IGF1R causes ligand-dependent transformation of fibroblasts to the malignant transformation of cells. Overexpression of insulin-like growth factor receptor (IGF1R) in several lines of normal and cancer cells has led to these effects being mediated by the type I IGF receptor (IGF1R). We have previously shown that LCC6 wild-type (WT) cells, a metastatic variant of MDA-MB-435 cancer cells originally derived from a breast cancer patient, exhibit enhanced motility in response to IGF-I compared with the parent MDA-MB-435 cells. To further understand the role of the type I insulin-like growth factor (IGF) receptor (IGF1R) in cancer metastasis we inhibited signaling via IGF1R using a C-terminal-truncated IGF1R. The truncated receptor retains the ligand binding domain but lacks the autophosphorylated tyrosine residues in the carboxyl terminus. Cells stably transfected with this truncated receptor (LCC6-DN cells) overexpressed the truncated IGF1R messenger RNA nearly 50-fold over endogenous receptor. The truncated receptor in the LCC6-DN cells behaved in a dominant negative manner to IGF-I, inhibiting IGF1R activation by IGF-I compared with the LCC6-WT cells. LCC6-DN cells failed to phosphorylate the adaptor proteins insulin receptor substrate-1 and -2 in response to IGF-I and did not activate Akt after exposure to IGF-I. Unlike LCC6-WT cells, LCC6-DN cells did not show enhanced motility in response to IGF-I. To assay for metastasis, LCC6-WT and LCC6-DN cells were injected into the mammary fat pads of mice, and the primary xenograft tumors were removed after 21 days. Mice sacrificed 5 weeks later showed multiple lung metastases derived from LCC-WT xenografts, whereas mice harboring LCC6-DN xenografts showed no lung metastases. Our data show that IGF1R can regulate several aspects of the malignant phenotype. In these cells, metastasis but not proliferation requires IGF1R function.

Insulin-like growth factors (IGFs) have pleiotropic effects on normal and cancer cells. These effects are mediated by the type I insulin-like growth factor receptor (IGF1R). Several lines of evidence suggest that the IGF system and IGF1R are relevant to the malignant transformation of cells. Overexpression of IGF1R causes ligand-dependent transformation of fibroblasts.

(1) Embryonic fibroblasts obtained from IGF1R knockout mice cannot easily be transformed by simian virus 40 large tumor antigen (SV40T) or activated ras (2). When these fibroblasts are then stably transfected with IGF1R, they can be transformed by SV40T, suggesting that a functional IGF1R is required for oncogenic transformation (3). IGF1R may be required for oncogenic transformation and tumorigenicity of cells (4). The IGF system has also been implicated in maintaining the malignant phenotype. Mice with low circulating levels of IGF-I have reduced incidence of colon tumor growth and metastasis of the colon adenocarcinoma to the liver (5). When IGF-I is expressed in mammary gland of mice, these animals have increased rates of developing breast cancer (6). Elevated levels of IGF-I are associated with increased risk of developing breast, prostate, and colon cancer (7, 8).

IGF1R has been reported to be involved in several different cancers including breast cancer, prostate cancer, liver cancer, colon cancer, melanoma, glioblastomas, Ewing’s sarcoma (9), and childhood malignancies. Activation of this receptor has been reported to be enhanced in breast cancer (10). IGF1R protects cancer cells from chemotherapy (11–13), causes radioresistance (14), and enhances proliferation (15, 16). Blocking antibodies that inhibit binding of IGF-I to IGF1R inhibit tumor growth in mice (17, 18), demonstrating the importance of this receptor in tumorigenesis. IGF-I has also been shown to enhance adhesion and motility of several cancer cell types (19–25). However, the role of IGF1R in the metastatic process is not fully defined.

IGF1R consists of two covalently linked polypeptide chains each with an extracellular α-subunit that binds ligand and a transmembrane β-subunit that contains tyrosine kinase activity. The IGF1R is transported to the membrane fully assembled in the dimeric form, and after ligand binding tyrosine kinase activity is initiated. Binding of ligand results in transphosphorylation of the β-subunit of one chain by the opposite β-subunit chain. This transphosphorylation is required for activation of the receptor and activation of downstream signaling pathways (26). Therefore, to study the role of the IGF1R in the metastasis of cancer cells we have overexpressed a C-terminal-truncated IGF1R that retains the ligand binding domain but lacks the autophosphorylated tyrosine residues in the carboxyl terminus. When the protein is assembled with either wild-type or truncated receptor construct such a receptor can bind ligand but not be transphosphorylated.

It has been shown that a truncated IGF1R when expressed in rat fibroblasts causes inhibition of tumorigenesis (27). Dunn et al. (20) have used the extracellular ligand binding domain of IGF1R as a soluble receptor to neutralize circulating IGFs. This soluble receptor has been reported to behave in a dominant negative manner (28) and inhibited the adhesion, motility, and metastasis of MDA-MB-435 cells (20). In this approach it was possible that these effects were due to inhibition of...
circuiting IGF effects on either host or tumor tissue. To directly study the effect of inhibiting IGF1R in the cancer cells and its impact on metastasis we used the MDA-MB-435/LCC6 cells (LCC6-WT) (29), which are a metastatic variant of the estrogen receptor-negative MDA-MB-435 cells (30) derived from a patient with breast cancer. Recent reports suggest that the MDA-MB-435 cells have a gene expression pattern consistent with malignant melanomas (31, 32).

We have previously shown that the LCC6-WT cells exhibit enhanced motility in response to IGF compared with the parent MDA-MB-435 cells (33). To examine the role of IGF1R in the metastasis of breast cancer cells we transfected the C-terminal-truncated IGF1R into the LCC6-WT cells. We found that LCC6 cells stably transfected with a truncated IGF1R (LCC6-DN) failed to activate IGF1R and downstream signaling pathways in response to IGF-I. LCC6-DN cells were unable to metastasize to the lungs in a xenograft model of tumor growth. These results suggest that IGF1R, in addition to its well known role in stimulating proliferation of breast cancer cells, plays an essential role in the metastasis of these cancer cells. Moreover, these metastatic events may be regulated independently of proliferative signals. Thus, targeting IGF1R function can affect the metastasis of cancer cells, and anti-IGF therapy may inhibit several characteristics of the malignant phenotype.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All reagents and chemicals were purchased from Sigma, and cell culture reagents were from Invitrogen unless otherwise noted. IGF-I was purchased from GroPep (Adelaide, Australia). Anti-phosphotyrosine antibody (PY-20) conjugated to horseradish peroxidase was from BD Biosciences (Lexington, KY). Antibodies against extracellular signal-regulated protein kinase (ERK)-1/2 (phospho-specific and total) and Akt (phosphospecific and total) were purchased from Cell Signaling (Beverly, MA). The rabbit polyclonal antibody against IRS-1 was produced by Alpha Diagnostics (San Antonio, TX), and the immune serum was affinity-purified on immobilized protein A as described previously (34). The antibody against IRS-2 was purchased from Upstate Biotechnology (Lake Placid, NY). The polyclonal antibody against the β-subunit of IGF1R (IGF1Rβ) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the polyclonal antibody against hemagglutinin A (HA) was from Covance (Berkeley, CA). Anti-rabbit secondary antibody conjugated to horseradish peroxidase was from Amersham Biosciences. Protein A-agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acrylamide, bisacrylamide and pre-stained molecular weight markers were from Bio-Rad.

**Cell Lines and Culture**—LCC6-WT cells were obtained from Dr. Robert Clarke at the Lombardi Cancer Center, Georgetown University, Washington D.C. LCC6-WT and LCC6-DN cells were routinely maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 20 μg/ml streptomycin, and 20% horse serum. Cells were plated in 24-well plates with 20,000 cells/well in regular growth medium. Cells were switched to SFM for 24 h and then treated as indicated in the figure legends. All treatments were done in triplicate. Growth was measured 4–5 days after treatment. Growth was assayed by the uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as described previously (40). 1 × 10^5 cells were plated in 24-well plates with 30,000 cells/well in regular growth medium. Cells were switched to SFM for 24 h and then treated as indicated in the figure legends. All treatments were done in triplicate. Growth was measured 4–5 days after treatment. Growth was assayed by the uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as described previously (39). 60 μl of 5 mg/ml MTT solution in phosphate-buffered saline was added to each well. After 4 h, media were aspirated, and formazan crystals were lysed with 500 μl of solubilization solution (95% Me2SO + 5% Iscove’s modified essential medium). Absorbance was measured with a plate reader at 570 nm using a 670-nm differential filter. Proliferation assays were repeated 3 times.

**Immunoprecipitation**—Cells were plated in 24-well plates with 30,000 cells/well in regular growth medium. Cells were switched to SFM for 24 h and then treated as indicated in the figure legends. All treatments were done in triplicate. Growth was measured 4–5 days after treatment. Growth was assayed by the uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as described previously (39). 60 μl of 5 mg/ml MTT solution in phosphate-buffered saline was added to each well. After 4 h, media were aspirated, and formazan crystals were lysed with 500 μl of solubilization solution (95% Me2SO + 5% Iscove’s modified essential medium). Absorbance was measured with a plate reader at 570 nm using a 670-nm differential filter. Proliferation assays were repeated 3 times.

**Anchorage-independent Growth**—Anchorage-independent growth assays were performed as described (40). 1 × 10^5 cells per well of a 6-well plate were used. 1 ml of 0.8% SeaPlaque-agarose (BioWhittaker, Rockland, ME) in culture medium was solidified in the bottom of each well as the bottom agar. Cells in growth media with 5% serum without or with 5 μM IGF-I were mixed with 0.45% agarose. The cells mixed with agarose were overlaid on the agarose. After 9–10 days colonies were counted using a microscope with a grid in the eyepiece. Three randomly selected fields were counted for each well, and the average number of colonies is shown. Results shown are representative of one experiment with triplicates for each treatment.

**Role of the Type I IGF Receptor in Metastasis**

**Cell Lysis**—Cells were washed 3 times with ice-cold PBS—buffered saline and lysed with 500 μl/10-cm dish lysis buffer TNESV (50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40, 2 mM EDTA, pH 8.0, 100 mM NaCl, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin). Lysates were clarified by centrifugation at 12,000 g for 20 min at 4 °C, and soluble cellular proteins were used for experiments or stored at −20 °C. Protein concentrations of the lysates were determined using the bicinchoninic acid (BCA) protein assay reagent kit (Pierce).

**Immunoblotting**—For immunoblotting, 40 μg of cellular proteins were subjected to reducing SDS-PAGE on 8% polyacrylamide gels following the Laemmli system (38). After SDS-PAGE proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST, 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.05% Tween 20) for 1 h at room temperature with gentle shaking. Membranes were then washed 5 times with TBST for 5 min each. Expression of truncated IGF1R in LCC6-DN cells was assayed by blotting with a 1:5000 dilution of HA antibody for 1 h at room temperature. To detect levels of IGF1R, membranes were blotted with a 1:1000 dilution of rabbit polyclonal antibody against IGF1Rβ. Membranes were washed five times with TBST for 5 min each and incubated with a 1:2000 dilution of anti-rabbit secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Membranes were washed as before, and chemiluminescence was done using SuperSignal West Pico substrate (Pierce).

**Immunoprecipitation**—Cells were lysed in 1 ml of protein A-agarose as described previously. The antibody against IRS-2 was purchased from Upstate Biotechnology (Lake Placid, NY). The polyclonal antibody against the β-subunit of IGF1R (IGF1Rβ) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the polyclonal antibody against hemagglutinin A (HA) was from Covance (Berkeley, CA). Anti-rabbit secondary antibody conjugated to horseradish peroxidase was from Amersham Biosciences. Protein A-agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acrylamide, bisacrylamide and pre-stained molecular weight markers were from Bio-Rad.

**RNA interference**—The interference vector pCMV-IRE2-HA/Flag-IRES-EGFP used in the experiments was a modified vector pCMV-IRE2-HA/Flag (40). 20 μg of plasmid encoding IGF1R mRNA, the autoradiograph was analyzed by densitometry using a video densitometer (Lasgel). Relative mRNA levels were calculated using the following formula: (Sample OD/Reference OD) x (Reference cDNA/Reference RNA) x 1,000. The expression of IGF1R was normalized to the expression of 18S ribosomal RNA. All experiments were performed at least 3 times with similar results.
Expression of C-terminal-truncated IGF1R in LCC6 Cells—LCC6 cells were stably transfected with the construct for truncated IGF1R. Expression of the truncated IGF1R was measured by RNAse protection assay (Fig. 1). The probe covers the full-length wild-type transcript (458 bp) and also extends over a natural splice site that leads to the alternately spliced transcript of 366 bp (36). Truncated IGF1R protected a fragment of 262 bp. RNA from MCF-7 cells was used as control, and the full-length wild-type 458-bp protected fragment and the alternately spliced wild-type variant fragment of 366 bp were detected (lane 2). Similarly, the LCC6-WT cells also expressed the full-length wild-type 458 bp and alternately wild-type spliced 366-bp fragments as shown in lane 3. Neither MCF-7 nor LCC6-WT expressed the message for the truncated receptor. Individual LCC6-DN clones expressed the message for the wild-type full-length and alternately spliced fragments as shown in lanes 4–8. In addition, several clones overexpressed the message for the truncated receptor (262 bp protected fragment in lanes 4–8). Several clones expressing the message for the truncated receptor were selected for further study. While there was some minor variation in IGF1R mRNA levels in the individual clones (Fig. 1), immunoblotting revealed levels of receptor protein equivalent to the wild-type cell line (data not shown). Densitometric analysis of the truncated receptor mRNA expressed by individual clones revealed a more than 5-fold increase in levels of the truncated mRNA compared with wild-type mRNA. All of the clones behaved in a similar manner to inhibit activation of endogenous IGF1R (see Fig. 3B). In addition to the similarity in the biochemical response to IGF-I among the individually selected clones, we also found that multiple clones behaved in a similar fashion in monolayer growth assays and cell motility studies (data not shown). Thus, a single clone was selected for further characterization. In this study we used clone number 6 (Fig. 1, lane 5) and referred to as LCC6-DN cell line. The levels of the truncated protein were assayed by HA immunoblotting as shown in Fig. 2A. In the first lane, LCC6-WT cells did not express the truncated HA-tagged IGF1R, whereas in Fig. 2A, second lane, LCC6-DN cells expressed the HA-tagged truncated IGF1R at 50 kDa. Some of the higher molecular weight unprocessed receptors were also detected in Fig. 2A, second lane. Levels of wild-type full-length receptor were detected using an antibody directed against the C terminus of IGF1Rβ. Fig. 2B shows that levels of endogenous IGF1R between LCC6-WT and LCC6-DN cells were similar.

To determine whether the truncated HA construct could heterodimerize with the wild-type receptor we immunoprecipi-
tated cell lysates with an anti-HA antibody then immuno- 
blotted with an anti-IGF1R antibody that only recognizes the C 
terminus of the β-subunit of IGF1R. Fig. 2C shows that trans- 
crated HA-tagged IGF1R construct could co-immunoprecipitate 
with the wild-type IGF1R only in LCC6-DN cells. These data 
suggest that the IGF1R-truncated receptor is capable of forming 
heterodimers with the wild-type receptor.

Truncated IGF1R Behaved in a Dominant Negative Manner 
To Inhibit IGF1R Activation by IGF-I—in LCC6-WT cells we 
have previously shown that IRS-2 is the major adaptor protein 
phosphorylated by IGF-I-mediated activation of IGF1R (33). As 
reported previously (37) and shown in Fig. 3A, IGF-I treatment 
of LCC6-WT cells resulted in detection of a 185-kDa phospho- 
rylated band in IGF-I-treated cells (lane 2) but not in untreated 
cells (lane 1). In contrast in LCC6-DN cells, IGF-I treatment 
did not result in significantly enhanced phosphorylation of the 
185-kDa protein (lane 4 compared with lane 3). As a control we 
used MCF-7 cells, which phosphorylated IRS-1 in response to 
IGF-I (lanes 5 and 6). All of the other clones expressing tran- 
cuted IGF1R showed similar inhibition of IGFIR activation, as 
shown in Fig. 3B. All of the in vitro studies on signaling via the 
IGF1R described below were done on four independent clones, 
and they all behaved similarly (data not shown).

To ensure that LCC6-DN had reduced phosphorylation of IRS 
species, we immunoprecipitated lysates with antibodies 
specific for IRS-1 (Fig. 3C) or IRS-2 (Fig. 3D) followed by 
anti-phosphotyrosine blotting. As shown in Fig. 3, LCC6-WT 
phosphorylated both IRS-1 (Fig. 3C, lane 2) and IRS-2 (Fig. 3D, 
lane 2) after IGF-I treatment. LCC6-DN cells, however, did not 
phosphorylate IRS-1 in response to IGF-1 (Fig. 3C, lane 4 
compared with lane 3). They also had much less phosphoryla- 
tion of IRS-2 (Fig. 3D, lane 4 compared with lane 3) after IGF 
treatment compared with LCC6-WT cells. As controls for the 
immunoprecipitations we used MCF-7 cells (Fig. 3C, lane 6 
compared with lane 5) for IRS-1 and MDA-231BO cells for 
IRS-2 (Fig. 3D, lane 6 compared with lane 5). We have previ- 
ously shown that in MCF-7 cells IRS-1 is the major species 
avtivated by IGF-I treatment (37), and in MDA-231BO cells IRS-2 
is the major protein phosphorylated by IGF-I treatment (33). These results indicate that 50-fold overexpression of tran- 
cuted IGF1R in LCC6-DN cells resulted in markedly dimin- 
ished activation of endogenous full-length IGF1R in these cells.

Dominant Negative IGF1R Inhibited Downstream Signaling 
Pathways—We next examined signaling pathways distal to 
IRS. We and others show that activation of IGF1R results in 
phosphorylation of p44/p42 ERK1/ERK2 of the mitogen-acti- 
vated protein kinase pathway (37) and activation of the phos- 
phatidylinositol 3-kinase 3-kinase target Akt (37, 41). LCC6-WT and 
LCC6-DN cells were treated with or without IGF-I, and phos- 
phorylation of Akt was measured (Fig. 4A). In LCC6-WT cells, 
IGF-I treatment resulted in the phosphorylation of Akt (Fig 4A, 
lane 4 versus lane 3). In LCC6-DN cells phosphorylation of Akt 
was not detected after IGF-I treatment (lane 6 versus lane 5). As 
controls we used MDA-231BO (lanes 1 and 2) and MCF-7 
cells (lanes 7 and 8), which we have previously shown activated 
the phosphatidylinositol 3-kinase pathway in response to IGF-I 
(37). Fig. 4B shows total levels of Akt were similar among the 
cell lines.

We next examined activation of ERK1/ERK2 using a phos- 
pho-specific p44/p42 antibody. In both LCC6-WT and 
LCC6-DN cells, p44/p42 ERK1/ERK2 was constitutionally phos- 
phorylated (Fig. 4C, lanes 3 and 5), and IGF-I did not cause any 
further phosphorylation in LCC6-WT (lane 4) or LCC6-DN 
(lane 6). Constitutive phosphorylation of ERK1/ERK2 in MDA-
231BO was also seen (Fig. 4B, lanes 1 and 2) as previously shown. As a control we show MCF-7 cells, in which IGF-I treatment resulted in phosphorylation of ERK1/ERK2 (lanes 1, 2, and 3) and total ERK1/ERK2 (lanes 4, 5, and 6). Both the p42 and p44 kDa ERK1/ERK2 are shown. In all panels LCC6-WT cells were untreated (lane 3) or treated with 5 nM IGF-I (lane 4), and LCC6-DN cells were untreated (lane 5) or treated with 5 nM IGF-I (lane 6) for 10 min. As controls we also used MDA-MB 231 BO (lanes 1 and 2) and MCF-7 cells (lanes 7 and 8).

**Fig. 5.** Monolayer growth of LCC6-WT and LCC6-DN cells are similar. LCC6-WT and LCC6-DN cells were grown in monolayers in serum-free conditions and treated with 5 nM IGF-I or 10% fetal bovine serum for 5 days. Cell number was estimated by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Cell number is shown as the absorbance at 570 nm, and the data represent the mean ± S.E. of four independent experiments with triplicate samples in each experiment.

**Fig. 6.** Dominant negative IGF1R inhibited IGF-I-mediated motility. LCC6-WT, LCC6-DN, and MDA-231BO (control) were assayed for motility using a modified Boyden chamber migration assay as described under “Experimental Procedures.” Cells were untreated (open bars) or treated with 5 nM IGF-I (filled bars). Results are shown as the total number of migrating cells, and the data represent the mean ± S.E. of three independent experiments with triplicate samples in each experiment. Unpaired t test was used to compare untreated versus treated cells.

231BO was also seen (Fig. 4B, lanes 1 and 2) as previously shown. As a control we show MCF-7 cells, in which IGF-I treatment resulted in phosphorylation of ERK1/ERK2 (lanes 1, 2, and 3) and total ERK1/ERK2 (lanes 4, 5, and 6). Both the p42 and p44 kDa ERK1/ERK2 are shown. In all panels LCC6-WT cells were untreated (lane 3) or treated with 5 nM IGF-I (lane 4), and LCC6-DN cells were untreated (lane 5) or treated with 5 nM IGF-I (lane 6) for 10 min. As controls we also used MDA-MB 231 BO (lanes 1 and 2) and MCF-7 cells (lanes 7 and 8).
serum-containing media were mixed without or with 5 nM IGF-I in independent growth. LCC6-WT and LCC6-DN cells in full fetal bovine serum have been reported to metastasize to lungs (42, 43), we examined the lungs for metastatic deposits to determine the effect of inhibition of IGF1R on this process. When mice were sacrificed at the end of the first two experiments (days 21–24), all mice (5/5) with LCC6-WT xenograft tumors showed numerous pulmonary micrometastases, whereas 0/5 mice with LCC6-DN xenograft tumors showed metastases (data not shown). To further explore this finding we surgically resected the primary tumors from two mice in each group at day 21. In this experiment the growth of the LCC6-WT was slightly greater than LCC6-DN, but as noted LCC6-DN cells had only minimally slower tumor growth when examined in three separate experiments. Moreover, after resection, both cell lines displayed regrowth of tumors at the resected site. By day 54, the LCC6-WT cells had slightly greater growth than LCC6-WT cells (Fig. 8). Thus, the ability to form tumors at the primary site of inoculation is not substantially affected by expression of IGF1R DN construct.

Mice were then sacrificed at day 54. When these mice were then examined for metastatic deposits, we found that both mice with LCC6-WT xenograft tumors had gross lung metastases, whereas the two mice with LCC6-DN xenograft tumors did not. Both mice with LCC6-WT tumors had numerous well-circumscribed lung metastases. Fig. 9 shows a heart and lung block removed from an animal harboring LCC6-WT tumor (left panel). Histomorphologically, the pulmonary metastases were similar to the primary tumor origin in the mammary fat pad (data not shown). In contrast, a lung from a mouse with LCC6-DN xenograft is shown in the right panel and showed no metastases. No gross metastases were seen in the lungs of all animals with LCC6-DN cells, although two micrometastatic cell clusters were found in the right lung of one mouse with LCC6-DN xenograft tumor at day 54. Thus, dominant negative IGF1R inhibited formation of lung metastases in athymic mice, suggesting that IGF1R may be essential for the metastasis of these cells.

DISCUSSION

Cancer is often an incurable disease because many patients already have metastatic spread of their disease at first diagnosis. Recently there has been a plethora of reports on the metastatic process, and a better understanding of this process is emerging (44). The process of metastasis involves the ability of cancer cells to invade the basement membrane of cells, intravasate the blood vessels and enter circulation, survive in the circulation to reach distant sites, extravasate from vessels into the surrounding tissue at the new site, and finally arrest in the metastatic sites and colonize them (45–47). Although it is clear that extracellular proteins involved in cell adhesion play a role in metastasis, it is not clear if these processes can be regulated by peptide growth factors.

The IGF system has been implicated in regulation of the malignant phenotype. IGFs promote the proliferation, survival, motility, and metastasis of cancer cells. The role of IGF1R in stimulating proliferation of tumor cells is well defined, and inhibition of IGF1R activation using an inhibitory antibody against IGF1R such as dIg3 has been shown to inhibit xenograft growth of cancer cells (17, 18). However, the role of IGF1R in the metastasis of breast cancer is incompletely defined even though this system has been implicated in the metastasis of several cancers.

IGFs and IGF1R have been shown to influence the metastasis of several cancers. Low circulating levels of IGF-I in liver-specific IGF-I deficient (LID) mice has been related to decreased metastasis of gastric and colon cancer in these mice (5). Using a mouse model of pancreatic islet cell tumorigenesis, it has been shown that IGF1R is up-regulated focally at the margins and in invasive regions of carcinomas (48). Gene array analyses show that IGF1R is significantly increased in a variant of a mouse breast cancer cell line that metastasizes to the brain compared with the parent cell line (49). Our laboratory has also shown that IGF1R levels are increased in a variant of MDA-MB-231 breast cancer cells that were selected in vivo for metastasis to the bone (33). Furthermore, IGF1R has been implicated in the metastasis of uveal melanomas (50) and lung carcinoma (51). It also promotes invasiveness of pancreatic cancer cells.

We and others show that in vitro interaction between IGF1R and integrins may be required for motility of cells (52–54). In MDA-231BO cells, which are a variant of the MDA-MB-231 cells selected for increased metastasis to bone in vivo, both IGF1R activation and α9β1 integrin occupancy are required for motility (52). It has also been shown that ligand occupancy of α9β1 is required for IGF-I-stimulated motility of smooth muscle cells (55). It has been suggested that IGF1R, integrins, and extracellular matrix may play a coordinated role in IGF-I-stimulated motility of cancer cells (19). We were interested in determining the role of IGF1R in the metastasis process and whether IGF1R by itself has an effect on metastasis. Therefore, we inhibited cancer cell IGF1R function by overexpressing a truncated IGF1R that lacks the autophosphorylated residues. We have shown here that this truncated receptor behaved in a functionally dominant negative manner to inhibit activation of endogenous IGF1R by IGF-I (Fig. 3). This dominant negative inhibition could potentially be achieved by several mechanisms; our data suggest that formation of heterodimers between the truncated construct and wild-type receptor occurred (Fig. 2C). This construct inhibited the IGF-I-stimulated phosphorylation of Akt. In LCC6 and MDA231BO cells ERK1/2 of the mitogen-activated protein kinase pathway are constitutively active, and IGF did not further enhance the activation. Our results suggest that the mitogen-activated protein kinase pathway is not involved in the metastatic processes regulated.

Fig. 7. Dominant negative IGF1R inhibited anchorage-independent growth. LCC6-WT and LCC6-DN cells in full fetal bovine serum-containing media were mixed without or with 5 nM IGF-I in 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates. Using a grid colonies formed were counted on a portion of the well. Three randomly selected fields were counted for each well and averaged. Each treatment was done in triplicate, and the results are shown as the average number of colonies ± S.E. The experiment was repeated three times with similar results, and a representative experiment is shown. An unpaired t test was used to compare untreated versus treated cells. n.s., not significant.
by IGF-I, as it is basally active in these cells and IGF-I treatment does not result in further activation of this pathway.

Furthermore, we have shown in this study that cells expressing the dominant negative IGF1R had decreased colony formation in agar, but lack of functional IGF1R did not affect their proliferation rates in vitro. This result was expected in view of the fact that IGFBPs do not promote proliferation of the parent MDA-MB-435 or the metastatic variant LCC6 cells (Fig. 5). We had previously shown that inhibition of IGF-I action with IGFBP-1 also inhibits anchorage-independent growth of LCC6 cells (56). Because IGFBP-1 interrupts integrin function and IGF1R activation, these experiments with dominant negative IGF1R show that disruption of IGF1R alone can inhibit anchorage-independent growth of these cells. In contrast, LCC6-DN cells did not show increased motility in response to IGF-I. However, we noticed a higher basal motility rate of LCC6-DN cells compared with LCC6-WT cells. We have seen this previously in MDA-231BO cells transfected with an antisense construct to IRS-2 compared with MBA-MB-231BO cells (33). It is possible that cells selected in vitro for increased metastasis such as LCC6 and MDA-231BO become less adhesive, and IGF-I regulates their adhesion and motility on specific substrates (33). When IGF-I signaling is interrupted in these cells they become more adhesive, and IGF-I does not enhance their motility. This increased adhesion then results in higher basal motility rates as measured by the Boyden chamber assay. In prostate cancer cells Plymate et al. (57) show that more aggressive tumors lose IGF1R expression. In this study cell lines with more aggressive tumor growth in vivo had diminished levels of IGF1R. These authors did not examine the ability of the cells to metastasize, and it is highly likely that IGF1R may serve different functions depending on the differentiation of the neoplasm. This differential function of IGF1R has been proposed in breast cancer cells (58). Although more aggressive tumors have lower levels of IGF1R, our data suggest that expression of IGF1R in more aggressive tumors may be linked to metastatic potential and not to growth at the primary site.

There are other reports of the ability of dominant negative IGF1R to inhibit cancer cell biology. Prager et al. (27) transfected a dominant negative IGF1R into rat fibroblasts and showed that it inhibited tumorigenesis. Our studies show that IGF1R is not required for tumorigenesis of LCC6 cells. In contrast, IGF1R is required for metastasis. Dunn et al. (59) reported that a IGF1R construct that behaves in a dominant negative manner inhibited motility and metastasis of MDA-MB-435 cells. Their approach, however, was different from that described in this work. Their construct created a point mutation in the a-subunit resulting in a stop codon in the extracellular domain. This resulted in the secretion of a soluble truncated IGF1R with only the extracellular domain of IGF1R (28). This soluble IGF1R then served to neutralize circulating ligands. In their approach it was possible that the effect on metastasis may be due to inhibiting the effects of the host IGF system, as this study did not show a direct effect on IGF signaling in the cells expressing the dominant negative construct. Our results clearly show that inhibition of IGF1R signaling within the cancer cell blocked pulmonary metastases.

Recently there have been other reports describing the role of IGF1R in metastasis of human colon cancer. Reinmuth et al. (59) have recently shown that IGF1R plays a role in multiple mechanisms that mediate angiogenesis and metastasis of human colon cancer cells. Using a dominant negative IGF1R, they have shown after splenic injection that cells expressing dominant negative IGF1R failed to form liver metastases, and they also failed to form liver tumors when injected into the livers. Our experiments did not involve direct injection of tumor cells into the vascular space. Thus, in these cells it appears that IGF1R plays an important role in the complete metastatic phenotype, from breaching the basement membrane to establishment of growth in distant sites.

The precise steps in the metastatic cascade that are regu-
lated by IGF1R are not yet clear. Our data suggest that IGF1R may be required for colonization at metastatic sites. It is possible that these cells cannot live in the metastatic environment without functional IGF1R. Alternately, cells with dominant negative IGF1R could be less invasive, suggesting that inhibition of IGF1R function has inhibited the ability of cells to invade through the basement membrane. However, this appears not to be the case because in our experiments both LCC6-WT and LCC6-DN tumors appeared to be equally aggressive locally. Because IGF1R has also been implicated in adhesion of cells and cell-cell contact (60, 61), it is possible that functional impairment of IGF1R in LCC6-DN cells has inhibited regulated adhesion to cell surfaces. Perhaps impairment of IGF1R function in these cells affected the ability of IGF-I to induce production of cytokines such as interleukin-8. It has recently been suggested that IGF-I can increase production of interleukin-8 in melanomas by increasing its transcription rate (62).

As IGF1R targeting strategies are being developed (34, 63), understanding the function and signaling pathways will be required to interpret the effects of an anti-IGF agent. In the models of cancer metastasis, it appears that IGF1R can regulate both growth regulatory and metastatic signals. Moreover, response to IGF1R activation may not be easily predicted by levels of receptor expression. Accounting for the differences in tumor cell biology regulated by IGF1R must be considered when designing clinical studies.

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