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Mengqian Chen
University of Central Florida

Ya-Yuan Fu
University of Central Florida

Chen-Yong Lin

Li-Mei Chen
University of Central Florida

Karl X. Chai
University of Central Florida

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Prostasin induces protease-dependent and independent molecular changes in the human prostate carcinoma cell line PC-3

Mengqian Chen [a], Ya-Yuan Fu [b,d], Chen-Yong Lin [c], Li-Mei Chen [b], Karl X. Chai [a,b,*]

[a] *Biomolecular Science Center, Burnett College of Biomedical Sciences, University of Central Florida, Orlando, FL, USA*
[b] *Department of Molecular Biology and Microbiology, Burnett College of Biomedical Sciences, University of Central Florida, Orlando, FL, USA*
[c] *Greenebaum Cancer Center, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD, USA*
[d] *State Key Laboratory of Reproductive Biology, Institute of Zoology and Graduate School, Chinese Academy of Sciences, Beijing, People's Republic of China*

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Abstract

Expression of prostasin in the PC-3 human prostate carcinoma cells inhibited in vitro invasion, but the molecular mechanisms are unknown. Wild-type human prostasin or a serine active-site mutant prostasin was expressed in the PC-3 cells. Molecular changes were measured at the mRNA and the protein levels. Cell signaling changes were evaluated by measuring phosphorylation of the extracellular signal-regulated kinases (Erk1/2) following epidermal growth factor (EGF) treatment of the cells. Protein expression of the EGF receptor (EGFR) was differentially down-regulated by the wild-type and the active-site mutant prostasin. The mRNA expression of EGFR and the transcription repressor SLUG was reduced in cells expressing wild-type prostasin but not the active-site mutant. Phosphorylation of Erk1/2 in response to EGF was greatly reduced by the wild-type prostasin but not by the active-site mutant. The mRNA expression of the urokinase-type plasminogen activator (uPA), the uPA receptor (uPAR), cyclooxygenase-2 (COX-2), and the inducible nitric oxide synthase (iNOS) was decreased by the wild-type and the active-site mutant prostasin. The mRNA or protein expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), matriptase, and E-cadherin was greatly increased by the active-site mutant prostasin. In conclusion, prostasin expression elicits both protease-dependent and independent molecular changes in the PC-3 cells.

Keywords: Serine Protease; Matriptase; Epidermal Growth Factor Receptor; Prostate Cancer

1. Introduction

Since its discovery more than a decade ago, prostasin, a glycosylphosphatidylinositol (GPI)-anchored extracellular serine protease has been shown to play important roles in epithelial physiology. These functional roles include suppression of invasion [1,2], regulation of gene expression during inflammation [3], and activation of the epithelial sodium channel (ENaC) [4].

Activation of ENaC by prostasin is mechanistically the best-defined function for prostasin, with very recent data consistent with a proteolytic cleavage of the gamma subunit of ENaC by prostasin [4]. The other in vitro and in vivo phenotypic changes induced by prostasin remain mechanistically undefined. We undertook the current study to probe into the potential molecular mechanisms by which prostasin impacts the cell's behavior. We used the PC-3 human prostate carcinoma cell line, which has a compromised prostasin expression due partly to promoter DNA hypermethylation [5]. Upon prostasin re-expression the PC-3 cells displayed reduced in vitro invasion through a Matrigel barrier [1]. We focused on the extracellular molecular players in tumor invasion previously established for this cell line, because prostasin is an active serine protease anchored to the outside of the plasma membrane [6].

The epidermal growth factor receptor (EGFR) was the first member of the erbB family of receptor tyrosine kinases to be discovered and the first cell surface receptor to be shown with a role in cancer biology [7]. EGFR overexpression is implicated for a causative role in ten different types of solid tumor [8].

* Corresponding author. Department of Molecular Biology and Microbiology, University of Central Florida, Building 20, Room 323, 4000 Central Florida Boulevard, Orlando, FL 32816-2364, USA. Tel.: +1 407 823 6122; fax: +1 407 823 0956.
E-mail address: kxchai@mail.ucf.edu (K.X. Chai).

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Cellular signals initiated by activation of this receptor tyrosine kinase confer cancer cells with advantages in angiogenesis, growth, and motility [9]. The PC-3 cell line is a high expresser of EGFR [10], which is a major mediator of prostate cancer cell motility and invasiveness [11]. The urokinase-type plasminogen activator (uPA), a serine protease, through interactions with its membrane receptor, the GPI-anchored uPAR, also promotes prostate cancer invasiveness [12]. The invasion promoting effect of the uPA-uPAR signaling route in the PC-3 cell line is modulated by an EGFR tyrosine kinase inhibitor (AG1478) [13], reducing uPA production [14]. E-Cadherin, a cell–cell adhesion molecule and a tumor suppressor, is down-regulated in the PC-3 cell line [15], resulting in increased invasive behavior [16]. The PC-3 cell line expresses the granulocyte-macrophage colony-stimulating factor (GM-CSF), which, acts in an autocrine loop to stimulate the tumor cells [17]. GM-CSF release is also regulated by the EGFR signaling pathway [18]. These target genes and proteins were investigated in PC-3 cells expressing either a wild-type human prostasin or a serine active-site mutant.

The prostasin serine protease can be activated by the type-II transmembrane extracellular serine protease matriptase in vitro and in vivo [19]. Inhibition of matriptase expression in the PC-3 cells resulted in suppression of invasion [20]. Prostasin expression in the prostate is regulated at the transcription level partly by promoter DNA methylation [5], and partly by transcription factors such as the sterol-regulatory element-binding proteins (SREBP's), the SNAIL and the SLUG [21]. SLUG is a well-known E-cadherin expression repressor and an inducer of epithelial–mesenchymal transition (EMT) [22], and is up-regulated by EGF [21]. The inflammation-associated expression of the inducible nitric oxide synthase (iNOS), but not of the cyclooxygenase-2 (COX-2) was attenuated in the mouse bladder by prostasin [3]. In PC-3 cells expressing the wild-type or the mutant prostasin, we also examined the expression states of these regulators of prostasin expression, and genes that are regulated by prostasin.

2. Materials and methods

2.1. Materials

The human prostate carcinoma cell line PC-3 (Passage 18) was obtained from the American Type Culture Collection (ATCC, Manassas, VA), and cultured in conditions previously described [1]. A polyclonal antibody against human prostasin was described previously [6]. Polyclonal antibodies to EGFR (sc-03), Erk1/2 (sc-94), and a monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-32233) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A monoclonal antibody to E-cadherin (C20820) was purchased from BD Biosciences (San Jose, CA). A monoclonal antibody to human matriptase (M32) was described previously [23]. A polyclonal antibody against phospho-Erk1/2 (V803A) was purchased from Promega (Madison, WI). A recombinant human EGF was purchased from Invitrogen (Carlsbad, CA).

2.2. Cell culture and transfection

PC-3 cells were transfected with episomal expression plasmids carrying a wild-type human prostasin cDNA, or an active-site mutant prostasin cDNA, using methods previously described [1]. Construction of the episomal expression plasmids and the mutant prostasin cDNA was described previously [1,3]. Transfectants harboring the control plasmid, expressing the wild-type prostasin, or expressing the mutant prostasin were plated in 60-mm dishes and cultured to confluence for harvest of the culture medium, and the cells.

2.3. RNA isolation, and reverse transcription-real-time polymerase chain reactions (RT-rtPCR)

Confluent monolayers were lysed with the Trizol reagent (Invitrogen) for total cellular RNA isolation per supplier's protocol. Reverse transcription was carried out for each sample using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) per supplier's protocol. Real-time polymerase chain reaction was carried out for each sample using the iQ SYBR Green Supermix (Bio-Rad) per supplier's protocol. PCR programs and methods of quantification were described previously [3]. The message number of the GAPDH was used as the reference for calculating specific gene messages. PCR primers for GAPDH and SLUG were described previously [3,21]. PCR primers for the following human gene transcripts are listed in the order of forward and reverse:

COX-2	5′-CCT CCT GTG CCT GAT GAT TG-3′
	5′-ACT GAT GCG TGA AGT GCT G-3′
E-Cadherin	5′-AGA ATG ACA ACA AGC CCG AAT-3′
	5′-CGG CAT TGT AGG TGT TCA CA-3′
EGFR	5′-CTG ACC AAA ATC ATC TGT GCC C-3′
	5′-CGT GGC TTC GTC TCG GAA TT-3′
GM-CSF	5′-AGC CAC TAC AAG CAG CAC-3′
	5′-ACA AGC AGA AAG TCC TTC AG-3′
iNOS	5′-ATC TCT GGT CAA GCT GGA TGC-3′
	5′-GCC TTA TGG TGA AGT GTG TCT TG-3′
Matriptase	5′-GTC CTG CTC ATC ACA CTG-3′
	5′-GTC AAT GTT GGG TGG GTA G-3′
uPA	5'-GAC ATT GCC TTG CTG AAG-3′
	5′-CGG ATA GAG ATA GTC GGT AG -3′
uPAR	5′-CAC TCA GAG AAG ACC AAC AG-3′
	5′-GCA CAA GTC TAA CCC ACA C-3′

2.4. Enzyme-linked immunosorbant assay (ELISA)

A Quantikine™ human GM-CSF immunoassay kit (R&D Systems, Minneapolis, MN) was used for determining the quantities of secreted GM-CSF in the medium collected from the PC-3 transfectant cultures. The medium was collected at 48 h after a fresh medium change over sub-confluent cultures. The assay was performed per supplier's protocol. Quantities of GM-CSF were expressed as pg/ml after normalizing with the cell number in the culture dishes.

2.5. Western blot analysis

Total cell lysate harvested from each cell type at equal amounts for all cell types, was subjected to western blot analysis with appropriate antibodies as described previously [6]. Each membrane was re-blotted with a GAPDH antibody as a control for protein loading. For each sample, 40 μg of protein were loaded, except for the phospho-Erk1/2 blot for which 20 μg of protein per sample were loaded. The antibodies were used at the following dilutions: prostasin at 1:5,000, GAPDH (sc-32233) at 1:5,000, EGFR (sc-03) at 1:4,000, phospho-Erk1/2 (V803A) and Erk1/2 (sc-94) at 1:5,000, matriptase (M32) at 1:2,500, and E-cadherin (C20820) at 1:2,000. Appropriate secondary antibodies conjugated to horse-radish peroxidase (HRP) were used at 1:10,000, and signals were developed by enhanced chemilluminescence (ECL). Specific target protein signals were digitally quantified and normalized against the quantity of the GAPDH signal.

2.6. EGF treatment of cells and analysis of EGF signaling pathway activation

Confluent PC-3 transfectants were placed under serum-free medium for overnight, and EGF treatment of the cultures was performed for 15 min at

50 ng/ml concentration of EGF, diluted in serum-free medium. The cells were then immediately applied to western blot analysis for total Erk1/2 and phospho-Erk1/2. Relative changes of the phosphorylation state of Erk1/2 were determined following digital signal quantification and normalization against total Erk1/2 levels.

2.7. Statistical analysis

Expression level evaluation using the western blot densitometry data, or the quantitative real-time PCR data was performed by comparing the "means", wherein the data graphed or listed in the table represent the Means±Standard Error (SE). The Student *t*-test (one-tailed, equal variance) was employed for assessing statistical difference (defined as when $p<0.05$) between data groups.

3. Results

3.1. EGFR signaling pathway is down-regulated by prostasin

The wild-type and the serine active-site mutant prostasin proteins were expressed in the PC-3 cells at equal quantities, as shown by the results of a western blot analysis (Fig. 1).

At the protein level, EGFR expression was down-regulated by the wild-type prostasin and the serine active-site mutant prostasin expressed in the PC-3 cells, by 66% and 48%, respectively, when compared to cells carrying the control plasmid (Fig. 2A). The wild-type prostasin expressed in the PC-3 cells also down-regulated EGFR mRNA expression by 34% when compared to cells carrying the control plasmid, while the mutant prostasin did not affect EGFR mRNA expression (Fig. 2B, and Table 1). At the cellular function level, only the wild-type prostasin was able to inhibit EGF-EGFR signaling, with a 61% reduction of Erk1/2 phosphorylation following EGF stimulation, when compared to cells carrying the control plasmid (Fig. 2C). The mutant prostasin did not have an effect on Erk1/2 phosphorylation after the EGF stimulation.

3.2. Expression of SLUG, uPA, and COX-2 is down-regulated differentially by the wild-type and the mutant prostasin

At the mRNA level, expression of SLUG was down-regulated by the wild-type prostasin expressed in the PC-3 cells, by 71%, when compared to cells carrying the control plasmid, while the mutant prostasin had no effect on SLUG mRNA expression (Fig. 3A, and Table 1).

For uPA or COX-2, however, both the wild-type and the mutant prostasin were able to down-regulate the mRNA expression, but with the wild-type prostasin displaying a more robust phenotype (i.e., statistically different from the mutant phenotype). The wild-type prostasin reduced uPA or COX-2 mRNA expression by 72.5% or 79%, respectively; while the mutant prostasin was only able to reduce uPA or COX-2 mRNA expression by 31% or 31%, respectively (Fig. 3B and C, and Table 1).

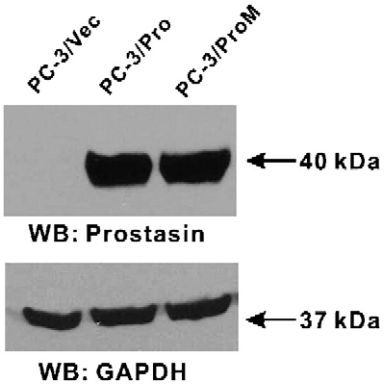

Fig. 1. Expression of the wild-type and a serine active-site mutant prostasin in PC-3. A representative western blot analysis of total lysate from PC-3 cells transfected with a vector control plasmid (PC-3/Vec), a wild-type human prostasin cDNA plasmid (PC-3/Pro), and a serine active-site mutant prostasin cDNA plasmid (PC-3/ProM).

3.3. Expression of uPAR and iNOS is down-regulated by both the wild-type and the mutant prostasin

At the mRNA level, expression of uPAR was down-regulated equally well, by 27%, by the wild-type prostasin, or the mutant prostasin expressed in the PC-3 cells, when compared to cells carrying the control plasmid (Fig. 4A, and Table 1).

For the iNOS mRNA, wild-type prostasin down-regulated its expression in the PC-3 cells by 70%, while the mutant prostasin down-regulated the iNOS mRNA by 68%, when compared to cells carrying the control plasmid (Fig. 4B, and Table 1). There is no statistical difference in iNOS mRNA expression levels of the cells expressing the wild-type prostasin and those expressing the mutant.

3.4. Expression of GM-CSF, matriptase, and E-cadherin is up-regulated by the serine active-site mutant prostasin

At the mRNA level, GM-CSF expression was up-regulated by the mutant prostasin expressed in the PC-3 cells, to 2.66 fold, when compared to cells carrying the control plasmid (Fig. 5A, and Table 1). On the other hand, wild-type prostasin expressed in the PC-3 cells produced a down-regulation effect on GM-CSF mRNA expression, reducing it to 79% of that in the control cells. The mRNA level changes of GM-CSF expression are reflected by the quantitative changes of the secreted GM-CSF in the culture medium (Fig. 5B). The mutant prostasin increased the amount of secreted GM-CSF to 3.2 fold of that of the control cells, while the wild-type prostasin reduced the amount to 69%.

For the matriptase mRNA, only the mutant prostasin expressed in the PC-3 cells had an effect on its expression, increasing it to 16.8 fold of that in the control cells (Fig. 5C, and Table 1). At the protein level, only in the mutant prostasin-expressing cells was the matriptase band detected in the western blot analysis (Fig. 5D).

For the E-cadherin mRNA, both the wild-type and the mutant prostasin expressed in the PC-3 cells had an inducing effect on its expression, increasing it to 1.9 fold and 16.6 fold, respectively, of that in the control cells (Fig. 5E, and Table 1). At the protein level, the changes corresponded to the mRNA changes, with the wild-type prostasin increasing the cellular E-cadherin protein to 2.1 fold, and the mutant prostasin, 7 fold,

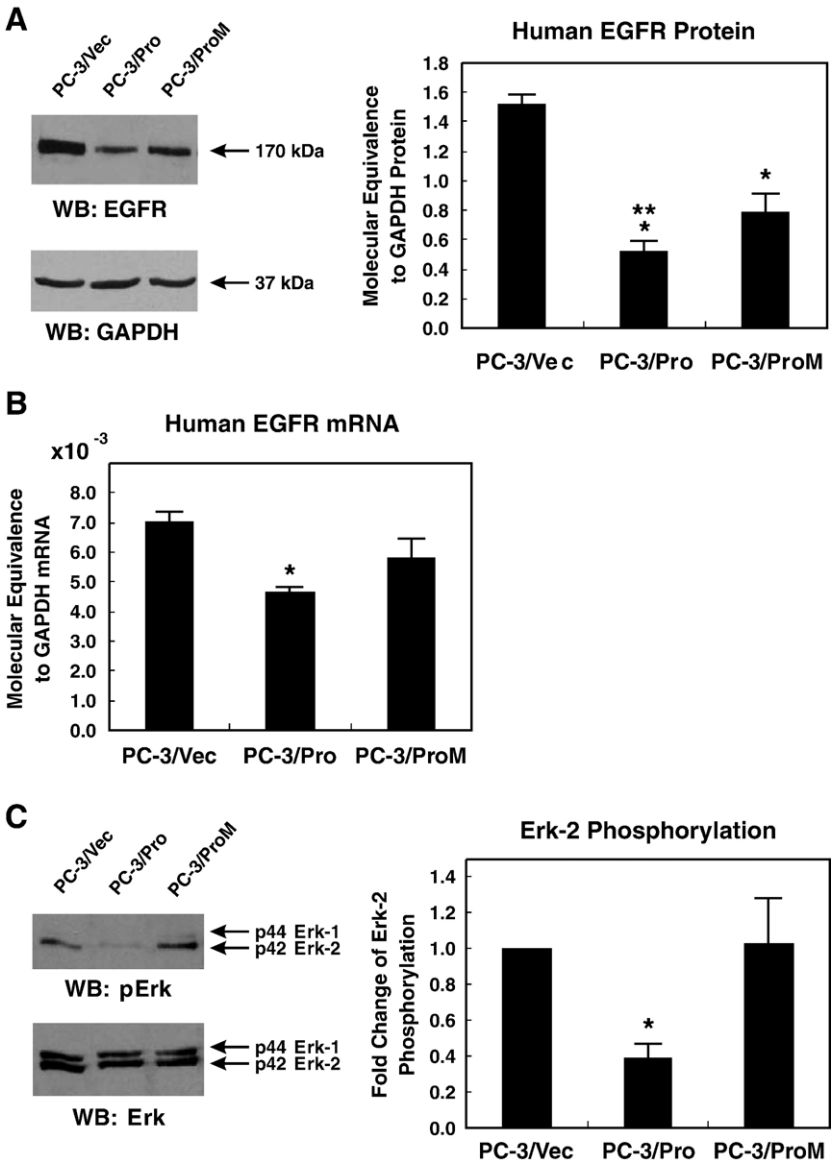

Fig. 2. Regulation of EGFR expression and EGF-EGFR signaling in PC-3 by the wild-type and the mutant prostasin. (A) Western blot analysis of EGFR in PC-3 transfected with a vector control plasmid (PC-3/Vec), a wild-type human prostasin cDNA plasmid (PC-3/Pro), and a serine active-site mutant prostasin cDNA plasmid (PC-3/ProM). Relative EGFR expression levels between PC-3/Vec, PC-3/Pro, and PC-3/ProM were determined following densitometric measurement of the specific protein bands and normalization against the GAPDH signals, as shown in the bar graph to the right. Calculations were based on data from three independent experiments. The single-asterisk indicates a statistical difference between the PC-3/Vec and the PC-3/Pro, or between the PC-3/Vec and the PC-3/ProM data groups ($p<0.05$). The double-asterisk indicates a statistical difference between the PC-3/Pro and the PC-3/ProM data groups ($p<0.05$). (B) Real-time PCR analysis of reverse-transcribed cellular RNA for expression of EGFR. Experimental groups are as indicated under each data column, representing relative levels of expression normalized to the mRNA level of GAPDH. The single-asterisk indicates a statistical difference between the PC-3/Vec and the PC-3/Pro data groups ($p<0.05$). Calculations were based on data from four independent experiments. (C) Western blot analysis of Erk1/2 phosphorylation in response to EGF stimulation. Relative Erk1/2 phosphorylation levels between PC-3/Vec, PC-3/Pro, and PC-3/ProM were determined following densitometric measurement of the phospho-Erk2 bands and normalization against the total Erk2 bands, as shown in the bar graph to the right. Calculations were based on data from three independent experiments. The single-asterisk indicates a statistical difference between the PC-3/Vec and the PC-3/Pro data groups ($p<0.05$).

over the level of E-cadherin protein in the vector control cells (Fig. 5F).

4. Discussion

In this report, we present our initial findings in the efforts of defining the molecular mechanisms by which prostasin serine protease, as an extracellular enzyme, impacts epithelial cell behavior. We have previously determined that in human prostate and breast cancer cells that lost prostasin expression, an anti-invasion phenotype was associated with the re-expression of prostasin, while cell proliferation in vitro or in vivo was not affected [1,2]. In this report, we focused our attention on the molecules that play a role in the invasive behavior of a model cell line, the human prostate cancer cell line PC-3.

Table 1
Gene expression changes in PC-3 evaluated by RT-rtPCR

Experiment group	Gene evaluated	Normalized to GAPDH control (fold)	Change versus control fold
PC-3/Vec	EGFR	$7.06\pm0.27\times10^{-3}$	1.0
PC-3/Pro	EGFR	$4.65\pm0.16\times10^{-3}$	0.66*
PC-3/ProM	EGFR	$5.84\pm0.65\times10^{-3}$	nsd
PC-3/Vec	SLUG	$1.42\pm0.08\times10^{-2}$	1.0
PC-3/Pro	SLUG	$0.41\pm0.02\times10^{-2}$	0.29*
PC-3/ProM	SLUG	$1.18\pm0.10\times10^{-2}$	nsd
PC-3/Vec	uPA	$2.29\pm0.10\times10^{-1}$	1.0
PC-3/Pro	uPA	$0.63\pm0.04\times10^{-1}$	0.275*$^{\phi}$
PC-3/ProM	uPA	$1.59\pm0.03\times10^{-1}$	0.69*
PC-3/Vec	uPAR	$2.87\pm0.10\times10^{-2}$	1.0
PC-3/Pro	uPAR	$2.09\pm0.12\times10^{-2}$	0.73*
PC-3/ProM	uPAR	$2.10\pm0.05\times10^{-2}$	0.73*
PC-3/Vec	COX-2	$9.69\pm0.51\times10^{-4}$	1.0
PC-3/Pro	COX-2	$1.99\pm0.23\times10^{-4}$	0.21*$^{\phi}$
PC-3/ProM	COX-2	$6.73\pm0.60\times10^{-4}$	0.69*
PC-3/Vec	iNOS	$5.55\pm0.97\times10^{-5}$	1.0
PC-3/Pro	iNOS	$1.65\pm0.22\times10^{-5}$	0.30*
PC-3/ProM	iNOS	$1.79\pm0.23\times10^{-5}$	0.32*
PC-3/Vec	GM-CSF	$3.60\pm0.21\times10^{-4}$	1.0
PC-3/Pro	GM-CSF	$2.84\pm0.12\times10^{-4}$	0.79*$^{\phi}$
PC-3/ProM	GM-CSF	$9.56\pm0.32\times10^{-4}$	2.66*
PC-3/Vec	Matriptase	$3.68\pm0.48\times10^{-4}$	1.0
PC-3/Pro	Matriptase	$3.53\pm0.45\times10^{-4}$	nsd
PC-3/ProM	Matriptase	$6.195\pm0.51\times10^{-3}$	16.8*
PC-3/Vec	E-Cadherin	$9.23\pm1.13\times10^{-4}$	1.0
PC-3/Pro	E-Cadherin	$1.79\pm0.10\times10^{-3}$	1.9*$^{\phi}$
PC-3/ProM	E-Cadherin	$1.53\pm0.18\times10^{-2}$	16.6*

Real-time PCR analysis of reverse-transcribed cellular RNA from PC-3 cells transfected with a control plasmid (PC-3/Vec), a wild-type human prostasin. cDNA plasmid (PC-3/Pro), and a serine active-site mutant prostasin cDNA plasmid (PC-3/ProM). Experiments were performed at least four times. Calculations in the table were based on data from four representative experiments.
* Indicates a statistical difference ($p<0.05$) between the PC-3/Vec and the PC-3/Pro, or between the PC-3/Vec and the PC-3/ProM data groups.
ϕ Indicates a statistical difference ($p<0.05$) between the PC-3/Pro and the PC-3/ProM data groups.
nsd: No statistical difference, between the data group indicated and PC-3/Vec.

The PC-3 cells were shown to display active prostasin protease on the membrane upon re-expression [6]. This cell line also presents the prostasin-activating enzyme, matriptase [20], and in our hands we confirmed an mRNA-level matriptase expression (Fig. 5C, and Table 1). We then used our sublines expressing the wild-type or the active-site mutant prostasin to investigate the molecular mechanisms that are dependent or independent of prostasin's protease function.

The first target molecules that we examined were the EGFR and those regulated by the EGFR during tumor invasion. The EGFR was identified as a major player in prostate cancer invasion, in vivo, and in vitro in the model cell line PC-3. The EGF-EGFR signaling initiates on the outside of the cell, where prostasin serine protease is located. We first evaluated the potential of prostasin to regulate EGFR protein expression in the PC-3 cells following re-expression. wild-type prostasin produced a 66% reduction of EGFR protein expression when compared to the control cells (Fig. 2A). At the mRNA level, a 34% reduction of EGFR expression was also observed in the cells expressing the wild-type prostasin (Fig. 2B, and Table 1). Moreover, the wild-type prostasin but not the serine active-site mutant down-regulated EGF-EGFR signaling, as EGF-stimulated Erk1/2 phosphorylation was reduced by 61% (Fig. 2C). It had been shown previously that EGF-EGFR signaling could regulate EGFR mRNA expression in prostate cancer cell lines, including the PC-3 [24]. Our observation on EGFR expression regulation by prostasin is consistent with a model of EGFR protein down-regulation followed by EGFR mRNA down-regulation. In the PC-3 cells, activation of the EGF-EGFR signaling pathway does not stimulate cell proliferation [25]. The

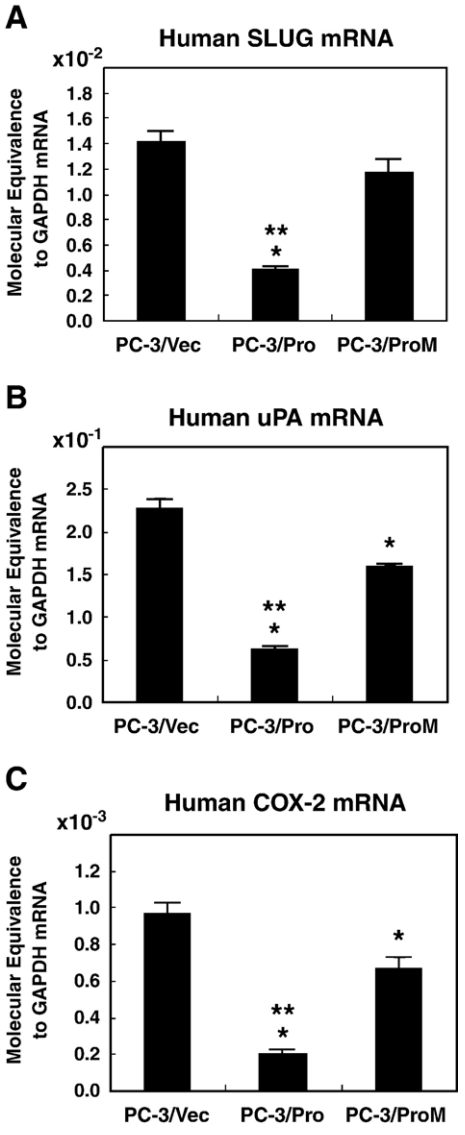

Fig. 3. Real-time PCR analysis of reverse-transcribed cellular RNA for expression of SLUG (A), uPA (B), and COX-2 (C). Experimental groups are as indicated under each data column, representing relative levels of expression normalized to the mRNA level of GAPDH. The single-asterisk indicates a statistical difference between the PC-3/Vec and the PC-3/Pro, or between the PC-3/Vec and the PC-3/ProM data groups ($p<0.05$). The double-asterisk indicates a statistical difference between the PC-3/Pro and the PC-3/ProM data groups ($p<0.05$). Calculations were based on data from four independent experiments.

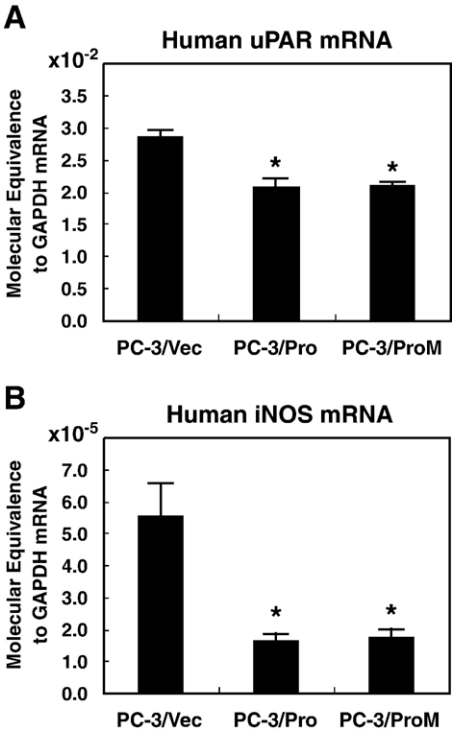

Fig. 4. Real-time PCR analysis of reverse-transcribed cellular RNA for expression of uPAR (A) and iNOS (B). Experimental groups are as indicated under each data column, representing relative levels of expression normalized to the mRNA level of GAPDH. The single-asterisk indicates a statistical difference between the PC-3/Vec and the PC-3/Pro, or between the PC-3/Vec and the PC-3/ProM data groups ($p<0.05$). Calculations were based on data from four independent experiments.

Erk1/2 phosphorylation down-regulation by the wild-type prostasin is consistent with its anti-invasion but non-anti-proliferation phenotype [1]. We have demonstrated previously that EGF stimulation up-regulates SLUG expression while SLUG is a repressor of prostasin expression [21]. Here we showed that the wild-type prostasin is capable of down-regulating SLUG expression (Fig. 3A and Table 1). The EGF-EGFR signaling pathway down-regulation by prostasin may very well be the underlying mechanism for the SLUG down-regulation. Prostasin is essential for maintaining tight junction protein expression and tight junction formation [26], while SLUG is a robust repressor of E-cadherin expression [22]. In this regard, the reciprocal expression regulation between prostasin and SLUG mediated by EGF-EGFR may play a functional role in epithelial differentiation.

The serine active-site mutant prostasin also produced a protein-level EGFR down-regulation, at 48% (Fig. 2A), but not as great as the down-regulation observed with the wild-type prostasin. The mutant prostasin, however, did not have an effect on the EGFR mRNA expression (Fig. 2B, and Table 1) or EGF-stimulated Erk1/2 phosphorylation (Fig. 2C). A possible explanation is that the extent of the mutant prostasin-associated EGFR protein down-regulation was not at a threshold point to affect the effective number of surface receptor presentation to impact receptor binding-activated signaling or EGFR mRNA expression.

The uPA mRNA was down-regulated by the wild-type prostasin in the PC-3 cells (Fig. 3B, and Table 1), a result that is consistent with the invasion-suppressing role of prostasin [1]. This result is also consistent with an EGF-EGFR-stimulated uPA expression [14] being down-regulated by the wild-type prostasin. Both the wild-type and the mutant prostasin were shown to down-regulate the uPAR mRNA expression (Fig. 4A, and Table 1), but the mechanism and the impact of this regulation are unclear. It is also difficult to speculate, at present, on the mechanism or impact of the uPA expression down-regulation by the mutant prostasin (Fig. 3B, and Table 1).

We have observed several molecular changes associated with the expression of the active-site mutant prostasin in PC-3 cells. It must be emphasized that these molecular changes are experimental artifacts because this form of the prostasin protein does not exist in nature. Its value to our investigation is to help identify the proteins that directly interact with prostasin, allowing us to establish whether they are potential substrates of the prostasin serine protease. Our results on the EGFR protein and mRNA expression following prostasin and mutant prostasin re-expression in the PC-3 cells are consistent with a proteolytic processing role for prostasin on a new candidate substrate, the EGFR. On the other hand, we cannot rule out at present that the EGFR expression changes resulted first from a transcriptional regulation. We did not observe an apparent proteolytically processed EGFR band in our western blots. But the potentially cleaved EGFR could be rapidly internalized and degraded. Further research is required to ascertain the timing and sequence of EGFR expression regulation by prostasin.

Bruns et al. [4] recently reported that the proteolytic activation of the gamma subunit of the ENaC is carried out by prostasin, but they also noted that the serine active-site mutant prostasin produced a similar proteolytic effect. A potential mechanism was offered to explain the apparent proteolytic effect of the serine active-site mutant prostasin on ENaCgamma, that it could have retained a residual serine protease activity. An alternative explanation was that the abundance of over-expressed prostasin, wild-type or mutant, could have exhausted the supply of serine protease inhibitors, allowing other proteases to take action. We have observed in the PC-3 cells, however, a dramatic inducing effect by the mutant prostasin, on the activating serine protease, matriptase. Matriptase and prostasin share a great deal in substrate preference, for example, cleaving the Gln–Ala–Arg-X type of substrates [5,27]. It is plausible that in the experiments performed to confirm prostasin cleavage of ENaCgamma, the mutant prostasin transfected into the cells also induced matriptase expression to cleave ENaCgamma.

The biological relevance of mutant prostasin-induced gene expression requires careful consideration as such is the case with the E-cadherin expression. The wild-type prostasin produced a moderate induction effect on E-cadherin expression, a result that is also consistent with prostasin's anti-invasion role. A robust induction of E-cadherin expression at the mRNA level and the protein level, however, was observed in cells expressing the mutant prostasin. This induction appears to correlate with

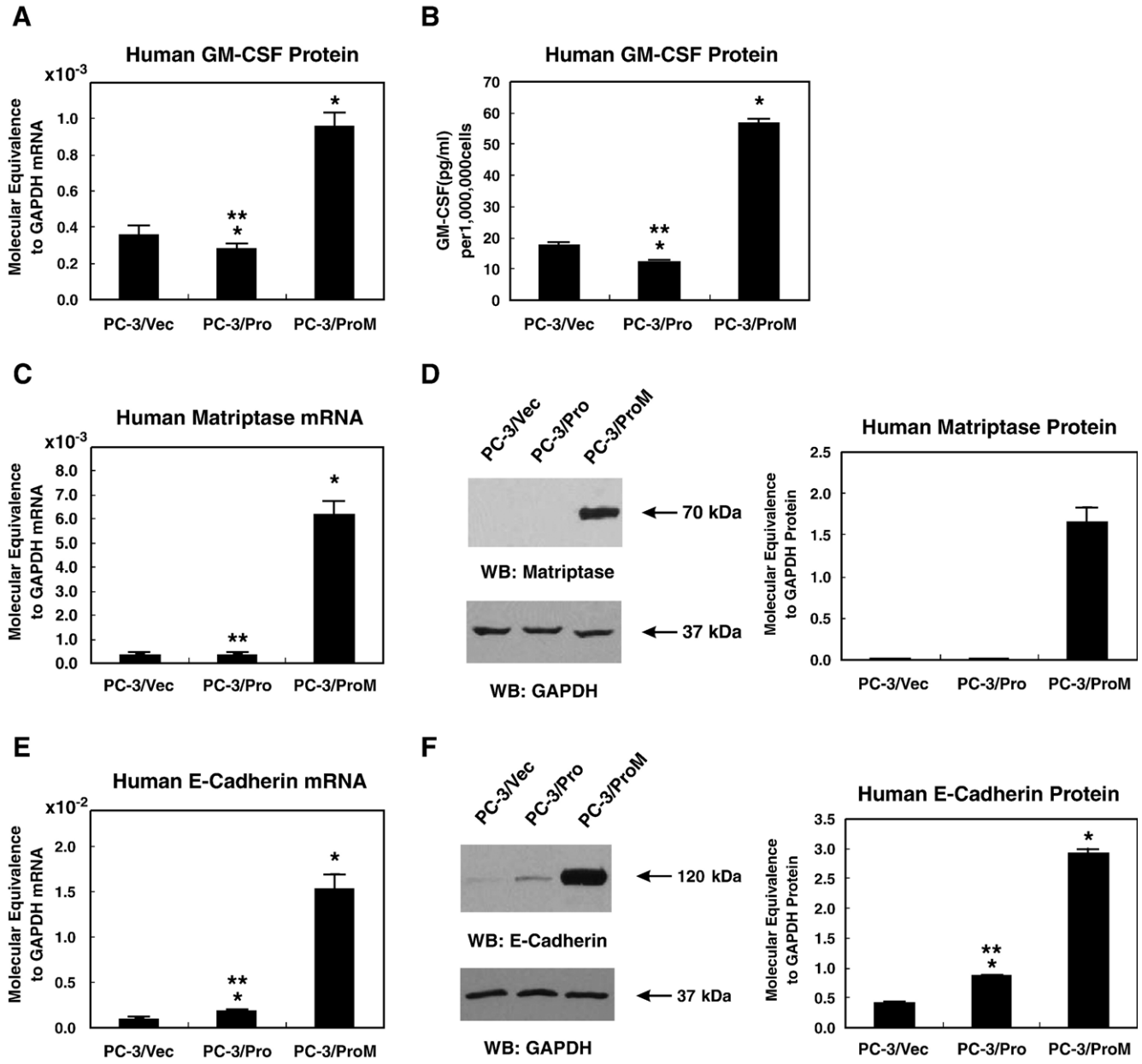

Fig. 5. The serine active-site mutant prostasin induces the expression of GM-CSF (A and B), Matriptase (C and D), and E-Cadherin (E and F). Experimental groups are as indicated under each data column, or over each sample, representing relative levels of expression normalized to the mRNA/protein level of GAPDH, whichever appropriate. The single-asterisk indicates a statistical difference between the PC-3/Vec and the PC-3/Pro, or between the PC-3/Vec and the PC-3/ProM data groups ($p<0.05$). The double-asterisk indicates a statistical difference between the PC-3/Pro and the PC-3/ProM data groups ($p<0.05$). Calculations of the mRNA expression levels were based on data from four independent experiments. Calculations of the protein expression levels were based on data from three independent experiments.

that of the matriptase expression, suggesting that matriptase and prostasin may have an overlapping point in the mechanisms and pathways by which they modulate cell signaling.

We also investigated two inflammation-induced genes for potential regulation by prostasin in the PC-3 cells, namely COX-2 and iNOS. Induction of bladder iNOS mRNA by inflammation was attenuated by prostasin, while induction of bladder COX-2 mRNA was not [3]. Prostasin did not regulate inflammation-induced COX-2 expression in the bladder because it is not co-expressed in the COX-2 producing cells [3]. We tested the expression of these two genes in response to prostasin re-expression in the PC-3 cells, and found that both the wild-type prostasin and the mutant prostasin produced a down-regulation on both COX-2 and iNOS (Figs. 3C and 4B, and Table 1). For COX-2 the down-regulation by the mutant prostasin was not as great as that by the wild-type prostasin, but for iNOS, the two versions of prostasin were equally effective. We could reason from these results that prostasin is also capable of regulating COX-2 expression, in cells where it is co-expressed with COX-2. We did not observe a down-regulation of iNOS expression by the mutant prostasin in the bladder inflammation model [3], but an effect was seen in the PC-3 cells in the present study. It is also possible that an induction of other membrane-type extracellular proteases, such as matriptase, was

responsible for the COX-2 or iNOS expression down-regulation in PC-3 cells expressing the mutant prostasin.

In conclusion, prostasin re-expression in the PC-3 human prostate carcinoma cells induced serine protease-dependent and apparently serine protease-independent molecular changes. The protease-dependent changes are considered biologically relevant, while it will require further research investigation to determine the biological relevance of the protease-independent changes.

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