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**ENHANCED INVASION OF HORMONE REFRACTORY PROSTATE CANCER
CELLS THROUGH HEPATOCYTE GROWTH FACTOR (HGF) INDUCTION OF**

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Enhanced Invasion of Hormone Refractory Prostate Cancer Cells Through Hepatocyte Growth Factor (HGF) Induction of Urokinase-Type Plasminogen Activator (u-PA)

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BACKGROUND. Increased expression of the hepatocyte growth factor (HGF) receptor (MET) is associated with high-grade prostatic adenocarcinoma and metastasis. However, the mechanism through which MET signaling contributes to prostate cancer (CaP) metastasis remains unclear.

METHODS. Human PC-3 CaP cells and in vivo selected, isogenic variant cells of increasing metastatic potential (PC-3M, PC-3M-Pro4, and PC-3M-LN4) were used to investigate the effect of HGF on CaP cell growth, protease production, and invasion. Cell-free urokinase-type plasminogen activator (u-PA) expression and function following HGF treatment were analyzed by Western blot, ELISA, and casein/plasminogen zymography. In vitro invasion stimulated by HGF was measured using Matrigel-coated invasion chambers.

RESULTS. Both mRNA and functional protein for MET were detected in each of the CaP cell lines. HGF treatment (0–40 ng/ml) weakly increase proliferation, however, HGF induced soluble u-PA protein and activity 3-fold in the metastatic variant cells. HGF significantly stimulated the invasion of highly metastatic PC-3M-LN4 cells through Matrigel and treatment with specific urokinase receptor inhibitors diminished the HGF-stimulated invasion in a dose-dependent manner.

CONCLUSIONS. These results demonstrate the biological significance of u-PA up-regulation in response to HGF in highly metastatic hormone refractory CaP cells. *Prostate* 59: 167–176, 2004. © 2003 Wiley-Liss, Inc.

KEY WORDS: prostate cancer; invasion; tyrosine kinase receptor; metastasis; proteolytic enzymes

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INTRODUCTION

Osteoblastic lesions of the pelvis and vertebral column are the most frequent distant metastases formed in human prostate cancer (CaP). Recent autopsy data suggest that more than 80% of all men who die of CaP have metastatic disease in the bone [1,2]. Analysis of factors present in the bone microenvironment may yield important information regarding the high prevalence of bone metastases in human CaP.

Hepatocyte growth factor (HGF) or scatter factor, is expressed in the bone microenvironment. HGF signals through the receptor tyrosine kinase MET to mediate (in a cell-type specific manner) invasion, survival, proliferation, migration, morphogenesis, and angiogenesis [3]. Human bone stromal cells have been shown to express HGF [4,5] and human bone marrow stromal fibroblasts specifically stimulate the growth of LNCaP cells in vivo [6]. HGF has also been reported to promote CaP colony formation on bone marrow stromal cells in vitro [7]. Immunohistochemical analysis of CaP surgical specimens has demonstrated a relationship between MET expression and CaP progression [8,9]. These analyses have revealed that bone and lymph node metastatic CaP cells have a higher frequency of MET expression compared to CaP metastases at other sites suggesting an increased significance of MET expression in the context of the bone [8]. Taken together, the data suggest that CaP cells are responsive to HGF, which may promote CaP cell growth in the bone environment.

HGF is secreted as an inactive, single chain protein and is sequestered in the extracellular space by heparin-sulfate proteoglycans [10,11]. To become active, HGF must be cleaved into a two-chain heterodimer by the action of serine proteases. Urokinase-type plasminogen activator (u-PA) is a serine protease that has been shown to activate HGF in vitro [12–15]. It is unclear whether HGF is a physiological substrate for u-PA in vivo. However, during liver regeneration, hepatocyte-derived u-PA is believed to initiate ECM degradation that results in the release and subsequent activation of HGF [16,17]. In a similar fashion, metastatic tumor cells may generate increased HGF levels in the organ microenvironment through the production of u-PA. Recent evidence has further demonstrated that HGF can in turn effect u-PA expression. A number of cell lines have been shown to up-regulate u-PA in response to HGF in vitro [18–26] presumably through increased transcription following Map kinase/AP-1 activation down stream of MET [27]. CaP cell lines have been shown to express u-PA in vitro and u-PA over expression in Mat Ly Lu CaP cells was shown to enhance bone metastasis following intracardiac injection. The demonstration that u-PA can activate HGF

and that HGF can in turn increase u-PA expression suggests that u-PA and HGF are part of a paracrine loop which may promote tumor cell invasion in vivo. However, the mechanism through which HGF/MET signaling contribute to CaP metastasis remains unclear.

To address this question, we examined whether HGF/MET signaling mediates the invasiveness of CaP cells through HGFs ability to induce the expression of proteases associated with metastasis. We report that HGF can up-regulate u-PA protein production and stimulate invasion through Matrigel of highly metastatic human CaP cells. Moreover, we provide evidence that the mechanism for this effect is u-PA dependent as inhibitors that target the urokinase receptor diminish HGF-stimulated invasion.

MATERIALS AND METHODS

Cells and Culture Conditions

The human PC-3 CaP cell line was obtained from the American Type Culture Collection (Rockville, MD). PC-3M was established from liver metastases produced in nude mice subsequent to intrasplenic injection of PC-3 cells [28]. Low-metastatic PC-3M-Pro4 cells were generated following the initial intraprostatic injection of PC-3M cells and subsequent orthotopic cycling of resultant prostate tumor cells four rounds into the prostate of nude mice [29]. Likewise, high-metastatic PC-3M-LN4 cells were isolated from the parental PC-3M cells subsequent to intraprostatic injection and in vivo recycling of lymph node tumor cells [29]. All cell lines were maintained on plastic in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 1× penicillin-streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 1× vitamin solution (GIBCO, Grand Island, NY), and incubated in 5% CO₂–95% air at 37°C. The cells used in all experiments, with the exception of PC-3 cells, were between passage 6 and 20. All were free of Mycoplasma.

Northern Blot Analysis

mRNA was extracted from subconfluent populations of cells using the FastTrackTM mRNA isolation kit (Invitrogen, Inc., San Diego, CA). Poly(A⁺) RNA (5 µg/well) was fractionated on a 1% formaldehyde/agarose gel, electrotransferred at 0.8 A to GeneScreen nylon membranes (DuPont, Boston, MA), and UV cross-linked with 120,000 µJ/cm² using a UV Stratallinker 1800 (Stratagene, La Jolla, CA).

Probes for Northern Blot Analysis

The probes used for Northern hybridization corresponded to full-length human c-Met cDNA (kind gift

from G. Vande Woude), to full-length EGF-R cDNA (courtesy of Dr F. Kern and Dr M. Lippman), and to rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Each cDNA fragment was radio labeled with the rediprime random primer labeling kit (Amersham Corp., Adington Heights, IL) using [α - 32 P] deoxycytidine 5'-triphosphate. All hybridizations were performed at 65°C with 5×10^6 cpm/blot for 4 hr in rapid-hyb buffer (Amersham Corp.) following at least a 1 hr pre-hybridization in rapid-hyb buffer (Amersham Corp.).

Immunoprecipitation and Western Blotting

Cells were seeded to 6-well plates at a density of 1.5×10^5 cells per 2.0 ml complete medium and allowed to attach 24 hr. Following this incubation, the medium was replaced with 2.0 ml serum-free medium/well and the cells serum starved for 24 hr. Human recombinant hepatocyte growth factor (R&D Systems, Minneapolis, MN) was then added directly to serum-free conditioned medium to a final concentration of 40 ng/ml and incubated for 15 min in 5% CO₂-95% air at 37°C. Serum starved cells were also treated with human epidermal growth factor (40 ng/ml, GIBCO-BRL) and FBS (5% v/v) to serve as negative controls for MET activation.

Following growth factor treatment, cells were washed once on ice with ice-cold PBS containing 5 mM EDTA and 1 mM sodium orthovanadate and scraped into 0.25 ml/well ice-cold lysis buffer [1% Triton X-100, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol (v/v), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 50 μ g/ml trypsin inhibitor, 1 mM sodium orthovanadate]. Cell lysates were transferred to 1.5 ml tubes and incubated 20 min on ice to allow complete lysis. Cell lysates were then clarified by centrifugation (5 min at 16,000g and 4°C) and aliquots of each were removed for protein determination by the DC protein assay (Bio-Rad, Hercules, CA).

Equal amounts of protein (200 μ g/sample) were diluted in 1.0 ml RIPA buffer [PBS with 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS] and incubated 1 hr at 4°C with 0.5 μ g/sample anti-human MET mAb UB2 (UBI, Charlottesville, VA). Following this incubation, the immune complexes were precipitated overnight at 4°C with 20 μ l/sample protein A/G sepharose (Onco-gene, Cambridge, MA). Samples were washed three times in RIPA buffer, boiled in 5 \times reducing sample buffer, and resolved by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). Separated proteins were transferred onto 0.45 μ m nitrocellulose membranes (Amersham Corp.). The filters were blocked with 3% BSA in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and probed with mouse

anti-phospho-tyrosine mAb (1:5,000, UBI) followed with horseradish-peroxidase conjugated donkey anti-mouse IgG (IgG-HRP) (1:2,000, Amersham Corp.). Protein bands were visualized using the ECL detection system (Amersham Corp.). To demonstrate equal loading, the blots were stripped and re-probed with rabbit anti-human MET pAb C-28 (1:1,000, Santa Cruz, Santa Cruz, CA) followed by donkey anti-rabbit IgG-HRP (1:2,000, Amersham Corp.).

Cell Proliferation Assay

Cells were seeded to 96-well plates at a density of 1.5×10^3 cells per 0.2 ml complete medium and allowed to attach 24 hr. Following this incubation, the cells were washed once with serum-free RPMI and replaced with medium containing 0 or 5% FBS (v/v) and 0 or 40 ng/ml of either EGF or HGF in triplicate. Cells were cultured for 96 hr. The last 2 hr, MTT was added to a final concentration of 1 mM. The formazan product was dissolved in DMSO and absorbencies were read on a Dynatech MR 5000 plate reader at 570 nm.

Analysis of Cell-Free u-PA

The amount of u-PA protein secreted into the culture medium (cell-free u-PA) was determined by Western blotting as described previously [22]. Briefly, cells seeded for Western blotting were washed and treated with 1% FBS-containing medium supplemented with 0 or 40 ng/ml EGF or HGF for 48 hr in 5% CO₂-95% air at 37°C. Conditioned medium was prepared for Western analysis by filtration through 0.2 μ m Acrodisc syringe filters (Gelman Sciences, Ann Arbor, MI). The underlying cells were trypsinized and the viable cell number determined by trypan blue exclusion. To control for differences in proliferation rates among each treatment group, aliquots of conditioned media from equal number of cells (4,000 cells/lane) were diluted with 5 \times non-reducing sample buffer and resolved by 10% SDS-PAGE. Filters were probed with polyclonal rabbit anti-human u-PA #389 (1:1,000, American Diagnostica, Greenwich, CT) followed with donkey anti-rabbit IgG-HRP (1:2,000, Amersham Corp.). Cell-free u-PA in 48 hr conditioned medium was also quantified by Immubind ELISA kit (American Diagnostica) according to manufacture's protocol using a 1:50 dilution of conditioned medium.

Cell-free u-PA activity was assessed by Casein/plasminogen zymography as described with modifications [30]. Briefly, 48 hr conditioned medium, adjusted for differences in proliferation rates (2,000 cells/lane), was separated under non-reducing conditions on a 10% SDS-PAGE containing 0.1% (w/v) casein (Sigma, St. Louis, MO) and 10 μ g/ml human plasminogen (Calbiochem, La Jolla, CA). Following electrophoresis,

the gels were incubated at 37°C for 2 hr in buffer A [2.5% (v/v) Triton X-100, 50 mM Tris-HCl (pH 7.5), 0.05% (w/v) sodium azide] followed by 10 hr at 37°C in buffer B [0.15 M NaCl, 100 mM Tris-HCl (pH 7.5)]. Plasminogen-dependent proteolysis was detected as a clear zone following staining with 0.25% Coomassie blue in 10% acetic acid/40% methanol.

Densitometry

The u-PA protein levels were quantified in the linear range of the film on a densitometer (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant software program. Sample measurements were calculated as the ratio of the average volume of the u-PA specific spot over the cell type untreated control.

In Vitro Invasion Assay

In vitro invasion through Matrigel-coated filters was performed as described with modifications [22]. Briefly, cells were harvested with trypsin, washed once with serum-free medium, and re-suspended in 5% FBS medium to a concentration of 5×10^4 viable cells/ml. 25,000 cells/0.5 ml were added to each well of a 24-well Matrigel-coated invasion chambers (Becton Dickinson, Bedford, MA). The chambers were placed in wells containing 0.5 ml of RPMI-1640 supplemented with 5% FBS and 0 or 40 ng/ml of EGF or HGF and incubated for 48 hr in 5% CO₂-95% air at 37°C. At the end of the incubation period, non-invading cells were removed with a cotton swab from the top of the filter and the invading cells stained with Hema 3[®] solution (CMS, Houston, TX). The total number of invading cells/filter was quantified under 10 \times magnification.

Where indicated, a final concentration of u-PA amino-terminal fragment (u-PA-ATF) of 2.5 μ M (corresponding to amino acids 1-143; kindly provided by J. Henkin, Abbott Laboratories, Abbott Park, IL) or a u-PA receptor binding antagonist peptide Å5 of 50-500 nM (corresponding to u-PA amino acids 20-30) was added to the plated cells. Å5 was synthesized and cyclized using a covalent linker [31] and inhibits the binding of ¹²⁵I-DFP-urokinase to RKO cells with an IC₅₀ of approximately 11 nM [32]. Each compound was added at the time of cell plating to demonstrate involvement of the u-PA proteolysis network in the HGF-stimulated invasion of CaP cells. u-PA-ATF and Å5 were non-toxic at the doses utilized and had no effect on cell viability or proliferation ([32], data not shown). In separate experiments, a final concentration of HGF neutralizing antibody (R&D systems) of 10 μ g/ml was added to the lower chamber to show HGF specific effect on invasion.

Statistical Analysis

Cellular proliferation rates, densitometric analyses, and cell-free u-PA production were compared by the unpaired Student's *t*-test.

RESULTS

mRNA and Protein Analysis of MET Expression in High Versus Low Metastatic Human CaP Cells

Given that MET expression directly correlates with high-grade prostatic adenocarcinomas and metastases in humans, we examined whether the PC-3 isogenic cell lines express the MET molecule. Northern blot analyses of steady-state transcripts demonstrated that each of the cell lines tested express high and equivalent amounts of the 7.0 kb Met message (Fig. 1, lanes A-D). Western blot analysis with anti-sera to MET also demonstrated that the MET protein was equally expressed among each cell line (Fig. 2). Biological activity was demonstrated following HGF treatment as shown by the specific phosphorylation of the 145 kDa β chain of MET (Fig. 2, lanes D,H,L,P). Only 5% serum and hEGF were included as negative controls for MET phosphorylation. These data demonstrate that the PC-3 isogenic cell lines express high levels of the functional HGF receptor, MET.

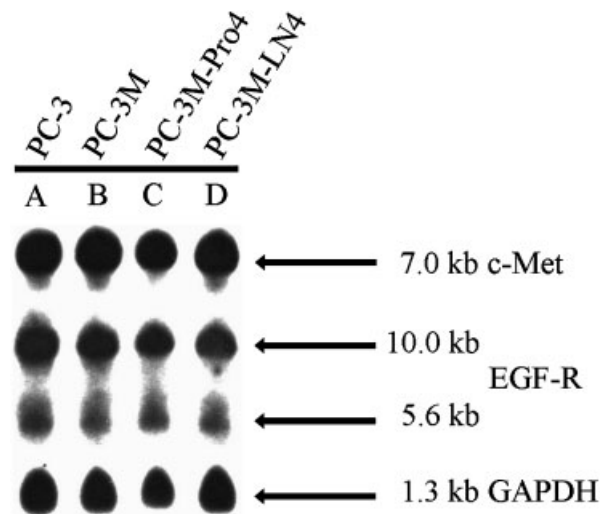


Fig. 1. Steady-state mRNA analysis of PC-3 isogenic prostate carcinoma cell lines. Poly(A⁺) mRNA was isolated from PC-3, parental PC-3M, low-metastatic PC-3M-Pro4, and high-metastatic PC-3M-LN4 CaP cells by oligo (dT)-cellulose chromatography. A 5 μ g/lane was fractionated on a 1% formaldehyde agarose gel, electrotransferred to nylon membrane, and hybridized with cDNA probes to MET, EGF-R, and as a loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

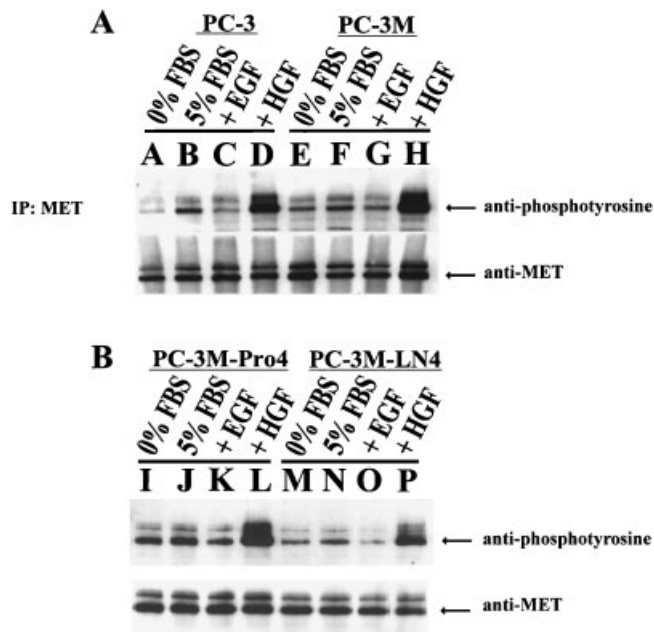


Fig. 2. Western blot analysis for functional MET protein. PC-3, parental PC-3M, low-metastatic PC-3M-Pro4, and high-metastatic PC-3M-LN4 CaP cells were plated in complete medium, serum-starved 18 hr, and treated with HGF or EGF at 40 ng/ml or serum (5% v/v) for 15 min prior to lysis. Two hundred micrograms of total protein was immunoprecipitated with anti-MET UB2 mAb and separated by 7.5% SDS-PAGE. Nitrocellulose filters were probed separately with anti-sera specific to phosphotyrosine for activity and MET C-28 pAb for loading. The immunoreactive bands were detected by incubation with peroxidase-conjugated secondary anti-sera and visualized using the ECL system. 5% serum and HGF were included as negative controls for MET phosphorylation.

Effect of HGF on the Proliferation of Metastatic Human CaP Cells

Despite the presence of functional MET protein, treatment with recombinant human HGF resulted in only weak proliferative capacity in each of the cell lines tested. Figure 3 shows the mean percent increase over untreated controls \pm standard deviation of triplicate wells from a representative MTT proliferation assay. The data indicate that each of the CaP cell types was similarly stimulated to a maximum of 30% above untreated controls following treatment with 40 ng/ml HGF in 5% serum containing medium. These data demonstrate that HGF weakly stimulates the anchorage-dependent growth of the PC-3 isogenic cell lines in vitro.

Induction of u-PA in Metastatic CaP Cells Following Ligand Activation of MET

As HGF treatment stimulated anchorage-dependent growth similarly in PC-3 CaP cells of different meta-

static potentials, we analyzed the relationship between HGF/MET signaling and u-PA protein production. Analyses of u-PA protein secreted into the culture medium (cell-free u-PA) in both the low metastatic PC-3M-Pro4 cells and the high metastatic PC-3M-LN4 cells is shown in Figure 4. Cell-free u-PA could be detected in untreated control cells after 48 hr. Consistent with similar levels of functional MET protein in both cell lines, exposure to HGF (40 ng/ml) resulted in a 2–3-fold elevation over untreated controls in both low metastatic PC-3M-Pro4 and highly metastatic PC-3M-LN4 cells, respectively (Fig. 4a, lanes D,G). EGF did not stimulate cell-free u-PA protein production. Enzyme-linked immunosorbant assay (ELISA) for cell-free u-PA protein confirmed the HGF-stimulated increase observed by Western blotting (Table I). Casein/plasminogen zymography for u-PA activity following HGF treatment showed enhanced protein activity similar to that observed in the Western blot and ELISA analyses. These data indicate that HGF increases cell-free u-PA protein levels directly correlating with enhanced activity of u-PA (Fig. 4b, lanes D,G). These results indicate that ligand activation of MET results in the up-regulation of functional, cell-free u-PA protein in metastatic variants of human PC-3 CaP cells.

Induction of In Vitro Invasion in Metastatic CaP Cells Following Ligand Activation of MET

To determine if a >3-fold increase in cell-free u-PA protein is physiologically relevant, in vitro invasion assays were performed. Figure 5a shows the fold increase over untreated controls, mean \pm the standard error, of three separate experiments comparing the high-metastatic PC-3M-LN4 cells. These data indicate that HGF, but not EGF or 5% serum, increased the invasion of highly metastatic PC-3M-LN4 cells greater than 59-fold over untreated control cells ($P < 0.002$). HGF similarly stimulated the invasion of low metastatic PC-3M-Pro4 cells 5-fold consistent with the observed increase in cell-free u-PA in these cells (data not shown). These results support a role for HGF in the invasion of metastatic CaP cells in vitro.

To test the direct involvement of u-PA in HGF-stimulated in vitro invasion, the assays were repeated in the presence of the specific u-PA receptor inhibitors u-PA-amino terminal fragment (u-PA-ATF) or the u-PA receptor binding antagonist peptide, $\Delta 5$. The addition of u-PA-ATF at 2.5 μ M resulted in greater than 50% inhibition of HGF-stimulated invasion ($P < 0.02$; Fig. 5B). The addition of $\Delta 5$ at 50–500 nM resulted in a dose-dependent inhibition to baseline of PC-3M-LN4 HGF-stimulated invasion (Fig. 5C). Further, the addition of HGF neutralizing antibody eliminated the HGF-stimulated invasion showing that HGF is responsible

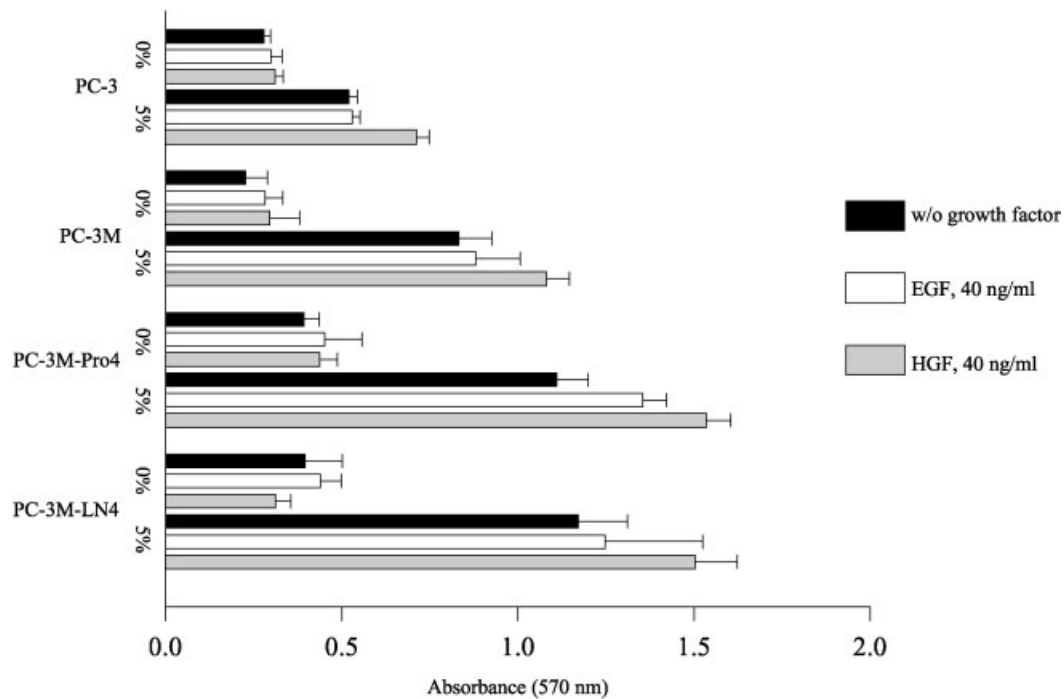


Fig. 3. Effect of HGF on proliferation in vitro. PC-3, parental PC-3M, low-metastatic PC-3M-Pro4, and high-metastatic PC-3M-LN4 CaP cells ($1,500/\text{well}$) were treated with 0 or 40 ng/ml HGF or EGF in 0 or 5% serum-containing medium for 96 hr. MTT (1 mM) was added for the last 2 hr of the incubation. Formazan product was solubilized in DMSO and measured at OD 570 nm. The data is presented as the mean percent increase over untreated controls \pm standard deviation of triplicate wells from a representative MTT proliferation assay.

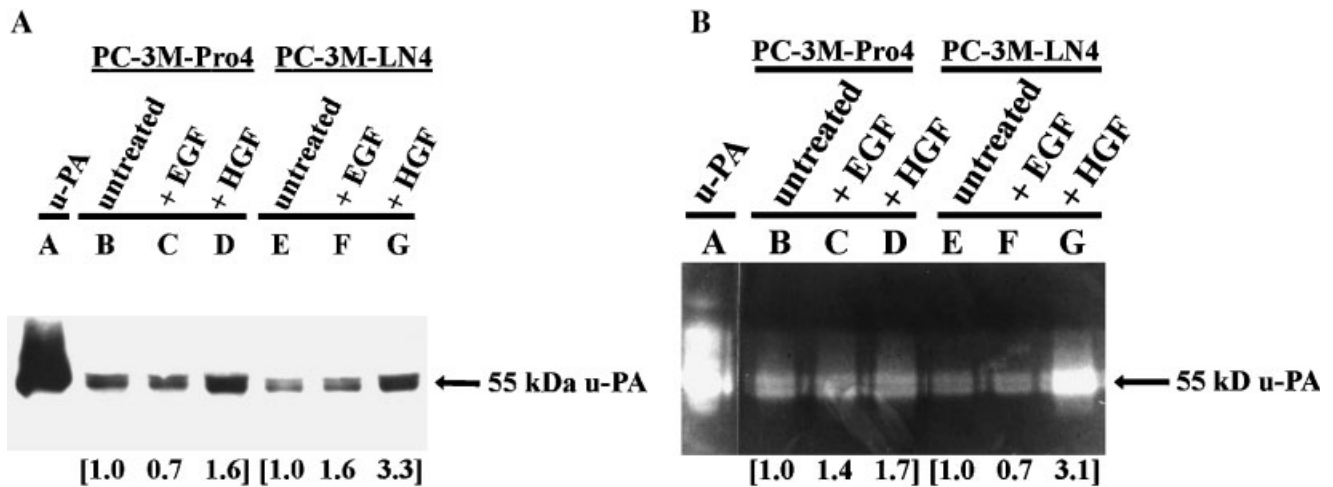


Fig. 4. HGF treatment increases cell-free urokinase-type plasminogen activator (u-PA) protein production. **A:** Low-metastatic PC-3M-Pro4 and high-metastatic PC-3M-LN4 CaP cells ($1.5 \times 10^5/\text{well}$) were treated with or 0 or 40 ng/ml HGF or EGF in 1% serum containing medium for 48 hr. Conditioned medium from equal numbers of cells (4,000/lane) was resolved under non-reducing conditions by 10% SDS-PAGE and transferred to nitrocellulose. Filters were probed with anti-serum specific to human u-PA, detected by incubation with peroxidase-conjugated secondary anti-serum, and visualized using the ECL system. **B:** Effect of HGF on u-PA activity. Forty-eight hours serum-free conditioned medium from equal numbers of cells (2,000/lane) was resolved under non-reducing conditions by 10% SDS-PAGE impregnated with casein and plasminogen. Following a brief renaturation period, gels were stained with coomassie blue. Zones of clearing visible after light destaining indicate active protease.

TABLE I. Effects of HGF on Cell-Free u-PA Production by Human CaP Cells^a

	PC-3	PC-3M	PC-3M-Pro4	PC-3M-LN4
w/o Growth Factor	1.0 ± 0.1 (576.2)	1.0 ± 0.1 (132.6)	1.0 ± 0.1 (948.8)	1.0 ± 0.2 (174.4)
EGF, 40 ng/ml	1.2 ± 0.2 (692.5)	0.7 ± 0.0 (98.6)	1.3 ± 0.1 (1213.9)	1.5 ± 0.1 (264.5)
HGF, 40 ng/ml	1.1 ± 0.0 (631.3)	1.6 ± 0.2 (215.1)	1.1 ± 0.2 (1006.9)	3.9 ± 0.0* (685.8)

^aThe data is presented as the fold increase over untreated control ± standard deviation of duplicate wells. The mean protein concentration of cell-free u-PA (ng/ml/10⁶ cells) appears in parentheses. One representative experiment of four.

**P* < 0.002, PC-3M-LN4 compared to PC-3, PC-3M, or PC-3M-Pro4.

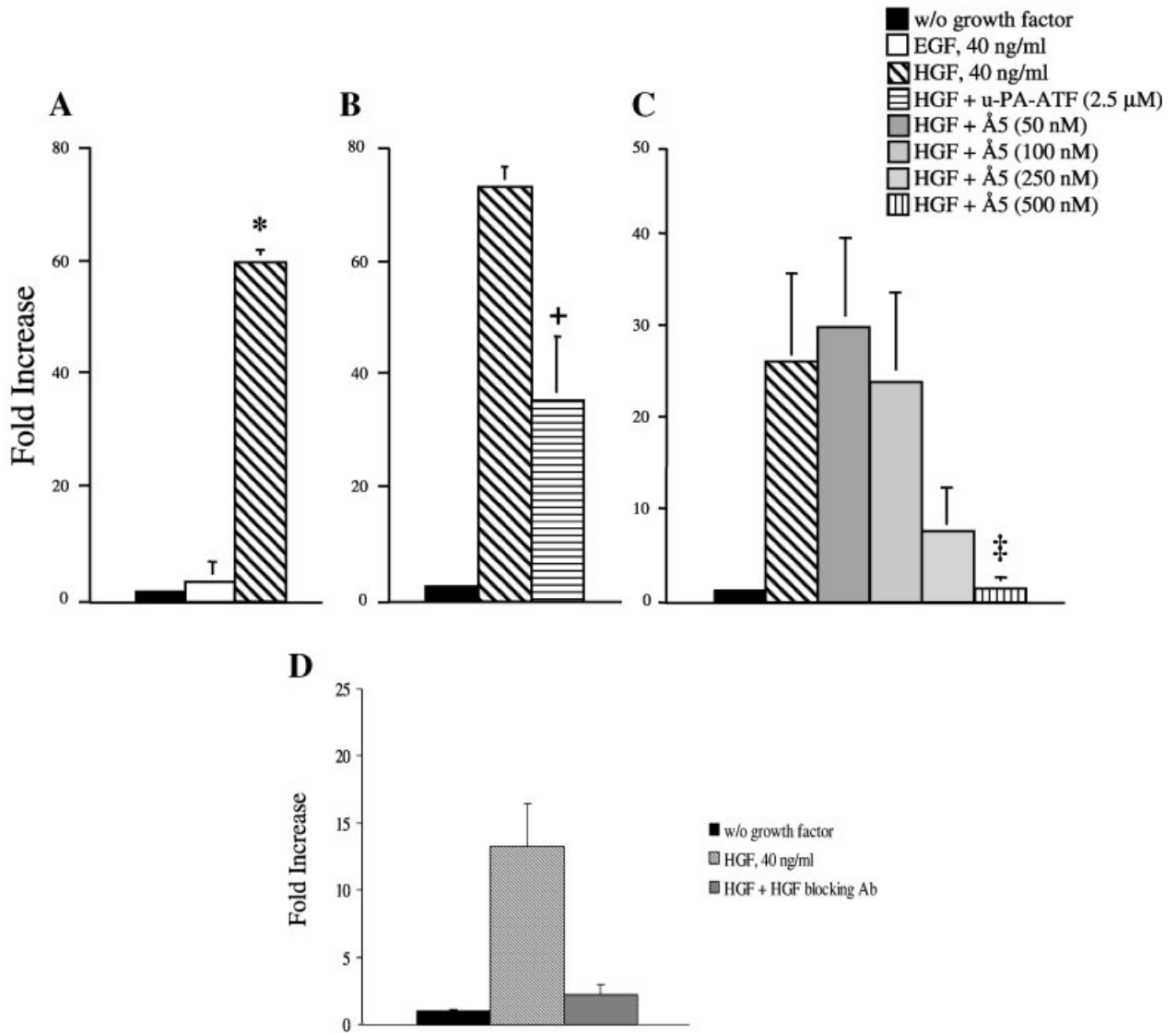


Fig. 5. HGF induces in vitro invasion via a u-PA dependent mechanism. **A:** High-metastatic PC-3M-LN4 CaP cells (25,000/0.5 ml) were seeded in serum-containing medium to the upper chamber of a Matrigel invasion chamber. Five percent serum-containing medium supplemented with 0 or 40 ng/ml HGF or EGF was placed in the lower chamber. After 48 hr, invading cells were stained and quantified. The data is presented as the fold increase over untreated controls, mean ± standard error, of three separate experiments. **B:** In a separate experiment, 2.5 μM u-PA receptor antagonist u-PA-amino terminal fragment (u-PA-ATF) was added to the upper chamber and the effect on invasion quantified. The data is presented as the fold increase over untreated control, mean ± standard deviation, of duplicate wells. **C:** In a separate experiment, 0 – 500 nM u-PA receptor antagonist peptide, Å5, was added and the effect on invasion quantified, mean ± standard deviation, of duplicate wells. **D:** A final concentration of HGF neutralizing antibody of 10 μg/ml was added to the lower chamber to show HGF specific effect on invasion. The data is presented as the fold increase over untreated controls, mean ± standard error, of three separate experiments.

for the u-PA dependent invasion (Fig. 5D). Taken together, these data demonstrate that HGF specifically increases the in vitro invasion of metastatic human CaP cells through the up-regulation of the u-PA proteolysis network.

DISCUSSION

In order for a tumor cell to colonize a distant site, it must successfully interact with the organ microenvironment. Chung et al. [4] hypothesized that the predilection of CaP cells to metastasize to the bone is a result of reciprocal interactions between tumor and stromal-derived growth factors [33]. The strong association of MET expression in bone lesions of CaP and the high expression of HGF in the bone stroma led us to examine the role of HGF/MET signaling in CaP invasion and metastasis.

In this study, human, isogenic CaP cells with different metastatic propensities were shown to express high and equal amounts of a functional MET protein. However, despite this functional receptor, treatment of these cells with HGF proved to be weakly mitogenic, stimulating each cell type approximately 30% above untreated controls. Analysis of protease production in response to HGF revealed that HGF increased the levels of cell-free u-PA, approximately 2–3-fold in both low metastatic PC-3M-Pro4 cells and highly metastatic PC-3M-LN4 cells. The modest increase in cell-free u-PA protein following HGF treatment, however resulted in a significant increase in in vitro invasion. In Matrigel-coated invasion chamber assays, chemo-attractant HGF, but not 5% serum or EGF, stimulated the invasion of the high metastatic PC-3M-LN4 cells 59-fold above untreated controls. Furthermore, treatment with non-toxic doses of the specific urokinase receptor inhibitors u-PA-ATF or A5 resulted in a dose-dependent reduction of the HGF-stimulated invasion. Taken together, these results support the biological significance of u-PA up-regulation in response to HGF in metastatic CaP cells.

Recently, others have reported that HGF enhances the in vitro attachment to and subsequent invasion through Matrigel of DU145 and PC-3 human CaP cells [34–36]. It has also been shown that the serum level of HGF in men with metastatic CaP is significantly elevated compared to men with localized disease [37]. These data are in agreement with our own and further support a role for HGF in CaP invasion and metastasis. The analyses presented herein support a mechanism where MET positive CaP cells utilize bone stromal-derived HGF to up-regulate u-PA and promote invasion of the skeleton.

In addition to mediating bone-specific metastasis in CaP, HGF/MET may also participate in the formation

of an osteoblastic lesion. This is a unique feature of CaP metastases and represents 95% of all bone metastases formed by CaP [38]. Recently, HGF was described as a factor coupling osteoblast/osteoclast functions to the re-organization of normal bone structure [39,40]. Both osteoblasts and osteoclasts were shown to express a functional MET. HGF is a strong osteoblast mitogen and it stimulates chemotactic migration and bone resorption by osteoclasts in the presence of osteoblasts. It is in this fashion that HGF may participate in the clinical manifestations of CaP, the formation of an osteoblastic metastasis.

Based on our in vitro findings, interfering with CaP metastasis by abrogating either the u-PA proteolysis network or HGF/MET signaling may be feasible. Indeed, the direct inhibition of u-PA by the enforced expression of the plasminogen activator inhibitor type 1 (PAI-1) or a u-PA dominant negative protein have both been shown to reduce or block primary tumor growth, tumor-associated angiogenesis, and experimental metastasis of transfected CaP cells versus controls [41–43]. The same has been described for small molecule u-PA inhibitors such as Amiloride, *p*-aminobenzamidine, and B-428 in various animal models [44–46]. Currently, experiments are underway to directly test the role of u-PA and MET in CaP metastasis in vivo through the use of u-PA specific inhibitors or MET specific ribozymes, respectively.

In conclusion, we provide evidence that HGF stimulates the secretion of biologically active u-PA and enhances in vitro invasion through Matrigel of human CaP cells with differing metastatic potential. These data support a role for u-PA in CaP cell invasion as specific inhibitors to the urokinase receptor inhibited the HGF-stimulated invasion. Collectively, these data support a model that HGF/MET participate in CaP invasion and metastasis.

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REFERENCES

1. Harada M, Iida M, Yamaguchi M, Shida K. Analysis of bone metastasis of prostatic adenocarcinoma in 137 autopsy cases. *Adv Exp Med Biol* 1992;324:173–182.
2. Bubendorf L, Schopfer A, Wagner U, Sauter G, Moch H, Willi N, Gasser TC, Mihatsch MJ. Metastatic patterns of prostate cancer: An autopsy study of 1,589 patients. *Hum Pathol* 2000;31(5): 578–583.
3. Jiang W, Hiscox S, Matsumoto K, Nakamura T. Hepatocyte growth factor/scatter factor, its molecular, cellular and clinical implications in cancer. *Crit Rev Oncol Hematol* 1999;29(3): 209–248.

4. Chung LW, Li W, Gleave ME, Hsieh JT, Wu HC, Sikes RA, Zhau HE, Bandyk MG, Logothetis CJ, Rubin JS. Human prostate cancer model: Roles of growth factors and extracellular matrices. *J Cell Biochem (Suppl)* 1992;16H:99–105.
5. Takai K, Hara J, Matsumoto K, Hosoi G, Osugi Y, Tawa A, Okada S, Nakamura T. Hepatocyte growth factor is constitutively produced by human bone marrow stromal cells and indirectly promotes hematopoiesis. *Blood* 1997;89(5):1560–1565.
6. Gleave ME, Hsieh JT, von Eschenbach AC, Chung LW. Prostate and bone fibroblasts induce human prostate cancer growth in vivo: Implications for bidirectional tumor-stromal cell interaction in prostate carcinoma growth and metastasis. *J Urol* 1992;147(4):1151–1159.
7. Lang SH, Clarke NW, George NJ, Testa NG. Scatter factor influences the formation of prostate epithelial cell colonies on bone marrow stroma in vitro. *Clin Exp Metastasis* 1999;17(4):333–340.
8. Pisters LL, Troncoso P, Zhau HE, Li W, von Eschenbach AC, Chung LW. C-met proto-oncogene expression in benign and malignant human prostate tissues. *J Urol* 1995;154(1):293–298.
9. Humphrey PA, Zhu X, Zarnegar R, Swanson PE, Ratliff TL, Vollmer RT, Day ML. Hepatocyte growth factor and its receptor (c-MET) in prostatic carcinoma. *Am J Pathol* 1995;147(2):386–396.
10. Naldini L, Weidner KM, Vigna E, Gaudino G, Bardelli A, Ponzetto C, Narsimhan RP, Hartmann G, Zarnegar R, Michalopoulos GK. Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. *EMBO J* 1991;10(10):2867–2878.
11. Ruoslahti E, Yamaguchi Y. Proteoglycans as modulators of growth factor activities. *Cell* 1991;64(5):867–869.
12. Naldini L, Tamagnone L, Vigna E, Sachs M, Hartmann G, Birchmeier W, Daikuhara Y, Tsubouchi H, Blasi F, Comoglio PM. Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. *EMBO J* 1992;11(13):4825–4833.
13. Mars WM, Zarnegar R, Michalopoulos GK. Activation of hepatocyte growth factor by the plasminogen activators uPA and tPA. *Am J Pathol* 1993;143(3):949–958.
14. Naldini L, Vigna E, Bardelli A, Follenzi A, Galimi F, Comoglio PM. Biological activation of pro-HGF (hepatocyte growth factor) by urokinase is controlled by a stoichiometric reaction. *J Biol Chem* 1995;270(2):603–611.
15. Mars WM, Kim TH, Stolz DB, Liu ML, Michalopoulos GK. Presence of urokinase in serum-free primary rat hepatocyte cultures and its role in activating hepatocyte growth factor. *Cancer Res* 1996;56(12):2837–2843.
16. Michalopoulos GK, DeFrances MC. Liver regeneration. *Science* 1997;276(5309):60–66.
17. Shimizu M, Hara A, Okuno M, Matsuno H, Okada K, Ueshima S, Matsuo O, Niwa M, Akita K, Yamada Y, Yoshimi N, Uematsu T, Kojima S, Friedman SL, Moriawaki H, Mori H. Mechanism of retarded liver regeneration in plasminogen activator-deficient mice: Impaired activation of hepatocyte growth factor after Fas-mediated massive hepatic apoptosis. *Hepatology* 2001;33(3):569–576.
18. Pepper MS, Matsumoto K, Nakamura T, Orci L, Montesano R. Hepatocyte growth factor increases urokinase-type plasminogen activator (u-PA) and u-PA receptor expression in Madin-Darby canine kidney epithelial cells. *J Biol Chem* 1992;267(28):20493–20496.
19. Grant DS, Kleinman HK, Goldberg ID, Bhargava MM, Nickoloff BJ, Kinsella JL, Polverini P, Rosen EM. Scatter factor induces blood vessel formation in vivo. *Proc Natl Acad Sci USA* 1993;90(5):1937–1941.
20. Rosen EM, Knesel J, Goldberg ID, Jin L, Bhargava M, Joseph A, Zitnik R, Wines J, Kelley M, Rockwell S. Scatter factor modulates the metastatic phenotype of the EMT6 mouse mammary tumor. *Int J Cancer* 1994;57(5):706–714.
21. Boccaccio C, Gaudino G, Gambarotta G, Galimi F, Comoglio PM. Hepatocyte growth factor (HGF) receptor expression is inducible and is part of the delayed-early response to HGF. *J Biol Chem* 1994;269(17):12846–12851.
22. Jeffers M, Rong S, Vande Woude GF. Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signalling in human cells concomitant with induction of the urokinase proteolysis network. *Mol Cell Biol* 1996;16(3):1115–1125.
23. Li H, Shimura H, Aoki Y, Date K, Matsumoto K, Nakamura T, Tanaka M. Hepatocyte growth factor stimulates the invasion of gallbladder carcinoma cell lines in vitro. *Clin Exp Metastasis* 1998;16(1):74–82.
24. Monvoisin A, Neaud V, De LV, Dubuisson L, Balabaud C, Bioulac-Sage P, Desmouliere A, Rosenbaum J. Direct evidence that hepatocyte growth factor-induced invasion of hepatocellular carcinoma cells is mediated by urokinase. *J Hepatol* 1999;30(3):511–518.
25. Moriyama T, Kataoka H, Hamasuna R, Yoshida E, Sameshima T, Iseda T, Yokogami K, Nakano S, Koono M, Wakisaka S. Simultaneous up-regulation of urokinase-type plasminogen activator (uPA) and uPA receptor by hepatocyte growth factor/scatter factor in human glioma cells. *Clin Exp Metastasis* 1999;17(10):873–879.
26. Okusa Y, Ichikura T, Mochizuki H, Shinomiya N. Urokinase type plasminogen activator and its receptor regulate the invasive potential of gastric cancer cell lines. *Int J Oncol* 2000;17(5):1001–1005.
27. Ried S, Jager C, Jeffers M, Vande Woude GF, Graeff H, Schmitt M, Lengyel E. Activation mechanisms of the urokinase-type plasminogen activator promoter by hepatocyte growth factor/scatter factor. *J Biol Chem* 1999;274(23):16377–16386.
28. Kozlowski JM, Fidler IJ, Campbell D, Xu ZL, Kaighn ME, Hart IR. Metastatic behavior of human tumor cell lines grown in the nude mouse. *Cancer Res* 1984;44(8):3522–3529.
29. Pettaway CA, Pathak S, Greene G, Ramirez E, Wilson MR, Killion JJ, Fidler IJ. Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. *Clin Cancer Res* 1996;2(9):1627–1636.
30. Simon C, Juarez J, Nicolson GL, Boyd D. Effect of PD 098059, a specific inhibitor of mitogen-activated protein kinase kinase, on urokinase expression and in vitro invasion. *Cancer Res* 1996;56(23):5369–5374.
31. Jones TR, Haney DN, Varga J. 1999. Patent 5,942,492.
32. Allgayer H, Wang H, Gallick GE, Crabtree A, Mazar A, Jones T, Kraker AJ, Boyd DD. Transcriptional induction of the urokinase receptor gene by a constitutively active Src. Requirement of an upstream motif (–152/–135) bound with Sp1. *J Biol Chem* 1999;274(26):18428–18437.
33. Koenen KS, Yeung F, Chung LW. Osteomimetic properties of prostate cancer cells: A hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. *Prostate* 1999;39(4):246–261.
34. Gmyrek GA, Walburg M, Webb CP, Yu HM, You X, Vaughan ED, Vande Woude GF, Knudsen BS. Normal and malignant

- prostate epithelial cells differ in their response to hepatocyte growth factor/scatter factor. *Am J Pathol* 2001;159(2):579–590.
35. Nishimura K, Kitamura M, Miura H, Nonomura N, Takada S, Takahara S, Matsumoto K, Nakamura T, Matsumiya K. Prostate stromal cell-derived hepatocyte growth factor induces invasion of prostate cancer cell line DU145 through tumor-stromal interaction. *Prostate* 1999;41(3):145–153.
 36. Parr C, Davies G, Nakamura T, Matsumoto K, Mason MD, Jiang WG. 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 Comm* 2001; 285(5):1330–1337.
 37. Naughton M, Picus J, Zhu X, Catalona WJ, Vollmer RT, Humphrey PA. Scatter factor-hepatocyte growth factor elevation in the serum of patients with prostate cancer. *J Urol* 2001; 165(4):1325–1328.
 38. Sharpe WS, McDonald JR. Reaction of bone to metastasis form carcinoma of the breast and prostate. *Arch Pathol* 1942;17: 321–325.
 39. Grano M, Galimi F, Zamboni G, Colucci S, Cottone E, Zallone AZ, Comoglio PM. Hepatocyte growth factor is a coupling factor for osteoclasts and osteoblasts in vitro. *Proc Natl Acad Sci USA* 1996;93(15):7644–7648.
 40. Fuller K, Owens J, Chambers TJ. The effect of hepatocyte growth factor on the behaviour of osteoclasts. *Biochem Biophys Res Comm* 1995;212(2):334–340.
 41. Soff GA, Sanderowitz J, Gately S, Verrusio E, Weiss I, Brem S, Kwaan HC. Expression of plasminogen activator inhibitor type 1 by human prostate carcinoma cells inhibits primary tumor growth, tumor-associated angiogenesis, and metastasis to lung and liver in an athymic mouse model. *J Clin Invest* 1995;96(6): 2593–2600.
 42. Jankun J, Keck RW, Skrzypczak-Jankun E, Swiercz R. Inhibitors of urokinase reduce size of prostate cancer xenografts in severe combined immunodeficient mice. *Cancer Res* 1997;57(4): 559–563.
 43. Evans CP, Elfman F, Parangi S, Conn M, Cunha G, Shuman MA. Inhibition of prostate cancer neovascularization and growth by urokinase-plasminogen activator receptor blockade. *Cancer Res* 1997;57(16):3594–3599.
 44. Iishi H, Tatsuta M, Baba M, Yano H, Uehara H, Nakaizumi A. Suppression by amiloride of bombesin-enhanced peritoneal metastasis of intestinal adenocarcinomas induced by azoxy-methane. *Int J Cancer* 1995;63(5):716–719.
 45. Billstrom A, Hartley-Asp B, Lecander I, Batra S, Astedt B. The urokinase inhibitor p-aminobenzamidine inhibits growth of a human prostate tumor in SCID mice. *Int J Cancer* 1995;61(4): 542–547.
 46. Xing RH, Mazar A, Henkin J, Rabbani SA. Prevention of breast cancer growth, invasion, and metastasis by antiestrogen tamoxifen alone or in combination with urokinase inhibitor B-428. *Cancer Res* 1997;57(16):3585–3593.