the additional presence of the A extracellular domain significantly increased the B-NRP1 domain interactions with VEGF₁₆₅ (40). To identify the VEGF₁₆₅ binding sites on NRP2, discrete Myc-His₆-tagged extracellular domains of NRP2, A, B, and AB, were cloned, expressed, and purified from HEK293 conditioned media (Fig. 2A). ¹²⁵I-VEGF₁₆₅ (5 ng/ml) was incubated in solution with equal amounts of each of the domains and the complexes were immunoprecipitated with an anti-Myc antibody (Fig. 2B). The A-domain (lane 2) did not pull down ¹²⁵I-VEGF₁₆₅, whereas both the B- (lane 3) and the AB-domains (lane 4) were able to do so. Whereas the B-NRP2 domain is the main site of interaction between NRP2 and VEGF₁₆₅, SEMA3F interacts only weakly with B-NRP2 and requires both the A and B domains of NRP2 for optimal interaction (Fig. 2C). Equal amounts of V5-His₆-tagged SEMA3F (5 μg) were incubated with equal amounts of each of the NRP2 domains and these proteins were co-immunoprecipitated with anti-Myc antibody, followed by Western blot with an anti-V5 antibody. Relatively little SEMA3F interaction was observed with A-NRP2 (lane 2) or with B-NRP2 (lane 3). However, a substantial amount of SEMA3F was pulled down with AB-NRP2, 15- and 10-fold more than with A- and B-NRP2, respectively (Fig. 2C, lane 4 versus 2 and 3, respectively).

The co-immunoprecipitation results (Fig. 2, B and C) were confirmed by analyzing the binding curves of the different NRP2 domains for the two ligands. For VEGF₁₆₅, a dose-dependent binding to B-NRP2 and AB-NRP2, but not to A-NRP2, was observed (Fig. 2D). The Kᵰ values were 5.9 ± 0.6 nM for B-NRP2 and 8.3 ± 1.3 nM for AB-NRP2 but not detectable for A-NRP2, suggesting that the A domain did not make a significant contribution to VEGF₁₆₅ binding. Interestingly, the B domain alone (5.9 nM) bound VEGF₁₆₅ as well as the whole NRP2 protein did (5.2 nM). Binding curves showed dose-dependent binding of SEMA3F to AB-NRP2, lower to B-NRP2 but none to A-NRP2 (Fig. 2E). The values of the Kᵰ values were 1.4 ± 0.5 and 2.6 ± 0.4 nM, respectively, for B and AB-NRP2, and not detectable for A-NRP2.

Even though the affinity of SEMA3F for B-NRP2 was 4-fold higher than VEGF₁₆₅ values, the capacity of B-NRP2 for VEGF₁₆₅ was significantly higher than for SEMA3F. Indeed, at saturation about 30% of the total VEGF₁₆₅ was bound to receptors or semaphorin receptors and can be engineered to overexpress one receptor at a time, for example, NRP2 or NRP1 (21, 34). The Kᵰ of VEGF₁₆₅ and SEMA3F for NRP2 were calculated from the binding curves of ¹²⁵I-VEGF₁₆₅ and ¹²⁵I-SEMA3F for PAEC NRP2. The Kᵰ for the 2 ligands were fairly similar, 5.2 ± 3.7 nM for VEGF₁₆₅ (Fig. 1A) and 3.9 ± 1.5 nM for SEMA3F (Fig. 1B), respectively. To determine whether VEGF₁₆₅ and SEMA3F are competitive inhibitors, ¹²⁵I-VEGF₁₆₅ (5 ng/ml, 0.12 nM) was incubated with PAEC NRP2 or PAEC NRP1 at increasing unlabeled SEMA3F concentrations (0 to 250 nM) (Fig. 1C). ¹²⁵I-VEGF₁₆₅ binding to PAEC NRP2 was inhibited by up to 80% at 100 nM cold SEMA3F (830-fold molar ratio VEGF₁₆₅/¹²⁵I-VEGF₁₆₅). On the other hand, inhibition of ¹²⁵I-VEGF₁₆₅ binding to PAEC NRP1 was only 15% at the same concentration of cold SEMA3F. The diminished ability of SEMA3F to inhibit ¹²⁵I-VEGF₁₆₅ binding to NRP1 compared with NRP2 is consistent with SEMA3F having a 10-fold lower affinity for NRP1 compared with NRP2 (10).

In a complementary experiment, PAEC NRP2 were incubated with ¹²⁵I-SEMA3F (25 ng/ml, 0.13 nM) and increasing concentrations of cold VEGF₁₆₅ (0 to 200 nM). VEGF₁₆₅ was able to compete with ¹²⁵I-SEMA3F for NRP2 binding sites on PAEC NRP2. At the highest concentration of unlabeled VEGF₁₆₅ (200 nM, about 1540-fold molar ratio VEGF₁₆₅/¹²⁵I-SEMA3F) 53% of ¹²⁵I-SEMA3F was displaced from the cell surface by VEGF₁₆₅ (Fig. 1D). The binding of ¹²⁵I-SEMA3F to PAEC NRP1 was so low, almost null as expected, that competition with cold VEGF₁₆₅ could not be measured and was moot.

![FIGURE 1. Dissociation constants for VEGF₁₆₅ and SEMA3F binding to PAEC NRP2 and competition of these 2 ligands for binding to NRP2. PAEC NRP2 were incubated with increasing amounts of ¹²⁵I-VEGF₁₆₅ in a 96-well plate (A) or ¹²⁵I-SEMA3F in a 48-well plate (B) for 1 h at 4 °C. The cells were lysed and the cell-associated radioactivity was measured in a γ-counter. The values at each concentration point were corrected for nonspecific binding to PAEC parental cells lacking NRP2. An average of at least three experiments is shown. C, increasing amounts of cold SEMA3F competed ¹²⁵I-VEGF₁₆₅ binding to PAEC NRP2 but not PAEC NRP1. D, increasing amounts of cold VEGF₁₆₅ competed ¹²⁵I-SEMA3F binding to PAEC NRP2; the low binding of ¹²⁵I-SEMA3F to PAEC NRP1, which precluded competition experiments, is shown in the closed box on the y axis. For panels C and D, cell-associated radioactivity was determined as in panels A and B.](image-url)
B-NRP2 Mutants with Selective Enhanced Affinity for VEGF<sub>165</sub>

**FIGURE 2.** NRP2 domain binding specificities for VEGF<sub>165</sub> and SEMA3F. A, schematic view of NRP2 domain structures (upper panel). NRP2 domains are Myc- and His<sub>6</sub>-tagged and the two SEMA3F constructs are His<sub>6</sub>-tagged and, respectively, Myc- or V5-tagged. Bottom panel, expression levels of the constructs shown above. The constructs were transfected in HEK293 cells, the conditioned media were collected after 48 h and analyzed by SDS-PAGE followed by Western blot with anti-Myc or anti-V5 antibody, respectively. From left to right: A-, B-, AB-NRP2, mock (--), SEMA3F-Myc-tagged (3F), SEMA3F-V5-tagged (3F), and mock (--). B, <sup>125</sup>I-VEGF<sub>165</sub> (5 ng/ml) was preincubated with purified A-, B, and AB Myc-tagged domains at room temperature for 2 h. The complexes were immunoprecipitated and immunocomplexes were analyzed by SDS-PAGE followed by autoradiography; the bottom panel shows a densitometric quantification of the bands (ImageJ); C, co-immunoprecipitation of V5-tagged SEMA3F with the NRP2 domains. V5-tagged SEMA3F (25 pg/ml) and equal amounts of A-, B-, and AB-NRP2 Myc-tagged domains were incubated for 2 h at room temperature and immunoprecipitated overnight at 4 °C with an anti-Myc antibody. The immunocomplexes were analyzed by SDS-PAGE and Western blotting with an anti-V5 antibody. The bottom panel shows a densitometric quantification of the bands (ImageJ); D and E, binding of <sup>125</sup>I-VEGF<sub>165</sub> and <sup>125</sup>I-SEMA3F to NRP2 domains. <sup>125</sup>I-VEGF<sub>165</sub> (5 ng/ml) – 70,000 cpm total (D) or <sup>125</sup>I-SEMA3F (25 ng/ml, ~55,000 cpm total) (E) were preincubated at room temperature for 2 h with increasing concentrations of the Myc-tagged A-, B-, and AB-NRP2 domains, immunoprecipitated by Myc antibody, and bound radioactivity was determined. The capacity of binding was also measured. At saturation, the percentages of total <sup>125</sup>I-VEGF<sub>165</sub> bound to B- and AB-NRP2 (about 27 and 32%, respectively) are both significantly higher than the corresponding percentages of bound <sup>125</sup>I-SEMA3F (3.5 and 15%, respectively). Thus the capacity of B-NRP2 for VEGF<sub>165</sub> is about 8-fold higher than for SEMA3F.

B-NRP2, whereas only about 4% of the total SEMA3F was bound to B-NRP2. Taken together these data suggest a preferential interaction of B-NRP2 with VEGF<sub>165</sub> rather than with SEMA3F.

Effects of NRP2 Domains on VEGF<sub>165</sub> and SEMA3F Binding to EC—Domain-mediated inhibition of VEGF<sub>165</sub> and SEMA3F binding to PAEC NRP2 was also tested out at a cellular level using PAEC NRP2 (Fig. 3). A-NRP2 was not effective in displacing <sup>125</sup>I-VEGF<sub>165</sub> from PAEC NRP2. On the other hand, about 80% of the <sup>125</sup>I-VEGF<sub>165</sub> was displaced from the cell surface by both B- and AB-NRP2 at the final concentration of 1000 nM (Fig. 3A). <sup>125</sup>I-SEMA3F displacement from the PAEC NRP2 was most effective using AB-NRP2 and much less effective using A-NRP2 or B-NRP2 (Fig. 3B). Similar results for both ligands were obtained on HUVEC (not shown). IC<sub>50</sub> values for these inhibitions are reported in Table 1. Taken together, the competition-binding experiments on EC confirm the immunoprecipitation results.

Asp-289, respectively, were exchanged with neutral alanine by site-directed mutagenesis to reduce the B-NRP2 electronegative potential to obtain B-NRP2 E284A,E291A (designated as POS) (Fig. 4A). The construct was transiently transfected into HEK293 cells and the collected conditioned media were purified as described under “Experimental Procedures.” The relative K<sub>d</sub> values of the mutant for VEGF<sub>165</sub> was determined by immunoprecipitation assays (Table 2). The double alanine mutation increased the K<sub>d</sub> for VEGF<sub>165</sub> by 2-fold, from 5.9 nM of the WT B-NRP2 to 12.0 nM (Fig. 4A and Table 2). By contrast, the K<sub>d</sub> of this mutant for SEMA3F was virtually unchanged (1.05 versus 1.44 nM of the WT B-NRP2) (Fig. 4C and Table 2).

On the other hand, when the electronegative potential of the B-domain was increased significant improvements in K<sub>d</sub> values for VEGF<sub>165</sub> occurred. In particular, arginine 287 (Arg-287) was changed to glutamate and asparagine 290 (Asn-290) was changed to serine or aspartate to obtain double mutants...
B-NRP2 mutants with selective enhanced affinity for VEGF\textsubscript{165}

B-NRP2 R287E,N290S and R287E,N290D (designated as NEG1 and NEG2, respectively) (Fig. 4A). The \(K_d\) values of the two mutants for VEGF\textsubscript{165} were 0.89 and 0.74 nm, respectively, showing an enhancement in affinity of 6.6- and 8-fold, respectively, compared with WT B-NRP2 (Fig. 4B and Table 2). Thus, these increases in electronegativity increase VEGF\textsubscript{165} affinity by almost a log.

The \(K_d\) values of these B-NRP2 domain mutants for SEMA3F were determined and were not significantly changed. The \(K_d\) values of NEG1 and NEG2 for SEMA3F were 1.78 and 0.98 nm, respectively, compared with 1.44 nm for WT B-NRP2, showing that SEMA3F binding was not affected (Fig. 4B and Table 2).

The ability of the mutants to displace \(^{125}\text{I}-\text{VEGF}_{165}\) and \(^{125}\text{I}-\text{SEMA3F}\) from the surface of PAEC NRP2 was determined as well. Consistent with the dissociation constants, the IC\(_{50}\) values of mutants NEG1 and NEG2 (166 and 184 nm, respectively) for \(^{125}\text{I}-\text{VEGF}_{165}\) showed an improvement of...
B-NRP2 Mutants with Selective Enhanced Affinity for VEGF165

TABLE 2

| B-NRP2 Mutants       | Kd (nM) | IC50 (nM) | Kd (nM) | IC50 (nM) |
|-----------------------|---------|-----------|---------|-----------|
| WT                    | 5.89 ± 0.57 | 265.4 ± 46.1 | 1.44 ± 0.45 | 1041.7 ± 136.2 |
| POS: E284A E291A      | 12.02 ± 1.67 | 534.2 ± 201.0 | 1.05 ± 0.58 | 1122.5 ± 289.6 |
| NEG1: R287E N290S     | 0.89 ± 0.51  | 165.7 ± 58.5  | 1.78 ± 1.09  | 1252.3 ± 135.1 |
| NEG2: R287E N290D     | 0.74 ± 0.31  | 183.8 ± 60.1  | 0.98 ± 0.44  | 1184.9 ± 310.2 |
| NEG1: R287E N290S     | 0.89 ± 0.51  | 165.7 ± 58.5  | 1.78 ± 1.09  | 1252.3 ± 135.1 |
| NEG2: R287E N290D     | 0.74 ± 0.31  | 183.8 ± 60.1  | 0.98 ± 0.44  | 1184.9 ± 310.2 |

* These values were extrapolated.

NRP1 and NRP2 play an important role in the normal development of the neuronal and vascular systems and in pathological tumor progression, tumor angiogenesis, and metastasis. NRP2s are atypical in that they bind two disparate families of ligands, the VEGFs that are pro-angiogenic and the class-3 semaphorins that, besides repelling axons, are anti-angiogenic. This ability to bind two unrelated ligands suggests that the NRP structure must have a defining role in mediating VEGF165 and semaphorin binding and activity. The pair of NRP1 ligands, VEGF165 and Collapsin-1 (chicken homolog for the human SEMA3A), competed for NRP1 binding and showed opposite effects on a dorsal root ganglia collapse assay and on an in vitro angiogenesis assay (23). The interactions of VEGF165 and semaphorins with NRP2 are not as well studied, thus, we decided to look more closely at these interactions, with an emphasis on NRP2 structural requirements for binding.

In this report we have 1) determined the $K_d$ values for VEGF165 and SEMA3F binding to NRP2-expressing cells and showed competition between these 2 ligands for NRP2 binding; 2) determined that whereas the B-NRP2 domain is sufficient for VEGF165 binding, both the A- and B-NRP2 domains are necessary for SEMA3F binding; 3) used site-directed mutagenesis to identify amino acid residues in the B-NRP2 domain involved in VEGF165, but not SEMA3F, binding; and 4) showed that these (up to 8-fold) higher affinity B-NRP2 mutants have enhanced inhibition of VEGF165 binding to endothelial cells but no effect on SEMA3F binding compared with WT B-NRP2.

PAEC NRP2 were used to determine the affinities of VEGF165 and SEMA3F for NRP2, because they have the advantage of expressing NRP2 but not any of the VEGF165 receptor tyrosine kinases that could compromise interpretation of VEGF165 binding results. The calculated dissociation constants for VEGF165 and SEMA3F were similar, 5.2 and 3.9 nM, respectively. These $K_d$ values were higher than the previously reported $K_d$ of 0.13 nm for VEGF165/NRP2 (45) and 0.09 nm for SEMA3F/NRP2 (10). For SEMA3F, one possibility is that Chen et al. (10) expressed the a5 isoform of NRP2, whereas we have expressed the a22 isoform. There are also differences in cell lines used for NRP2 overexpression, namely PAEC versus COS-7. Different cell lines with different cell surface proteoglycan expression patterns might result in different dissociation constants. It should be noted that there have also been discrepancies reported for VEGF165 binding to NRP1, with reported $K_d$ values of 0.18 (45) and 14.4 nm (46), respectively.

SEMA3A and VEGF165 compete for binding to NRP1 (23). Accordingly, the possibility of competition between SEMA3F or VEGF165 with NRP2 was examined. Cold SEMA3F could efficiently compete with $^{125}$I-VEGF165 for binding to PAEC.
NRP2, with 80% displacement at 100 nM, whereas binding to PAEC NRP1 was displaced by only 15% at the same concentration. This result is consistent with previous observations that SEMA3F is a much more specific ligand, by 10-fold, for NRP2 than for NRP1 (10). In complementary experiments, cold VEGF165 was not as effective in inhibiting 125I-SEMA3F binding to PAEC NRP2. The maximal inhibition was 53%. Previously it has been reported that VEGF165 did not inhibit SEMA3F binding to PAEC NRP2 at all (47). It may be that because SEMA3F, but not VEGF165, binds A-NRP2, VEGF165 may not be an optimal competitor of SEMA3F-NRP2 interaction because it cannot compete at the A domain level.

The competition of VEGF165 and SEMA3F for binding to NRP2 suggested that there are overlapping binding sites on this receptor. The extracellular domain of NRP2 has 3 subdomains, A, B, and C, the last involved in receptor dimerization (35–41). To determine NRP2 ligand binding sites, discrete NRP2 extracellular domains (A-, B-, and AB-NRP2) were cloned and used in pull-down experiments. The conclusion was that VEGF165 requires only the B-NRP2 domain for binding, whereas SEMA3F requires both the A and B domain. Previously we had shown similar results for NRP1 (40). VEGF165 binding to NRP1 showed that the B-NRP1 domain was sufficient for binding VEGF165. However, differently than NRP2, the additional presence of A-NRP1 (AB-NRP1) resulted in markedly improved VEGF165 binding to NRP1. Our VEGF165 NRP2 binding data are consistent with results recently published by Kärpänen et al. (48).

Semaphorin 3A has a SEMA domain that binds to A-NRP1, an immunoglobulin (Ig) domain, and a C-terminal basic domain that interacts with both A- and B-NRP1 (37). Mutations in the N-terminal a-domain repeat of NRP1 (a1-NRP1) completely disrupts interaction with SEMA3A while maintaining VEGF165 binding capacity (11). In our analysis of SEMA3F-NRP2 interaction, both A and B (AB-NRP2) were necessary for optimal interaction with SEMA3F consistent with previous analysis showing that SEMA3A requires both A-NRP1 and B-NRP1 for optimal binding (41). Comparisons of affinity constants showed that VEGF165 affinity for the B domain alone was about the same as full-length NRP2 expressed on cells. Similarly, the $K_d$ values for SEMA3F binding to the isolated AB domain and full-length NRP2 were very similar. These results show that data obtained by binding of discrete domains can be quite accurate. Further support for identifying the role of the A and B domains in VEGF165 and SEMA3F binding came from IC$_{50}$ values of cell-based competition binding studies. Both AB- and B-NRP2 domains inhibited binding of VEGF165 to PAEC NRP2, whereas only AB-NRP2 inhibited SEMA3F binding. Similar results were obtained with HUVEC, a non-engineered EC type expressing NRP2 (not shown).

Affinity is not the only measure of efficacy of ligand/NRP2 interactions. Our binding experiments show that the percentages of VEGF165 bound to B and AB-NRP2 at saturation (about 27 and 32%, respectively) were both significantly higher than the corresponding percentages of bound SEMA3F (3.5 and 15%, respectively). Taken together these data suggest a preferential interaction of B-NRP2 with VEGF165 rather than with SEMA3F.

By all accounts, VEGF165 binds to the B-NRP2 domain. To identify amino acids in B-NRP2 involved in VEGF165 binding, advantage was taken of the published crystal structure of the b1 domain of NRP1 (42) and of the high sequence identity (52%) between b1-NRP1 and b1-NRP2. The b1 domain has the characteristic folding of discoidin family protein members. It consists of eight $\beta$ strands, a five-stranded anti-parallel $\beta$-sheet packed against a three-stranded anti-parallel $\beta$-sheet, giving rise to a $\beta$-sandwich fold (42). Six loops (L1–L6) extend from the top of the sandwich and define a groove (42), which has been recently demonstrated to be the binding site for Tuftsin (a VEGF exon 8 peptide analogue) (44).

The b1 structure reveals a marked polarity in charge distribution, with electropositive and electronegative regions. Because VEGF165 and SEMA3F are basic, the electronegative region is a possible binding site for these two ligands. Site-directed mutagenesis of B-NRP2 in the 280–290 loop was used to substitute amino acids with the aim of altering B-NRP2 charge and looking for effects on VEGF165 binding. Mutagenesis sites in b1-NRP2 were chosen so that the corresponding positions in b1-NRP1 were occupied by identical/homologous residues, and thus less likely to induce structural alterations. Exchange of electronegative residues to neutral ones showed a 2-fold reduced affinity for VEGF165 compared with B-NRP2 WT. The affinity for SEMA3F was unchanged. On the other hand, increasing the electronegative charge by two different amino acid substitutions decreased the $K_d$ to 0.89 and 0.74 nM, respectively, compared with 5.9 nM for B-NRP2 WT, an increase in VEGF165 affinity of 6.6- and 8-fold, respectively. In contrast, the $K_d$s of the mutants for SEMA3F (1.78 and 0.98 nM, respectively) were only slightly altered compared with B-NRP2 WT (1.44 nM). These results are significant in identifying NRP2 amino acid residues involved in VEGF165 binding. 2 glutamate residues (Glu-284 and Glu-291) in b1-NRP2. Interestingly, they are involved in VEGF165 binding but not SEMA3F binding. Thus, whereas VEGF165 and SEMA3F are competitive inhibitors for NRP2 binding, there appear to be some non-overlapping residues.

The co-crystallization of B-NRP1 with Tuftsin has been recently reported (44). Tuftsin (TKPR) is partially homologous to the VEGF exon 8 sequence (CDKPR). The co-crystallization data suggest that Tuftsin establishes contacts with residues Tyr-297, Asp-320, Ser-346, Thr-349, and Tyr-353. No contacts were observed with residues of the 280–290 loop. However, VEGF165 interaction with NRP1 involves not only VEGF exon 8 but the 44-amino acid VEGF exon 7 as well (49, 50). In particular, residues 22–44 of VEGF exon 7 with the addition of the first cysteine of VEGF exon 8 have been shown to be critical for VEGF165-NRP2 interactions (50). Thus, it is likely that the B-NRP2 mutants we have generated interact with VEGF exon 7. A VEGF exon 7/NRP co-crystal has not yet been reported. Future site-directed mutagenesis studies will identify other amino acid residues involved in VEGF165 binding.

VEGF inhibitors are currently being developed for anti-angiogenesis cancer therapy. Examples showing clinical efficacy include VEGFR-2 kinase inhibitors (PTK787, Novartis), antibodies against VEGF (Bevacizumab, Genentech), and VEGF-Traps, a decoy soluble receptor consisting of the second Ig
domain of VEGFR-1 fused to the third Ig domain of VEGFR-2 (Aventis/Regeneron) (51–53). These antiangiogenesis therapies target VEGF/VEGFR interactions. However, it is evident that NRPs promote tumor progression in animal models (24, 25). In addition, high expression levels of NRP1 and NRP2 correlate with poor outcomes clinically (27–30). Therefore, targeting NRPs might be a viable strategy. A peptide (ATWLPPR, A7R) that inhibits VEGF165 interaction with NRP1 inhibited VEGF165-induced in vitro angiogenesis and tumor growth of MDA-MB231 (54). Furthermore, a bicyclic peptide corresponding to the sequence of VEGF165 exons 7 and 8 was able to inhibit VEGF165 interaction with NRP1 and VEGF165 signaling, e.g., VEGFR-2, phospholipase Cγ, and extracellular signal-regulated kinase (ERK) phosphorylation (55). Recently, it has been shown that targeting tumors with a combination of NRP1-blocking antibodies together with anti-VEGF antibodies resulted in a significantly increased tumor growth inhibition compared with anti-VEGF alone (26). Previously, we showed that soluble NRP1 (essentially the A and B domains) inhibited VEGF165 binding to ECs, and VEGF165-inhibited VEGF-2 phosphorylation (56). Overexpression of sNRP1 inhibited tumor growth and induced apoptosis, possibly by sequestering VEGF (56).

We have developed a strategy of increasing the electronegative potential of B-NRP2 to generate mutant proteins with increased affinity for VEGF165 and unaffected SEMA3F binding. This is a crucial property because these mutants could be selective inhibitors of the pro-angiogenic and pro-tumor progression properties of VEGF165 without affecting the reported anti-angiogenic and anti-metastasis features of SEMA3F. The mutants are better inhibitors of VEGF165 binding to EC than WT B-NRP2. Mutant Kd values for VEGF165 so far are as low as 0.74 nM, lower than soluble Flk (16 nM) (57) but 1–2 logs higher than soluble Flt (20 pM) (58). Future goals are to improve the selective inhibitors of the pro-angiogenic and pro-tumor growth and induced apoptosis, possibly by sequestering VEGF (56).

ACKNOWLEDGMENTS—We thank Dr. Silvia Coma, Dr. Andrew Dudley, Dr. Kashi Javaherian, Dr. Cathy Korsgren, and Dr. Jiang Yang for critical reading of the manuscript. We thank Melissa Herman for proofreading the manuscript and Ethan Bickford for technical assistance in figure preparation.

REFERENCES

1. Fujisawa, H. (2004) J. Neurobiol. 59, 24–33
2. Eichmann, A., Mäkinen, T., and Alitalo, K. (2005) Genes Dev. 19, 1013–1021
3. Klagesbrun, M., and Eichmann, A. (2005) Cytokine Growth Factor Rev. 16, 535–548
4. Klagesbrun, M., Takashima, S., and Mamluk, R. (2002) Adv. Exp. Med. Biol. 515, 33–48
5. Ellis, L. M. (2006) Mol. Cancer Ther. 5, 1099–1107
6. Takahashi, T., Fournier, A., Nakamura, F., Wang, L. H., Murakami, Y., Kalb, R. G., Fujisawa, H., and Strittmatter, S. M. (1999) Cell 99, 59–69
7. Nakamura, F., Kalb, R. G., and Strittmatter, S. M. (2000) J. Neurobiol. 44, 219–229
8. Tamagno, L., and Comoglio, P. M. (2000) Trends Cell Biol. 10, 377–383
9. He, Z., and Tessier-Lavigne, M. (1997) Cell 90, 739–751
10. Chen, H., Chedotal, A., He, Z., Goodman, C. S., and Tessier-Lavigne, M. (1997) Neuron 19, 547–559
11. Gu, C., Rodriguez, E. R., Reimert, D. V., Shu, T., Fritzsch, B., Richards, L. J., Kolodkin, A. L., and Ginty, D. D. (2003) Dev. Cell 5, 45–57
12. Gu, C., Yoshida, Y., Livet, J., Reimert, D. V., Mann, F., Merte, J., Henderson, C. E., Jessell, T. M., Kolodkin, A. L., and Ginty, D. D. (2005) Science 307, 265–268
13. Yuan, L., Moyon, D., Pardanaud, L., Breant, C., Karkkainen, M. J., Alitalo, K., and Eichmann, A. (2002) Development 129, 4797–4806
14. Eichmann, A., Yuan, L., Moyon, D., Libel, F., Pardanaud, L., and Breant, C. (2005) Int. J. Dev. Biol. 49, 259–267
15. Herzog, Y., Kalcheim, C., Kahane, N., Reshef, R., and Neufeld, G. (2001) Mech. Dev. 109, 115–119
16. Yaniv, K., Isogai, S., Castranova, D., Dye, L., Hitomi, J., and Weinstein, B. M. (2006) Nat. Med. 12, 711–716
17. Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T., and Fujisawa, H. (1999) Development 126, 4895–4902
18. Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., Niwa, H., Miyazaki, J., Hirota, S., Kitamura, Y., Kitsukawa, T., Fujisawa, H., Klagesbrun, M., and Horii, M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3657–3662
19. Lee, P., Gu, C., Yoshida, Y., Livet, J., Mann, F., Merte, J., Hendry, J., and Ginty, D. D. (2003) J. Neurosci. 23, 735–745
20. Bielenberg, D. R., Pettaway, C. A., Takashima, S., and Klagesbrun, M. (2006) Exp. Cell Res. 312, 584–593
21. Miao, H. Q., Soker, S., Feiner, L., Alonso, J. L., Raper, J. A., and Klagesbrun, M. (1999) J. Cell Biol. 146, 233–242
22. Miao, H. Q., Lee, P., Lin, H., Soker, S., and Klagesbrun, M. (2000) FASEB J. 14, 2523–2539
23. Parkh, A. A., Fan, F., Liu, W. B., Ahmad, S. A., Stoeltzing, O., Reimnuth, N., Bielenberg, D., Bucana, C., Klagesbrun, M., and Ellis, L. M. (2004) Am. J. Pathol. 164, 2139–2151
24. Pan, Q., Chantler, Y., Liang, W. C., Stawicki, S., Mak, J., Rathore, N., Tong, R. K., Kowalski, J., Yee, S. F., Pacheco, G., Ross, S., Cheng, Z., Le Coutler, J., Plowman, G., Peale, F., Koch, A. W., Wu, Y., Bagri, A., Tessier-Lavigne, M., and Watts, R. J. (2007) Cancer Cell 11, 53–67
25. Osada, H., Tokunaga, T., Nishi, M., Hatanaka, H., Abe, Y., Tsug, A., Kijima, H., Yamazaki, H., Ueyama, Y., and Nakamura, M. (2004) Anticancer Res. 24, 547–552
26. Hansel, D. E., Wilentz, R. E., Zon, L., Hansel, D. E., Wilentz, R. E., Yeo, C. J., Schulick, R. D., Montgomery, E., and Fishman, M. C., Childs, S., Epstein, J. A., and Weinstein, B. M. (2004) Am. J. Surg. Pathol. 28, 347–356
27. Lantuejoul, S., Convent, B., Drabkin, H., Brambilla, C., Roche, J., and Brambilla, E. (2003) J. Pathol. 206, 336–347
28. Kawakami, T., Tokunaga, T., Hatanaka, H., Kijima, H., Yamazaki, H., Abe, Y., Osamura, Y., Inoue, H., Ueyama, Y., and Nakamura, M. (2002) Cancer Res. 59, 4291–4297
29. Takagi, S., Hirata, T., Agata, K., Mochii, M., Eguchi, G., and Fujisawa, H. (1999) Neuron 7, 295–307
30. Giger, R. J., Urquhart, E. R., Gillespie, S. K., Levengood, D. V., Ginty, D. D., and Kolodkin, A. L. (1998) Neuron 21, 1079–1092
31. Nakamura, F., Tanaka, M., Takahashi, T., Kalb, R. G., and Strittmatter, S. M. (1998) Neuron 21, 1093–1100
32. Remzi, M. J., Feiner, L., Koppel, A. M., and Raper, J. A. (1999) J. Neurosci.
39. Nakamura, F., and Goshima, Y. (2002) Adv. Exp. Med. Biol. 515, 55–69
40. Mamluk, R., Gechtman, Z., Kutcher, M. E., Gasiunas, N., Gallagher, J., and Klagsbrun, M. (2002) J. Biol. Chem. 277, 24818–24825
41. Gu, C., Limberg, B. J., Whitaker, G. B., Perman, B., Leahy, D. J., Rosenbaum, J. S., Ginty, D. D., and Kolodkin, A. L. (2002) J. Biol. Chem. 277, 18069–18076
42. Lee, C. C., Kreusch, A., McMullan, D., Ng, K., and Spraggon, G. (2003) Structure 11, 99–108
43. Oberg, C., Waltenberger, J., Claesson-Welsh, L., and Welsh, M. (1994) Growth Factors 10, 115–126
44. Vander Kooi, C. W., Jusino, M. A., Perman, B., Neau, D. B., Bellamy, H. D., and Leahy, D. J. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 6152–6157
45. Gluzman-Poltorak, Z., Cohen, T., Herzog, Y., and Neufeld, G. (2000) J. Biol. Chem. 275, 29922
46. Yamazaki, Y., Takani, K., Atoda, H., and Morita, T. (2003) J. Biol. Chem. 278, 51985–51988
47. Gluzman-Poltorak, Z., Cohen, T., Shibuya, M., and Neufeld, G. (2001) J. Biol. Chem. 276, 18688–18694
48. Karpanen, T., Heckman, C. A., Keskitalo, S., Jeltsch, M., Ollila, H., Neufeld, G., Tamagnone, L., and Alitalo, K. (2006) FASEB J. 20, 1462–1472
49. Soker, S., Fidder, H., Neufeld, G., and Klagsbrun, M. (1996) J. Biol. Chem. 271, 5761–5767
50. Soker, S., Gollamudi-Payne, S., Fidder, H., Charmahelli, H., and Klagsbrun, M. (1997) J. Biol. Chem. 272, 31582–31588
51. Hicklin, D. J., and Ellis, L. M. (2005) J. Clin. Oncol. 23, 1011–1027
52. Holash, J., Davis, S., Papadopoulos, N., Croll, S. D., Ho, L., Russell, M., Boland, P., Leidich, R., Hylton, D., Burova, E., Ioffe, E., Huang, T., Radziejewski, C., Bailey, K., Fandl, J. P., Daly, T., Wiegand, S. J., Yancopoulos, G. D., and Rudge, J. S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11393–11398
53. Willett, C. G., Boucher, Y., di Tomaso, E., Duda, D. G., Munn, L. L., Tong, R. T., Chung, D. C., Sahani, D. V., Kalva, S. P., Kozin, S. V., Mino, M., Cohen, K. S., Scadden, D. T., Hartford, A. C., Fischman, A. J., Clark, J. W., Ryan, D. P., Zha, A. X., Blazkowsky, L. S., Chen, H. X., Shellito, P. C., Lauwers, G. Y., and Jain, R. K. (2004) Nat. Med. 10, 145–147
54. Starzec, A., Vassy, R., Martin, A., Lecouvey, M., Di Benedetto, M., Crepin, M., and Perret, G. Y. (2006) Life Sci. 79, 2370–2381
55. Jia, H., Bagherzadeh, A., Hartzoulakis, B., Jarvis, A., Lohr, M., Shaikh, S., Aqil, R., Cheng, L., Tickner, M., Esposito, D., Harris, R., Driscoll, P. C., Selwood, D. L., and Zachary, I. C. (2006) J. Biol. Chem. 281, 13493–13502
56. Gagnon, M. L., Bielenberg, D. R., Gechtman, Z., Miao, H. Q., Takashima, S., Soker, S., and Klagsbrun, M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2573–2578
57. Lin, P., Sankar, S., Shan, S., Dewhirst, M. W., Polverini, P. J., Quinn, T. Q., and Peters, K. G. (1998) Cell Growth & Differ. 9, 49–58
58. Kendall, R. L., and Thomas, K. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10705–10709