Featured Article

Reduced c-Met Expression by an Adenovirus Expressing a c-Met Ribozyme Inhibits Tumorigenic Growth and Lymph Node Metastases of PC3-LN4 Prostate Tumor Cells in an Orthotopic Nude Mouse Model

Sun Jin Kim, Marjorie Johnson, Kristen Koterba, Matthew H. Herynk, Hisanori Uehara, and Gary E. Gallick[1]

Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas [S. J. K., M. J., K. K., H. U.], and The Program in Cancer Biology, University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas [M. H. H., G. E. G.]

Abstract

Purpose: **The expression of c-Met, the receptor protein tyrosine kinase for hepatocyte growth factor/scatter factor, frequently increases during prostate tumor progression. However, whether reduced c-Met expression inhibits tumor growth and metastasis has not been ascertained.**

Experimental Design: **c-Met expression was reduced by infection of an adenovirus expressing a c-Met ribozyme into the highly metastatic human prostate cancer cell line PC3-LN4. *In vitro*, effects on c-Met, Akt, and extracellular signal-regulated kinase 1/2 expression and phosphorylation, Src expression and activity, and vascular endothelial growth factor expression were determined, as were effects on cell migration and invasion. Prostate tumor formation and metastasis to regional lymph nodes in nude mice were examined after both *ex vivo* and *in vivo* infection of cells.**

Results: **Infection of PC3-LN4 cells with the Ad-c-Met-expressing ribozyme decreased steady-state c-Met levels, decreased Src kinase activity, decreased vascular endothelial growth factor expression, and decreased migration and invasion *versus* the pU1 (control) virus. Significant inhibition of tumorigenicity (histologically confirmed tumors in only 1 of 10 mice) and consequent lymph node metastasis were observed upon *ex vivo* infection of Ad-c-Met. Similarly, gene therapy experiments led to complete inhibition of tumor growth in 7 of 8 mice.**

Conclusions: **Reduction in c-Met expression substantially inhibits both tumor growth and lymph node metastasis of PC3-LN4 cells in orthotopic nude mouse models. Therefore, targeting the c-Met signaling pathways may be important in controlling tumor growth and metastasis in human prostate cancers.**

Introduction

Cancer of the prostate is the most common cancer in men in North America and ranks second in cancer-related deaths in this population (1, 2). Despite efforts to promote early detection, >50% of patients have metastatic disease at diagnosis and initiation of treatment (3). As a result, metastases of hormone-refractory cells develop and ultimately lead to mortality. Currently, conventional treatments such as taxane-based chemotherapy (4, 5) are ineffective in treating patients with advanced disease. Thus, the identification of novel targets and new treatment regimens are critical for future control of advanced prostate cancer. Inhibitors of RPTKs,[2] often overexpressed in prostate cancer, may provide such targets for development of novel therapies.

One potential RPTK target in prostate cancer is c-Met. c-Met is expressed primarily in epithelial tissues (6). HGF, also known as SF, is the primary ligand of c-Met (6–8). Upon stimulation of HGF/SF, c-Met is tyrosine phosphorylated and initiates a cascade of signals that lead to activation of cellular behaviors such as mitogenesis, cell migration, matrix adhesion and invasion, angiogenesis, morphogenesis, survival, or combination of these events depending on the cell types stimulated. Mutations and aberrant overexpression of c-Met and HGF/SF have been correlated with disease progression and clinical outcome in a variety of cancers (9–14).

Aberrant signaling through c-Met has been associated with prostate tumorigenesis and progression in rodents and man. In the Nobel rat, overexpression of HGF and c-Met occurs during sex hormone-induced prostatic carcinogenesis (15). In the human disease, c-Met overexpression occurs as early as prostate intraepithelial neoplasia (16). Furthermore, strong correlative evidence also suggests a role for HGF/SF/c-Met signaling in the progression and metastasis of human prostate cancers. Analysis of prostate cancer surgical specimens demonstrates a direct

Received 1/8/03; revised 6/10/03; accepted 6/23/03.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
This research was supported in part by NIH Grants U54 CA 090810 and 2RO-1 CA65527 and DOD 17-00-1-0524 and The Gillson Longenbaugh Foundation (to G. E. G.).
[1] To whom requests for reprints should be addressed, at Department of Cancer Biology-179, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3657; Fax: (713) 745-1927; E-mail: ggallick@mdanderson.org.

[2] The abbreviations used are: RPTK, receptor protein kinase; HGF, hepatocyte growth factor; SF, scatter factor; VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; MOI, multiplicity of infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Erk, extracellular signal-regulated kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

association between increased c-Met expression and the formation of bone and lymph node metastases (17–19). More than 80% of all men who develop prostate cancer will develop metastatic disease in the bone, especially in the pelvis, femur and vertebrae and human bone stroma, a rich source of HGF/SF (20, 21). The possible role of HGF/SF in the preferential metastasis of prostate cancer to the bone was implicated by demonstrating the importance of this growth factor on the formation of prostate epithelial cell colonies on bone marrow stroma with epithelial cells from benign and malignant prostate tissues (22). HGF/SF and c-Met are also biomarkers of invasion of prostate cancer cells and progression of disease in latent and clinical prostate cancer, including prostatic intraepithelial neoplasia (23–29). Parr *et al.* (30) demonstrated that NK4, an HGF/SF variant, suppressed the HGF/SF-induced phosphorylation of paxillin, matrix adhesion, and invasion of prostate cancer cells. However, although substantial evidence implicates c-Met overexpression with prostate tumor progression, no studies have assessed the effects of reducing c-Met expression on tumor growth and metastasis.

To examine the role of c-Met in other tumor types in which it is overexpressed, the strategy of ribozyme-mediated-c-Met down-regulation has been used (31–33). In the glioblastoma cell lines U-373 (expressing c-Met only) and U-87 (expressing c-Met and HGF), Abounader *et al.* (32) demonstrated that stable ribozyme-expressing clones were reduced in c-Met expression by 95%. c-Met down-regulation in U-373 did not affect tumor growth or incidence. Reduction in tumor incidence and growth in the brain resulted only when an autocrine HGF/c-Met loop was present (32). Therapeutic treatment of orthotopically implanted U-87 tumors with adenovirus and/or liposomes containing the c-Met or HGF specific ribozyme increased the median survival of treated animals compared with untreated animals (31). Thus, in this model, c-Met down-regulation reduces tumorigenicity only when c-Met is activated by autocrine production of HGF. In two human breast cancer cell lines, MDA-MB 231 and MCF-7, ribozyme-mediated c-Met down-regulation significantly reduced HGF-induced migration and invasion (33) relative to a control (PU1) construct. Thus, expression of this c-Met ribozyme has proven successful in determining the biological effects of reducing c-Met expression in a highly specific manner. In this study, we report that reduction of c-Met expression by either *ex vivo* or *in vivo* infection with a defective adenovirus expressing a c-Met ribozyme of PC3P-LN4 prostate cancer cells, a metastatic variant of PC3P, inhibits tumorigenic growth and lymph node metastasis in orthotopic models in male nude mice. Previous studies have demonstrated that signaling through c-Met induces angiogenesis (34, 35). In accord with these results, we demonstrate that down-regulation of c-Met strongly down-regulates VEGF expression and, furthermore, that c-Src tyrosine kinase activity is, in part, responsible for reduced VEGF expression.

Materials and Methods

Cell Lines and Cell Culture Conditions. The PC-3 human prostate cancer cell line was originally obtained from the American Type Culture Collection (Manassas, VA). The PC-3M cell line was derived from a liver metastasis produced by parental PC-3 cells growing in the spleen of a nude mouse. PC-3M cells were implanted orthotopically into the prostate of nude mice, and after several cycles of *in vivo* selection through prostate and regional lymph nodes, the highly metastatic cell line to lymph node and distant organ, PC-3P-LN4, was isolated (36). The PC-3P-LN4 cell line was maintained as monolayer cultures in DMEM/F12 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, a 2-fold vitamin solution (Life Technologies, Inc.) in the absence of antibiotics. Cell cultures were maintained and incubated in 5% CO_2/95% air at 37°C. Cultures were free of *Mycoplasma* and the following murine viruses: reovirus type 3; pneumonia virus; K virus; Theiler's encephalitis virus; Sendai virus; minute virus; mouse adenovirus; mouse hepatitis virus; lymphocytic choriomeningitis virus; ectromelia virus; and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD).

Animals. Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were housed and maintained in specific pathogen-free conditions. The facilities were approved by the American Association for Accreditation of Laboratory Animal Care and meet all current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with Institutional guidelines when they were 8–12 weeks old.

Adenovirus Ribozyme Construct Targeting Human c-Met. A plasmid expressing the c-Met ribozyme was constructed by Abounader *et al.* (32). Briefly, pZeoU1EcoSpe parent vector was derived from wild-type U1snRNA, which is an essential component of the spliceosome complex and is stable and abundant in the nucleus. The U1 promoter was cloned in a *Bam*HI site of a modified pZeo-EcoSpe vector, which is zeocin resistant. A hammerhead ribozyme that recognizes and cleaves the GUC sequence was used. The complementary pair of ribozyme oligonucleotides that span between 540 and 560 with a GUC sequence at position 560 of human c-Met was synthesized, annealed at 40°C, and ligated into pU1 at the *Eco*RI and *Spe*I site to create the vector. As a control, an irrelevant sequence was also cloned into pU1 between *Eco*RI and *Spe*I sites and was designated (Ad PU1).

Replication-defective adenoviral vectors of the ribozyme expressing (Ad-c-Met) and control (Ad-PU1) constructs were constructed as previously described by Abounder *et al.* (32), and purified viruses were a generous gift of Dr. John Laterra (Kennedy Krieger Research Institute). For viral stock, adenoviruses were infected into 293 embryonic kidney cells. Cells were harvested 36–40 h after infection, pelleted, resuspended in PBS, and lysed by three freeze-thaw cycles. Cell debris was removed, and resulting cellular lysates were subjected to double CsCl centrifugation. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. Adenovirus titer represented as optical particles units was determined by optical absorbance as described previously (28). Infectious viral titers were determined by quick cytopathic effect assays. Viral particles/ml were calculated by using the following formula: [(number of cells/well) × (dilution with cytopathic effect, 10^4) × (10 virus/cell)]/0.3 ml viral

sample/well. For infection of PC3-LN4 cells, cultures at 70% confluency were infected with at the indicated MOI. MOI was determined by plaque forming units in 293 s cells after serial dilution.

Immunoblotting. Cells (1×10^6) were plated in monolayer on a 10-cm dish and infected with either Ad-PU-1 or Ad-c-Met for 24 h. Cells were washed twice with Ca^{2+}, Mg^{2+}-free ice-cold PBS [135 mM NaCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4 (pH 7.4), and 2.5 mM KCl] and lysed on ice in lysis buffer [50 mM HEPES (pH 7.0), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM Na PP_i, 10% glycerol, 1% Triton X-100, and 1 mM Na_2VO_4] plus 1 tablet/10 ml lysis buffer consisting of Complete Mini, EDTA-free protease inhibitor mixture tablets (Roche) for 10 min before clarification by centrifugation at 15,000 rpm at 4°C for 15 min. Protein concentrations were quantitated by using the Detergent-Compatible Protein Assay system (Bio-Rad, Hercules, CA) and absorbance measured by a spectrophotometer at 750 nm. Whole cell lysates were boiled for 5 min then loaded in Laemelli's sample buffer (2% SDS, 5% mercaptoethanol, 125 mM Tris (pH 6.8), 1 mM EDTA, 10% glycerol, and 0.02% bromphenol blue] and resolved by SDS-PAGE on an 8% polyacrylamide gel. Proteins were transferred to NitroPure nitrocellulose membranes (Fisher Scientific). Membranes were blocked for 1 h in blocking buffer (5% dry milk; Kroger, Houston, TX) in Tris-buffered saline pH 7.5 (TBS-T, 100 mM NaCl, 50 mM Tris, and 0.05% Tween 20). Blots were probed with anti-c-Met (clone C-28; Santa Cruz Biotechnology, Santa Cruz, CA), anti-actin (clone AC-15; Sigma-Aldrich, St. Louis, MO), anti-GAPDH antibody (Chemi-Con, Temecula, CA) anti-Src (clone 327; Oncogene Research Products, Cambridge, MA) mouse monoclonal antibodies, and/or anti-Erk1/2 (Ab-2; Oncogene Research Products), anti-phospho-p44/42 mitogen-activated protein kinase (Thr^{202}/Tyr^{204}; Cell Signaling Technology, Beverly, MA), anti-Akt (Cell Signaling Technology), and anti-phospho-Akt (Ser^{473}; Cell Signaling Technology) rabbit antisera as indicated. Antibodies were diluted in TBST [Tris-buffered saline-0.1% Tween 20 (v/v)] with 5% dried milk. Peroxidase-conjugated secondary antisera, goat antimouse antisera (Bio-Rad), or goat antirabbit (Bio-Rad) were used to detect the respective primary antibodies. Immunoreactive proteins were visualized with Chemiluminescence Reagent Plus detection system (NEN, Boston, MA), followed by and exposing the membrane to Kodak Biomax MR film (Amersham, Piscataway, NJ). Bands were scanned using a Hewlett-Packard ScanJet scanner and quantitated using Scion Image. The densitometry readings of the chemiluminescent bands were normalized to GAPDH or actin expression. Control lanes were normalized to 1.0.

Src Immune Complex Kinase Assay. Cells were rinsed with ice-cold PBS then detergent lysates were made as described above. Cells were homogenized and clarified by centrifugation at $10{,}000 \times g$. Cell lysates (250 μg protein) were reacted for 2 h with the Src monoclonal antibody 327 (Oncogene Research Products). Immune complex kinase assays were performed as previously described by Windham *et al.* (37). Briefly, immune complexes were formed by the addition of 6 μg of rabbit antimouse IgG (Organon Teknika, Durham, NC) for 1 h, followed by 50 μl of 10% (v/v) formalin-fixed Pansorbin (*Staphylococcus aureus*, Cowan strain; Calbiochem, La Jolla, CA) for 30 min. Pellets were then washed three times in a buffer consisting of 0.1% Triton X-100, 150 mM NaCl, and 10 mM sodium phosphate. Reactions were initiated at 22°C by the addition to each sample of 10 μCi of [γ^{32}P]ATP, 10 mM Mg^{2+}, and 100 μM sodium orthovanadate in 20 mM HEPES buffer. To analyze phosphorylation of an exogenous substrate, 10 μg of rabbit muscle enolase (Sigma-Aldrich) were added to the reaction buffer. After 10 min, reactions were terminated by the addition of SDS sample buffer. Proteins were separated by SDS-PAGE on 8% polyacrylamide gels, and radioactive proteins were detected by autoradiography.

Cell Proliferation. The MTT assay was used to estimate growth rates of cells. PC3-LN4 cells (4×10^5) were plated in monolayer on a 10-cm dish and infected with Ad-Pu1 or Ad-c-Met at MOIs of 25 and 50. Cells were trypsinized, and viable cells were counted using a hemacytometer after trypan blue staining. One thousand cells for each line were plated in triplicate each on a 96-well plate. Every 24 h, 50 μl of MTT (Sigma-Aldrich) were added to each well. After 2 h of incubation, all media were removed, and the cells were solubilized with the addition of 200 μl of DMSO (Fisher Scientific). The absorbance readings were performed on a spectrophotometer at 750 nm. The optical densities readings were divided by the reading at time 0 to obtain the percent increase in growth and plotted on a log scale *versus* time.

Migration and Invasion Assays. Migration assays were performed using a modified Boyden chamber system with a filter containing 8-μm pores (Control Inserts; Fisher Scientific). Invasion chambers (Biocoat Matrigel Invasion Chambers; Fisher Scientific) are identical to the migration chambers, with the addition of Matrigel layered on the top of the insert. For both assays, the bottom chamber was filled with 500 μl of conditioned media and then the insert was placed in the conditioned media. Conditioned media were obtained by growing NIH 3T3 fibroblasts to 80% confluency at 72 h in DMEM:Ham's F-12 + 10% FBS. After 72 h, the media were removed from the cells and filter sterilized. For the migration and invasion assays, equal numbers of cells in a volume of 500 μl for each cell line were suspended in their growing media and placed in the top chamber. The chamber was placed at 37°C for the length of the assay. To assess the cells that had migrated, the cells on the top of the filter were removed with a cotton swab. The cells that migrated through and adhered to the bottom of the filter were fixed and stained with the Hema 3 Stain Set (Fisher Scientific) according to the manufacturer's instructions. The stained cells on each insert were counted in five ×200 fields and averaged to give $n = 1$/insert. The sample size was therefore the number of inserts.

VEGF Expression. To determine expression of VEGF protein, conditioned medium from PC3-LN4 control and infected cells were harvested after 48 h from cells growing in 1% FBS-containing medium. The VEGF concentration was determined using an ELISA kit for human VEGF (Biosource) as we have previously reported (38, 39) [the ELISA kit recognizes the VEGF121 and VEGF165 isoforms of the protein (40)].

***Ex Vivo* Infection and Orthotopic Implantation of PC3 LN-4 Cells.** Tumor cells (1×10^6) were infected for 24 h with either Ad-PU1 or Ad-c-Met at an MOI of 25. Cells were harvested after a brief exposure to 0.25% trypsin:0.1% EDTA

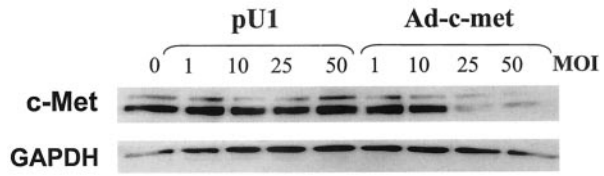

Fig. 1 Effects of Ad-PU1 and Ad-c-Met infection on c-Met expression in PC3-LN 4 cells. Cells were mock infected or infected with the indicated MOI of adenovirus, and immunoblotting was performed for c-Met expression as described in "Materials and Methods."

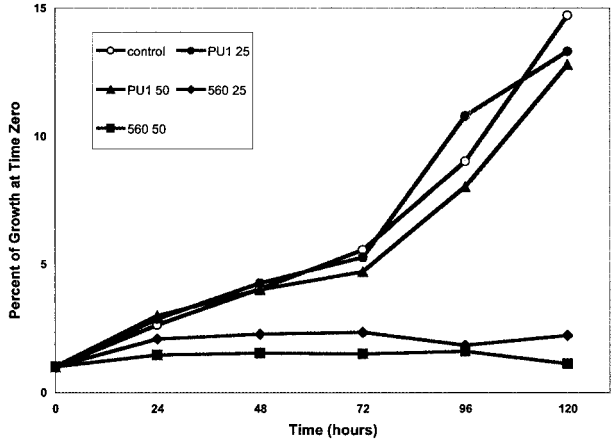

Fig. 2 Effects of infection with Ad-PU1 and Ad-c-Met on proliferation of PC3-LN4 cells. Cells (4×10^5) were seeded in 10-cm tissue culture dishes and mock infected or infected with adenovirus at the indicated MOI. Proliferation was estimated by the incorporation of MTT, as described in "Materials and Methods."

solution (w/v). After dislodging cells, the cell suspensions in culture media were pipetted to obtain single-cell suspensions and counted for cell viability in trypan blue with a hemacytometer. A total of 2.5×10^6 cells/group was counted, centrifuged, and resuspended in sterile Ca^{2+}- and Mg^{2+}-free HBSS at 5×10^5 cells/50 μl. Orthotopic injection was done as described previously (36). In brief, nude mice were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL) and placed in a supine position. A low midline incision was made, and the prostate was exposed. Fifty μl of HBSS containing 5×10^5 cells were injected into a lateral lobe of this prostate. The wound was closed with surgical metal clips. A total of 10 mice/group was injected.

Gene Therapy Studies. Subconfluent cultures of PC3P-LN4 cells were harvested by trypsinization. Cells were counted and resuspended in Ca^{2+}- and Mg^{2+}-free HBSS at 50,000 cells/50 μl. Cells were then implanted in the prostate of nude mice as described above, and the wound was closed with surgical metal clips. Tumors were treated at 7 and 14 days after implantation. Tumors were exposed by the same procedure by low midline incision and exposure of the prostate. Tumors were injected with PBS or 1.5×10^9 plaque forming units of Ad-c-Met or Ad-PU1, each in a total volume of 20 μl. Tumors and lymph nodes were harvested 14 days after the final gene intratumoral injection. Each experimental condition was performed on groups of 8 mice.

Necropsy Procedures and Histological Studies. The mice were euthanized at 4 weeks after tumor cell injection, and body weights were determined. Primary tumors in the prostate were excised, measured, and weighed. Specimens were formalin-fixed and paraffin-embedded for H&E staining. Macroscopically enlarged lymph nodes were harvested, and the presence of metastatic disease was confirmed by histology.

Statistical Analyses. Tumor incidence and incidence of lymph node metastasis (χ^2 test), tumor weight (Mann-Whitney test), total and phosphorylated c-Met protein expression (Student's *t* test), and cellular proliferation (Student's *t* test) were compared statistically.

Results

Reduction of c-Met Protein Levels in PC3-LN4 Cells by Ad-c-Met Infection. PC3-LN4 human prostate cancer cells express high c-Met levels, with constitutive tyrosine phosphorylation and high intrinsic tyrosine kinase activity, characteristic of cell lines with activated c-Met. Although c-Met can be activated by mutation in the tyrosine kinase domain (12, 13), no mutations in this region were observed in c-Met in PC3-LN4 cells (data not shown). To examine the effects of c-Met down-regulation, PC3-LN4 cells were mock infected or infected with PU1 and Ad c-Met adenoviruses. As shown in Fig. 1, infection with pU1 (control) virus did not change the expression of c-Met at MOIs as high as 50. In contrast, infection with MOIs of 25 and 50 decreased c-Met expression by 95% relative to PU1 infection at similar MOIs and corrected for loading by GAPDH expression.

Inhibition of Cellular Proliferation of PC3-LN4 Cells by Ad-c-Met Infection. To determine the *in vitro* growth rates of c-Met down-regulated cells, MTT analysis was performed at 24, 48, 96, and 120 h after infection. The results are shown in Fig. 2. Infection with pU1/control adenovirus did not affect cellular proliferation of PC3-LN4 cells, but c-Met ribozyme adenovirus significantly inhibited the cellular proliferation of PC3-LN4 cells. Growth inhibition rates (percentage) at 24, 48, 72, 96, and 120 h were 33.0 ± 7.3, 52.5 ± 3.0, 64.5 ± 5.2, 82.8 ± 0.9, and 87.2 ± 0.8 with 25 MOI and 44.6 ± 2.7, 62.1 ± 4.4, 73.1 ± 1.0, 82.3 ± 1.4, and 92.4 ± 0.8 with 50 MOI, respectively ($P < 0.01$, paired Student's *t* test). There was no significant difference in inhibitory effect of cellular proliferation between the two different MOIs. These results suggest that c-Met expression is important to proliferation and/or survival of PC3-LN4 cells.

Suppression of Migration and Invasion by Ad-c-Met Infection. One of the principal functions of c-Met is promotion of migration, a process also important in metastasis. To examine the effects on migration of reduced c-Met expression in PC3-LN4 cells, single cells were seeded in the upper chamber of a standard two-chamber migration assay (Boyden chamber), and cells migrating to the bottom of the insert were counted as described in "Materials and Methods." As shown in Fig. 3*A*, PC3-LN4 cells were substantially reduced in migratory capacity when infected with Ad c-Met but not Ad-PU1, with almost no cells migrating upon infection with an MOI of 50 (a 97%

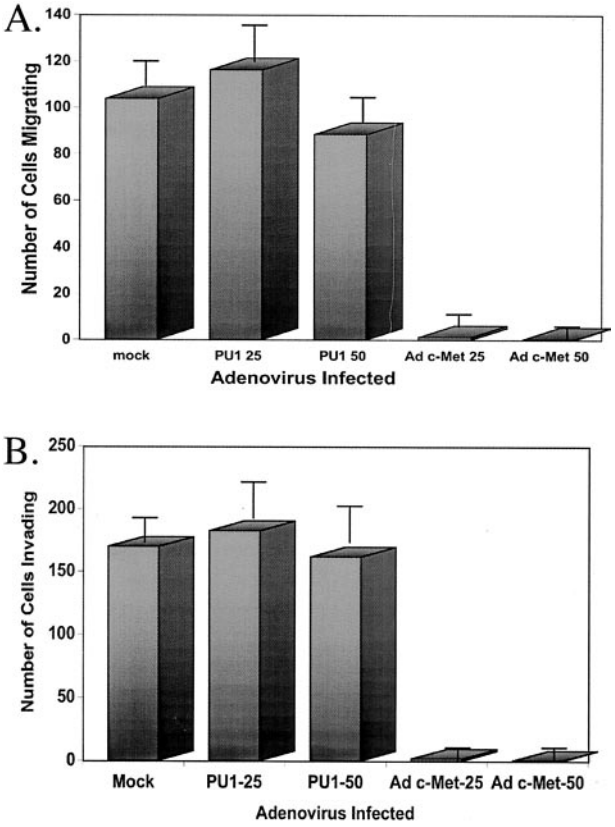

Fig. 3 Effects of infection of Ad-PU1 and Ad-c-Met on migratory and invasive potentials of PC3-LN4 cells. Migration and Invasion assays were performed using a modified Boyden chamber system with a filter containing 8-μm pores. NIH 3T3 conditioned medium was used as a chemoattractant in the bottom chamber, and equal numbers of infected cells or mock-infected cells were placed on the top chamber and allowed to grow for 72 h. Cells that migrated to the bottom of the insert were counted as described in "Materials and Methods." *A*, migration assays in which Boyden chambers were uncoated; *B*, invasion assays in which inserts were coated with Matrigel.

decrease to an average of <1 cell ± 0.31, $P < 0.01$, unpaired Student's *t* test). Parallel assays in which cells on the top of the insert were stained with trypan blue indicated that the cells were viable. To assess the ability of cells to invade through an extracellular matrix-like environment, Boyden chambers were used in which inserts were coated with Matrigel, and invasion assays were also performed as described in "Materials and Methods." The results are shown in Fig. 3*B*. Very similar results were seen to that of the migration assay, with no significant effect of the PU1 [control virus on invasion and an almost complete inhibition of invasive capacity at an MOI of 50 of Ad c-Met (99% decrease in cells invading to an average of <1 cell ± 0.11, $P < 0.001$, unpaired Student's *t* test)]. Thus, reduction of c-Met corresponded with reduced biological functions resulting from c-Met signaling.

Tumorigenicity in the Prostate after *ex Vivo* Infection of Ad-c-Met. As c-Met down-regulation decreased the *in vitro* growth rates, migration and invasion activity, *in vivo* growth rates, and metastatic behavior were analyzed. Cells were infected with adenovirus or mock infected and were implanted orthotopically into male nude mice as described in "Materials and Methods." Four weeks after injection, prostates were harvested and analyzed. In mock-infected PC3-LN4 cells 88.9% (8 of 9) mice developed tumors. Similarly, 88.9% (8 of 9) mice formed prostate tumors with pU1/control virus-infected PC3-LN4 cells (Table 1). There was no significant difference in tumor weight between mock-infected PC3-LN4-injected group (median: 0.58g) and pU1/50 adenovirus-infected PC3-LN4-injected group (median: 0.47g; $P > 0.05$, Mann-Whitney test). In mock-infected and the Ad-pU1/50 control virus-infected group, lymph nodes of tumor-bearing mice were enlarged and macroscopically confirmed of their tumor involvement by H/E staining (Figs. 4 and 5). In contrast, infection with Ad-c-Met significantly reduced tumor incidence and tumor growth with 10% of animals (1 of 10) developing tumors, and the one macroscopically detectable tumor was very small (0.07g) with largely normal histology by H&E staining (Fig. 5*D*). There were no mice with detectable lymph node metastases (Fig. 4*C*). Statistical comparison in tumor weight was inappropriate because of the low tumorigenicity induced by Ad-c-Met-infected cells.

Table 1 Tumorigenicity and lymph node metastases induced by PC3-LN4 cells after *ex vivo* infection of adenovirus or mock infection

Group[a]	Tumor incidence[b]	Tumor size (g) Median (range)	Lymph node involvement[c]
Mock	8/8	0.58 (0.31–1.01)	8/8
Ad-Pu-1	9/9	0.47 (0.01–2.45)	9/9
Ad-c-Met	1/10	0.07	0/1

[a] PC3-LN4 cells were mock infected, or infected with the indicated adenovirus at an MOI of 50, and 5×10^5 cells were injected into the prostates of nude mice, as described in "Materials and Methods."

[b] Number of mice with tumors/number of mice surviving surgery for prostate tumor cell implantation.

[c] Number of mice with lymph node metastases/number of mice with tumors.

Effects of Intraprostatic Injection of Adenoviruses into Preestablished PC3-LN4 Tumors. To determine whether reduced c-Met expression could affect the growth of PC3-LN4 cells growing in the prostate, gene therapy experiments were performed by allowing the tumor cells to grow in the prostate for 7 days, then injecting Ad-c-Met, Ad-PU1, or sham-injecting mice in the prostate 7 and 14 days later as described in "Materials and Methods." The results of these experiments are shown in Table 2. In 7 of 8 mice infected with Ad-PU1 and 8 of 8 sham-injected mice, tumors developed in the prostate. In contrast, only 1 of 8 mice injected with Ad-c-Met developed a tumor and that tumor was substantially smaller than tumors forming in control mice (Table 2). These results suggest that c-Met is performing an important survival function in PC3-LN4-induced tumors, and c-Met reduction is incompatible with development of macroscopic tumors.

Decreased c-Met Decreases VEGF Expression and Src Kinase Activity. Ample evidence has implicated c-Met signaling in angiogenesis and especially expression of VEGF (34, 35, 41, 42). To determine whether decreased VEGF expression along with decreased proliferation rates might explain, in part, the growth inhibition of tumors after decreased c-Met expres-

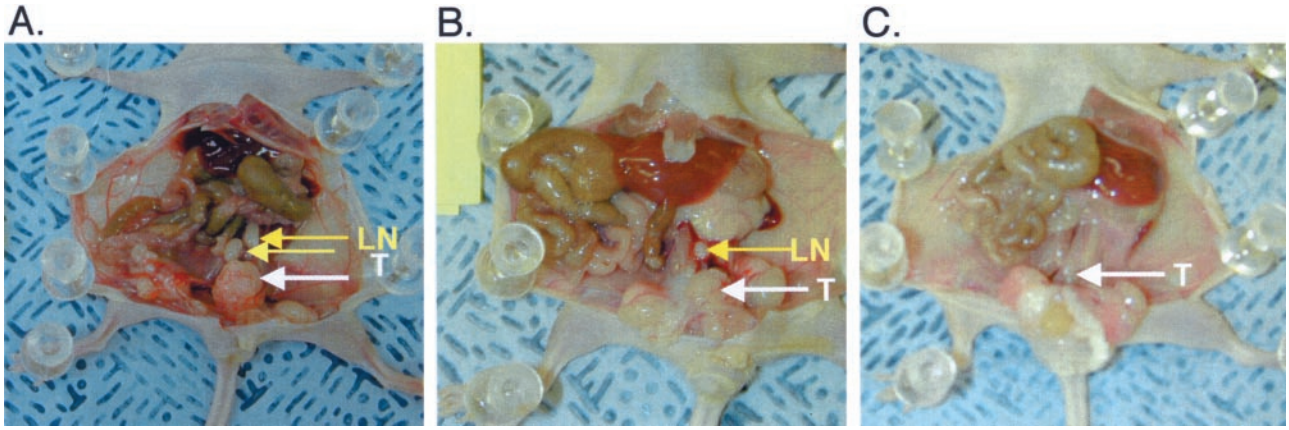

Fig. 4 Tumor formation in nude mice following: mock infection (*A*); *ex vivo* infection with Ad-PU-1 control adenovirus (*B*); or *ex vivo* infection with Ad-c-Met (*C*). PC3 LN-4 cells were mock infected or infected with given adenoviruses at an MOI of 50, after which cells were implanted into the prostate of nude mice as described in "Materials and Methods." Mice were killed 4 weeks later and examined for presence of tumors. *C* displays the only mouse implanted with PC3 LN-4 Ad c-Met-infected cells with macroscopic evidence of tumor.

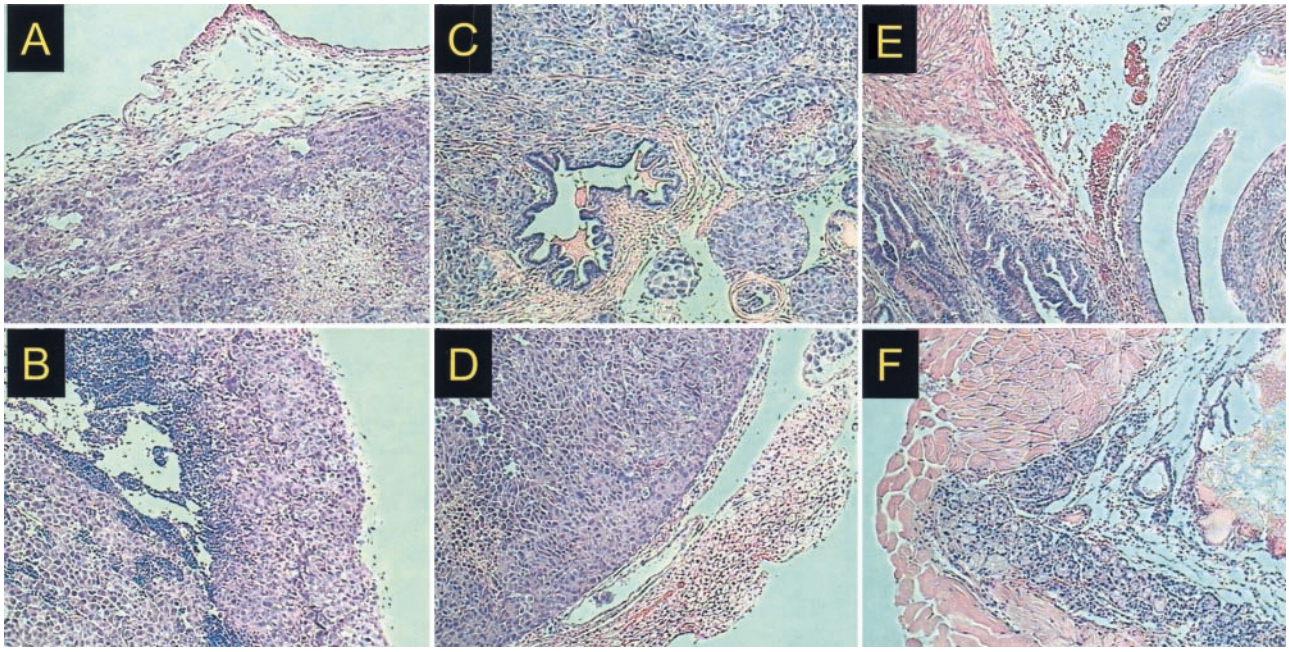

Fig. 5 H&E staining of nude mouse prostate tumors after orthotopic injection of mock, Ad-PU 1 control virus, or Ad-c-Met-infected PC3-LN4 cells. Primary tumor (*A*) and lymph node metastasis (*B*) from mice with mock-infected cells; primary tumor (*C*) and lymph node metastasis (*D*) from PU-1-infected cell; H&E of possible primary tumor from Ad-c-Met-infected cells (*D*); normal prostate (*E*).

sion, we examined VEGF expression in the media of PC3-LN4 cells 48 h after infection with ribozyme-expressing (Ad-c-Met) or control (PU-1) virus. The results are shown in Fig. 6. Significant reduction in VEGF expression (per 10^6 cells as measured by ELISA) was observed in PC3-LN4 cells in Ad-c-Met-infected cells relative to Ad-PU1 (control)-infected cells.

To begin to examine signaling pathways that might be affected by c-Met expression and are important to VEGF expression, we determined the status of Erk1/2, Akt, and Src after down-regulation of c-Met by Ad-c-Met. The activity of Src, (Src autophosphorylation and phosphorylation of the exogenous substrate enolase) in the immune complex kinase assay is reduced in PC3-LN4 cells infected with Ad-c-Met, with >50% reduction observed in autophosphorylation at an MOI of 25 and >80% reduction of autophosphorylation observed at an MOI of 50, relative to uninfected controls (Fig. 7). Phosphorylation of the exogenous substrate enolase was also reduced relative to uninfected controls in a dose-dependent manner, although to a lesser extent than autophosphorylation. Only a small reduction in Src activity was observed upon infection with high MOIs of the

Table 2 Tumorigenicity and lymph node metastases after mock or intraprostatic infection of adenovirus into nude mice injected intraprostatically with PC3-LN4 cells

Group[a]	Tumorigenicity[b]	Tumor size (g) Median (range)	Lymph node involvement[c]
Sham	8/8	0.66 (0.48–0.99)	8/8
Ad-PU1 (50MOI)	7/8	0.48 (0.4–0.59)	7/7
Ad-c-Met (25MOI)	1/8	0.04	0/1
Ad-c-Met (50MOI)	1/8	0.08	0/1

[a] PC3-LN4 cells (5×10^5) were injected into the prostates of nude mice as described and allowed to grow for 7 days, after which indicated adenovirus (or sham injection) was delivered 7 and 14 days after tumor cell implantation.

[b] Number of mice with tumors/number of mice injected.

[c] Number of mice with lymph node metastases/number of mice with tumors.

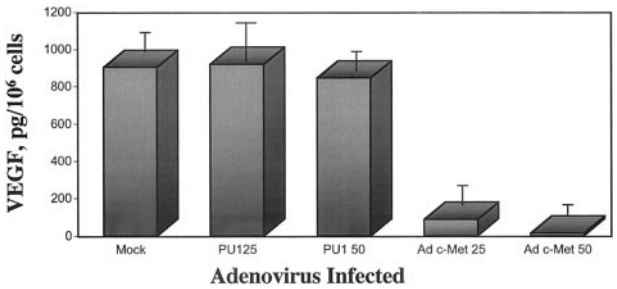

Fig. 6 Expression of VEGF in PC3 Ln-4 cells infected with Ad-c-Met or Ad-PU1. Conditioned medium from PC3-LN4 control and infected cells was harvested after 48 h from cells growing in 1% FBS-containing medium, and VEGF expression was determine by ELISA, as described in "Materials and Methods." To account for differences in growth rates, VEGF expression is expressed as pg VEGF/10^6 cells.

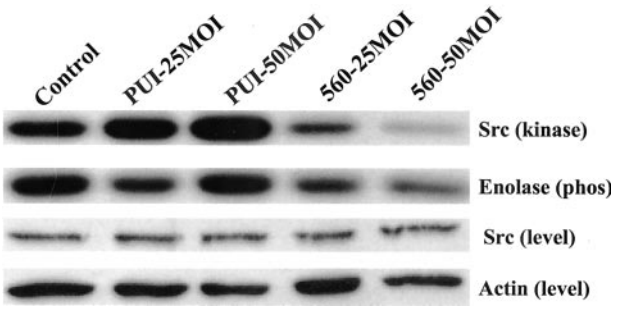

Fig. 7 Expression and activity of c-Src in mock, Ad-c-Met, and Ad-Pu1-infected PC3 LN-4 cells. Cells were mock-infected or infected with adenoviruses at indicated MOIs. To determine Src activity, cell lysates were immunoprecipitated with the Src-specific antibody mAb 327, and the immune complex kinase assay was performed with addition of enolase as an exogenous substrate as described in "Materials and Methods." To determine expression of Src, immunoblotting with mAb 327 was performed as described previously.

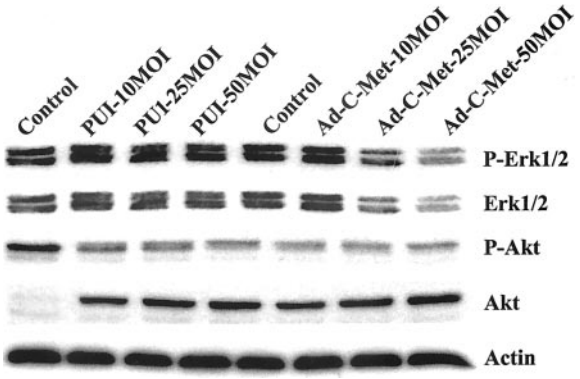

Fig. 8 Expression of phosphorylated and total Erk1/2 and Akt in mock, Ad-c-Met, and Ad-PU-1-infected PC3 LN-4 cells. Cell lysates were subject to SDS-PAGE, electrotransferred to NitroPure nitrocellulose membranes, and incubated with phospho-Akt-specific or phospho-Erk1/2-specific antibodies. Blots were stripped and reprobed with antibodies recognizing phosphorylated and nonphosphorylated Akt or Erk 1/2 as previously described in "Materials and Methods."

control virus, and no obvious changes in Src expression were observed at the MOIs used for control or ribozyme-expressing virus (Fig. 7). These results demonstrate that down-regulation of c-Met down-regulates Src activity. In contrast, adenovirus infection (*i.e.*, either Ad-c-Met or Ad-PU1) appeared to increase expression of Akt, but no differences in phosphorylation were observed between control (Ad-PU1) and ribozyme-expressing (Ad-c-Met) viruses (Fig. 8). Erk1/2 expression appeared somewhat decreased by infection with Ad-c-Met, but this decrease was $<$2-fold and likely reflects the slower proliferation rate of these cells. Thus, decreased VEGF expression observed after c-Met down-regulation correlates with decreased c-Src activity.

Discussion

Despite surgical removal of organ-confined lesions, the biological unpredictability of prostate cancer does not guarantee patient survival. As with most solid tumors, patients that succumb to prostate cancer almost invariably do so because of metastatic spread of the disease. Metastasis of androgen-independent prostate cancer to the bone causes devastating symptoms such as intractable bone pain, nerve compression and pathological fractures, and at this stage, tumors rarely respond to conventional chemotherapies (4, 5). Therefore, development of new treatment modalities inhibiting progression and metastasis of the disease are clearly needed. Inhibition of receptor protein tyrosine kinases has shown considerable promise in the treatment of some tumors. In prostate tumors, aberrant expression of RPTKs is relatively common. For example, members of the ErbB family, specifically HER1, HER2, HER3 (43, 44) but not HER4 (45), are frequently overexpressed. This overexpression is associated with androgen-independent growth of prostate tumor cells, suggesting that overexpression of this gene family promotes tumor progression. One of the most consistently overexpressed RPTKs in prostate cancer is c-Met. Analysis of clinical specimens has linked c-Met overexpression to prostate tumor progression (16–18, 23, 24, 26); however, no studies have examined the cause/effect relationship between c-Met tumor growth and metastasis in an orthotopic model.

In this study, we used PC3-LN4 cells, a variant of PC3-P

cells that by passage through nude mice is increased in metastatic potential to the lymph nodes. Reduction of c-Met expression was achieved by an adenovirus expressing a highly specific c-Met ribozyme as described by Abounader *et al.* (32). The approach of using adenoviruses expressing ribozymes has been highly successful in specifically reducing the expression of diverse gene products such as HER2/neu (46) and Bcl-2 (47). The selectivity of this approach ensures that c-Met alone is targeted, thus allowing specific conclusions to be derived with respect to c-Met function. In our studies, >90% reduction of c-Met expression resulted from adenovirus infection at an MOI of 50, and inhibition of c-Met alone was sufficient to affect biological functions of prostate tumor cells. In colon tumor cells, we have demonstrated that reductions of only ~30% in c-Met expression are sufficient to decrease the ability to form tumors in the livers of nude mice (48). In contrast, in glioblastoma cells, Abounader *et al.* (32) required inhibition of both c-Met and its ligand, HGF, to affect tumor growth. Thus, overexpression alone in PC3P-LN4 cells appears to be sufficient to activate signaling pathways important to the biological properties of these cells. Furthermore, similar *in vitro* effects were observed with PC3P cells (data not shown), suggesting that c-Met overexpression is not important only to selected variants of high metastatic potential.

The main focus of this study was to examine the effect of c-Met down-regulation in tumor cells on *in vivo* growth and metastasis of tumor cells. Therefore, we compared the tumor growth in orthotopic site and development of metastases to the regional lymph nodes. We used two approaches for these studies: *ex vivo* infection of adenoviruses into PC3-LN4P cells followed by orthotopic implantation and orthotopic implantation of tumor cells followed by *in vivo* injection of adenoviruses into the prostate. Down-regulation of c-Met expression after *ex vivo* infection significantly reduced tumor formation or delayed growth in the prostate. Strikingly, *in vivo* delivery of the ribozyme by intraprostatic injection of Ad-c-Met led to highly similar results. Thus, overexpression of c-Met in prostate tumor cells alone appears to play an important role in the ability of these cells to grow. As c-Met can be activated by HGF/SF in the stromal cells of the prostate (49), these results suggest that inhibition of c-Met in the tumor cells may also inhibit receptor activation induced by HGF in the tumor microenvironment. *In vitro* studies also demonstrated that down-regulation of c-Met reduced not only proliferation rates but also cellular migration and cellular invasion and VEGF expression, suggesting that c-Met may play multiple roles in prostate tumor progression.

Although a large number of signaling cascades are induced upon c-Met activation [reviewed by Furge *et al.* (50) and Maulik *et al.* (51)], we demonstrated that decreased c-Src activity, but not Akt or Erk1/2 phosphorylation, occurred upon c-Met down-regulation. This result is consistent with Src constitutively docking with c-Met when c-Met is overexpressed and therefore activated. Strong biochemical (52) and genetic evidence (53) support a role for Src in several c-Met-mediated functions, including migration and proliferation, and activated c-Met requires functional Src for tumorigenic growth of NIH 3T3 cells in nude mice (54). As Src is involved in a number of survival pathways [reviewed by Schlessinger (55)], it is likely that Src activation also contributes to critical survival pathways in prostate tumor growth and/or progression. However, the multifunctional roles of c-Met in activating signal transduction pathways suggest that additional pathways important to tumor progression may be down-regulated as well and that decreased Src activity may be only one of the factors contributing to decreased VEGF expression. Nevertheless, we have demonstrated previously with this model system that ectopic expression of PTEN inhibited the development of lymph node metastases but not that of primary tumors (56). Furthermore, vascularization of tumors was unchanged (56). Ectopic expression of PTEN does not affect c-Src activity (G. E. Gallick, unpublished observations), suggesting that promotion of angiogenesis by c-Met through Src activation may be early event in prostate tumor progression. Although it is difficult to determine whether the down-regulation of c-Met significantly reduced metastatic potential of the PC3-LN4 cells because of low tumorigenicity and low tumor burden (delayed growth), the *in vitro* data showing reduced migration and invasion activity suggests that suppression of metastatic activity of the cells by down-regulation of c-Met is highly probable. Thus, altering gene expression in prostate tumor cell lines and examining the effects in orthotopic models is providing insights as to not only what roles specific gene products play in tumor growth and progression but also where in progression of the disease these alterations are important. These results have potential important therapeutic implications as protein tyrosine kinase inhibitors continue to reach the clinic. Understanding which pathways are required for various stage of prostate tumor progression should lead ultimately to understanding which inhibitors and combinations thereof may be of efficacy in different state tumors.

Acknowledgments

We thank Drs. Roger Abounader and John Laterra (Kennedy Krieger Research Institute) for their generous gift of the control and c-Met ribozyme-expressing adenoviruses, Dr. Isaiah Fidler for his helpful advice in the design of the experiments, and Nila U. Parikh for her excellent technical help.

References

1. Landis, S. H., Murray, T., and Bolden Wingo, P. A. Cancer statistics 1999. Cancer (Phila.), *49:* 8–31, 1999.

2. Soh, S., Kattan, M. W., Berkman, S., Wheeler, T. M., and Scardino, P. T. Has there been a recent shift in the pathological features and prognosis of patients treated with radical prostatectomy? J. Urol., *157:* 2212–2218, 1997.

3. Jacobs, S. C. Spread of prostatic cancer to bone. Urology, *21:* 337–344, 1983.

4. Petrylak, D. P., MacArthur, R. B., O'Conor, J., Shelton, G., Judge, T., Balog, J., Pfaff, C., Bagiella, E., Heitjan, D., Fine, R., Zuech, N., Sawczuk, I., Benson, M., and Olsson, C. A. Phase I trial of docetaxel with estramustine in androgen-independent prostate cancer. J. Clin. Oncol., *17:* 958–967, 1999.

5. Smith, D. C., Esper, P., Strawderman, M., Redman, B., and Poenta, K. J. Phase II trial of oral estramustine, oral etoposide, and intravenous paclitaxel in hormone-refractory prostate cancer. J. Clin. Oncol., *17:* 1664–1671, 1999.

6. Michalopoulos, G. K., and DeFrances, M. C. Liver regeneration. Science (Wash. DC), *276:* 60–66, 1997.

7. Niji, S., Tashiro, K., Koyama, E., Nohno, T., Ohyama, K., Tanuguchi, S., and Nakamura, T. Expression of hepatocyte growth factor gene in endothelial and Kupffer cells of damaged rat livers, as revealed by in

situ hybridization. Biochem. Biophys. Res. Commun., *173:* 42–47, 1990.

8. Vigna, E., Naldini, L., Tamagnone, L., Longati, P., Bardelli, A., Maina, F., Ponzetto, C., and Comoglio, P. M. Hepatocyte growth factor and its receptor, the tyrosine kinase encoded by the c-MET proto-oncogene. Cell Mol. Biol., *40:* 597–604, 1994.

9. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. Molecular cloning and expression of human hepatocyte growth factor. Nature (Lond.), *342:* 440–443, 1989.

10. Gherardi, E., and Stoker, M. Hepatocyte and scatter factor. Nature (Lond.), *346:* 228, 1990.

11. Di Renzo, M. F., Olivero, M., Giacomini, A., Porte, H., Chastre, E., Miroassay, L., Nordlinger, B., Bretti, S., Bottardi, S., and Giordano, S. Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. Clin. Cancer Res., *1:* 147–154, 1995.

12. Schmidt, L., Duh, F. M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S. W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U. R., Feltis, J. T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C. J., Walther, M. M., Tsui, L. C., Geil, L., Orcutt, M. L., Stackhouse, T., Zbar, B., *et al*. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinoma. Nat. Genet., *16:* 68–73, 1997.

13. Rusciano, D., Lorenzoni, P., Burger, M. M. Expression of constitutively activated hepatocyte growth factor/scatter factor receptor (c-met) in B16 melanoma cells selected for enhanced liver colonization. Oncogene, *11:* 1979–1987, 1995.

14. Ferracini, R., Di Renzo, M. F., Scotlandi, K., Baldini, N., Olivero, M., Lollini, P., Cremona, O., Campanacci, M., and Comoglio, P. M. The Met/HGF receptor is overexpressed in human osteosarcomas and is activate by either a paracrine or an autocrine circuit. Oncogene, *10:* 739–749, 1995.

15. Tam, N. N., Chung, S. S., Lee, T., and Wong, Y. C. Aberrant expression of hepatocyte growth factor an its receptor, c-Met, during sex hormone-induced prostatic carcinogenesis in the Noble rat. Carcinogenesis (Lond.), *21:* 2183–2191, 2000.

16. Pisters, L. L., Troncoso, P., Zhau, H. E., Li, W., von Eschenbach, A. C., and Chung, L. W. c-Met proto-oncogene expression in benign and malignant human prostate tissues. J. Urol., *154:* 293–298, 1995.

17. Inoue, K., Chikazawa, M., Karashima, T., Iiyama, T., Kamada, M., Shuin, T., Furihata, M., and Ohtsuki, Y. Overexpression of c-Met/hepatocyte growth factor receptors in human prostatic adenocarcinoma. Acta Med. Okayama, *52:* 305–310, 1998.

18. Zhu, X., and Humphrey, P. A. Overexpression and regulation of expression of scatter factor/hepatocyte growth factor in prostates carcinoma. Urology, *56:* 1071–1074, 2000.

19. Kurimoto, S., Moriyama, N., Horie, S., Sakai, M., Kameyama, S., Akimoto, Y., Hirano, H., and Kawabe, K. Co-expression of hepatocyte growth factor and its receptor in human prostate cancer. Histochem. J., *30:* 27–32, 1998.

20. Chung, L. W., Li, W., Gleave, M. E., Hsieh, J. T., Wu, H. C., Sikes, R. A., Zhau, H. E., Bandyk, M. G., Logothetis, C. J., and Rubin, J. S. Human prostate cancer model: roles of growth factors and extracellular matrices. J. Cell. Biochem. Suppl., *16H:* 99–105, 1992.

21. Takai, K., Hara, J., Matsumoto, K., Hosoi, G., Osugi, Y., Tawa, A., Okada, S., and Nakamura, T. Hepatocyte growth factor is constitutively produced by human bone marrow stromal cells and indirectly promotes hematopoiesis. Blood, 1; *89:* 1560–1565, 1997.

22. Lang, S. H., Clarke, N. W., George, N. J., and Testa, N. G. Scatter factor influences the formation of prostate epithelial cell colonies on bone marrow stroma *in vitro*. Clin. Exp. Metastasis, *17:* 333–340, 1999.

23. Zhau, H. E., Pisters, L. L., Hall, M. C., Zhao, L. L., Troncoso, P., Pollack, A., and Chung, L. W. Biomarkers associated with prostate cancer progression. J. Cell. Biochem. Suppl., *19:* 208–216, 1994.

24. Myers, R. B., and Grizzle, W. E. Changes I biomarker expression in the development of prostatic adenocarcinoma. Biotech. Histochem., *72:* 86–95, 1997.

25. Nishimura, K., Kitamura, M., Takada, S., Nomomura, N., Tsujimura, A., Matsumiya, K., Miki, T., Matsumoto, K., and Okuyama, A. Regulation of invasive potential of human prostate cancer cell lines by hepatocyte growth factor. Int. J. Urol., *5:* 276–281, 1998.

26. Watanabe, M., Fukutome, K., Kato, H., Murata, M., Kawamura, J., Shiraishi, T., and Yatani, R. Progression-linked overexpression of c-Met in prostatic intraepithelial neoplasia and latent as well as clinical prostate cancers. Cancer Lett., *141(1–2):* 173–178, 1999.

27. Davies, G., Jiang, W. G., and Mason, M. D. Cell-cell adhesion molecules and signaling intermediates and their role in the invasive potential of prostate cancer cells. J. Urol., *163:* 985–992, 2000.

28. Mittereder, N., March, K. L., and Trapnell, B. C. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J. Virol., *70:* 7498–7509, 1996.

29. van Leenders, G., van Balken, B., Aalders, T., Hulsbergen-Van De Kaa, C., Ruiter, D., and Schalken, J. Intermediate cells in normal and malignant prostate epithelium express c-Met: implications for prostate cancer invasion. Prostate, *51:* 98–107, 2002.

30. Parr, C., Davies, G., Nakamura, T., Matsumoto, K., Mason, M. D., and Jiang, W. G. The HGF/SF-induced phosphorylation of paxillin, matrix adhesion and invasion of prostate cancer cells were suppressed by NK4, an HGF/SF variant. Biochem. Biophys. Res. Commun., *285:* 1330–1337, 2001.

31. Abounader, R., Lal, B., Luddy, C., Koe, G., Davidson, B., Rosen, E. M., and Laterra, J. *In vivo* targeting SF/HGF and c-met expression via U1snRNA/ribozymes inhibits glioma growth and angiogenesis and promotes apoptosis. FASEB J., *16:* 108–110, 2002.

32. Abounader, R., Ranganathan, S., Lal, B., Fielding, K., Book, A., Dietz, H., Burger, P., and Laterra, J. Reversion of human glioblastoma malignancy by U1 small nuclear RNA/ribozyme targeting of scatter factor/hapatocyte growth factor and c-met expression. J. Natl. Cancer Inst. (Bethesda), *91:* 1548–1556, 1999.

33. Jiang, W. G., Grimshaw, D., Lane, J., Martin, T. A., Abounader, R., Laterra, J., and Mansel, R. E. A hammerhead ribozyme suppresses expression of hepatocyte growth factor/scatter factor receptor c-Met and reduces migration and invasiveness of breast cancer cells. Clin. Cancer Res., *7:* 2555–2562, 2001.

34. Dong, G., Chen, Z., Li, Z. Y., Yeh, N. T., Bancroft, C. C., and Van Waes, C. Hepatocyte growth factor/scatter factor-induced activation of MEK and PI3K signal pathways contributes to expression of proangiogenic cytokines interleukin-8 and vascular endothelial growth factor in head and neck squamous cell carcinoma. Cancer Res., *61:* 5911–5918, 2001.

35. Horiguchi, N., Takayama, H., Toyoda, M., Otsuka, T., Fukusato, T., Merlino, G., Takagi, H., and Mori, M. Hepatocyte growth factor promotes hepatocarcinogenesis through c-Met autocrine activation and enhanced angiogenesis in transgenic mice treated with diethylnitrosamine. Oncogene, *21:* 1791–1799, 2002.

36. Pettaway, C. A., Pathak, S., Greene, G., Ramirez, E., Wilson, M. R., Killion, J. J., and Fidler, I. J. Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. Clin. Cancer Res., *2:* 1627–1636, 1996.

37. Windham, T. C., Parikh, N. U., Siwak, D. R., Summy, J. M., McConkey, D. J., Kraker, A. J., and Gallick, G. E. Src activation regulates anoikis in human colon tumor cell lines. Oncogene, *21:* 7797–7807, 2002.

38. Reinmuth, N., Fan, F., Liu, W., Parkh, A. A., Stoeltzing, O., Jung, Y. D., Bucana, C. D., Radinsky, R., Gallick, G. E., and Ellis, L. M. Impact of insulin-like growth factor receptor-1 function on angiogenesis, growth, and metastasis of colon cancer. Lab. Investig., *82:* 1377–1389, 2002.

39. Reinmuth, N., Liu, W., Jung, Y. D., Ahmad, S. A., Shaheen, R. M., Fan, F., Bucana, C. D., McMahon, G., Gallick, G. E., and Ellis, L. M. Induction of VEGF in perivascular cells defines a potential paracrine

mechanism for endothelial cell survival. FASEB J., *15:* 1239–1241, 2001.

40. Cheng, S. Y., Nagane, M., Huang, H. S., and Cavenee, W. K. Intracerebral tumor-associated hemorrhage caused by overexpression of the vascular endothelial growth factor isoforms VEGF121 and VEGF165 but not VEGF189. Proc. Natl. Acad. Sci. USA, *94:* 12081–12087, 1997.

41. Van Belle, E., Witzenbichler, B., Chen, D., Silver, M., Chang, L., Schwall, R., and Isner, J. M. Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor: the case for paracrine amplification of angiogenesis. Circulation, *97:* 381–390, 1998.

42. Kuba, K., Matsumoto, K., Date, K., Shimura, H., Tanaka, M., and Nakamura, T. HGF/NK4, a four-kringle antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppresses tumor growth and metastasis in mice. Cancer Res., *60:* 6737–643, 2000.

43. Zhau, H. E., Wan, D. S., Zhou, J., Miller, G. J., and von Eschenbach, A. C. Expression of c-erb B-2/neu proto-oncogene in human prostatic cancer tissues and cell lines. Mol. Carcinog., *5:* 320–327, 1992.

44. Kuhn, E. J., Kurnot, R. A., Sesterhenn, I. A., Chang, E. H., and Moul, J. W. Expression of the c-erbB-2 (HER-2/neu) oncoprotein in human prostatic carcinoma. J. Urol., *150:* 1427–1433, 1993.

45. Robinson, D., He, F., Pretlow, T., and Kung, H. J. A tyrosine kinase profile of prostate carcinoma. Proc. Natl. Acad. Sci. USA, *93:* 5958–5962, 1996.

46. Suzuki, T., Anderegg, B., Ohkawa, T., Irie, A., Engebraaten, O., Halks-Miller, M., Holm, P. S., Curriel, D. T., Kashani-Sabet, M., and Scanlon, K. J. Adenovirus mediated ribozyme targeting of HER-2/neu inhibits *in vivo* growth of breast cancer cells. Gene Ther., *7:* 241–248, 2000.

47. Gibson, S. A., Pellenz, C., Huchison, R. E., Davey, F. R., and Shillitoe, E. J. Induction of apoptosis in oral cancer cells by an anti-bcl-2 ribozyme delivered by an adenovirus vector. Clin. Cancer Res., *6:* 213–222, 2000.

48. Herynk, M. H., Stoeltzing, O., Reinmuth, N., Parikh, N. U., Abounader, R., Laterra, J., Radinsky, R., Ellis, L. M., and Gallick, G. E. Down-regulation of c-Met inhibits growth in the liver of human colorectal carcinoma cells. Cancer Res., *63:* 2990–2996, 2003.

49. Gmyrek, G. A., Walburg, M., Webb, C. P., Yu, H. M., You, X., Vaughan, E. D., Vande Woude, G. F., and Knudsen, B. S. Normal and malignant prostate epithelial cells differ in their response to hepatocyte growth factor/scatter factor. Am. J. Pathol., *159:* 579–590, 2001.

50. Furge, K. A., Zhang, Y. W., and Vande Woude, G. F. Met receptor tyrosine kinase: enhanced signaling through adapter proteins. Oncogene, *19:* 5582–5589.

51. Maulik, G., Shrikhande, A., Kijima, T., Ma, P. C., Morrison, P. T., and Salgia, R. Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. Cytokine Growth Factor Rev., *13:* 41–59, 2002.

52. Rahimi, N., Hung, W., Tremblay, E., Saulnier, R., and Elliott, B. c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. J. Biol. Chem., *273:* 33714–33721, 1998.

53. Maina, F., Pante, G., Helmbacher, F., Andres, R., Porthin, A., Davies, A. M., Ponzetto, C., and Klein, R. Coupling Met to specific pathways results in distinct developmental outcomes. Mol. Cell, *7:* 1293–1306, 2001.

54. Nakaigawa, N., Weirich, G., Schmidt, L., and Zbar, B. Tumorigenesis mediated by MET mutant M1268T is inhibited by dominant-negative Src. Oncogene, *19:* 2996–3002, 2000.

55. Schlessinger, J. New roles for Src kinases in control of cell survival and angiogenesis. Cell, *100:* 293–296, 2000.

56. Davies, M. A., Kim, S. J., Parikh, N. U., Dong, Z., Bucana, C. D., and Gallick, G. E. Adenoviral-mediated expression of MMAC/PTEN inhibits proliferation and metastasis of human prostate cancer cells. Clin. Cancer Res., *8:* 1904–1914, 2002.