

Competing Autocrine Pathways Involving Alternative Neuropilin-1 Ligands Regulate Chemotaxis of Carcinoma Cells¹

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Abstract

Neuropilin-1 (NP1), in conjunction with plexins, promotes axon repulsion by binding to semaphorin 3A (SEMA3A). Although NP1 is expressed in carcinoma cells, its functions have remained elusive, and neither SEMA3A nor plexin expression has been explored in cancer. Here we provide evidence that breast carcinoma cells support an autocrine pathway involving SEMA3A, plexin-A1, and NP1 that impedes their ability to chemotax. Reducing SEMA3A or NP1 expression by RNA interference or inhibiting plexin-A1 signaling enhanced migration. Conversely, expression of constitutively active plexin-A1 impaired chemotaxis. The paradox of how breast carcinoma cells expressing these endogenous chemotaxis inhibitors are able to migrate is explained by their expression of vascular endothelial growth factor (VEGF), a NP1 ligand that competes with SEMA3A for receptor binding. Finally, we establish that the ratio of endogenous VEGF and SEMA3A concentrations in carcinoma cells determines their chemotactic rate. Our findings lead to the surprising conclusion that opposing autocrine loops involving NP1 regulate the chemotaxis of breast carcinoma cells. Moreover, our data indicate a novel autocrine function for VEGF in chemotaxis.

Introduction

In addition to the classical VEGF³ tyrosine kinase receptors, KDR and Flt-1, NP1 serves as a high-affinity VEGF receptor (1). NP1 expression on endothelial cells enhances VEGF signaling by increasing the affinity of VEGF for the classical VEGF receptor tyrosine kinase KDR (1). Interestingly, NP1 expression has also been reported in a variety of tumors in the absence of KDR or Flt-1 (1, 2). On the basis of the established importance of VEGF in tumor progression, our previous studies investigated a role for NP1 in carcinoma cells as a VEGF receptor, in the absence of classical VEGF receptor tyrosine kinases. These studies indicated that NP1 supports a VEGF autocrine signaling pathway that is critical for breast carcinoma cell survival (2).

Of note, NP1 was identified originally in neurons as a receptor for SEMA3A, a soluble member of the semaphorin family that plays a critical role in axon guidance (3, 4). The ability of NP1, which lacks consensus signaling domains, to deliver SEMA3A-associated chemorepulsive signals is dependent on NP1 associations with plexins, proteins displaying Met homologies (5, 6). Although functions for

NP1 as a VEGF receptor in tumor cells have been reported (2, 7), the possibility that NP1 influences tumor function by supporting signaling through its alternative ligand, SEMA3A, has not been examined. Here, we provide the first evidence for expression of SEMA3A and plexin-A1 in carcinoma cells and demonstrate that these molecules are autocrine inhibitors of breast carcinoma migration. Importantly, we also identify a novel function for VEGF in carcinoma cell migration involving its inhibition of SEMA3A activity.

Materials and Methods

mRNA Detection. mRNA was purified from the indicated cell lines using the RNEasy kit (Qiagen) according to the manufacturer's recommended protocol. RNA (2 μg) was added to RT-PCR reactions containing the indicated primers at a concentration of 0.6 μM. Alternatively, cDNA was generated from carcinoma cells purified from human breast tumors (provided by K. Polyak, Dana-Farber Cancer Institute). The conditions for amplifying SEMA3A and NP1 cDNA were as follows: 35 cycles, 95°C, 15 min; 95°C, 30 s; 55°C, 1 min; and 72°C, 1 min, followed by a 72°C, 10-min final extension step. The conditions for amplifying plexin-A1 cDNA were as follows: 35 cycles, 95°C, 15 min; 95°C, 30 s; 58°C, 1 min; and 72°C, 2.5 min, followed by a 72°C, 10-min extension step. The sequences of amplification primers are as follows: SEMA3A Forward, GACTTTGCTATCTTCCGAACCTTGGGCAC; SEMA3A Reverse, GCTATACATACACACGGCTGATCCCTTG; NP1 Forward, ATGGAGAGGGGCTGCGG; NP1 Reverse, CTATCGCGTGTGCGGTGTA; Plexin-A1 Forward, GAGGATGCCGACATGTTTCGGCTTCG; and Plexin-A1 Reverse, AGGGCGTCATGGGCACGCACGG.

RNAi Transient Transfections. RNAis were designed and synthesized by Dharmacon, Inc. (see below for sequences). Cells at 60% confluency were transfected in penicillin/streptomycin-free medium with the indicated RNAi using TKO lipid (Mirus), following the manufacturer's recommended protocol. The following RNAi concentrations were determined to be optimal for inhibiting protein expression: 200 nM RNAi for all cell lines; 200 nM SEMA3A RNAi for MDA-231 cells; 100 nM SEMA3A RNAi for MDA-435 and MCF-7 cells. After 20 h, RNAis were removed, and the cells were maintained in complete medium with the indicated antibodies for an additional 24 h: NP1 RNAi, GAGAGGUCCUGAAUGUUCCTT; Scrambled NP1 control, AGAUGUAGUCGUCGCUTT; SEMA3A RNAi: AAAGUUCAUUAGUGCCACCU; and Scrambled SEMA3A control, AAGUGCACGCCUCUAUAUAUC.

SEMA3A and SCR SEMA3A RNAi Retrovirus Generation. To create SEMA3A-pSUPER and SCR SEMA3A-pSUPER expression vectors, the following oligonucleotides (Invitrogen, Grand Island, NY) were cloned into pSUPER (a gift from R. Agami, The Netherlands Cancer Institute, Amsterdam, the Netherlands): SEMA3A, 5'-gatccccAGTTCATTAGTGCCACCTtcaagagaAGGTGGGCACTAATGAACTttttgaaa-3' and 5'-agcttttcaaaaaAGTTCATTAGTGCCACCTtctctgaaAGGTGGGCACTAATGAACTggg-3'; SCR SEMA3A, 5'-gatccccGTGCACGCCTCTATATATCtcaagagaGATATATAGAGCGTGCACtttttgaaa-3' and 5'-agcttttcaaaaaGTGCACGCCTCTATATATCtctctgaaGATATATAGAGGCGTGCACggg-3'. EcoRI- and XhoI-digested inserts containing the H1-RNA promoter and targeting oligonucleotides from pSUPER were then subcloned into pSUPER.retro (Oligoengine, Seattle, WA). All plasmids were sequenced to confirm that they were correct.

To generate retroviruses, SEMA3A-pSUPER or SCR SEMA3A-pSUPER,

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³ The abbreviations used are: VEGF, vascular endothelial growth factor; NP1, neuropilin-1; SEMA3A, semaphorin 3A; RNAi, RNA interference; ZVAD-FMK, benzylloxycarbonyl-VAD-fluoromethyl ketone; β-gal, β-galactosidase; VSV, vesicular stomatitis virus; AS, antisense.

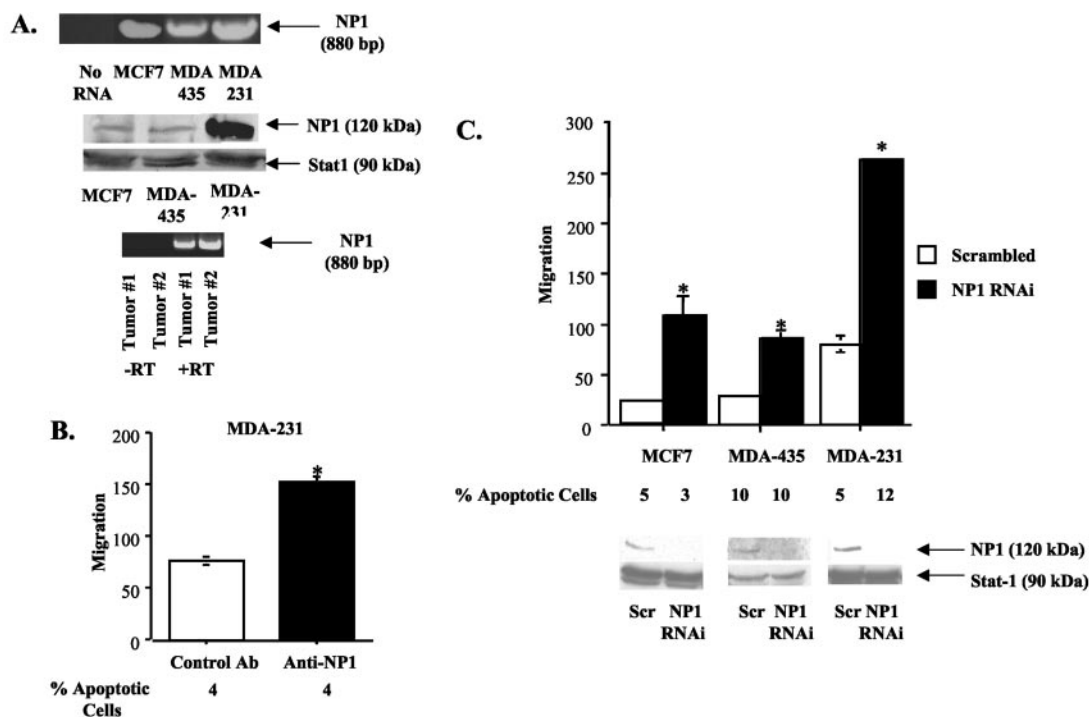


Fig. 1. NP1 suppresses chemotaxis of breast carcinoma cells. *A*: *top panel*, NP1-specific RT-PCR reactions were performed using the indicated mRNAs; *middle panel*, protein extracts were immunoblotted with the indicated antibodies (mouse anti-NP1 and rabbit anti-Stat1; Santa Cruz Biotechnology); *bottom panel*, NP1 was PCR amplified from cDNA obtained from carcinoma cells isolated from breast tumors. *B*, MDA-231 cells were incubated for 6 h (+ ZVAD-FMK) with either a rabbit IgG or NP1-specific polyclonal antibody (*Ab*; provided by Alex Kolodkin, Johns Hopkins University School of Medicine, Baltimore, MD), and their chemotaxis toward conditioned 3T3 medium was assessed in a 3-h assay in the continued presence of antibody (+ ZVAD-FMK). *C*, cells were transfected with a scrambled (*Scr*) or NP1-specific RNAi, and their ability to migrate toward conditioned 3T3 medium was assessed in a 24-h (MCF7), 15-h (MDA-435), or 3-h (MDA-231) assay. The mean number (bars, \pm SD) of migrated cells from two wells (four fields/well) is indicated. *, $P < 0.02$ in a Student's *t* test. Inhibition of NP1 expression by NP1 RNAi was assessed by immunoblotting, as described in *A*. The percentage of apoptotic cells was assessed using Annexin V-FITC, as described previously (17). Similar results were observed in three independent experiments.

retro and expression plasmids containing proteins required for viral propagation (Imgenex, San Diego, CA) were transfected into 293T cells. Viral supernatants were harvested, and MDA-MB-435 recipient cells were infected in the presence of 8 μ g/ml of Polybrene (Sigma, St. Louis, MO). After infection for 24 h, resistant cells were selected with puromycin (2 μ g/ml).

DNA Transfections. Cells were transfected in the presence of Lipofectamine (Life Technologies, Inc.) and ZVAD-FMK with a β -gal-expressing plasmid (1 μ g), and either VSV-tagged, dominant-negative human plexin-A1 (plexin-A1 Δ Cyt, provided by P. Comoglio, University of Torino, Italy) or myc-tagged constitutively active murine plexin-A1 (1 μ g of PlexA1 Δ Sem, provided by S. Strittmatter, Yale University School of Medicine, New Haven, CT). The ability of these transfectants to migrate toward conditioned medium was assessed after 48 h in the presence of ZVAD-FMK.

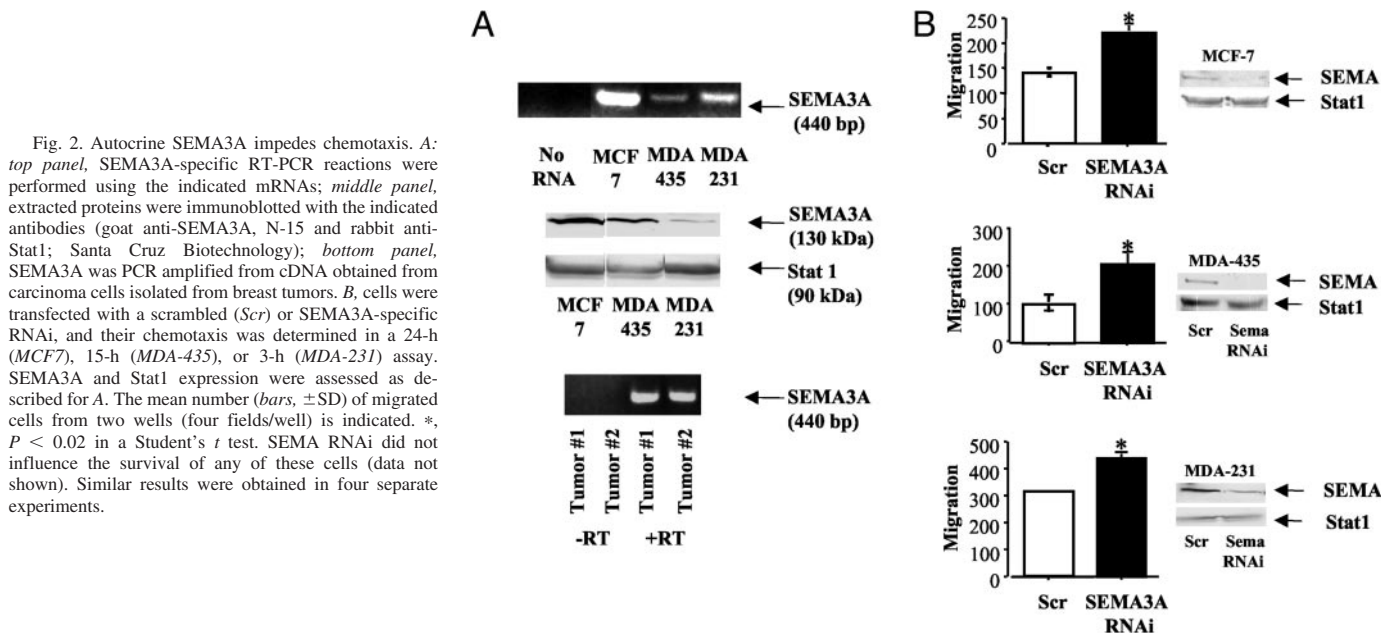
Chemotaxis Assays. Chemotaxis toward conditioned NIH3T3 medium was assessed using collagen (Cohesion; 15 μ g/ml)-coated Transwell chambers, as described previously (8).

Results and Discussion

Given that NP1 is expressed in breast carcinoma cell lines (Refs. 1 and 2; Fig. 1A) and tumors (Fig. 1A), we assessed the potential involvement of this receptor in carcinoma chemotaxis. Surprisingly, a NP1-neutralizing antibody increased the chemotaxis of MDA-231 cells toward NIH 3T3 conditioned medium 2-fold (Fig. 1B). To confirm and extend this finding, we implemented an RNAi strategy to diminish NP1 expression in each of three breast carcinoma cell lines. Our previous data indicate that NP1 is essential for breast carcinoma survival because it supports VEGF autocrine survival signaling (2). To evaluate the role of NP1 in migration separately from its requirement for breast carcinoma cell survival (2), NP1 RNAi transfections were performed in the presence of the general caspase inhibitor, ZVAD-FMK. Under these conditions, the inhibition of NP1 expres-

sion did not impact cell survival (Fig. 1C). Of note, this RNAi abolished NP1 expression in MDA-435 and MDA-231 cells, and it increased their chemotaxis by 1.5- and 2.3-fold, respectively (Fig. 1C). In addition, this RNAi reduced NP1 expression in MCF-7 cells, a poorly migratory breast carcinoma line, and enhanced their chemotaxis 5-fold (Fig. 1C).

SEMA3A inhibits axon outgrowth by binding to NP1 and the NP1 coreceptor, plexin-A1 (5, 6). On the basis of our finding that NP1 is inhibitory for breast carcinoma migration, we hypothesized that these cells express SEMA3A and plexin-A1. In fact, SEMA3A and plexin-A1 mRNA were detected in each of three breast carcinoma cell lines, as well as in primary breast tumors (Figs. 2A and 3A). We also identified SEMA3A and plexin-A1 protein by immunoblotting proteins extracted from these samples with a SEMA3A- or plexin-A1-specific antibody (Figs. 2A and 3A). To elucidate a function for SEMA3A in breast carcinoma cells, we reduced SEMA3A expression using a SEMA3A RNAi. This RNAi, which reduced SEMA3A expression significantly (Fig. 2B), increased the migration of these cells (Fig. 2B) without influencing cell survival (data not shown). To assess the importance of plexin-A1 in migration, MDA-231 cells were transfected with a plexin-A1 cytoplasmic domain deletion mutant that inhibits SEMA3A signaling (6). Expression of this mutant in MDA-231 cells enhanced their migration significantly (Fig. 3B). Conversely, expression of a semaphorin homology domain deletion mutant of plexin-A1 that exhibits constitutive activity in neurons (9) inhibited MDA-231 migration (Fig. 3C). None of these reagents influenced cell survival in the presence of ZVAD-FMK (Fig. 3, B and C). These data indicate that an autocrine pathway involving



SEMA3A, NP1, and plexin-A1 impedes the chemotaxis of breast carcinoma cells. Our ability to increase breast carcinoma migration by expressing a dominant-negative plexin-A1 suggests that other plexins, if expressed in these cells, cannot support SEMA3A signaling in the absence of plexin-A1 function.

Genes that are inhibitory for cell growth are frequently subject to chromosomal deletion, mutational inactivation, or gene silencing in tumor cells (10–12). The ability of breast carcinoma cells to migrate and invade, despite their expression of molecules involved in SEMA3A signaling, suggested that they support a novel mechanism for repressing SEMA3A function. Increased VEGF expression is a hallmark of breast carcinoma progression (13, 14). Until recently, the function of VEGF in tumor progression was thought to relate solely to its angiogenic activity. We were intrigued by the reported ability of

recombinant VEGF and recombinant SEMA3A, which exhibit similar affinities for NP1 and NP1/plexin complexes, respectively (1, 5), to compete for NP1 binding (15, 16). On the basis of these findings, we postulated that endogenous VEGF and SEMA3A compete for NP1 binding, and that the ratio of the concentration of these proteins in carcinoma cells is a critical determinant of their chemotactic rate. To determine this ratio, we measured the relative amounts of SEMA3A and VEGF protein in these cells (Fig. 4A). We then compared the ratio of these concentrations to the relative chemotactic rate of these carcinoma cells. As shown in Fig. 4B, MCF-7 cells, which exhibited the lowest chemotactic rate, displayed the highest ratio of SEMA3A to VEGF protein. MDA-435 cells, which were more chemotactic than MCF-7 cells, demonstrated a lower SEMA3A:VEGF concentration ratio (Fig. 4B). The lowest SEMA3A:VEGF ratio was observed in

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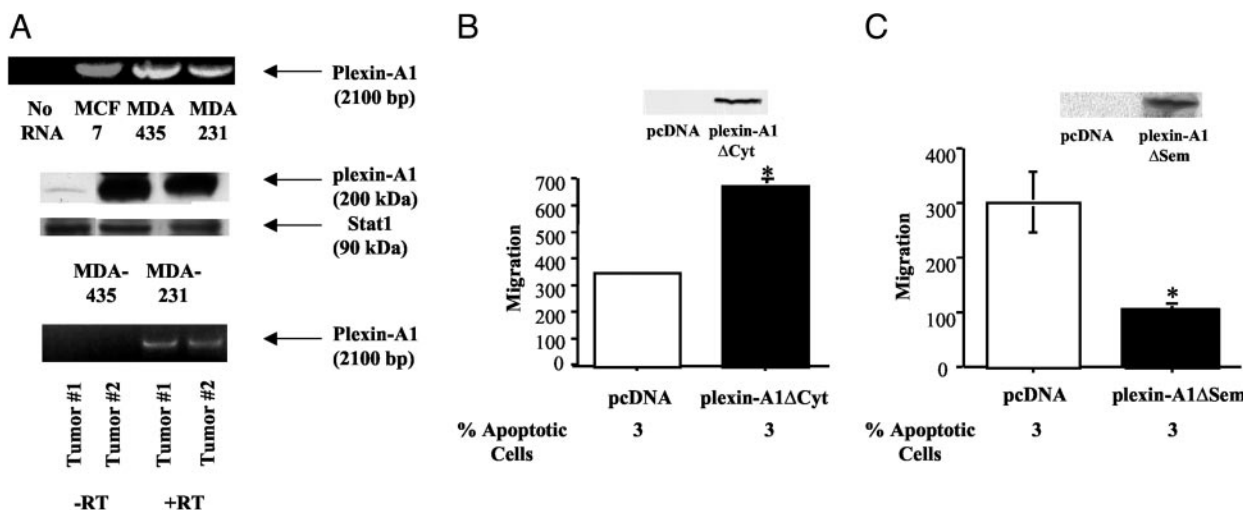


Fig. 3. Plexin-A1 signaling inhibits chemotaxis. *A*: top panel, plexin-A1-specific RT-PCR reactions were performed using the indicated mRNAs; middle panel, plexin-A1 and Stat1 proteins were detected by immunoblotting (rat anti-plexin-A1 provided by H. Fujisawa, Nagoya University Graduate School of Science, Nagoya, Japan; rabbit anti-Stat1 provided by Santa Cruz Biotechnology); bottom panel, plexin-A1 fragments were amplified from cDNA isolated from carcinoma cells purified from breast tumors. *B*, MDA-231 cells were cotransfected with a β -gal-expressing plasmid in addition to either a control plasmid or a VSV-tagged plexin-A1 cytoplasmic domain deletion mutant (*plexin-A1ΔCyt*), and their migration was measured after 48 h by 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside staining. Expression of the VSV-tagged construct was confirmed by immunoblotting with a VSV-specific antibody (Sigma). *C*, MDA-231 cells were cotransfected with a β -gal-expressing vector and either a control plasmid or a constitutively active, myc-tagged plexin-A1 construct (*plexin-A1ΔSem*). The number of migrated cells after 48 h was assessed by 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside staining. Expression of the myc-tagged construct was confirmed by immunoblotting with a myc-specific antibody (Sigma). For *B* and *C*, the mean number (bars, \pm SD) of migrated cells from two wells (four fields/well), as well as the percentage of apoptotic cells, is indicated. *, $P < 0.02$ in a Student's *t* test. Similar results were obtained in two trials.

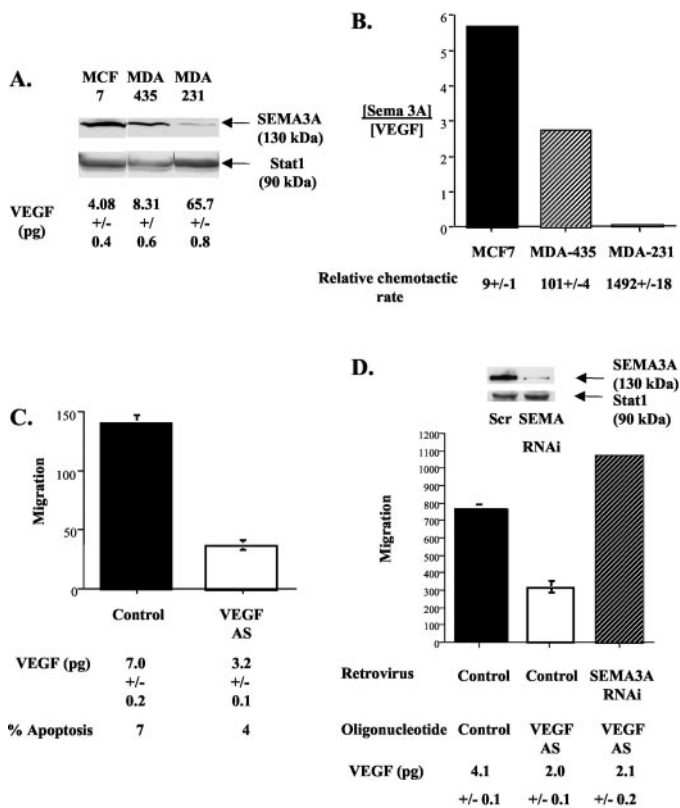


Fig. 4. Relative concentration of VEGF and SEMA3A determines chemotaxis rate. *A*, SEMA3A expression in equivalent amounts of total cellular protein extracted from MCF7, MDA-435, and MDA-231 cells was assessed by immunoblotting and quantified by densitometry. VEGF expression (pg) in 100 μ g of total proteins extracted from these cells was measured by ELISA (Research and Diagnostic Systems). *B*, the ratio of relative levels of SEMA3A and VEGF was determined for the indicated breast carcinoma cell lines. In addition, the rates of chemotaxis for these carcinoma lines were assessed by determining the mean number of cells (bars, \pm SD) that had migrated toward conditioned medium in a 16-h assay. Similar trends were observed in three trials. *C*, MDA-435 cells were transfected with either a VEGF AS or sense (Control) oligonucleotide as described previously (Ref. 2; + ZVAD-FMK), and their migration toward conditioned medium was assessed in a 15-h assay. The percentage of apoptotic cells was measured as described for Fig. 1. *D*, MDA-435 cells were infected stably with either a SEMA3A RNAi-expressing or control retrovirus. These retroviral cells were then transfected transiently with either a control or VEGF AS oligonucleotide (+ ZVAD-FMK). The ability of these cells to migrate toward conditioned medium was determined in a 15-h assay. For *C* and *D*, the mean number (bars, \pm SD) of migrated cells from two wells (four fields/well) was determined. *, $P < 0.02$ in a Student's *t* test. VEGF and SEMA3A expression were assessed as described for *A*. Similar results were obtained in two separate experiments.

MDA-231 cells, which exhibited the most robust rate of chemotaxis (Fig. 4B).

If the relative concentrations of endogenous VEGF and SEMA3A in breast carcinoma cells determine their chemotactic rate, then altering expression of these NP1 ligands should influence chemotaxis. To reduce VEGF expression, MDA-435 cells were transfected with either a VEGF AS or control oligonucleotide. VEGF AS transfection reduced VEGF expression by \sim 50% relative to transfection with the control oligonucleotide (Fig. 4C). Importantly, VEGF AS transfection did not reduce SEMA3A expression in these cells (data not shown). Strikingly, the ability of VEGF AS transfectants to migrate toward conditioned medium was reduced relative to that of the control transfectants (Fig. 4C). These transfectants were maintained in the presence of ZVAD-FMK, and the reduction in VEGF expression did not influence their survival (Fig. 4C).

If a major role for VEGF in carcinoma cells involves its antagonism of autocrine SEMA3A, then reducing SEMA3A expression in VEGF AS transfectants should offset the decrease in chemotaxis caused by reduced VEGF expression. To decrease SEMA3A expression stably,

MDA-435 cells were infected with retroviruses expressing either a SEMA3A-specific or scrambled RNAi (control). Stable infectants that expressed SEMA3A RNAi exhibited a significant decrease in SEMA3A expression relative to cells infected with the control retrovirus (Fig. 4D). These infectants were then transfected transiently with either the VEGF AS or control oligonucleotide. Confirming the data in Fig. 4C, VEGF expression in VEGF AS transfectants was reduced by 50%. We then determined the ability of these cells to chemotax toward conditioned NIH 3T3 medium. Confirming the data in Fig. 4C, the chemotaxis of cells infected with the control retrovirus was significantly reduced by VEGF AS transfection. Strikingly, the migration of VEGF AS transfectants was restored upon reducing SEMA3A expression with the SEMA3A RNAi-expressing retrovirus. In the presence of ZVAD-FMK, we did not observe an effect of reducing either SEMA3A or VEGF expression on cell survival (data not shown). These data identify VEGF and SEMA3A as antagonistic, autocrine NP1 ligands that regulate breast carcinoma migration.

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