

Award Number: W81XWH-05-1-0618

TITLE: Novel Role of Prostate- Specific Membrane Antigen in Prostate Cancer Invasion and Metastasis

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REPORT DATE: April 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE 01-04-2007			2. REPORT TYPE Final		3. DATES COVERED 22 Sep 2005 – 21 Mar 2007	
4. TITLE AND SUBTITLE Novel Role of Prostate- Specific Membrane Antigen in Prostate Cancer Invasion and Metastasis					5a. CONTRACT NUMBER	
					5b. GRANT NUMBER W81XWH-05-1-0618	
					5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Arundhati Ghosh, Ph.D. Email: ghosha@ccf.org					5d. PROJECT NUMBER	
					5e. TASK NUMBER	
					5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cleveland Clinic Foundation Cleveland, OH 44195					8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					10. SPONSOR/MONITOR'S ACRONYM(S)	
					11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited						
13. SUPPLEMENTARY NOTES Original contains colored plates: ALL DTIC reproductions will be in black and white.						
14. ABSTRACT See page 5						
15. SUBJECT TERMS Prostate Cancer						
16. SECURITY CLASSIFICATION OF:				17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	19b. TELEPHONE NUMBER <i>(include area code)</i>			
				UU	21	

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Abstract:

Title of Proposal: Role of Prostate Specific Membrane Antigen in suppressing prostate cancer invasion and metastasis.

Prostate Specific Membrane Antigen, PSMA is a type II transmembrane glycoprotein, overexpressed in prostate carcinoma (PCa) including androgen sensitive and independent disease, increased in expression with early relapse following therapy. PSMA is a carboxypeptidase with two important enzymatic functions, namely, folate hydrolase and NAALADase. We have used *in vitro* invasion assays to explore the possible role of PSMA in PCa cells. Androgen dependent PCa lines, which express PSMA endogenously (e.g., LNCaP, CWR22) are less invasive compared to androgen independent PSMA negative PC3 or DU145 cells. Ectopic expression of PSMA in PC3 cells reduced the invasiveness of these cells, suggesting that this reduction in the invasion capability of PSMA expressing cells is due to PSMA expression, not due to intrinsic properties of different cell lines. Expression of PSMA mutants lacking carboxypeptidase activity reduced the impact of PSMA expression on invasiveness. Thus it appears that the enzymatic activity is associated with PSMA's effect on cellular invasiveness.

To test our experimental results *in vivo*, we have further co-expressed PSMA and luciferase reporter gene in highly invasive PC3MM2 lines. We obtained several expressors with high luciferase and PSMA expression. Tumor formation ability and metastatic potential of these lines will be tested in mice.

Keywords: Prostate Cancer, PSMA, Carboxypeptidase, Invasion, Metastasis.

Proposal Main Body:

Title: Role of Prostate Specific Membrane Antigen in suppressing prostate cancer invasion and metastasis.

Background: Cell surface peptidases are known to play key roles in the control of growth and differentiation by modulating the activities of peptide factors and by regulating their access to adjacent cell. Effects may include either inactivating stimulatory peptides or activating inhibitory peptides [Nanus, 2003]. It is known that small peptides could be potential chemotactic factors and modulate migration and invasion of cells through extracellular matrix. Prostate Specific Membrane Antigen (PSMA) is a carboxypeptidase expressed on the membrane of prostate cancer (PCa) cells, which may release be potential modulators or inhibitors by its enzymatic activity. According to our previous observation, enzymatically active PSMA can markedly inhibit invasiveness of PCa cells. Our hypothesis is that PSMA modulates the invasiveness of PCa cells both *in vitro* and *in vivo* by targeting specific effectors. Here, I propose to delineate the pathway through which PSMA acts and identify the putative effectors of diminished invasiveness. I plan to address these questions through the following specific aims.

Specific Aim I: To study PSMA's role in prostate cancer invasion and metastasis in a bioluminescent, orthotopic metastatic model of prostate cancer.

Our previous observation was that expression of PSMA on the cell membrane reduces the invasion of PCa cells through extracellular matrix and PSMA's enzymatic activity is responsible for this novel function. We would like to verify this observation *in vivo* by expressing PSMA and its mutants in a bioluminescent orthotopic metastatic model in nude mice and measure the effect of PSMA in modulating the invasion and metastasis of the PCa cells.

Specific Aim II: To identify and characterize the involvement of putative factor(s) involved in modulating the invasion capacity of prostate cancer cells.

Under this specific aim, we will explore the pathway of PSMA-mediated inhibition of invasion by isolating and characterizing the potential chemotactic factor(s) secreted by PCa cells which could be inactivated by PSMA, thereby slowing down the invasion of PCa cells.

Concept: Prostate cancer (PCa) is one of the most commonly diagnosed forms of cancer among men in United States and is second only to lung cancer. The number of new prostate cancer cases in the US alone in the year 2004 was 230,110 and number of deaths was 22,010 as reported by American Cancer Society surveillance research [Jemal et al., 2004]. Deaths from PCa occur mostly in patients with aggressive androgen independent metastatic disease and PSMA has been correlated with aggressive disease [Sweat et al., 1998]. PSMA is a membrane-glycoprotein and a carboxypeptidase [Pinto et al., 1996; Robinson et al., 1987], negatively regulated by androgens, present in normal prostate epithelium and found to be upregulated many-fold in PCa, from androgen sensitive to androgen insensitive as well as its bony metastatic sites [Israeli et al., 1994; Israeli et al., 1993]. Overexpression of PSMA in primary PCa correlates with other traditional prognostic factors and can independently predict the disease outcome such as early relapse after therapy [Ross et al., 2003]. We started with the working hypothesis that PSMA expression is associated with biological processes of tumor invasion and metastasis. By expressing PSMA within prostate cancer cells that normally do not express PSMA or its various mutants [for enzymatic function [Ghosh and Heston, 2003] and internalization function [Ghosh and Heston, 2004]], we have shown that PSMA-expression lowers the invasion capability of aggressive prostate cancer cells (PC3) (Figure 1 A) and that this PSMA-mediated effect on invasiveness of PC3 cells is dependent on its enzymatic activity [Ghosh et al., 2005] (Figure 2 A,B)). Furthermore, abolishing endogenous PSMA expression from low invasive prostate cancer cells (LNCaP), by RNA interference (Figure 2), enhanced their invasiveness by 5 fold (Figure 1B). Based on those observations, my hypothesis is that PSMA is proteolytically modulating the function of invasion promoting peptides by cleaving them or generating

amino acids as its by-products, which is suppressing invasion of PSMA-expressing cells through extracellular matrix.

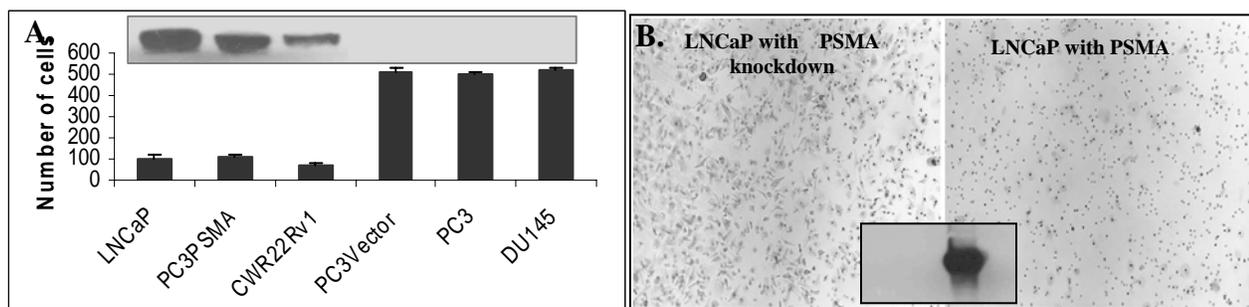


Figure 1: A. Invasion assay with different prostate cancer cells in Boyden chambers with matrigel-coated membranes. Quantification of the number of cells invaded to the other side of the membrane was done by visually counting the cells in randomly chosen fields. PSMA protein expression profiles of different lines have been shown in the inset. B. Invasion assay using LNCaP cells and LNCaP cells stably expressing shRNA to abolish the endogenous PSMA expression. Inset showing the PSMA protein expression in two different lines.

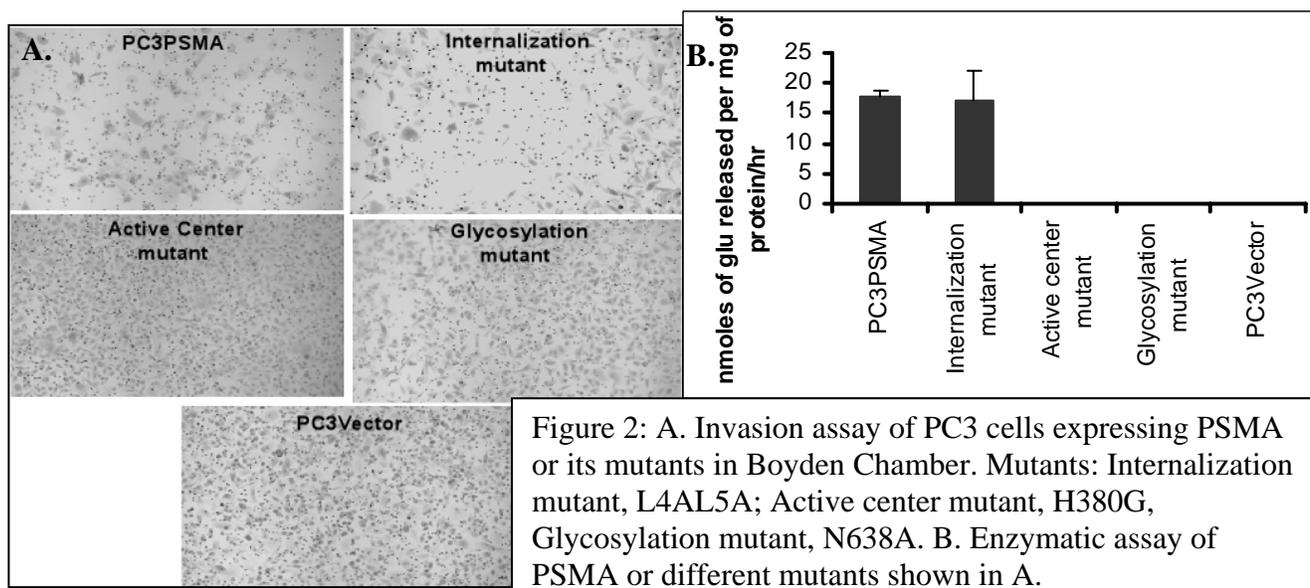


Figure 2: A. Invasion assay of PC3 cells expressing PSMA or its mutants in Boyden Chamber. Mutants: Internalization mutant, L4AL5A; Active center mutant, H380G, Glycosylation mutant, N638A. B. Enzymatic assay of PSMA or different mutants shown in A.

Feasibility/plausibility: We have established that ectopic expression of PSMA (on the surface of non-expressing prostate cancer cells reduced the level of invasiveness of those cells, and or ablation of endogenous PSMA expression from low-invasive prostate cancer cells enhances their invasiveness. My hypothesis is that PSMA is proteolytically modulating the function of invasion promoting peptides by cleaving them or generating amino acids as its by-products, which could be responsible for inhibition of the invasion of PSMA-expressing cells through extracellular matrix *in vitro*. We plan to further extend our experimental findings to animal model *in vivo* using bioluminescent cells expressing different proteins of interest.

Specific Aim I: To study PSMA's role in prostate cancer invasion and metastasis in a bioluminescent orthotopic metastatic model of prostate cancer cells.

Animal experiments with bioluminescent PC3MM2 cells expressing PSMA or its different mutants:

Establishment of bioluminescent PC3MM2 line: We are planning to investigate the PSMA-mediated anti-invasive effect on PCa cells *in vivo* by expressing PSMA or its mutants in a bioluminescent derivative of the PC3MM2 cell line and implanting those cells orthotopically on the ventral lobes of the prostate in nude mice. PC3MM2 is a highly metastatic subline of the PC3 cell line. It will be obtained from Dr. Isiah Fidler at MD Anderson Cancer Center. We will express a luciferase reporter gene in this line to make it bioluminescent [Jenkins et al., 2003], subsequently, one such luciferase expresser line will be used for expressing PSMA or its enzymatically inactive mutants (H380G, N638A) or its internalization mutant (L4AL5A) or vector DNA.

Metastatic animal model using bioluminescent LNCaP cell and its derivative lines: We will test the behavior of LNCaP cells with knocked down expression of PSMA [Ghosh et al., 2005] in tumor progression and metastasis compared to parental LNCaP cells. Another LNCaP cell line with mismatched shRNA, which does not reduce the expression of PSMA, will be used as a negative control. We have established this line in the laboratory and this line behaves in the same way as parental LNCaP line.

Animal experiments: Orthotopic injection of various cell lines will be done in collaboration with Dr. Heston's laboratory and with Dr. Dan Lindner, director of the animal tumor core located at Taussig Cancer Center, CCF according to the protocol described earlier [Garzotto et al., 1997]. Briefly 5×10^4 cells in 50 μ l volume will be injected into a ventral prostate lobe after exposing the prostate gland surgically. We will use 15 animals per group as we obtain a variable take rate of 80-90% in nude mice of these human transplanted xenografts. 15 animals per group is the least number of animals that can be used to obtain reliable significance. 15 mice for each cell line with a total of 75 mice will be used for 5 different lines for PC3MM3 cell line-based experiments. We will use total 45 mice (15 x 3) for 3 cell lines for LNCaP-cell line based experiments. Animals will be allowed to recover from surgery and bioluminescent imaging will be performed with an IVIS camera system (Xenogen, Alameda, CA) in collaboration with Dr. Zhenghong Lee (Assistant Professor, Dept of Radiology, Case Western Reserve University, Cleveland, OH) 10 days post surgery and every 24 day interval for a period of 96 days. Regions of interest from displayed images will be quantified as total photon counts using Living Image software from Xenogen. Furthermore, following necropsy 96 days post surgery, total tumor volume will be measured (14) and histological examination will be done following paraffin embedding, sectioning and staining at the Histology section of CCF imaging core,

Potential problems: 1) Establishment of a bioluminescent cell line and using such line in an orthotopic tumor model has been successfully shown earlier [Jenkins et al., 2003]. We have sufficient expertise in making and maintaining various stable lines in our laboratory for various experimental purposes.

2) The technique of injection of cell lines to establish an orthotopic prostate tumor model has been mastered coinvestigator W. Heston, a prominent scientist in the Prostate research field, and, the PI has gained extensive expertise in this technique [Garzotto et al., 1997; Vieweg et al., 1994]. While we do not expect difficulties with the orthotopic model, we will also establish an experimental metastasis model by intracardiac injection of cells [Jenkins et al., 2003].

Other supportive experiments: This PSMA-mediated invasion suppression effect will be tested in invasive cell lines of other tissue types, such as breast, melanoma, renal carcinoma, by *in vitro* invasion assays, to test the generality of the PSMA-mediated effect.

Experimental plans: The expression of PSMA and its mutants will be accomplished by co-transfection of PSMA or its various mutants or the vector pIRES-puro encoding cDNAs at the ratio of 20:1 in cells and the expressing clones will be selected with puromycin and highest expressing clone will be used.

Specific Aim II: Identification and characterization of putative factor(s) involved in modulating the invasion capacity of prostate cancer cells.

According to our previous observation, conditioned media from PC3 cells (added to the upper chamber of standard boyden chambers for the matrigel invasion assay) could visibly enhance the invasiveness of PSMA expressing cells by 4 fold (obtained by visually counting the cells from randomly chosen fields) (Figure 3). In the present aim, we will isolate, identify and characterize the invasion-enhancing putative factor(s) from PC3 conditioned media.

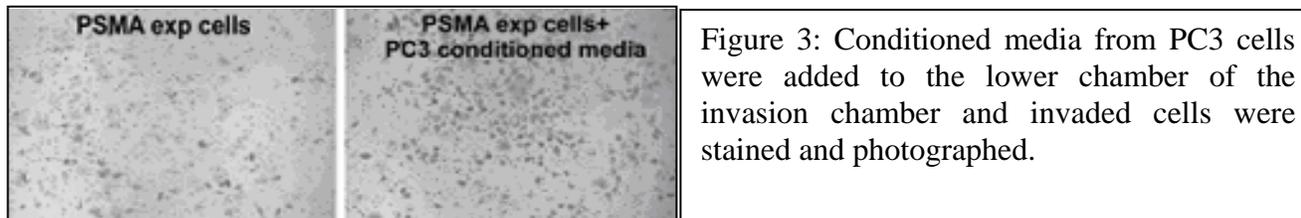


Figure 3: Conditioned media from PC3 cells were added to the lower chamber of the invasion chamber and invaded cells were stained and photographed.

Purification of the putative factor(s): Conditioned media from PC3 cells will be precipitated with 40% ammonium sulfate. Supernatant from the precipitation will be checked for the presence of activation factor by in vitro invasion assay. Supernatant will be further concentrated by ultrafiltration through 30 kd cut off membrane PTTK minitan plate (Millipore, Bedford, MA). The concentrate will be assayed for invasion property. If the active peptide is present in the concentrate, then it will be separated by RP-HPLC and tested for invasion activity in each fraction. The active fraction will be directly used to identify the peptide/peptides by mass-spectroscopy. First, we will determine the molecular weight of the components using MALDI-TOF, if the peptides are sufficiently small in size they can be directly used for sequencing by tandem MS-MS, or they will be trypsin-digested and sequenced.

Innovation: This proposal is highly innovative. Firstly, it plans to further establish the invasion suppressive function of a membrane-bound carboxypeptidase *in vivo*. Expression of PSMA on established PCa cell lines causes less invasiveness of these cell lines and this function is dependent on the enzymatic function of PSMA. This study will establish the fact that PSMA is an invasion suppressor for PCa cells and possibly can be used further for suppressing invasiveness of other type of cancers as well. Metastatic suppressors are known, but very few invasion suppressors have been reported. Since invasion is the first step before metastasis, establishment of this function of PSMA *in vivo* is very important. Secondly, this proposal also aims to isolate and characterize the putative peptide factor (s) secreted by PC3 cells that can inhibit PSMA function. This will lead to the opening of new avenues for finding specific ways to inhibit secretion of such factors and thereby inhibit invasion.

Relevance: PSMA protein is overexpressed in all forms of prostate cancer and is associated with bad prognosis, yet this protein serves as an invasion suppressor. Again, PCa is less aggressive than most of the cancers, and in this study we will establish that prostate cancer is less invasive because of PSMA expression on the membrane of the cancer cells. The metastatic lines of prostate cancer, namely PC3 (bone metastatic) and DU145 (brain metastatic), are non-expressing and highly invasive. This study will help us establish the fact for the first time why this cancer is less invasive. Once we establish the PSMA-mediated anti-invasive effect *in vivo*, we will further explore the invasion-suppressive function of this protein by expressing it in invasive lines of other tissue types. This will further establish the general invasion suppressor function of this protein. Furthermore, in the future, it will be pertinent to identify possible enhancers of this peptidase activity, not inhibitors, so as to prevent the spread of cancer. Identification of a putative invasive factor from PC3 cell conditioned media will help us identify putative chemoattractants generated by these cells and will lead to potential projects to further inhibit secretion of such factors from PCa cells

Supplementary data:

Reduced PSMA expression increases the invasiveness of CWR22 cells

Knockdown of PSMA expression by using shRNA in CWR22 cells:

This was achieved by using a specific construct which contained two shRNA sequence targeted against PSMA transcript. This construct was used along with another vector DNA containing a puromycin resistant marker to cotransfect CWR22 cells. The antibiotic resistant colonies were picked up and expanded and PSMA expression was tested. One such line with reduced PSMA expression showed increased invasiveness in a matrigel coated Boyden chamber assay.

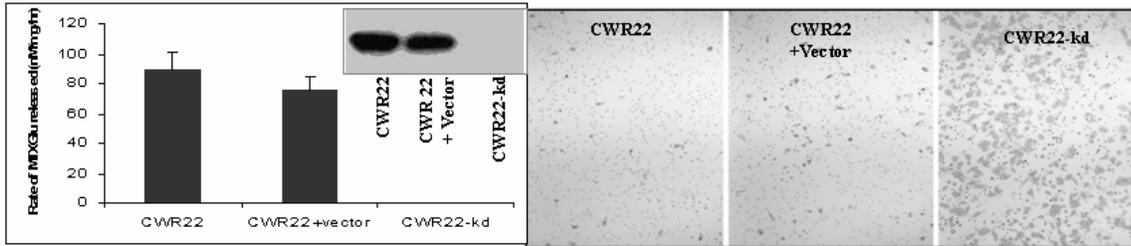


Figure 4 Legend: Reduced PSMA expression increased the invasiveness of the CWR22 cells. Western blot (inset) showing the reduced PSMA expression from CWR22 cells; the enzymatic activity of PSMA using these different cell line specific extracts have been expressed in bar graph. Increased invasiveness of the PSMA knockdown line has been shown by Boyden chamber invasion assay (right hand panel).

Enzymatic activity of PSMA modulates its cellular invasiveness:

This was studied by using various PSMA specific monoclonal antibodies (mAb) on the enzymatic activity of PSMA as well as cellular invasiveness on a cell line endogenously expression PSMA protein. For this we used LNCaP cell line which expresses a relatively high level of endogenous PSMA. We isolated the cell membranes from the LNCaP cells (as source of PSMA protein) and incubated a fixed amount of protein with various mAbs specific to different regions of PSMA. Most of the antibodies did not have any effect on PSMA’s enzymetic function, whereas antibody 5.4 showed strong inhibition. When cells were incubated with this antibody 5.4 used in standard matrigel coated boyden chamber assay, showed increase in invasiveness of the LNCaP cells, whereas other mAbs did not have much of an effect on LNCaP cell invasiveness.

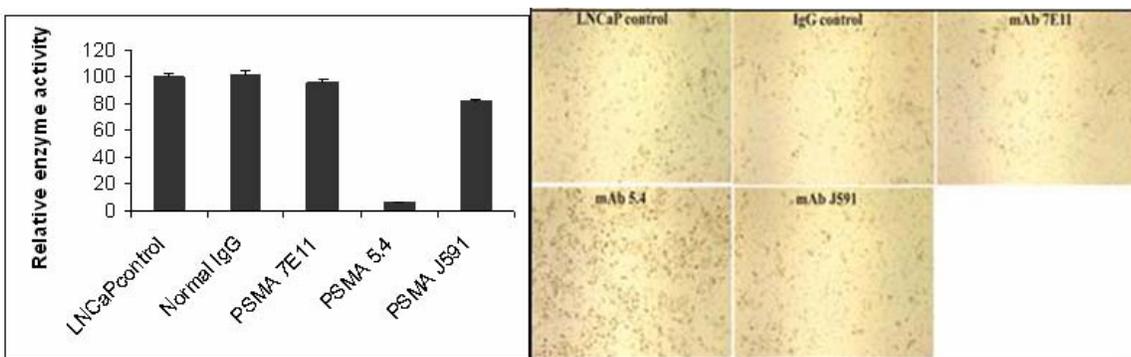


Figure 5 Legend: PSMA enzyme activity has a role of its effect on cellular invasiveness. Effect of different mAbs on PSMA enzymatic activity has been shown on the left panel and on cellular invasiveness has been shown in right hand panel.

Implication of PSMA and Filamin A interaction on the invasiveness of the prostate cancer cells:

It has been known earlier that PSMA interacts with Filamin A (FLNA) within cells. FLNA is an actin binding protein, which maintains the cellular stiffness and enables the cell migration. FLNA also interacts with various other membrane-bound and cytoplasmic protein, thereby plays a significant role in signaling events leading to cell migration. FLNA is dimeric protein and PSMA interacts with the dimerization domain of FLNA. Interaction with FLNA stabilizes PSMA to the membrane surface. We investigated whether PSMA-FLNA interaction has any role in this PSMA mediated effect on cellular invasiveness. For this purpose, we used shRNA to knockdown the expression of FLNA from PC3 cells which abundantly express FLNA. One such knockdown PC3 cell line with significant reduction of Filamin A protein was selected and used in the invasion assay. It was found that reduction of FLNA significantly reduced the invasiveness of cells. Same result was found in FLNA null melanoma cell line, M2 which does not express FLNA and A7 cell line, which is a FLNA cell line in M2 background. This indicates that FLNA itself has a role in cell invasion. We further expressed PSMA protein and its enzymatically inactive mutant within FLNA knockdown PC3 cell line and FLNA-null M2 cell lines and checked there invasiveness.

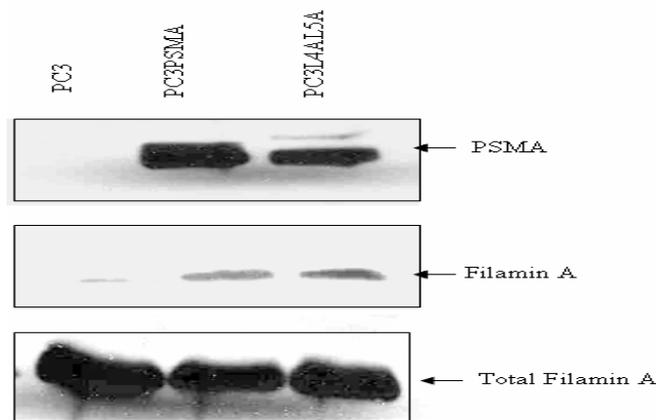


Figure 6: Legend: Interaction between PSMA and FLNA in PC3 cells is shown. PSMA, PSMA-internalization mutant (L4AL5A) proteins are shown in the middle panel. Total PSMA and its mutant expressed in PC3 cells are shown in top panel and total amounts of FLNA expressed by these three lines are shown in the bottom panel.

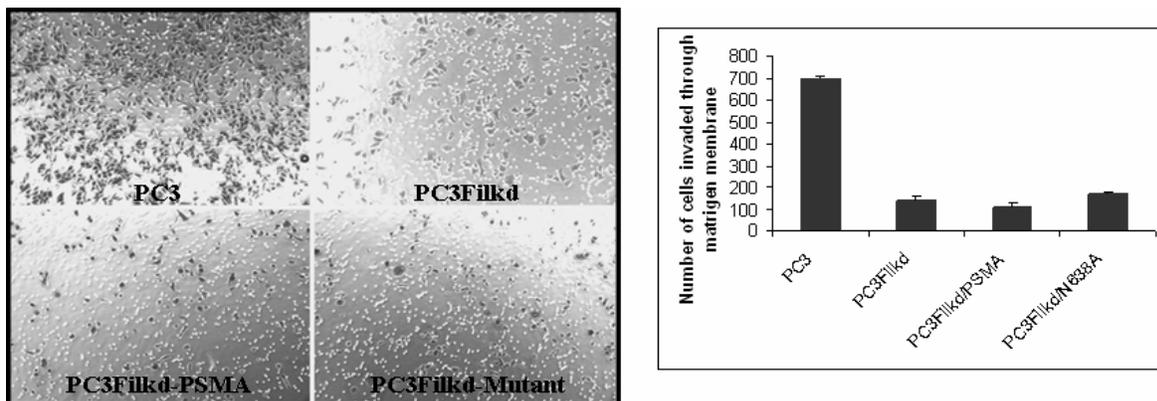


Figure 7: Legend: PSMA modulates cellular invasion independent of FLNA. Invasion assay of FLNA knockdown (Filkd), Filkd cell expressing PSMA or its enzymatically inactive mutant within PC3 cell background (shown in left panel) and quantitation of the invasion assay (shown in the right panel).

Similar results were obtained with FLNA null M2 cells expressing PSMA, indicated that this PSMA-mediated modulation of cellular invasion is independent of its interaction with FLNA

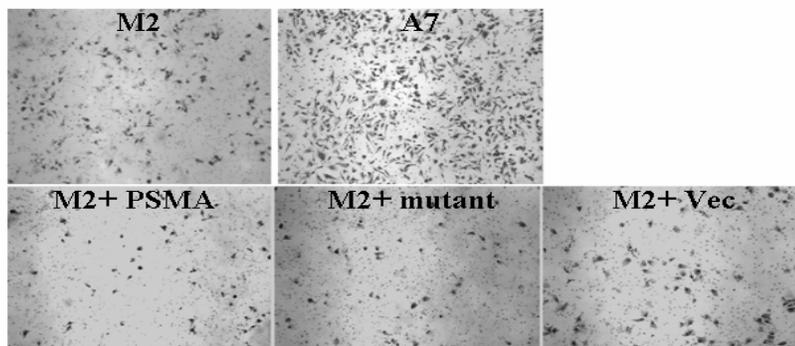


Figure 8 Legend: PSMA modulates cellular invasion independent of FLNA. M2, FLNA null cells; A7, FLNA expressed in M2 cells; M2+PSMA, PSMA expressed in M2 cells; M2+Mutant, enzymatically inactive mutant expressed in M2 cells; M2+Vec, empty vector expressed in M2 cells.

Study of PSMA-mediated effect on cellular invasion by orthotopic implantation of cell lines (expressing PSMA or its various mutants within) ventral prostate of nude mice.

Establishment of Bioluminescent cell lines expressing PSMA or its various mutants:

We wanted to achieve this goal by using a highly metastatic cell line derived from PC3 cells named PC3MM2 line. This cell line was obtained from Dr Isiah Fidler, MD Anderson Cancer Center. We have successfully established PC3MM2 cell line expressing PSMA protein and Renilla luciferase together.

We have selected 3 such cell lines which are high expresser lines for PSMA expression (measured by western blot) as well as express Renilla Luciferase reporter protein in abundantly (measured by luciferase activity). We are at present studying the tumor formation and metastatic ability of these lines using nude mice.

Concluding Remark:

This is the first time we have shown that PSMA modulate the migration/invasion capacity of the PCa cells. Cell lines endogenously or ectopically expressing PSMA have low invasiveness compared to non-PSMA expressing lines. Expression of shRNA within cell lines targeted against the PSMA transcript showed increased cellular invasiveness compared to control PSMA expressing lines. This function of PSMA is dependent on the enzymatic function of PSMA. Expression of enzymatically inactive mutant of PSMA within cell lines increases the migration/invasion capability of the cells. This function of PSMA on the cell lines is not dependent on its interaction with FilaminA protein. PSMA interacts with FilaminA with its N-terminal cytoplasmic tail. By using FilaminA null cells (M2) or expressing PSMA within FilaminA expressing M2 cells (A7) and by knocking down FilaminA from PC3 cells, we have shown that PSMA can modulate cell migration of prostate cancer cells independent of FilaminA. We have

successfully established PSMA and luciferase gene within PC3 MM2 cell line. Few lines have high PSMA and Luciferase levels. We have tested migration/invasion capability of these lines. Presently we are in the process of testing metastatic ability of these lines in nude mice.

ACRONYM LIST

Ac: Acetylated

Asp: Aspartate

CCF: Cleveland Clinic Foundation

cDNA: Complementary DNA

ECM: Extracellular Matrix

Exp: Expressing

Glu: Glutamate

Hyg: Hygromycin

i.p: Intra-peritoneal

kd: Kilodalton

Met: Methionine

mg/kg: milligram/kilogram

nt: Nucleotide

PCa: Prostate Cancer

PCR: Polymerase Chain Reaction

PSMA: Prostate Specific Membrane Antigen

Puro: Puromycin

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

shRNA: Short hairpin loop RNA

FLNA: Filamin A

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Publication and meeting abstracts :

Research Articles:

1. **Ghosh, A.**, Wang, X., Klein, E., and Heston, W. D.W. (2005) Novel role of prostate-specific membrane antigen in suppressing prostate cancer invasiveness. *Cancer Res* **65**, 727-731.
2. **Ghosh, A. et al.**, Effect of Prostate Specific Membrane Antigen and Filamin A interaction on the migration and invasion of Prostate Cancer cells (2007) Manuscript in preparation.

Meeting abstracts:

1. **Ghosh A.**, Wang, X., Harsch, K., Wang, D., London, D., Peterca, A., Magi-Galluzzi, C. and Heston, WDW. (2006) Implication of PSMA and Filamin A interaction on migration of cells. Poster presentation at Fall SBUR meeting, Phoenix, Arizona.
2. **Ghosh, A.**, Wang, X., Wang, D., London, A., Magi-Galluzzi, C. and Heston, WDW. (2006) Role of Filamin A and PSMA interaction on the invasiveness of prostate cancer cell. 97th AACR proceedings, Washington DC.
3. **Ghosh, A.**, Wang, D., London, D., Peterca, A. and Heston, WDW. (2005) Effect of Prostate Specific Membrane Antigen (PSMA) and Filamin A interaction on Prostate Cancer Invasion and Metastasis. Poster presentation at annual SBUR meeting, Miami Beach, Florida.

APPENDIX: Ghosh et al., 2005; Cancer Res; 65(3): 727-731

Novel Role of Prostate-Specific Membrane Antigen in Suppressing Prostate Cancer Invasiveness

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Abstract

Prostate-specific membrane antigen (PSMA), a type II transmembrane glycoprotein, is overexpressed in prostate cancer. PSMA is a unique cell surface marker, negatively regulated by androgen and extensively used for imaging of hormone refractory carcinomas and metastatic foci. PSMA is a carboxypeptidase with two important enzymatic functions, namely, folate hydrolase and NAALADase. PSMA also exhibits an endocytic function, in which it spontaneously recycles through endocytic vesicles. PSMA is overexpressed at various stages of prostate cancer, including androgen-sensitive and -independent disease, increased in expression with early relapse after therapy. We have used *in vitro* invasion assays to explore the possible role of PSMA in the metastasis of prostate cancer cells. Androgen-dependent prostate cancer lines, which express PSMA endogenously (e.g., LNCaP, MDA PCa2b, and CWR22Rv1) are less invasive compared with androgen-independent PC3 or DU145 cells, neither of which expresses PSMA. Ectopic expression of PSMA in PC3 cells reduced the invasiveness of these cells, suggesting that this reduction in the invasion capability of PSMA-expressing cells is due to PSMA expression and not to intrinsic properties of different prostate cancer cell lines. Furthermore, knockdown of PSMA expression increased invasiveness of LNCaP cells by 5-fold. Finally, expression of PSMA mutants lacking carboxypeptidase activity reduced the impact of PSMA expression on invasiveness. Thus, it seems that the enzymatic activity is associated with the effect of PSMA on invasiveness. (Cancer Res 2005; 65(3): 727-31)

Introduction

Prostate cancer is the most commonly diagnosed form of cancer among men in the United States and is second only to lung cancer as a cause of cancer-related death. Death from prostate cancer occurs largely in patients with aggressive androgen-insensitive metastatic disease (1). Prostate-specific membrane antigen (PSMA) protein has been correlated with aggressive disease (2). PSMA is a transmembrane-carboxypeptidase (3, 4), which is up-regulated 10-fold or more in prostate cancer, from androgen-sensitive to androgen-independent prostate cancer, and in metastatic deposits. PSMA expression is negatively regulated by androgen (5). Overexpression of PSMA in primary prostate cancer correlates with other traditional prognostic factors and can independently predict the disease outcome such as early relapse after therapy (6). It is still not

known whether PSMA has any effect on prostate cancer metastasis. Thus, PSMA overexpression is associated with a poor prognosis and we therefore hypothesized that PSMA expression is associated with biological processes of tumor invasion and metastasis. In our present report, we have tested our hypothesis that PSMA expression modulates the invasion capability of prostate cancer cells. We used prostate cancer cell lines that do not express PSMA and expressed PSMA or PSMA mutants within them or knocked down expression of the cell lines that normally express PSMA with small interfering RNA approach. We examined the invasiveness of these lines on a Matrigel invasion chamber. We report here the results of these studies.

Materials and Methods

Cell Lines and Reagents. PC3 cells (CRL-1435), CWR22Rv1 (CRL-2505), LNCaP (CRL-1740), and DU145 (HTB-81) were obtained from American Type Culture Collection (Manassas, VA). Retrovirally transformed PC3PSMA and the transfection control PC3Vector cells were obtained from Dr. Michel Sadelain (Laboratory of Gene Transfer and Gene Expression, Gene Transfer and Somatic Cell Engineering Facility, Memorial-Sloan Kettering Cancer Center, New York, NY, ref. 7). The cells were maintained in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 2 mmol/L of L-glutamine and 10% fetal bovine serum (United States Biochemical, Cleveland, OH). PSMA cDNA was originally cloned in our laboratory (5). Monoclonal antibodies used in this study (J591 and PM1T 485.5) were obtained from Dr. Neil Bander (Division of Hematology and Medical Oncology, Department of Medicine, Weill Medical College of Cornell University, New York, NY) and Hybritech (subdivision of Beckman Coulter, Inc., San Diego, CA), respectively. For making stable lines, different constructs were precipitated with pIRES-puro at the ratio of 20:1 and transfected to the cells. The stable clones were selected with puromycin.

PSMA Mutants. Generation of cDNA encoding N336A, N459A, N476A, and N638A mutants of PSMA have been described earlier (8). The constructs L4AL5A and H380G were generated by using Stratagene (La Jolla, CA) QuickChange site-directed mutagenesis kit as described before (8). Various mutants are summarized below:

Mutant	Internalization	Enzymatic activity	Location/domain
H380G	Yes	No	Active site, E
L4AL5A	No	Yes	Internalization motif, A
N336A	No	No	Glycosylation site, E
N459A	No	No	Glycosylation site, E
N476A	No	No	Glycosylation site, E
N638A	Yes	No	Glycosylation site, F

Cell Membrane Preparation and Folate Hydrolase Assay. Cell membrane preparation was done as described in Ghosh and Heston (8). Twenty micrograms of protein were used for Western blot and 2 µg of protein were used for folate hydrolase assay. Folate hydrolase

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assay was done using high-performance liquid chromatography separation and spectrometric analysis of reaction products as described before (8). The released glutamate from MTXglu2 was measured and expressed as nanomoles of glutamate released per milligram of protein per hour.

RNA Interference Using Short Hairpin RNA Construct. Short hairpin RNA generating hairpin primers were designed by using RNA interference oligo retriever (available from www.cshl.org/public/SCIENCE/hannon.html). A pGEM1 plasmid containing the human U6 promoter was used as a template for the PCR reaction. The vector contains ~500 bp of upstream U6 promoter sequence with an SP6 sequence that flanks the upstream portion of the U6 promoter sequence. Therefore, the 5' primer was SP6 universal primer oligo sequence, and the 3' primer was composed of the hairpin loop forming a sequence along with the 3' region of the U6 promoter. Two different PCR products containing two different hairpin sequences (against two different regions of PSMA sequence) under the U6 promoter were cloned in two different regions of pCDNA3.1 vector sequence. The vector carrying the short hairpin RNA sequences were transfected into LNCaP cells along with pIRES-puro vector DNA at the ratio of 20:1. The clones expressing short hairpin RNA were selected against puromycin.

Invasion Assays Using Boyden Chamber. Matrigel invasion assays were done with cells expressing or nonexpressing PSMA as described by Albini et al. (9). A fixed number of cells (5×10^5 cells/mL) in serum-free medium was added to the upper chamber of the Boyden chamber coated with Matrigel (coated on a polyvinyl pyrrolidone-free polycarbonate filter with 8- μ m pore size) inserts (BD Pharmingen, San Diego, CA) and the lower chamber contained 10% serum-containing medium. The cells were incubated for 48 hours. Invasion of cells to the underside of the Matrigel-coated membrane was detected by staining the cells with Mayer's hematoxylin solution and visualizing the cells under

a microscope. After staining, cells were counted under a microscope in four random fields (magnification $\times 100$) and results were expressed in the form of a bar graph.

Results

PSMA Expression Reduces Invasiveness of Prostate Cancer Cell *In vitro*. LNCaP and CWR22Rv1 cells endogenously express PSMA, whereas PC3 and DU145 do not (Fig. 1A). LNCaP cells have a higher PSMA expression compared with CWR22Rv1 or PC3PSMA (ectopically expressing PSMA in PC3 cells), as shown in Fig. 1A. The enzymatic activity profile of these different cell lines corresponds with their protein profile (Fig. 1B). PC3 and DU145 cells are non-PSMA-expressing and highly aggressive, whereas PSMA-expressing cell lines show less invasion through a Matrigel membrane (Fig. 1C). Quantification of cells that invaded through the Matrigel matrix showed that PSMA expression in PC3 cells reduces the invasion by 5-fold (Fig. 1D). LNCaP and CWR22Rv1 cells are likewise minimally invasive. Inducible expression of PSMA using the tetracycline system in PC3 cells also supported this finding. These PC3 (Tet+) cells with tetracycline off-regulatory system did not express PSMA protein (Fig. 2A and B) and were as invasive as wild-type PC3 cells (10). The same cells when induced to synthesize PSMA (Tet-, i.e., upon removal of tetracycline) attenuated their invasion through the Matrigel-coated membrane and this reduction level was comparable to that of PC3PSMA cells (with comparable amounts of expression of enzymatically active PSMA protein) as described earlier (Fig. 2C).

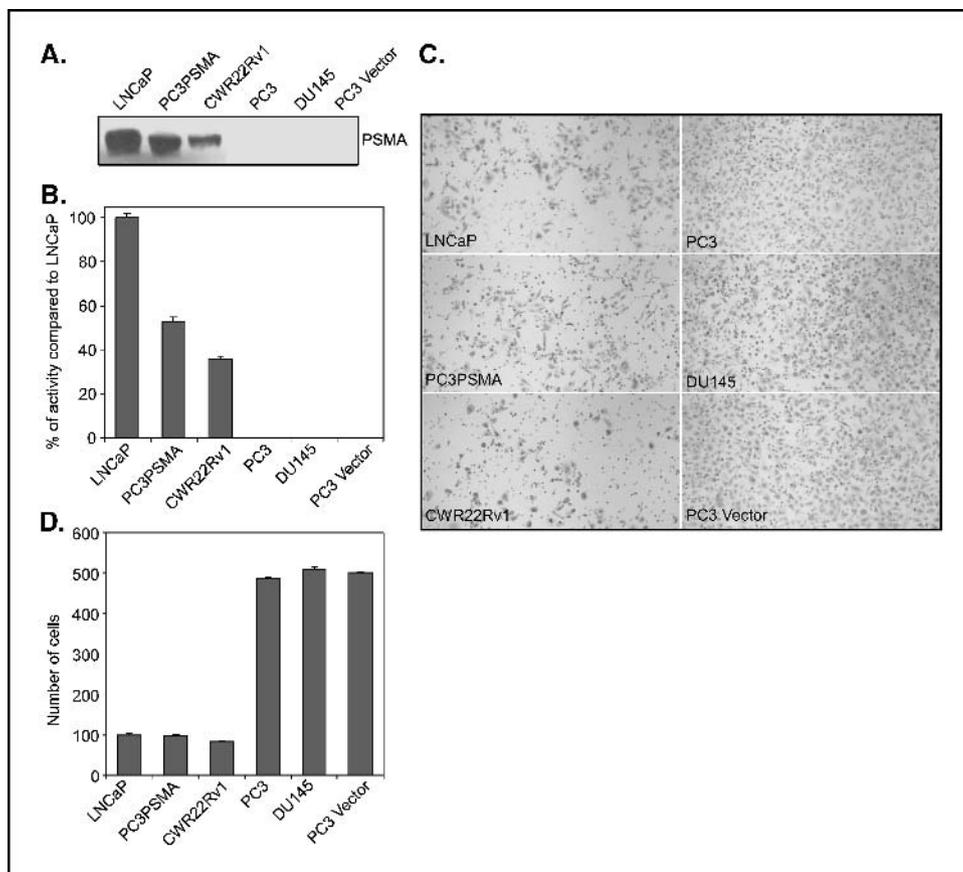
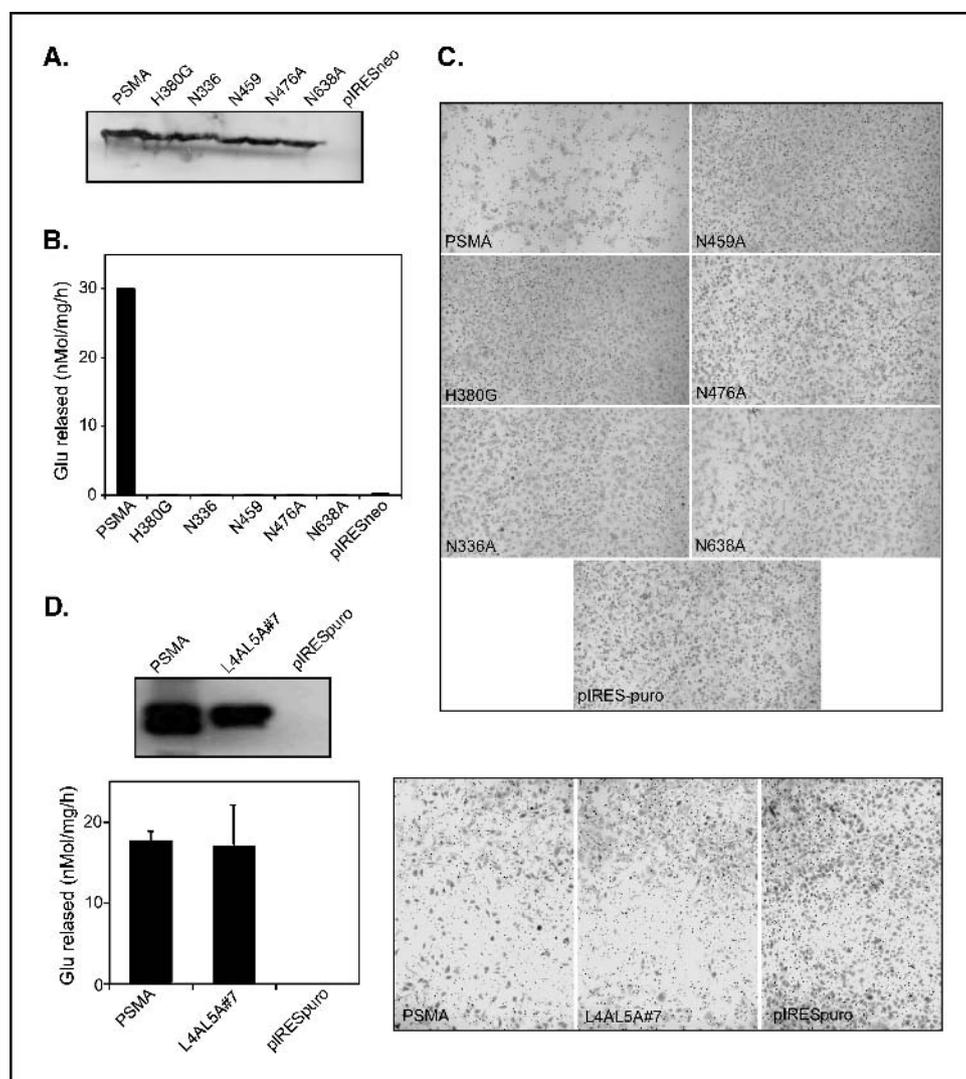


Figure 1. PSMA-expressing prostate cancer cells show reduction in invasiveness compared with nonexpressing cells. **A**, Western blot analysis of the PSMA protein from different cell lines, namely, LNCaP, CWR22, PC3PSMA, PC3, DU145, and PC3Vector. Fifty micrograms equivalent proteins from different cell lines were analyzed by SDS-PAGE and Western blotted onto the membrane. The PSMA protein was detected by J591 antibody. **B**, enzyme assay of PSMA obtained from 2 μ g protein equivalent of membrane preparations of different cell lines and glutamate released from MTXglu2 was measured by high-performance liquid chromatography and expressed as nanomoles of glutamate released per milligram of protein per hour. The enzyme activity thus obtained was finally plotted as percentage of LNCaP cell activity (100% control). **C**, Matrigel invasion assay. A fixed number of cells (5×10^5 /mL) of LNCaP, CWR22, PC3PSMA, PC3, DU145, and PC3Vector were seeded onto the upper chamber of the Matrigel insert in serum-free medium. After 48 hours post seeding, the invasion of cells to the other side of the Matrigel membrane was monitored. Cells attached to the lower surface were fixed, stained by 10% hematoxylin solution, and photographed. **D**, the number of cells that invaded the lower side of the membrane was scored by counting the hematoxylin-stained cells and plotted.

Figure 2. Enzyme activity of PSMA is important for invasion assay. *A*, Western analysis of 50 μ g equivalent protein PC3-PSMA and different PSMA mutants or vector-transfected cell lines (N380G, N336A, N459A, N476A, N638A, and pIRES-neo). *B*, folate hydrolase assay of 2 μ g equivalent protein from different cell lines expressing different mutant proteins. *C*, invasion assay using 5×10^5 cells/mL from different cell lines PC3-PSMA, H380G, N336A, N459A, N476A, N638A, and pIRES-neo. *D*, *top*, Western analysis of 50 μ g equivalent protein PC3-PSMA and PC3 cells expressing L4AL5A mutant of PSMA and parental PC3 cells. *Bottom, left*, enzyme assay of 2 μ g equivalent protein of PC3-PSMA and PC3-L4AL5A and parental PC3 cells expressed in terms of rate of Glu released from MTXGlu2 (nmol/L/mg of protein/h); *right*, Matrigel invasion assay using 5×10^5 cells of the different cell lines mentioned above.



Enzymatic Activity of PSMA Correlates with Reduced Cell Invasiveness. Several proteases have been found to play a role in cell invasion and metastasis. To explore whether PSMA enzymatic activity or intracellular alteration of cellular rigidity or internalization was involved, we used various mutant-expressing cell lines (lacking one or the other of these properties) for invasion assay. PC3 cells expressing H380G mutant of PSMA (where the mutation is located at the zinc binding site at the active center, renders it enzymatically inactive; ref. 11), invaded through the Matrigel membrane very efficiently. Other enzymatically inactive PSMA mutants express PSMA protein with mutation at the various sugar-attachment sites (8), that is, N336A, N459A, N476A, and N638A mutants, with the first three having mutations in the three putative glycosylation sites located at the catalytic domain and the fourth one expressing protein with mutation at domain F. The N638A mutant behaves like PSMA in terms of protein half-life and membrane insertion and internalization but lacks enzymatic activity. All of the cell lines expressing different mutants are as invasive as parental PC3 cells (Fig. 3B and C). This result indicated that the enzymatic activity of PSMA may be responsible for this function. We examined the role of another PSMA mutant, L4AL5A, which is defective in its endocytic function, yet enzymatically

active (12, 13). PC3 cells expressing this mutant showed less invasiveness, which was comparable to the wild-type, PSMA-expressing cells (Fig. 3D).

PSMA Knockdown from LNCaP Cells Restores the Invasiveness of LNCaP Cells. LNCaP cells are poorly invasive and express PSMA endogenously in large quantity on its cell surface (Fig. 1). PSMA protein expression was abolished from LNCaP cells by means of short hairpin RNA targeted against two different regions of PSMA-encoding messages. This method could efficiently block protein expression, which was confirmed by Western blot analysis as well as the enzymatic activity of the cell membrane preparations of LNCaP knockdown cells (Fig. 4A and B). LNCaP cells lacking PSMA protein increased the invasiveness of LNCaP cells (Fig. 4C) by 5-fold (Fig. 4D) as compared with parental LNCaP cells endogenously expressing PSMA protein.

Discussion

Association of PSMA with progression of disease at various stages of prostate cancer suggests a positive role in prostate cancer tumor cell growth and metastasis. Contrary to our expectation, in our studies with prostate cancer cell lines we

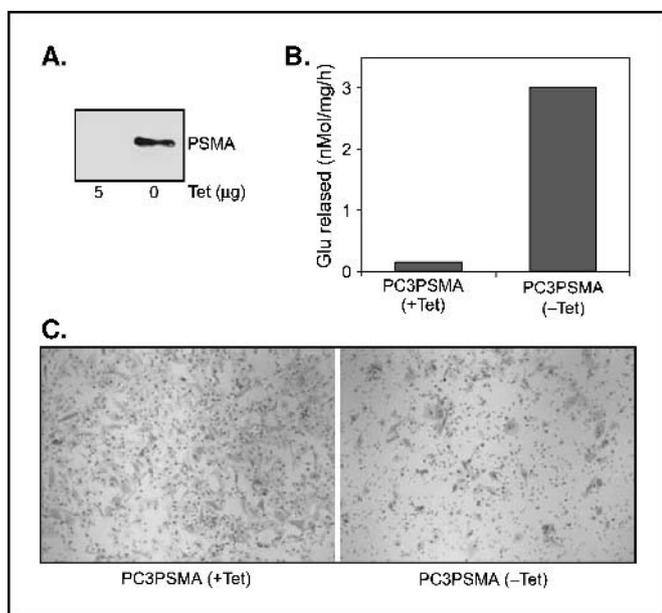


Figure 3. Invasion assay of inducible cell line of PSMA expressed in PC3 cells with or without tetracycline (*Tet*)-containing medium. **A**, Western blot analysis of 50 µg equivalent proteins from PC3-PSMA cells (with or without tetracycline) analyzed by SDS-PAGE. **B**, enzymatic analysis of PC3-PSMA (+*Tet*) and (-*Tet*) in the presence or absence of tetracycline, respectively. The tetracycline-off situation expressed the protein (-*Tet*). **C**, Matrigel invasion assay showing the invading capability of PC3-PSMA cell line with and without tetracycline.

found PSMA expression to have a strong role in decreasing the invading capacity of cells across the Matrigel membrane. Expression of cell surface peptidases are known to play a key role in the control of growth and differentiation by modulating the activity of peptide factors and by regulating their access to adjacent cells by two basic mechanisms; either by inactivation of stimulatory peptide or by activation of inhibitory factors (14). The enzymatic function of PSMA seems to decrease the ability of prostate cancer cells to invade the extracellular matrix. The pathway through which PSMA functions in controlling the invasiveness is still unidentified.

To detect whether the enzymatic function of PSMA has any role in the invasiveness of the PC3 cells, we have used enzymatically inactive mutants of PSMA with mutations located in different regions of PSMA protein and used them for invasion assays. All such mutants ectopically expressed in PC3 cells show invasiveness comparable to parental PC3 cells, indicating that enzyme activity of PSMA expressed on the surface of PC3 cells plays an important role in determining its ability to invade the extracellular matrix. We have also used well-established enzymatic inhibitors of PSMA and found them to make low invasive lines more invasive (data not shown). This indicates that when PSMA is overexpressed, the enzymatic activity of PSMA is involved in decreasing cell invasiveness.

We also used an inducible line of PSMA in our invasion assay and we have found that such a line has an attenuated invading capacity when induced to express PSMA protein. Knockdown of PSMA expression from LNCaP cells showed a similar result in which removal of PSMA expression could increase the ability of LNCaP cells to invade the extracellular matrix, indicating that the enzymatic activity of PSMA is altering the invasiveness of LNCaP cells as well. Knockdown of PSMA from LNCaP cells will similarly allow these cells to invade more efficiently because the peptidase

function of PSMA is not available anymore to modulate putative factor(s). Similarly, expressing wild-type PSMA into PC3 cell surface made these cells less invasive.

PSMA is known to internalize spontaneously like a number of well-established cell surface receptors (15). The internalization of PSMA requires an MXXXL motif. The cytoplasmic tail of PSMA interacts with actin-binding protein filamin A. Filamin binding to PSMA reduces its internalization and enzyme activity (16). We used a PC3PSMA cell line as contrasted with the PC3-L4AL5A (PC3 cells expressing PSMAL4AL5A mutant) cell line for invasion assays. The PSMAL4AL5A mutant does not internalize (13), and it is enzymatically as active as wild-type PSMA. We found that both PC3PSMA and PC3PSMAL4AL5A show similar patterns of decreased invasiveness as opposed to parental PC3 cells, which are highly invasive. It is known that PSMAL4AL5A interacts with filamin A (data not shown) but this does not resolve whether filamin A provides any role in invasion. This finding is in contrast with a similar function shown by matrix metalloprotease MT1-MMP, in which dynamic turnover by internalization of the protein regulates cell surface levels of enzyme during cell migration and invasion (17). Future planned experiments will examine the effect

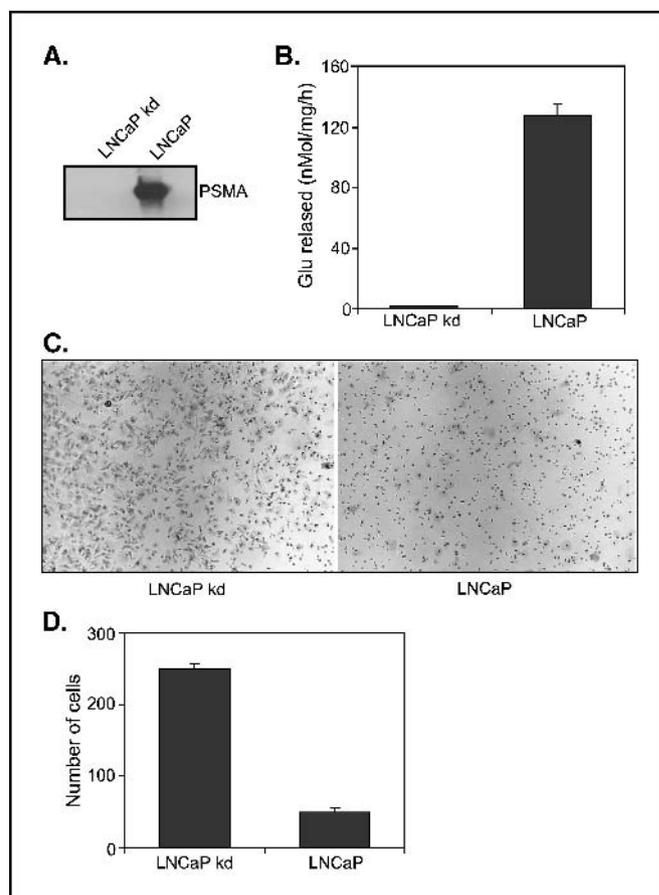
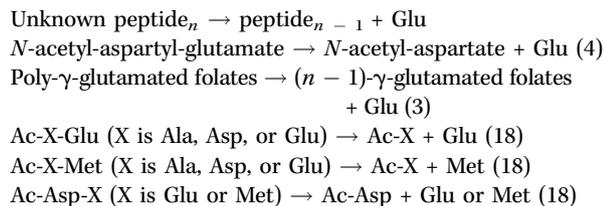


Figure 4. PSMA knockdown from LNCaP cells by RNA interference method increases their invasion activity. **A**, Western blot analysis of LNCaP cells and PSMA knockdown line from LNCaP knockdown cells (*LNCaP kd*) detected with J591 antibody. **B**, enzymatic activity of PSMA protein expressed on the membrane of LNCaP or LNCaP knockdown cells. **C**, invasion assay of a fixed number (5×10^5 cells/mL) of LNCaP knockdown cells and parental LNCaP cells were analyzed and the number of hematoxylin-stained cells that have invaded through the Matrigel membrane were photographed. **D**, graphical representation of cell invasion assay shown in C.

of knockdown of filamin A expression to further delineate the role of PSMA-filamin interaction.

Therefore, the carboxypeptidase function of PSMA contributes to the low level of invasiveness of PSMA-expressing cells. One conclusion is that PSMA is proteolytically modulating invasion-promoting peptides or amino acids. Following is a list of PSMA-specific substrates:



One of the constant products of PSMA enzymatic reaction is glutamate, which might also be a potential modulator and functions through glutamate receptors. Glutamate receptors have been reported to be present in prostate and LNCaP cells as well (19, 20). Alternatively, folates, which are produced as a by-product of this enzymatic reaction, could be a potential modulator for PSMA enzymatic activity. The role of folates and glutamates on the invasiveness of PSMA-expressing cells across the Matrigel membrane needs to be investigated further. However, discovery of the last three groups of substrates of PSMA open the possibility of existence of many potentially important yet unidentified sub-

strates, which could contribute to this effect on the invasiveness of PSMA-expressing prostate cancer cells.

Because of this interplay between proteases and peptidases and the receptors, it will be important to understand what modifiers are being generated that affect this process of cell invasion. We are beginning to examine whether we can extend our finding *in vitro* to an *in vivo* model system. If it is shown to have the same effect as observed with prostate cancer cell lines *in vitro*, then one would want to identify factors that enhance PSMA enzymatic activity and not those that inhibit its enzymatic activity. We also plan to study the detailed mechanism behind this anti-invasive role of PSMA. Indeed, if there are nutritional or other environmental modifiers, it may be possible that they would contribute to the aggressiveness or lack of aggressiveness of prostate cancer by modifying PSMA enzymatic activity and as such may help to explain the extreme range of biological aggressiveness of prostate cancer.

Acknowledgments

Received 10/3/2004; revised 11/6/2004; accepted 11/21/2004.

Grant support: NIH grant CA101069-01.

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We thank Drs. Neil Bander and Michel Sadelain for providing J591 monoclonal antibody and PC3PSMA-expressing cells, respectively, Dr. Saumen Sarkar (Department of Molecular Biology, Cleveland Clinic Foundation, Cleveland, OH) for vector and primers regarding short hairpin RNA, and Dr. Thomas Powell for helpful discussions.

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