PRSS3/Mesotrypsin Is a Therapeutic Target for Metastatic Prostate Cancer

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Abstract

PRSS3/mesotrypsin is an atypical isoform of trypsin that has been associated with breast, lung, and pancreatic cancer cell malignancy. In analyses of open source transcriptional microarray data, we find that PRSS3 expression is upregulated in metastatic prostate cancer tissue, and that expression of PRSS3 in primary prostate tumors is prognostic of systemic progression following prostatectomy. Using a mouse orthotopic model with bioluminescent imaging, we show that PRSS3/mesotrypsin is critical for prostate cancer metastasis. Silencing of PRSS3 inhibits anchorage-independent growth of prostate cancer cells in soft agar assays, and suppresses invasiveness in Matrigel transwell assays and three-dimensional (3D) cell culture models. We further show that treatment with recombinant mesotrypsin directly promotes an invasive cellular phenotype in prostate cancer cells and find that these effects are specific and require the proteolytic activity of mesotrypsin, because neither cationic trypsin nor a mesotrypsin mutant lacking activity can drive the invasive phenotype. Finally, we show that a newly developed, potent inhibitor of mesotrypsin activity can suppress prostate cancer cell invasion to a similar extent as PRSS3 gene silencing. This study defines mesotrypsin as an important mediator of prostate cancer progression and metastasis, and suggests that inhibition of mesotrypsin activity may provide a novel modality for prostate cancer treatment. Mol Cancer Res; 10(12): 1555–66. ©2012 AACR.

Introduction

Prostate cancer is the most commonly occurring cancer and second leading cause of cancer-related death in American men (1). Mortality results from metastasis of hormone-refractory cancer cells to bone, lung, liver, and other vital organs (2). Prostate cancer is also a very heterogeneous disease, in some cases lying latent for decades, whereas in other cases advancing rapidly to systemic metastasis and death (3). Elucidating the molecular pathways that distinguish between indolent cancers and those that progress more rapidly is critical, both for risk stratification of patients to guide clinical management, and for development of better therapies to treat patients at high risk for progression or with existing metastatic disease. Therapeutic strategies that inhibit cancer cell invasiveness, or reduce establishment or growth of metastases, could be of considerable benefit.

The metastatic cascade of prostate cancer involves a defined sequence of events, first neoangiogenesis or lymphangiogenesis, followed by loss of tumor cell adhesion, local invasion of host stroma, and escape of tumor cells into the vasculature or lymphatics, and finally dissemination, extravasation, and colonization of specific metastatic sites (4–6). Many proteases, upregulated in tumor cells or secreted by tumor-associated stromal cells, perform specific functions to facilitate the various steps of this cascade. Proteases release angiogenic factors, shed cell-adhesion molecules, degrade basement membranes, stimulate epithelial–mesenchymal transition, aid extravasation, and can also be required for colonization of metastatic sites (6–8). Identifying which proteases drive the specific steps of tumor progression, and defining their specific modes of action, can reveal novel druggable targets and unexplored avenues for cancer intervention (9).

The serine protease mesotrypsin, encoded by the PRSS3 gene, has recently been implicated as an important mediator of progression and metastasis in pancreatic cancer (10). Ectopic expression of PRSS3 transcripts has also been reported in other epithelial cancers and cancer cell lines including prostate, colon, and lung (11–14). PC-3 cells, derived from a bone metastasis of a grade 4 prostate adenocarcinoma (15), show very high upregulation of PRSS3 (16). Here, we show that PRSS3/mesotrypsin expression is a critical effector of prostate cancer malignancy, and that it plays a key functional role in promoting metastasis.

Materials and Methods

Cell culture and reagents

RWPE-1 cells and the derivative cell lines NB-14, NB-11, and NB-26 were obtained from American Type Culture
Collection (ATCC) and were maintained according to ATCC specifications in keratinocyte serum free media (K-SFM, Invitrogen) supplemented with 0.05 mg/mL bovine pituitary extract (BPE) and 5 ng/mL human recombinant EGF. PC3-M cells were kindly provided by Dr. Raymond Bergan from Northwestern University, Robert H. Lurie Medical Research Center (Chicago, IL). PC3-M cells were maintained in RPMI-1640 (Invitrogen) supplemented with 10% FBS (Invitrogen).

**RNA extraction, cDNA synthesis, and quantitative real-time PCR**

RNA was isolated from cultures using TRIZol reagent (Invitrogen) according to manufacturer’s protocols. cDNA was synthesized according to kit specifications using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was conducted using TaqMan gene expression assays (Applied Biosystems) on an Applied Biosystems 7900HT Fast Real-Time PCR System according to manufacturer’s protocols. TaqMan assays used included, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hs99999905_m1 and PRSS3, Hs00605637_m1.

**Lentiviral shRNA knockdowns**

A validated lentiviral short hairpin RNA (shRNA) construct NM_002771.2-454s1c1 targeting human PRSS3 was obtained from the MISSION TRC-Hs1.0 library (Sigma). A non-target control lentiviral vector containing a short hairpin that does not recognize any human genes (NT) was used as a negative control in all RNA interference (RNAi) experiments. Conditioned media containing infective lentivirus particles were produced using HEK293FT cells and following supplier protocols. For lentiviral transduction, PC3-M cells were seeded at 1.5 × 10^6 cells per 10-cm dish. After 24 hours, medium was replaced with a mixture of 3.6 mL RPMI containing 10% FBS and 10 μg/mL polybrene (Fisher), and 2.4 mL conditioned lentiviral media. Media was changed after 24 hours and transduced cells were selected with 2 μg/mL puromycin. Cells were maintained under puromycin selection for up to a week before use in invasion assays or transduction with lentiviral construct pSIN-luc [(gift of Dr. Yasuhiro Ikeda, Mayo Clinic (Rochester, MN)] conferring expression of firefly luciferase for use in the orthotopic tumor model. Knockdown was routinely assessed at the transcript level by qRT-PCR as described earlier, and in some experiments was also assessed at the protein level in cell lysates by Western blotting using a custom rabbit polyclonal antiserum (Cocalico Biologicals) raised against mesotrypsin peptide acetyl-TQAECKASYPGKITNS-NH2 conjugated to key-hole limpet hemocyanin (KLH; EZ Biolab), as described previously (17).

**Animals and orthotopic model of spontaneous metastasis**

Six- to 10-weeks old Nod/LtSz-prkds(scid) males from Jackson Laboratory (Bar Harbor, ME) were maintained under the guidance of approved Mayo Clinic Institutional Animal Care and Use Committee protocol A19709. Mice were housed in a specific pathogen-free barrier facility with 12 hours light/dark cycle. Food (Harlan Irradiated Diet T.7912.15), water, and bedding (corncob) were sterilized and cages changed at least once a week. For the orthotopic implantation experiment, PC3-M cells transduced with PRSS3-targeted or nontarget control shRNA lentivirus and superinfected with pSIN-luc, conferring expression of firefly luciferase, were trypsinized, quenched with RPMI containing 10% FBS, counted, and resuspended in 50% growth factor–reduced Matrigel and 50% serum-free RPMI-1640 medium. Mice were anesthetized with Avertin (200 mg/kg) by intraperitoneal injection and shaved. A small vertical incision in the lower abdominal region was made and the prostate exteriorized by exposing the bladder and seminal vesicle. PC3-M cells (3 × 10^6 cells in 10 μL) were injected into the dorsolateral side of the prostate using a 27.5-gauge needle attached to a 50 μL glass Hamilton Series 1700 gastight syringe. The peritoneum was closed with a suture and the incision closed with 7 mm wound clips. Saline was administered subcutaneously during recovery on the warming bed. The study initially included 9 mice in the control group and 10 mice in the PRSS3 knockdown group. One mouse in the nontarget control group failed to form a tumor and was excluded from all analyses.

**Bioluminescent imaging**

*In vivo* and *ex vivo* bioluminescent imaging was conducted using the IVIS Spectrum three-dimensional (3D) imaging system (Xenogen). Twice per week, all mice were injected intraperitoneally with 150 mg/kg potassium D-luciferin (Gold Biotechnologie) and anesthetized with 2.5% isoflurane. In the imaging chamber, mice remained sedated with isoflurane administered through nose cones. Up to 5 mice were imaged *in vivo* at a time with image exposures ranging from 1 second to 3 minutes. Regions of interest (ROI) were drawn around the primary tumor and quantified as photons/second, using Living Image software (Xenogen). For *ex vivo* imaging of tumors and organs harboring metastases, all mice were injected with 150 mg/kg potassium D-luciferin 5 minutes before euthanization by CO2 asphyxiation at 2 weeks postimplantation. Tissues (lungs, heart, liver, diaphragm, kidneys, spleen, and primary tumor) were excised and placed into 15 mm Petri dishes containing 300 μg/mL potassium D-luciferin in Dulbecco’s PBS. The lungs were imaged first (1–60 seconds exposure), followed by the liver, diaphragm, kidney, spleen (1–2 minutes exposures), and the primary tumor (0.5–1 seconds exposure). All 8 control group mice and 10 PRSS3 knockdown group mice were included in analyses of *in vivo* and *ex vivo* bioluminescence and of tumor weight.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded primary tumors and lungs were sectioned and stained with hematoxylin and eosin (H&E). Sections were also stained for human cytokeratins using monoclonal mouse anti-human cytokeratin clones AE1/AE3 (Dako) at 1:200 dilution and for human vimentin using antivimentin clone V9 (EMD Millipore) at 1:1,500 dilution.

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Invasion assays
replicates of the experiment, each containing 4 technical
threshold. Data shown represent the results of 4 biologic
number of colonies detected increased with decreasing
thresholds down to 3
shown; identical trends were observed using alternative
colony size threshold was set at 7

Anchorage-independent growth assays
Anchorage-independent growth was assayed by the ability
cells to form colonies in soft agar following a published
procedure (18). PC3-M control cells or PRSS3 knockdown
cells (2 × 10^5 cells/well) were plated in medium containing
10% FBS and 0.75% agarose in 6-well plates. Cells were
incubated at 37°C in 5% CO2 for 14 days, and then fixed
with 100% methanol, stained with Giemsa stain, and each
well was photographed at 960 × 960 pixel resolution.
Colonies were quantified using Image-Pro Plus 6.3 software
(Media Cybernetics), using automatic intensity range selec-
tion for dark objects on bright background. Minimum
(meanSEM) section through all lung lobes
was aspirated, and cells were overlaid with medium supple-
mented with 10% Matrigel as well as with 100 nmol/L
recombinant mesotrypsin, mesotrypsin-S195A, or cationic
trypsin where indicated. Cultures were maintained at 37°C
in 5% CO2 for up to 5 days, with the media/10% Matrigel
overlay (containing fresh protease as appropriate) replaced
on day 3. Recombinant proteases were expressed in bacteria,
purified to homogeneity, refolded, activated, and assayed as
previously described (21). Photographs shown are representa-
tive of 6 biologic replicates for PC3-M cells, 2 biologic
replicates for NB26 cells, and 4 biologic replicates for NB11
cells.

Invasion assays
PC3-M cells transduced with lentiviral shRNA constructs
were seeded at 1 × 10^6 cells per 10-cm dish the day before
assay set-up. BD Falcon 24-well cell culture inserts (8.0 µm)
were coated with 50 µg Matrigel basement membrane
matrix in 100 µL of serum-free RPMI and placed at 37°C
for 4 hours. Cells were rinsed with cold 1× PBS and lifted
with 0.05% trypsin, and then suspended in 1 mL of RPMI
containing 0.1% BSA and counted using a Countess auto-
mat ed cell counter (Invitrogen). In 24-well invasion cham-
bors, the bottom chambers contained 750 µL of NIH/3T3
cell conditioned serum-free medium (Dulbecco’s modified
Eagle’s medium supplemented with 50 µg/mL ascorbic acid)
as chemoattractant (19). Suspend ed cells (2 × 10^5 in 400 µL
media) were placed into the upper chambers, and then
allowed to invade for 18 hours at 37°C in 5% CO2. A
similar protocol was followed for invasion assays with NB26
cells, except that 2 × 10^5 cells were added to each chamber
and allowed to invade for 24 hours. In some experiments,
potential mesotrypsin inhibitor bovine pancreatic trypsin
inhibitor (BPTI)–K15R/R17G (0–100 nmol/L), prepared
and purified as previously described (17), was added to cell
suspensions in the upper chamber. Quadruple technical
replicates were set per treatment. Noninvading cells were
removed from the insert by scrubbing with a cotton swab,
and then cells on the lower surface of the filter were fixed with
100% methanol for 30 minutes at −20°C, stained with
0.1% crystal violet in 20 mmol/L MES, pH 6.0, for 1 hour at
room temperature, and air dried. Stained filters were photo-
graphed at ×2 magnification and cells were counted using
Image-Pro 6.3 software (Media Cybernetics). Consistent
results were obtained from 4 experiments using NB26 cells
and from more than 10 experiments using PC3-M cells with
slightly varying conditions and inhibitor concentrations.
Data shown represent 4 biologic replicates ± SEM.

3D culture assays
3D cultures in Matrigel were established using the “on-
top” protocol (20). Briefly, in 6-well plates, a base layer of
500 µL 100% Matrigel was polymerized, WPE-1 NB-11 or
NB-26 cells (4 × 10^5 cells/well) or PC3-M cells (5 × 10^4
cells/well) were seeded and allowed to attach, excess medium
was aspirated, and cells were overlaid with medium supple-
mented with 10% Matrigel as well as with 100 nmol/L
recombinant mesotrypsin, mesotrypsin-S195A, or cationic
trypsin where indicated. Cultures were maintained at 37°C
in 5% CO2 for up to 5 days, with the media/10% Matrigel
overlay (containing fresh protease as appropriate) replaced
on day 3. Recombinant proteases were expressed in bacteria,
purified to homogeneity, refolded, activated, and assayed as
previously described (21). Photographs shown are representa-
tive of 6 biologic replicates for PC3-M cells, 2 biologic
replicates for NB26 cells, and 4 biologic replicates for NB11
cells.

Statistical analysis
Data for association of PRSS3 expression and prostate
cancer progression were obtained from http://www.ncbi.
nlm.nih.gov/sites/GDSbrowser?acc=GDS1439 (probe
213421_s_at; ref. 22) and http://www.ncbi.nlm.nih.gov/
sites/GDSbrowser?acc=GDS2545 (probe 40043_at; ref. 23).
Data for PRSS3 expression in patients with prostate
cancer following prostatectomy (24) were obtained from
http://microarray-pubs.stanford.edu/prostateCA/images/
fig1data.txt and clinical information was obtained from
http://microarray-pubs.stanford.edu/prostateCA/images/
supptab2.pdf. Patients were divided for survival analysis into
groups of high PRSS3 expression (above median; n = 11)
and low PRSS3 expression (below median; n = 11). All
statistical analyses were conducted using Prism 4 (GraphPad
Software). Pairwise comparisons for gene expression levels,
soft agar colony formation, and transwell cellular invasion
assays were made using the unpaired t test. Pairwise com-
parisons of bioluminescence or tumor number between
mouse groups were made using the Mann–Whitney test.
Significance in the survival analysis was determined using the
log-rank test.

Results
Mesotrypsin gene PRSS3 is upregulated in advanced
prostate cancer and is prognostic of recurrence
While expression of the PRSS3 gene encoding mesotryps-
in is largely restricted to the pancreas and brain, PRSS3 is
transcriptionally upregulated with cancer progression in a
number of epithelial cancers, including lung, breast, and
www.aacrjournals.org Mol Cancer Res; 10(12) December 2012 1557

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pancreas (10, 13, 25). To explore a connection between PRSS3 expression and prostate cancer progression, we examined expression profiles in publicly available microarray data from previously reported clinical studies. We identified a correlation between PRSS3 expression and prostate cancer progression in a microarray dataset (22) in which benign prostate (n = 5), clinically localized prostate cancer (n = 5), and metastatic prostate cancer tissue specimens (n = 5) were transcriptionally profiled. We found a trend of increasing PRSS3 expression with progression, and significant upregulation of PRSS3 in metastatic tumors compared with benign and clinically localized tumors (Fig. 1A). We also analyzed a second transcriptional microarray study (23) comparing normal prostate tissue (n = 25), primary prostate tumors (n = 65), and castration-resistant metastatic samples (24 samples obtained from 4 patients). In this study, we found a similar trend of increasing PRSS3 transcription with progression and significant upregulation in metastases relative to normal tissue (Fig. 1B). Another open source microarray study reported clinical follow-up data for patients with prostate cancer following prostatectomy to remove localized prostate cancer following prostatectomy to remove localized study reported clinical follow-up data for patients with normal tissue (Fig. 1B). Another open source microarray study reported clinical follow-up data for patients with prostate cancer metastasis, and the evidence that PRSS3 expression in primary tumors is prognostic of recurrence, suggest that mesotrypsin may play a critical functional role in prostate cancer progression.

**PRSS3 silencing suppresses metastasis of prostate cancer cells in an orthotopic model**

To evaluate a potential functional role for PRSS3 in prostate cancer metastasis, we implemented an orthotopic implantation model of human prostate cancer cells in non-obese diabetic/severe combined immunodeficient NOD/SCID mice to faithfully replicate multiple stages of the metastatic cascade. Our model used PC3-M cells, a subline of PC-3 human prostate adenocarcinoma cells that were selected for enhanced metastatic potential (26). PC3-M cells were transduced with a lentiviral construct conferring expression of firefly luciferase that enabled bioluminescent detection of as few as 19 cells (Fig. 2A and B), allowing tumor progression to be monitored in real time using *in vivo* bioluminescence imaging (Fig. 2C). Similarly to previous studies in nude mice (27), we found that PC3-M cells implanted orthotopically in NOD/SCID mice formed rapidly growing tumors characterized by widespread metastasis. In preliminary studies to characterize the model, we observed metastases first in the lungs; 2 of 5 mice euthanized at 9 days postimplantation had pulmonary metastases detectable by *ex vivo* bioluminescence imaging at necropsy, and 9 of 9 mice euthanized at 14 days postimplantation had pulmonary metastases. In mice euthanized at 2 weeks or longer postimplantation, we have detected additional metastases to
liver, kidney, diaphragm, and spleen (Fig. 2D). We note that lung and liver are common sites of human prostate cancer metastases (2), although like most mouse models of prostate cancer metastasis (28), our model does not recapitulate metastasis to bone.

To measure the impact of PRSS3 silencing in this model, PC3-M cells were stably transduced either with a nontarget control virus (NT) or with a specific lentiviral shRNA targeting PRSS3 (KD), which effectively suppressed expression both at the transcript level (Fig. 3A) and at the protein level (Fig. 3B). Cells were superinfected with the firefly luciferase construct and then surgically implanted into the mouse prostate (3 × 10^5 cells per animal). In a study of 2-week duration, with 10 mice in the PRSS3 knockdown group and 8 in the control group, it seemed that PRSS3 knockdown had minimal impact on tumor growth, based on total in vivo bioluminescence (Fig. 3C). At the time of euthanasia, assessment of PRSS3 transcript levels by qRT-PCR in a sampling of the primary tumors confirmed that PRSS3 expression remained suppressed in tumors of the knockdown group for the duration of the study (Fig. 3D). Tumor burden from cells in which PRSS3 was silenced, was
reduced in comparison with NT control; although the difference did not reach significance as assessed by ex vivo bioluminescence (Fig. 3E), it was significant when assessed by weight of the resected prostate ($P = 0.0044$; Fig. 3F).

We observed a striking effect of PRSS3 silencing on suppression of metastasis; when metastatic tumor burden was assessed at necropsy, 2 weeks postimplantation, by ex vivo bioluminescence imaging of resected organs (Fig. 4A and B), all 8 control mice showed extensive pulmonary metastases, 2 had metastasis to liver, and 2 had metastasis to the spleen. In contrast, of the 10 mice bearing tumors in which PRSS3 expression had been suppressed, only 4 showed visible evidence of lung metastasis by bioluminescent imaging, and none showed evidence of metastasis to liver or spleen. When we assessed tumor burden in the lungs as total flux and compared the 2 groups with a Mann–Whitney test, the difference in mean number of lung metastases per section in the PRSS3 knockdown group (KD; $n = 8$) compared with the control group (NT; $n = 8$) was highly significant ($P = 0.0022$; Fig. 4E). We assessed PRSS3 expression in lung metastases of both groups, and found that PRSS3 remained suppressed in cells that did metastasize to lung in the KD group (Fig. 4F), suggesting that metastasis can still occur, although with reduced incidence, in the absence of mesotrypsin.

**Figure 4.** PRSS3 silencing inhibits prostate to lung metastasis in orthotopic xenografts. A, mice implanted with PC3-M cells in which PRSS3 was knocked down with viral shRNA (KD; $n = 10$) showed significantly reduced pulmonary metastasis at 2 weeks postimplantation, as assessed by ex vivo imaging at necropsy, relative to control mice (NT; $n = 8$; $P = 0.0021$). B, lungs from a mouse implanted with nontarget control virus-transduced PC3-M cells (NT) show flux measurement near the group median with extensive metastasis in all lobes (left), in contrast with lungs from a representative mouse implanted with PC3-M cells in which PRSS3 was knocked down with viral shRNA (KD), which show no evidence of metastasis (right). C and D, lung section from a mouse implanted with NT PC3-M cells (left) showed the presence of many small metastases such as that indicated by the black arrow in panels showing H&E staining (C) and immunohistochemical staining for human cytokeratins (D). Lung section from a mouse implanted with PRSS3 KD PC3-M cells (right) illustrates normal lung morphology (C) and background staining for human cytokeratins (D). E, histologic and immunohistochemical analyses found a significantly lower mean number of lung metastases per section in the PRSS3 knockdown group (KD; $n = 8$) compared with the control group (NT; $n = 7$; $P = 0.0022$). F, lung tissue bearing metastases from mice with PRSS3 KD tumors ($n = 5$) showed significantly lower expression of human PRSS3 when normalized to human GAPDH, relative to lung tissue from mice bearing NT control tumors ($n = 5$; bars, SEM; $^*$, $P = 0.043$).
role in the tumor-propagating phenotype of prostate cancer cells.

**PRSS3 silencing suppresses invasion in prostate cancer cells**

Another essential characteristic of prostate cancer cell metastasis is the capacity to invade through basement membrane and interstitial connective tissue (4). To experimentally probe the role of mesotrypsin in this phenotype, we identified a series of prostate-derived cell lines spanning a phenotypic spectrum from benign to aggressively invasive. The RWPE-1 methyl-nitrosourea (MNU) cell series shares a common genetic background and features progressive phenotypic changes that model prostate cancer progression from preneoplastic epithelial cells to prostatic intraepithelial neoplasia (PIN) to invasive prostatic carcinoma (29). These cell lines were derived from benign immortalized RWPE-1 human prostate epithelial cells by treatment with the chemical carcinogen MNU followed by selection for tumorigenicity in nude mice, and have been extensively characterized with respect to their karyotype, expression of cancer markers, and growth phenotypes in culture and in mice. They model a spectrum of phenotypes with regard to loss of cell polarity, loss of differentiation, and acquisition of invasive behavior, from NB14 (least progressed) through NB11 to NB26 (most progressed; ref. 29). We included in this comparison the PC3-M cell line as a representative of highly aggressive, androgen-independent metastatic prostate cancer (26). Using qRT-PCR to assess transcript levels of *PRSS3* in these cell lines, we found that nonmalignant RWPE-1 progenitor cells express very low levels of *PRSS3*, whereas the MNU-transformed cell lines show increasing levels of *PRSS3* expression correlating with their reported invasiveness and malignancy (Fig. 6A). The metastatic PC3-M cells expressed yet higher levels of *PRSS3* (Fig. 6A), consistent with the pattern seen in human tumor samples (Fig. 1A and B).

We evaluated the invasiveness of cells in this series using Matrigel transwell invasion assays, which confirmed previously reported phenotypes (29). NB14 cells were minimally invasive, NB11 cells were slightly more invasive, and NB26 cells, possessing higher levels of *PRSS3* expression, were the most invasive among the MNU cells series, although still far less invasive than PC3-M cells. Knockdown of *PRSS3* by stable transduction with a lentiviral shRNA construct led to a dramatically reduced invasion in the invasive cell lines PC3-M (Fig. 6B) and NB26 (Fig. 6C). We also assessed the impact of *PRSS3* knockdown on migration through uncoated transwell filters, finding significant differences albeit of lesser magnitude than the impact on invasion (Supplementary Fig. S1).

A novel inhibitor of mesotrypsin suppresses invasion of prostate cancer cells

Our results showing that *PRSS3* silencing inhibits metastasis in a mouse orthotopic model and anchorage-independent growth and invasiveness in cultured prostate cancer cells suggest that endogenous mesotrypsin activity may help drive invasion and metastasis, and therefore that this protease may offer a potential target for novel antimetastatic therapies. Mesotrypsin is notoriously resistant to inhibition and is unaffected by a number of drugs targeting other serine proteases; however, we have recently used structure-guided mutagenesis to modify the polypeptide trypsin inhibitor BPTI (aprotinin, Trasylol) to generate a novel mesotrypsin inhibitor (17). BPTI binds to the active site and blocks activity of most serine proteases of trypsin-like specificity, but deleterious interactions between BPTI and mesotrypsin residue Arg-193 greatly diminish binding affinity (21, 30).

We have incorporated 2 mutations into the binding loop of BPTI, a Lys-to-Arg mutation at residue 15, which strengthens a stabilizing salt bridge in the enzyme primary specificity pocket (30), and an Arg-to-Gly mutation at residue 17, which renders the inhibitor binding loop much more sterically complementary to the enzyme active site (Fig. 6D; ref. 17). In doing so, we have generated a prototype mesotrypsin inhibitor with 2,400-fold improved mesotrypsin affinity (4$	imes$ = 5.9 nmol/L; ref. 17).

In Matrigel transwell invasion assays, comparing PC3-M cells in which *PRSS3* has been silenced by shRNA with control cells and cells treated with varying concentrations of BPTI-K15R/R17G, we find that the inhibitor suppresses invasion in a dose-dependent fashion, in which 100 nmol/L inhibitor treatment is as equally effective as *PRSS3* knockdown (Fig. 6E). These results suggest that cell autonomous

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**Figure 5.** *PRSS3* silencing inhibits anchorage independent growth of prostate cancer cells. PC3-M cells transduced with shRNA virus targeting *PRSS3* (KD) or with a nontarget control virus (NT) were plated in soft agar and grown for 2 weeks. Cells in which *PRSS3* expression was suppressed showed significantly fewer colonies per well. Photographs of representative fields are shown above graphical results. *p < 0.0001; bars, SEM; n = 4 biologic replicates.
endogenous expression of mesotrypsin is a critical mechanism by which prostate cancer cells invade, and that targeting mesotrypsin activity may be a method to suppress prostate cancer invasion.

**PRSS3/mesotrypsin promotes an invasive morphology in prostate cancer cells**

To further explore the functional impact of PRSS3/mesotrypsin expression on prostate cancer cells in a physiologically relevant environment, we grew cells in 3D culture in Matrigel artificial basement membrane. To examine the impact of mesotrypsin proteolytic activity on cellular morphology, active purified recombinant mesotrypsin was added to the media of some cultures. PC3-M cells, which revealed a native invasive morphology characterized by spiky protrusions when grown in 3D (Fig. 7A), reverted to a less invasive morphology characterized by small-rounded cell clusters when PRSS3 was silenced using a lentiviral shRNA construct (Fig. 7B). The reciprocal phenomenon was observed in cells of the MNU series, which grow natively in 3D as a mixture of small amorphous cell clusters and spherical acini as previously described (31), shown for NB26 cells in Fig. 7C and for NB11 cells in Fig. 7E. Treatment with 100 nmol/L mesotrypsin stimulated a dramatically altered growth phenotype characterized by invasive protrusions and a web-like branching morphology (Fig. 7D and F). Similar effects of mesotrypsin were observed on the 3D growth phenotype of NB14 cells (not shown). Critically, we found that stimulation of invasive growth morphology was dependent upon the proteolytic activity of mesotrypsin as the catalytically inactive mesotrypsin-S195A mutant had no effect (Fig. 7G), and that the effect was highly specific to mesotrypsin as it was not recapitulated by the closely homologous human cationic trypsin at equivalent concentration (Fig. 7H). These observations further suggest a specific and critical function of mesotrypsin in prostate cancer invasion and metastasis, an activity that can potentially be targeted with therapeutic outcome.

**Discussion**

Here, we have found that the PRSS3 gene, encoding the serine protease mesotrypsin, is a gene that is upregulated in association with prostate cancer metastasis and poor outcome. Our studies using a mouse orthotopic model of metastatic human prostate cancer show that PRSS3 expression is a critical factor in prostate to lung metastasis in this...
metastasis is an important future direction. Evaluation of a potential role for PRSS3 in bone is particularly common and is a prominent cause of morbidity and mortality of patients with prostate cancer. Studies in additional model systems will be required to evaluate this possibility. Because metastasis to bone is particularly common and is a prominent cause of morbidity and mortality of patients with prostate cancer, evaluation of a potential role for PRSS3 in bone metastasis is an important future direction.

Our orthotopic model, in which extensive metastasis to lung is typically observed within a 2-week window, may be broadly useful for evaluation of genes important for metastasis generally and for metastasis to lung specifically. This model may also prove advantageous for preclinical evaluation of a variety of antimetastatic therapeutics for prostate cancer. An additional feature of our model is the use of tumor cells modified to express firefly luciferase, which enabled tracking of tumor growth in vivo and identification of sites of tumor metastasis ex vivo by bioluminescent imaging. The quantification from ex vivo imaging of primary tumors (Fig. 3E) and of organs containing metastases (Fig. 4B) was highly consistent with traditional methods of assessing tumor burden (Fig. 3F, 4C and D). The key advantage of this technology for our study was the ease of identifying sites of distant small metastases for further study, which otherwise would have required much more labor intensive microscopic histologic examination of all possible metastatic sites.

Our cell culture studies indicate that the role of PRSS3 in metastasis is pleiotropic; knockdown of PRSS3 in the aggressively metastatic PC3-M cell line compromised both the capacity for anchorage-independent growth, indicating a potential function in survival at metastatic sites, and the capacity for cellular invasion, suggestive of functions in breaching the integrity of basement membrane barriers, a function often aided by protease activity. The PRSS3 gene encodes the serine protease mesotrypsin, and we find that active recombinant mesotrypsin, but not an inactive mutant, can stimulate prostate cancer cell invasive morphology, suggesting that the effects of PRSS3 expression in metastasis are mediated through the proteolytic activity of mesotrypsin. That the invasive phenotype observed in our 3D culture model cannot be recapitulated by cationic trypsin suggests that we are not merely seeing an effect of indiscriminate matrix degradation, but rather a phenotype induced by cleavage of one or more specific substrates of mesotrypsin.

An obvious next question will be, what are those specific mesotrypsin substrates? In prior investigations seeking to identify biologic mesotrypsin substrates, we identified cleavage of the cell surface protein CD109 as a mechanism by which mesotrypsin promotes loss of basal polarity and increased proliferation in malignant breast cells (25), whereas in prostate cancer cells we have identified the amyloid precursor protein (APP) Kunitz protease inhibitor domain as a specific mesotrypsin substrate with unclear physiologic consequences (33). As candidate mesotrypsin substrates, we have individually silenced CD109 and APP in cells of the WPE-1 MNU series; knockdown of these genes neither sensitized cells to recombinant mesotrypsin treatment nor blocked responsiveness to mesotrypsin in 3D culture (A. Hockla and E.S. Radisky, unpublished data). This suggests that neither of these candidates is likely to be the sole mesotrypsin substrate responsible for mediating the invasive phenotype in prostate cancer cells, and that the immediate effector through which mesotrypsin drives prostate cancer progression has yet to be discovered.

The protease-activated receptors (PAR) represent another group of candidate mesotrypsin substrates. PAR signaling

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**Figure 7.** Endogenous and recombinant mesotrypsin stimulate invasive 3D growth morphology. A and B, PC3-M cells transduced with nontarget control virus (A) or with PRSS3-targeted shRNA virus (B) were grown in Matrigel in serum-free media for 3 days and then photographed. C and D, NB26 cells were grown in Matrigel and treated with (C) buffer only or (D) 100 nmol/L active recombinant human mesotrypsin for 5 days and then photographed. E–H, NB11 cells were grown in Matrigel and treated with (E) buffer only, (F) 100 nmol/L active recombinant human mesotrypsin, (G) 100 nmol/L catalytically inactive mesotrypsin-S195A mutant, or (H) 100 nmol/L active recombinant human cationic trypsin for 5 days and then photographed. Scale bar, 100 μm.
has been implicated in the proliferation, migration, invasion, and metastasis of a number of tumor types (34, 35). In prostate cancer, PAR-1, -2, and -4 are upregulated (36, 37) and have been shown to enhance proliferation (38) and to stimulate cell migration (39) and RhoA-induced cytoskeletal reorganization (40), processes important for invasion and metastasis. There have been conflicting reports of the ability of mesotrypsin to cleave and activate PAR-1, -2, and -4 on the surface of epithelial cells; 1 group has reported significant cleavage and downstream signaling, albeit with greatly reduced potency compared with other trypsins (11, 41), whereas another group has reported that mesotrypsin cannot activate epithelial PARs (42). Further study will be required to determine whether PAR signaling may be involved in promotion of prostate cancer cell invasion and metastasis by mesotrypsin.

Another mechanistic question brought up by our data concerns the regulation of PRSS3 itself; what drives increased expression of this gene in prostate cancers of increasing malignancy? The PRSS3 gene encodes several precursor splice isoforms of the serine protease mesotrypsin, initiated from 2 different promoters resulting in different first exons (43–45). The transcript encoding mesotrypsinogen, most abundant in normal pancreas, is transcribed from a promoter regulated by pancreatic transcription factor PTF1, whereas the transcripts encoding trypsinogens 4a, 4b, and 5, more commonly found outside the pancreas, are transcribed from an alternative promoter that is likely responsible for expression of PRSS3/mesotrypsin in tumors (43, 46). Little is known about the mechanisms of transcriptional regulation of the extrapancreatic PRSS3 isoforms, but in colon cancer cells, PRSS3 transcription seems to be upregulated in response to the oncogenic zinc finger transcription factor ZKSCAN3 (14). Another clue may be found in cell surface protein cytookeratin-associated protein in cancer (CAPC), also known as LRR2C6, a protein with tumor suppressor activities that is highly expressed in normal prostate cells (47). Overexpression of CAPC in MDA-MB-231 breast cancer cells suppressed tumor development and lung metastasis in mice; transcriptional profiling showed 10- to 20-fold reduced expression of PRSS3 along with several other poten
tially important genes in the CAPC-expressing cultured cells and tumors (47).

The suppression of prostate cancer metastasis through silencing of PRSS3, and the finding that pharmacologic inhibition of mesotrypsin can recapitulate the effect of PRSS3 knockdown on cellular invasion, suggests mesotrypsin as a potential target for antimetastatic therapies. The human proteome contains over 500 distinct proteolytic enzymes, and in fact proteases represent one of the largest classes of druggable targets (48–50). A caveat is that the protease inhibitors will probably require fine selectivity toward the right cancer-promoting proteases, and the ability to spare “antitarget” proteases involved in critical biologic processes or tumor suppressive functions, for efficacy as cancer therapeutics (9, 51). Examples of the dangers of poorly selective protease inhibitors are found in the cancer clinical trials of matrix metalloproteinase (MMP) inhibitors; these drugs inhibited too wide a swath of this large protease family, and in doing so, caused troubling side effects and failed to improve clinical outcome (49, 52, 53). Serine proteases also represent a large family, with more than 100 members; as with MMPs, identification and selective targeting of the right cancer promoting serine proteases will likely be essential, because these enzymes also have essential functions in the coagulation, fibrinolysis, and complement pathways. In addition, similar to the case with the MMP family, some serine proteases have been found to have tumor suppressing activities (54–56).

Toward development of potent and selective mesotrypsin inhibitors, we have undertaken structural and biochemical studies to define the binding specificity of mesotrypsin. The mesotrypsin active site possesses stere and electrostatic features unique among human serine proteases that modulate interactions with the “primed-side” residues (those residues located proximal to the cleavage site in the direction of the protein C-terminus) of a polypeptide substrate or inhibitor (21, 30, 57). These distinctive active site features are responsible for the resistance of mesotrypsin to many polypeptide trypsin inhibitors (58, 59), and are also likely responsible for the unique substrate specificity of meso
trypsin that we infer from the inability of cationic trypsin to substitute for mesotrypsin (Fig. 7). Significantly, these distinctive features may also provide an opportunity to develop new inhibitors, complementary in shape and charge to the mesotrypsin active site, which will selectively target mesotrypsin for therapeutic purposes. Our protot
type inhibitor, while capable of potently inhibiting mesotrypsin catalytic function and biologic activity, does not possess the selectivity that would ultimately be desirable in a therapeutic inhibitor (17); additional efforts to optimize selectivity are required. Finally, mesotrypsin inhibition as a therapeutic strategy would also require evaluation of the prevalence of mesotrypsin expression as a mechanism driving progression and metastasis in patients with prostate cancer, and identification of the subset of patients most likely to benefit from such molecularly targeted therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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