A Dominant Negative Mutant of the Insulin-like Growth Factor-I Receptor Inhibits the Adhesion, Invasion, and Metastasis of Breast Cancer

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ABSTRACT

The 5-year survival rate for women with metastatic breast cancer is only 25–30%; thus, the need to improve treatment is apparent. Overexpression of insulin-like growth factor-I receptor (IGF-IR) correlates with poor prognosis and local recurrence. In this study, we addressed whether functional impairment of IGF-IR affects adhesion, invasion, and metastasis of breast cancer. Impairment of IGF-IR function was achieved by transfecting a dominant negative form of the receptor, termed 486stop, into MDA-MB-435 metastatic breast cancer cells. The protein product of 486stop is secreted extracellularly, resulting in a bystander effect. Cellular adhesion to laminin and collagen was inhibited 94 and 88%, respectively. Furthermore, 486stop inhibited insulin-like growth factor-I-stimulated invasion through collagen IV by 75%. The dominant negative receptor was secreted, as evidenced by the observation that MDA-MB-435 and MDA-MB-231 cells were prevented from binding to laminin by 90% when treated with conditioned medium (CM) from 486stop-transfected cells. CM also inhibited the invasion of MDA-MB-231 cells across collagen IV by 80%. Finally, CM made MDA-MB-231 cells 30% more sensitive to Taxol®-induced cell death. Growth in soft agar was suppressed by 486stop, but growth in monolayer was unaffected. When injected into the mammary fat pad, 486stop did not significantly suppress growth of the metastatic breast carcinoma cell line MDA-MB-435 was used because IGF-IR and its ligands, IGF-I and IGF-II, are increased in patients with breast cancer. IGF-IR is a member of the tyrosine kinase family and is comprised of two α and two β subunits. The α subunits serve as the ligand-binding domain, and the β subunits span the plasma membrane and transmit cellular signals. IGF-IR is commonly overexpressed in breast cancer (4), but this is not because it is constitutively activated (5), as is the case for Erb-B2 (6). One possible mechanism for IGF-IR overexpression is the loss of its repression following mutations of the tumor suppressor gene p53 (5). A second possibility that is particularly relevant to breast cancer is hormonal regulation of IGF-IR by estrogens (7). In addition to overexpression of IGF-IR, serum IGF-I levels are reported to be 25% higher in breast cancer patients compared to normal age-matched controls (8). These data suggest that an enriched endocrine supply of IGF-I develops in women with breast cancer for unknown reasons. Ligand activation of IGF-IR can also occur in a paracrine manner by IGF-II. Stromal IGF-II levels are above normal in 50% of invasive breast cancer cases, which correlates with increased stromal cell proliferation (9). The consequence of enhanced IGF-II signaling in transgenic mice is the development of mammary cancer (10). IGF-IR signaling may contribute to the pathogenesis of breast cancer through modulation of tumor growth by stimulation of cell proliferation and through inhibition of apoptosis. IGF-I and IGF-II are mitogenic for a number of breast cancer cell lines (11). In addition, our laboratory reported that IGF-I also protects preneoplastic breast cancer cells from apoptosis (12).

We propose that IGF signaling may also affect malignancy by modulation of cell adhesion and invasion, independent of tumor growth. Clinical studies suggest that IGF-IR is a prognostic for cancer recurrence and reduced survival (13). Turner et al. (13) found that 52% of breast cancers have high levels of IGF-IR. Patients with high IGF-IR levels relapse more frequently within 4 years after diagnosis than do patients without high IGF-IR expression in their breast cancers. On the basis of the conclusion from that study, IGF-IR is associated with a decline in short-term survival but has no predictive value for long-term survival. In contrast, Railo et al. (14) reported that long-term survival is inversely correlated with IGF-IR levels (14). It is noteworthy that survival is less likely if a tumor is IGF IR positive and ER negative than if the converse is true (14). It is not clear how overexpression of IGF-IR influences local recurrence. IGF-IR signaling may increase invasion of the breast cancer cells at local and distant sites. IGF-I stimulates the migration of a variety of cancer cells (15), including the breast cancer cell lines MCF-7 and MDA-MB-231 (16). Disruption of IGF-IR using antibodies or chemical inhibitors reverses breast cancer invasion. Antibodies to IGF-IR (16) and genistein, a tyrosine kinase inhibitor (17), suppress the invasive ability of breast carcinomas cells. However, no study to date has addressed the effect of the functional impairment of IGF-IR on breast cancer metastasis in vivo.

Here, we evaluated the role of IGF signaling at different steps in the metastatic cascade, including adhesion, invasion, and metastasis. The metastatic breast carcinoma cell line MDA-MB-435 was used because...
it is one of the few models for metastatic breast cancer using human cells (18). IGF-IR was inhibited in the MDA-MB-435 cells by stable transfection with a dominant negative form of IGF-IR, termed 486stop. The 486stop plasmid contains a point mutation in the coding region of the α subunit at codon 486, resulting in a premature stop signal. A truncated protein is secreted by cells expressing the 486stop plasmid (19). A bystander effect of the 486stop protein is evident because CM from stably transfected rat glioblastoma cells inhibits growth of untransfected rat glioblastoma cells in soft agar (19). IGF-IR signal transduction is also perturbed in 486stop-expressing cells because phosphorylation of insulin receptor substrate-1 and mitogen-activated protein kinase is attenuated (19). We report here that the functional impairment of IGF-IR by introduction of 486stop results in decreased cellular adhesion to the ECM proteins laminin and collagen I, suppression of cellular invasion through collagen IV, bystander inhibition of adhesion and invasion, and attenuated formation of metastasis in vivo. These data suggest that IGF signaling is an important target for several steps along the metastatic cascade and provide a mechanistic rationale for the targeting of IGF-IR in the treatment of metastatic breast cancer.

MATERIALS AND METHODS

Cell Culture and Transfection. MDA-MB-435 human breast carcinoma cells were obtained from Dr. Janet Price (M. D. Anderson Cancer Center, Houston, TX) and maintained in DMEM-F12 supplemented with 10% FCS (Summit, Ft. Collins, CO) at 37°C and 5% CO2. The plasmid containing 486stop was created by introduction of a point mutation into codon 486 of IGF-IR. This mutation results in a premature stop signal, as described previously (19). To generate the vector control, 486stop was cut with XbaI and BamHI restriction enzymes to remove the insert. Plasmids were introduced into MDA-MB-435 cells by liposome-mediated gene transfer using Lipofectin (Life Technologies, Inc., Gaithersburg, MD) according to manufacturer's instructions. After 48 h, cells were treated with 400 µg/ml G418 (Life Technologies). Transfected and vector control cells were harvested with 0.25% trypsin in PBS, neutralized with DMEM-F12 supplemented with 10% PCS, IGF-I, or IGF-II (50 ng/ml; Collaborative Biomedical Products) for 1 h at room temperature. The lower chamber was filled with DMEM-F12 supplemented with 10% PCS, IGF-I, or IGF-II (50 ng/ml; Collaborative Biomedical Products). Invasion assays were performed according to Doerr and Jones (16). MDA-MB-435 and MDA-MB-231 cells were allowed to invade for 16 and 4 h, respectively. These times were empirically determined to be optimal for each cell line. The CM was used to test invasion of MDA-MB-231 cells as described above. The only deviation from the above protocol was that CM was isolated from two individual clones (SR22 and SR26) rather than from a pooled clone population of cells.

Cell Doubling Time. Cells were plated at 50,000 cells/well in six-well dishes (n = 6/time point) and cultured in 10% FCS/DMEM-F12 for 24, 48, 72, and 96 h. Fresh medium was added every other day to ensure that nutrients were not limited. The number of cells was determined by measurement on a Coulter counter (Coulter Electronics, Miami, FL).

Soft Agar Assays. Vector control and 486stop-transfected cells were plated at a density of 50,000 cells/60-mm dish containing a bottom layer of 0.6% agar and a top layer of 0.3% agar according to Annab el al. (21). Colonies were allowed to develop for 2 weeks. The average number of colonies was calculated by counting each sample three times in random fields using an eyepiece with a grid at a ×200 magnification.

Experimental Metastasis. Female nude mice (Taconic, Germantown, NY) that were 4 weeks of age received 1.5 × 10^7 vector control or 486stop cells in the mammary fat pad (22). Mice were housed in a pathogen-free environment and cared for according to guidelines set by the Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals. After 10 weeks, mice were sacrificed, and the tumors were removed, weighed, and frozen at –80°C. Lungs, livers, lymph nodes, and gastrointestinal mesentery-containing lymph vessels were collected and analyzed for metastases.

PCR Detection of 486stop. Tumor DNA (100 ng/ml) was analyzed by PCR to confirm the presence of plasmid DNA. Random samples were selected to assess the presence of the plasmid DNA. PCR amplification was performed as described previously (23), with brief modifications. Genomic DNA was subjected to a 95°C hot start for 2 min, followed by 37 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min and a 4°C hold. Amplification was achieved using 5′ primer CAT TOC TTC AGA GCT GGA GA and 3′ primer GCT AGG TAA CCA GCC AG. The PCR product was separated on a 7% polyacrylamide gel and stained with ethidium bromide.

Immunoprecipitation. The soluble receptor was immunoprecipitated according to Richards et al. (24), with the following modifications. Cells were plated at a concentration of 5 × 10^7/T150 dish in 15 ml of DMEM-F12; 48 h later, the CM was spun at 1000 × g for 30 min, 6000 × g for 30 min, and centrifuged at 16000 × g for 1 h and then 1 ml of the concentrated CM was transferred to a 1.5-ml tube along with α-IR3 antibody (0.1 µg/ml). CM from two different clones, clones 22 and 26, was evaluated. To determine whether the soluble receptor could be detected in CM without concentrating the media, CM was also subjected to immunoprecipitation. Proteins were eluted from the protein A beads in 50 µl of 2X sample buffer, yielding concentration factors of 20X and 160X for the neat and concentrated CM, respectively. The samples were run on a 7.5% acrylamide gel and stained with Coomassie blue or transferred to nitrocellulose and detected as described previously (23). Detection of the soluble receptor was achieved by incubating with the antibody to the NH2-terminal end of the α subunit, N20 (Santa Cruz Biotechnology, Santa Cruz, CA), at a 1:5000 dilution.

Cell Survival. CM from the SR22 and SR26 clones was prepared as described above and added to MDA-MB-231 cells (5 × 10^3 cells/well). The cells were treated with Taxol® (10 nm; Sigma), and cell survival was measured after 72 h using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, as described previously (12). CM contained 400 µg/ml G418 for selection purposes; therefore, that amount of G418 was added to 5% FCS/DMEM-F12 as a toxicity control. In addition, the toxicity-negative control was CM from the vector control cell line without the addition of Taxol®.

Statistical Analysis. Data were analyzed using one of the following tests for significance: Student’s t test, Mann-Whitney U test, or Fisher’s exact test (25).
RESULTS

IGF-I Stimulates Adhesion of Breast Cancer Cells. Adhesion is the first step in the metastatic cascade; therefore, we addressed the role of IGF signaling in cellular adhesion. Both IGF-I and IGF-II increased adherence of the metastatic breast cancer MDA-MB-435 to laminin by ~3-fold (Fig. 1a). IGF-I was more effective at stimulating adhesion than IGF-II at a concentration of 10 ng/ml. This is likely because IGF-I has a higher affinity for the IGF-IR than for IGF-II. There was no difference in adhesion between 10 and 100 ng/ml IGF-I.

Expression of 486stop Reduces Adhesion of Breast Tumor Cells to ECM Proteins. We next addressed whether disruption of IGF-IR would inhibit adhesion to ECM proteins. Functional inhibition of IGF-IR was achieved by stable transfection with the dominant negative mutant of the IGF-IR, termed 486stop. The extent and specificity of inhibition by 486stop was tested on laminin, collagen IV, collagen I, and vitronectin. These ECM proteins were selected because they are expressed in the basement membrane (laminin and collagen IV), interstitial matrix (collagen I), and vasculature (vitronectin). Down-regulation of IGF-IR function by 486stop-expressing cells inhibited adhesion to collagen I and laminin by 88 and 94%, respectively (Fig. 1b). There was no effect on adhesion to collagen IV or vitronectin. Similar results were observed using αIR3, a neutralizing antibody specific for the α subunit of IGF-IR. Adhesion to collagen I and laminin was inhibited 89 and 70% by αIR3, whereas binding to collagen IV and vitronectin was unaffected. The use of the neutralizing antibody confirmed that the protein product from 486stop-expressing cells was specific for IGF-IR-mediated adhesion.

CM Blocks Adhesion of Metastatic Breast Cancer Cell Lines MDA-MB-435 and MDA-MB-231. Cells expressing the 486stop plasmid produce a secreted protein resulting in a bystander effect. The bystander effect of 486stop was tested by collecting CM from 486stop-expressing cells and testing whether it inhibited the adhesion of untransfected breast cancer cells (MDA-MB-435 and MDA-MB-231). The MDA-MB-231 cell line is similar to MDA-MB-435 in that it is an ER-negative metastatic breast cancer cell line but it is more adhesive and invasive than the MDA-MB-435 cell line. CM inhibited adhesion of MDA-MB-435 cells to laminin by 90% (Fig. 2a). In addition, CM from the 486stop-expressing cells inhibited adhesion of MDA-MB-231 cells to laminin by >90% (Fig. 2b). Although MDA-MB-231 cells were more adhesive to laminin than MDA-MB-435 cells, 486stop CM inhibited adhesion to the same degree.

486stop Suppresses Cellular Invasion. Invasion through the basement membrane is an important step in the metastatic cascade, allowing cancer cells to move beyond the confines of the primary tumor environment. IGF-I stimulated both MDA-MB-231 and MDA-MB-435 cells to invade through collagen IV (Fig. 3a). Similar results were observed with IGF-II (data not shown). MDA-MB-231 cells served as a positive control for invasion stimulated by IGF-I, as reported previously (16). The presence of 486stop suppressed invasion of MDA-MB-435 cells through collagen IV (Fig. 3b). Invasion was suppressed ~3-fold; this correlated with the 3-fold stimulation of invasion by IGF-I (Fig. 3a). The bystander effect of 486stop was next tested by treating untransfected MDA-MB-231 cells with CM from two different clones, SR22 and SR26. Media collected from both clones inhibited invasion of MDA-MB-231 cells by ~80% (Fig. 3c). Because the CM also contained G418, we tested the selection media to determine whether it had any influence on invasion as a negative control. Cellular adhesion was not affected by G418; thus, the inhibition of invasion was due to the 486stop-soluble receptor and not the reagent used for selection.

Growth Characteristics of 486stop-expressing Cells in Vitro. The consequence of blocking IGF-IR via 486stop was investigated for changes in monolayer growth and the ability to grow in soft agar. We observed that 486stop-expressing cells grew at the same rate as the
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Fig. 2. The potential bystander effect of 486stop was evaluated using CM to determine whether the soluble receptor inhibited cellular adhesion of cells that were not transfected with the plasmid. MDA-MB-435 (a) and MDA-MB-231 (b) were preincubated with CM for 10 min and then added to collagen I-coated 96-well plates. Control cells were tested in the presence of 5% FCS/DMEM-F12. Adhesion assay was as described in the legend to Fig. 1a. Replicates of 12 were performed for each treatment. Student's t test was used to determine statistical significance (P < 0.001).

MDA-MB-435 vector control (Fig. 4). These data further confirm that the functional inhibition of IGF-IR has negligible effects on the cell doubling times (reviewed in Ref. 26). Other growth factors in 5% PCS saturated the ability of 486stop to suppress growth. The ability of cells to grow in suspension depends upon their anchorage independence and ability to grow as aggregates. Functional impairment of IGF-IR by 486stop suppressed the ability of cells to grow in soft agar (Fig. 5). Growth in monolayer and growth in three dimensions are therefore dependent upon different factors. Similar growth characteristics are observed when IGF-IR is blocked by antisense oligonucleotides in human melanoma cells (27).

Suppression of Metastasis by 486stop-expressing Cells in Vivo. Metastasis studies were performed by orthotopic transplantation of MDA-MB-435 cells expressing either 486stop or the vector control plasmids. After 10 weeks, the mice were sacrificed, and tissues were collected for analysis. The soluble receptor did not significantly change the tumor incidence: vector control and 486stop had 100 and 90% tumor incidence, respectively. Tumors from the 486stop mice were 47% smaller, although this difference was not statistically significant (Table 1). Mice that received the vector control-expressing cells developed lung metastases (6 of 10, macroscopic; 8 of 9, microscopic), whereas none of the mice that received the cells expressing 486stop had lung tumors. In the liver, 3 of 10 mice from the vector control group had metastases, whereas no metastases were observed in the mice that were injected with cells expressing 486stop. Upon gross necropsy, large swollen mesenteric lymph vessels were evident in all of the vector control mice, although only 3 of 10 mice that received cells expressing 486stop had gross evidence of metastasis to this region. Microscopic examination of the lymph nodes showed metastases in 10 of 10 mice that received the vector control whereas signs of lymph node involvement were seen in only 5 of 10 mice that received 486stop.

Mice from each group were compared for overall metastasis. Four different sites were evaluated for metastases: lymphatic vessels, lymph nodes, lungs, and livers. Metastases were detected in three of four sites in the mice that received the vector control cells. In contrast, the mean number of metastases positive site on average was <1 (0.8 of 4) when mice where injected with the 486stop plasmid (Table 1). This comparison demonstrated that cells expressing the 486stop truncated protein suppressed metastasis (Table 1). Suppression of metastases was independent of the primary tumor size; for example, the A13 mouse received the cells expressing the vector control plasmid and developed a primary tumor that was 4.44 g, with metastases detected in the lung, liver, lymph vessel, and lymph nodes. In contrast, the SR3 mouse received the cell expressing the 486stop plasmid and developed a primary tumor that was 4.59 g in weight, although there were no metastases detected in the lung, liver, or lymph vessel. There was indication of metastases to the lymph node, although it is noteworthy that in the SR3 mouse tumor cells were only located in the marginal sinus (grade I) and not in the intermediary sinus (grade II) or throughout the parenchyma (grade III; Ref. 28). The lymph node from A13 was largely infiltrated with tumor cells, making it a grade III metastasis. In general, the degree of infiltration into the lymph node was less in the mice that received 486stop compared to those that received the vector control.

PCR Confirmation of 486stop in Tumors. Tumors were analyzed by PCR using primers that specifically amplified only the 486stop insert and not the endogenous IGF-IR (Fig. 6). This was done to confirm that tumors possessed the 486stop insert. A 600-bp fragment was detect in samples collected from four separate mice. Sequencing of one of the PCR products (SR11) confirmed that tumors contained the 486stop plasmid (amplification of 1153–1783 bp).

The Soluble Receptor Was Immunoprecipitated from CM. Individual clones were isolated and screened by PCR (Fig. 7a). Two of the clones, SR22 and SR26, produced a single 600-bp PCR product that comigrated with the 486stop plasmid control. The PCR product was sequenced for confirmation (data not shown). An immunoprecipitation method was next developed to monitor the production of the soluble receptor into the medium. A M, 90,000 protein was detected by Coomassie Blue staining of an acrylamide gel (Fig. 7b) and by immunoblotting (Fig. 7c). These samples were obtained from CM taken from the SR22 and SR26 clones. The M, 90,000 protein was
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Fig. 3. a, IGF-I stimulated the invasion of MDA-MB-231 and MDA-MB-435 cells through collagen IV. Invasion assays were conducted using a modified Boyden chamber. The upper chamber contained 4 x 10^5 cells/well, and the lower well contained either serum-free DMEM-F12 or IGF-I (10 ng/ml). Cells were allowed to invade, and then the membrane was stained with Diff Quik, and the number of cells that invaded were counted (×200 magnification using an eyepiece with a grid). b, invasion of MDA-MB-435 was inhibited by 486stop transfection. IGF-I (10 ng/ml) was added to the lower well, and cells were incubated for 16 h. c, CM inhibited invasion of MDA-MB-231 cells. The invasion assay was performed according to the "Materials and Methods." Replicates of six were performed for each treatment. The positive control for invasion was the addition of IGF-I as shown in a. Addition of IGF-I reproducibly stimulates invasion of MDA-MB-231 cells by 4-fold, and this control was run with each experiment. As a negative control, CM from the vector control cell line was tested but did not inhibit invasion (data not shown).

Fig. 4. Cell growth on plastic was not affected by 486stop. MDA-MB-435 cells transfected with either the vector control plasmid or the 486stop plasmid were plated at a density of 1 x 10^4/60 mm dish. The cell number was determined at 24, 48, 72, and 96 h after plating by counting on a Coulter counter. Data points, means of six replicates; bars, SD. △, vector control; ▲, 486stop.

evident in 160X concentrated samples, as well as in the 20X concentrated sample upon longer exposure. These data demonstrate that the soluble receptor is produced into the medium and can be detected using two different antibodies to the α subunit of IGF-IR. The antibody used for the immunoprecipitation was αIR3, which recognizes the α subunit but does not react with the protein upon Western blotting. To improve the stringency of the experiment, a second antibody to the α subunit was used, N20. This antibody reacts with the NH2 terminus of the α subunit and is very responsive to detecting the α subunit of IGF-IR by Western blot analysis.

CM Sensitized MDA-MB-231 Cells to Taxol-induced Cytotoxicity. IGF-I protects breast cancer cells from cell death induced by a variety of anticancer drugs (12). It follows that blocking the IGF-I pathway via the soluble receptor may reverse this process. MDA-MB-231 cells were treated with the anticancer drug Taxol® in the presence or absence of CM from the clones SR22 and SR26 (Fig. 8). In the presence of CM, Taxol® decreased cell survival by 30% compared to Taxol® alone. The decrease in cell survival is via induction of apoptosis because we have observed DNA ladders after exposing MDA-MB-231 cells to Taxol® (data not shown).

DISCUSSION

The hallmark of malignancy is the ability of cancer cells to invade (29). Local invasion begins when cancer cells adhere to the basement
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Fig. 5. Growth in soft agar was suppressed in cells expressing 486stop. Cells expressing either the vector control or 486stop were seeded at a concentration of $3 \times 10^4$ cells/60-mm dish. a. after two weeks, the number of colonies was counted. Replicates of 10 were performed for each cell type. The average number of colonies for each cell type is demonstrated, and a visual representation of this is shown in b. c, representative photomicrograph of soft agar colonies formed by the cells expressing the vector control or the 486stop plasmid. Magnification of photomicrograph was ×250.

membrane and invade into the interstitial matrix. Here, we found that the functional impairment of IGF-IR by a dominant negative mutant, 486stop, dramatically suppressed adhesion and invasion of two different ER-negative breast cancer cell lines. These results are particularly intriguing because 486stop inhibited cellular interactions with proteins that are expressed in different tissue compartments. Laminin and collagen IV are expressed in the basement membrane, whereas collagen I is a component of the interstitial matrix. Cells expressing 486stop were inhibited from adhering to laminin, migrating across a collagen IV coated membrane and, ultimately, adhering to collagen I in the interstitial matrix. Taken together, these suggest that inhibition of IGF-IR function can potentially suppress adhesion and invasion at more than one point in the metastatic cascade. Our results correlate with reports indicating that IGF regulates membrane ruffling, an architectural change associated with cellular adhesion (30). We demonstrate that IGF signaling is important for adhesion to laminin, which is interesting because IGF-I also stimulates invasion through laminin-coated membranes (16). The ability of cancer cells to migrate is essential to their ability to metastasize. We demonstrated that IGF signaling positively regulates the coordinated cellular processes of adhesion and invasion in vitro. Furthermore, our data showed that the functional disruption of IGF-IR by 486stop resulted in fewer distant metastases in vivo.

The bystander effect of 486stop is a powerful strategy for the functional impairment of IGF-IR. Using CM from 486stop-expressing cells, we demonstrated a strong inhibition of adhesion and cellular invasion. It is noteworthy that both MDA-MB-435 cells, which are moderately adhesive, and MDA-MB-231 cells, which have a high avidity for adhesion, were inhibited to the same degree. These data suggest that when clonal variants develop with an advantage for metastasis, 486stop can act to suppress metastasis of variant cells with different metastatic ability. Suppression of metastasis in our study may also be a result of the bystander effect of 486stop. It would be interesting to introduce 486stop into breast cancer cells growing in the mammary fat pad to determine whether 486stop represses tumor metastases ectopically. The clear advantages to the use of the soluble receptor are that it has a bystander effect and does not have the limitations associated with the nonspecific nature of antisense oligonucleotides. In addition, it does not have the short half-life of neutralizing antibodies in vivo. Finally, we demonstrated that the 486stop
Nude mice received $1.5 \times 10^6$ cells expressing either the vector control or the 486stop plasmids into the mammary fat pad. Ten weeks later, mice were sacrificed, and primary tumors were weighed. Lungs were inflated, and metastases were counted under a compound microscope. Metastases to the lymph nodes were determined by evaluation of H&E-stained tissue sections. The lymph vessel metastases were determined by gross necropsy.

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486stop

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<td>SR2</td>
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<td>0</td>
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<td>Median</td>
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**Table 1** Metastatic suppression by cells expressing 486stop

- Lung metastases quantified macroscopically.
- Lung metastases quantified microscopically.
- LN, lymph node; LV, lymph vessel; N/A, not available due to improper inflation; NS, no significant difference.
- Rank score of metastasis to any site was determined by the sum of the affected sites evaluated.
- Tumor weight comparisons, vector control vs. 486stop, Mann-Whitney U test.
- Lung metastasis incidence, vector control vs. 486stop; Fisher's exact test, $P < 0.001$.
- Liver metastasis, vector control vs. 486stop; Fisher's exact test.
- Lymph node metastasis, vector control vs. 486stop; Fisher's exact test, $P < 0.001$.
- Lymph vessel metastasis, vector control vs. 486stop; Fisher's exact test, $P < 0.05$.
- Metastasis to any site, vector control vs. 486stop; Fisher's exact test, $P < 0.001$.

Bystander effect sensitized MDA-MB-231 cells to cytotoxicity induced by the anticancer drug Taxol®; thus, expression of 486stop could be used in combination with chemotherapeutic drugs to improve therapeutic efficacy. In summary, the bystander effect of 486stop provides a novel approach for potential gene therapy applications.

Other IGF-IR dominant negative mutants exist (31-33), although their activity is not attributed to the secretion of a soluble receptor. A truncation mutation at codon 952 of the β-subunit also acts as a functional inhibitor of IGF-IR, but its protein product is not secreted. Cells expressing 952stop have attenuated growth in soft agar and are not tumorigenic in nude mice when injected into s.c. tissue. Several other dominant mutants of IGF-IR demonstrate the ability to suppress growth in soft agar (33), but these mutants have not previously been studied for effects on cancer invasion and metastasis. The 486stop dominant negative mutant is similar to the other mutants in that it also inhibits growth in soft agar but it stands alone as the only secreted product that exhibits an effect on cellular adhesion, invasion, and metastasis.

IGF signaling also regulates the invasion and metastasis of other types of cancers in vivo. We previously reported that elevation of serum IGF-I results in the development of highly invasive transitional cell carcinomas of the bladder (34). This is particularly important because tumor invasion carries a poor prognosis for patients with bladder cancer (35). A reduction in serum IGF-I by hypophysectomy has a similar effect in inhibiting the metastatic behavior of murine bladder cancer xenografts.
INHIBITION OF IGF-I SUPPRESSES BREAST CANCER METASTASIS

Our study is the first to demonstrate that functional disruption of IGF signaling results in a suppression of metastasis from an orthotopic site. We demonstrate here that IGF signaling is intrinsic to the ability of breast cancer cells to move beyond their primary site. Thus, targeting IGF may prevent further metastasis and possibly prevent breast cancer recurrence. One approach is to target IGF-IR, and another is to target the IGF-IR ligands, IGF-I and IGF-II. Tamoxifen is a widely used anticancer drug for breast cancer. Most of the pharmacological activity of tamoxifen is associated with its potent antiestrogen activities, but tamoxifen also reduces serum IGF-I (39) (40) in patients with breast cancer. In addition, tamoxifen reduces IGF-I mRNA in the rat livers and lungs (41), potentially reducing tumor growth at secondary sites where IGF-I is normally expressed at high levels. Patients with breast cancer often benefit from taking tamoxifen, by the fact that disease recurrence (25%, P < 0.00001) and death (17%, P < 0.0001) are significantly attenuated (42). Although tamoxifen is a promising drug for the treatment of metastatic breast cancer, it is not particularly effective for the treatment of ER-negative breast cancers. We conclude that the functional impairment of the IGF-IR inhibits adhesion, invasion, and metastasis of ER-negative breast cancer cells. Our study implicates that targeting IGF-IR may improve therapy for metastatic breast cancers, particularly those that are insensitive to antiestrogens.

osteosarcoma cells (36). Inhibition of IGF-IR also inhibits invasion and metastasis of prostate and liver cancers. In the case of prostate cancer cells, inhibition of IGF-IR results in a suppression of invasion into the brain parenchyma (37). Murine liver cancer cells are inhibited from lung colonization when IGF-IR is inhibited by antisense produced by an expression plasmid (38). It is, therefore, possible that blocking IGF-IR will suppress the metastatic phenotype of a number of cancers. One of the shortcomings of other studies is that the cancers did not develop in the natural site of origin, i.e., the orthotopic site. This is an important issue because the vasculature and lymphatic drainage of a primary tumor are central to metastatic dissemination.

Fig. 7. PCR detection and immunoprecipitation of soluble receptor. DNA was isolated from clones SR22, SR24, SR25, and SR26 then amplified by PCR. a, a single PCR product of 600 bp was detected in the SR22 and SR26 clones. CM was harvested from SR22 and SR26 and then concentrated either 20X or 160X, as described in “Materials and Methods.” The soluble receptor was immunoprecipitated from CM produced by SR22 and SR26. A protein of Mr ~90,000 was detected by Coomassie Blue staining of an acrylamide gel (b) or by transferring the proteins to nitrocellulose and immunoblotting with an antibody to IGF-IR (c). The soluble receptor was also detectable in the 20X samples upon longer exposure, whereas no protein was found in the CM from the vector control cell line (data not shown).

Fig. 8. The soluble receptor sensitizes MDA-MB-231 cells to Taxol®. MDA-MB-231 cells were treated with Taxol® for 72 h, and then cell survival was measured by the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Taxol® alone had little effect (10%) on cell survival compared to the G418 control. In contrast, cell survival was significantly decreased (40%) by Taxol® when the cells were also treated with CM from clones SR22 and SR26 (P < 0.01, Student’s t test). The negative control for cytotoxicity was CM from the vector control cell line.
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A Dominant Negative Mutant of the Insulin-like Growth Factor-I Receptor Inhibits the Adhesion, Invasion, and Metastasis of Breast Cancer


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