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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). 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. In the past year, we have demonstrated in orthotopic mouse model systems that inhibition of both pathways greatly decreases tumorigenicity and metastatic potential of human prostate tumor cells. By inhibiting c-Met expression with an Adenovirus expressing a c-Met ribozyme, tumorigenicity of PC3-LN4 cells (with high metastatic potential) was greatly reduced, and no metastases were formed. Inhibition of uPAR (urokinase plasminogen activator receptor), by the competitive inhibitor for uPA binding, A6, reduced tumorigenicity and metastatic potential. A6 also reduced cellular invasion. Thus, in the past year, we have made substantial progress in demonstrating the effectiveness of inhibition of two critical pathways in metastasis in mouse models, and providing new insights into the role of these molecules in the metastatic phenotype.

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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 previous 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 indicated in that report, the focus of this year would be 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) and uPAR (urokinase Plasminogen Activator receptor) for which uPa (urokinase plasminogen activator) is the ligand. In last year's progress report, I indicated we would focus this year's efforts on the in vivo work in Task 2 and initiate Task 1. We have made substantial progress in each of these areas, as described below.

BODY

The major thrust of Task 2 was to examine the effects on metastatic potential in an orthotopic model of inhibiting c-Met and uPaR with the antagonist termed A6, then examine the combination of the inhibitors to see if better anti-tumor efficacy could be achieved. The inhibitors alone have achieved some remarkable efficacy, as described below.

Inhibition of c-Met

As indicated in the previous progress report, technological advances have made this task easier. The original approach in inhibiting c-Met was to use a plasmid harboring a c-Met hammerhead ribozyme, developed by Drs. Roger Abounader and John Laterra, Johns Hopkins University. More recently, these investigators developed an Adenovirus expressing this ribozyme; thus facilitating both in vitro and animal studies. Our first experiment with this virus was to determine the multiplicity of infection required to achieve inhibition of c-Met. The metastatic prostate tumor cell line PC3-Ln4 was employed for these studies, as described in the original proposal. Results are shown in Figure 1. Cells were infected with increasing multiplicities of infection (MOI) of a control adenovirus lacking the ribozyme (PU-1) or containing the ribozyme (560). No affect of c-Met expression was observed with the control virus, but decreased expression to 90% could be achieved with the ribozyme-expressing virus. Thus, this strategy appeared ideal for testing the ability to inhibit tumorigenicity and metastatic potential in orthotopic nude mouse models.

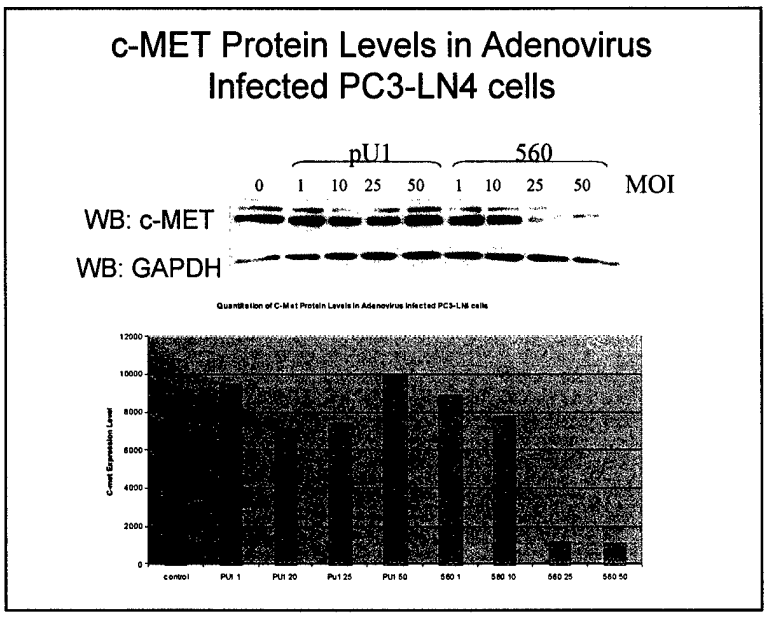

Fig. 1. Inhibition of c-Met in Adenovirus-infected PC3-LN4 cells. Cells were infected with control (Pu-1) or ribozyme containing (560) virus, and c-Met expression was assessed by western blotting. Quantitation of results is shown in the bar graph below

Effect of c-Met inhibition in nude mice

Next, the results of ex vivo infection of PC3Ln4 cells were examined for tumor growth in nude mice. Hematoxylin and eosin staining of the tumors is shown in Figure 2. Under these conditions, only one animal ectopically infected with Ad-560 developed a very small tumor, as compared to 8/9 with the control virus, as shown in Figure 3. We have just finished a gene therapy approach, where one million cells were implanted into the prostate, allowed to grow for a week, and intraprotatic injections of Ad-560 were given twice at two week intervals. Only 1/10 tumors developed after 30 days under these conditions, whereas 6/8 mock-treated and 8/10 control virus-treated animals developed tumors. Therefore, reduction of c-Met appears very effective in inhibiting tumor growth. No lymph node metastases were observed under any of the experimental conditions. The gene therapy experiment has been performed only once, and so must be considered preliminary, but is promising. To finish this task, we must know if the gene therapy will lead to tumor formation, such that combinations of Adenovirus and uPAR inhibitors (see below) can be employed.

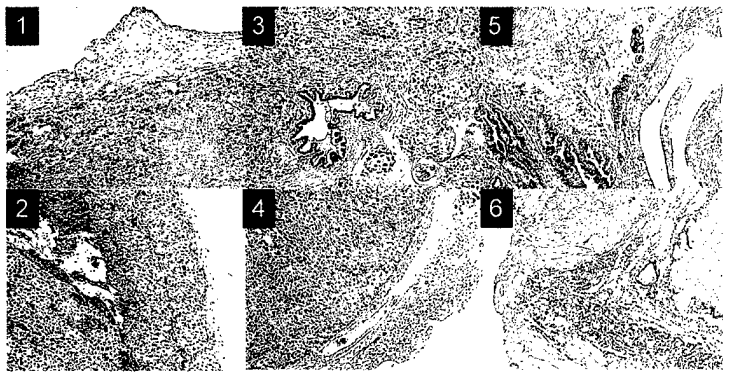

Fig.2 H&E stains of nude mouse prostate after direct injection of c-MET ribozyme or PU1 control virus via adenoviral infection of PC3-LN4 cells. (1) control tumor (2) control lymph node (3) Pu1 control virus tumor (4) Pu1 control virus lymph node (5) prostate of 560 (6) healthy prostate.

Effect of A6 on uPar

As described in Task 2, we are performing experiments to determine the effect of the u-PAR antagonist on *in vitro* prostate cancer invasion. We employed Å6, an Å5 congener (with increased solubility), which also is a potent inhibitor of urokinase binding to its receptor (Guo *et al.* 2000). To determine that the Å6 concentration we proposed for the *in vivo* studies was indeed effective in blocking urokinase binding to its receptor we did the following experiment. Nude mice bearing orthotopic tumors generated with PC-3M- LN4 cells were treated with Å6 or vehicle. Subsequently, tumors were harvested and urokinase bound to membrane u-PAR recovered by an acid treatment and assayed by

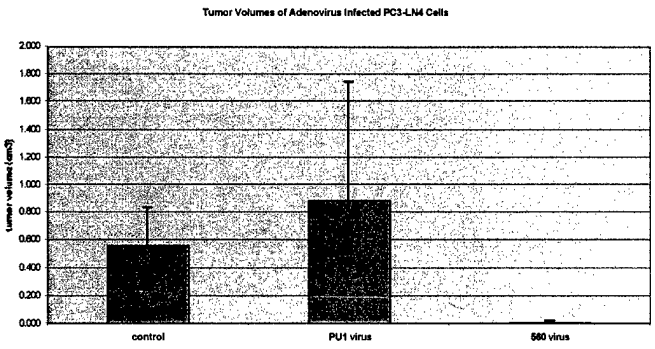

Fig. 3. Inhibition of tumors in an by an Adenovirus expressing a c-Met ribozyme in orthotopic model of prostate tumor progression

Cell Line	No. of mice with tumor/ No. of mice receiving injection	Tumor volume, cm^3, mean ± standard deviation
PC3-LN4:		
Control	8/9	.555 ± .284
PU1	9/9	.886 ± .864
560	1/10	.007 ± .022

Table 1. Å6 inibits urokinase binding to its receptor in PC-3M- LN4 tumors *in vivo*

	Control		Å6 -treated	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Membrane-bound Urokinase (ng/mg protein)	33	42	7	9

PC-3M- LN4 cells (50,000) were injected orthotopically into the prostate of nude mice. After 21 days, the mice were injected twice i/p with Å6 (or vehicle) at a dosage of 75 mg/kg. After 24 h, tumors were harvested and the membrane fraction purified by ultracentrifugation (100,000 X G). The purified membranes (equal protein) were acid treated to dissociate urokinase from u-PAR. The acid eluant was pH-neutralized and assayed for urokinase by ELISA.

ELISA. We found (Table 1) that Å6-treated mice showed a >75 % decrease in the amount of urokinase associated with tumor membrane receptors. Thus, the concentration of Å6 we propose to use effectively competes for binding of the endogenous protease with its receptor in prostate tumors *in vivo*.

Effect of A6 on tumorigenicity in an orthotopic model

Towards determining the ability of Å6 to reduce metastases, we performed a preliminary experiment using morbidity as an endpoint. PC-3M-LN4 cells were injected into nude mice to form orthotopic tumors and 3 days later, Å6, or carrier only, was injected twice daily thereafter. Expectedly, mice treated with the carrier had rapid disease progression with 100 % morbidity after 57 days (not shown). Conversely, all of the Å6-treated mice were viable and healthy after an equivalent time.

We next extended these findings to show that Å6 reduces the metastatic spread of PC-3M-LN4 to the lymph nodes. PC-3M-LN4 cells were injected orthotopically into nude mice. After tumor establishment, mice were treated daily with the indicated doses of Å6 daily. Subsequently, mice were sacrificed and analyzed for tumor spread to the lymph nodes. While prostate tumor masses are clearly obvious in both Å6 (75 mg/kg) treated- and untreated mice, enlarged lymph nodes (with histologically-confirmed tumor cells- Figure Panel B) are only evident in the control mouse (Figure 4, Panel A). Analysis of control and treatment groups indicated that Å6 caused a dose-dependent decrease in the spread of the prostate cancer cells to the lymph nodes (Table 2). The higher concentration of Å6 resulted in over a 75 % reduction in the size of the tumor nodules in the lymph nodes ($p<0.01$).

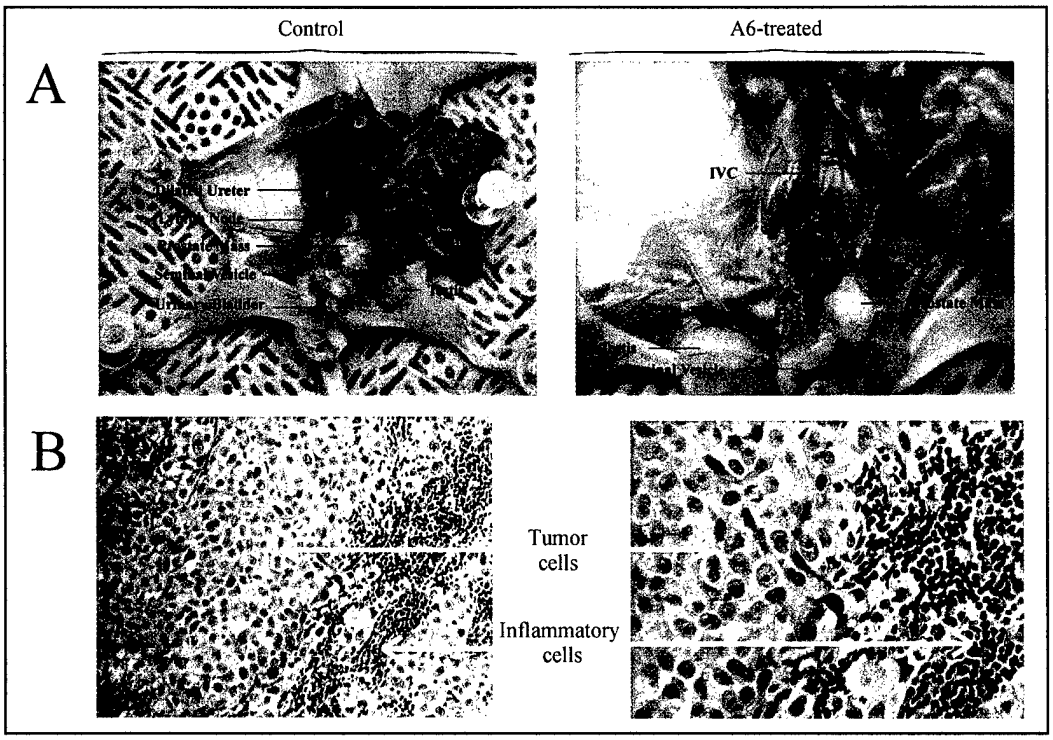

Figure. 4. Å6 treatment decreases PC-3M-LN4 spread to the lymph nodes. Nude mice were injected orthotopically with PC-3M-LN4 (5 X 10^5) and after 9 days treated with 75 mg/kg Å6, or carrier, for 5 weeks. PANEL A; gross examination indicates prostatic tumor mass. IVC, inferior vena cava. PANEL B; histological examination of enlarged lymph nodes from the control group.

Table 2. Å6 treatment decreases PC-3M-LN4 spread to the lymph nodes.

Data analysis from the experiment described for the Figure. Lymph node volumes were averaged across each group and differences tested by the Mann-Whitney U-Test. S.E. standard error.

	# Mice	Tumor Weight (g)	Range	Lymph Node Tumor Volume (mm3)	S.E.	p value
Control	6	0.58	0.2-1.1	8	3.8	
Å6 (25 mg/kg)	7	0.50	0.1-0.5	2.5	0.8	0.05
Å6 ((75 mg/kg)	6	0.50	0.1-0.7	1.4	0.8	0.006

These data demonstrate the efficacy of the chosen Å6 doses in reducing prostate cancer spread over an identical dose range that interferes with u-PAR. Thus, we have nearly completed Task 2, with the exception of the possible combination of the two inhibition strategies.

Task 1

As indicated in the last progress report, Task 1 was to be initiated in the last year. We have done so. Most of Task 1 involves 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. We were waiting to initiate this task until a successful strategy was developed to inhibit c-Met, which as indicated above

has now been demonstrated. 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. The results of this study are shown in Figure 5. Reduction of c-Met by Adenovirus expressing the c-Met ribozyme greatly reduced the ability of the cells to migrate. In contrast, no effect was observed with the control adenovirus. These results demonstrate directly that c-Met regulates migration.

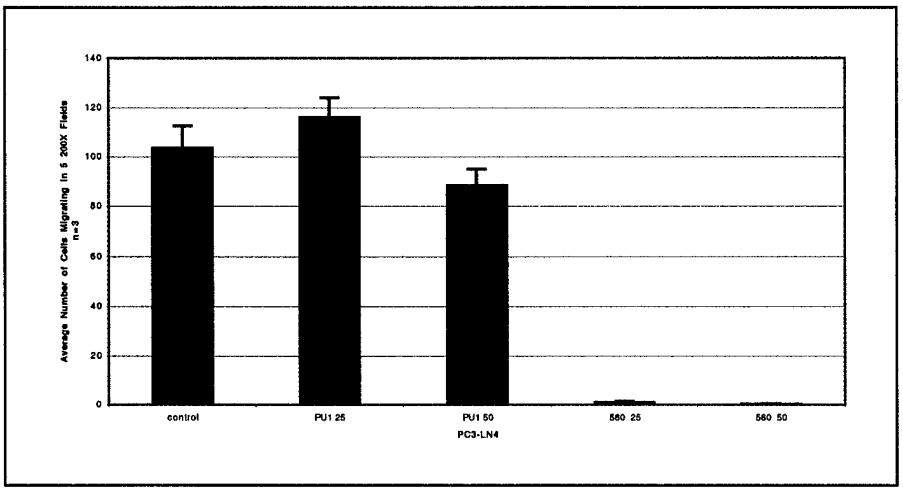

Fig. 5 Migration of PC3-Ln4 cells after reduction of c-Met. The highly metastatic PC3-Ln4 cells were infected with increasing MOI of control Adenovirus (PU-1) or ribozyme-containing Adenovirus (560). Migration was assessed by counting the number of cells on the bottom of a Boyden chamber.

Next, invasion through matrigel was determined. This assay is performed identically to that described above, except the Boyden chamber is coated with matrigel as representative of the extracellular matrix. Thus, this three-dimensional matrix requires the invasion of cells for them to be able to traverse the pores in the Boyden chamber. Results are shown in Figure 6.

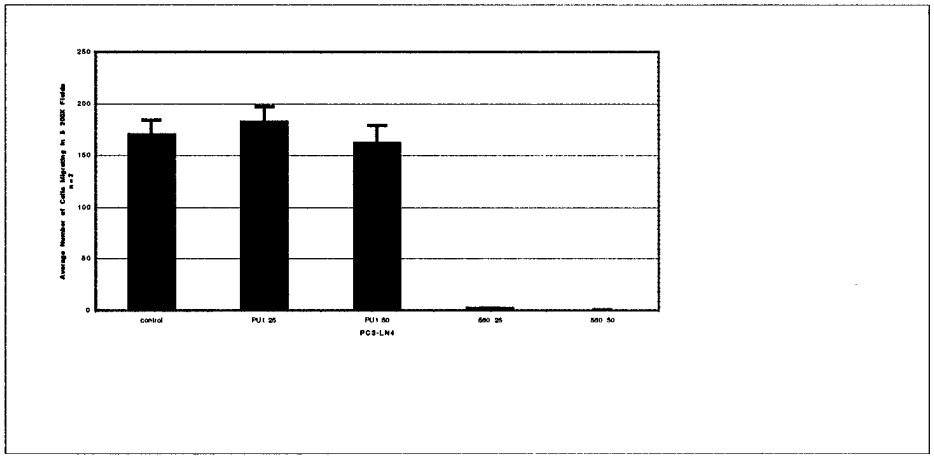

Fig. 6 Invasion of PC3-Ln4 cells after reduction of c-Met. The highly metastatic PC3-Ln4 cells were infected with increasing MOI of control Adenovirus (PU-1) or ribozyme-containing Adenovirus (560). Invasion was assessed by counting the number of cells on the bottom of a Boyden chamber with matrigel at the upper surface.

Finally, the effect of the uPAR inhibitor A6 was examined. Increasing concentrations of A6 were used to examine the invasiveness of PC3-Ln4 cells in an identical assay to that described above. The results are shown in Figure 7. A6 was highly efficient in inhibiting cellular invasion in this assay. Thus, Task

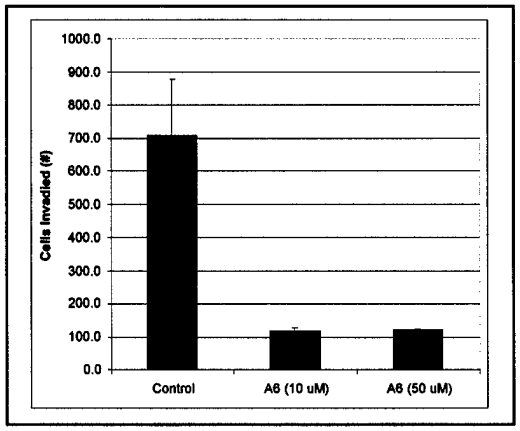

Figure 7. Inhibition of in vitro invasion by a u-PAR antagonist

PC-3M- LN4 cells (25,000) were plated on Matrigel-coated porous filters. After 6 h to allow cell attachment, Å6 was added where indicated. After an additional 48 h, cells on the lower aspect of the filter were counted. The Å6 did not alter the growth rate of the cells.

1 is nearly completed, although a combination of inhibition strategies will be required to complete the initially proposed task.

Inhibition of c-Met-mediated Signaling by PTEN/MMAC

In the last Progress Report, an alternate strategy to inhibit c-Met signaling, through use of re-expression of the tumor suppressor gene PTEN/MMAC was proposed. As the ultimate goal of this project is to inhibit tumor spread, and non-DOD sources of funding were available for this study, we have continued to pursue the effects of expression of this tumor gene in the cell line systems described above. Results from our first study are striking, with ectopic expression of MMAC/PTEN inhibiting tumor metastasis, but not tumor establishment of PC3 cells in an orthotopic nude mouse model. The first publication on these results (Davies et al., 2002) is provided in the APPENDIX, along with a commentary by Drs. Fernandez and Eng (SEE APPENDIX) indicating the importance of our observations. Thus, it is possible that re-introduction of MMAC/PTEN will be an excellent combination strategy to those that are the main focus of the Statement of Work. Further, as MMAC/PTEN is the negative regulator of survival pathways activated by c-Met, this study is well within the focus of the goals of this proposal. However, should we move in the direction of combining MMAC/PTEN with c-Met and uPAR inhibition, we would seek permission of the Grants Officer. As some of the same personnel were involved in the MMAC/PTEN project, I thought it appropriate to acknowledge the DOD support on this exciting work.

Task 3

This task involves examination of surgical specimens. This task was not proposed to begin until year 2, and as indicated in last year's report, will be delayed do to the late start of the grant. This task will be undertaken in the upcoming year, but a no cost extension of the grant will be requested to complete it.

KEY RESEARCH ACCOMPLISHMENTS

- Reduction of c-Met expression decreases tumorigenicity and metastatic potential of human prostate cells in an orthotopic model
- Reduction of c-Met decrease uPAR expression
- Reduction of c-Met decreases migratory and invasive ability of prostate tumor cells
- Inhibition of uPAR function by the competitive inhibitor for uPA, A6, leads to decreased tumor growth and metastases in an orthotopic nude mouse model for prostate tumor progression

- Inhibition of uPAR function by A6 decreases the invasive ability of human prostate tumor cells
- Ectopic expression of MMAC/PTEN inhibits metastatic potential of the same prostate tumor cells by reversing some of the same molecular pathways activated by overexpression of c-Met

REPORTABLE OUTCOMES

Downregulation of c-Met expression decreases tumorigenic growth and inhibits metastasis of PC3 Ln-4 human prostate tumor cells in an orthotopic nude mouse model-Manuscript in preparation

Targeted Inhibition of uPAR function by the uPA antagonist A6 decreases the growth and invasiveness of PC3 Ln-4 human prostate cells in an orthotopic nude mouse model-Manuscript in preparation

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-see APPENDIX

CONCLUSIONS

Work in year 1 (actually only four months due to change of PI's) established that the strategies proposed for inhibition of uPAr and c-Met could be successful. Work in year 2, as presented in this progress report, has proven the effectiveness of the strategies in *in vivo* models. Inhibition of c-Met dramatically decreased tumorigenicity of human prostate tumor cells grown in the prostates of nude mice, and completely inhibited spread to the lymph nodes. Inhibition of uPAR by the uPA anatagonist a6 was highly effective in reducing tumorigenic potential in similar model systems. These experiments nearly complete task 2. Both compounds were highly successful in inhibiting cellular invasion, the principal requirement of task 1. Thus, many of the initial goals of the grant have been accomplished, by demonstrating the efficacy of inhibition of two critical pathways important to tumor progression. Further, the work demonstrates the relatedness of the pathways, as inhibition of c-Met inhibits uPAR expression. Thus, I believe we are making excellent progress in completing the tasks, which are providing new insights into prostate tumor progression, and possible strategies to interfere with metastasis, the process that kills most patients afflicted with the disease.

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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).

APPENDIX

1. Magali Fernandez and Charis Eng. The Expanding Role of PTEN in Neoplasia: A Molecule for All Seasons? : Commentary re: M. A. Davies, *et al.*, Adenoviral- mediated Expression of MMAC/PTEN Inhibits Proliferation and Metastasis of Human Prostate Cancer Cells. Clin. Cancer Res., *8:* 1904–1914, 2002. Clin. Cancer Res. 2002 8: 1695-1698.

2. Michael A. Davies, Sun J. Kim, Nila U. Parikh, Zhongyun Dong, Corazon D. Bucana, and Gary E. Gallick. Adenoviral-mediated Expression of MMAC/PTEN Inhibits Proliferation and Metastasis of Human Prostate Cancer Cells. Clin. Cancer Res. 2002 8: 1904-1914.

Adenoviral-mediated Expression of MMAC/PTEN Inhibits Proliferation and Metastasis of Human Prostate Cancer Cells[1]

Michael A. Davies, Sun J. Kim, Nila U. Parikh, Zhongyun Dong, Corazon D. Bucana, and Gary E. Gallick[2]

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ABSTRACT

Purpose: **The purpose of this study was to determine the effects of adenoviral transgene expression of MMAC/PTEN on the *in vitro* and *in vivo* growth and survival of PC3 human prostate cancer cells.**

Experimental Design: **Adenoviruses expressing MMAC/PTEN or green fluorescent protein as a control were introduced into PC3 cells, and effects on signal transduction pathways and growth of tumors in an orthotopic nude mouse model were determined.**

Results: **MMAC/PTEN expression in PC3 cells decreased the level of phospho Akt but not that of phospho Mapk or FAK. Expression of MMAC/PTEN inhibited the *in vitro* growth of PC3 cells primarily by blocking cell cycle progression. *Ex vivo* introduction of MMAC/PTEN expression did not inhibit the tumorigenicity of orthotopically implanted PC3 cells, but it did significantly reduce tumor size and completely inhibited the formation of metastases. *In vivo* treatment of pre-established orthotopic PC3 tumors with adenoviral MMAC/PTEN did not significantly reduce local tumor size, but it did diminish metastasis formation.**

Conclusions: **MMAC/PTEN functionally regulates prostate cancer cell metastatic potential in an *in vivo* model system and may be an important biological marker and therapeutic target for human prostate cancer.**

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths in men in the United States, with a predicted 32,000 patients succumbing to the disease in 2001 (1). Currently, there is no effective treatment for advanced stages of this disease. To improve our understanding of prostate cancer progression, many studies have examined the chromosomal alterations that characterize its different stages (2, 3). Although many alterations have been observed, those involved most frequently include chromosomes 7q, 8p, 10, 13q, and 16q (4–6). Allelic deletions associated with chromosome 10q, specifically the q23–25 region, have been observed to preferentially occur in the advanced stages of disease (7, 8). Reintroduction of this chromosomal region into rat prostate cancer cells significantly inhibited their metastatic capabilities but failed to alter their tumorigenicity (9). Many studies have implicated the tumor suppressor gene, *MMAC/PTEN,* as critical to the biological effects associated with deletions in (or reintroduction of) chromosome 10. *MMAC/PTEN* was initially identified because of homozygous deletions of the gene in human glioma and breast cancer cell lines, as well as by its homology to protein phosphatases (10–12). These and subsequent studies demonstrated that deletions and mutations of the gene occur in a wide variety of cancers, including glioma, prostate, breast, melanoma, endometrial, and ovarian (reviewed in Ref. 13). With the exception of endometrial cancer, alterations to MMAC/PTEN are detected almost exclusively in advanced stages of disease. Thus, the loss of *MMAC/PTEN* function does not appear to be required for tumor initiation in these diseases but instead was a hallmark of tumor progression.

One of the critical functions for the tumor suppressor activity of MMAC/PTEN is its intrinsic lipid phosphatase activity (14), which results in removal of the phosphate moiety at the 3′ position of phosphatidylinositol. By this activity, MMAC/PTEN antagonizes signaling mediated by PI3K[3] and negatively regulates the phosphorylation and activation of a number of important mitogenic and/or prosurvival signaling molecules. Our group and others have shown that enforced MMAC/PTEN expression decreases the phosphorylation and kinase activity of Akt/PKB, a proto-oncogene that is activated by PI3K-mediated signaling events (15, 16). Akt/PKB, a serine-threonine protein kinase, has been shown to be a critical regulator of many different cellular processes, including apoptosis, cell cycle progression, angiogenesis, and metabolism. Other reports (17, 18) have demonstrated that MMAC/PTEN may also dephosphorylate FAK, a protein tyrosine kinase involved in the regulation of cellular adhesion, motility, and survival.

Numerous studies have implicated the loss of MMAC/

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[1] Supported by DOD 17-00-1-0524 and The Gillson Longenbaugh Foundation (to G. E. G.).
[2] To whom requests for reprints should be addressed, at Department of Cancer Biology, Box 179, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3657; Fax: (713) 745-1927; E-mail: ggallick@mdanderson.org.

[3] The abbreviations used are: PI3K, phosphatidylinositol-3′ kinase; MAPK, mitogen-activated protein kinase; Ad-GFP, adenovirus expressing the enhanced green fluorescent protein; Ab, antibody; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; MOI, multiplicity of infection; FAK, focal adhesion kinase; FACS, fluorescence-activated cell sorter; PKB, protein kinase B.

PTEN in prostate cancer progression. In the initial identification of the gene, homozygous genetic inactivation of MMAC/PTEN was observed in the LNCaP and PC3 human prostate cancer cell lines, both of which were established from metastatic lesions (10, 11). Multiple cytogenetic studies of prostate tumors detected low rates of mutation and deletion of MMAC/PTEN in organ-confined prostate cancers (19–21). However, ~60% of metastatic prostate cancers demonstrated loss of heterozygosity for *MMAC/PTEN,* accompanied by a significant rate of alterations of the second allele (22, 23). In several tumors that retain the *MMAC/PTEN* gene, decreased expression of the protein may be observed, *e.g.,* Whang *et al.* (24) demonstrated that *MMAC/PTEN* mRNA and protein levels were down-regulated in >50% of prostate tumors in which the *MMAC/PTEN* coding region was not mutated. Furthermore, immunohistochemical analysis of human prostate cancer specimens detected down-regulated expression of MMAC/PTEN protein in a heterogeneous pattern (25). Interestingly, the foci in which MMAC/PTEN expression was lost were generally high-grade lesions, suggesting that these foci might give rise to the aggressive components of these tumors.

In contrast, studies using genetically engineered mice suggest a potential role for MMAC/PTEN at an earlier stage of prostate tumor development. The loss of both alleles of *MMAC/PTEN* results in embryonic lethality in mice (26–28). *MMAC/PTEN*$^{+/-}$ mice are viable and spontaneously develop lymphoid hyperplasia, several types of tumors, and an autoimmune syndrome (29). The prostates of these mice demonstrate global hyperplasia and frequently develop prostatic intraepithelial hyperplasia, the precursor of invasive prostate cancer. The global dysregulated growth suggests that the loss of a single allele of *MMAC/PTEN* causes this phenotype (26). Breeding these mice with *p27*$^{+/-}$ mice, leading to progeny that are genetically *MMAC/PTEN*$^{+/-}$ and *p27*$^{+/-}$ or $^{-/-}$, resulted in 100% penetrance of frank prostate cancer, including invasive tumors (30). Thus, these knockout studies suggest that *MMAC/PTEN* is a potent regulator of prostate proliferation and demonstrate in this system a potential role in the earlier stages of prostate cancer tumorigenesis.

To further examine the ability of MMAC/PTEN expression to regulate prostate tumor development and/or progression, we have studied the effects of MMAC/PTEN expression in PC3 human prostate cells. The PC3 cell line was originally derived from a prostate cancer metastasis and represents a model for androgen-insensitive disease. The PC3 cells lack MMAC/PTEN expression because of deletion of both alleles (10, 11) and also lack wild-type p53 function and androgen receptor expression (31). When injected orthotopically into the prostate of nude mice, the PC3 cells grow aggressively locally (32, 33) and readily metastasize to regional lymph nodes (31). In this study, we expressed MMAC/PTEN in PC3 cells via an adenovirus vector Ad-MMAC (15) and examined the effects on cell signaling pathways, growth, and survival of PC3 cells *in vitro.* Additionally, the effects of MMAC/PTEN expression on the *in vivo* growth of PC3 cells was tested both by orthotopic implantation of cells infected with Ad-MMAC before their inoculation into nude mice (*ex vivo* treatment) and by the treatment of preestablished tumors with Ad-MMAC (*in vivo* treatment). These studies provide further insight into the ability of MMAC/PTEN to regulate specific cell signaling pathways and cellular behaviors in prostate cancer cells and demonstrate for the first time the regulation of *in vivo* prostate cancer metastatic capability.

MATERIALS AND METHODS

Cell Lines and Adenoviruses. The PC3 cell line was obtained originally from the American Type Culture Collection, Rockville, MD. The cells were maintained in culture in media supplemented with 10% FCS and incubated in 5% CO_2/95% air at 37°C. The recombinant adenovirus Ad-MMAC expresses the human wild-type MMAC cDNA under the control of the human cytomegalovirus immediate-early promoter/enhancer (34) and was generously provided by Robert Bookstein (Canjii, Inc., San Diego, CA). Ad-GFP was derived from the same vector as Ad-MMAC; the replication-deficient virus without a transgene (Ad-DE1/Ad5-δE1) has been described previously (35), along with the virus expressing the p53 transgene (36). Adenoviruses were harvested after infection of 293 cells and isolated by cesium chloride gradient, and titer was determined by absorbance. Briefly, viral stock was propagated in 293 cells. Cells were harvested 36–40 h after infection, pelleted, resuspended in PBS, and lysed by three freeze-thaw cycles. Cell debris was removed, and resulting cellular lysates were subjected to double CsCl centrifugation. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. Adenoviral titer represented as optical particles units was determined by optical absorbance as described previously (37).

Tumorigenicity Studies. Cells (500,000) were plated on 10-cm tissue culture dishes. Approximately 18 h later, cells were infected with indicated amounts of adenovirus in 4 ml of media. Cells were gently agitated every 15 min for 1 h, then 6 ml of fresh media were added to each plate. Cells were infected with Ad-MMAC, or Ad-GFP at the indicated MOI, or mock infected by incubation with media alone. Twenty-four h after infection, cells were harvested by trypsinization. Cells were counted and resuspended in Ca^{2+} and Mg^{2+}-free HBSS at 100,000 cells/50 μl. Cells were then implanted in the prostate of nude mice as described previously (33). In brief, nude mice were anesthetized with Nembutal and placed in a supine position. A low-midline incision was made, and the prostate was exposed. Fifty μl of HBSS containing 100,000 cells were injected into a lateral lobe of the prostate. The wound was closed with surgical metal clips. Each experimental condition was performed on groups of eight mice.

Gene Therapy Studies. Subconfluent cultures of PC3 cells were harvested by trypsinization. Cells were counted, then were resuspended in Ca^{2+} and Mg^{2+}-free HBSS at 50,000 cells/50 μl. Cells were then implanted in the prostate of nude mice as described above, and the wound was closed with surgical metal clips. Tumors were treated at 7 and 14 days after implantation. Tumors were exposed by the same procedure, by a low-midline incision, and exposure of the prostate. Tumors were injected with PBS, or 1.5×10^9 plaque-forming units of Ad-GFP or Ad-MMAC, each in a total volume of 20 μl. Tumors and lymph nodes were harvested 14 days after the final gene intratumoral injection. Each experimental condition was performed on groups of eight mice.

Necropsy Procedures and Histological Studies. The mice were euthanized at the times indicated above. Primary tumors in the prostate were excised and weighed. For immunohistochemistry and H&E staining procedures, some tumors were embedded in OCT compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C. Other tumors were formalin fixed and paraffin embedded. Lymph nodes were harvested, and the presence of metastatic disease was determined histologically.

For BrdUrd staining, the mice were injected with 250 μg of BrdUrd. Two h later, the mice were sacrificed, and the prostates were formalin fixed and embedded in paraffin. For staining, tissue sections were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were then treated with 2N HCl for 30 min at 37°C. For CD31 staining, sections of frozen tissues were fixed with cold acetone and transferred to PBS. The slides were then rinsed twice with PBS, and endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 min. The samples were then washed three times with PBS and incubated for 10 min at room temperature with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with 1:100 dilution of monoclonal mouse anti-BrdUrd Ab (Becton Dickinson, Mountain View, CA) or a 1:100 dilution of monoclonal rat anti-CD31 Ab (PharMingen, San Diego, CA). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of peroxidase-conjugated antimouse IgG1 or antirat IgG. The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics, Huntsville, AL). The sections were then washed three times with distilled water and counterstained with Gill's hematoxylin.

For TUNEL staining, frozen sections were fixed with 4% paraformaldehyde in PBS for 20 min, rinsed twice with PBS, and rinsed with double distilled water containing 0.04% Brij-35 (Fisher Scientific, Pittsburgh, PA). The sections were treated with 20 μg/ml Proteinase K (Boehringer Mannheim) for 10 min, rinsed twice with PBS for 5 min, and incubated with terminal deoxynucleotidyl transferase buffer for 10 min. Terminal deoxynucleotidyl transferase and biotinylated 16-dUTP (Boehringer Mannheim) were then added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 h. The slides were washed with buffer two times in 5 min, followed by double distilled water. The sections were incubated with a protein block consisting of 2% normal goat serum in PBS for 10 min, followed by peroxidase-labeled streptavidin diluted 1:400 for 30 min at 37°C. The slides were rinsed three times with PBS and incubated with the Stable 3,3′-diaminobenzidine (Research Genetics) for 30 min at 37°C. The sections were rinsed with distilled water, air dried, mounted with Universal Mount Research Genetics), and examined by bright field microscopy.

Cell Growth. Cells (500,000) were plated on 10-cm tissue culture dishes. Approximately 18 h later, cells were infected with indicated amounts of adenovirus in 4 ml of media. Cells were gently agitated every 15 min for 1 h, then 6 ml of fresh media were added to each plate. Cells were infected with Ad-GFP, Ad-MMAC, or Ad-p53 at the indicated MOI or mock infected with media alone. At the indicated time points, cells were harvested by collecting the supernatant and trypsinizing attached cells. Viable cells were counted by microscopic analysis using a hemocytometer. The significance of differences in cell number was analyzed by unpaired Student's *t* test.

Protein Analysis. Cells (500,000) were plated on 10-cm tissue culture dishes. Approximately 18 h later, cells were infected with indicated amounts of adenovirus in 4 ml of media. Cells were gently agitated every 15 min for 1 h, then 6 ml of fresh media were added to each plate. Cells were infected with Ad-GFP or Ad-MMAC at the indicated MOI or mock infected by incubation with media alone. At the indicated time points, cells were harvested on ice. For analysis of signal transduction pathways, 24 h after infection, cells were washed with serum-free media twice, then incubated overnight in serum-free media. After ~18 h of serum starvation, cells were harvested either with ("stimulated") or without ("starved") a 10-min incubation with 1% FCS-enriched media. Harvesting of cell lysates was performed on ice. After two washes with cold 1× PBS, cells were harvested in lysis solution containing 50 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM EGTA, 100 mM NaF, 10 mM $NaPP_i$, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 μM pepstatin, 10 μg/ml aprotinin, 5 mM iodoacetic acid, and 2 μg/ml leupeptin. The homogenates were clarified by centrifugation at 15,000 × *g* for 15 min at 4°C.

Western analysis was performed as described previously (16). Immunoblotting was performed using antibodies against total and phospho-specific Akt and MAPK (New England Biolabs, Boston, MA) and MMAC/PTEN (Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase-conjugated secondary Ab (Amersham, Chicago, IL). Protein-Ab complexes were detected by chemiluminescence (enhanced chemiluminescence; Amersham). For detection of MMAC/PTEN in mouse tumors, 500 μg of tumor lysate were first immunoprecipitated with the MMAC/PTEN Ab (Santa Cruz Biotechnology); then Western blotting was performed as described above.

For analysis of FAK phosphorylation, 250 μg of lysate were incubated with 2 μg of α-FAK monoclonal Ab (Upstate Biotechnology) for 2.5 h at 4°C, with 6 μl of rabbit antimouse IgG Ab for 1 h, then with 35 μl of Pansorbin Cells for 30 min. Complexes were centrifuged for 1 min at 15,000 × *g*, washed three times with immune complex kinase assay wash buffer, boiled for 5 min in 1× SDS-PAGE buffer, then run on 7.5% gels and transferred to Immobilon-P membranes as described above. Immunoblotting was performed using antibodies specific for phospo-tyrosine residues (4G10; Upstate Biotechnology) and FAK (Transduction Labs).

Apoptosis and Cell Cycle Analysis. Cells (500,000) were plated on 10-cm tissue culture dishes. Approximately 18 h later, cells were infected with indicated amounts of adenovirus in 4 ml of media. Cells were gently agitated every 15 min for 1 h; then 6 ml of fresh media were added to each plate. Cells were infected with Ad-GFP, Ad-DE1, Ad-MMAC, or Ad-p53 at the indicated MOI or mock infected with media alone. At the indicated time points, cells were harvested by collecting the supernatant and trypsinizing attached cells. Cells were fixed in 1% paraformaldehyde and stored in 70% ethanol at 4°C. An equal number of cells was then processed and analyzed for

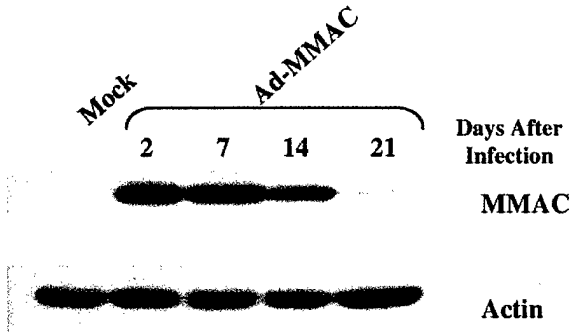

Fig. 1 MMAC/PTEN expression in PC3 cells. Western blotting analysis of PC3 treated with media alone (*Lane 1*) or infected with 25 MOI Ad-MMAC (*Lanes 2–5*). Ad-MMAC-infected cells were harvested at 2, 7, 14, and 21 days after infection to determine induction and duration of MMAC/PTEN expression (*top*). Immunoblotting for actin (*bottom*) performed to confirm protein loading.

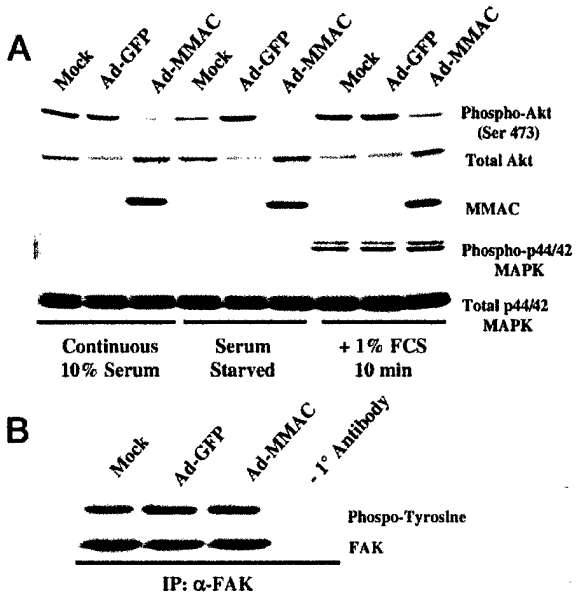

Fig. 2 Effects of ectopic MMAC/PTEN expression in PC3 cells on expression and phosphorylation of signaling intermediates. In *A*, immunoblotting was performed 48 h after mock infection or infection with 25 MOI of Ad-GFP or Ad-MMAC. Cells were harvested after continuous incubation in complete tissue culture media (*Lanes 1–3*) or after a 24-h serum starvation with (*Lanes 7–9*) or without (*Lanes 4–6*) acute 1% FCS stimulation. Phosphorylation of Akt/PKB and p44/42 MAPK (*bottom*) was detected by phosphorylation site-specific antibodies. MMAC/PTEN protein expression also shown (*middle*). *B*, phosphorylation status of FAK in PC3 cells mock treated with media alone (*Lane 1*) or infected with 25 MOI of Ad-GFP (*Lane 2*) or Ad-MMAC (*Lane 3*). Cells were harvested 48 h after indicated treatments. Cell lysates were immunoprecipitated with α-FAK, immunoblotted with α-phosphotyrosine (*top*), and reprobed with α-FAK (*bottom*).

apoptosis by TUNEL assay using the Apo-BrdUrd kit (Phoenix Flow Systems, San Diego, CA) or stained solely with propidium iodide (50 μg/ml in PBS). Apoptotic cells were detected and quantitated by flow cytometry. The significance of differences between different treatment groups was analyzed by unpaired Student's *t* test.

RESULTS

***In Vitro* Studies.** PC3 cells were chosen for these studies because of their ability to form lymph node metastases after orthotopic implantation into nude mice (33). Before the animal studies, we first examined whether the effects of ectopic expression of PTEN/MMAC were similar to those observed in other cell lines. Flow cytometric analysis of PC3 cells infected with an adenovirus engineered to express the Ad-GFP determined that 25 MOI was the minimum concentration of adenovirus required to infect >95% of the cells. To express MMAC/PTEN, PC3 cells were infected with a replication-deficient adenovirus engineered to express wild-type *MMAC/PTEN* cDNA under the control of the human cytomegalovirus promoter. Western blotting analysis showed that PC3 cells did not express MMAC/PTEN protein after mock treatment or infection with a control adenovirus but express high levels after infection with Ad-MMAC (Fig. 1). MMAC/PTEN expression was maintained for at least 21 days in cultured cells, although a significant decrease in the amount of protein was observed at later time points, as compared with 48 h after infection. It is not clear if this represents a decrease in expression by cells infected with Ad-MMAC or a dilutional effect of the proliferation of cells that were not infected.

To further characterize the function of MMAC/PTEN in PC3 cells, the effects of its expression on the phosphorylation status of intermediates of cell signaling pathways were examined by Western blotting. Analysis was performed on cells maintained in FCS-enriched media, as well as in cells that were serum starved or acutely stimulated. For all three tissue culture conditions, MMAC/PTEN expression inhibited phosphorylation of Akt/PKB at its activating residues without diminishing total Akt/PKB protein levels (Fig. 2*A*). These results are consistent with MMAC/PTEN's lipid phosphatase activity and are identical to our previous results in both LNCaP prostate cancer cells and human glioma cells (15, 16). The Ad-GFP virus also led to small reductions in phospho Akt expression; however, this reduction was substantially less than that observed with the Ad-MMAC virus. MMAC/PTEN expression did not decrease the mitogen-stimulated phosphorylation of p44/42 MAPK, as compared with mock or control adenovirus treatments. This also is consistent with our previous results. The effect of MMAC/PTEN expression on the phosphorylation of FAK was also examined. No decrease was observed in tyrosine phosphorylation, or total protein levels, of FAK in PC3 cells (Fig. 2*B*).

The ability of MMAC/PTEN to regulate PC3 cellular growth *in vitro* was determined by counting viable cell numbers at various time points after infection with control adenoviruses or Ad-MMAC or mock treatment with media alone. Infection with Ad-MMAC decreased the rate of PC3 proliferation at all time points examined (Fig. 3*A*). Treatment with Ad-MMAC was significantly more growth inhibitory than treatment with a control adenovirus (Fig. 3*B*; $P < 0.05$ on day 4, $P < 0.01$ on day 6). In previous studies, we had observed that MMAC/PTEN expression was more growth inhibitory than p53 expression in

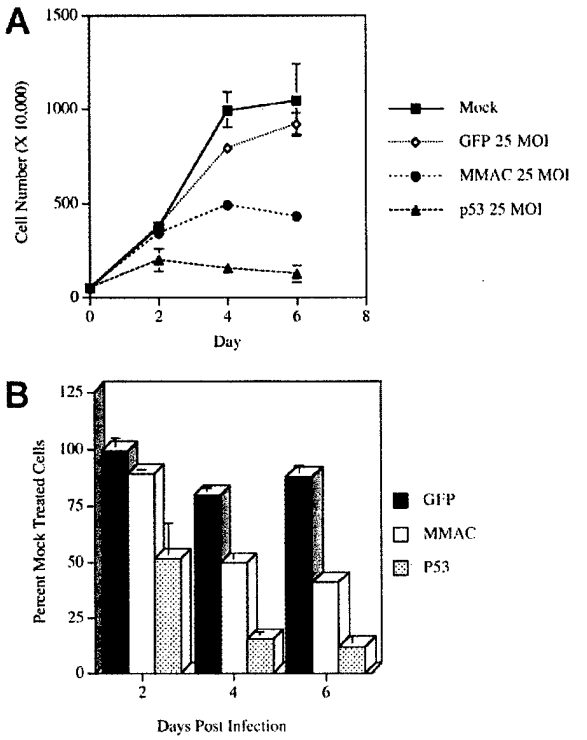

Fig. 3 Effects of ectopic MMAC/PTEN and p53 expression on *in vitro* PC3 cell growth. *A*, viable number of PC3 cells mock infected (■) or infected with 25 MOI of Ad-GFP (◇), Ad-MMAC (●), or Ad-p53 (▲) at 0, 2, 4, and 6 days after infection. *B*, growth of PC3 cells infected with 25 MOI of Ad-GFP (*solid bars*), Ad-MMAC (*open bars*), or Ad-p53 (*shaded bars*), as compared with mock-infected cells. *Columns*, average; *bars*, SD.

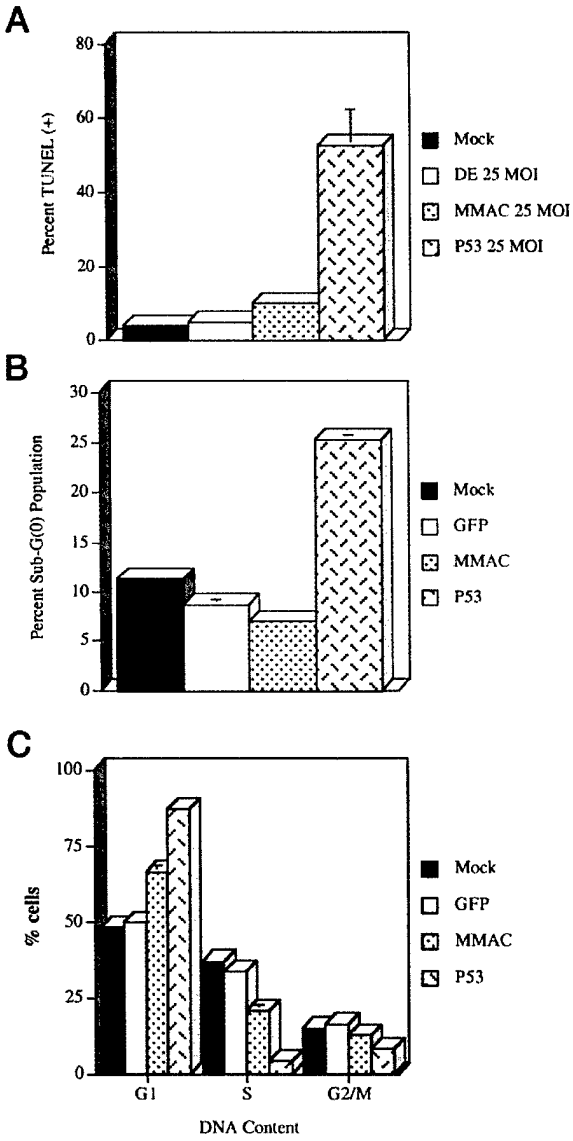

Fig. 4 Effects of ectopic MMAC/PTEN and p53 expression on *in vitro* PC3 apoptosis. In *A*, graph shows the percentage of PC3 cells that stain TUNEL positive after mock infection (*solid bars*) or infection with 25 MOI of Ad-GFP (*open*), Ad-MMAC (*shaded*), or Ad-p53 (----) at day-4 postinfection. In *B*, graph shows the percentage of PC3 cells with Sub-G_0 DNA content at day 6 after infection, as determined by propidium iodide staining followed by FACS analysis. Identical labels as in *A*. *Columns*, average; *bars*, SD. *C*, cell cycle distribution of PC3 cells harvested 58 h after infection, as determined by phosphatidylinositol staining followed by FACS analysis. Cells were mock infected (*solid bars*) or infected with 25 MOI of Ad-GFP (*open*), Ad-MMAC (*shaded*), or Ad-p53 (----). *Columns*, average; *bars*, SD.

LNCaP prostate cancer cells (16). Therefore, the effect of Ad-MMAC treatment on the growth of PC3 cells was compared with treatment with adenoviral p53 (Ad-p53). In contrast to LNCaP cells, PC3 cells were more sensitive to p53 expression than to ectopic MMAC/PTEN expression. Infection with Ad-p53 resulted in an 85% reduction in viable cell number at day 4 and ~90% at day 6 after infection. Both of these effects were significantly greater than was seen with Ad-MMAC ($P < 0.05$ for both days).

To determine the mechanism of MMAC/PTEN-mediated growth inhibition, the effects of these treatments on apoptosis and proliferation were examined by flow cytometry (Fig. 4). Four days after infection with Ad-MMAC, 10% of cells were TUNEL positive, as compared with 5% with control adenovirus, suggesting only a minor increase in MMAC/PTEN-induced apoptosis. In contrast, infection with Ad-p53 produced 52% TUNEL positivity, indicating a significant induction of apoptosis by p53 expression (Fig. 4*A*). As an independent measure of apoptosis, propidium iodide staining followed by FACS analysis was performed to determine the percentage of cells with sub-G_0 DNA content (*i.e.*, cells likely to be undergoing apoptosis). On day 6 after infection, only 7% of Ad-MMAC-infected cells had sub-G_0 DNA content, as opposed to 25% of Ad-p53-treated cells (Fig. 4*B*). Similar differences in relative apoptosis were observed in both assays, suggesting that the growth-inhibitory effect induced by MMAC/PTEN expression cannot be attributed to the induction of apoptosis.

The effect of MMAC/PTEN expression on cellular proliferation was examined by cell cycle analysis (Fig. 4*C*). Infection

with Ad-MMAC produced an increase in the G_1 population of ~20%, with a concurrent decrease in the S phase population *versus* mock-treated cells. The changes in both the G_1 and S phase populations after MMAC/PTEN expression were significant when compared with either mock treatment or infection with a control adenovirus ($P < 0.05$). This G_1 arrest is consistent with experiments examining MMAC/PTEN expression in other cell types (38–42). p53 expression also induced a G_1 arrest in the PC3 cells. Similar to the assays of cell growth and apoptosis, the arrest induced by p53 expression was significantly greater than that induced by MMAC/PTEN, as quantified by G_1 and S phase cell populations ($P < 0.05$).

***In Vivo* Studies.** Previous studies in a number of human cancer cell types have demonstrated that the orthotopic implantation of cells in nude mice more closely resembles the biological behaviors of these cells in humans, particularly in regards to the development of metastases. This has proven particularly true of human prostate cancer cells, which form primary tumors and metastases with much lower efficiency when implanted ectopically in nude mice.

To assess the ability of MMAC/PTEN expression to regulate PC3 tumorigenicity and *in vivo* growth, the effects of *ex vivo* treatment of the cells were examined. PC3 cells were mock infected, or infected with 25 MOI of Ad-GFP or Ad-MMAC, as had been performed for the *in vitro* studies. After (24 h) these treatments, equal numbers of cells were injected into the prostates of nude male mice. The mice were then sacrificed 21 days after implantation, as was necessitated by the morbidity resulting from the tumors that had formed. The mice were dissected and analyzed for the incidence and size of prostate tumors and for the incidence of metastases to regional lymph nodes. A 100% incidence of prostate tumors was observed for all three treatments, indicating that MMAC/PTEN expression did not inhibit PC3 tumorigenicity. Although MMAC/PTEN expression did not abolish tumor formation, the size of tumors resulting from Ad-MMAC-infected cells was significantly smaller than tumors of the other treatment groups (Fig. 5*A*). Tumors formed by PC3 cells infected with Ad-MMAC were 77% smaller by mass than tumors formed from mock-infected PC3 cells, whereas Ad-GFP-infected cells were only 25% smaller. In addition to its effect on local tumor growth, MMAC/PTEN expression completely inhibited the development of PC3 lymph node metastases. Although 100% of the mice in the mock treatment group and 80% of the mice in the Ad-GFP treatment group demonstrated enlarged lymph nodes, with the presence of PC3 cells confirmed histologically, none of the mice in the Ad-MMAC treatment group demonstrated lymph node metastases (Fig. 5*B*). Thus, MMAC/PTEN expression did not inhibit primary tumor formation, but it did reduce primary tumor size and inhibited the development of lymph node metastases.

Immunohistochemical analysis was performed on the harvested tissues to determine the effects of MMAC/PTEN expression on the *in vivo* behavior of PC3 cells. MMAC/PTEN expression did not affect the morphology of the PC3 cells, or the general architecture of the tumors formed by these cells, as demonstrated by H&E staining (Fig. 6*A*). These sections also demonstrate the histological evidence of lymph node invasion by mock- and Ad-GFP-infected PC3 cells, whereas the lymph nodes of Ad-MMAC-infected cells demonstrate only lymphoid

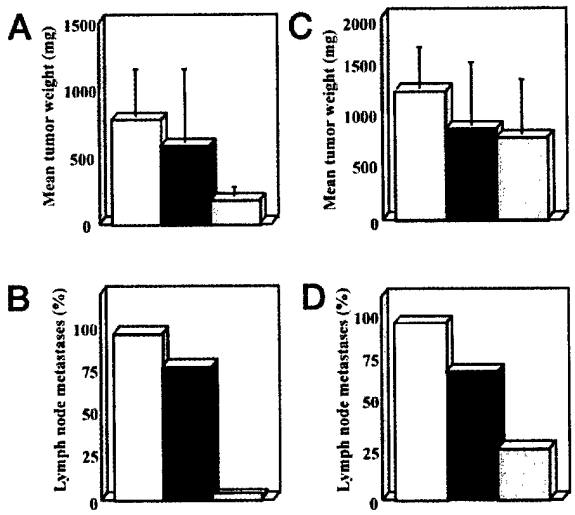

Fig. 5 Effects of MMAC/PTEN expression on the growth and metastasis of orthotopically implanted PC3 cells. For *ex vivo* studies, 24 h before implantation, PC3 cells were mock infected with media alone (*open bar*) or were infected with 25 MOI of Ad-GFP (*solid bar*) or Ad-MMAC (*shaded bar*). Tumors were harvested 21 days after implantation. *A*, mean tumor weight; *B*, percentage of mice with histologically confirmed lymph node metastases. *Columns*, average; *bars*, SD. For *in vivo* Ad-MMAC treatment of pre-established orthotopic PC3 tumors on growth and metastasis, tumors were injected with indicated treatments at days 7 and 14 after implantation and were harvested 17 days after the final treatment. Graphs demonstrate mean tumor weight (*C*) and percentage of mice with lymph node metastases (*D*) of PC3 tumors treated *in vivo* with PBS (*open bar*), Ad-GFP (*solid bar*), or Ad-MMAC (*shaded bar*). *Columns*, average; *bars*, SD.

cells (Fig. 6*B*). To determine the mechanism of MMAC/PTEN's inhibitory effect on primary tumor size, the tumors were stained for markers of apoptosis, angiogenesis, and proliferation. TUNEL staining for apoptotic cells did not demonstrate differences between the three treatment groups (Fig. 7*A*). To assess tumor angiogenesis, the tumors were stained for CD31, a marker of endothelial cells. Again, no significant differences were observed between the different treatments (Fig. 7*B*). Finally, tumor proliferation was assessed by BrdUrd incorporation. Tumors of both mock- and Ad-GFP-treated PC3 cells showed generalized, strongly positive staining for BrdUrd, indicating that the tumor cells were proliferating globally (Fig. 7*C*). In contrast, most of the areas of the Ad-MMAC treatment group tumors were negative for BrdUrd incorporation, although there were some positively stained regions around the tumor margin. Thus, *ex vivo* MMAC/PTEN expression significantly inhibited PC3 cellular proliferation *in vivo*, without significantly affecting apoptosis or angiogenesis. Western blotting analysis of immunoprecipitated proteins indicated that the tumors of PC3 infected with Ad-MMAC *ex vivo* did express elevated levels of MMAC/PTEN at the time of tumor harvest (data not shown).

As *ex vivo* treatment of PC3 cells with Ad-MMAC reduced primary tumor size and inhibited metastasis formation, we next tested the effects of *in vivo* Ad-MMAC treatment of pre-established PC3 orthotopic tumors. PC3 cells were injected into the prostate of nude mice. The tumors were injected on days 7 and

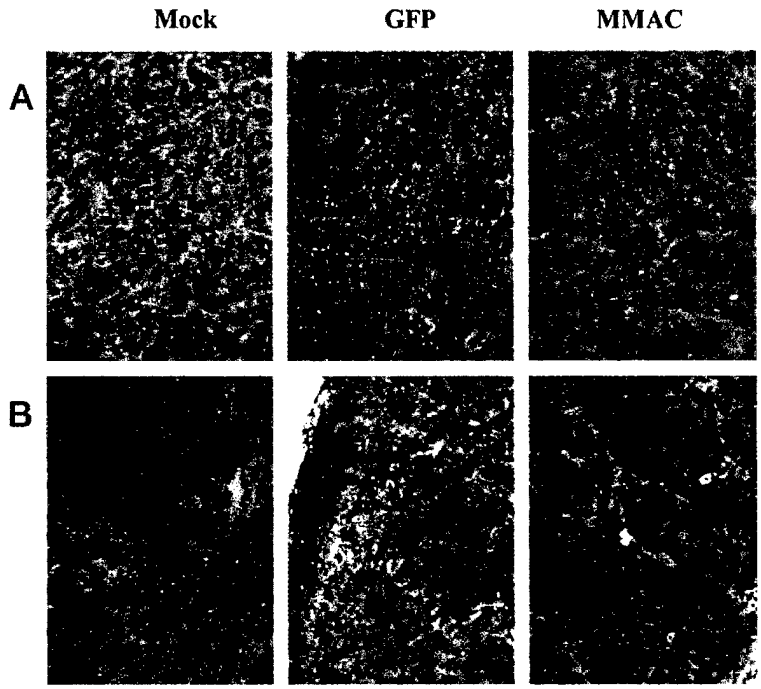

Fig. 6 Effect of *ex vivo* MMAC/PTEN expression on morphology of orthotopic PC3 tumors and histological examination of regional lymph nodes. H&E staining of frozen prostate tumors (*A*) and regional lymph nodes (*B*) harvested 21 days after implantation. Prostates for all treatment groups demonstrate replacement of normal tissue by tumor cells. Lymph nodes of mock- and Ad-GFP-infected PC3 cells demonstrate evidence of metastasis, as most of the tissue consists of prostate cancer cells, with few normal lymphoid cells (small, *blue* cells) remaining. The lymph node of the Ad-MMAC treatment group demonstrates no evidence of metastasis.

14 after implantation with 1.5×10^9 plaque-forming units of Ad-GFP or Ad-MMAC or an equal volume of PBS. Mice were sacrificed at day 31 after implantation, because of the morbidity of the tumor burden. None of the treatment groups demonstrated tumor regression, as 100% of the mice demonstrated large primary tumors (Fig. 8, *A* and *B*) despite the presence of MMAC/PTEN expression in the tumors (Fig. 8*C*). Treatment with Ad-MMAC resulted in a 35% decrease in primary tumor mass as compared with treatment with PBS ($P < 0.05$; Fig. 5*C*). However, treatment with Ad-GFP resulted in a similar inhibition of tumor mass ($P = 0.78$ *versus* Ad-MMAC). This suggested that the observed reduction in tumor size by *in vivo* Ad-MMAC treatment was largely because of the toxicity of the multiple injections of adenovirus and not because of MMAC/PTEN expression. However, although *in vivo* treatment with Ad-MMAC did not have a significant effect on primary tumor growth, it markedly inhibited the formation of lymph node metastases (Figs. 5*D* and 8*B*). Although the presence of metastases in lymph nodes or at other sites was not examined, any enlarged nodes surrounding the primary tumor were examined for the presence of tumors. Lymph node metastases were grossly detected in 87% of PBS-injected tumors, 63% of Ad-GFP-treated tumors, and 25% of Ad-MMAC-treated tumors. These findings were confirmed by histological analysis. Thus, the *in vivo* treatment of pre-established tumors of PC3 cells with Ad-MMAC did not induce tumor regression or significantly reduce tumor size, but it did diminish the formation of metastases. To determine that the primary tumors that formed in Ad-MMAC-infected cells were not because of loss of expression of MMAC, Western blotting was performed after immunoprecipitation of tumor cell lysates with MMAC/PTEN-specific antibodies as described in "Materials and Methods." As is observed in Fig. 8*C*, primary tumors from Ad-MMAC-infected cells clearly demonstrate the presence of the *MMAC/PTEN* gene product. Thus, the lack of tumor regression and/or reduction in size of tumors cannot be attributed to loss of MMAC/PTEN expression.

DISCUSSION

In the studies reported here, we have examined the impact of adenovirus-mediated MMAC/PTEN expression on the behavior of human prostate cancer cells under both *in vitro* and *in vivo* conditions in the human prostate tumor cell line, PC3. The goal of the studies was to determine whether MMAC/PTEN inhibited the development of lymph node metastases in cells implanted orthotopically in the prostate tissue of nude mice. This model more closely mimics the biology of human prostate cancer than s.c. implantation, as previous studies have demonstrated that the tissue microenvironment of the mouse prostate promotes rapid growth of the human prostate cancer cells locally and the development of spontaneous metastases (32, 33).

First, the effects of ectopic expression of MMAC/PTEN on signaling pathways, proliferation, and survival were determined. Our results are consistent with the now well-established model in which MMAC/PTEN regulates the PI3K signaling pathway by decreasing the pools of 3′-phosphorylated phosphatidylinositol (reviewed in Ref. 43). In contrast, no effects of MMAC/PTEN expression on phosphorylation of FAK were observed. These results are similar to those observed in several tumor cell lines and in MMAC/PTEN$^{-/-}$ fibroblasts, in which increased phosphorylation of Akt/PKB, but no differences in FAK phosphorylation, are observed relative to *MMAC/PTEN*$^{+/+}$ cells. Thus, although multiple lines of evidence support the activity of

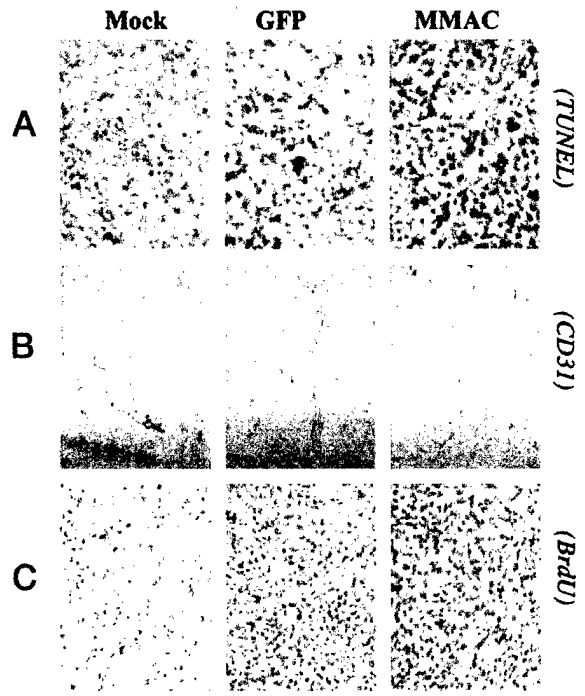

Fig. 7 Effect of *ex vivo* MMAC/PTEN expression on *in vivo* apoptosis, angiogenesis, and proliferation of orthotopically implanted PC3 cells. Immunohistochemical analysis of the same primary prostate tumors. *A*, TUNEL staining for apoptotic cells (*top row*, ×10 magnification). *B*, CD31 staining for endothelial cells, indicating vessel formation (*middle*, ×4 magnification). *C*, BrdUrd incorporation, indicating cellular proliferation (*bottom*, ×10 magnification). In each section, positively staining cells appear *red*. Sections shown are representative of the general appearance of individual tumors in multiple animals in each treatment group.

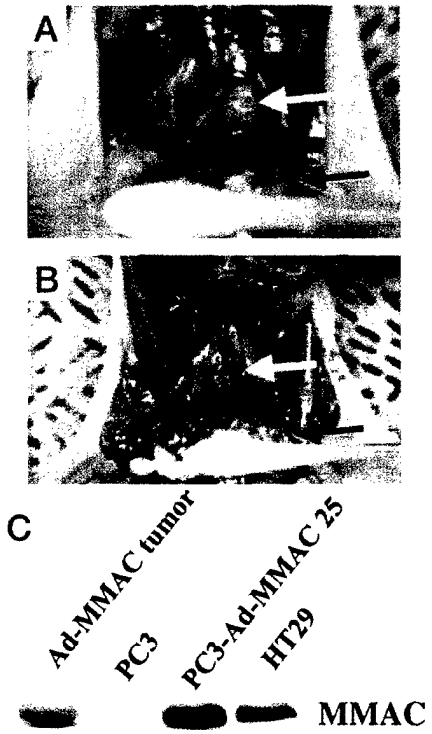

Fig. 8 PC3 tumors treated *in vivo* with Ad-GFP or Ad-MMAC. Photographs show representative gross anatomy of mice bearing PC3 tumors that were injected twice with Ad-GFP (*A*) or Ad-MMAC (*B*). *Green arrows*, the prostate tumors; *yellow arrows*, para-aortic lymph nodes. Prostate tumors treated with Ad-GFP demonstrated large, hardened lymph nodes as seen in *A*, which histological examination confirmed represented metastases of the primary prostate tumor. Tumors treated with Ad-MMAC showed small, soft lymph nodes that did not contain prostate tumors cell. *C*, expression of MMAC/PTEN. MMAC/PTEN was immunoprecipitated from 500 μg of lysates from mouse tumors (Ad-MMAC tumor) or cell lines (PC3, PC3 infected with Ad MMAC at 25 MOI, and HT29 cells, a colon tumor cell line expressing MMAC/PTEN). Western blotting was performed to detect MMAC/PTEN as described in "Materials and Methods."

MMAC/PTEN as a phosphatidylinositol phosphatase, the studies presented here do not support the identification of FAK as a substrate of MMAC/PTEN's putative protein phosphatase activity.

The expression of MMAC/PTEN in PC3 cells resulted in a significant inhibition of growth as compared with mock and control adenovirus treatments. This growth inhibition is not because of increased induction of apoptosis. These biological effects are similar to those we observed previously in experiments using the LNCaP human prostate cancer cell line (16). Although LNCaP cells demonstrate some apoptosis after high levels of MMAC/PTEN expression, quantitatively, the amount of apoptosis induced did not explain the extent of growth inhibition observed. Additionally, overexpression of Bcl-2 protein blocked MMAC/PTEN-induced apoptosis in LNCaP cells but not its growth inhibitory effects. The predominant regulation of prostate proliferation by MMAC/PTEN was also reported in the analyses of hyperplastic prostate tissue of *MMAC/PTEN*$^{+/-}$ mice and of prostate tumors in *MMAC/PTEN*$^{+/-}$ *Cdkn1b* +/− mice. In both of these studies, no changes in apoptotic indices were detected in the *MMAC/PTEN*$^{+/-}$ prostate tissues, but significant increases in proliferative indices were, as compared with *MMAC/PTEN*$^{+/+}$ mice (28).

The tumor suppressor gene *p53* is also inactivated frequently in metastatic prostate cancer (2, 44–47) and is currently being used in clinical trials in prostate cancer patients (48). In the studies in LNCaP cells, the expression of MMAC/PTEN was significantly more growth inhibitory than ectopic expression of p53. However, in the studies presented here, p53 expression inhibited the growth and survival of PC3 cells significantly more than the expression of MMAC/PTEN. The extent of growth inhibition induced by p53 observed here is very consistent with earlier published reports (31). The difference in comparative sensitivities to these two treatments between these two prostate cell lines could be because of a number of factors. The most likely explanation for this difference is that LNCaP cells harbor wild-type p53 function, whereas PC3 cells do not. Thus, p53 would be expected to be more growth inhibitory in the latter cells. A similar pattern of differing sensitivity to MMAC/PTEN and p53 has also been observed in human glioma cell lines. We reported previously that the expression of MMAC/PTEN in the

U251 human glioma cell line, which harbors mutant p53, resulted in a minimal inhibition of growth, whereas p53 expression potently inhibited U251 growth and survival (16, 36). In contrast, the U87 human glioma cell line, which retains wild-type p53 function, is more sensitive to adenoviral MMAC/PTEN expression than to adenoviral p53 (34). Paramio *et al.* (49) have demonstrated that MMAC/PTEN-mediated growth inhibition is not p53 dependent. Although these experiments were not conducted in prostate cells, MMAC/PTEN expression was growth inhibitory to PC3 cells in these experiments, suggesting that MMAC/PTEN-induced growth inhibition in prostate cells also is not p53 dependent. Therefore, there appears to be no direct relationship between the status of p53 mutation and sensitivity to ectopic expression of MMAC/PTEN.

Two previous studies have examined the effects of MMAC/PTEN expression in PC3 cells *in vitro*. Similar to this report, Sharrard *et al.* (50) observed an inhibition of PC3 cell growth after MMAC/PTEN expression without an induction of apoptosis. In contrast, Persad *et al.* (51) reported that the expression of MMAC/PTEN in PC3 cells resulted overwhelmingly in the induction of apoptosis. However, apoptosis was demonstrated only in cells that were maintained in serum-free media for 48 h. Thus, these results do not contradict the results obtained here, as we examined cells maintained in mitogen-enriched tissue culture media. It is clear by the Western blotting presented in this report (Fig. 2) that the mitogenic pathways of PC3 cells are sensitive to the mitogen content of the media, as cells starved for only 18 h demonstrated a decrease in Akt/PKB phosphorylation. Thus, in the studies of Persad *et al.* (51), the expression of MMAC/PTEN in addition to serum starvation may have inhibited this prosurvival pathway sufficiently to induce the apoptotic cascade.

***In Vivo* Studies.** Having established that ectopic expression of MMAC/PTEN in PC3 cells resulted in biological and signaling effects similar to those reported for many tumor cell lines, the main goal of this study was to examine the *in vivo* function of MMAC/PTEN in human prostate cancer cells. Two different experimental approaches were used. In the first experiments described, MMAC/PTEN expression was introduced *ex vivo* by treating the cells with Ad-MMAC before implanting the cells in nude mice. This technique has the advantage of allowing uniform gene expression with a single treatment of the cells and involves minimal mechanical toxicity. However, it is a somewhat artificial system to evaluate a gene for potential therapy applications, as treatment of patients will involve expressing the gene in tumor cells already present in the prostate. Therefore, the effects of *in vivo* treatment of pre-established PC3 tumors with Ad-MMAC were also examined. This allows for a more relevant examination of the potential for clinical gene therapy. However, this technique produces more heterogeneous and limited gene expression in the tumor cells and intrinsically involves more mechanical trauma to the tumor. Thus, the *in vivo* expression technique may be somewhat limited in accurately representing the true *in vivo* function of the gene.

The *ex vivo* expression of MMAC/PTEN in PC3 cells did not inhibit the tumorigenicity of these cells when they were implanted orthotopically. However, *ex vivo* expression of MMAC/PTEN did significantly reduce local tumor size by inhibiting cellular proliferation, a result that is consistent with the *in vitro* experiments. Additionally, *ex vivo* expression of MMAC/PTEN completely inhibited the development of lymph node metastases by PC3 cells. Thus, these experiments suggest that MMAC/PTEN is not a critical regulator of prostate tumor formation but is a critical regulator of aggressive local prostate tumor growth and metastasis. This model is consistent with the pattern of mutations and deletions of *MMAC/PTEN* that have been detected in human prostate cancer clinical specimens but represents the first *in vivo* demonstration of this proposed role. However, this finding does contrast with the observation in genetically engineered mice that loss of a single *MMAC/PTEN* allele promotes prostate hyperplasia and tumor formation (27). Although those murine lesions and tumors may have also included other genetic alterations not recapitulated in the PC3 cell line, it is notable that there is no report of an increased incidence of abnormal prostate proliferation in the familial syndromes that harbor germ-line *MMAC/PTEN* mutations. Thus, the results in the genetically engineered mice may reflect a different regulation of prostate homeostasis in that species than is functional in the human tissue.

The *in vivo* treatment of pre-established tumors of PC3 cells with Ad-MMAC did not induce tumor regression but did moderately inhibit localized prostate tumor size. This reduction in tumor size could not be attributed to MMAC/PTEN expression, however, as infection with a control adenovirus resulted in a similar degree of inhibition. Substantial variation in the size of the tumors, particularly in the Ad-MMAC treatment group, indicates that repeated injections into the prostate tumors resulted in nonspecific toxicity. Furthermore, it is expected that not all tumor cells will be infected by this procedure. Thus, in this experimental setting, it is difficult to assess the effect of MMAC/PTEN expression on the local growth of the cancer cells. However, *in vivo* treatment of these tumors with Ad-MMAC did have an inhibitory effect on the metastatic behavior of these cells. This effect was apparent in the rate of lymph node metastasis, which was confirmed by histological analysis. The potent inhibition of the metastasis of PC3 cells by MMAC/PTEN is consistent with the effects of *ex vivo* expression and, again, with the detected pattern of genetic inactivation of *MMAC/PTEN* in clinical specimens.

The inhibition of PC3 prostate cancer cell metastasis after both *ex vivo* and *in vivo* expression of MMAC/PTEN is striking. Although these studies did not determine the specific mechanism of this effect, previous studies have demonstrated that MMAC/PTEN can regulate a number of *in vitro* behaviors characteristic of metastatic cancer cells. These regulated behaviors include anoikis, motility, and invasion (reviewed in Ref. 13). Although MMAC/PTEN expression clearly regulates signaling pathways responsible for the above biological behaviors, it is also possible that MMAC/PTEN's inhibitory effect on metastasis may be because of the regulation of factors released into the circulation by the tumor cells in the prostate. This hypothesis should also be investigated, as Ad-MMAC treatment of pre-established tumors likely resulted in heterogeneous expression of MMAC/PTEN but still inhibited the formation of metastases.

The effects of adenoviral p53 on the *in vivo* growth of PC3 cells have also been investigated previously. Two separate reports have demonstrated that the *ex vivo* expression of p53 in

PC3 cells inhibits the tumorigenicity of these cells (52, 53). More recently, Eastham *et al.* (31) have reported that a single injection of adenoviral p53 into pre-established orthotopic PC3 tumors resulted in the complete regression of several tumors, a 75% reduction in local tumor size, and a 55% reduction in metastasis formation. Although different numbers of cells and amounts of adenovirus were used, the reported increased *in vivo* growth-suppressive effects of p53 expression in PC3 cells as compared with MMAC/PTEN agree with the increased inhibitory effect that we observed *in vitro*. Although it was not determined *in vivo*, each of these studies demonstrated clear evidence *in vitro* of a significant induction of apoptosis after p53 expression. Given that MMAC/PTEN-mediated growth inhibition does not seem to be predominantly mediated by apoptosis, a combined gene therapy of MMAC/PTEN and p53 might provide an additive effect.

As yet, few studies have examined the effects of *in vivo* treatment of pre-established tumors with adenoviral MMAC/PTEN. Sakurada *et al.* (54) examined the effects of MMAC/PTEN gene therapy on pre-established tumors of human endometrial cancer cells and reported a cytostatic effect. However, the cells were injected s.c. into the mice instead of orthotopically, and the effects of MMAC/PTEN expression on metastasis formation were not reported. Recently, Wen *et al.* (55) reported the effects of stable *ex vivo* expression of MMAC/PTEN on the growth of orthotopically implanted U87 human glioma cells. Similar to the results reported here in PC3 cells, MMAC/PTEN expression did not inhibit the tumorigenicity of the U87 cells but did reduce tumor size and prolonged survival.

In summary, the results presented here functionally support the proposed role of MMAC/PTEN as a regulator of prostate cancer progression. Specifically, they have demonstrated that MMAC/PTEN inhibits the metastatic potential of PC3 cells without inhibiting local prostate tumor formation. These studies also suggest that additional investigations are indicated to assess MMAC/PTEN's potential as a prognostic marker for staging and treatment purposes and also as a potential therapeutic tool.

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The Biology Behind

The Expanding Role of PTEN in Neoplasia: A Molecule for All Seasons?[1]

Commentary re: M. A. Davies, *et al.*, Adenoviral-mediated Expression of MMAC/PTEN Inhibits Proliferation and Metastasis of Human Prostate Cancer Cells. Clin. Cancer Res., *8:* 1904–1914, 2002.

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Abstract

Not since the discovery of p53 has another molecule received as much attention as PTEN. In the 5 years since the discovery of *PTEN*, encoding a dual specificity phosphatase tumor suppressor on 10q23, it has been shown to be a susceptibility gene for an inherited cancer syndrome, Cowden syndrome, and for several developmental disorders; it has been shown to play a prominent role in normal murine and human development; and it has been shown to be instrumental in cell cycle arrest, apoptosis, and/or possibly cell migration and cytoskeletal affairs. Initial work on cancer cell lines had suggested that *PTEN* caused every type of cancer because it was reported that a relatively high frequency of a variety of cancer cell lines, whether derived from solid tumors or hematological malignancies, had homozygous or compound heterozygous genetic alterations involving *PTEN*. Such data, together with the germ-line human and murine model data, suggested that *PTEN* mutations occurred "early" in sporadic tumorigenesis. However, subsequent painstaking work in noncultured primary tumors and in careful *in vitro* overexpression studies over the last 4 years demonstrated that the mechanism of PTEN inactivation can be varied and might be cell type dependent. Furthermore, apart from sporadic endometrial carcinoma, *PTEN* alteration in noncultured sporadic neoplasias likely occurs "late," promoting progression and metastasis. The article by Davies *et al.* (Clin Cancer Res., *8:* 1904–1914, 2002) sheds light on all of these issues when they report on data that derive from a "triple threat" strategy, *i.e., in vitro, in vivo,* and *ex vivo,* to demonstrate that adenoviral infection of PTEN into PTEN-null PC3 prostate cancer cell lines results in decreased metastatic potential without significantly altering tumor size via the predominant mechanism of G_1 cell cycle arrest but not apoptosis.

Introduction

In this issue of *Clinical Cancer Research*, Davies *et al.* (1) use an "*in vitro-in vivo-ex vivo*" system to demonstrate that adenoviral infection of the tumor suppressor gene *PTEN* into the PTEN-null PC3 prostate cancer line results in decreased metastatic potential without altering tumor size. A literature search using "PTEN" as a keyword reveals at least 721 publications, spanning such broad topics as normal development, glycemic control, cardiovascular disease, and carcinogenesis. Is PTEN a molecule for all seasons?

The important discovery of *PTEN* is intimately tied to the seemingly obscure story of the inherited hamartoma-tumor syndromes. The first putative locus for an inherited hamartoma syndrome, CS[3] (MIM 158350), characterized by multiple hamartomas and a risk of breast and thyroid cancers, was mapped to 10q22–q23 in 1996 (2). *PTEN/MMAC1/TEP1* (MIM 601728) was isolated by three different groups (3–5). Using positional cloning strategies, two groups isolated *PTEN/MMAC1* at 10q23.3 (3, 4). The third group isolated *TEP1* when searching for molecules with homology to protein tyrosine phosphatases (5). By nucleotide and predicted protein sequence alone, PTEN was shown to have a large region of homology to chicken tensin and bovine auxilin and a protein tyrosine-phosphatase domain.

Protean *PTEN:* One Gene—Many Syndromes

Because the putative locus for CS was mapped previously to 10q22–q23, *PTEN* became an excellent candidate susceptibility gene. Germ-line mutations in *PTEN* have been identified in 80% of probands with CS (6, 7). Subsequently, germ-line *PTEN* mutations were found in 60% of patients with Bannayan-

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[3] The abbreviations used are: CS, Cowden syndrome; PS, Proteus syndrome; MMAC, mutated in multiple advanced cancer; PTEN, phosphatase, tensin homologue, deleted on chromosome ten; TEP1, transforming growth factor-β-induced epithelial cell-derived protein tyrosine phosphatase; PI3K, phosphatidylinositol 3-kinase.

Riley-Ruvalcaba syndrome (MIM 153480; Ref. 8) and in a proportion of individuals with PS (MIM176920) and *Proteus*-like syndromes (9). CS is an autosomal dominant inherited cancer syndrome characterized by multiple hamartomas and risks of breast, thyroid, and endometrial neoplasias (10). Bannayan-Riley-Ruvalcaba syndrome is characterized by macrocephaly, lipomatosis, hemangiomatosis, and speckled penis (11). PS is characterized by hamartomas, lipomas, and overgrowth with a mosaic distribution of affected tissues (12). Because it is of sporadic occurrence and mosaic distribution, many originally believed that either somatic mutation or germ-line mosaic mutation to be its origin, hence, the significance of finding germ-line *PTEN* mutations in a subset of PS (9). Remarkably, germ-line mutations in *PTEN* have been found in an individual with hydrocephaly with VATER (vertebral and anal malformations, tracheoesophageal atresia, and radial and renal malformations) association (13) and an individual with megaencephaly and autistic features (14). It has been suggested that syndromes that are characterized by the presence of germ-line *PTEN* mutations may be grouped by molecular definition and referred to as the *PTEN* hamartoma-tumor syndromes (8). Thus, *PTEN* hamartoma-tumor syndromes encompass *PTEN* mutation-positive cases regardless of the presenting clinical syndrome. This molecular-based diagnosis is important because it impacts clinical surveillance recommendations: presence of a germ-line *PTEN* mutation irregardless of syndrome name, even in syndromes that, in the past, were not known to be associated with cancer risk, should trigger the clinician to institute cancer surveillance similar to those recommended for CS (10). Similar to most tumor suppressor genes, the spectrum of mutations includes truncating and missense mutations scattered throughout the gene [reviewed by Waite and Eng (15)]. In at least one large series of CS probands, approximately two-thirds of the mutations were in exons 5, 7, and 8, with 40% within exon 5, although this exon only represented 20% of the coding sequence of the gene (7). Exon 5 encodes the phosphatase core motif, and the preponderance of mutations in that exon reflects the biology.

Somatic *PTEN* Alterations—Master Molecule for Sporadic Carcinogenesis?

Somatic *PTEN* mutations have been found, to a greater or lesser extent, in a wide variety of solid tumors and hematological malignancies, with the highest frequencies observed in cell lines. A review of the literature by Bonneau and Longy (16) found 332 somatic *PTEN* mutations in primary tumors and metastases. Initial work performed mainly on cell lines suggested a high frequency of intragenic somatic mutations, homozygous deletions, and biallelic loss of *PTEN* (3, 17). However, as further somatic genetics were performed, it became obvious that in noncultured primary solid tumors, only endometrial carcinomas and glioblastoma multiforme harbored frequent somatic mutations and biallelic structural alterations (18, 19). In the case of endometrial carcinomas, somatic *PTEN* mutation or epigenetic silencing occurs as one of the earliest events, perhaps even in normal-appearing glands (20). In contrast, somatic mutation and allelic loss of *PTEN* in brain tumors of the glioneural line occurs with any frequency only in glioblastoma multiforme, the highest grade among these tumors (19). Interestingly, in a proportion of solid tumors that have a low frequency (<10%) of somatic intragenic *PTEN* mutation, epigenetic (*i.e.*, beyond genetic) silencing of PTEN can be a major mechanism of inactivation, *e.g.*, melanomas (21), and in thyroid tumors and islet cell tumors, perhaps inappropriate subcellular compartmentalization (22–24). In other words, multiple mechanisms of somatic PTEN inactivation occur depending on the type of neoplasia involved.

PTEN in Prostate Carcinogenesis—Master Switch for Metastasis?

Apart from rare exceptions, *i.e.*, endometrial carcinomas, it would appear that somatic *PTEN* mutations and deletions occur as a late event in the majority of noncultured solid tumors. The human *in vivo* data for sporadic adenocarcinomas of the prostate are no different. Overall, the intragenic mutation frequency ranges from 0% to >60%, with those in the 40–65% range for prostate cancer cell lines (17, 25–27). Loss of heterozygosity frequency averages 40% whether in cultured or noncultured prostate cancers. However, it would appear that loss of *PTEN* expression, believed to be secondary to promoter hypermethylation, tends to occur in advanced prostate cancers (28). Furthermore, the highest frequencies of 10q loss of heterozygosity and biallelic structural alterations occur in metastatic prostate cancer samples (25, 26). Indeed, complete loss of PTEN protein expression in noncultured primary prostate cancers was shown to be associated with a high Gleason score of 7 or higher and with advanced pathological stage (American Joint Committee on Cancer T3b and T4; Ref. 29). In other words, a nonworking PTEN is associated with poor outcome, and in prostate cancer, poor outcome is always tied with metastasis.

In this issue of *Clinical Cancer Research*, Davies *et al.* (1) report on data from experiments planned by leaping from the stage set by the genetic and molecular pathology data described above, and the historical observation that reintroduction of the human 10q23–q25 region into rat prostate cancer cells failed to alter tumorigenicity but significantly inhibited metastatic potential (30). The investigators use the PC3 prostate cancer model, which is known to be PTEN null. When they infected PC3 with an adenoviral construct harboring wild-type *PTEN*, phosphorylation of the proapoptotic factor Akt/PKB was inhibited, an observation similar to that seen after ectopic expression of PTEN in a variety of cell lines, whether PTEN wild-type or null [Refs. 31–35; reviewed by Waite and Eng (15)]. This downstream consequence is consistent with the known lipid 3-phosphatase activity of PTEN, which dephosphorylates its major substrates, phosphoinositide-3,4,5-triphosphate and phosphoinositide-4,5-diphosphate, thus acting in opposition to PI3K [Refs. 36, 37; reviewed by Waite and Eng (15)]. The Akt/PKB pathway lies downstream of the PI3K pathway. Therefore, when PTEN is wild type and functional, phosphorylation of Akt is inhibited. Depending on cell type, functioning PTEN induces cell cycle arrest at G_1 and/or apoptosis, which are mediated by the D cyclins and p27 [reviewed by Waite and Eng (15)]. Davies *et al.* (1) demonstrate that the mechanism involved in their models is G_1 arrest and not apoptosis.

Of significance, Davies *et al.* (1) have demonstrated *in vitro*, *in vivo*, and *ex vivo* that introduction of wild-type PTEN into established PC3 cells decreased metastatic potential but did

not induce growth inhibition. The *ex vivo* model involved orthotopic implantation of PC3 cells infected with the adenoviral construct with wild-type *PTEN* (Ad-MMAC), Ad-GFP, and sham into nude mice. For all treatment groups, a 100% incidence of prostate carcinoma was observed. Ectopic PTEN expression did not inhibit tumor growth, but it inhibited the development of lymph node metastases. The *in vivo* model involved PC3 cells implanted in the prostate of nude mice followed by treatment of resultant tumors with the adenoviral construct with wild-type *PTEN* (Ad-MMAC) and controls at 7 and 14 days after implantation. They found that none of the treatment groups demonstrated tumor regression even in the presence of PTEN expression. The treatment with Ad-MMAC and Ad-GFP led to a 35% decrease in tumor mass as compared with the PBS-treated control group that was attributed to toxicity of multiple injections of adenovirus. However, the treatment with Ad-MMAC markedly inhibited the formation of lymph node metastases. Similar results were obtained with their *ex vivo* experiments as well. The authors commented that their observations diverge from constitutional *Pten*+/− murine models where prostate hyperplasia and tumor formation have been observed (38). In other words, the observations from constitutional knock-out murine models would suggest that prostate neoplasia results from early *Pten* dysfunction. The great value of Davies' "humanized" models is that they do recapitulate the genetic data in human prostate cancer samples, and they are consistent with the human inherited hamartoma-tumor syndromes that are characterized by germ-line *PTEN* mutations. *PTEN* mutation positive CS patients, *e.g.,* do not have an increased incidence of prostate cancer (10).[4]

PTEN-based Therapy for Prostate Cancer?

This study proposes an important therapeutic tool for the treatment of prostate cancer: gene therapy with exogenous wild-type *PTEN*. The authors also provide some evidence that combined gene therapy with wild-type *PTEN* and *TP53* for prostate cancers, which are presumably PTEN and p53 deficient, might provide an additive effect. Although gene therapy has captured the imagination of scientist, clinician, and lay public alike for several decades, there has yet to be a gene-based therapy that has been successful and used as clinical routine to treat cancer. Thus, aside from choice of vectors, proposing *PTEN* as gene therapy requires budding gene therapists to consider the following. If wild-type *PTEN* is introduced into a tumor with a mutant *PTEN*, would the resultant dominant-negative effect (39) cause more harm? Furthermore, if the data in the Davies *et al.* (1) report is correct, then would the presence or the introduction of wild-type *PTEN* into prostate cancer metastases (which raises other issues as well) prevent more metastases because it presumably would not shrink existing metastases? Given the data presented in their report and the natural history of resected localized, early-stage prostate cancer, then it would also seem inappropriate to use *PTEN* gene therapy in early stages. So when would *PTEN* gene therapy be used for prostate cancer? Nonetheless, proposing an "upstream" (*i.e.,* PTEN) replacement therapy may hold further promise than targeting downstream of a dysfunctional PTEN. Many have believed that PTEN-mediated growth suppression is only mediated by its lipid phosphatase activity and thus have proposed targeting downstream of the lipid phosphatase activity (40). However, because there is accumulating evidence that PTEN-mediated growth suppression is also dependent on its protein phosphatase activity and could be independent of PI3K/Akt [reviewed by Waite and Eng (15)], such downstream targeting either might not be 100% efficacious or might even cause harm.

[4] C. Eng *et al.,* unpublished observations.

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