Androgen-induced TMPRSS2 activates matriptase and promotes extracellular matrix degradation, prostate cancer cell invasion, tumor growth and metastasis

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Abstract

Dysregulation of androgen signaling and pericellular proteolysis are necessary for prostate cancer (PCa) progression but the links between them are still obscure. In this study, we show how the membrane-anchored serine protease TMPRSS2 stimulates a proteolytic cascade that mediates androgen-induced PCa cell invasion, tumor growth and metastasis. We found that matriptase serves as a substrate for TMPRSS2 in mediating this pro-invasive action of androgens in PCa. Further, we determined that higher levels of TMPRSS2 expression correlates with higher levels of matriptase activation in PCa tissues. Lastly, we found that the ability of TMPRSS2 to promote PCa tumor growth and metastasis was associated with increased matriptase activation and enhanced degradation of extracellular matrix nidogen-1 and laminin β1 in tumor xenografts. In summary, our results establish that TMPRSS2 promotes the growth, invasion and metastasis of prostate cancer cells via matriptase activation and extracellular matrix disruption, with implications to target these two proteases as a strategy to treat prostate cancer.
Introduction

Prostate cancer (PCa) is the most frequent male malignancy and a leading cause of cancer-related death in many Western countries (1). In PCa, androgen signaling generally regulates the expression of genes associated with cancer cell growth and survival (2). Since androgen signaling has been shown to be involved in PCa development and progression (3), androgen deprivation therapy is an option for the patients. Initially, most PCa cells respond to this therapy, but eventually tumors relapse and take on a castration-therapy-resistant PCa phenotype that correlates with poor prognosis and high metastatic potential (4). Moreover, androgen signaling has been shown to be involved in PCa cell invasion (5,6), and strongly implicated in metastasis (3). However, the detailed molecular mechanisms through which androgen signaling can induce PCa cell invasion and metastasis still need more investigation.

Deregulation of pericellular proteolysis has been proposed to be involved in cancer progression because of its role in the degradation of the extracellular matrix and the alteration of the microenvironment (7). Recently, several lines of evidence have shown that dysregulation of some membrane-anchored serine proteases (MASPs) contribute to the progression of many human disorders including tumor growth, invasion and metastasis (8). Among them, matriptase has been focused on because it plays crucial roles in carcinogenesis and cancer cell invasion including PCa (9-12). In PCa cells, androgens can induce matriptase activation and shedding (13). However, the molecular mechanism through which androgens induce matriptase activation and whether the androgen-induced proteolytic cascade plays a role in PCa progression and metastasis are still unknown.
TMPRSS2 is a member of the MASP family (14) and predominantly expressed in prostate (15). Recent studies have indicated that a gene fusion of the 5′ untranslated region of TMPRSS2 to ETS transcription factors (ERG and ETV1) is often observed in PCa tissues (16). In addition, TMPRSS2 protein level has been shown to be correlated with PCa progression (17,18). However, the in vivo substrate(s) of TMPRSS2 and the TMPRSS2-initiated proteolytic cascade are unknown and the exact role of TMPRSS2 in PCa progression is still unclear. In this report, we addressed the functional role of TMPRSS2 in androgen-induced PCa cell invasion, tumor growth and metastasis, and identified the substrates of TMPRSS2 thus providing a mechanistic explanation of the role of TMPRSS2 in PCa progression.
Materials and Methods

Cell culture. LNCaP FGC, CWR22Rv1, PC-3, DU145, VCaP, HEK293A and CHO cells were originally obtained from American Type Culture Collection (Rockville, MD, USA) (19). C-81 LNCaP cells were a gift from Dr. Ming-Fong Lin (Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, USA) (20). Cells were tested and authenticated by genetic profiling using short tandem repeat (STR) analysis.

DHT treatment. Twenty-four hours after seeding, cells were then steroid-starved in a phenol red-free RPMI 1640 medium with 5% charcoal/dextran-stripped FBS (cFBS) for 24 h and serum-starved for another 24 h. Steroid- and serum-starved cells were treated with the indicated concentrations of DHT or an anti-androgen, bicalutamide (Casodex) for 18 h. Control cells were added with an equal amount of solvent EtOH.

Lentiviral particle preparation and infection. Small hairpin RNAs [shTM2-1 (TRCN0000000266) and shTM2-2 (TRCN0000000265)] for TMPRSS2 knockdown, and small hairpin RNAs [shMTX-1 (TRCN0000038052) and shMTX-2 (TRCN0000038053)] for matriptase knockdown were obtained from the National RNAi Core Facility of Academia Sinica, Taiwan. An shRNA against luciferase (shLuc) was used as a control. The production of lentiviral particle was described in the supplementary information. After infection for 24 h, the medium was refreshed and the infected cells were selected by 2 mg/mL puromycin.

Protein identification using in-gel digestion and LC/MS/MS. Equal volumes of the concentrated conditioned media under non-boiling and non-reducing conditions were resolved on 4-20% gradient SDS-PAGE. After silver staining, the target protein bands were cut and subjected to in-gel tryptic digestion. The detail procedure of In-gel
digestion was described in the supplementary information. Peptides were extracted with 50% acetonitrile and dried. The protein identity was revealed by LC/MS/MS (Thermo LTQ-Velos and ESI-QUAD-TOF).

**Cell migration, invasion and wound healing assays.** Cell migration and invasion assays were carried out as previously described (11). For invasion assay, 15 µg Matrigel diluted in 100 µL phenol red- and serum-free RPMI1640 medium were coated to the upper filter of each insert. No Matrigel was coated on the filter for migration assay. Cells were steroid-starved in a phenol red-free RPMI 1640 medium with 5% cFBS for 24 h and serum-starved for another 24 h. Steroid- and serum-starved cells were seeded at a density of $4 \times 10^5$ cells per 200 µl phenol red- and serum-free media into the upper well of each transwell, and the lower wells were added with 1 mL of phenol red-free RPMI1640 medium with 10% cFBS as chemoattractants. Wound healing assay was used as follows: A scratch was made using a micropipette tip and cells were washed to remove detached cells and debris. Photographs of the same area of the wound were taken at 0 and 48 h for measuring the closure of the wound after the treatment of the indicated concentrations of DHT. For the DHT treatment on transwell assays, the indicated concentrations of DHT or bicalutamide were added to both the upper and lower wells for 48-hour incubation.

**Cell proliferation assay.** Cell proliferation was measured by MTT assays according to the manufacturer’s instructions (Sigma, USA). Cells were seeded in triplicate in 96-well plates and media were refreshed every two days. For DHT treatment, steroid-starved cells were treated with the indicated concentrations of DHT or bicalutamide for the
various times. SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA) was used to measure the absorbance at 570 nm.

**Western blot.** Western blotting was carried out using a standard protocol as previously described (11). Samples for detection of matriptase (M24 and M69) and HAI-1 (M19) (a gift from Dr. Chen-Yong Lin, Georgetown University Medical Center, Washington DC, USA) were prepared under non-boiling and non-reducing conditions, because these antibodies mainly recognize native protein structures. M24 recognized total matriptase and activated matriptase/HAI-1 complex, and M69 specifically recognized the activated matriptase/HAI-1 complex. M19 recognized free HAI-1 and activated matriptase/HAI-1 complex (21). To analyze the shedding of matriptase and HAI-1, the conditioned media were collected and concentrated using Amicon Ultra-4 centrifuge filter devices (Millipore), and then analyzed by western blot. For other protein determination, samples were prepared under reducing and denaturing conditions. For the determination of the protease domain of matriptase and TMPRSS2, two polyclonal antibodies against their protease domains were obtained from Calbiochem (CA, USA) and Abcam (MA, USA), respectively. Other primary antibodies used in this study were listed in Table S2.

**Quantitative Real-Time PCR.** Quantitative real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo, IL, USA), and the expression levels of target genes were statistically calculated with normalization to GAPDH. The primers used in this study were listed in supplemental Table 1.

**Coimmunoprecipitation.** HEK293A cells were co-transfected with matriptase and TMPRSS2 plasmids in the presence of HAI-1 plasmids using lipofetamine 2000 (Invitrogen, CA, USA). After transfection, cells were cultured for 48 h and then lyzed in
RIPA buffer. Cell lysates were mixed with antibodies against flag or V5 tag and gently rotated at 4°C. Next day, protein A magnetic beads (GE, CT, USA) were added to the mixture for isolating the complexes by rotation for 1 h. After isolation, the protein-bead complexes were washed five times using 0.1 % Tween20 TBS. The precipitated proteins were boiled in 2x Laemmli sample buffer for 10 minutes and subjected to SDS-PAGE and western blot analysis.

**TMPRSS2 protease assay.** The production of recombinant TMPRSS2 and matriptase proteins were described in detail in supplementary information. The insoluble fractions of LNCaP cells were prepared as described in previous study (22). For *in vitro* protease activity and Matrigel degradation assays, recombinant TMPRSS2 proteins (25 ng) were incubated with 100 ng of recombinant matriptase proteins or insoluble fractions of LNCaP cells, or 10 μg Matrigel (BD Biosciences, MA, USA) for 1 h at 37°C in PBS. After electrophoresis, proteins in gel were stained with Coomassie Brilliant Blue G-250 or used for western blot analysis.

**Proteolytic activity assay.** TMPRSS2-activated matriptase proteolytic activities were examined by the measurement of a fluorescence resonance energy transfer (FRET) of a protease substrate (Boc-Gln-Ala-Arg-AMC, ENZO life science, NY, USA). Purified recombinant matriptase proteins (50 ng) were incubated with or without recombinant wild-type or S441A TMPRSS2 proteins (10 ng) for 1 h at 37°C in PBS. After the incubation, the substrate was added into each sample with a final concentration of 5 μM for the protease activity assay. The proteolytic enzymatic reaction was measured by using an ELISA reader (PARADIGM detection platform, Beckman Coulter, CA, USA)
under an excitation wavelength of 380 nm and an emission wavelength of 460 nm for
the indicated times.

**Tumor xenografts.** All procedures for animal experimental protocols were approved by
the institutional Animal Care and Use Committee (IACUC) of the College of Medicine,
National Taiwan University. For subcutaneous xenograft study, 6-week-old male nude
mice were inoculated subcutaneously into the dorsal flank with $10^6$ cells in 100 μl Opti-
medium plus 100 μl regular Matrigel (BD Biosciences, MA, USA). Tumor volume was
monitored weekly by Vernier caliper measurement of two perpendicular tumor
diameters (L and W) and then calculated using the formula: $LW^2/2$. For analysis of the
role of TMPRSS2 in the tumor growth in an androgen-ablated condition, male mice
received casodex at a dosage of 25 mg/kg intraperitoneally (i.p.) daily until scarification.
After eight (LNCaP TMPRSS2-knockdown group) or sixteen weeks (LNCaP TMPRSS2-
overexpression group), the mice were scarified and individual tumors were taken and
weighed. For analyzing metastasis, SCID mice were anesthetized by 1.2% of avertin
and then $2 \times 10^5$ of shLuc or shTM2 LNCaP cells were injected into the anterior prostate
lobes of each mouse, since intraprostatic injection of LNCaP cells in SCID mice has
shown to serve as a useful animal model to investigate mechanisms of metastasis (23).
Sixteen weeks after orthotopic injection, the mice were euthanized and the end-point
tumor masses were weighed and statistically calculated with mean±SEM. LNCaP cells
with the passage numbers below 40 were used for the experiments since the cells still
kept their androgen sensitivity. The passage numbers of the stable pools used for the
experiments were within 10 passages, when the first selection of the stable pools was
denoted as the first passage for the stable pools.
**Immunofluorescence Microscopy.** The tumor sections on slides were fixed in 4% paraformaldehyde, and permeabilized using 0.1% Triton-X100 in PBS. Samples were stained with primary antibodies TMPRSS2 (1:500, Abcam), M24 (1:250) and M69 (1:250), and then followed by secondary FITC or Alexa568-conjugated mouse or rabbit antibodies. Nuclei were counterstained with DAPI. Slides were examined and photographed using a Zeiss Axiophot fluorescence microscope.

**Immunohistochemical staining of prostate tissue sections.** Three human prostate tissue arrays were purchased from US Biomax (Ijamsville, MD). The details about tissue assays were described in the supplementary information. Immunohistochemical staining was carried out as previously described (24). The immunohistochemical images were scored by the percentage (P) of staining of tumour cells or prostate epithelial cells (0, <10%; 1, 10%-25%; 2, 25%-50%; 3, 50%-75%; 4, >75%) and by the intensity of staining (I) (0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining). The scoring results of each sample were the combination of P plus I values. To define protein expression levels, the scores of ≤3 were classified as belonging to the low-expression group and the scores above 3 were classified to the high-expression group. Chi-squared test was used to evaluate the correlation between activated matriptase and TMPRSS2 expression. A two-sided P value of less than 0.05 was considered statistically significant.

**Statistical analysis.** The intensities of images or bands were measured using ImageJ software, analyzed using GraphPad Prism 4.0 (GraphPad Software, CA, USA), expressed as mean±SEM and statistically calculated using ANOVA program. P values
of less than 0.05 were considered significant in all studies. * denotes a P value less than 0.05; ** denotes a P value less than 0.01; *** denotes a P value less than 0.001.
Results

Involvement of TMPRSS2 in androgen-induced PCa cell invasion- To analyze the effect of androgens on PCa cell motility, androgen-sensitive LNCaP cells were used for cell migration and invasion assays since the cells are the most representative model for studying androgen signaling in PCa cells (20). Fig. 1A&1B showed that dihydrotestosterone (DHT) induced the cell invasion and migration in a dose-response manner, reaching to the induction plateau by 10 nM DHT. Hereafter, the concentrations of 10 nM DHT were used for following experiments. Bicalutamide, an anti-androgen reagent, could block this DHT-induced PCa cell motility (Fig. 1C&S1A). To further identify which MASP was involved in androgen-induced PCa cell invasion, we first analyzed the effects of DHT on the expression of MASPs in LNCaP cells. The results showed that DHT significantly induced the expression of TMPRSS2 but not the other MASPs, and that the induction of TMPRSS2 was antagonized by bicalutamide; a similar induction phenomenon as seen in the well-known androgen-regulated gene, PSA (Fig. S1B&S1C). Moreover, DHT not only significantly induced the expression of TMPRSS2 protein with two molecular masses of 60 (full-length) and 38 kDa (N-terminus) in a dose-responsive manner (Fig. 1D), but also dramatically induced the expression of PSA protein and promote cell growth (Fig. S1D&S1E), indicating that the LNCaP cells still keep androgen sensitivity. We further explore the role of TMPRSS2 in androgen-induced PCa cell invasion and found that TMPRSS2 silencing significantly abrogated DHT-induced PCa cell invasion, returning it to basal levels (Fig. 1E, 1F&S1F), while this silencing did not significantly affect DHT-induced LNCaP cell proliferation within 48 hours after the treatment in comparison with shLuc LNCaP cells (Fig. S1G). These data
together indicate that TMPRSS2 plays an important role in androgen-induced invasion of PCa cells.

**Identification of TMPRSS2 substrates in PCa cells** - To identify putative TMPRSS2 substrate(s) in human PCa cells, we established a stable clone of doxycycline-inducible TMPRSS2-overexpressing LNCaP cells. Upon induction, the expression of exogenous TMPRSS2 proteins was dramatically increased (Fig. 2A). The conditioned media after the induction were collected for SDS-PAGE and gel staining. The intensities of the four main protein bands were increased in the conditioned media of TMPRSS2-overexpressing cells (Fig. 2B, #1~4). These four bands were cut out from the gel for in-gel protein digestion and LC/MS/MS analysis. Several membrane-anchored proteins were identified as listed in Fig. 2C and Table S3. Among them, MASP-matriptase caught our attention as a putative TMPRSS2 substrate owing to its high frequency of appearance and having the same membranous localization as TMPRSS2.

**TMPRSS2 induces matriptase activation** - To further examine whether TMPRSS2 could induce matriptase activation in PCa cells, we selected two stable clones of TMPRSS2-overexpressing LNCaP cells, and found that the overexpression of TMPRSS2 increased the activated levels of matriptase which formed a 120-kDa complex with HAI-1 and occurred along with reduced levels of latent matriptase (70 kDa) and free HAI-1 (50 kDa) (Fig. 3A) with no effect on matriptase and HAI-1 gene expression (Fig. S2A). The data indicate that TMPRSS2 can induce matriptase activation in PCa cells. We then used doxycycline-inducible TMPRSS2-expressing LNCaP cells to analyze the role of TMPRSS2 in matriptase activation. TMPRSS2 was expressed starting at 2 hours after induction and the activation of matriptase followed at
4 hours after induction (Fig. S2B). Together, these results indicate that TMPRSS2 can induce matriptase activation in LNCaP cells. Similar results for TMPRSS2-induced matriptase activation were also observed in CWR22Rv1, PC-3 and C-81 LNCaP cells (Fig. S2C), suggesting that TMPRSS2-induced matriptase activation is a common phenomenon in PCa cells. Overexpression of TMPRSS2 in CWR22Rv1 cells also could enhance the cell invasion and anchorage-independent growth (Fig. S2D&S2E), suggesting that TMPRSS2 plays roles in PCa cell invasion and tumor growth. Moreover, TMPRSS2 silencing significantly decreased the levels of activated matriptase (Fig. 3B). To avoid the off-target effects of shRNAs and to confirm the specific role of TMPRSS2 in matriptase activation, we constructed a TMPRSS2-overexpression plasmid with a resistance to shTM2-1 by generating a silent mutation on the shRNA-targeting site. The data showed that the overexpression of TMPRSS2 was able to rescue matriptase activation in the TMPRSS2-knockdown cells (Fig. S2F). Together, the data strongly indicate that TMPRSS2 plays a role in matriptase activation in human PCa cells.

Matriptase is a substrate of TMPRSS2- To investigate whether matriptase is a substrate of TMPRSS2, we first used pull-down assays to analyze if TMPRSS2 could form a complex with matriptase. The results showed that matriptase could be pulled down by wild-type TMPRSS2 with less affinity by S441A TMPRSS2, and TMPRSS2 could be brought out by matriptase (Fig. 3C). This suggests that TMPRSS2 can form a complex with matriptase. To further investigate the direct role of TMPRSS2 in the activation of matriptase, we purified recombinant TMPRSS2 proteins with the extracellular regions of wild-type or S441A TMPRSS2 for proteolytic assays. The results showed that recombinant wild-type TMPRSS2 proteins could undergo auto-activation
after purification, which was indicated by the release of its protease domain, while S441A TMPRSS2 mutant lost this autoproteolytic property (Fig. 3D, left panel). Then, the insoluble fraction of LNCaP cell lysate containing matriptase zymogen was mixed with these two recombinant TMPRSS2 proteins. Using a specific antibody against the matriptase protease domain, we found that recombinant wild-type TMPRSS2 but not its protease-null mutant could induce matriptase activation, indicated by the released matriptase’s protease domain (26 kDa) (Fig. 3D, right panel). To further analyze if TMPRSS2 could directly activate matriptase via proteolysis, we performed in vitro proteolytic assays, and found that wild-type recombinant TMPRSS2, but not its protease-null mutant could release the protease domain (26 kDa) of purified recombinant wild-type matriptase (Fig. 3E). Furthermore, the role of TMPRSS2 in activating the proteolytic activity of matriptase was determined using an in vitro proteolytic assay with a substrate Boc-Gln-Ala-Arg-AMC. The results showed that active TMPRSS2 robustly increased the proteolytic activity of recombinant matriptase, compared to matripase or TMPRSS2 alone (Fig. 3F). In addition, these two recombinant TMPRSS2 proteins were added into shLuc and shTM2 LNCaP cell cultures. The wild-type recombinant TMPRSS2, but not its protease-null mutant could enhance the activation and shedding of matriptase in shLuc LNCaP cells, and rescue matriptase activation and shedding in shTM2 LNCaP cells (Fig. S3A). Taken together, these data indicate that TMPRSS2 can activate its substrate matriptase via a direct proteolytic process in PCa cells.

**TMPRSS2 has an important role in DHT-induced matriptase activation in PCa cells** - To further explore whether TMPRSS2 is involved in androgen signaling to...
enhance matriptase activation in PCa cells, we used immunoblotting to analyze the DHT effect on the activated levels of matriptase in shLuc or shTM2 LNCaP cells. As shown in Fig. 4A and S3C, DHT dramatically induced the expression of TMPRSS2 and increased the activated levels of matriptase in shLuc LNCaP cells, while TMPRSS2 silencing reduced the induction effect of DHT on matriptase activation. Furthermore, the role of TMPRSS2 in androgen-induced matriptase activation was examined in VCaP cells because this cell line presumably exhibits TMPRSS2 knockout effect due to the gene fusion of TMPRSS promoter with ERG (16). Unexpectedly, the results showed that DHT could dramatically induce TMPRSS2 and ERG expression and reached the induction plateau by 1 nM DHT, and simultaneously promoted matriptase activation (Fig. 4B). The results for androgen-induced TMPRSS2 expression and matriptase activation in VCaP cells were very similar to the phenomena observed in LNCaP cells. Together these data indicate that TMPRSS2 functions as an androgen-induced protease that is important for the promotion of matriptase activation and PCa cell invasion.

**Role of matriptase in DHT-induced LNCaP cell invasion**- To further explore the role of matriptase in DHT-induced PCa cell invasion, we analyzed the effect of DHT on the invasion of matriptase-knockdown LNCaP cells. As shown in Fig. 4C, 4D&S3D, matriptase silencing reduced matriptase protein levels by approximately 80% and 30% in shMTX-1 and shMTX-2 LNCaP cells, leading to approximate 60% and 20% decrease in cell invasion. To further explore if matriptase was involved in androgen-induced PCa cell invasion and TMPRSS2 expression, we examined the DHT effect on the invasion of shMTX-1 LNCaP cells, and found matriptase knockdown significantly reduced DHT-induced PCa cell invasion (Fig. 4E), but did not interrupt DHT-induced TMPRSS2
expression (Fig. 4F). Together, the results indicate that matriptase is important for androgen-induced TMPRSS2 expression that promotes PCa cell invasion.

Identification of extracellular components as substrates of TMPRSS2- To further explore if TMPRSS2 can also proteolytically cleave some components of the extracellular matrix (ECM), the recombinant wild-type or S441A TMPRSS2 proteins (Fig. 5A) were incubated with matrigel. The result (Fig. 5B) showed that the 160-kDa band was absent after the wild-type TMPRSS2 reaction but not after its protease-null mutant. The 160-kDa band in the gel was cut out for LC/MS/MS analysis and laminin β1, laminin γ1 and nidogen-1 were identified as putative TMPRSS2’s substrates according to their molecular weights (Fig. 5C and Table S4). Immunoblot analysis (Fig. 5D) showed that wild-type TMPRSS2 but not its S441A mutant dramatically decreased the protein levels of nidogen-1 (right panel), partly degraded laminin β1 (left panel) and had no significant effect on laminin γ1 (middle panel). These results imply that TMPRSS2 can proteolytically degrade laminin β1 and nidogen-1 but not laminin γ1, leading to a loose ECM network. To clarify whether there was a role of ECM degradation in TMPRSS2-induced PCa cell invasion or growth, ECM was pre-incubated with recombinant wild-type TMPRSS2 (rTM2) or protease-null TMPRSS2 (rS441A) proteins and then used to coat on transwells for cell invasion assays or for cell proliferation assays. Since PC-3 cells expressed none or little TMPRSS2 (Fig. S3E), PC-3 cells were then selected to investigate the role of ECM degradation in TMPRSS2-elicited PCa cell invasion and proliferation. As shown in the Fig. 5E, PC3 cell invasion capability was significantly increased by approximately 1.7 folds in the transwells coated with rTM2-treated matrigel, compared to those in the transwells coated with ECM without any treatment (control) or
treated with protease-null rTM2 (S441A). Moreover, TMPRSS2-degraded ECM had no significant effect on PC3 cell proliferation (Fig. 5F) and matriptase activation (Fig. S3F). Thus, the results indicate that the degradation of extracellular matrix by TMPRSS2 plays important role in promoting PCa cell invasion but had no role in TMPRSS2-elicited cell proliferation. Together, the data suggest that in addition to direct induction of matriptase activation, TMPRSS2 also can degrade ECM components, laminin β1 and nidogen-1, contributing to PCa cell invasion.

**Examination of TMPRSS2 role in tumor growth in vivo**- To further investigate the role of TMPRSS2 in prostate tumor growth, shLuc and shTM2 LNCaP cells were subcutaneously injected into nude mice. Tumor growth in xenograft mice was monitored by measuring tumor volumes weekly and tumor weights at the end point. Tumor volumes derived from shTM2 cells were significantly smaller than those from shLuc cells (Fig. 6A), while the body weights of shTM2 mice were greater than those of shLuc mice (Fig. S4A). After scarification, the average of the tumor weights was significantly reduced in the shTM2 mice, compared to the control group (Fig. 6B). We further analyzed the expression of TMPRSS2 and matriptase in the xenograft tumors, and found that the expression level of TMPRSS2 and the activated level of matriptase were remarkably decreased in the whole lysates of shTM2 tumors (Fig. 6C). Immunofluorescence results further confirmed that a decreased level of TMPRSS2 was observed in shTM2 tumors and occurred in parallel with a reduced level of total and activated matriptase, compared to shLuc tumors (Fig. 6D and S4B). These results indicate that TMPRSS2 plays a role in PCa tumor growth and matriptase activation during the cancer progression. To further test whether increased TMPRSS2 expression
could promote androgen-independent tumor growth, LNCaP cells with overexpression of wild-type TMPRSS2 (TM2), its protease-null mutant (S441A), and control cells (PLKO) were subcutaneously injected into nude mice. To mimic PCa patients receiving androgen ablation therapy, mice were administrated daily with casodex, an anti-androgen reagent. As shown in Fig. 6E, the tumor volumes of TM2 cells grew more quickly than those of PLKO and S441A cells, while the body weights among these three groups showed no significant difference (Fig. S4C). The average of the tumor volumes was significantly increased in the TM2 group from week 13, compared to the other two groups (Fig. 6E). Interestingly, the average of S441A tumor weight was significantly reduced in comparison with the control group, suggesting that this TMPRSS2 mutant may exhibit a dominant negative function (Fig. 6F). We further found that the expression of TMPRSS2 and the activated level of matriptase were increased in TM2 but not S441A tumors (Fig. 6G). The immunofluorescence results further showed that both levels of TMPRSS2 and activated matriptase were increased in TM2 tumor tissues compared to PLKO tumors (Fig. 6H and S4D). Interestingly, the immunofluorescence images in S441A xenografted tumors showed that the higher expression of S441A TMPRSS2 correlated with a lower level of activated matriptase (Fig. 6H), suggesting that S441A TMPRSS2 mutant lacked the ability to proteolytically activate matriptase in tumor lesions, as shown in the in vitro proteolytic assays (Fig. 3E&3F). Ki67 IHC staining showed that the amounts of Ki67-positive cells were significantly increased in TM2 tumors (Fig. S4E). In the xenograft tumor lesions, TMPRSS2 significantly reduced the protein level of nidogen-1 and induced the degradation of laminin β1 (80-kDa fragment), while the S441A TMPRSS2 mutant did not act on both proteins (Fig. 6I&S4F).
The data together indicate that TMPRSS2 plays roles in the tumor growth of PCa and promotes androgen-independent cancer growth, which occur in parallel with increased levels of activated matriptase in the tumor lesions.

**Role of TMPRSS2 in prostate cancer metastasis**- To further evaluate the role of TMPRSS2 in PCa metastasis, shLuc and shTM2 LNCaP cells were orthotopically injected into the anterior prostates of SCID mice. After 16 weeks, 62.5% of orthotopic tumors (5/8) showed cancer metastases to adrenal glands in the shLuc group, compared to the shTM2 group [1/7(14.2%)] (Fig. 6J). The average of the orthotopic tumor weights was significantly reduced in the shTM2 group compared to the control group (Fig. S4G). To further analyze the expression of TMPRSS2 and matriptase in orthotopic and metastatic lesions, the IHC results (Fig. 6K) showed that compared to the primary tumors, increased levels of TMPRSS2 and activated matriptase were observed in metastatic tumor lesions of shLuc mice. The intensities of activated matriptase were reduced in parallel with TMPRSS2 reduction in the primary tumors of the shTM2 group compared to shLuc mice. The effect of TMPRSS2 knockdown on the proliferation of shTM2-1 LNCaP cells was further examined. The results (Fig. S4I) showed that TMPRSS2 silencing exhibited no significant effect on the cell proliferation in a regular culture condition, suggesting what TMPRSS2 knockdown cells exhibited low metastatic rate is apparently not due to the slower growth rate of shTM2-1 cells. The results together indicate that TMPRSS2 plays a role in PCa metastasis and occurs along with the activation of matriptase.

**Correlation of TMPRSS2 protein levels with the levels of activated matriptase in archival PCa specimens**- To evaluate the clinical relevance of TMPRSS2 expression...
and the status of matriptase activation, we used IHC to examine the levels of TMPRSS2 and activated matriptase in human PCa specimens. The protein levels of TMPRSS2 were positively correlated with the levels of activated matriptase in the PCa specimens (Fig. 6L&S5A). Moreover, the levels of TMPRSS2, total matriptase and activated matriptase were increased following cancer progression up to a moderate stage of PCa, but showed a reduced level in poorly differentiated tissues, similar to non-cancerous tissues (Fig. S5B). Moreover, the results (Fig. S5C) from the overall correlation analysis further showed the levels of TMPRSS2, total matriptase and activated matriptase were significantly increased in the cancerous group compared to the normal group. These findings suggest a strong correlation between the levels of TMPRSS2 and activated matriptase during PCa progression.
Discussion

Recent reports have indicated that androgen signaling can induce the invasive ability of PCa cells (5). However, the molecular mechanisms by which androgen signaling enhances PCa cell invasion, tumor growth and metastasis are still unclear. In this study we found that TMPRSS2 is an important androgen-regulated gene that can promote PCa tumor growth and metastasis, via matriptase activation and degradation of ECM laminin β1 and nidogin-1. Thus, TMPRSS2 is implicated in the acceleration of PCa progression.

Although androgen signaling has been strongly implicated in PCa cell invasion and metastatic potential (25), levels of the androgen receptor (AR) have a controversial role in PCa progression. Several studies have indicated that AR signaling plays a positive role in PCa cell invasion, at least via up-regulation of MMP-2/-9 activity and the PI3-kinase pathway (5,6). Conversely, restoration of AR expression in PC3 cells decreases cell invasion (26), tumorigenicity and metastasis (27) and also results in the suppression of cell proliferation by androgens (28). One possibility is that PC-3 cells are not a typical adenocarcinoma of prostate since PC3 cells have been shown with the neuroendocrine cell phenotype (29). However, the reasons why AR overexpression in PC3 cells reduces the malignant behavior of the cells still remain largely unknown. In this study, our data suggest that androgen signaling exhibits an induction effect on PCa cell invasion via up-regulation of its target gene TMPRSS2. In PCa, the expression level of TMPRSS2 has shown a significant increase in malignant cancer tissues compared to benign tissues (18,30), while TMPRSS2 expression level in metastatic cancer tissues is not significantly different from that in benign prostatic tissues (18). Our data also indicate
that the protein level of TMPRSS2 is significantly increased in moderately and intermediately differentiated PCa tissues (GS5-6&7) compared to normal prostate tissues. However, there is no statistical difference in TMPRSS2 expression between poorly differentiated tissues (GS8-10) and benign tissues. The results thus indicate that the expression of TMPRSS2 is correlated with PCa progression up to an aggressive but not a metastatic stage, and raised the question of whether TMPRSS2 plays a role in PCa tumor growth and metastasis. Our results from in vivo experiments further verify that TMPRSS2 has a positive role in the tumor growth and metastasis of PCa, and indicate that the elevation of TMPRSS2 in PCa cells also contributes to tumor growth under androgen-ablated conditions. Moreover, in the metastatic lesions of orthotopic xenograft mice, the levels of PSA (Fig S4H), TMPRSS2 proteins, and activated matriptase were much higher than those in their primary tumors. These data suggest that when PCa cells gain additional androgen signaling to induce TMPRSS2 expression and activated matriptase, those cancer cells are highly metastatic and move out their primary regions to other places. The remaining cancer cells in the primary regions of poorly differentiated PCa only exhibit normal or low androgen signaling that cannot further induce TMPRSS2 expression and matriptase activation. This may explain why the levels of TMPRSS2 expression and matriptase activation in the in situ poorly differentiated PCa tissues become no difference from those in normal prostate tissues. The data, therefore, suggest that TMPRSS2 has an oncogenic potential to promote PCa progression and metastasis, even in androgen-ablated conditions.

Tmprss2-/- TRAMP mice have shown with reduced metastasis but larger primary tumors, compared to Tmprss2+/+ TRAMP mice. However, knockdown of TMPRSS2 in LNCaP
C4-2B cells suppressed their cell growth (31). Our results (Fig. 6B&S1G) showed that TMPRSS2 silencing reduced PCa tumor growth and androgen-induced LNCaP cell growth. The discrepancy of the tumor growth between TRAMP and xenograft mouse models, and the role of TMPRSS2 in androgen-induced PCa cell growth may be due to the difference of PCa cell types, mouse neuroendocrine/poor differentiated tumors vs. human prostate adenocarcinoma. This is because the TRAMP mouse models have been shown to often develop neuroendocrine and poor differentiated tumors accompanied by diminished AR activity following the disease progression (32,33). Thus, our data indicate that in human prostate cancers, TMPRSS2 plays a positive role in androgen-induced PCa cell invasion, tumor growth and metastasis. In TRAMP model, TMPRSS2 mainly play a role in cell invasion and metastasis. The differential roles of TMPRSS2 in the tumor growth between the TRAMP and xenograft mouse models may be due to the differences of PCa cell types or TMPRSS2’s substrates, etc. and need more investigation.

In this report, we also identified matriptase as a substrate of TMPRSS2 in PCa cells and delineated a proteolytic cascade from TMPRSS2 to matriptase that mediates androgen-induced PCa cell invasion. Our results support that TMPRSS2 is a molecular linkage through which androgens can induce matriptase activation. Although the previous report indicated that matriptase activation undergoes an antoactivation process in vitro (22), this is the first report of matriptase activation by another membrane-anchored serine protease, TMPRSS2, in response to androgens. Moreover, recent reports have also shown that downregulated expression of two cognate inhibitors of matriptase (HAI-1 and HAI-2) is correlated with the progression of PCa and other cancers (34-37), and
occurs in parallel with matriptase activation and cancer cell invasion (24,38). Thus, reduced levels of HAI-1 or HAI-2 contribute to matriptase activation and cancer progression. In this study, we also observed that TMPRSS2 could promote HAI-1 shedding in PCa cells (Fig. S3B). HAI-1 has been shown to play an important role in the biosynthesis, intracellular trafficking, activation and inhibition of matriptase, with functions as a matriptase’s body guard to prevent inappropriate matriptase proteolytic activity during the protease synthesis and trafficking to plasma membrane, as well as a cognate inhibitor for matriptase inhibition after this protease activation (39). The proper balance between matriptase and HAI-1 is quite important for matriptase life cycle. The HAI-1 shedding induced by TMPRSS2 may lower down the inhibition function of HAI-1 on matriptase and concomitantly prolong the proteolytic activity of matriptase. The data together suggest that in addition to the direct role of TMPRSS2 in the proteolytic activation of matriptase, TMPRSS2 also simultaneously reduces cellular HAI-1 by shedding to further enhance matriptase proteolytic activity. Moreover, since HAI-1 can also inhibit other pericellular serine proteases including hepsin and prostasin (40), the shedding of HAI-1 by TMPRSS2 may also cause the activation of these pericellular proteases. Together, androgen-induced TMPRSS2 expression or its overexpression following PCa progression can activate matriptase and/or other HAI-1-modulated serine proteases leading to promoting PCa progression.

The microenvironment is important for tumor development and progression. The ECM in the microenvironment plays an important role in the maintenance of tissue homeostasis (41). Disruption of ECM integrity renders the microenvironment invasion-permissive and allows cancer cell growth and invasion (42,43). Cancer cells often secrete proteolytic
enzymes to degrade the ECM and invade the surrounding tissue (44). Our results indicate that up-regulation of TMPRSS2 can directly activate matriptase and degrade ECM components nidogen-1 and laminin β1, leading to PCa progression. Nidogen-1 functions as a key component for basement membrane assembly by connecting laminin and collagen networks and integrating other ECM components (45), and decreased expression of nidogen-1 can weaken the strength of the basement membrane and the interaction between cancer cells and the ECM, leading to the promotion of cancer cell invasion and metastasis (46). Moreover, matriptase activation has been shown to alter the microenvironment by degradation of fibronectin and laminin (47), or by activation of pro-HGF, PAR-2, uPA, and MMP-1/-3 (10), all contributing to tumor growth and metastasis. Thus, the data strongly suggest that dysregulation of the proteolytic cascade of TMPRSS2 to matriptase can result in ECM degradation and alteration of the tumor microenvironment in favor of cancer cell penetration and PCa progression.

In conclusion, our results suggest that the expression of TMPRSS2 induced by androgens can initiates a pericellular proteolytic cascade to activate matriptase that promotes PCa cell invasion. Moreover, TMPRSS2 can also promote PCa tumorigenicity and metastasis, which occurs along with degradation of ECM components, nidogen-1 and laminin β1. Thus, TMPRSS2 can mediate matriptase activation by androgens and disrupt the extracellular matrix network integrity, leading to PCa cell invasion, tumor growth, and metastasis (Fig. 7). We may explain why TMPRSS2 overexpression or increased matriptase activity following PCa progression is associated with a high metastatic potential and poor prognosis. These data further provide compelling evidence of a serine protease cascade regulated by androgen signaling to promote PCa
progression and metastasis. Hence, TMPRSS2 and matriptase may be valuable molecular targets for the development of novel therapeutic approaches to suppress PCa progression and metastasis.
Acknowledgments

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References


Figure Legends

Fig. 1. DHT induces LNCaP cell invasion and TMPRSS2 expression.  
A. Analysis of LNCaP (P33) cell invasion upon the treatment of different concentrations of DHT for 48 hours using matrigel-coated transwell assays. B. Analysis of LNCaP (P34) cell migration after the treatment of DHT (0, 1, 10, 50 nM) by wound-healing assays. Representative photographs (200x) were taken using a contrast-phase microscope at 0 and 48 h after the treatment (left panel). The wound closure was statistically quantified by measuring the empty area using ImageJ software (right panel). C. Analysis of LNCaP cell (P33) invasion upon the treatment of DHT and Bicalutamide at the indicated concentration. D. Immunoblot analysis of TMPRSS2 upon DHT or bicalutamide treatment at the indicated concentration, using an α-TMPRSS2 Ab (AL20). E. Immunoblot analysis of TMPRSS2 in shLuc, shTM2-1 and shTM2-2 LNCaP cells (P36) using AL20 Ab upon DHT treatment. F. Invasion assays of shLuc, shTM2-1 and shTM2-2 LNCaP cells upon DHT treatment. *, P < 0.05. **, P < 0.01. ***, P < 0.001.

Fig. 2. Identification of matriptase as a TMPRSS2 substrate.  
A. Western blot analysis of TMPRSS2 in doxycycline-induced TMPRSS2 LNCaP cells (P36). Cells were induced with 0 or 1 μg/ml doxycycline for 16 h and lysates were detected by western blot with an α-flag Ab. B. Silver staining of the conditioned media from doxycycline-induced TMPRSS2 LNCaP cells. Arrows indicate the increased signals under TMPRSS2 overexpression. C. Protein identity in #1~4 bands after LC/MS/MS analysis.

Fig. 3. TMPRSS2-induced matriptase activation.  
A. Western blot analysis of TMPRSS2, total matriptase, activated matriptase and HAI-1 using α-TMPRSS2 (AL20), α-total matriptase (M24), activated matriptase (M69) and HAI-1 (M19) Abs in two stable...
clones (TM2#1 and TM2#2) of TMPRSS2-overexpressing LNCaP cells (P38). B. Immunoblot analysis of TMPRSS2, total matriptase, activated matriptase and HAI-1 in TMPRSS2-knockdown LNCaP (shTM2-1 and shTM2-2) and control cells (shLuc) (P36). C. Interaction between TMPRSS2 and matriptase analyzed by co-immunoprecipitation and immunoblot analysis (matriptase with V5 tag and TMPRSS2 with flag tag). D. Proteolytic cleavage of matriptase by TMPRSS2 in insoluble fractions of LNCaP lysates. Purified rTM2 proteins were detected using an anti-His Ab. The matriptase levels in the insoluble fractions of LNCaP cells after the treatment of wild-type and S441A rTM2 were examined by an antibody (IM1014) against the protease domain of matriptase, indicated by the arrow (right panel). E. Direct proteolytic cleavage of TMPRSS2 on matriptase. Purified recombinant TMPRSS2 proteins were incubated with purified recombinant matriptase proteins at 37°C for 1 h. Samples were used for immunoblot analysis using an α-matriptase Ab. F. Analysis of TMPRSS2-induced matriptase proteolytic activity by monitoring the fluorescence signal of Boc-Gln-Ala-Arg-AMC substrate. Measurements were performed in triplicate and represent as means ± SEM. *, P < 0.05. **, P < 0.01. ***, P < 0.001.

**Fig. 4. Role of matriptase in DHT-induced PCa cell invasion.** A. Immunoblot analysis of the DHT effect on matriptase in shTM2-1 LNCaP cells (P36). B. Western blot analysis of TMPRSS2, ERG and matriptase upon DHT treatment in VCaP cells (P61). VCaP cells were treated with the indicated concentrations of DHT and then analyzed with the antibodies against TMPRSS2 (AL20), ERG, total matriptase (M24) and anti-activated matriptase (M69). β-actin was used as a control. C. Immunoblot analysis of matriptase in matriptase-knockdown LNCaP (shMTX-1 and shMTX-2) and
control cells (shLuc) (P37). **D.** Effect of matriptase silencing on the cell invasion. **E.** Analysis of matriptase role in DHT-induced cell invasion. ShLuc and shMTX-1 LNCaP cells (P37) were used for cell invasion assays upon DHT treatment. **F.** Immunoblot analysis of TMPRSS2 and matriptase upon DHT treatment in shLuc and shMTX-1 LNCaP cells (P37). ***, P < 0.001

**Fig. 5.** **TMPRSS2 cleaves laminin β1 and nidogen-1.** **A.** Western blot analysis of purified recombinant wild-type (WT) and S441A TMPRSS2 proteins using an α-His Ab. **B.** Coomassie blue staining of TMPRSS2-cleaved matrigel. Black arrows indicated TMPRSS2-cut proteins and were extracted for LC/MS/MS analysis. **C.** List of protein identities in the 160-kDa band of matrigel (indicated by arrow) after LC/MS/MS analysis. **D.** Immunoblot analysis of laminin β1, laminin γ1 and nidogen-1 in TMPRSS2-cleaved matrigel using α-laminin β1, α-laminin γ1 and α-nidogen-1 Abs. **E.** Effect of TMPRSS2-degraded extracellular matrix on PC3 cell invasion. 20 μg Matrigel was pre-incubated with PBS, 50 ng rTM2 or protease-null rTM2 (S441A) protein for 1 hour and then were coated into each transwell for cell invasion assays or used for cell proliferation analysis by MTT assays. **F.** For cell proliferation analysis, each set of PC3 cells was treated with or without 20 μg/ml of pre-treated matrigel for the indicated times. *, P < 0.05

**Fig. 6.** **TMPRSS2 promoted tumor growth and metastasis in xenograft models.**

**A.** Analysis of tumor growth using the measurement of tumor volumes. shLuc or shTM2 LNCaP cells (P39) were subcutaneously inoculated into the dorsal flank. Tumor volumes were measured weekly, plotted and statistically calculated as mean±SEM. n=6. **B.** Examination of tumor weights in the xenograft mice. After 8 weeks, tumor lesions were weighed, plotted and statistically calculated as mean±SEM. n=6. **C.** Immunoblot
analysis of TMPRSS2 and matriptase in the extracts of xenograft tumors using AL20, M24 and M69 mAbs. 

D. Immunofluorescence images of TMPRSS2 (red, upper panel) and activated matriptase (M69) (green, middle panel). Merged images are shown in the bottom panel. Scale bar, 20 μm. 

E. Analysis of TMPRSS2 role in androgen-independent tumor growth. PLKO (n=6), TMPRSS2-overexpressing (TM2, n=6) and TMPRSS2 S441A-overexpressing (S441A, n=4) LNCaP cells (P40) were inoculated subcutaneously into the dorsal flank. Mice were intraperitoneally administered 25 mg/kg casodex daily. Tumor volumes were measured weekly, plotted and statistically calculated as mean±SEM. 

F. After 16 weeks, tumor tissues were weighed, plotted and statistically calculated as mean±SEM. 

G. Immunoblot analysis of TMPRSS2 and matriptase in extracts of xenograft tumors using AL20 and M69 mAbs. 

H. Immunofluorescence images of TMPRSS2 (red, upper panel) and activated matriptase (M69) (green, middle panel). Merged images are shown in the bottom panel. 

I. Immunoblot analysis of nidogen-1 in extracts of xenograft tumors (PLKO, TMPRSS2 and S441A) using an α-nidogen-1 Ab. The levels of nidogen-1 were quantified by Image J software and statistically calculated from three mice each group with means±SEM. 

J. TMPRSS2 silencing reduced metastasis in an orthotopic xenograft model. shLuc (n=8) or shTM2 (n=7) LNCaP cells (P40) were introduced into the anterior prostates of SCID mice. After 16 weeks, tumor lesions from metastatic adrenal glands (metastatic lesion) are shown in the upper panel and the metastatic ratios were listed in the lower table. Arrows indicated metastatic PCa lesions in adrenal glands. 

K. Immunohistochemical analysis of TMPRSS2 (upper panel) and activated matriptase (M69, lower panel) in the primary or metastatic lesions in shLuc and shTM2 LNCaP xenograft mice using AL20
and M69 mAbs. Nuclei were counterstained with haematoxylin. L. Correlation of TMPRSS2 protein levels with the activated levels of matriptase in archival PCa specimens. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \).

**Figure 7. Schematic model for TMPRSS2-promoted PCa cell invasion, tumor growth and metastasis.** In PCa cells, androgens can induce TMPRSS2 expression via activating AR to bind the androgen response element (ARE). Androgen-induced TMPRSS2 initiates a pericellular proteolytic cascade to activate matriptase that promotes PCa cell invasion. Moreover, TMPRSS2 can also promote PCa tumorigenicity and metastasis, which occurs along with degradation of ECM components, nidogen-1 and laminin \( \beta 1 \). Thus, TMPRSS2 can mediate matriptase activation by androgens and disrupt the extracellular matrix network, leading to PCa cell invasion, tumor growth, and metastasis.
Figure 1

A

Number of invasive cells (per field)

DHT (nM) 0 1 10 50

* **

B

Wound closure (%)

DHT (nM) 0 1 10 50

* 

C

Number of invasive cells (per field)

DHT (nM) 0 10 10 0 10

Bica. (μM) 0 10 10 10 0

*** ***

D

TM2

N-terminus (38 kDa)

β-actin

E

shLuc shTM2-1 shTM2-2

DHT (kDa) 0 10 0 10 0 10

55

Full length (60 kDa)

TM2

N-terminus

β-actin

F

Number of invasive cells (per field)

DHT (nM) 0 10 0 10 0 10

shLuc shTM2-1 shTM2-2

*** *** ***
Figure 2

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The MS/MS spectra are searched using Mascot search engine against the NCBInr database.
Figure 3
Figure 4

A

B

C

D

E

F

Cell Invasion (ratio to shLuc)
Figure 5

**A**

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**B**

Colormagel

**C**

List of protein identifications in the 160-kDa band of matrigel after LC/MS/MS analysis

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The MS/MS spectra are searched using Mascot search engine against the NCBI nr database.

**D**

**E**

Coating matrigel with rTM2 or rS441A treatment

**F**

PC-3 cell proliferation (ratio to Day 0) vs. Day (s)
Figure 6

A. Tumor volume (mm³)

B. Tumor weight (mg)

C. Western blot analysis of shLuc and shTM2 groups.

D. Immunofluorescence images of TM2 and shTM2 groups.

E. Tumor volume (mm³) over time.

F. Tumor weight (mg) comparison across different groups.

G. Western blot analysis for different groups.

H. Immunofluorescence images for different groups.

I. Western blot analysis for nidogen expression.

J. Metastasis analysis for shLuc and shTM2 groups.

K. Primary and metastatic tissue samples for different groups.

L. TMPRSS2 and matrilysin expression in human prostate cancer tissues.

Table L:

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N=165

Significance of association was determined using a X² test.
**Figure 7**

- **ECM degradation**
  - nidogen-1, laminin β1

- **Cell Invasion**
  - Tumor Growth
  - Metastasis

- **Matriptase**
  - zymogen
  - Active matriptase

- **TMPRSS2**

- **mRNA**
  - nucleus

- **androgen**
  - HSP
  - AR

- **plasma membrane**

- **ARE**
  - TMPRSS2
Androgen-induced TMPRSS2 activates matriptase and promotes extracellular matrix degradation, prostate cancer cell invasion, tumor growth and metastasis


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