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TITLE: Cellular Mechanisms Regulating Urokinase-Type Plasminogen Activator in Hormone Refractory Prostate Cancer: A Novel Therapeutic Target

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## INTRODUCTION

DOD Award number DAMD17-00-1-0524, "Cellular Mechanisms Regulating Urokinase-Type Plasminogen Activator in Hormone Refractory Prostate Cancer: A Novel Therapeutic Target" has its goal inhibiting the expression of two pathways critical to prostate cancer progression, one mediated by the tyrosine kinase receptor c-Met, and the second mediated by urokinase plasminogen activator (uPA). Each pathway may be important to prostate tumor progression, and further, they may be related, i.e., increased c-Met expression leads directly to increased uPa expression. The purpose of the research is to provide a novel therapeutic basis for the development of prostate tumor metastases through inhibition of these growth regulatory pathways. DAMD17-00-1-0524 was originally awarded to Robert Radinsky, Ph.D. with the award date of July 1, 2000. Dr. Radinsky subsequently left the University of Texas M.D. Anderson Cancer Center for Amgen Pharmaceuticals and I (Gary E. Gallick, Ph.D.) was approved to become the Principal Investigator of this grant. As indicated in the first progress report, a thorough review of the project was undertaken when I was named PI, new animal protocols were written, in response to concerns of the U.S. Army veterinarians; thus the project was initiated only four months prior to the submission of the "year 1" progress report. As a result, I requested and received a one-year no cost extension on this Award. As indicated in last year's report "year 2", we focused on the most crucial aspect of the success of this work, the in vivo test of the requirement of c-Met, the protein tyrosine kinase receptor for hepatocyte growth factor (HGF) to affect prostate tumor progression and uPAR (urokinase Plasminogen Activator receptor) for which uPa (urokinase plasminogen activator) is the ligand to affect the same properties. In this year's progress report, I am pleased to note that one publication has resulted from each of the areas of focus last year- demonstration that a uPa inhibitor could inhibit metastatic growth of prostate tumor cells as proposed in the original tasks (Boyd et al. *Am. J. Pathol.* 162: 619-626, 2003); and inhibition of c-Met could inhibit growth and metastatic potential of prostate tumor cells in an orthotopic model (Kim et al., *Clin Ca Res*, *in Press*). Both of these manuscripts are provided in the APPENDIX to this progress report, which will be largely devoted to describing these results that we consider very exciting. As indicated in last year's progress report, I indicated that we would complete the critical in vivo work (Task 2) and then initiate Task 1. As most work on both tasks is now finished, in this year's progress report, they will be presented in order.

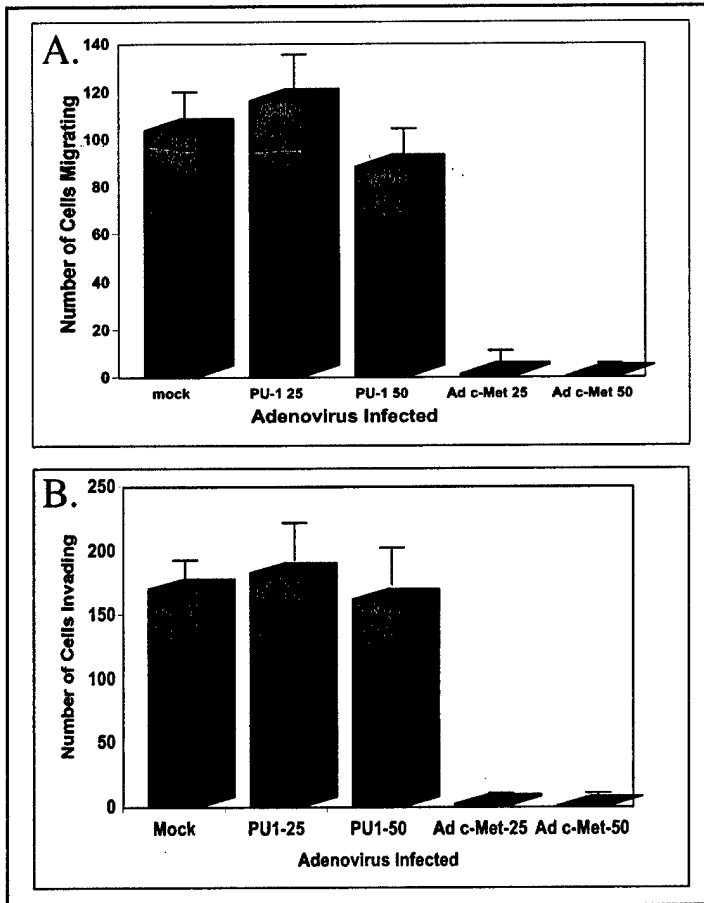
## BODY

### Task 1:

The major thrust of task 1 involved evaluation of high and low metastatic prostate cancer cells to invade a three dimensional matrix after HGF treatment and in the presence of uPa antagonists. The approach to this task is almost identical to that outlined in the original proposal. First, we tested the ability of c-Met inhibited cells to migrate in a Boyden chamber assay. Migration does not require a three dimensional matrix, but is one of the key properties of metastasis. Preliminary results on this assay were shown in last year's

submission; completed results are shown in Figure 1A (from Kim et al, see APPENDIX, Fig. 3). Invasion of cells was examined through the use of a Boyden chamber on which

the surface on which cells are grown was coated with Matrigel; thus cells must degrade the equivalent of a basement membrane. Results of the highly invasive PC3-LN4 cells is shown in Fig. 1B.



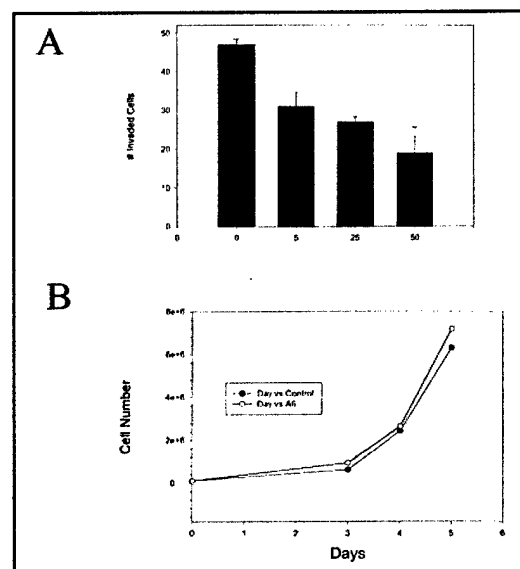
**Figure 1. Effects of infection of Ad-PU 1 and Ad-c-Met on migratory and invasive potentials of PC3-LN4 cells.** Migration and invasion assays were performed using a modified Boyden chamber system with a filter containing 8 mm pores. NIH 3T3 conditioned medium was used as a chemoattractant in the bottom chamber, and equal numbers of infected cells or mock-infected cells were placed on the top chamber and allowed to grow for 72h. 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.

Inhibition of c-Met was accomplished by the use of an Adenovirus expressing a c-Met ribozyme obtained from Roger Abounader and John Lattera (Abounader et al., 2002). As indicated in Figure 1, cells in which c-Met was reduced by increasing expression of ribozyme were greatly inhibited in their ability to migrate and invade.

### A6 Also Inhibits Cellular Invasion

Similar invasion assays were performed with

**Fig. 2 A6 reduces in vitro invasiveness in PC3-LN4 cells.** Cells were plated on matrigel-coated filters. After 6hr attachment, A6 was added at indicated concentration. Two days later, the cells on the upper aspect of the filter were removed and invaded cells stained, photographed and enumerated. Data are presented as mean +/- SD from three independent assays. B. Growth curve of PC3-LN4 cells in the presence or absence of 50µM/L A6. (from Boyd et al-SEE APPENDIX)



A6, affecting uPAR function (Guo et al., 2000). These results are now also published (Boyd et al, 2003-see Appendix Fig. 2), and are displayed in Figure 2A. To ensure that decreased invasion did not result from decreased proliferation (hence fewer cells to invade, a growth curve was performed in the presence of A6. As indicated in Fig. 2B, A6 did not affect proliferation at concentrations at which invasion was inhibited. Thus, results from Task 1 demonstrate that inhibition of either uPAR or c-Met inhibits cellular invasion. Determining results of the combination of the inhibitors will essentially complete the task.

## Task 2

Task 2 focuses on determining if inhibition of uPAR and c-Met effects growth and progression of prostate tumor cells in an orthotopic nude mouse model. For these experiments, PC3-LN4 (highly metastatic) prostate tumor cells were implanted into the prostates of nude mice, and "treatment" regimens were performed by use of uPAR antagonist (A6) of an Adenovirus expressing a c-Met ribozyme, exactly as proposed in the initial application. As indicated in the introduction, both strategies were not only successful, they have led to publications (see APPENDIX), and so only the critical results will be presented here.

## Inhibition of uPAR

For this study, A6 was injected daily into tumor-bearing mice. Mice were sacrificed when signs of morbidity were evident, in accord with the guidelines of the Institutional Animal Care and Use Committee and those in response to the DOD veterinary staff. Results of these experiments are shown in Figure 3 (Boyd et al., Figure 3 in APPENDIX).

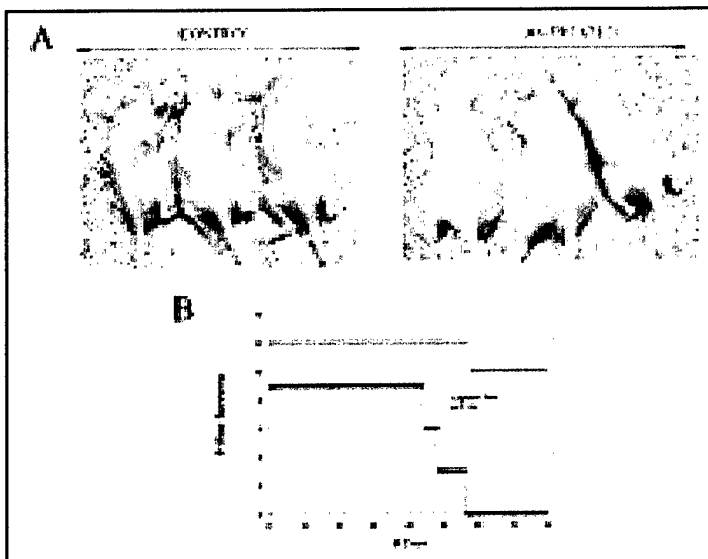


Figure 3. Treatment of tumor-bearing mice with the uPAR antagonist, A6. A; Pictures of mice bearing PC3-LN4 tumors after daily treatment with carrier PBS control) or 27mg/Kg A6. B. Mice were orthotopically injected with 2 million cells. When primary tumors were palpable, mice were divided into two groups with one receiving PBS alone and the other administered with 75 mg/KG A 6 on a daily basis. Mice were sacrificed when moribund as determined by standard animal use and care criteria. Differences in survival rates were statistically significant ( $p < 0.0001$ )

Not only was time to morbidity significantly enhanced by A6 treatment, lymph node involvement of tumor was significantly reduced by up to 70% (P=0.004-See Boyd et al, Fig. 5-APPENDIX. These results demonstrate inhibition of uPAR has therapeutic potential for the treatment of prostate cancer.

In last year's progress report and from work of others, upregulation of uPAR was demonstrated to result from increased c-Met expression. Thus, one of the other parts of Task 2 was to determine the effect of c-Met inhibition on growth and metastasis of prostate cells in the identical orthotopic model. Prior to performing "gene therapy" experiments, to examine the feasibility of c-Met inhibition, PC3-LN 4 cells were infected *ex vivo* with a control or ribozyme-expressing virus, and implanted orthotopically in nude mice. Results from this set of experiment are shown in Table 1 (Kim et al., Table 1-see Appendix).

Table 1 *Tumorigenicity and lymph node metastases induced by PC3-LN4 Cells following ex vivo Infection of Adenovirus or Mock Infection*

Group <sup>a</sup>	Tumor Incidence <sup>b</sup>	Tumor size (g) median (range)	Lymph node Involvement <sup>c</sup>
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

<sup>a</sup>PC 3-LN4 cells were mock infected, or infected with the indicated adenovirus at an MOI of 50, and  $5 \times 10^5$  cells were injected into the prostates of nude mice, as described in Materials and Methods

<sup>b</sup>Number of mice with tumors/number of mice surviving surgery for prostate tumor cell implantation

<sup>c</sup>Number of mice with lymph node metastases/number of mice with tumors

The striking results of this experiment were that growth of tumors in 9/10 mice was completely inhibited. The next set of experiments (see Kim et al-APPENDIX for complete details) was to implant one million cells into the prostate, allow tumors to grow for one week, then perform two intraprostatic injections of ribozyme-expressing or control virus at two week intervals. The results of these experiments are shown in Table 2 (also Table 2 of Kim et al.-APPENDIX). Surprisingly, intratumoral injection was nearly as effective as *ex vivo* infection with adenoviruses, as only 1/8 animals had palpable tumors after 30 days with multiplicity of infections of either 25 or 50 with the ribozyme-expressing virus. In marked contrast, 7/8 animals developed tumors in control virus treatments, and each of these animals developed lymph node metastases.

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

Group <sup>a</sup>	Tumorigenicity <sup>b</sup>	Tumor size (g) Median (range)	Lymph node Involvement <sup>c</sup>
Sham	8/8	0.66 (0.48-0.99)	8/8
Ad-PU-1 (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

<sup>a</sup>PC3-LN4 cells ( $5 \times 10^5$ ) were injected into the prostates of nude mice as described, and allowed to grow for seven days, after which indicated adenovirus (or sham injection) was delivered 7 and 14 days post-tumor cell implantation.

<sup>b</sup>Number of mice with tumors/number of mice injected

<sup>c</sup>Number of mice with lymph node metastases/number of mice with tumors

These results demonstrate that two key elements in signal transduction-activation of a critical growth factor receptor (c-Met), and activation by c-Met of a downstream protein (uPAR) critical to cell invasiveness are both attractive targets for therapeutic strategies for the treatment of prostate cancer. To finish task 2, the original proposal described experiments to determine the effect of inhibiting both pathways simultaneously. This presents somewhat of a "problem", as the inhibition of c-Met itself almost completely abolished tumor growth; thus, no additive effects could possibly be obtained by a combination of inhibitors. However, to test the efficacy of the strategy, we will in the final year of the proposal attempt to use lower MOIs of virus, in which tumor invasion is not completely abolished, to determine if inhibition of c-Met and uPAR does have additive effects. These studies may be important, because in mouse models, it is easier to achieve higher MOIs of adenovirus-expressed c-Met ribozyme than in humans. Therefore, we will complete task 2 in year 3.

### Task 3

This task involves examination of surgical specimens. This task was not proposed to begin until year 2. We have begun to acquire samples for this task. This task will be undertaken in the upcoming year, and as we were granted a no cost extension to the studies, we anticipate no difficulty in completing this task.

### KEY RESEARCH ACCOMPLISHMENTS

- Reduction of c-Met decreases uPAR expression (from last year's report)
- Inhibition of uPAR function alone is sufficient to inhibit migration and invasion of human prostate tumor cells
- Inhibition of c-Met expression alone is sufficient to inhibit migration and invasion of human prostate tumor cells

- Reduction of c-Met expression decreases tumorigenicity and metastatic potential of human prostate cells in an orthotopic model (from last year's report)
- Gene therapy experiments in nude mice demonstrate that c-Met reduction not only inhibits growth of prostate tumors, but blocks development of lymph node metastasis
- Treatment of mice with the uPAR antagonist A6 significantly increases survival (as measured by onset of morbidity) of nude mice harboring tumors induced by human prostate tumor cells

## REPORTABLE OUTCOMES

**Many of the hypotheses posed in the original submission with respect to c-Met regulating uPAR and promoting invasion and metastasis of prostate cancer appear correct. In addition, as described last year, we have begun to define the relationship between c-Met, uPAR, and the metastasis suppressor, MMAC/PTEN. These data are now in publication form in three manuscripts:**

Adenoviral-mediated expression of MMAC/PTEN inhibits proliferation and metastasis of human prostate cancer cells. Davies, MA, Kim SJ, Parikh NU, Dong, Z., Bucana CD, and Gallick, GE *Clinical Cancer Res*, 8, 1904-1914, 2002 (from last year's report)

A Urokinase-Derived Peptide (A6) Increases Survival of Mice Bearing Orthotopically Grown Prostate Cancer and Reduces Lymph Node Metastasis. Boyd, D.D., Kim, S.-J., Wang, H., Jones, T.R., and Gallick, G.E. *Am. J. Pathol.* 162, 619-626, 2003.

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. Kim S.-J., Johnson, M., Koterba K., Uehara H., and Gallick, G.E. *Clinical Cancer Research*, in Press

## CONCLUSIONS

Both uPAR and c-Met contribute to the invasive potential of prostate tumor cells; c-Met also contributes to the migratory potential of these cells. Both c-Met and uPAR are targets for therapeutic strategies to inhibit prostate cancer. Inhibition of c-Met results in inhibition of growth at the primary tumor site (prostate), and significantly, prevents development of lymph node metastases in an orthotopic nude mouse model.

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

Boyd, D.D., Kim, S.-J., Wang, H., Jones, T.R., and Gallick, G.E. A Urokinase-Derived Peptide (A6) Increases Survival of Mice Bearing Orthotopically Grown Prostate Cancer and Reduces Lymph Node Metastasis. *Am. J. Pathol.* 162, 619-626, 2003.

Guo, Y., Higazi, A. A., Arakelian, A., Sachais, B.S., Cines, D., Goldfard, R.H., Jones, T.R., Kwaan, H., Mazar, A.P., and Rabbani, S.A. A peptide derived from the binding region of urokinase plasminogen activator (uPA) inhibits tumor progression and angiogenesis and induces tumor cell death *in vivo*. *FASEB J.* 14: 1400-1410, (2000).

Kim S.-J., Johnson, M., Koterba K., Uehara H., and Gallick, G.E. 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. *Clinical Cancer Research*, in Press.

# A Urokinase-Derived Peptide (Å6) Increases Survival of Mice Bearing Orthotopically Grown Prostate Cancer and Reduces Lymph Node Metastasis

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**The high rate of prostate cancer mortality invariably reflects the inability to control the spread of the disease. The urokinase-type plasminogen activator and its receptor (u-PAR) contribute to prostate cancer metastases by promoting extracellular matrix degradation and growth factor activation. The current study was undertaken to determine the efficacy of a urokinase-derived peptide (Å6) in reducing the lymph node metastases of prostate cancer using a model in which prostatic tumors established in nude mice from orthotopically implanted PC-3 LN4 prostate cancer cells disseminate to the lymph nodes. As a first step in evaluating the *in vivo* effectiveness of Å6, we determined its effect on *in vitro* invasiveness. *In vitro*, Å6 reduced the invasiveness of PC-3 LN4 cells through a Matrigel-coated filter without affecting growth rate. A first *in vivo* survival experiment showed that all Å6-treated mice were alive after 57 days, and half of them tumor-free, whereas all control mice receiving vehicle had died. In a second experiment with a larger tumor inoculum and a longer delay until treatment, whereas 71% of control mice and 83% of mice treated with a scrambled peptide developed lymph node metastases, only 22 to 25% of Å6-treated mice had positive lymph nodes. Further, lymph node volume, reflective of tumor burden at the secondary site, was diminished 70% in Å6-treated mice. In conclusion, we provide definitive evidence that a peptide spanning the connecting region of urokinase suppresses metastases and, as a single modality, prolongs the life span of prostate tumor-bearing mice. (Am J Pathol 2003, 162:619–626)**

Prostate cancer afflicts 209,000 American men every year and is second only to lung cancer as the leading cause of cancer deaths in the male population. The high rate of mortality invariably reflects spread of this disease to the secondary sites and consequently, effective treat-

ments in the future will require a means of combating prostate cancer dissemination.

It is well established that the spread of virtually all malignancies require the expression of one or more proteases which serve to cleave extracellular matrix and activate growth factors.<sup>1</sup> The urokinase-type plasminogen activator<sup>2</sup> contributes to tumor progression by converting plasminogen into plasmin a widely acting serine protease that cleaves several basement membrane components including laminin and fibronectin<sup>3</sup> as well as type IV collagen indirectly via activation of metalloproteinases.<sup>4</sup> Urokinase, achieves this by binding to a cell surface receptor (u-PAR)<sup>5,6</sup> which increases the efficiency by which plasminogen is converted into plasmin.<sup>7</sup> Further, urokinase cleaves u-PAR thereby promoting chemotaxis.<sup>8,9</sup>

There is currently strong evidence implicating the urokinase-u-PAR axis in prostate cancer progression. For example, the urokinase gene is amplified in some hormone-refractory prostate cancers<sup>10</sup> and overexpression of this protease increases skeletal metastases of this malignancy.<sup>11</sup> Additionally, in two separate studies,<sup>12,13</sup> the expression of an exogenous plasmid encoding urokinase lacking an enzyme active site, prevented metastases of human (PC-3) and murine (MAT-LyLu) prostate cancers. Further, independent studies by Festuccia et al<sup>14</sup> and Hollas et al<sup>15</sup> reported that urokinase-u-PAR complexes characterized the invasive phenotype of cultured prostate cancer cells and that antibodies that prevented this interaction blocked *in vitro* invasion. Moreover, high u-PAR levels in the serum is predictive of metastatic prostate cancer and shortened survival time.<sup>16</sup> Taken together, these reports would suggest that the urokinase-u-PAR axis represents a therapeutic target for controlling prostate cancer metastases. We therefore determined the potential of a urokinase-derived peptide (Ac-KPSSPPEE-amide, hereafter referred to as Å6) spanning amino acids 136–143 to counter the metastases of orthotopically grown prostate cancer. This peptide, which non-competitively blocks the interaction of urokinase with

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soluble u-PAR *in vitro*,<sup>17</sup> has proven efficacious in reducing glioblastoma growth and angiogenesis.<sup>18</sup>

## Materials and Methods

### Cell Culture

Establishment of the highly metastatic PC-3 LN4 cell line has been described elsewhere.<sup>19</sup> Cells were grown in DMEM/F12 culture medium containing 10% FBS.

### Western Blotting for u-PAR

Western blotting for u-PAR was performed as described previously.<sup>20</sup> Briefly, cells were extracted into a Triton X-100-containing buffer supplemented with protease inhibitors. Insoluble material was removed by centrifugation and the cell extract immunoprecipitated with a polyclonal anti-u-PAR antibody. The immunoprecipitated material was then subjected to standard Western blotting and the blot probed with 5 µg/ml of an anti-u-PAR monoclonal antibody (no. 3931, American Diagnostica, Greenwich, CT) and an HRP-conjugated goat anti-mouse IgG. Bands were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

### Northern Blotting

The level of steady state mRNAs was determined by Northern analysis.<sup>20</sup> Total cellular RNA was extracted from 90% confluent cultures using 5.0 mol/L guanidinium isothiocyanate and purified on a cesium chloride cushion (5.7 mol/L) by centrifugation at 150,000 × *g* for 20 hours. Purified RNA was electrophoresed in a 1.5% agarose-formaldehyde gel and transferred to Nytran-modified nylon by capillary action using 10X SSC. The Northern blot was probed at 42°C with a random primed radiolabeled u-PAR cDNA which starts at

the transcription start site and extends 0.65 kb downstream. The blots were then washed at 65°C using 0.25X SSC in the presence of 0.75% SDS.

### In Vitro Invasion Assays

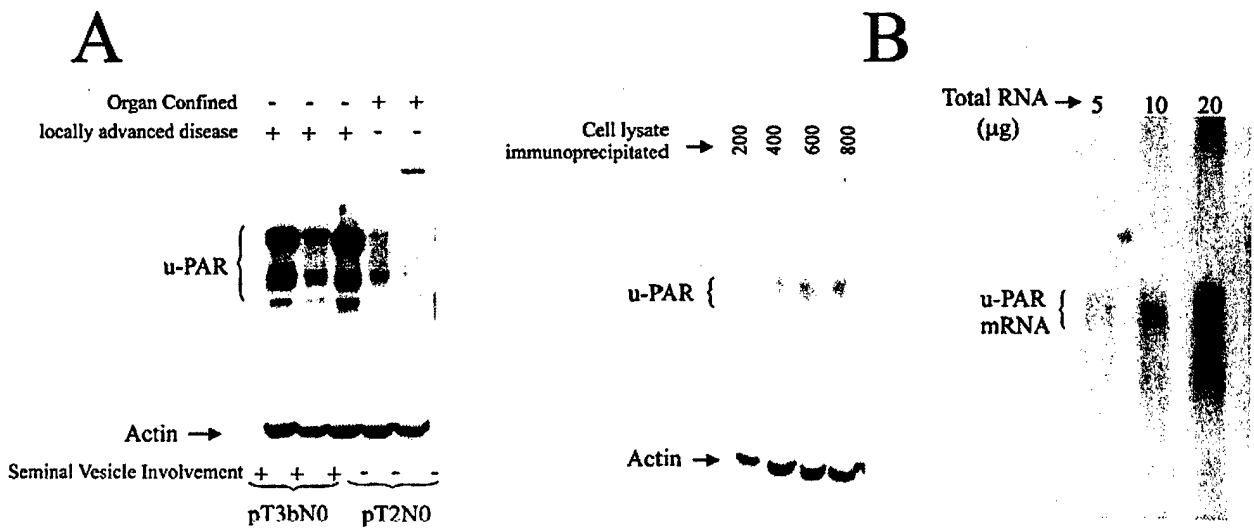
These were performed as described by this laboratory previously,<sup>21</sup> but with modifications. Briefly, cells are dispersed with 4 mmol/L EDTA and 250,000 cells dispensed into a BD BioCoat Falcon cell culture chamber (BD Biosciences Bedford, MA). The chamber was subsequently inserted into the outer well, the latter also containing culture medium. The cells were incubated at 37°C for 2 days after which the cells on the upper aspect of the filter were removed with a cotton swab and cells on the lower aspect stained using the DifQuik kit. Invaded cells were enumerated.

### Orthotopic Model to Assess Prolongation of Life Span

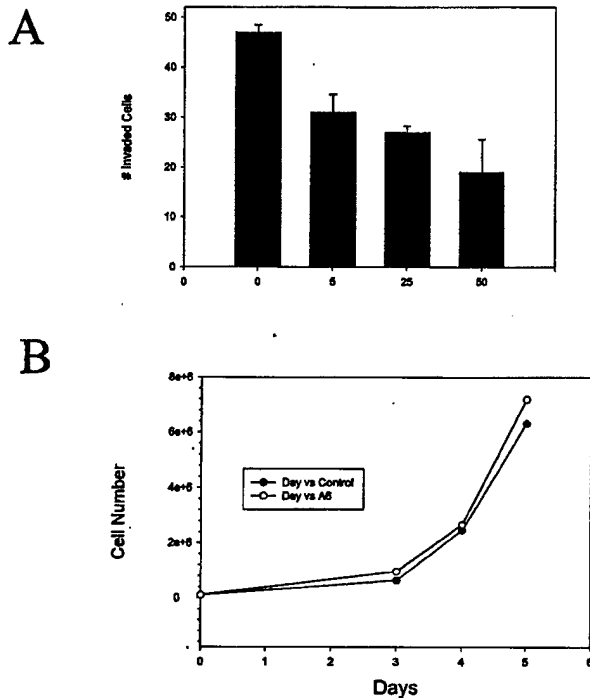
Nu/Nu mice (8–12 weeks of age) were injected with 2 × 10<sup>5</sup> PC-3 LN4 cells/50 µl in Ca<sup>2+</sup>, Mg<sup>2+</sup>-free HBSS into the prostate as described previously.<sup>19</sup> After 3 days to allow for tumor establishment, mice were injected every 12 hours with either vehicle (PBS) or Å6. Each Å6 injection contained 37.5 mg/kg such that the daily dose was 75 mg/kg.

### Orthotopic Model for Measuring in Vivo Metastases

These were carried out as above, except that the tumor inoculum was 5 × 10<sup>5</sup> PC-3 LN4 cells. After 7 days, when primary tumors were palpable, Å6 or Å16 was administered i.p. twice daily at 25 or 75 mg/kg/day body weight. In the male mouse, the prostate is located in the bladder



**Figure 1.** u-PAR expression is increased in locally advanced prostate cancer. **A:** Equal protein (500 µg) from resected prostate tumor extracts was immunoprecipitated with an anti-u-PAR antibody and analyzed for u-PAR protein by Western blotting. The residual supernatant from the immunoprecipitated was immunoblotted for actin. **B:** PC-3 LN4 cells were analyzed for u-PAR protein (left panel) or for mRNA (right panel). Western blotting was as described for panel A with the exception that the amount of protein immunoprecipitated was varied. For the Northern blotting, total RNA (20 µg) from PC-3 LN4 cells was subjected to Northern blotting for u-PAR mRNA.



**Figure 2.** Å6 reduces *in vitro* invasiveness of PC-3 LN4 cells. **A:** PC-3 LN4 ( $2.5 \times 10^5$  cells) were plated on Matrigel-coated porous filters. After 6 hours to allow for cell attachment, Å6, was added at the indicated concentration. Two days later, the cells on the upper aspect of the filter were removed and invaded cells stained, photographed and then enumerated. Data are presented as mean  $\pm$  SD of three independent assays. **B:** Growth curve of PC-3 LN4 grown in the presence, or absence, of 50  $\mu\text{mol/L}$  Å6.

neck (outlet), allowing for easy palpation of an enlarged prostatic mass from the outside.<sup>22</sup> The prostate mass is round, fixed and hard. At the end of the experiment, mice were sacrificed and prostate and lymph nodes examined both macroscopically and microscopically for the presence of tumor cells. For histological examination, tissues were stained with hematoxylin and eosin.

**Statistics**

Statistical analysis was performed using the InStat (version 3.05) and Prism statistical software (GraphPad, San Diego, CA). The Mann-Whitney non-parametric test (two-tailed) was used to test for statistical significant differences between Å6-treated and untreated cells in the *in vitro* invasion assays as well as reductions in lymph node volumes in the *in vivo* model. Survival curves were tested for statistically significant differences using the log rank test.

**Results**

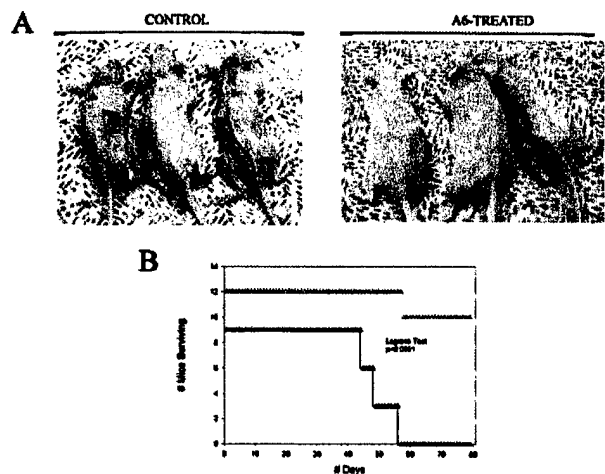
**Elevated u-PAR Expression in Locally Advanced Prostatic Cancer**

Previous studies with prostate cancer have strongly suggested a role for the urokinase-u-PAR axis in the progression of this disease. Therefore, u-PAR protein levels were

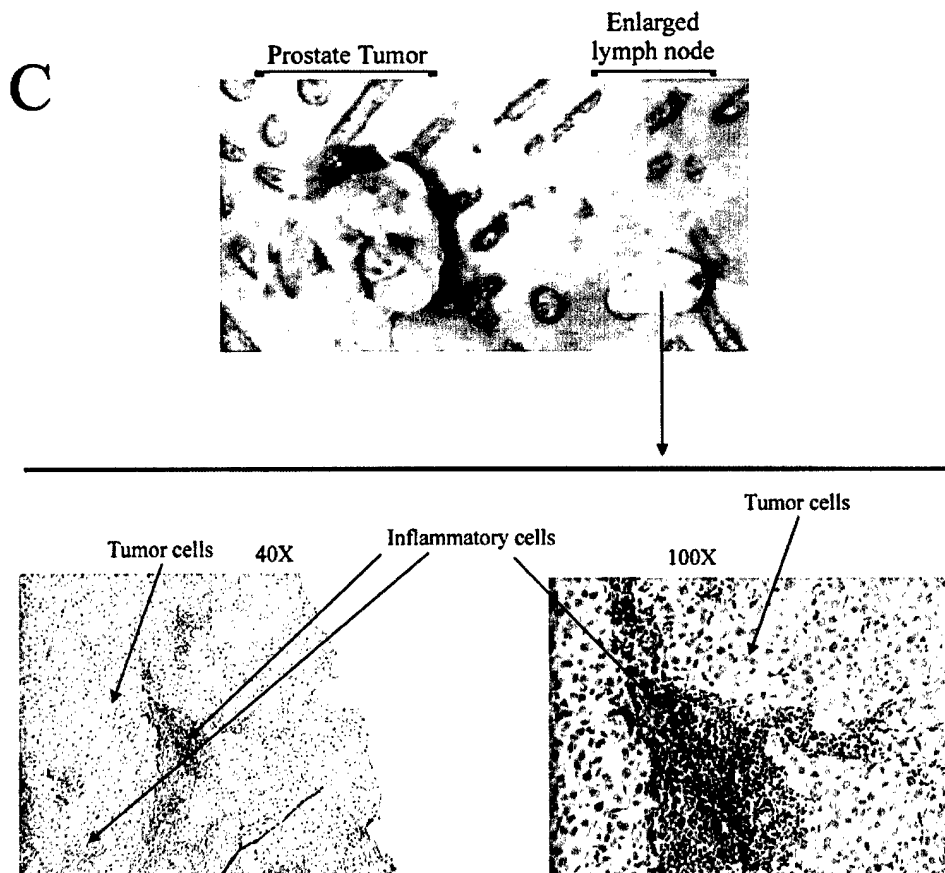
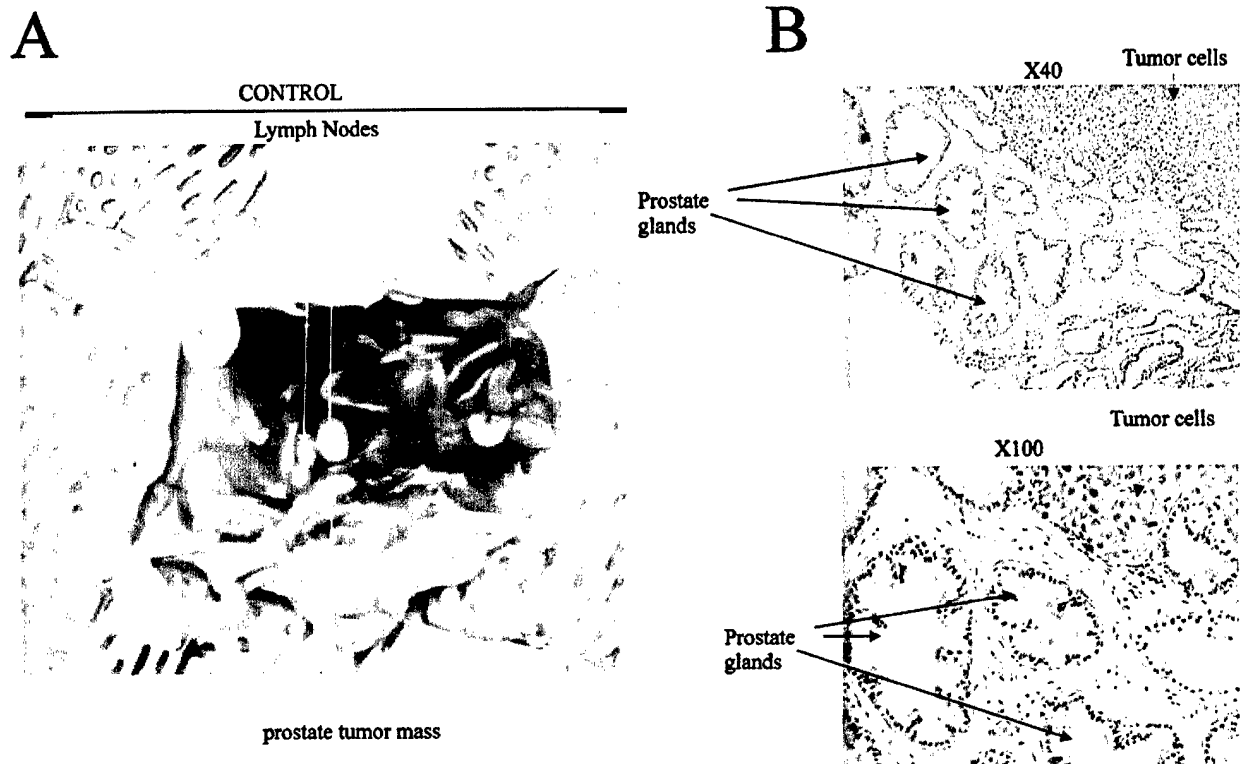
compared in resected prostate cancer derived from patients with organ confined or locally advanced (demonstrating seminal vesicle involvement) disease. All tumors were resected at the M.D. Anderson Cancer Center. Three tumors had bilateral extracapsular tumor extension without regional lymph node metastases (pT3bN0) while two other tumors were palpable but confined to the prostate (pT2N0). While u-PAR protein was abundant in tumor extracts from three patients with locally advanced disease (Figure 1A), the amount of this protein derived from patients with organ-confined disease was at, or below, the detection limit of Western blotting. These results are consistent with previous studies demonstrating that the urokinase/u-PAR axis contributes to prostate tumor progression, and that inhibition of this proteolytic axis might limit this process.

**Reduction of in Vitro PC-3 LN4 Invasion by Å6**

We first determined the ability of Å6 to diminish *in vitro* invasiveness of PC-3 LN4 cells. These cells express both u-PAR as evidenced by both Northern and Western blotting (Figure 1B) and urokinase as shown previously.<sup>14,15</sup> Cells were plated on Matrigel-coated porous filters and after 6 hours to allow for cell attachment, Å6 added at varying concentrations shown previously to reduce *in vitro* breast cancer invasiveness.<sup>17</sup> In the absence of the peptide, PC-3 LN4 showed a pronounced invasiveness through the extracellular matrix-coated barrier (Figure 2A). However, addition of the urokinase-derived peptide resulted in a dose-dependent decrease in the number of cells traversing the Matrigel-coated filter (Figure 2A). At 50  $\mu\text{mol/L}$ , Å6 caused about a 60% diminution in *in vitro* invasion by PC-3 LN4 cells. This difference was statistically significant ( $P < 0.05$ ). The reduced invasiveness



**Figure 3.** Untreated, but not Å6-treated, PC-3 LN4-bearing mice show severe cachexia and decreased survival. **A:** Pictures of mice bearing orthotopically grown PC-3 LN4 tumors after daily treatment for 50 days with carrier (PBS-control) or 75 mg/kg Å6. **B:** Mice were orthotopically injected with  $2 \times 10^5$  cells. When primary tumors were palpable, mice were divided into two groups with one group receiving PBS alone and the other administered with 75 mg/kg Å6 on a daily basis. Mice were sacrificed when moribund as determined by standard IACUC criteria. Differences in the survival rates were tested for statistical significance using the log rank test.



was not due to a diminished cell proliferation (Figure 2B). The diminished invasion of PC-3 LN4 cells was less impressive than previously published for the urokinase inhibitor amiloride.<sup>10</sup> It may be that the greater effect with amiloride reflects its multiple mechanisms of action. Indeed, in addition to inhibiting urokinase activity,<sup>23</sup> amiloride also reduces mRNA levels for this plasminogen activator and its receptor.<sup>24,25</sup>

### Å6 Prolongs the Survival Time of PC-3 LN4-Bearing Mice

To assess the efficacy of Å6 in prolonging life span, we used an *in vivo* model in which  $2 \times 10^5$  highly metastatic PC-3 LN4 cells are injected orthotopically into the prostate. After 3 days to allow for tumor establishment, mice were injected every 12 hours with either vehicle (PBS) or Å6 (75 mg/kg/day). After 38 days, all of the mice in the control group had palpable prostatic tumors whereas none of the mice in the Å6-treated group had palpable tumors. The severe cachexia demonstrated by the control group, but not by the Å6-treated mice, is apparent at day 50 (Figure 3A). The control mice were sacrificed when moribund (Figure 3B) to keep the study within IACUC guidelines. While there were 2 deaths in the Å6-treated mice after 59 days (Figure 3B), these deaths were not due to tumor, since at autopsy, both the prostate and lymph nodes were observed to be tumor-free. Deaths in these Å6-treated mice were due to systemic infection originating at the injection site as a consequence of inadequate sterile technique compounded by the frequent injection schedule. The surviving Å6-treated mice were sacrificed at day 80 and autopsied. Of these, 4 of 10 mice were deemed to be tumor-free. Of the 6 mice that developed primary tumors, only 2 showed lymph nodes positive for disease. It is noteworthy that the reduced incidence (50%) of primary tumor development in the Å6-treated mice compares with 100% tumor development in mice receiving the vehicle alone. Log rank analysis indicated that the prolonged survival of the Å6-treated mice compared with the controls was statistically significant ( $P < 0.0001$ ).

### Å6 Attenuates the Metastasis of Prostate Cancer in an Orthotopic Model

Since Å6 effectively prevented the establishment of primary prostatic tumors in the model described above, it was not possible to assess the drug's activity against their metastasis. Therefore, a second study was conducted using the above model, but, with a larger tumor inoculum and withholding treatment for 7 days until the primary tumor mass was definitely palpable. Inoculation of  $5 \times 10^5$  PC-3 LN4 cells, with no treatment, resulted in the formation of an enlarged prostatic tumor mass at the primary site (Figure 4, A and B) at a rate of between 85

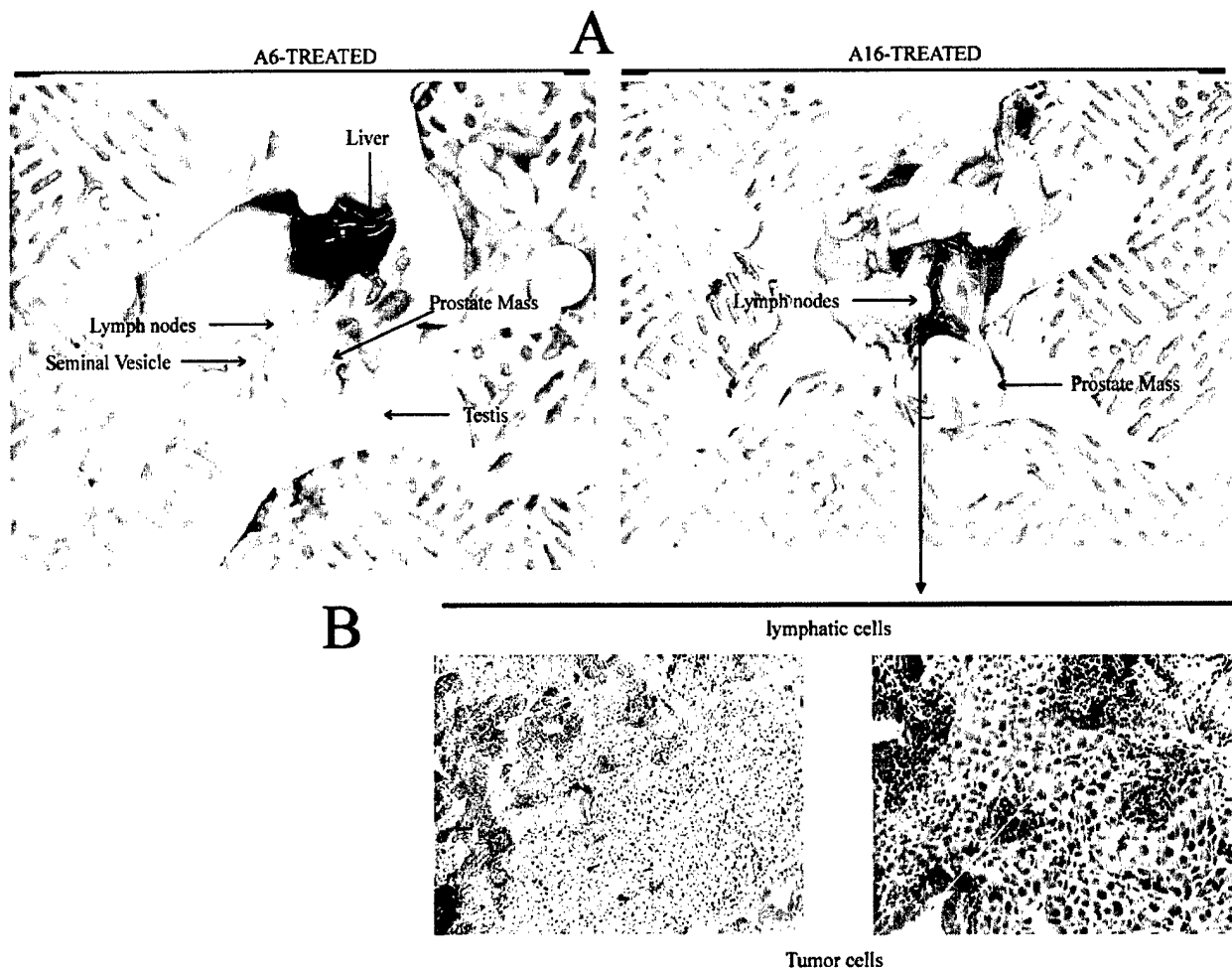
and 100% after 6 weeks. The majority of these mice (71%) demonstrated spread of the disease to the regional lymph nodes as evidenced both macroscopically (Figure 4A) and microscopically (Figure 4C). In the treated group, mice were injected twice daily with 25 or 75 mg/kg/day of Å6 or as a control, Å16, a peptide comprised of the scrambled Å6 amino acid sequence (Ac-PSESPEKP-NH<sub>2</sub>). After 6 weeks, mice were sacrificed and tumor weights and lymph node involvement determined. Å6 treatment had little effect on primary tumor mass (Figure 5, A and C). However, Å6, but not the control peptide Å16, reduced the percentage of mice with tumor cell-positive lymph nodes from 71 to as low as 22% (Figure 5, B and C). Further, while Å6 diminished lymph node volume (Figure 5, A-C) by up to 70% ( $P = 0.004$ ), the control peptide, Å16, had no effect on this parameter.

### Discussion

Effective therapy of prostate cancer is hampered by the lack of suitable agents for controlling the spread of the disease. Indeed the poor prognosis of those patients who present with metastatic disease compared with those individuals with organ-confined prostate cancer (81% and 25% disease-free at 10 years, respectively) indicate the urgent need for strategies to combat the metastatic disease. We demonstrate, herein, the efficacy of Å6, a urokinase-derived peptide previously shown to interfere with the urokinase-u-PAR system,<sup>17</sup> in reducing the metastatic spread of orthotopically grown prostate cancer and prolonging the life span of prostate cancer-bearing mice.

Å6 has previously been shown to have anti-tumor effects on other experimental cancers including mammary adenocarcinoma<sup>17,26</sup> and glioblastoma.<sup>18</sup> However, while the previous study of Guo and co-workers<sup>17</sup> indicated an anti-metastatic effect of Å6, since the primary tumor mass was reduced in both cases, it was difficult to determine whether reduced metastases was a direct effect of the agent on the metastatic process or secondary to a smaller primary tumor size. Our study was intended to resolve this issue and determine whether Å6 has an anti-metastatic effect. In our first experiment with the PC-3 LN4 highly metastatic orthotopic prostatic carcinoma model that used treatment 3 days after a tumor inoculum of  $2 \times 10^5$  cells, we fully expected the primary tumor to establish itself and metastasize despite treatment with Å6. It was therefore a surprise to note that the treated animals fared very well compared to the untreated controls, and we therefore extended the duration of treatment of 10 mice to 80 days. It is noteworthy that 4 of these 10 mice were tumor-free on autopsy. Of the 6 mice that did have a primary tumor, only 2 had lymph

**Figure 4.** Extra-prostatic spread of orthotopically grown PC-3 LN4 to the lymph nodes. PC-3 LN4 cells ( $5 \times 10^5$ ) were injected into the prostate of male nu/nu mice. After 6 weeks, mice were sacrificed and prostate and lymph node tissues fixed and stained for histological examination. **A** depicts the prostatic tumor mass and enlarged lymph nodes while histological evidence of tumor cells in the prostatic mass are illustrated in **B**. The presence of tumor cells in the enlarged lymph nodes is apparent by histology (**C**). These data confirm the spread of the orthotopically implanted prostate cancer cells to the lymph nodes.



**C**

	# Mice	Mean Primary Tumor Mass (g)	% Mice with Positive Lymph Nodes	Lymph Node Volume (cm <sup>3</sup> )		
				Average	Range	Probability
Control	8	0.58	71	7.1	0-56	
A6 (25 mg/kg)	8	0.44	25	2.2	0-6	p = 0.004
A6 (75 mg/kg)	7	0.49	22	2.4	0-6	p = 0.05
A16 (75 mg/kg)	6	0.7	83	9.6	0-19	p = 0.146

**Figure 5.** A6 reduces the lymph node-spread of orthotopically grown PC-3 LN4 cells. **A, B:** PC-3 LN4 cells ( $5 \times 10^5$  cells) were injected into the prostates of nu/nu mice. When primary tumors were established, as determined by palpation, mice were injected twice daily with the indicated doses of A6 or A16. After 6 weeks, mice were sacrificed, and examined macroscopically and microscopically for spread of the prostate cancer to the lymph nodes. **C:** Statistical analysis of the effect of A6 and A16 on the spread of PC-3 LN4 to the lymph nodes. Probability levels are given with respect to the control.

node metastases. This result was in pronounced contrast to the result from the untreated control animals, all of which succumbed to metastatic disease and exhibited severe cachexia before their sacrifice. These findings are consistent with other studies demonstrating an attenuating effect of A6 on primary tumor volume.<sup>18,26</sup> In a second experiment, we therefore made a determined effort

to establish the primary tumor before start of treatment. This was achieved with a larger tumor inoculum ( $5 \times 10^5$  cells) and waiting until the primary tumor was clearly palpable; this took 7 days. A6 treatment started on day 8, continued for 6 weeks, and resulted in little effect on the primary tumor mass relative to controls but a marked effect on lymph node metastasis.

Thus, our findings are the first to show that Å6 administered alone effectively prolongs the survival of tumor-bearing mice, in this case orthotopically grown prostate cancer. We can only speculate as to how Å6 prolongs survival of the tumor-bearing mice. Certainly, in the clinical situation in humans, tumor progression is generally associated with protein wasting (negative nitrogen balance) and reduced food intake, all events that are self-reinforcing, thereby hastening the demise of the individual. Additionally, renal failure and bilateral ureter obstruction from lymph node enlargement and hepatic failure from spread of the tumor cells to the liver may also contribute to the increased morbidity of the control animals.

Our results contrast with a study<sup>18</sup> where it was reported that Å6 combined with cisplatin, but not as a single modality, increased survival of glioblastoma-bearing mice. Second, our findings demonstrate that in the second, more difficult to treat, model the effect of inhibiting the urokinase-u-PAR axis is entirely on the development of lymph node metastases and not growth of tumor at the primary site. A third, broad, conclusion that can be drawn by comparison of the two experiments is that earlier treatment with Å6 on smaller PC-3 LN4 tumors was much more effective than later treatment on larger tumors. This may have implications for how Å6 may best be used in cancer patients.

At the present time, there are few studies to identify candidate metastases-suppressing drugs. One exception is a previous report by Rabhani and colleagues,<sup>27</sup> who demonstrated the ability of the nucleoside analogue  $\beta$ -L-(–)dioxolane-cytidine to reduce the incidence and spread of the highly metastatic Dunning R3227 Mat Ly Lu cells to the adrenals. However, as in previous studies with Å6, it was not clear whether the benefit of this agent was due to its effect on the metastatic process or secondary to the marked decrease in tumor volume.

Considering the role of the urokinase-u-PAR axis in promoting extracellular matrix degradation, cell migration and chemotaxis<sup>8,21,28</sup> as well as preventing tumor dormancy,<sup>29,30</sup> antagonism of the urokinase-u-PAR axis represents one potential means of controlling spread of cancer. Some evidence for this possibility has been derived from the study of various malignancies including prostate cancer. In one study, the epidermal growth factor domain of murine urokinase fused to the Fc portion of human IgG proved to be a high affinity antagonist of the murine u-PAR and inhibited neovascularization and growth of B16 melanoma in syngeneic mice.<sup>31</sup> In another study, Goodson and others isolated a family of u-PAR-binding ligands by affinity selection of a 15-mer random peptide library displayed on bacteriophage M13.<sup>32</sup> Further, small molecular weight inhibitors of urokinase such as amiloride and *p*-aminobenzamidide<sup>33</sup> have proven effective in reducing LNCaP and DU145 prostate tumor growth in mice. While it is not possible at the present time to determine which of these agents would prove most efficacious in blocking the urokinase-u-PAR axis, one practical benefit of Å6 is its high aqueous solubility compared with the bacteriophage peptides isolated previously.<sup>32</sup>

The mechanism by which Å6 exerts its anti-tumor and anti-metastatic effects is presently unclear. In a previous

study,<sup>17</sup> it was shown that Å6, which is derived from the connecting peptide of urokinase (amino acids 136–143) acts as a non-competitive antagonist of the u-PAR using a non-cell based biochemical assay in which soluble u-PAR and immobilized urokinase were used. However, it is also possible that Å6 mediates its anti-metastatic effects via other mechanisms. For example, phosphorylation of urokinase at serine 138 has previously been shown to modulate cell adhesion and motility<sup>34</sup> and Å6 might act as a phosphorylation substrate in competition with endogenous urokinase.

In conclusion, we have demonstrated the ability of Å6, a urokinase-derived peptide, to increase the survival of mice bearing orthotopically grown prostate cancer and to reduce the metastatic spread of this cancer to lymph nodes. Å6 can be added to a short list of agents that perturb the urokinase-u-PAR axis to counter tumor invasiveness. Our study sets the stage for future investigations to determine the clinical utility of Å6, or congeners, in reducing prostate cancer dissemination.

### Acknowledgments

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RE: Manuscript #030066, 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

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We have completed evaluation of your above-referenced manuscript. I am pleased to inform you that it has been accepted for publication in *Clinical Cancer Research*.

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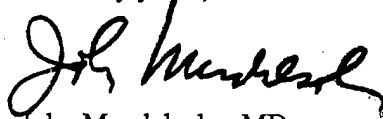
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January 6, 2003

Dear Dr. Mendelsohn:

Enclosed are four originals of our manuscript entitled “**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**” for consideration for publication in *Clinical Cancer Research*. The manuscript presents original data that has not been submitted elsewhere, and all authors agree to its submission. There are no financial relationships with any commercial company, as this work was funded entirely by federal grants.

The data in the manuscript demonstrate the importance of overexpression of the receptor protein tyrosine kinase, c-Met, in progression of prostate tumor cells, and key signaling pathways required for the biologic effects of c-Met in vitro and in vivo. While past studies have correlated c-Met expression with poor prognosis, we sought to determine a cause/effect relationship with c-Met and tumorigenic growth and metastatic potential by downregulating c-Met expression in a highly specific manner using an Adenovirus expressing a c-Met ribozyme. Using this strategy, we reduced steady-state c-Met levels in PC3P-Ln4 cells prostate tumor cells, and demonstrated that proliferation, migration and invasion were inhibited in vitro. Ex vivo infection of the adenovirus resulting in lowered c-Met expression nearly completely inhibited growth of these cells in the prostate of nude mice as well as ability to metastasize to the lymph nodes. Strikingly, in vivo injection of the Adenovirus expressing the c-Met ribozyme into the prostates of nude mice in which PC3P-LN4 cells were growing (i.e. a “gene therapy” experiment) also resulted in significant inhibition tumor outgrowth and development of lymph node metastases. We determined that one of the key signaling molecules downregulated by decreased c-Met expression is Src activation, and in accord with Src inducing VEGF expression, that downregulation of c-Met greatly decreased VEGF expression in vitro. We therefore believe that the studies in this paper add considerable insights into prostate tumor progression, demonstrating that c-Met activation is important in early stages of progression, that Src is a key intermediate in biologic functions induced by c-Met, and

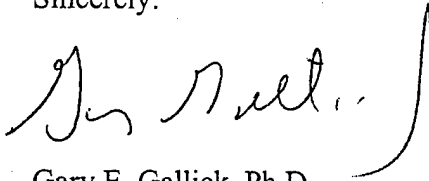
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that one of the important roles of c-Met/Src activation is inducing expression of VEGF. Finally, the data strongly suggest that a c-Met inhibitor could have therapeutic efficacy in the treatment of prostate cancer. We believe that these results will be of high interest to the readers of Clinical Cancer Research. If I may provide any additional information with respect to the manuscript, please do not hesitate to contact me.

Sincerely:

A handwritten signature in black ink, appearing to read "Gary Gallick". The signature is fluid and cursive, with a large, sweeping flourish at the end.

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**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**

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**Key words:** prostate cancer, receptor protein tyrosine kinases, c-Src, tumor progression

**Running title:** c-Met Downregulation Inhibits Prostate Tumor Growth

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## Abstract

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

**Methods:** 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 Erk1/2 expression and phosphorylation, Src expression and activity, and VEGF 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 following 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 VEGF expression and decreased migration and invasion versus the pU1 (control) virus. Significant inhibition of tumorigenicity (histologically confirmed tumors in only one of ten 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 7/8 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, more than 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 receptor protein kinases (RPTKs), 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). Hepatocyte growth factor (HGF), also known as scatter factor (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 (PIN) (16). Further, 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 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, specially 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, while 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 downregulation has been employed (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 downregulation 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 downregulation 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 downregulation 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 biologic 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 downregulation of c-Met strongly downregulates VEGF expression, and further, 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 (Rockville, MD). 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 Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 1mM sodium pyruvate, 0.1mM nonessential amino acids, 2mM L-glutamine, a two-fold vitamin solution (GIBCO), in the absence of antibiotics. Cell cultures were maintained and incubated in 5% CO<sub>2</sub>/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 U. S. Department of Agriculture, U. S. Department of Health and Human Services, and the National Institute of Health. The mice were used in accordance with Institutional guidelines when they were 8 to 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 BAMHI 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 EcoRI and SpeI site to create the vector. As a control, an irrelevant sequence was also cloned into pU1 between EcoRI and SpeI sites and was designated PU-1.

Replication-defective adenoviral vectors of the ribozyme expressing (Ad-c-Met) and control (Ad-PU-1) constructs were constructed as described by Abounder et al. (32) and purified viruses were a generous gift of Dr. J. 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 phosphate-buffered saline, 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 (OPU), was determined by optical absorbance as described previously (28). Infectious viral titers were determined by quick cytopathic effect (CPE) assays. Viral particles/ml were calculated by using the following formula: [(number of cells/well)x(dilution with CPE, 10<sup>4</sup>)x(10 virus/cell)]/0.3ml viral sample/well. For infection of PC3-LN4 cells, cultures at 90% confluency were infected with at the indicated multiplicity of infection (MOI).

### **Immunoblotting**

Cells (1x10<sup>6</sup>) were plated in monolayer on a 10cm dish and infected with either Ad-PU-1 or Ad-c-Met for twenty-four hours. Cells were washed twice with Ca<sup>2+</sup>, Mg<sup>2+</sup>-free ice cold phosphate-buffered saline (135 mM NaCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8.0 mM Na<sub>2</sub>HPO<sub>4</sub>, pH7.4, 2.5 mM KCl) and lysed on ice in lysis buffer (50 mM Hepes, pH 7.0, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM ethylene glycol-bis(beta-amminiethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM sodium pyrophosphate (NaPPi), 10% glycerol, 1% Triton X-100, 1 mM Na<sub>2</sub>VO<sub>4</sub>) plus 1 tablet per 10 mL lysis buffer consisting of Complete Mini, EDTA-free Protease Inhibitor Cocktail 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 (DC) Protein Assay system (BioRad; Hercules, CA) and optical density measured by a spectrophotometer at 570 nm. Whole cell lysates were boiled for 5 min then loaded in Laemelli's sample buffer (2% SDS, 5% -mercaptanol, 125 mM Tris, pH 6.8, 1 mM EDTA, 10% glycerol, 0.02% bromophenol blue) and resolved by SDS-PAGE on an 8% polyacrylamide gel. Proteins were transferred to NitroPure nitrocellulose membranes (Fisher Scientific). Membranes were blocked for one hour in blocking buffer (5% dry

milk, Kroger; Houston, TX) in tris-buffered saline pH 7.5 (TBS-T, 100 mM NaCl, 50 mM Tris, 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-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Chemicon; Temecula, CA) anti-Src (clone 327) (Oncogene Research Products; Cambridge, MA) mouse monoclonal antibodies; and/or anti-Erk1/2 (Ab-2) (Oncogene Research Products; Cambridge, MA), anti-Phospho-p44/42 MAP Kinase (Thr202/Tyr204) (Cell Signaling Technology; Beverly, MA), anti-Akt (Cell Signaling Technology; Beverly, MA), and anti-Phospho-Akt (Ser473) (Cell Signaling Technology; Beverly, MA) 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 anti-mouse antisera (Bio-Rad; Hercules, CA), or goat anti-rabbit (Bio-Rad; Hercules, CA) 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 Scio Image. The densitometry readings of the chemiluminescent bands were adjusted to consider for GAPDH as a loading control. 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 x g. Cell lysates (250 $\mu$ g protein) were reacted for 2 hours with the Src monoclonal antibody 327 (Oncogene Research Products, Cambridge, MA). Immune complex kinase assays

were performed as described by Windham et al. (37). Briefly, immune complexes were formed by the addition of 6 $\mu$ g of rabbit anti-mouse IgG (Organon Teknika, Durham, NC) for 1 hour, followed by 50 $\mu$ l 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, 150mM NaCl, and 10mM sodium phosphate. Reactions were initiated at 22<sup>0</sup> C by the addition to each sample of 10 $\mu$ Ci of [ $\gamma^{32}$ P] ATP, 10 mM Mg<sup>2+</sup>, and 100  $\mu$ M sodium orthovanadate in 20mM HEPES buffer. To analyze phosphorylation of an exogenous substrate, 10  $\mu$ g rabbit muscle enolase (Sigma) was 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 employed to estimate growth rates of cells. PC3-LN4 cells (4 x 10<sup>5</sup>) were plated in monolayer on a 10 cm dish and infected with Ad-Pu-1 or Ad-c-Met at MOIs of 25 and 50. Cells were trypsinized and cell viable cells were counted using a hemacytometer following trypan blue staining. One thousand cells for each line were plated in triplicated each on a 96 well plate. Every twenty-four hours, 50  $\mu$ l MTT {3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Thiazolyl blue, Sigma} were added to each well. After two hours of incubation, all media was removed and the cells were solubilized with the addition of 200  $\mu$ l DMSO (Fisher Scientific). The optical densities were read on a spectrophotometer at 570 nm. The optical densities readings were divided by the reading at time zero 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  $\mu\text{m}$  pores (Control Inserts; Fisher Scientific). Invasion chambers (Biocoat Matrigel Invasion Chambers; Fisher Scientific) are identical to the migration chambers, with the addition of a layer of matrigel layered on top of the pores. For both assays, the bottom chamber was filled with 500  $\mu\text{l}$  of conditioned media and then the insert was placed in the conditioned media. Conditioned media was obtained by growing NIH 3T3 fibroblasts to 80% confluency at 72 hours in DMEM/F12 + 10% FBS. After 72 hours, the media was removed from the cells and filter sterilized. For the migration and invasion assays, equal numbers of cells in a volume of 500  $\mu\text{l}$  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 200x fields and averaged to give  $n=1$  per 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 hours from cells growing in 1% FBS-containing medium. The VEGF concentration was determined using an enzyme-linked immunosorbent assay (ELISA) kit for human VEGF (R&D System) 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 ( $1 \times 10^6$ ) were infected for twenty-four hours with either Ad-PU-1 or Ad-c-Met at an MOI of 25. Cells were harvested after a brief exposure to 0.25% trypsin:0.1% EDTA solution (w/v). After dislodging cells, the cell suspensions in culture media was pipetted to obtain single cell suspensions and counted for cell viability in trypan blue with a hemacytometer.  $2.5 \times 10^6$  cells per group were counted, centrifuged, and resuspended in sterile  $\text{Ca}^{2+}$  - and  $\text{Mg}^{2+}$  - free Hank's Balanced Salt Solution (HBSS) at  $5 \times 10^5$  cells per 50  $\mu\text{l}$ . 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 microliters of HBSS containing  $5 \times 10^5$  cells was injected into a lateral lobe of this prostate. The wound was closed with surgical metal clips. 10 mice/group were injected.

#### **Gene Therapy Studies**

Subconfluent cultures of PC3P-LN4 cells were harvested by trypsinization. Cells were counted and resuspended in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS at 50,000 cells/50ml. 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  $1.5 \times 10^9$  plaque forming units (pfu) of Ad-c-Met or Ad-PU-1, each in a total volume of 20  $\mu\text{l}$ . 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 Hematoxylin/Eosin (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 (chi-squared 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 the total and phosphorylated c-Met protein levels of PC3-LN4 cells by pU1/ribozyme of adenovirus (560) infection**

PC3-LN4 human prostate cancer cells express high c-Met levels. To examine the effects of c-Met downregulation, PC3-LN4 cells were mock infected, or infected with PU-1 and Ad c-Met adenoviruses. As Shown in Figure 1, infection with pU1 (control) virus did not change the expression of c-Met at MOIs as high as 50. In contrast, decreased c-Met expression (decreased by 90% at an MOI of 50) was observed when cells were infected with the ribozyme-expressing Ad-c-Met virus (Fig. 1.)

### **Inhibition of cellular proliferation of PC3-LN4 cells by Ad-c-Met infection**

To determine the in vitro growth rates of c-Met downregulated cells, MTT analysis was performed at 24, 48, 96 and 120 hours post-infection. The results are shown in Figure 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 (%) at 24, 48, 72, 96 and 120 hours were  $33.0 \pm 7.3$ ,  $52.5 \pm 3.0$ ,  $64.5 \pm 5.2$ ,  $82.8 \pm 0.9$ , and  $87.2 \pm 0.8$  with 25 MOI and  $44.6 \pm 2.7$ ,  $62.1 \pm 4.4$ ,  $73.1 \pm 1.0$ ,  $82.3 \pm 1.4$ , and  $92.4 \pm 0.8$  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 Figure 3A, PC3 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% decrease to an average of  $<1 \text{ cell} \pm .31$ ,  $p < 0.01$ , 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 was also performed as described in Materials and Methods. The results are shown in Figure 3B. 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  $<1 \text{ cell} \pm 0.11$ ,  $p < 0.001$ , unpaired Student's T test). Thus, reduction of c-Met corresponded with reduced biologic functions resulting from c-Met signaling.

#### **Tumorigenicity in the prostate following ex vivo infection of Ad-c-Met**

As c-Met downregulation 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% (8/9)

mice developed tumors. Similarly, 88.9% (8/9) 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 (Fig. 4 and 5). In contrast, infection with Ad-c-Met significantly reduced tumor incidence and tumor growth with 10% of animals (1/10) developing tumors, and the one macroscopically detectable tumor was very small (0.07g) with largely normal histology by H&E staining (Fig. 5D). There were no mice with detectable lymph node metastases (see Fig. 4C). Statistical comparison in tumor weight was inappropriate due to the low tumorigenicity induced by Ad-c-Met infected cells.

#### **Effects of Intraprostatic Injection of Adenoviruses into Pre-established PC3-LN4 Tumors**

To determine if 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-PU-1 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 7/8 mice infected with Ad-PU-1 and 8/8 sham-injected mice, tumors developed in the prostate. In contrast, only 1/8 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 if decreased VEGF expression along with decreased proliferation rates might explain, in part, the growth inhibition of tumors following decreased c-Met expression, we examined VEGF expression in the media of PC3-LN4 cells 48 hours after infection with ribozyme expressing (Ad-c-Met) or control (PU-1) virus. The results are shown in Figure 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-PU-1 (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 following downregulation 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 greater than 10 fold in PC3-LN4 cells infected with Ad-c-Met at an MOI of 50, with consistent reductions in activity observed at lower MOIs relative to the control virus (Fig. 7). Only a small reduction in Src activity was observed upon infection with high MOIs of the control virus (Fig. 7). Further, only very small reductions in Src expression were observed at MOIs as high as 100 (Fig. 7), demonstrating that downregulation of c-Met downregulates Src activity. In contrast, adenovirus infection (i.e. either Ad-c-Met or Ad-PU-1) appeared to increase expression of Akt, but no differences in phosphorylation were observed between control (Ad-PU-1)

and ribozyme-expressing (Ad-c-Met) viruses (Fig. 8). Erk 1/2 expression appeared somewhat decreased by infection with Ad-c-Met, but this decrease was less than 2 fold and likely reflects the slower proliferation rate of these cells. Thus, while adenovirus infection itself has some effects on signal transduction, the decrease in VEGF expression observed following c-Met downregulation likely results from 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 pathologic 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 HER 1, HER 2, HER 3 (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 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 biologic functions of prostate tumor cells, where 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 biologic properties of these cells. Further, 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 downregulation 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. Downregulation of c-Met expression following 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 appears to play an important role in the ability of these cells to grow. In vitro studies also demonstrated that downregulation 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.

While a large number of signaling cascades are induced upon c-Met activation [reviewed by Furge et al.(48), Maulik et al., (49)], we demonstrated that decreased c-Src activity, but not Akt or Erk1/2 phosphorylation occurred upon c-Met downregulation. This result is consistent with Src constitutively docking with c-Met when c-Met is overexpressed and therefore activated. Strong biochemical (50) and genetic evidence (51) 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 (52). As Src is involved in a number of survival pathways (reviewed by Schlessinger, 53), 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 downregulated as well. 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 (54). Further, vascularization of tumors was unchanged (54). Ectopic expression of PTEN does not affect c-Src activity (Gallick, unpublished), suggesting that promotion of angiogenesis by c-Met through Src activation may be early event in prostate tumor progression. While it is difficult to determine whether the downregulation of c-Met significantly reduced metastatic potential of the PC3-LN4 cells due to 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 downregulation 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.

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Table 1 *Tumorigenicity and lymph node metastases induced by PC3-LN4 Cells following ex vivo Infection of Adenovirus or Mock Infection*

Group <sup>a</sup>	Tumor Incidence <sup>b</sup>	Tumor size (g) median (range)	Lymph node Involvement <sup>c</sup>
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

<sup>a</sup>PC 3-LN4 cells were mock infected, or infected with the indicated adenovirus at an MOI of 50, and  $5 \times 10^5$  cells were injected into the prostates of nude mice, as described in Materials and Methods

<sup>b</sup>Number of mice with tumors/number of mice surviving surgery for prostate tumor cell implantation

<sup>c</sup>Number of mice with lymph node metastases/number of mice with tumors

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

Group <sup>a</sup>	Tumorigenicity <sup>b</sup>	Tumor size (g) Median (range)	Lymph node Involvement <sup>d</sup>
Sham	8/8	0.66 (0.48-0.99)	8/8
Ad-PU-1 (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

<sup>a</sup>PC3-LN4 cells ( $5 \times 10^5$ ) were injected into the prostates of nude mice as described, and allowed to grow for seven days, after which mice were sham injected or injected with indicated adenovirus two times in two week intervals as described in Materials and Methods.

<sup>b</sup>Number of mice with tumors/number of mice injected

<sup>c</sup>Number of mice with lymph node metastases/number of mice with tumors

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## Figure Legends

**Figure 1. Effects of Ad-PU-1 and Ad-c-Met infection on c-Met expression in PC3-LN4 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.

**Figure 2. Effects of infection with Ad-PU-1 and Ad-c-Met on proliferation of PC3-LN4 cells.** Cells ( $4 \times 10^5$ ) were seeded in 10cm 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.

**Figure 3. Effects of infection of Ad-PU-1 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  $\mu$ m 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 72h. 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.

**Figure 4. Tumor formation in nude mice following: (A) mock infection; (B), ex vivo infection with Ad-PU-1 control adenovirus; or (C) ex vivo infection with Ad-c-Met.** PC3 LN-4 cells were mock infected, or infected with given adenoviruses at 25 MOI, after which cells were implanted into the prostate of nude mice as described in Materials and Methods. Mice were killed four weeks later and examined for presence of tumors. Panel C displays the only mouse implanted with PC3 LN-4 Ad c-Met-infected cells with macroscopic evidence of tumor.

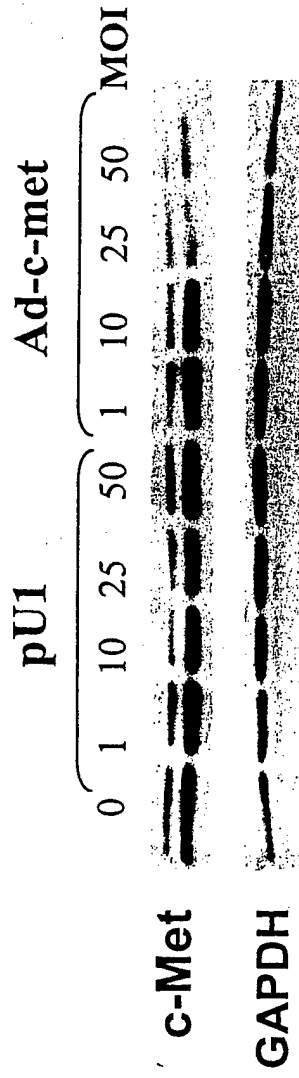
**Figure 5. H&E staining of nude mouse prostate tumors after orthotopic injection of mock, Ad-PU-1 control virus or Ad-c-Met-infected PC3-LN4 cells.** (A) Primary tumor and (B) Lymph node metastasis from mice with mock-infected cells; (C) Primary tumor and (D) lymph node metastasis from PU-1-infected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

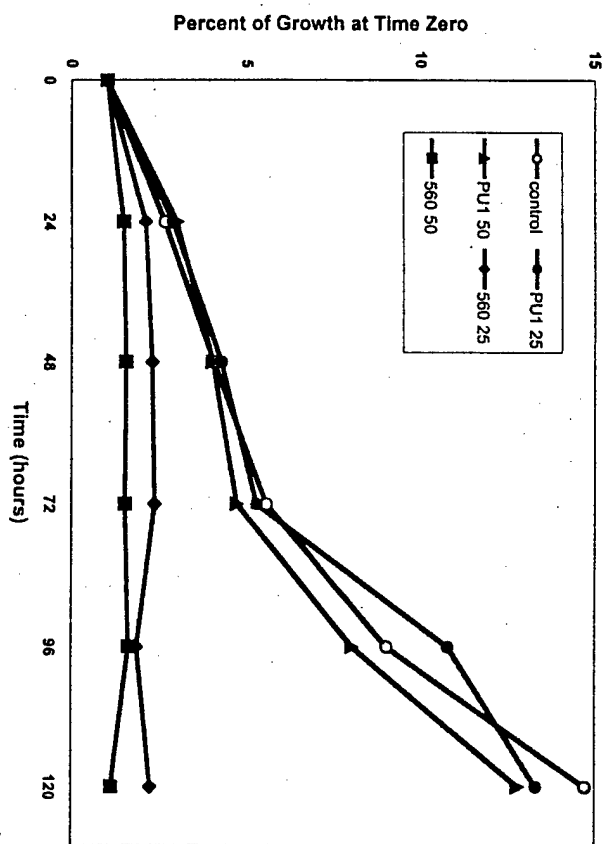
**Figure 6. Expression of VEGF in PC3 Ln-4 Cells infected with Ad-c-Met or Ad-PU-1.** Conditioned medium from PC3-LN4 control and infected cells were harvested after 48 hours from cells growing in 1% FBS-containing medium, and VEGF expression was determined by ELISA, as described in Materials and Methods.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

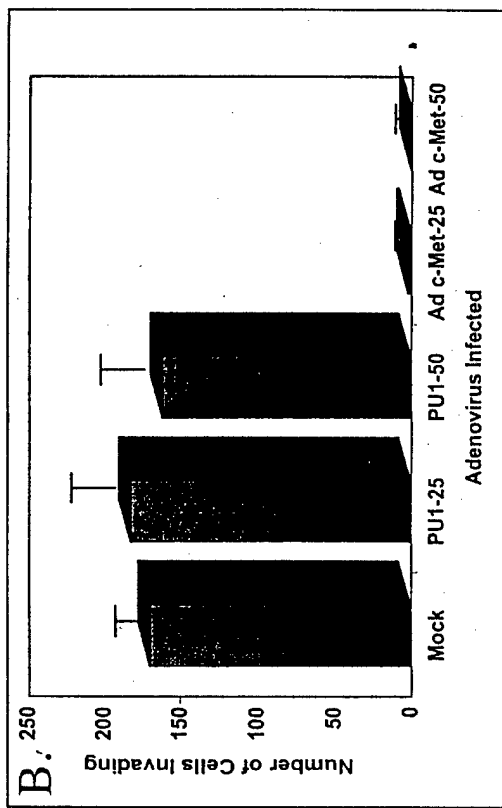
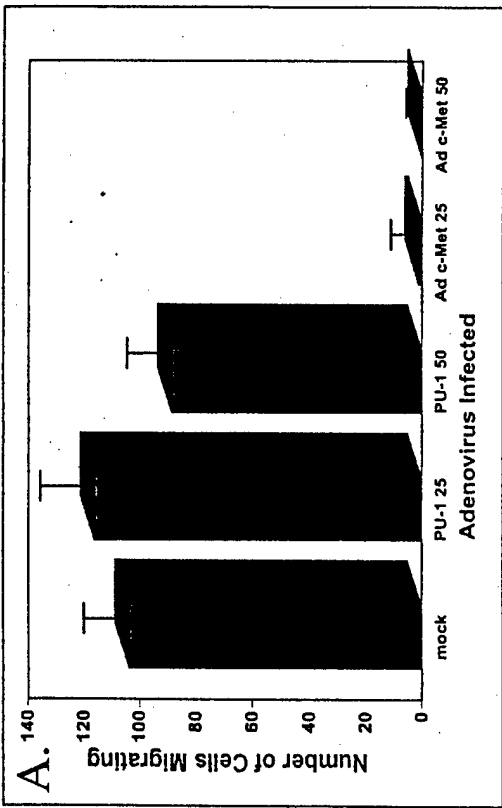
**Figure 7. Expression and Activity of c-Src in mock, Ad-c-Met and Ad-Pu-1-infected PC3 LN-4 cells.** Cells were mock infected or infected with adenoviruses at

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.

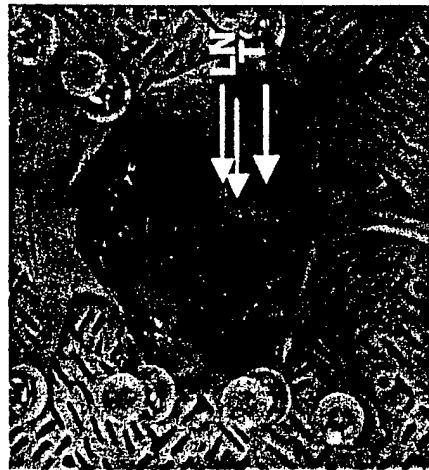
**Figure 8. Expression of phosphorylated and total Erk1/2 and Akt in mock, Ad-c-Met and Ad-PU-1-infected PC3 LN-4 cells.** Cell lysates were subject to SDS-PAGE, electrotransferred to PVD membranes, and incubated with phospho Akt-specific or phospho Erk1/2-specific antibodies. Blots were stripped and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







A.



B.



C.



