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13. ABSTRACT (Maximum 200 Words)

Our group recently characterized a novel autocrine survival pathway in breast carcinoma cells specifically mediated by binding of vascular endothelial growth factor to neuropilin-1 (NP1). Because NP1 promotes tumor cell survival, the goal of this proposal is to address the importance of NP1 in breast cancer progression. Thus far, we have determined that the level of NP1 does not increase in human tumors as a function of breast cancer progression. We have also demonstrated that the cytoplasmic domain of NP1 is not necessary for either its survival function or its role as a chemotaxis inhibitor. Unfortunately, the use of RNA interference to reduce NP1 expression has proven to be unsuccessful in two independent breast carcinoma cell lines suggesting a critical role for NP1 expression in the survival of these cells. To circumvent this problem and to allow for the study of NP1 in the progression of metastatic breast cancer, our laboratory is currently generating cell lines that are inducible for the expression of NP1 small-interfering oligonucleotides. The development of this system will enable us to address the importance of NP1 in metastasis following tumor formation in future studies.

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INTRODUCTION:

The specific aims outlined in this proposal seek to address the importance of neuropilin-1 (NP1) in breast cancer progression. Importantly, our group recently characterized a novel autocrine survival pathway in breast carcinoma cells specifically mediated by binding of vascular endothelial growth factor (VEGF) to NP1[1]. These studies were the first to identify a functional role for NP1 in tumor cells and they led us to hypothesize that NP1 plays a critical role in breast cancer metastasis. To further investigate the role of NP1 in breast cancer progression, we have proposed to evaluate the expression of NP1 as a function of tumor grade, to determine the contribution of NP1 in tumor formation and metastasis, and to assess the importance of the NP1 cytoplasmic domain in downstream signaling events. The results of these experiments will determine whether NP1 is a critical mediator in the progression of metastatic breast cancer.

BODY:

Specific Aim #1: Investigate the expression of NP1 as a function of breast cancer progression.

Summary: Specific Aim #1 was completed in the first year of funding as outlined in the Annual Summary Report 2003. To summarize, we found that NP1 was expressed at a low level in human breast tumor cells but that the level of NP1 expression did not increase with disease progression.

Specific Aim #2: Demonstrate that NP1 is sufficient and necessary for the progression of tumorigenic breast carcinoma cells to the metastatic phenotype.

Summary: To explore the significance of NP1 on metastasis *in vivo*, we generated MDA-MB-231 and SUM-159 breast carcinoma cell lines that retrovirally expressed siRNA oligonucleotides specific to NP1 or control scrambled-sequence oligonucleotides. We found that we were unable to generate NP1-deficient cell lines using this strategy even though the selected oligonucleotide sequence was efficient at reducing NP1 expression in transient transfection experiments. Consistent with its role as a pro-survival gene[1], we concluded that NP1 is essential to the survival of these cells and that cells with stably decreased NP1 expression likely do not survive the retroviral selection process but instead undergo apoptosis. To circumvent this problem, our laboratory is currently generating breast carcinoma cell lines that are inducible for the expression of siRNA oligonucleotides.

We initially attempted to stably reduce NP1 expression in the MDA-MB-231 breast carcinoma cell line. Because this approach was unsuccessful and to unsure that this phenomenon was not cell line specific, we next tried to decrease NP1 expression in the highly invasive SUM-159 breast carcinoma cell line[2;3]. Although this endeavor also failed to generate a NP1-deficient breast carcinoma cell line, we have found the SUM-159 cell line to be an excellent model system for investigating signaling pathways *in vitro* as well as for studying tumor formation and metastasis *in vivo*. Given the importance of integrin as well as growth factor receptor signaling in breast cancer progression[4], we have generated a SUM-159 cell line that lacks expression of the β4 integrin using siRNA strategies initially intended to inhibit NP1 expression. The data generated with this β4-deficient cell line are summarized below and suggest that the β4 integrin plays a role in tumor formation in a VEGF-dependent manner.

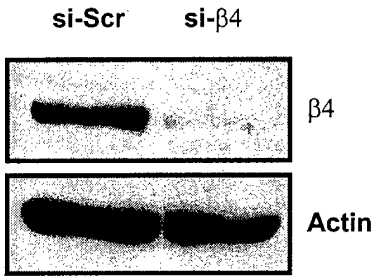

Fig. 1. Generation of a β4-deficient SUM-159 breast carcinoma cell line. SUM-159 cells were stably infected with a retrovirus expressing siRNA oligonucleotides to the β4 sequence (si-β4) or as control scrambled-sequence oligonucleotides (si-Scr). Protein extracts (50 μg) were resolved under reducing conditions by 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted for β4 and β-actin. Note the almost undetectable level of β4 protein expression in the β4-deficient cell line as compared to the si-Scr or control cell line.

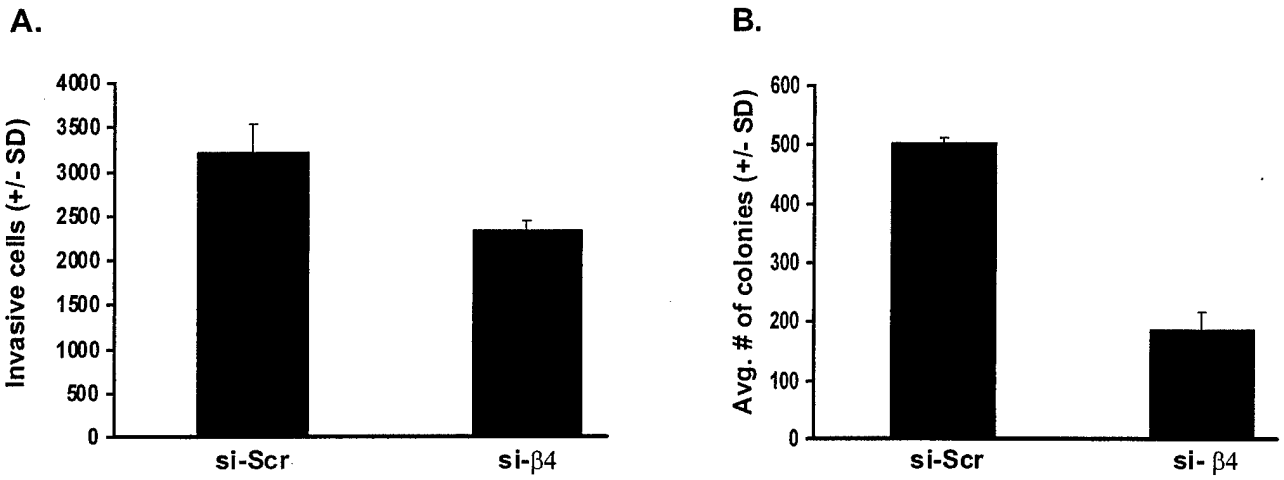

Fig. 2. Reduced β4 expression leads to decreased invasion and soft-agar colony formation. (A) The ability of si-Scr and si-β4 SUM-159 cells to invade Matrigel towards conditioned fibroblast medium was investigated in a 2 h assay. The results represent the mean number of invasive cells (+/- SD) from three wells (five fields per well). The number of invasive cells was decreased by 28% for the si-β4 cell line compared to the control si-Scr expressing cells. Similar data were obtained in three separate experiments. **(B)** To assess anchorage-independent growth, soft agar assays layered with complete growth medium were grown for 2 weeks. The results represent the mean number of colonies (+/- SD) from three wells (100 fields per well). The β4-deficient SUM-159 cell line produced 63% fewer colonies than the si-Scr control cell line. Similar results were obtained in three separate experiments.

Table 1. Expression of the β4 integrin promotes tumor formation. Female immunocompromised mice were injected in the mammary fat pad with either the si-Scr or si-β4 SUM-159 breast carcinoma cell line (2 x 10^6 cells per injection) in the presence of Matrigel. Tumors were harvested and weighed 7-10 weeks following injection.

SUM-159 Cells	Number of Mice	Number of Tumors	Tumor Weight (g)
si-Scr	16	13	0.64 +/- 0.43
si-β4	14	3	0.18 +/- 0.15

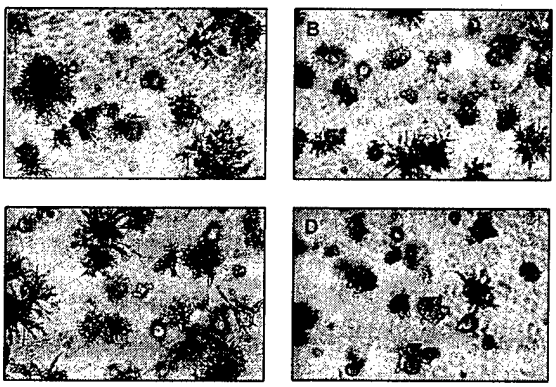

Fig 3. Rescue of the stellate morphology of the si-β4 SUM-159 cell line by VEGF. The si-Scr **(A)** or si-β4 **(B-D)** SUM-159 cell lines were grown in a three dimensional Matrigel matrix for 10 days. Matrices were layered with complete growth media (**A** and **B**) or in complete growth medium supplemented with VEGF (**C**) or as a negative control epidermal growth factor (**D**). Note that the si-β4 cells look unhealthy and largely devoid of the stellate morphology either in the absence of exogenous growth factor (**B**) or in the presence of EGF (**D**) whereas the presence of VEGF (**C**) largely restores the stellate morphology observed with the si-Scr cells (**A**). Future studies will quantitatively assess this data by performing survival analysis on dispase treated cells.

Specific Aim #3: Define the downstream signaling events mediated by NP1 that are responsible for metastasis.

Summary: Our laboratory has previously demonstrated that the cytoplasmic domain of NP1 is not required for its survival function (outlined in Annual Summary Report 2003). However, the recently identified role of NP1 as a chemotaxis inhibitor prompted us to investigate whether the cytoplasmic domain of NP1 is essential for its migratory function[5]. In three independent migration assays towards 3T3 conditioned medium, the level of migration was comparable for breast carcinoma cells transfected with either full-length or truncated NP1 indicating that the cytoplasmic domain is not required for this function.

KEY RESEARCH ACCOMPLISHMENTS:

- Generated MDA-MB-231 and SUM-159 breast carcinoma cell lines that are β4-deficient by retroviral infection with siRNA oligonucleotides followed by flow cytometry to isolate the population of cells with the lowest level of β4 expression (Fig. 1)
- Implicated the β4 integrin in tumor formation in an orthotopic mouse model (Table 1)
- Developed protocols for soft agar and three dimensional matrix assays for breast carcinoma cell lines routinely used in our laboratory as tools to predict *in vivo* cell behavior (Fig. 2B and Fig. 3)
- Demonstrated that the α6β4 integrin influences the invasion of breast carcinoma cells independent of the Met growth factor receptor {Chung et al., APPENDIX}
- Substantiated the importance of the α6β4 integrin in invasion and migration of breast carcinoma cells using RNA interference in *in vitro* assays {Lipscomb et al., APPENDIX}
- Identified NP1 as a regulator of chemotaxis in breast carcinoma cells {Bachelder et al., APPENDIX}

REPORTABLE OUTCOMES:

Publications:

E.A. Lipscomb, K.J. Simpson, S. Lyle, J.E. Ring, A.S. Dugan, and A.M. Mercurio. The α6β4 integrin promotes tumor formation *in vivo* by regulating the expression of vascular endothelial growth factor and tumor cell survival. *(In Preparation 6/04)*

J. Chung, S.O. Yoon, **E.A. Lipscomb**, and A.M. Mercurio. The Met receptor and the α6β4 integrin can function independently to promote carcinoma invasion. *(In Press 6/04, J. Biol. Chem.)*

E.A. Lipscomb, A.S. Dugan, I. Rabinovitz, and A.M. Mercurio. Use of RNA interference to inhibit integrin (α6β4)-mediated invasion and migration of breast carcinoma cells. *Clin. Exp. Met.* 20(6), 569-76, 2003.

R.E. Bachelder, **E.A. Lipscomb**, X. Lin, M.A. Wendt, N.H. Chadborn, B.J. Eickholt, and A.M. Mercurio. Autocrine regulation of carcinoma migration: A novel Semaphorin3A/Neuropilin-1 inhibitory pathway is suppressed by VEGF. *Cancer Res.* 63 (17), 5230-3, 2003.

Presentations: (November 2003)

"The α6β4 integrin and tumor formation in mice". Division of Cancer Biology and Angiogenesis Data Club, Beth Israel Deaconess Medical Center, Boston, MA 02215.

CONCLUSIONS:

The goal of this proposal is to define the mechanism by which NP1 contributes to the survival and metastasis of breast carcinoma cells. To date, we have determined that the level of NP1 does not increase in human tumors as a function of breast cancer progression (Aim 1). We have also shown that the cytoplasmic domain of NP1 is not required for its survival function and more recently, we have shown that this domain of NP1 is also not essential for its role as a chemotaxis inhibitor (Aim 3). To evaluate the importance of NP1 in tumor formation and metastasis, we attempted to implement RNA interference to reduce NP1 expression in two different breast carcinoma cell lines (Aim 2). Unfortunately, this approach has thus far been unsuccessful likely due to the importance of NP1 expression to the survival of these cells. Future studies will involve development of a system for inducible expression of siRNA oligonucleotides that will enable us to address the importance of NP1 in metastasis following tumor formation.

Importantly, the models and methodologies initiated to study NP1 function *in vivo* have already proven to be invaluable for studying other aspects of breast cancer progression. In particular, we have demonstrated that the SUM-159 cell line is a useful model for studying tumor formation and metastasis following injection in the mammary fat pad. Using this model system in conjunction with RNA interference, we have implicated the β4 integrin in tumor formation. Furthermore, we have begun to address the mechanism of the β4 integrin in tumor progression by using soft agar and three-dimensional matrix assays. These assays will also enable us in future studies to explore the importance of NP1 in tumorigenesis and metastasis once a cell line for inducible expression of NP1 siRNA oligonucleotides is generated and characterized.

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4. A. M. Mercurio et al., *J.Mammary.Gland.Biol.Neoplasia.* 6, 299-309 (2001).
5. R. E. Bachelder et al., *Cancer Res.* 63, 5230-5233 (2003).

APPENDICES:

J. Chung, S.O. Yoon, **E.A. Lipscomb**, and A.M. Mercurio. The Met receptor and the α6β4 integrin can function independently to promote carcinoma invasion. *(In Press 6/04 J. Biol. Chem.)*

E.A. Lipscomb, A.S. Dugan, I. Rabinovitz, and A.M. Mercurio. Use of RNA interference to inhibit integrin (α6β4)-mediated invasion and migration of breast carcinoma cells. *Clin. Exp. Met.* 20(6), 569-76, 2003.

R.E. Bachelder, **E.A. Lipscomb**, X. Lin, M.A. Wendt, N.H. Chadborn, B.J. Eickholt, and A.M. Mercurio. Autocrine regulation of carcinoma migration: A novel Semaphorin3A/Neuropilin-1 inhibitory pathway is suppressed by VEGF. *Cancer Res.* 63(17), 5230-3, 2003.

The Met Receptor and α6β4 Integrin Can function Independently to Promote Carcinoma Invasion

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Summary

It has been proposed that a constitutive, physical association of the Met receptor and the α6β4 integrin exists on the surface of invasive carcinoma cells and that HGF-mediated invasion is dependent on α6β4 (1). The potential impact and significance of these results prompted us to re-examine this hypothesis. Using three different carcinoma cell lines that express both Met and α6β4, we were unable to detect the constitutive association of these receptors by co-immunoprecipitation. Moreover, carcinoma cells that lack expression of α6β4 exhibited Met-dependent invasion towards HGF and increasing Met expression by viral infection of these cells enhanced invasion without inducing α6β4 expression. Although expression of α6β4 in such cells enhanced their invasion to HGF, it also enhanced their ability to invade towards other chemoattractants such as LPA and this latter invasion was not inhibited by a function blocking Met antibody. Finally, depletion of β4 by RNAi in invasive carcinoma cells that express both receptors reduced the ability of these cells to invade towards HGF by approximately 25% but it did not abrogate their invasion. These data argue that the invasive function of Met can be independent of α6β4 and that α6β4 has a generic influence on the invasion of carcinoma cells that is not specific to Met.

Introduction

Understanding the receptor-mediated mechanisms that underlie invasive carcinoma is a timely and significant endeavor. The involvement of specific integrins and growth factor receptors in the invasive process is established and several lines of evidence indicate that these two classes of surface receptors may cooperate to effect a wide-range of biological functions, including the migration and invasion of tumor cells (2-4). The available data indicate that integrin and growth factor signaling can be synergistic and, in some cases, physical association may occur between these receptor types. Insight into the nature of such receptor interactions has important implications not only for understanding the biology of tumor invasion but also for the design and use of therapeutics targeted to these receptors (5).

An integrin of particular relevance to invasive carcinoma is $\alpha 6\beta 4$ (6) (7) (8). This integrin, which is expressed primarily on the basal surface of most epithelia and in most carcinoma cells, is defined as an adhesion receptor for most of the known basement membrane laminins (6) (9). Indeed, a primary function of $\alpha 6\beta 4$, revealed by studies on knockout mice, is to maintain the integrity of epithelia (10,11). This critical role for $\alpha 6\beta 4$ derives from its ability to mediate the formation of stable adhesive structures termed hemidesmosomes on the basal cell surface that link the cytokeratin network with laminins in the basement membrane (12). Recent studies have revealed novel and important functions for this integrin in the migration and invasion of carcinoma cells (13). The expression of $\alpha 6\beta 4$ is maintained or often increased in invasive and metastatic carcinomas and its expression level correlates with the progression of these carcinomas (14). More recently, compelling data were reported that the $\alpha 6\beta 4$ integrin is essential for squamous carcinogenesis (15).

Given the potential importance of the α6β4 integrin to invasive carcinoma, extensive efforts are being made to define the mechanisms by which it facilitates the invasive process. Advances include the observation that α6β4 is localized to the leading edge of migrating carcinoma cells where it can contribute to the formation and stabilization of actin protrusions (16) (17). In addition, there is evidence from several labs that α6β4 stimulates the activity of phosphoinositide 3-OH kinase (PI3-K) in invasive carcinoma cells and that PI3-K is essential for migration and invasion (7,18). Interestingly, it has been suggested that α6β4 activates PI-3K and mediates invasion through its ability to cooperate with specific growth factor receptors (1,4,18). For example, α6β4 has been shown to associate with erbB2 on the surface of breast carcinoma cells and this interaction appears to facilitate activation of PI3-K and invasion (18,19).

More recently, it was argued that α6β4 functions as an essential 'adaptor' protein for the Met receptor in invasive carcinoma cells (1). The impact of this finding is amplified by the fact that substantial evidence exists for the importance of Met in the scattering, invasion and metastasis of tumor cells (20,21). If α6β4 were an essential, specific adaptor for Met function in these events, the consequences for carcinoma biology and therapy would be profound. The potential impact and significance of these results prompted us to re-examine the central findings of this study, i.e., a selective physical association of Met and α6β4 exists on the surface of invasive carcinoma cells and Met cannot promote invasion in the absence of α6β4 expression.

Experimental Procedures

Cells: MDA-MB-231 and MDA-MB-435 breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University, and A431 cells were purchased from ATCC. Cells were grown in low glucose DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 25 mM Hepes. The generation of stable transfectants of MDA-MB-435 cells that express the $\alpha 6\beta 4$ integrin has been described previously (7). For Met expression studies, a VSV-coated retrovirus containing Met cDNA was obtained from Dr. Morag Park (McGill University; Montreal). SUM-159 cells were obtained from Dr.Stephen Ethier at the University of Michigan Cancer Center and maintained in Hams F12 containing 5% FBS, 5 μg/ml insulin, 1mg/ml hydrocortisol, 1% penicillin-streptomycin, and 25 mM Hepes (22).

To create β4 si-RNA-pSUPER and β4 SCR -pSUPER expression vectors, the following oligonucleotides (Invitrogen; Grand Island, NY) were annealed and ligated into pSUPER (a gift from R. Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands) between the BglII and HindIII sites: β4 si-RNA, 5'gatccccGAGCTGCACGGAGTGTGTCttcaagagaGACACACTCCGTGCAGCTCtttttggaaa 3' and 5'agcttttccaaaaaGAGCTGCACGGAGTGTGTCtctcttgaaGACACACTCCGTGCAGCTCggg 3'; β4 SCR, 5'gatccccGTTGTCGAGGAACGGTGCCttcaagagaGGCACCGTTCCTCGACAACttttttggaaa 3' and 5'agcttttccaaaaaGTTGTCGAGGAACGGTGCCtctcttgaaGGCACCGTTCCTCGACAACggg 3'. EcoRI- and XhoI- digested inserts containing the H1-RNA promoter and either β4 si-

RNA or β4 SCR targeting oligonucloetides from pSUPER were then subcloned into pSUPER.retro (Oligoengine; Seattle, WA) to generate β4 si-RNA and β4 SCR –pSUPER.retro. All plasmids were sequenced to confirm that they were correct.

To generate retroviruses, β4 si-RNA or β4 SCR –pSUPER.retro and expression plasmids containing envelope and packaging proteins required for viral propagation (Imgenex; San Diego, CA) were tranfected into 293T cells (3×10^6 cells per 100-mm plate) with Lipofectamine (Invitorgen; Carlsbad, CA) as described by the manufacturer. Three days following transfection, viral supernatants were harvested and SUM-159 recipient cells were infected in the presence of serum-containing media supplemented with 8 μg/ml polybrene (Sigma; St. Louis, MO). Following infection for 24 hours, resistant cells were selected with 4.0 μg/ml puromycin (Clontech; Palo Alto, CA) and cell lines that stably expressed β4 si-RNA or β4 SCR were generated. The stable β4 si-RNA SUM-159 cell line was then sorted by flow cytometry and the population of cells that expressed the lowest level of β4 was isolated. The sorting and subsequent surface labeling analysis of the β4-deficient cell line was performed with 3E1, a mouse anti-β4 integrin antibody (Chemicon; Temecula, CA). The decreased level of β4 expression in this cell line was further confirmed by western blotting with a rabbit polyclonal anti-β4 integrin antibody (505) (17).

Biochemical Analyses: For the co-immunoprecipitation studies, cells were extracted for 15 minutes at 4°C with a buffer containing 50 mM HEPES (pH 7.4), 5 mM EDTA, 2 mM EGTA, 150 mM NaCl, 10% glycerol, and 1% NP40 in the presence of protease and phosphatase inhibitors (2 mM phenylmethylsulfonyl fluoride, 5 mg/ml each of aprotinin, leupeptin, pepstatin, sodium fluoride, and sodium orthovandate). Extracts were clarified by

centrifugation at 13,000 rpm for 10 minutes, the supernatants were collected, and their protein concentration was determined using the Bio-Rad DC protein assay kit (Hercules, CA). Immunoprecipitations were performed with equal amounts of total protein. Extracts were pre-absorbed overnight using protein A and G-Sepharose beads (Amersham; Piscataway, NJ) to prevent non-specific binding. After centrifugation at 2000 rpm for 5 min to pellet these beads, the supernatants were incubated with 1μg of the following antibodies overnight at 4°C: a rat β4 mAb, 439-9B (obtained from Rita Falcioni, Regina Elena Cancer Institute, Rome, Italy); a polyclonal anti-human Met, C-12 (Santa Cruz, CA); a mouse β4 mAb, 3E1 (Chemicon; Temecula, CA); a mouse Met mAb, DO-24 (Upstate Biotechnology; Lake Placid, MA) as well as the appropriate rat, rabbit and mouse IgGs (Sigma). Immune complexes were precipitated with protein A and G-Sepharose, washed four times with extraction buffer, and eluted in 1X reducing sample buffer.

For immunoblotting, cell extracts were prepared as described above. These extracts or the immune complexes were separated by SDS-PAGE and transferred to Hybond-C nitrocellulose membranes (Amersham). Membranes were blocked for 1 hour using a 50 mM Tris buffer (pH 7.5) containing 0.15 M NaCl, 0.05% Tween-20 (TBS-T) and 5% Carnation dry milk. The membranes were incubated overnight in the same buffer containing antibodies specific for either the β4 integrin (505), anti-actin (Sigma; St.Louis, MO), Met (C12; Santa Cruz, CA) or (DL-21; Upstate Biotechnology; Lake Placid, MA). After three, ten-minute washes in TBS-T, the membranes were incubated for one hour in blocking buffer containing HRP-conjugated secondary antobodies. After three ten-minute washes in TBS-T, proteins were detected by enhanced chemiluminescence (Pierce; Rockford, IL).

For immunoblots involving the phospho-tyrosine Ab (anti-phosphotyrosine, clone PY99, Cell Signaling Technology; Beverly, MA), the membranes were blocked for 1 hour using a 10 mM Tris buffer (pH 7.5) containing 0.5 M NaCl, 0.1% Tween-20 and 2% BSA (w/v). The membranes were washed briefly and then incubated overnight at 4°C in the same blocking buffer containing the phospho-tyrosine Ab. After washing, the filters were incubated for 1 hour in blocking buffer containing HRP-conjugated secondary Ab and the proteins were detected by enhanced chemiluminescence.

Invasion assays: To prepare the Transwell membranes (Corning Incorporation; Corning, NY) for the invasion assays, 0.5 μg of Matrigel (Collaborative Research; Bedford, MA) was diluted with cold water and dried onto the upper side of the membranes overnight at 25°C. On the following day, the lower sides of the membranes were coated with 10 μg/ml laminin-1 (Calbiochem) overnight at 4°C. The membranes were blocked with DMEM (or Ham's F12 for SUM159) for 1 hour at 37°C. Cells were trypsinized on the following day and re-suspended in DMEM or Ham's F12 containing 0.25% heat inactivated lipid-free BSA (HI-BSA), and a total of 5×10^4 cells was added to the upper chamber of each well. Chemoattractants [HGF (50 ng/ml) (R & D system; Minneapolis, MN), 100 ng/ml LPA (Sigma) or 3T3 conditioned medium] were added to the bottom wells in DMEM-BSA. In some assays, cells were pre-incubated with a function-blocking Met mAb, clone 95309 (R & D systems, Inc.) for 1 hour at 4°C before plating. After incubating for 2 or 4 hours at 37°C, non-migrating cells were mechanically removed with a cotton swab from the upper chamber. Cells that had migrated to the lower side of the Transwell were fixed with 100% methanol and stained with 0.2% crystal violet in 2% ethanol. Invasion was quantified by counting the number cells per square millimeter using a reticule with bright-field optics.

Results

Lack of evidence for constitutive association between Met and α6β4 integrin in invasive carcinoma cells. Evidence to support a physical association between Met and the α6β4 integrin has been shown in co-immunoprecipitation experiments using COS cells transfected to express high levels of both Met and α6β4, as well as in carcinoma cells that express both receptors endogenously (1). In particular, it was reported that β4 immunoprecipitates of A431 cells contain significant amounts of Met. To confirm these findings, we performed co-immunopreciptation assays using A431 cells, as well as MDA-MB-231 breast carcinoma cells, which express high levels of both α6β4 and Met. In addition, MDA-MB-435 cells that stably express the α6β4 integrin were examined because they had been used previously to demonstrate the necessity of α6β4 for HGF-dependent invasion (1). To assess a putative physical association of these two receptors, cells were extracted with the same NP-40 (1%) buffer used previously (1), and extracts were 'pre-cleared' and then immunoprecipitated with Abs specific for either the β4 integrin subunit (439-9B) or Met (C12). Subsequently, the precipitates were analyzed by immunoblotting with either a β4 specific polyclonal Ab (505) or a Met Ab (C12). As shown in Fig. 1A, the β4 integrin subunit was evident in the β4 immunoprecipitates and Met was evident in the Met immunoprecipitates. Of note, however, there was no indication of Met in the β4 immunoprecipitates, or of β4 in the Met immunoprecipitates. To exclude the possibility that the co-immunoprecipitation of these two receptors is dependent on the detergent used for extraction, we also used a Triton-X 100 (1%) based buffer but failed to detect evidence for their physical association (data not shown). In addition, other Abs were used for the co-

immunoprecipitation studies in A431 cells. Two different Met Abs (C-12 and DO-24) immunoprecipitated Met effectively, but not the β4 integrin subunit (Fig. 1B). Similarly, β4 integrin subunit Abs (439-9B and 3E1) immunoprecipitated the β4 integrin subunit but not Met (Fig. 1B).

Met can function independently of the α6β4 integrin in MDA-MB-435 cells. To evaluate the hypothesis that the invasive function of Met is dependent on the α6β4 integrin, we used MDA-MB-435 cells that express Met but not α6β4 (Fig. 2). In addition, the level of Met expression was increased in these cells by retroviral infection with a Met cDNA. This infection increased Met expression by approximately two-fold (Fig. 2A), but it did not induce expression of α6β4 (Fig. 2B). The ability of Met to signal in the absence of α6β4 expression was examined. In the control cells, HGF stimulation induced a rapid and marked increase in tyrosine phosphorylation of Met as assessed by immunoprecipitating Met followed by immunblotting these immunoprecipitates with a phospho-tyrosine specific Ab (Fig. 2C). The intensity of this phosphotyrosine signal was increased in the Met infectants, in agreement with the fact that these cells express significantly more Met than do the control infectants (Fig. 2A).

Subsequently, we assessed the ability of the control and Met infectants to invade towards HGF. Invasion assays were performed for 4 hours without prior serum deprivation. As shown in Fig. 2D, HGF induced the invasion of the control cells significantly and this invasion was abrogated by a function blocking Met-specific antibody. A two-fold induction of HGF-dependent invasion was observed in the Met infectants compared to control cells substantiating the conclusion that Met promotes invasion in the absence of α6β4 integrin (Fig 2D).

The α6β4 integrin has a generic influence on carcinoma invasion that is not specific to Met. A key finding in the previous study, which concluded that α6β4 is necessary for the invasive function of Met, was that expression of α6β4 in MDA-MB-435 cells induced their ability to invade towards HGF (1). Although our results confirm the observation that expression of the α6β4 integrin increases the invasion of these cells towards HGF, we observed that the mock transfectants, which lack α6β4, exhibited significant migration towards HGF (Fig. 3A). Moreover, the level of invasion induced by expression of α6β4 is comparable to the increased invasion that results from increased Met expression and that this latter mode of invasion occurs in the absence of α6β4 (Fig. 2D). Furthermore, expression of α6β4 in MDA-MB-435 cells also enhanced their ability to invade towards other chemoattractants such as LPA and this invasion is not inhibited by a function blocking Met antibody (Fig 3B). This latter result indicates that the expression of α6β4 can enhance the ability of MDA-MB-435 cells to invade independently of Met expression. Invasion assays were performed for 4 hours in serum-free medium and, under these conditions, no significant increase in apoptosis was observed for any cell population as assessed by annexin-V-FITC staining (data not shown).

To assess the putative functional dependence of Met on the α6β4 integrin from a different perspective, we used SUM-159 cells (22). These invasive breast carcinoma cells express both α6β4 and Met (Fig. 4A). Using this cell line, we generated a SUM-159 cell line deficient in β4 integrin expression using siRNA strategies (23). Stable infectants that exhibited a reduction in β4 expression were sorted by FACS using a β4 -specific antibody and a population of cells was isolated that exhibited no detectable β4 expression as

evidenced by immunoblotting (Fig. 4A). Of note, loss of β4 expression had no effect on Met expression in these cells (Fig. 4A). SUM-159 cells exhibit a robust invasion towards HGF (Fig. 4B). Loss of α6β4 expression reduced the ability of these cells to invade toward HGF by approximately 25% but it did not abrogate their invasion (Fig. 4B). Moreover, loss of α6β4 expression also diminished the invasion of SUM-159 cells towards 3T3 cell conditioned medium by approximately 30%.

Discussion

The report that the invasive function of the Met receptor is dependent upon a physical association with the α6β4 integrin, which provides a 'signaling adapter function' afforded a compelling model for invasive carcinoma that linked these two receptors (1). The data obtained in our study, unfortunately, do not support the central tenets of this model. Rather, they argue that the invasive function of Met can be independent of α6β4 and that α6β4 has a generic influence on the invasion of carcinoma cells that is not specific for HGF-dependent invasion.

The demonstration of a physical association between an integrin and a growth factor receptor provides *prima facie* evidence for cooperativeness of function. For this reason, the previous finding that Met and α6β4 could be co-immunoprecipitated from GTL-16 cells, which over-express a constitutively active form of Met, from COS cells engineered to express both receptors at high levels and from A431 cells, which also express both receptors, strengthened the possibility of a functional dependency (1). Using three different carcinoma cell lines that express both Met and α6β4 (A431, MDA-MB-231 and MDA-MB-435 engineered to express α6β4), however, we were unable to detect any evidence for the constitutive association of these receptors by co-immunoprecipitation. The reason for the difference in our results and those of Trusilino et al. (1) is unclear. It is worth noting, however, that in the previous study co-immunoprecipitation data were not provided for either MDA-MB-231 cells or MDA-MB-435/β4 cells, and an association between Met and α6β4 in A431 cells was detected only by immunoprecipitation with a β4 integrin antibody and immunoblotting with a Met Ab and not *vice versa.* In addition, the use of another, purified β4 integrin Ab (439-9B) failed to co-immunoprecipitate Met. Although

our data refute the existence of a constitutive association of Met with α6β4 in carcinoma cells, they do not exclude the occurrence of a transient association between these two receptors in certain physiological situations or that a spurious association may occur upon their gross over-expression.

The lack of evidence for a physical association between α6β4 and Met does not negate the possibility that they exhibit functional cooperativeness. To evaluate the hypothesis that the invasive function of Met depends upon α6β4, we assessed the invasion of MDA-MB-435 cells, which express Met but not α6β4. These cells exhibited significant invasion towards HGF and their rate of invasion increased in response to increasing Met expression by retroviral infection. The fact that these cells are capable of significant HGF-dependent invasion in the absence of α6β4 expression argues against the necessity of this integrin for Met function. Moreover, a key finding in the previous study was that expression of α6β4 in MDA-MB-435 cells induced their ability to invade towards HGF but not towards EGF. We note, however, EGF is not a suitable negative control because MDA-MB-435 cells lack expression of the EGF receptor (25) and R. Bachelder, personal communication). Additional support for the hypothesis that the invasive function of Met can occur independently of α6β4 is provided by our data on SUM-159 breast carcinoma cells. These invasive cells express both Met and α6β4, and they exhibit a robust invasion towards HGF. However, our finding that elimination of α6β4 expression using a β4 specific siRNA reduced but did not abrogate invasion towards HGF argues against the conclusion that α6β4 is an essential adaptor for Met in promoting carcinoma invasion. The strength of the SUM-159 data, in contrast to MDA-MB-435 cells, is that this

cell line exhibits endogenous expression of both receptors and the assumption can made that if Met function were dependent on $\alpha 6\beta 4$, it should be evident in such a cell line.

Based on several studies, as well as the findings reported here, a consensus is emerging that $\alpha 6\beta 4$ cooperates with growth factor receptors to promote carcinoma invasion and other functions (7,18,26). Perhaps, the most conclusive evidence in this regard is the finding that macrophage-stimulating protein upon binding to its receptor, the Ron tyrosine kinase, promotes an association between Ron and $\alpha 6\beta 4$ that results in PI3-K activation and consequent migration (4). There is also evidence that $\alpha 6\beta 4$ can cooperate with erbB2 in breast carcinoma cells to activate PI3-K and promote invasion (18). However, the conclusion that the function of one specific growth factor receptor (Met) is absolutely dependent on $\alpha 6\beta 4$ for promoting invasion is not supported by our data. A more appropriate assessment of the relationship between Met and $\alpha 6\beta 4$ would be that expression of $\alpha 6\beta 4$ can enhance invasion towards several growth factors including HGF, LPA, as well as those present in 3T3 conditioned medium (Figs 3 and 4). At the same time, our results indicate that the ability of Met to promote invasion is not dependent on $\alpha 6\beta 4$ in these cells and that increasing Met expression in the absence of $\alpha 6\beta 4$ enhances HGF-mediated invasion.

The mechanism that underlies the ability of $\alpha 6\beta 4$ to promote invasion likely involves its ability to stimulate PI3-K. Compelling evidence exists for $\alpha 6\beta 4$-mediated activation of this enzyme by mechanisms that include phosphorylation of IRS proteins (27) cooperation with erbB2 (18,28) and Ron (4), and the elaboration of VEGF autocrine signaling (24). In addition, the regulated expression of specific transcription factors such as

NFAT by $\alpha 6\beta 4$ may contribute to the invasive phenotype (29). Clearly, Met is one of several growth factor receptors whose function may be enhanced by $\alpha 6\beta 4$ expression but that can signal and promote invasion in the absence of $\alpha 6\beta 4$ expression. The challenge ahead is to define the mechanisms by which expression of $\alpha 6\beta 4$ enhances the function of multiple growth factor receptors.

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Figure Legends

Figure 1. Lack of evidence for constitutive association between Met and the α6β4 integrin. (**A**) Extracts from the indicated cell lines were immunoprecipitated with antibodies against Met (clone C12), β4 integrin (clone 439-9B) or the indicated IgG control (rb IgG: rabbit IgG and rIgG: rat IgG). Immunoblot analysis of these immunoprecipitates was performed using anti-Met (clone C-12) and anti-β4 integrin polyclonal (505) antibodies. (**B**) Extracts from A431 cells were immunoprecipitated with β4 integrin specific antibodies: mouse monoclonal clone 3E1, rat monoclonal clone 439-9B), Met specific antibodies: mouse monoclonal clone DO-24, rabbit polyclonal clone C-12) or IgG controls (mIgG: mouse IgG and rIgG: rat IgG), and the immunoprecipitates were analyzed by immunoblotting as described in A.

Figure 2. Met can function independently of the α6β4 integrin in MDA-MB-435 cells. (**A and B**) MDA-MB-435 mock and Met infectants, as well as A431 cells, were extracted and equal amounts of protein extracts were analyzed by immunoblot analysis using Met (clone C12), β4 (clone 505) and actin specific antibodies. (**C**) MDA-MB-435 mock and Met infectants were extracted at the designated time points upon HGF treatment and extracts were immunoprecipitated with anti-Met antibody (clone C12). Immunoblotting was done with either an anti-Met antibody (clone C12, upper panel) or an anti-pTyr antibody (clone p-Y-100, lower panel). (**D**) The ability of MDA-MB-435 mock and Met transfectants to invade Matrigel towards HGF was investigated in a 4 hour assay. Prior to these assays, cells were incubated for 30 minutes with either a control IgG (-) or a Met-specific antibody

(clone 95309). The mean number of invasive cells (+/- SD) from 5 independent fields per well is indicated on the y-axis. Similar data were obtained in 5 separate experiments.

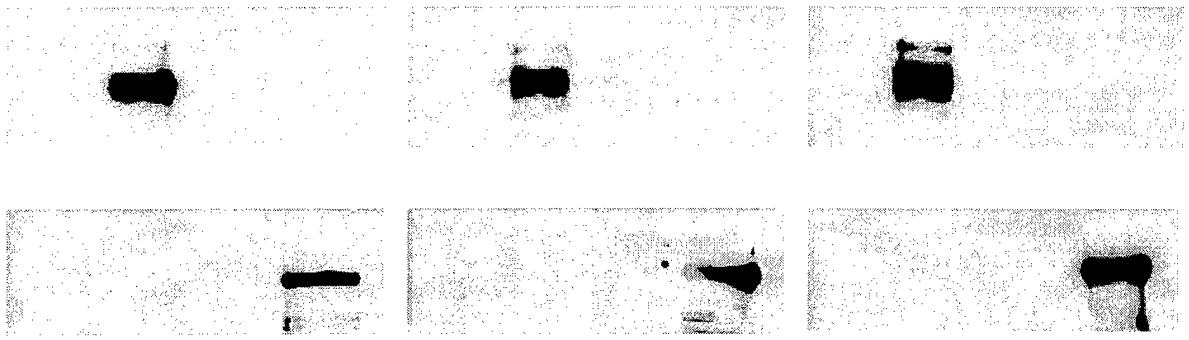
Figure 3. Expression of $\alpha 6\beta 4$ integrin enhances invasion towards multiple growth factors. The ability of MDA-MB-435 mock and β4 integrin transfectants to invade Matrigel towards HGF (25 ng/ml) **(A)** or LPA (100nM) **(B)** was investigated in a 4 hour assay. Prior to these assays, cells were incubated for 30 minutes with either a control IgG (-) or a Met-specific Ab (clone 95309). The mean number of invasive cells (+/- SD) from 5 independent fields per well is indicated on the y-axis. Similar data were obtained in 3 separate experiments.

Figure 4. Loss of $\alpha 6\beta 4$ integrin expression in SUM-159 cells reduces but does not abrogate invasion towards HGF and 3T3 conditioned medium. (A) SUM-159 cells stably expressing either scrambled (scr) or β4 integrin siRNA oligonucleotides were extracted and equal amounts of protein extracts were analyzed by immunoblot analysis using Met (C-12), β4 (505) and actin specific Abs. **(B)** The ability of the cells described in (A) to invade Matrigel towards HGF (50 ng/ml) or 3T3 conditioned medium was investigated in a 2 hour assay. The mean number of invasive cells (+/- SD) from the 5 independent field per well is indicated on the y-axis. Similar data were obtained in 3 separate experiments.

Figure 1

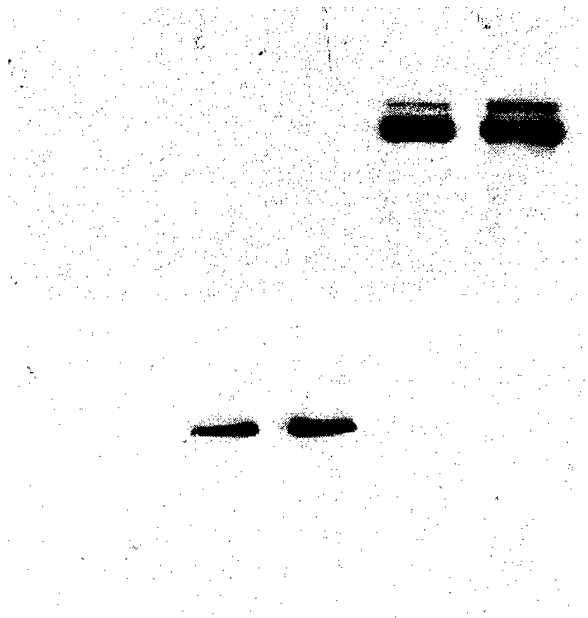
A.

Blot:



IP Ab	:	rbIgG	Met	rIgG	β4	rbIgG	Met	rIgG	β4	rbIgG	Met	rIgG	β4
Cell Line	:	β4 transfectant 435				231				A431			

B. **Blot:**



IP Ab	:	mIgG	rIgG	3E1	439-9B	DO24	C-12
Cell Line	:	A431					

Figure 2

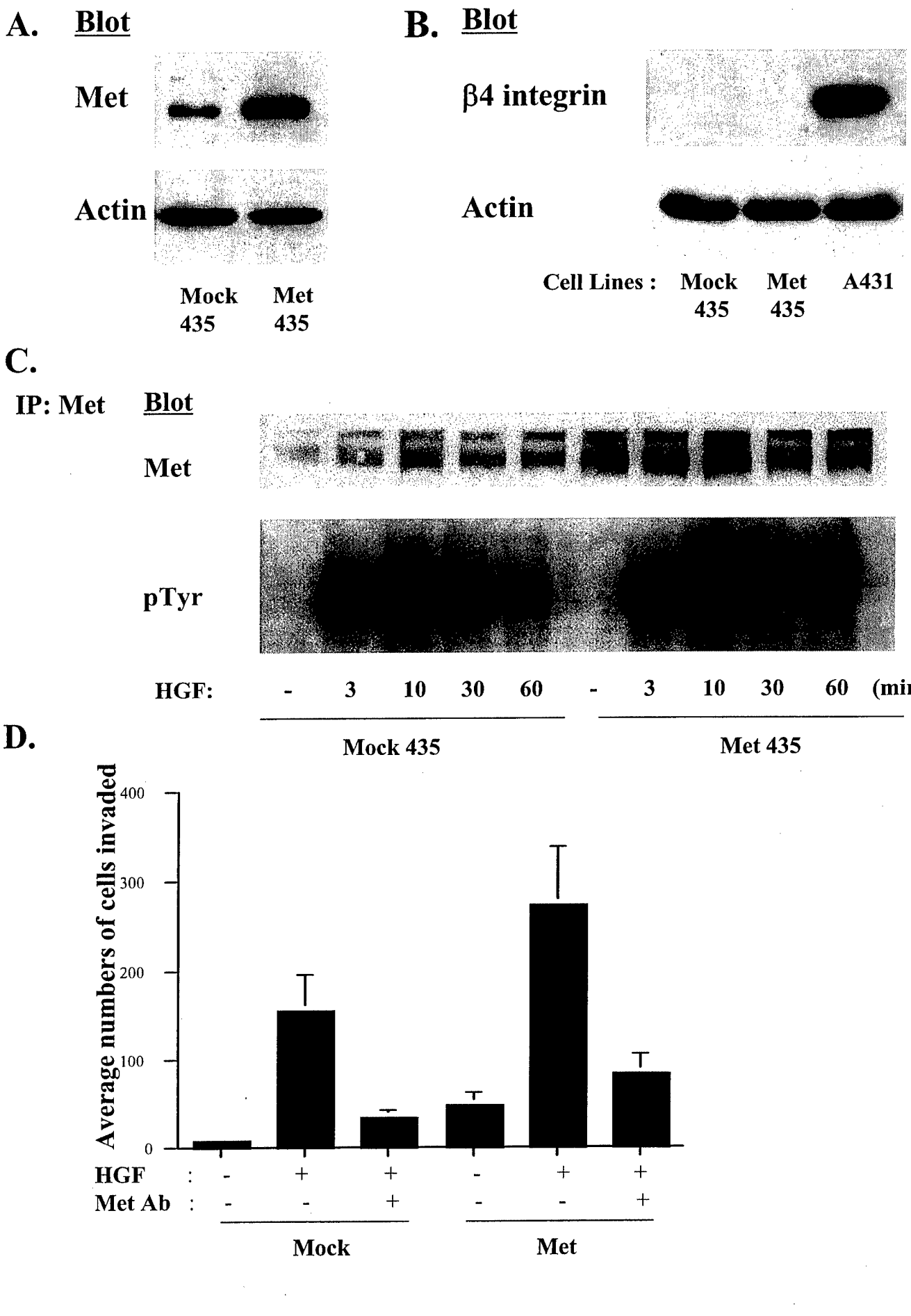
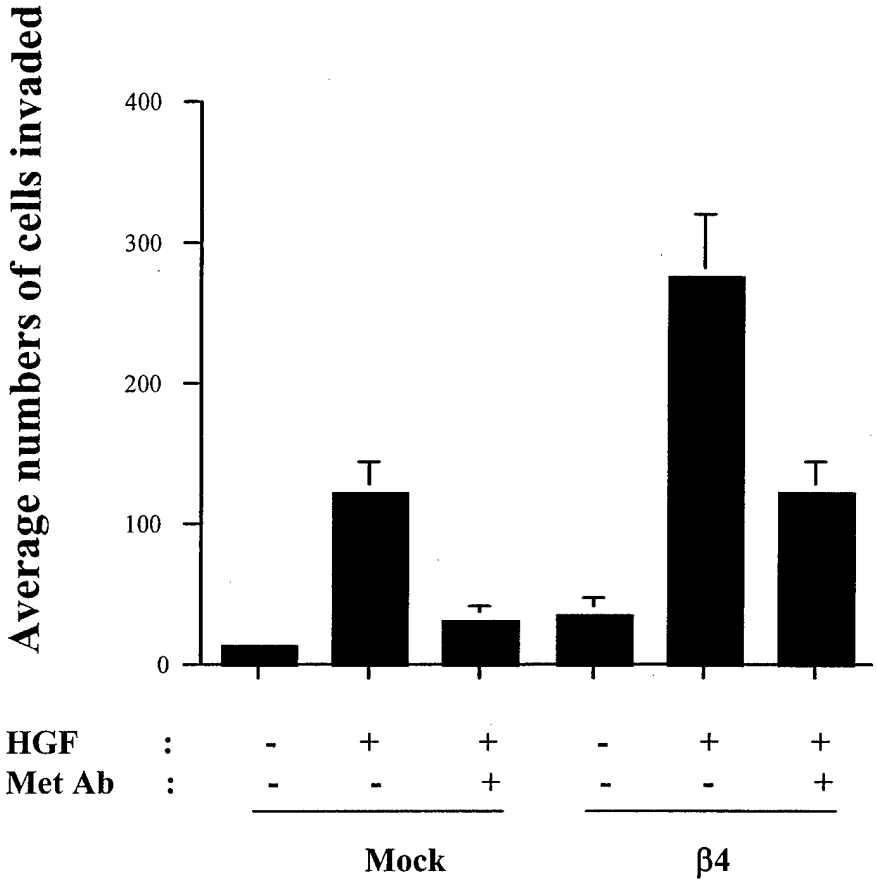

A.
Blot
Met
Actin
Mock 435
Met 435
B.
Blot
β4 integrin
Actin
Cell Lines : Mock 435
Met 435
A431
C.
IP: Met
Blot
Met
pTyr
HGF: - 3 10 30 60 - 3 10 30 60 (min)
Mock 435
Met 435
D.
Average numbers of cells invaded
400
300
200
100
0
HGF : - + + - + +
Met Ab : - - + - - +
Mock
Met

Figure 3

A.


Average numbers of cells invaded
400
300
200
100
0
HGF : - + + - + +
Met Ab : - - + - - +
Mock
β4

B.

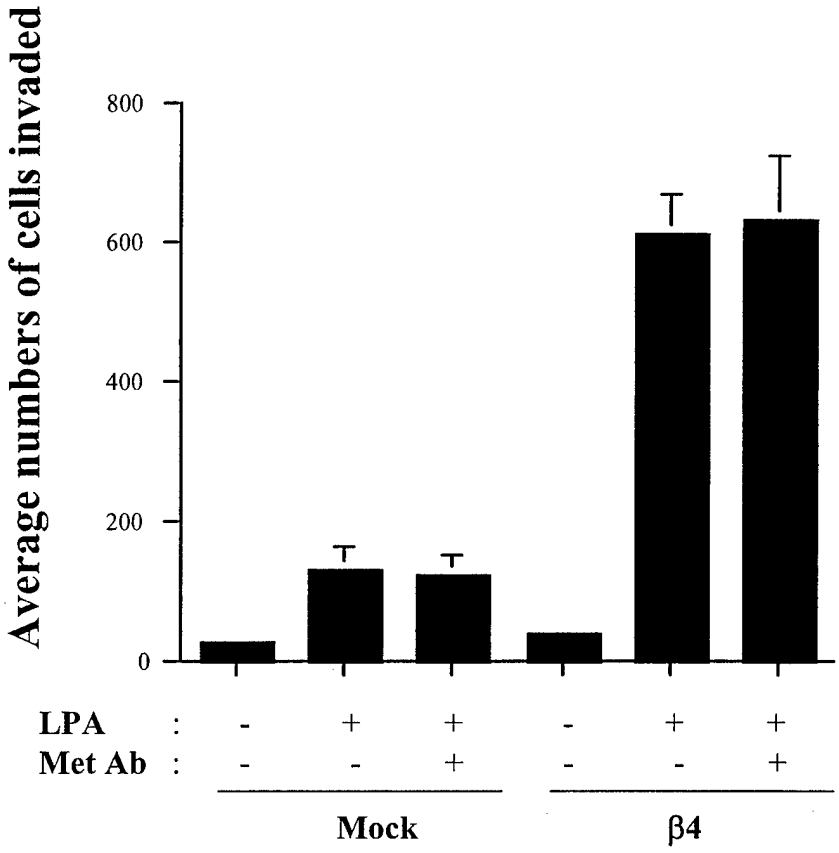
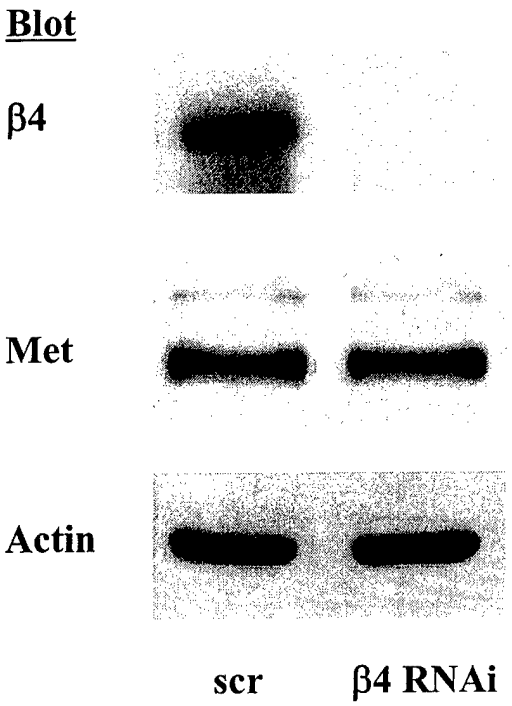
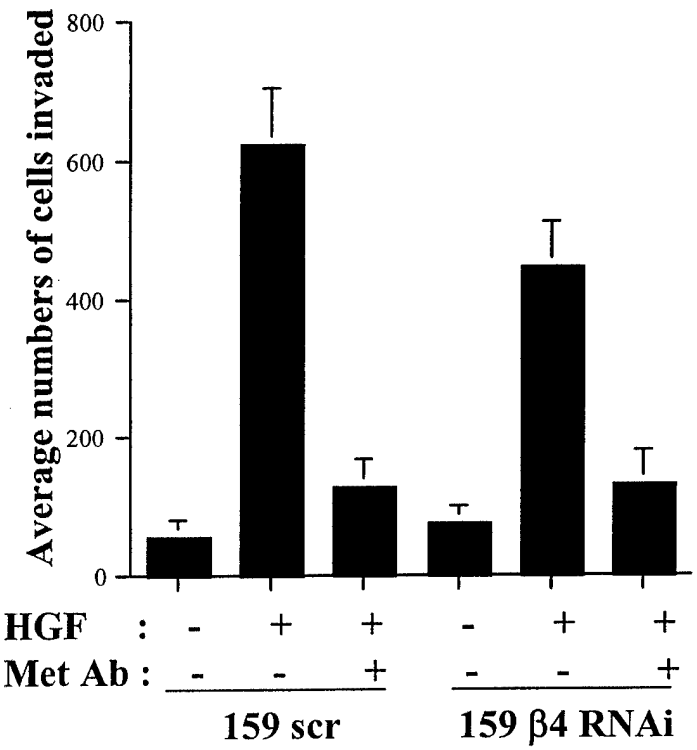

Average numbers of cells invaded
800
600
400
200
0
LPA : - + + - + +
Met Ab : - - + - - +
Mock
β4

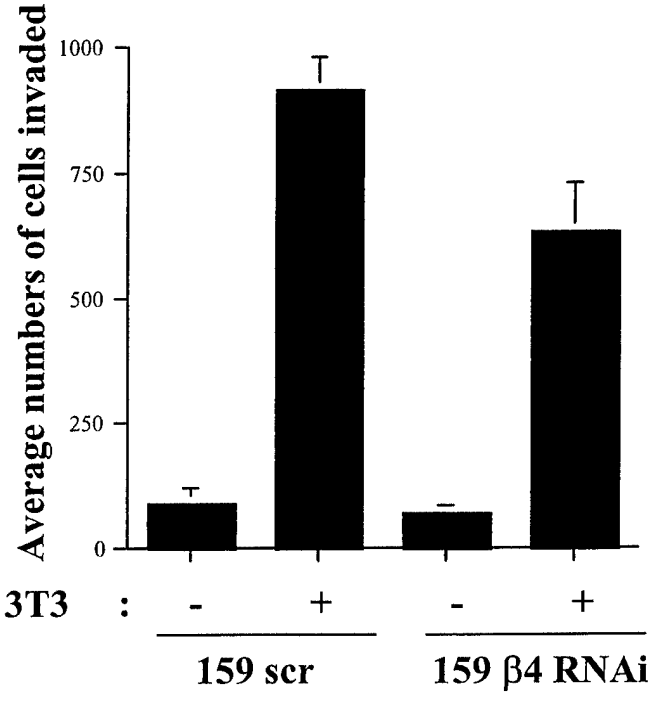
Figure 4

A.


Blot
β4
Met
Actin
scr
β4 RNAi

B.


Average numbers of cells invaded
800
600
400
200
0
HGF : - + + - + +
Met Ab : - - + - - +
159 scr
159 β4 RNAi


Average numbers of cells invaded
1000
750
500
250
0
3T3 : - + - +
159 scr
159 β4 RNAi

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Use of RNA interference to inhibit integrin ($\alpha6\beta4$)-mediated invasion and migration of breast carcinoma cells

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Key words: $\alpha6\beta4$, adhesion, integrin, invasion, ligand-independent, migration, small interfering RNA, RNA interference

Abstract

The application of small interfering RNA (siRNA) oligonucleotides to silence gene expression has profound implications for the intervention of human diseases including cancer. Using this technique, we explored the possibility that the $\alpha6\beta4$ integrin, a laminin adhesion receptor with a recognized role in the invasive phenotype of many carcinomas, represents a potential therapeutic target to inhibit the migration and invasion of carcinoma cells. We found that siRNA oligonucleotides targeted to either subunit of the $\alpha6\beta4$ integrin reduced cell surface expression of this integrin and resulted in decreased invasion of MDA-MB-231 breast carcinoma cells. Interestingly, reduced $\alpha6\beta4$ expression also promoted decreased migration on non-laminin substrata indicating that this integrin can function in a ligand-independent manner. In addition, the absence of $\beta4$ expression in these cells augmented the formation of $\alpha6\beta1$ heterodimers and increased adhesion to laminin-1. Taken together, these results substantiate the importance of the $\alpha6\beta4$ integrin in invasion and migration that has been demonstrated previously by expression of the $\beta4$ subunit in $\beta4$-deficient cell lines and by function blocking antibodies. Furthermore, these data suggest that the utilization of siRNA oligonucleotides to reduce the expression of the $\alpha6\beta4$ integrin may be a useful approach to prevent carcinoma cell progression.

Abbreviations: BSA – bovine serum albumin; DMEM – Dulbecco's modified Eagle's medium; IRS – insulin receptor substrate; LPA – lysophosphatidic acid; PBS – phosphate-buffered saline; PE – phycoerythrin; PI3-K – phosphatidylinositol 3-kinase; RIPA – radioimmune precipitation buffer; RNAi – RNA interference; siRNA – small interfering RNA; si-$\alpha6$ – siRNA oligonucleotides for $\alpha6$; si-$\beta4$ – siRNA oligonucleotides for $\beta4$; si-Inv – inverted-sequence oligonucleotides for $\beta4$; si-Scr – scrambled-sequence oligonucleotides for $\alpha6$; TBS – tris-buffered saline

Introduction

Double-stranded RNA triggers sequence-specific post-transcriptional gene silencing in a wide variety of organisms [1–3]. This naturally occurring process, referred to as RNA interference (RNAi), has recently been established as a powerful technique for reducing the expression of specific genes in mammalian systems. RNAi is induced in mammalian cells by introducing exogenous 21-nucleotide RNA duplexes or small interfering RNA (siRNA) oligonucleotides that are homologous to the desired gene [4–6]. Because RNAi induction offers great promise as a gene-specific therapeutic tool for a host of disease conditions, we investigated whether gene silencing by siRNA oligonucleotides could be a novel approach to inhibit the migration and invasion of carcinoma cells. As an initial step to addressing this question, we utilized siRNA oligonucleotides for the $\alpha6\beta4$ integrin, a receptor that has been implicated in the progression of many carcinomas [7].

The $\alpha6\beta4$ integrin is expressed primarily on the basal surface of most epithelia, and in a few other cell types [7, 8]. $\alpha6\beta4$ is defined as an adhesion receptor for most of the known laminins [9], although increasing evidence indicates that it can also signal independently of ligand binding [10, 11]. The $\beta4$ extracellular domain associates exclusively with the $\alpha6$ subunit to form $\alpha6\beta4$ complexes [12] whereas the $\alpha6$ subunit also associates with the $\beta1$ subunit to form $\alpha6\beta1$ heterodimers [9]. A primary function of $\alpha6\beta4$ is to maintain the integrity of epithelia through its ability to mediate the formation of stable and rigid structures termed hemidesmosomes on the basal surface that link the intermediate filament cytoskeleton with laminins in the basement membrane [13].

Although the involvement of $\alpha6\beta4$ in hemidesmosome organization and function has dominated the study of this integrin, the $\beta4$ subunit was initially identified as a tumor-related antigen associated with metastasis [14]. More recently, it has been demonstrated that the expression of $\alpha6\beta4$ is maintained or even increased in several types of invas-

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ive and metastatic carcinomas and that the $\alpha6\beta4$ expression level actually correlates with the progression of these carcinomas [7]. These correlative data have been substantiated by functional studies that have defined a pivotal role for $\alpha6\beta4$ in migration and invasion of carcinoma cells through its ability to interact with F-actin and activate key signaling pathways [15]. Furthermore, $\alpha6\beta4$ promotes survival in carcinoma cells lacking functional p53 protein [16]. The importance of $\alpha6\beta4$ in survival has recently been extended by the report that $\alpha6\beta4$ stimulates translation of vascular endothelial growth factor and that this pathway is a mechanism of survival in carcinoma cells [17].

The role of $\alpha6\beta4$ in migration and invasion was initially shown by exogenous expression of this integrin in $\beta4$-deficient colon and breast carcinoma cells [18, 19]. In breast carcinoma studies, $\alpha6\beta4$ promoted invasion in a phosphatidylinositol 3-kinase (PI3-K)-dependent manner [19]. Since these initial studies, $\alpha6\beta4$ has been shown to activate PI3-K and stimulate migration and invasion by both growth factor receptor-dependent and -independent signaling mechanisms. For instance, $\alpha6\beta4$ and ErbB-2, an orphan receptor of the epidermal growth factor family, associate in carcinoma cells [20] and this interaction stimulates PI3-K activity and invasion in a fibroblast model system [21]. An association of $\alpha6\beta4$ with the Met tyrosine kinase in carcinoma cells has also been demonstrated and $\alpha6\beta4$ was reported to be necessary for the invasive functions of Met by acting as an adapter protein to recruit PI3-K for enhanced Met signaling [11]. In a growth factor receptor-independent manner, the insulin receptor substrates (IRS-1 and IRS-2) act as signaling intermediates to link activated $\alpha6\beta4$ with PI3-K, a pathway that leads to an increase in carcinoma invasion [22]. In addition to stimulation of PI3-K signaling, the $\alpha6\beta4$ integrin has also been shown to promote migration by activation of the MAPK pathway [23, 24], the Rac and RhoA GTPases [19, 25], and the nuclear factor of activated T-cells family of transcription factors [26].

Although the data summarized above indicate that $\alpha6\beta4$ plays a pivotal function in the aggressive behavior of carcinoma cells, it is worth noting that this role was deduced from either expression of the $\beta4$ subunit into $\beta4$-negative cells or the use of antibodies to inhibit $\alpha6\beta4$ function. In the current study, we used the more definitive approach of RNAi to target the $\alpha6$ and $\beta4$ subunits of the $\alpha6\beta4$ integrin in breast carcinoma cells. Using this highly specific and efficient approach, we observed a significant reduction in the surface expression of this integrin that correlated with an inhibition of migration and invasion. Interestingly, the impact of reduced $\alpha6\beta4$ expression was apparent on non-laminin substrates, a finding that substantiates the ligand-independent function of $\alpha6\beta4$. These findings indicate that inhibition of integrin expression by siRNA oligonucleotides may be an effective approach to assess integrin function in carcinoma cells, as well as to impede tumor progression *in vivo*.

Materials and methods

Cells

The MDA-MB-231 human breast carcinoma cell line was obtained from the Lombardi Breast Cancer Depository at Georgetown University and maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Rockville, Maryland) supplemented with 10% fetal bovine serum (Sigma, St. Louis, Missouri), 100 U/ml penicillin, and 100 μg/ml streptomycin (each from Life Technologies).

siRNA transfections

Oligonucleotide sequences for $\beta4$ integrin were designated as si-$\beta4$ (GAGCUGCACGGAGUGUGUC) and as si-Inv (CUGUGUGAGGCACGUCGAG), an inverted control. $\alpha6$ integrin oligonucleotides included si-$\alpha6$ (GGUCGUGACAUGUGCUCAC) and a scrambled-sequence control, si-Scr (AUGCAGAGUGGCGCUCUCU). Oligonucleotides were synthesized by Dharmacon Research, Inc. (Lafayette, Colarodo). Cells ($0.5–2.0 \times 10^5$) were plated onto 35-mm tissue culture dishes 24 h prior to transfection with 200 nM of siRNA duplex using 25 μg of TransIT-TKO transfection reagent (Mirus, Madison, Wisconsin) in the presence of serum as described by the manufacturer. One day following transfection, the transfection medium was aspirated from the cells and replaced with fresh complete growth medium and incubated for an additional 48–72 h. For each transfection, either immunoblotting or flow cytometry was used to confirm reduced protein expression of the targeted gene.

Cell Surface Biotinylation and Immunoprecipitation

Following transfection for 3–4 days, MDA-MB-231 cells were washed two times each with phosphate-buffered saline (PBS) and HEPES buffer (20 mM HEPES, 130 mM NaCl, 5 mM KCl, 0.8 mM Mg Cl_2, 1.0 mM $CaCl_2$, pH 7.45). The cells were then incubated on ice with HEPES buffer containing EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, Illinois) at 0.5 mg/ml for 30 min. Each dish was washed three times with HEPES buffer and the cells were lysed in ice-cold radioimmune precipitation (RIPA) buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1% Nonidet-P40, 1% deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, leupeptin, and pepstatin) for 15 min at 4 °C. Cell lysates were clarified by centrifugation at $10,000 \times g$ for 10 min, the supernatants collected, and the total protein concentration of each lysate determined by the Bio-Rad DC protein assay (Hercules, California).

Immunoprecipitations following cell surface labeling were performed with equal amounts of total protein or 60 μg for $\alpha3$ and $\beta1$ integrin reactions and 125–150 μg for $\alpha6$ and $\beta4$ integrin samples. Lysates were preabsorbed with either rat IgG whole molecule agarose (Sigma) or, for mouse antibodies, protein G-Sepharose (Amersham, Piscataway, New

Jersey) and then incubated with 1 μg of an anti-integrin antibody overnight at 4 °C. The following antibodies were used for immunoprecipitation: 439-9B, rat anti-β4 integrin mAb (obtained from Rita Falcioni, Regina Elena Cancer Institute, Rome, Italy); GoH3, rat anti-α6 integrin mAb (Immunotech, Westbrook, Maine); MC13, mouse anti-β1 integrin mAb (obtained from Steve Akiyama, NIH, Research Triangle Park, North Carolina); P1B5, mouse anti-α3 integrin mAb (Life Technologies); as well as rat and mouse IgG (Sigma). Immune complexes were precipitated with rat IgG agarose or protein G-Sepharose, washed four times with RIPA buffer, and eluted in 1X reducing sample buffer (biotinylated α6 and β4 integrin immunoprecipitations) or 1X non-reducing sample buffer (biotinylated α3 and β1 integrin immunoprecipitations).

Immunoblotting

For preparing whole cell lysates, cells were rinsed twice with PBS and lysed in RIPA buffer as described above. Lysates and immune complexes were separated by SDS-PAGE and transferred to Hybond-C nitrocelluose membranes (Amersham). Membranes were incubated in block buffer consisting of Tris-buffered saline (TBS) containing 5% nonfat dry milk followed by antibody incubation in TBS containing 1% nonfat dry milk and 0.05% Tween-20. The blots were incubated with a 1:5000 dilution of rabbit polyclonal anti-β4-integrin (505) [15] or 0.5 μg/ml anti-β-actin (Sigma) followed by 0.04 μg/ml peroxidase-conjugated donkey anti-rabbit secondary antibody (Jackson Immnoresearch, West Grove, Pennsylvania). For biotinylation studies, the membranes were incubated in block buffer overnight and then labeled with 0.2 μg/ml peroxidase-conjugated streptavadin in block buffer containing 0.05% Tween-20 for 2 hours at 25 °C. All membranes were visualized by chemiluminescence (SuperSignal West Pico, Pierce). Densitometry was performed using IP Lab Spectrum (Webster, New York) computer software.

Apoptosis assay

Four days following transfection, cells were collected and their level of apoptosis was assessed using AnnexinV-phycoerythrin (PE) (Pharmingen, San Diego, California). Briefly, cells were washed one time each in 1X PBS and IX annexin buffer (10 mM Hepes-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and then incubated for 15 minutes at room temperature with 5 μg/ml AnnexinV-PE. Following incubation, cells were washed once with 1X annexin buffer and analyzed by flow cytometry.

Migration and invasion assays

For chemotaxis assays, the upper and lower surface of the membrane in each Transwell chamber (Costar, Cambridge, Massachusetts) were coated overnight at 4 °C with 15 μg/ml of collagen I (Vitrogen, Palo Alto, California). To prepare the Transwell membranes for invasion assays, 0.5 μg of Matrigel (Collaborative Research, Bedford, Massachusetts) was diluted with cold water and dried onto each filter overnight at 25 °C. For both chemotaxis and invasion assays, Transwell membranes were blocked on the following day with DMEM for 1 hour at 37 °C. Cells were trypsinized and resuspended in DMEM containing 0.25% heat-inactivated lipid free bovine serum albumin and a total of 1×10^5 cells was added to the upper chamber of each well. NIH3T3 conditioned medium or 100 nM lysophosphatidic acid (LPA) (Sigma) was added to the lower chamber of each well. After incubating for 1 hour (chemotaxis assays) or 4 hours (invasion assays) at 37 °C, nonmigrating cells were removed from the upper chamber with a cotton swab. Cells that had migrated to the lower surface of the membrane were fixed with 100% methanol and stained with 0.2% crystal violet in 2% ethanol. Migration was quantified by counting cells per square millimeter using bright-field optics.

Adhesion assays

For adhesion assays, 96-well tissue culture plates were coated overnight at 4 °C with 20 μg/ml laminin-1 (prepared from the EHS sarcoma provided by Dr Hynda Kleinman, NIDR, Bethesda, Maryland) or 1% BSA as a negative control. The wells were then washed 3 times with PBS and blocked for 10 min at 37 °C with 250 μg/ml heat-inactivated lipid free BSA in DMEM. Cells (3×10^4) were resuspended in DMEM and added to the protein-coated wells. After a 1 hour incubation at 37 °C, the wells were washed 3 times with PBS, fixed for 10 min with methanol, stained with a solution of 0.2% crystal violet in 2% ethanol, and washed 3 times with water. The crystal violet stain was solubilized with a 1% SDS solution and the adhesion was quantified by measuring the absorbance at 595 nm. To control for nonspecific adhesion, the mean absorbance (four wells per transfection condition) of the BSA-coated wells was subtracted from the mean absorbance of the laminin-coated wells in each experiment. The BSA-corrected adhesion obtained in the cells transfected with sequence-inverted β4 siRNA oligonucleotides was designated as equal to one and used to determine the fold induction of untransfected and β4 siRNA oligonucleotide transfected cells.

Results

siRNA oligonucleotides for β4 (si-β4) decrease $\alpha6\beta4$ cell surface expression

We used MDA-MB-231 cells to assess the potential usefulness of RNAi as an approach to inhibit $\alpha6\beta4$ function in carcinoma cells. These invasive and metastatic breast carcinoma cells express relatively high levels of $\alpha6\beta4$, as well as a small population of $\alpha6\beta1$ (L. Shaw, personal communication). The total level of endogenous β4 subunit was reduced by approximately 75% 4 days following transfection with si-β4 oligonucleotides compared to control inverted-sequence oligonucleotides (si-Inv) or untransfected (Unt) cells (Figure 1A). Reduced β4 expression was not detected earlier than 96 h (data not shown) indicating that siRNA oligonucleotides directed at β4, and possibly other membrane

spanning receptors, require several days to suppress protein expression effectively. These data demonstrate that si-$\beta4$ oligonucleotides reduce endogenous $\beta4$ expression in a breast carcinoma cell line.

To determine whether expression of the $\alpha6\beta4$ integrin was suppressed on the cell surface of MDA-MB-231 cells following transfection with si-$\beta4$ oligonucleotides, cells were biotinylated and immunoprecipitated for $\beta4$. $\beta4$ expression was decreased on the cell surface of si-$\beta4$ transfected cells by 70% compared to si-Inv cells and the amount of $\alpha6$ that co-immunoprecipitated with $\beta4$ in these cells was decreased by 66% (Figure 1B). These findings indicate that cell surface expression of $\alpha6\beta4$ is diminished in si-$\beta4$ transfected breast carcinoma cells.

We next investigated whether the surface expression of the $\alpha6$ subunit was altered in si-$\beta4$ transfected cells. Immunoprecipitation of biotin-labeled lysates revealed no significant difference in $\alpha6$ expression between si-$\beta4$ and si-Inv transfected cells (Figure 1C). Moreover, the surface expression of the $\alpha3$ and $\beta1$ integrin subunits was maintained in cells transfected with si-$\beta4$ oligonucleotides (Figure 1C). Isotype-control immunoprecipitations revealed the specificity of the immunoprecipitated protein for each integrin antibody (data not shown). Additional experiments using flow cytometry to identify cell surface integrin expression confirmed these results (data not shown). These data demonstrate that si-$\beta4$ oligonucleotides reduce the expression of $\alpha6\beta4$ on the cell surface of breast carcinoma cells.

si-$\beta4$ oligonucleotides decrease invasion and ligand-independent migration of MDA-MB-231 breast carcinoma cells

To address the functional consequence of reduced $\alpha6\beta4$ expression, we evaluated the ability of MDA-MB-231 cells to invade through Matrigel-coated Transwells following transfection with si-$\beta4$ oligonucleotides. The invasion of si-$\beta4$ transfected cells towards fibroblast conditioned medium was inhibited by 27% compared to cells transfected with the inverted control (Figure 2A). Based on reports that the $\alpha6\beta4$ integrin can impact the survival of carcinoma cells in stress conditions [16, 17], it was important to evaluate the effect of these oligonucleotides on apoptosis. The level of apoptosis was 5% for both si-$\beta4$ and si-Inv transfected cells as determined by the percentage of AnnexinV-PE$^+$ cells (data not shown). This data indicate that reduction of $\alpha6\beta4$ expression does not result in increased apoptosis under these conditions (10% serum) and that the decrease in invasion of si-$\beta4$ transfected cells cannot be attributed to increased levels of cell death.

The ability of $\alpha6\beta4$ to influence the functions of carcinoma cells can occur independently of ligand (laminin) binding [10, 11]. In particular, $\alpha6\beta4$ stimulates the chemotactic migration of carcinoma cells on collagen I but appears to have little impact on their haptotactic migration towards collagen, a process that involves only collagen adhesion receptors [10]. For this reason, we assessed the ability of MDA-MB-231 cells transfected with si-$\beta4$ and

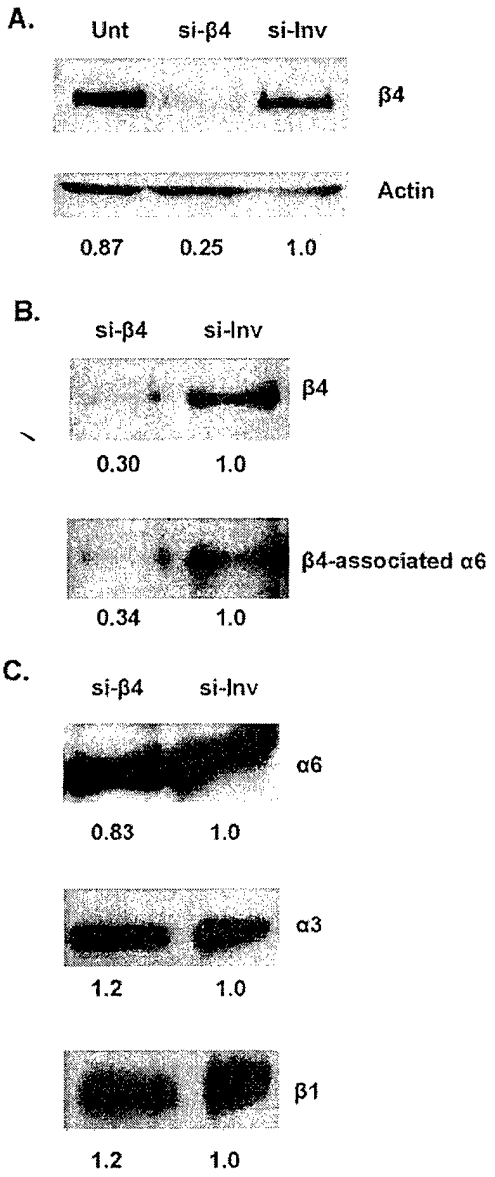

Figure 1. $\alpha6\beta4$ expression is reduced by si-$\beta4$ oligonucleotides. (A) MDA-MB-231 breast carcinoma cells were either untransfected (Unt) or transfected with $\beta4$ siRNA oligonucleotides (si-$\beta4$) or sequence-inverted siRNA oligonucleotides (si-Inv). RIPA extracts were obtained 96 hours post-transfection and equal amounts (30 μg) of total protein were resolved under reducing conditions by 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted for $\beta4$ and β-actin. The fold change in $\beta4$ expression was determined by normalizing each band to β-actin and designating the si-Inv lane as equal to 1. Similar results (25–75% reduction in $\beta4$ expression) were observed in greater than 5 independent experiments. Four days following transfection with si-$\beta4$ or si-Inv oligonucleotides, MDA-MB-231 cells were biotinylated, extracted in RIPA buffer, and immunoprecipitated for $\beta4$ (B) or $\alpha6$, $\alpha3$, and $\beta1$ (C), and transferred to nitrocellulose. Immunoprecipitations for $\alpha6$ and $\beta4$ (150 μg of total protein) were separated by 6% SDS-PAGE under reducing conditions whereas $\alpha3$ and $\beta1$ immunoprecipitations (60 μg of total protein) were eluted in non-reducing sample buffer. The membranes were incubated with peroxidase-conjugated streptavadin. The fold change in integrin expression following si-$\beta4$ transfection, when the si-Inv lane is set equal to 1, is indicated. Shown are representative blots from 3 separate experiments.

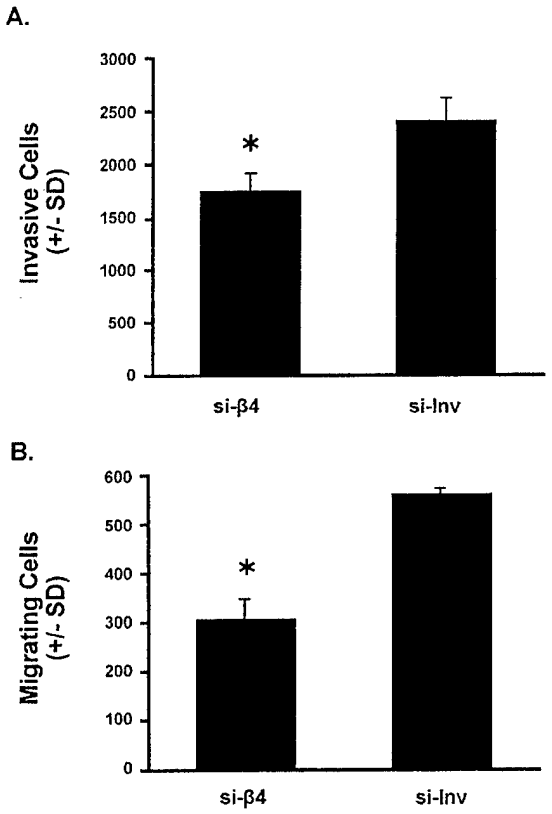

Figure 2. si-$\beta 4$ oligonucleotides decrease invasion and migration in MDA-MB-231 cells. (A) Four days following transfection, the ability of si-$\beta 4$ and si-Inv transfected MDA-MB-231 cells to invade Matrigel towards conditioned fibroblast medium was investigated in a 4 h assay. The results represent the mean number of invasive cells (+/− SD) from two wells (five fields per well). The number of invasive cells was significantly less for si-$\beta 4$ transfected cells than for si-Inv transfected cells (*two-tailed *t*-test, $P = 0.03$). Similar data were obtained in four separate experiments. (B) At 96 hours post-transfection, MDA-MB-231 cells were allowed to migrate through collagen I-coated Transwell membranes for 1 hour towards LPA (100 nM). The data represent the mean number of migrating cells (+/− SD) from 2 wells (5 fields per well). The number of migrating cells was significantly less for si-$\beta 4$ transfected cells than for si-Inv transfected cells (*two-tailed *t*-test, $P = 0.004$). Similar results were obtained in three independent trials.

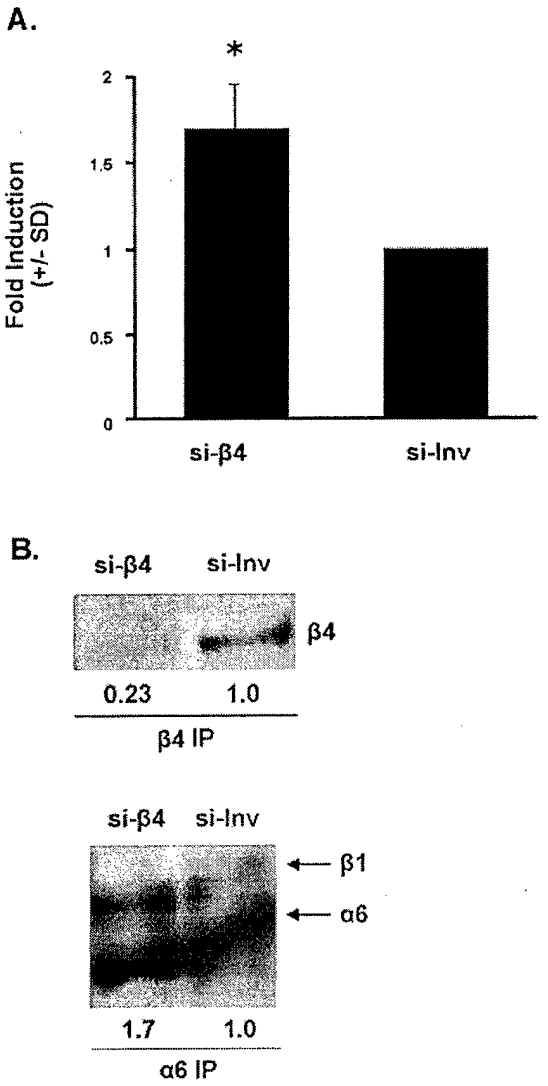

Figure 3. Reduced $\beta 4$ expression promotes adhesion to laminin-1 by enhancing $\alpha 6\beta 1$ formation. (A) MDA-MB-231 cells transfected with si-$\beta 4$ or si-Inv oligonucleotides were incubated for 1 hour in laminin-1 coated wells. Data are the mean fold induction (+/− SD) from three independent experiments, corrected for nonspecific adhesion to BSA, where the adhesion of si-Inv transfected cells was designated as equal to 1. The fold induction of si-$\beta 4$ transfected cells was significantly greater than observed for si-Inv transfected cells (*, two-tailed *t*-test, $P = 0.04$). (B) si-$\beta 4$ and si-Inv transfected MDA-MB-231 cells were biotinylated and lysed 96 h post-transfection and 150 μg of total protein was immunoprecipitated for either the $\beta 4$ (top blot) or $\alpha 6$ (bottom blot) subunit. Immune complexes were separated by 6% SDS-PAGE under reducing conditions and transferred to nitrocellulose. The membranes were then incubated with peroxidase-conjugated streptavadin and visualized by chemiluminescence. The level of $\beta 4$ (top) and $\alpha 6$-associated $\beta 1$ (bottom) expression following immunoprecipitation with the indicated antibodies is shown below each lane where the amount of $\beta 4$ (top) and $\beta 1$ (bottom) in the si-Inv sample is set equal to 1.

si-Inv oligonucleotides to migrate towards LPA, a known chemoattractant of breast carcinoma cells [10], on collagen I. The ligand-independent migration of MDA-MB-231 cells was inhibited by 45% when transfected with si-$\beta 4$ oligonucleotides compared to si-Inv oligonucleotides (Figure 2B). Taken together, these observations confirm the importance of $\alpha 6\beta 4$ expression in the migration and invasion of carcinoma cells as demonstrated previously by antibody inhibition experiments and by exogenous expression of this integrin in $\beta 4$-deficient carcinoma cell lines [18, 19].

Loss of β4 expression leads to increased adhesion to laminin-1 in MDA-MB-231 breast carcinoma cells

Because $\alpha 6\beta 4$ can function as a receptor for laminin-1 in some cells [9], we hypothesized that reduced $\alpha 6\beta 4$ expres-

sion by siRNA oligonucleotide transfection would disrupt adhesion to this laminin. However, the level of adhesion to laminin-1 in si-β4 transfected cells was significantly higher (1.7 fold) than observed in si-Inv transfected cells (Figure 3A). Considering that the expression of the α6 subunit remains unchanged in si-β4 transfected cells (Figure 1C and Figure 3B), we reasoned that the reduction of $\alpha6\beta4$ might lead to redistribution of the α6 subunit towards $\alpha6\beta1$, also a laminin-1 receptor [9]. We found that cells with decreased β4 expression (Figure 3B) had almost twice the level of α6-associated β1 as compared to si-Inv transfected cells as shown by immunoprecipitation of the α6 subunit in the same biotinylated lysates (Figure 3B). Co-immunoprecipitation of the α6 subunit in si-β4 and si-Inv transfected cells followed by immunoblotting for the β1 subunit yielded similar results (data not shown). These findings indicate that in the absence of sufficient levels of β4, α6 forms additional heterodimers with β1 in breast carcinoma cells. These findings indicate that in the absence of sufficient levels of β4, α6 forms additional heterodimers with β1 in breast carcinoma cells.

α6 siRNA oligonucleotides (si-α6) inhibit invasion and migration by reducing $\alpha6\beta4$ cell surface expression

MDA-MB-231 cells were transfected with si-α6 oligonucleotides and α6 scrambled-sequence oligonucleotides (si-Scr) to determine whether reduced α6 expression alters β4 cell surface levels and consequently decreases invasion and migration. Three days following transfection, cell surface receptors were biotinylated and cell lysates were immunoprecipitated for α6, β4, and β1. The si-α6 oligonucleotides decreased the expression of α6 by 61% compared to cells transfected with control oligonucleotides (Figure 4A). Interestingly, β4 expression was decreased by 59% in si-α6 transfected cells compared to si-Scr transfected cells whereas the cell surface expression of β1 was unchanged in cells transfected with either si-α6 or si-Scr oligonucleotides. Isotype-control immunoprecipitations performed for each antibody were negative (data not shown). These data indicate that siRNA oligonucleotides for α6 effectively reduce the level of $\alpha6\beta4$ expression on the cell surface of breast carcinoma cells. In addition, these findings substantiate the fact that most, if not all, β4 expressed on the cell surface is associated with α6.

Given our finding that si-α6 oligonucleotides efficiently decrease the level of $\alpha6\beta4$ on the cell surface of MDA-MB-231 cells, we investigated the ability of si-α6 oligonucleotides to inhibit their invasion and migration. The invasion of si-α6 transfected cells through Matrigel towards LPA was decreased by 53% compared to cells transfected with si-Scr oligonucleotides (Figure 4B). In additional experiments, we found a similar reduction in invasion when fibroblast conditioned medium was used as the chemoattractant (data not shown). The level of apoptosis was comparable for both si-α6 and si-Scr transfected cells as determined by the percentage of AnnexinV-PE$^+$ cells (data not shown). To assess the ability of si-α6 and si-Scr transfected cells to migrate in a ligand-independent manner, we performed migration assays towards LPA on collagen I-coated Transwells. The mean

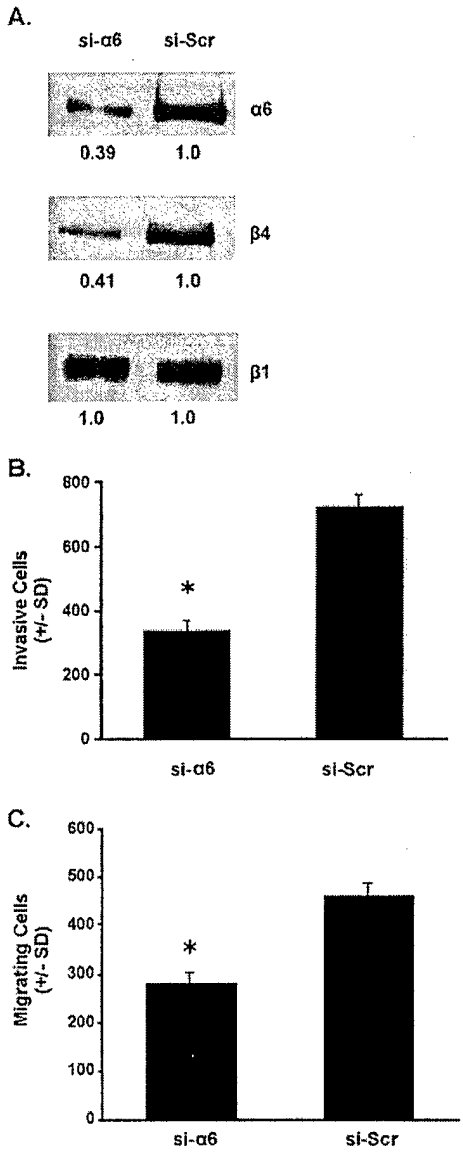

Figure 4. si-α6 oligonucleotides decrease invasion and migration of MDA-MB-231 cells. (A) Three days following transfection with si-α6 or si-Scr oligonucleotides, MDA-MB-231 cells were biotinylated, extracted in RIPA buffer, and immunoprecipitated for α6, β4, and β1. Each immunoprecipitation was initiated with 125 μg of total protein and separated by 6% SDS-PAGE. The membranes were incubated with peroxidase-conjugated streptavadin. The fold change in integrin expression following si-α6 transfection is shown in comparison to the si-Scr transfected band. (B) The ability of si-α6 and si-Scr transfected MDA-MB-231 cells to invade Matrigel towards LPA in a 4 h assay was assessed. The results represent the mean number of invasive cells (+/− SD) from two wells (five fields per well). The mean number of invasive cells was significantly decreased for si-α6 transfected cells compared to si-Scr transfected cells (*two-tailed t-test, $P = 0.0002$). Similar results were obtained in three independent trials. (C) At 72 h post-transfection with si-α6 or si-Scr oligonucleotides, the migration of these cells through collagen I-coated Transwell membranes towards LPA (100 nM) for 1 h was determined. The data represent the mean number of migrating cells (+/− SD) from two wells (five fields per well). The number of migrating cells was significantly less for si-α6 transfected cells than for si-Scr transfected cells (*two-tailed t-test, $P = 0.001$). Similar results were obtained in two separate experiments.

number of migrating cells was decreased by 39% in the si-α6 transfected cells compared to the si-Scr transfected cells (Figure 4C). These results provide more definitive evidence for the ability of $\alpha6\beta4$ to stimulate migration on non-laminin substrata. Overall, these data emphasize the importance of $\alpha6\beta4$ in the invasive phenotype of breast carcinoma cells.

Discussion

The relatively new technique of RNAi is a potentially powerful tool to assess the contribution of specific molecules to invasion and metastasis. In particular, the specificity and efficacy of this approach may be especially valuable for studying the integrin family members that exhibit complex structures and multiple functions. To assess the feasibility of RNAi in this capacity, we focused on the $\alpha6\beta4$ integrin. The involvement of this integrin in invasion and migration has been previously established by expression of the $\beta4$ subunit in $\beta4$-deficient cell lines and by function blocking antibodies. In this study, we provide the first evidence that the integrins can be inhibited by the more definite approach of RNAi and suggest that targeting of the $\alpha6\beta4$ integrin may be an effective strategy to assess the functions of this integrin and impede carcinoma progression.

The utilization of siRNA oligonucleotides targeted to the $\alpha6$ and $\beta4$ subunits of the $\alpha6\beta4$ integrin provided valuable information not only for the usefulness of RNAi to inhibit surface receptors but also on the mechanistic function of this integrin. We found that the endogenous expression of $\alpha6\beta4$ was not decreased until 72 or 96 hours post-transfection with si-$\alpha6$ and si-$\beta4$ oligonucleotides, respectively. This observation indicates that surface receptors may require considerable time for the RNAi approach to be effective and suggests that the half-life of the target protein and the rate of surface receptor recycling are major determinants in the ability of RNAi to inhibit expression. In contrast, our laboratory has shown that soluble proteins are inhibited efficiently by RNAi, usually within 24–48 hours after transfection (R.E. Bachelder, unpublished observation). In functional assays, reduced $\alpha6\beta4$ expression resulted in significant decreases in invasion and migration even though the inhibition of $\alpha6\beta4$ expression was less than complete. If the expression of $\alpha6\beta4$ were further diminished, the effects would likely be more dramatic. Because the reduction in $\alpha6\beta4$ expression in these studies is dependent on both transfection efficiency and the specific gene sequence selected for RNAi inhibition, manipulation of either of these parameters may ultimately lead to greater reduction in $\alpha6\beta4$ expression. For example, the use of different siRNA oligonucleotides targeted to either of the $\alpha6\beta4$ subunits may provide more complete inhibition. In addition, the recently developed DNA vector-based RNAi technology [27–29] which allows for stable inhibition of the targeted gene will eliminate transfection efficiency concerns and should also permit the generation of $\alpha6\beta4$ deficient cell lines. The implementation of these strategies will likely enable us to achieve greater reduction in $\alpha6\beta4$ expression in future studies.

Data from our laboratory and others have recently implicated the $\beta4$-subunit in ligand-independent signaling. For example, the $\alpha6\beta4$ integrin promotes migration and invasion, as well as lamellae formation, on non-laminin substrata such as collagen and these functions cannot be blocked by antibodies that inhibit $\alpha6\beta4$ adhesive interactions, an observation that discounts the possibility of adhesion to laminins deposited by the cells [10]. More recently, it was demonstrated that a truncated form of $\beta4$ that was unable to bind laminins could promote invasion [11]. In this study, the migration of MDA-MB-231 cells on collagen I, a non-ligand for $\alpha6\beta4$, was significantly inhibited by si-$\beta4$ and si-$\alpha6$ oligonucleotides thus providing further evidence that this integrin can function in the absence of ligand. This concept has profound implications for migration and invasion because it implies that the ability of $\alpha6\beta4$ to stimulate these pathways is not limited to specific matrix environments. Although the mechanism by which $\alpha6\beta4$ functions in this manner is unknown, the observation that $\beta4$ cytoplasmic domains can self-associate may explain this ligand-independent signaling [30]. Furthermore, $\alpha6\beta4$ has been shown to regulate the function of $\alpha3\beta1$, a dual collagen/laminin receptor, in keratinocytes [31, 32]. Even though we did not observe a change in either $\alpha3$ or $\beta1$ subunit expression in our studies, we cannot rule out the possibility that $\alpha6\beta4$ affects $\alpha3\beta1$-mediated signaling to cause decreased migration on collagen I in si-$\beta4$ and si-$\alpha6$ transfected cells.

Because $\alpha6\beta4$ is an adhesion receptor for laminin-1 as well as other laminins [9], we hypothesized that loss of this integrin would promote a decrease in adhesion to this substrata. However, we observed an increase in adhesion to laminin-1 following transfection with si-$\beta4$ oligonucleotides. This seeming paradox is explained by our finding that the $\alpha6$ subunit forms additional $\alpha6\beta1$ complexes in the absence of the $\beta4$ subunit. This finding is further supported by the observation that the $\alpha6\beta4$ integrin is decreased with either si-$\beta4$ or si-$\alpha6$ oligonucleotides but that cells transfected with si-$\alpha6$ oligonucleotides have reduced levels of the $\beta4$ subunit whereas the si-$\beta4$ oligonucleotides do not significantly decrease the expression of the $\alpha6$ subunit. Thus, the $\alpha6$ subunit is stabilized on the cell surface by associating with $\beta1$ subunits that are found expressed in excess in many cells but the $\beta4$ subunit, that associates exclusively with the $\alpha6$ subunit [12], is lost from the cell surface when the $\alpha6$ subunit is removed.

Several groups have recently demonstrated that siRNA oligonucleotides can inhibit gene expression *in vivo*. For example, mouse tail vein injections with siRNA oligonucleotides for luciferase, delivered as naked siRNA oligonucleotides or as plasmid DNA, in combination with a luciferase expression vector significantly reduced luciferase expression in adult mice [33]. In similar studies, the endogenous expression of the green fluorescent protein was decreased in the liver of a transgenic mouse strain that expresses this gene in nearly all organs [34]. Furthermore, the therapeutic potential of this technique to inhibit cancer progression was specifically shown by the lack of tumor formation in nude mice that were injected with cells that had been selected for stable

and reduced expression of oncogenic K-RAS [35]. Taken together, these studies indicate that RNAi may be a viable approach to treat human diseases including cancer.

In summary, we have shown that siRNA oligonucleotides can reduce the endogenous surface expression of the $\alpha 6\beta 4$ integrin in carcinoma cells. In the absence of $\alpha 6\beta 4$, we observed a significant reduction in the invasion and ligand-independent migration of these cells. These results demonstrate the validity of this technique to inhibit integrin expression in carcinoma cells and suggest that the $\alpha 6\beta 4$ integrin may be a potential gene target to prevent tumor progression *in vivo*. Future studies by our laboratory will directly explore this possibility in mouse cancer models.

Acknowledgements

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Advances in Brief

Competing Autocrine Pathways Involving Alternative Neuropilin-1 Ligands Regulate Chemotaxis of Carcinoma Cells[1]

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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 VEGF[3] 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, GACTTTGCTATCTTCCGAACTCTTGGGCAC; SEMA3A Reverse, GCTATACATACACACGGCTGATCCCTTG; NP1 Forward, ATGGAGAGGGGGCTGCCG; NP1 Reverse, CTATCGCGCTGTCGGTGTA; Plexin-A1 Forward, GAGGATGCCGACATGTTCGGCTTCG; 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, AGAGAUGUAGUCGCUCGCUTT; SEMA3A RNAi: AAAGUUCAUUAGUGCCCACCU; 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′-gatccccAGTTCATTAGTGCCCACCTttcaagagaAGGTGGGCACTAATGAACTttttggaaa-3′ and 5′-agcttttccaaaaaAGTTCATTAGTGCCCACCTctcttgaaAGGTGGGCACTAATGAACTggg-3′; SCR SEMA3A, 5′-gatccccGTGCACGCCTCTATATATCttcaagagaGATATATAGAGGCGTGCACttttggaaa-3′ and 5′-agcttttccaaaaaGTGCACGCCTCTATATATCtctcttgaaGATATATAGAGGCGTGCACggg-3′. *Eco*RI- and *Xho*I-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, benzyloxycarbonyl-VAD-fluoromethyl ketone; β-gal, β-galactosidase; VSV, vesicular stomatitis virus; AS, antisense.

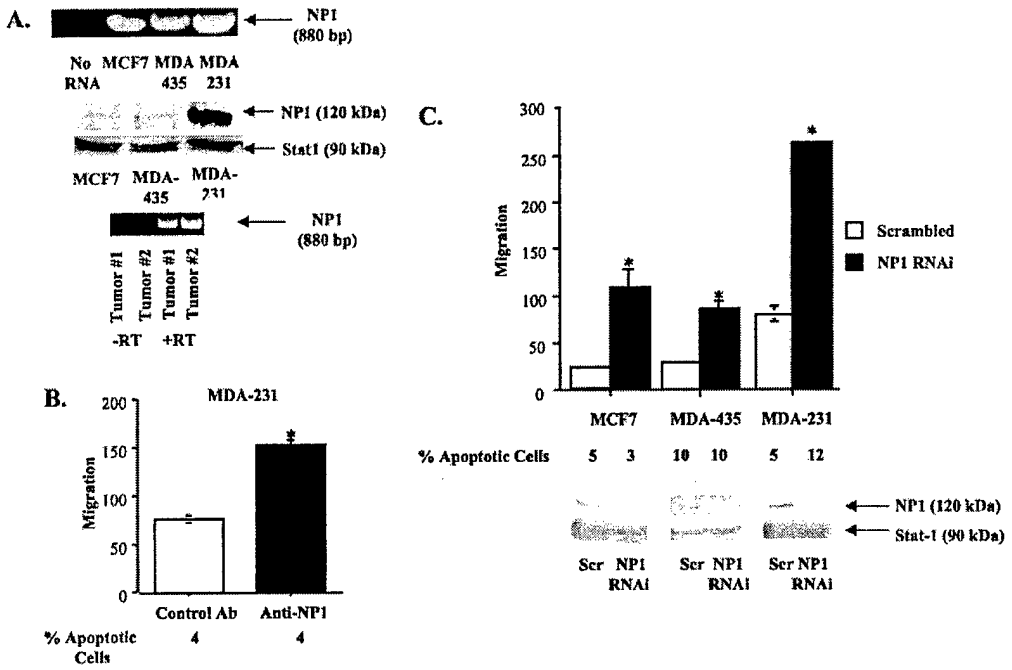

Fig. 1. NP1 suppresses chemotaxis of breast carcinoma cells. *A: top panel,* NP1-specific RT-PCR reactions were performed using the indicated mRNAs; *middle panel,* protein extracts were immunoblotted with the indicated antibodies (mouse anti-NP1 and rabbit anti-Stat1; Santa Cruz Biotechnology); *bottom 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,* ±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 μg/ml of Polybrene (Sigma, St. Louis, MO). After infection for 24 h, resistant cells were selected with puromycin (2 μg/ml).

DNA Transfections. Cells were transfected in the presence of Lipofectamine (Life Technologies, Inc.) and ZVAD-FMK with a β-gal-expressing plasmid (1 μg), and either VSV-tagged, dominant-negative human plexin-A1 (plexin-A1ΔCyt, provided by P. Comoglio, University of Torino, Italy) or myc-tagged constitutively active murine plexin-A1 (1 μg of PlexA1ΔSem, 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 μg/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. 1*A*) and tumors (Fig. 1*A*), 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. 1*B*). 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 expression did not impact cell survival (Fig. 1*C*). 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. 1*C*). In addition, this RNAi reduced NP1 expression in MCF-7 cells, a poorly migratory breast carcinoma line, and enhanced their chemotaxis 5-fold (Fig. 1*C*).

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. 2*A* and 3*A*). We also identified SEMA3A and plexin-A1 protein by immunoblotting proteins extracted from these samples with a SEMA3A- or plexin-A1-specific antibody (Figs. 2*A* and 3*A*). 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. 2*B*), increased the migration of these cells (Fig. 2*B*) 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. 3*B*). Conversely, expression of a semaphorin homology domain deletion mutant of plexin-A1 that exhibits constitutive activity in neurons (9) inhibited MDA-231 migration (Fig. 3*C*). 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

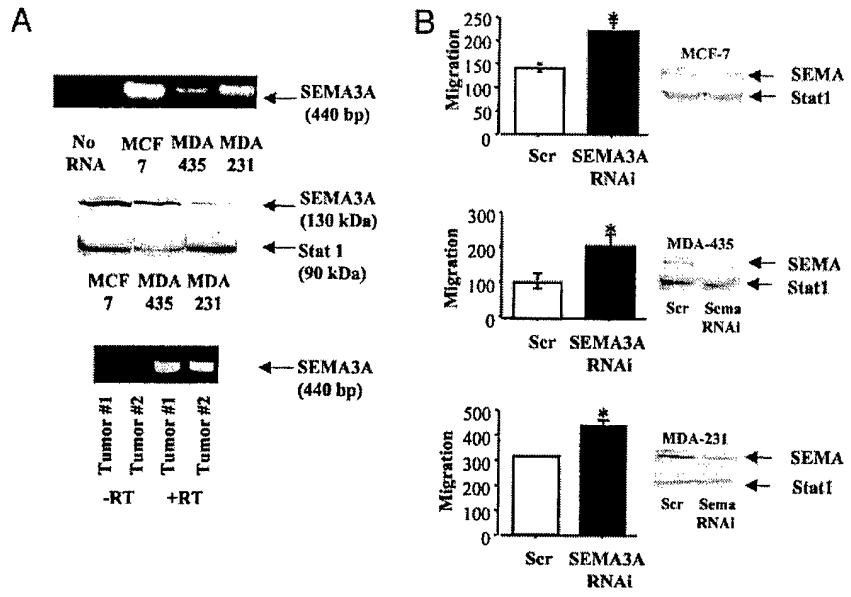

Fig. 2. Autocrine SEMA3A impedes chemotaxis. *A: top panel,* SEMA3A-specific RT-PCR reactions were performed using the indicated mRNAs; *middle panel,* extracted proteins were immunoblotted with the indicated antibodies (goat anti-SEMA3A, N-15 and rabbit anti-Stat1; Santa Cruz Biotechnology); *bottom panel,* SEMA3A was PCR amplified from cDNA obtained from carcinoma cells isolated from breast tumors. *B,* cells were transfected with a scrambled (*Scr*) or SEMA3A-specific RNAi, and their chemotaxis was determined in a 24-h (*MCF7*), 15-h (*MDA-435*), or 3-h (*MDA-231*) assay. SEMA3A and Stat1 expression were assessed as described for *A*. The mean number (*bars,* ±SD) of migrated cells from two wells (four fields/well) is indicated. *, $P < 0.02$ in a Student's *t* test. SEMA RNAi did not influence the survival of any of these cells (data not shown). Similar results were obtained in four separate experiments.

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. 4*A*). We then compared the ratio of these concentrations to the relative chemotactic rate of these carcinoma cells. As shown in Fig. 4*B,* 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. 4*B*). The lowest SEMA3A:VEGF ratio was observed in

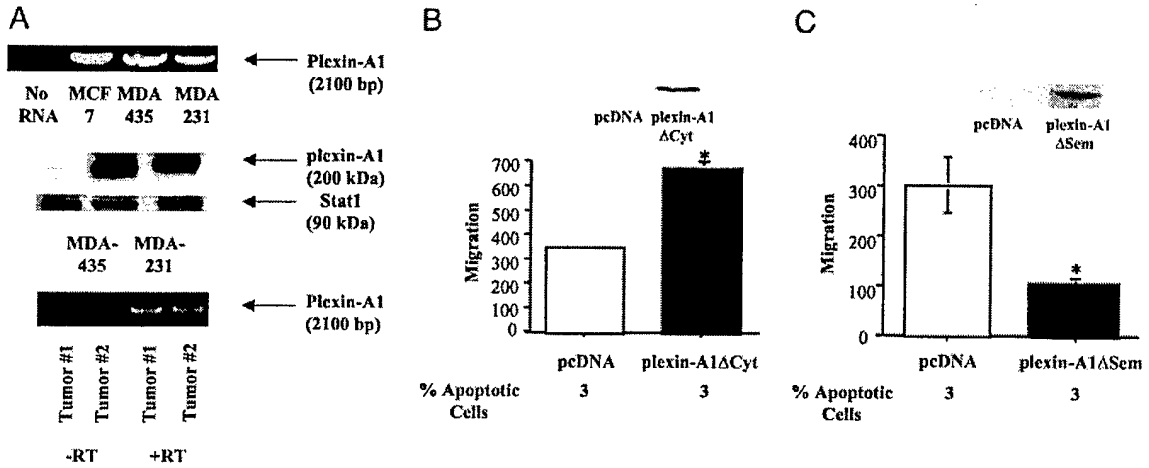

Fig. 3. Plexin-A1 signaling inhibits chemotaxis. *A: top panel,* plexin-A1-specific RT-PCR reactions were performed using the indicated mRNAs; *middle panel,* plexin-A1 and Stat1 proteins were detected by immunoblotting (rat anti-plexin-A1 provided by H. Fujisawa, Nagoya University Graduate School of 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,* ±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.

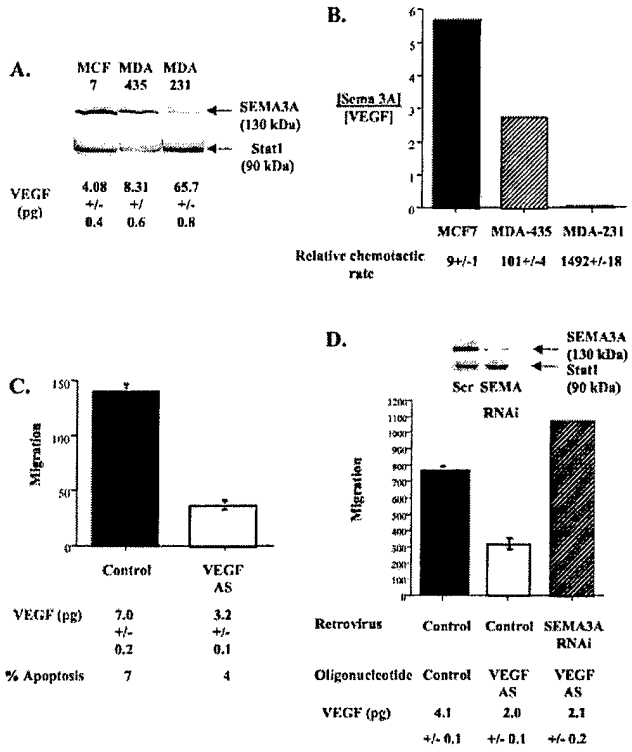

Fig. 4. Relative concentration of VEGF and SEMA3A determines chemotaxis rate. *A*, SEMA3A expression in equivalent amounts of total cellular protein extracted from MCF7, MDA-435, and MDA-231 cells was assessed by immunoblotting and quantified by densitometry. VEGF expression (pg) in 100 μg of total proteins 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. 4*B*).

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 ~50% relative to transfection with the control oligonucleotide (Fig. 4*C*). 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. 4*C*). These transfectants were maintained in the presence of ZVAD-FMK, and the reduction in VEGF expression did not influence their survival (Fig. 4*C*).

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. 4*D*). These infectants were then transfected transiently with either the VEGF AS or control oligonucleotide. Confirming the data in Fig. 4*C*, 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. 4*C*, 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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