Androgen-Induced TMPRSS2 Activates Matriptase and Promotes Extracellular Matrix Degradation, Prostate Cancer Cell Invasion, Tumor Growth, and Metastasis

Chun-Jung Ko1, Cheng-Chung Huang1, Hsin-Ying Lin1, Chun-Pai Juan1, Shao-Wei Lan1, Hsin-Yi Shyu1,2, Shang-Ru Wu1, Pei-Wen Hsiao3, Hsiang-Po Huang1, Chia-Tung Shun5, and Ming-Shyue Lee1

Abstract

Dysregulation of androgen signaling and pericellular proteolysis is necessary for prostate cancer 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 prostate cancer cell invasion, tumor growth, and metastasis. We found that matriptase serves as a substrate for TMPRSS2 in mediating this proinvasive action of androgens in prostate cancer. Further, we determined that higher levels of TMPRSS2 expression correlate with higher levels of matriptase activation in prostate cancer tissues. Lastly, we found that the ability of TMPRSS2 to promote prostate cancer 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. Cancer Res; 75(14): 2949–60. ©2015 AACR.

Introduction

Prostate cancer is the most frequent male malignancy and a leading cause of cancer-related death in many Western countries (1). In prostate cancer, androgen signaling generally regulates the expression of genes associated with prostate cancer growth and survival (2). Because androgen signaling has been shown to be involved in prostate cancer development and progression (3), androgen deprivation therapy is an option for the patients. Initially, most prostate cancer cells respond to this therapy, but eventually tumors relapse and take on a castration therapy-resistant prostate cancer phenotype that correlates with poor prognosis and high metastatic potential (4). Moreover, androgen signaling has been shown to be involved in prostate cancer cell invasion (5, 6) and strongly implicated in metastasis (3). However, the detailed molecular mechanisms through which androgen signaling can induce prostate cancer 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 (ECM) and the alteration of the microenvironment (7). Recently, several lines of evidence have shown that dysregulation of some membrane-anchored serine proteases (MASP) contributes 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 prostate cancer (9–12). In prostate cancer 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 prostate cancer 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 E26 transformation-specific (ETS) transcription factors (ERG and ETV1) is often observed in prostate cancer tissues (16). In addition, TMPRSS2 protein level has been shown to be correlated with prostate cancer progression (17, 18). However, the in vivo substrates of TMPRSS2 and the TMPRSS2-initiated proteolytic cascade are unknown, and the exact role of TMPRSS2 in prostate cancer progression is still unclear. In this report, we addressed the functional role of TMPRSS2 in androgen-induced prostate cancer cell invasion, tumor growth, and metastasis and identified the
substrates of TMPRSS2, thus providing a mechanistic explanation of the role of TMPRSS2 in prostate cancer progression.

**Materials and Methods**

**Cell culture**

LNCaP FGC, CWR22Rv1, PC-3, DU145, VCaP, HEK293A, and CHO cells were originally obtained from the American Type Culture Collection (19). C-81 LNCaP cells were a gift from Dr. Ming-Fong Lin (Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE; ref. 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-striped FBS (cFBS) for 24 hours and serum-starved for another 24 hours. Steroid- and serum-starved cells were treated with the indicated concentrations of DHT or an androgen, bicalutamide (Casodex) for 18 hours. 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 Supplementary Information. After infection for 24 hours, the medium was refreshed and the infected cells were selected by 2 μg/mL puromycin.

**Protein identification using in-gel digestion and LC/MS/MS**

Equal volumes of the concentrated conditioned media under nonboiling and nonreducing conditions were resolved on 4% to 100% gradient SDS-PAGE. After staining, the target protein bands were cut and subjected to in-gel tryptic digestion. The detail procedure of in-gel digestion was described in Supplementary Information. Peptides were extracted with 50% acetonitrile and dried. The protein identify 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 was coated to the upper filter of each chamber. 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 hours and serum-starved for another 24 hours. Steroid- and serum-starved cells were seeded at a density of 4 × 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 chemotactants. 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 hours 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). Cells were seeded in triplicate in 96-well plates, and media were refreshed every 2 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) 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) were prepared under nonboiling and nonreducing 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 and Abcam, respectively. Other primary antibodies used in this study were listed in Supplementary Table S2.

**Quantitative real-time PCR**

Quantitative real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo), and the expression levels of target genes were statistically calculated with normalization to GAPDH. The primers used in this study were listed in Supplementary Table S1.

**Coimmunoprecipitation**

HEK293A cells were cotransfected with matriptase and TMPRSS2 plasmids in the presence of HAI-1 plasmids using lipofectamine 2000 (Invitrogen). After transfection, cells were cultured for 48 hours and then lysed 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) were added to the mixture for isolating the complexes by rotation for 1 hour. After isolation, the protein–bead complexes were washed 5 times using 0.1% Tween20 TBS. The precipitated proteins were boiled in 2× 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 was 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 matrix metalloproteinase or soluble fractions of LNCaP cells, or 10 μg Matrigel (BD Biosciences) for 1 hour 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 matrix metalloproteinase activities were examined by the measurement of a fluorescence resonance energy transfer of a protease substrate (Boc-Gln-Ala-Arg-AMC, ENZO Life Sciences). Purified recombinant matrix metalloproteinase (50 ng) were incubated with or without recombinant wild-type or S441A TMPRSS2 proteins (10 ng) for 1 hour at 37°C in PBS. After the incubation, the substrate was added into each sample with a final concentration of 5 μmol/L for the protease activity assay. The proteolytic enzymatic reaction was measured by using an ELISA reader (PARADIGM Detection Platform; Beckman Coulter) 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 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 10⁶ cells in 100 μL Opti-medium plus 100 μL regular Matrigel (BD Biosciences). Tumor volume was monitored weekly by Vernier caliper measurement of two perpendicular tumor diameters (L and W) and then calculated using the formula: LW²/2. 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 i.p. daily until sacrifice. After 8 (LNCaP TMPRSS2-knockdown group) or 16 weeks (LNCaP TMPRSS2-overexpression group), the mice were sacrificed and individual tumors were taken and weighed. For analyzing metastasis, SCID mice were anesthetized with two molecular masses of 60 (full-length) and 38 kDa (N-terminal) in a dose-responsive manner (Fig. 1D), but also dramatically induced the expression of TMPRSS2 in the induction plateau by 10 nmol/L DHT. Hereafter, the concentrations of 10 nmol/L DHT were used for following experiments. Bicalutamide, an antiandrogen reagent, could block this DHT-induced prostate cancer cell motility (Fig. 1C; Supplementary Fig. S1A). To further identify which MASP was involved in androgen-induced prostate cancer 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 (Supplementary Fig. S1B and 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 (Supplementary Fig. S1D and S1E), indicating that the LNCaP cells still keep androgen sensitivity. We further explore the role of TMPRSS2 in androgen-induced prostate cancer cell invasion and found that TMPRSS2 silencing significantly abrogated DHT-induced prostate cancer cell invasion, returning it to basal levels (Fig. 1E and F; Supplementary Fig. S1F), while this silencing did not significantly affect DHT-induced LNCaP proliferation within 48 hours after the treatment in comparison with shLuc

Immunohistochemical staining of prostate tissue sections

Three human prostate tissue arrays were purchased from US Biomax. The details about tissue assays were described in Supplementary Information. Immunohistochemical staining was carried out as previously described (24). The immunohistochemical images were scored by the percentage (P) of staining of tumor cells or prostate epithelial cells (0, <10%; 1, 10%–25%; 2, 25%–50%; 3, 50%–75%; 4, >75%) and by the intensity of staining (1, negative staining; 2, weak staining; 3, moderate staining; and 4, 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. The χ² test was used to evaluate the correlation between activated matrix metalloproteinase 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), 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 prostate cancer cell invasion

To analyze the effect of androgens on prostate cancer cell motility, androgen-sensitive LNCaP cells were used for cell migration and invasion assays because the cells are the most representative model for studying androgen signaling in prostate cancer cells (20). Figure 1A and B showed that DHT induced the cell invasion and migration in a dose-response manner, reaching to the induction plateau by 10 nmol/L DHT. Hereafter, the concentrations of 10 nmol/L DHT were used for following experiments. Bicalutamide, an antiandrogen reagent, could block this DHT-induced prostate cancer cell motility (Fig. 1C; Supplementary Fig. S1A). To further identify which MASP was involved in androgen-induced prostate cancer 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 (Supplementary Fig. S1B and 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 (Supplementary Fig. S1D and S1E), indicating that the LNCaP cells still keep androgen sensitivity. We further explore the role of TMPRSS2 in androgen-induced prostate cancer cell invasion and found that TMPRSS2 silencing significantly abrogated DHT-induced prostate cancer cell invasion, returning it to basal levels (Fig. 1E and F; Supplementary Fig. S1F), while this silencing did not significantly affect DHT-induced LNCaP cell proliferation within 48 hours after the treatment in comparison with shLuc
Identification of TMPRSS2 substrates in prostate cancer cells

To identify putative TMPRSS2 substrate(s) in human prostate cancer 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 to 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 Supplementary 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.

Identification of TMPRSS2 substrates in prostate cancer cells

To identify putative TMPRSS2 substrate(s) in human prostate cancer 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 to 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 Supplementary 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 prostate cancer 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 (Supplementary Fig. S2A). These data indicate that TMPRSS2 can induce matriptase activation in prostate cancer 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 (Supplementary 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 (Supplementary Fig. S2C), suggesting that TMPRSS2-induced matriptase activation is a common phenomenon in prostate cancer cells. Overexpression of TMPRSS2 in CWR22Rv1 cells also could enhance the cell invasion and anchorage-independent growth (Supplementary Fig. S2D and S2E), suggesting that TMPRSS2 plays roles in prostate cancer 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. These data showed that the overexpression of TMPRSS2 was able to rescue matriptase activation in the TMPRSS2-knockdown cells (Supplementary Fig. S2F). Together, these data strongly indicate that TMPRSS2 plays a role in matriptase activation in human prostate cancer cells.
**Figure 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 1μg/mL doxycycline for 16 hours, 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, increased signals under TMPRSS2 overexpression. C, protein identity in #1–4 bands after LC/MS/MS analysis.

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 autoactivation after purification, which was indicated by the release of its protease domain, whereas S441A TMPRSS2 mutant lost this autoproteolytic property (Fig. 3D, left). 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). 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-Ctn-Ala-Arg-AMC. The results showed that active TMPRSS2 robustly increased the proteolytic activity of recombinant matriptase, compared with matriptase 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 (Supplementary Fig. S3A). Taken together, these data indicate that TMPRSS2 can activate its substrate matriptase via a direct proteolytic process in prostate cancer cells.

TMPRSS2 has an important role in DHT-induced matriptase activation in prostate cancer cells

To further explore whether TMPRSS2 is involved in androgen signaling to enhance matriptase activation in prostate cancer 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 Supplementary Fig. S3C, DHT dramatically increased the expression of TMPRSS2 and increased the activated levels of matriptase in shLuc LNCaP cells, whereas 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 nmol/L 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 prostate cancer cell invasion.

Role of matriptase in DHT-induced LNCaP cell invasion

To further explore the role of matriptase in DHT-induced prostate cancer cell invasion, we analyzed the effect of DHT on the invasion of matriptase-knockdown LNCaP cells. As shown in Fig. 4C and D and Supplementary Fig. S3D, matriptase silencing reduced matriptase protein levels by approximately 80% and 30% in shMTX-1 and shMTX-2 LNCaP cells, leading to approximately 60% and 20% decrease in cell invasion. To further explore if matriptase was involved in androgen-induced prostate cancer cell invasion, we examined the DHT effect on the invasion of shMTX-1 LNCaP cells and found matriptase knockdown significantly reduced DHT-induced prostate cancer 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 prostate cancer cell invasion.

Identification of extracellular components as substrates of TMPRSS2

To further explore if TMPRSS2 can also proteolytically cleave some components of the 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 γ1, laminin γ1, and nidogen-1 were identified as putative TMPRSS2’s substrates according to their molecular weights (Fig. 5C; Supplementary 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), partly degraded laminin γ1 (left), and had no significant effect on laminin γ1 (middle). These results imply that TMPRSS2 can proteolytically degrade laminin γ1 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-elicited prostate cancer cell invasion or growth, ECM was preincubated with recombinant wild-type TMPRSS2 (rTM2) or protease-null TMPRSS2 (S441A) proteins and then used to coat on transwells for cell invasion assays or for cell proliferation assays. Because PC-3 cells expressed none or little TMPRSS2 (Supplementary Fig. S3E), PC-3 cells were then selected to investigate the role of ECM degradation in TMPRSS2-elicited prostate cancer cell invasion or growth.
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 with 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 (Supplementary Fig. S3F). Thus, the results indicate that the degradation of ECM by TMPRSS2 plays important role in promoting prostate cancer cell invasion but had no role in TMPRSS2-elicited cell proliferation. Together, these data suggest that in addition to direct induction of matriptase activation, TMPRSS2 also can degrade ECM components, laminin β1, and nidogen-1, contributing to prostate cancer 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), whereas the body weights of shTM2 mice were greater than those of shLuc mice (Supplementary Fig. S4A). After scarification, the average of the tumor weights was significantly reduced in the shTM2 mice, compared with 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 with shLuc tumors (Fig. 6D; Supplementary Fig. S4B). These results indicate that TMPRSS2 plays a role in prostate cancer 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 prostate cancer patients receiving androgen ablation therapy, mice were administrated daily with casodex, an antiandrogen reagent. As shown in Fig. 6E, the tumor volumes of TM2 cells grew more quickly than those of PLKO and S441A cells, whereas the body weights among these three groups showed no significant difference (Supplementary Fig. S4C). The average of the tumor volumes was significantly increased in the TM2 group from week 13, compared with 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 with PLKO tumors (Fig. 6H; Supplementary Fig. 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).
suggested that S441A TMPRSS2 mutant lacked the ability to proteolytically activate matriptase in tumor lesions, as shown in the in vitro proteolytic assays (Fig. 3E and F). Ki67 IHC staining showed that the amounts of Ki67-positive cells were significantly increased in TM2 tumors (Supplementary Fig. S4E). In the xenograft tumor lesions, TMPRSS2 significantly reduced the protein level of nidogen-1 and induced the degradation of laminin B1 (80-kDa fragment), whereas the S441A TMPRSS2 mutant did not act on both proteins (Fig. 6I; Supplementary Fig. S4F). These data together indicate that TMPRSS2 plays roles in the tumor growth of prostate cancer 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 prostate cancer metastasis, shLuc and shTM2 LNCaP cells were orthotopically injected into the anterior prostates of SCID mice. After 16 weeks, 62.5% of orthotopic tumors of shLuc mice and 26% of orthotopic tumors of shTM2 mice were signifi cantly increased in TM2 tumors (Supplementary Fig. S5C) from the overall correlation analysis further showed that the levels of TMPRSS2, total matriptase, and activated matriptase were significantly increased in the cancerous group compared with the normal group. These findings suggest a strong correlation between the levels of TMPRSS2 and activated matriptase during prostate cancer progression.

Discussion

Recent reports have indicated that androgen signaling can induce the invasive ability of prostate cancer cells (5). However, the molecular mechanisms by which androgen signaling enhances prostate cancer 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 prostate cancer tumor growth and metastasis, via matriptase activation and degradation of ECM laminin B1 and nidogen-1. Thus, TMPRSS2 is implicated in the acceleration of prostate cancer metastasis and occurs along with the activation of matriptase.

Correlation of TMPRSS2 protein levels with the levels of activated matriptase in archival prostate cancer 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 prostate cancer specimens. The protein levels of TMPRSS2 were positively correlated with the levels of activated matriptase in the prostate cancer specimens (Fig. 6L; Supplementary Fig. S5A). Moreover, the results (Supplementary Fig. SSC) from the overall correlation analysis further showed that the levels of TMPRSS2, total matriptase, and activated matriptase were significantly increased in the cancerous group compared with the normal group. These findings suggest a strong correlation between the levels of TMPRSS2 and activated matriptase during prostate cancer progression.
TMPRSS2 Proteolytic Cascade in Prostate Cancer

Figure 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, top) and activated matriptase (M69; green, middle). Merged images are shown in the bottom plot. 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 i.p. 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, top) and activated matriptase (M69; green, middle). Merged images are shown in the bottom plot. 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 mean ± 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 prostate of SCID mice. After 16 weeks, tumor lesions from metastatic adrenal glands (metastatic lesion) are shown in the top plot, and the metastatic ratios are listed in the lower table. Arrows, metastatic prostate cancer lesions in adrenal glands. K, immunohistochemical analysis of TMPRSS2 (top) and activated matriptase (M69, bottom) in the primary or metastatic lesions in shLuc and shTM2 LNCaP xenograft mice using AL20 and M69 mAbs. Nuclei were counterstained with hematoxylin. L, correlation of TMPRSS2 protein levels with the activated levels of matriptase in archival prostate cancer specimens. *P < 0.05; **P < 0.01; ***P < 0.001.

cancer progression. Several studies have indicated that AR signaling plays a positive role in prostate cancer cell invasion, at least via upregulation 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 prostate cancer cell invasion via upregulation of its target gene TMPRSS2. In prostate cancer, the expression level of TMPRSS2 has shown a significant increase in malignant cancer tissues compared with benign tissues (18, 30), whereas 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 moderately differentiated prostate cancer tissues (GS5-6/6) compared with 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 prostate cancer progression up to an aggressive but not a metastatic stage, and raised the question of whether TMPRSS2 plays a role in prostate cancer 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 prostate cancer, and indicate that the elevation of TMPRSS2 in prostate cancer cells also contributes to tumor growth under androgen-ablated conditions. Moreover, in the metastatic lesions of orthotopic xenograft mice, the levels of PSA (Supplementary Fig S4H), TMPRSS2 proteins, and activated matriptase were much higher than those in their primary tumors. These data suggest that when prostate cancer 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 prostate cancer 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 prostate cancer tissues become no difference from those in normal prostate tissues. These data, therefore, suggest that TMPRSS2 has an oncogenic potential to promote prostate cancer progression and metastasis, even in androgen-ablated conditions.

TMPRSS2 in prostate cancer cells and delineated a proteolytic cascade from TMPRSS2 to matriptase that mediates androgen-induced prostate cancer 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 autoactivation 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 prostate cancer 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 prostate cancer cells (Supplementary 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 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. These 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 prostate cancer progression can activate matriptase and/or other HAI-1–modulated serine proteases, leading to promoting prostate cancer 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 upregulation of TMPRSS2 can directly activate matriptase and degrade ECM components nidogen-1 and laminin β1, leading to prostate cancer 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 ECM 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, these 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 prostate cancer progression.

In conclusion, our results suggest that the expression of TMPRSS2 induced by androgens can initiate a pericellular proteolytic cascade to activate matriptase that promotes prostate cancer cell invasion. Moreover, TMPRSS2 can also promote prostate cancer 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 ECM network integrity, leading to prostate cancer cell invasion, tumor growth, and metastasis (Fig. 7). We may explain why TMPRSS2 overexpression or increased matriptase activity following prostate cancer 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 prostate cancer progression and metastasis. Hence, TMPRSS2 and matriptase may be valuable molecular targets for the development of novel therapeutic approaches to suppress prostate cancer progression and metastasis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: C.-J. Ko, M.-S. Lee

Development of methodology: C.-J. Ko

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-J. Ko, H.-Y. Shyu, C.-T. Shun, M.-S. Lee

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.-J. Ko, P.-W. Hsiao, H.-P. Huang, M.-S. Lee

Writing, review, and/or revision of the manuscript: C.-J. Ko, H.-P. Huang, M.-S. Lee

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.-J. Ko, C.-C. Huang, H.-Y. Lin, C.-P. Juan, S.-W. Lan, H.-Y. Shyu, S.-R. Wu, P.-W. Hsiao, C.-T. Shun, M.-S. Lee

Study supervision: C.-T. Shun, M.-S. Lee

**Acknowledgments**

The authors thank Dr. Ming-Fong Lin (Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center) for LNCaP cells and Dr. Chen-Yong Lin (Georgetown University) for his gifts of antibodies.

**Grant Support**

This study was supported by Taiwan National Science Council Grant NSC 100-2628-B-002-004-MY4, Ministry of Science and Technology grant MOST 103-2321-B-002-096, National Health Research Institutes grants NHRI-EX102-99098C and NHRI-EX104-10401BL, National Taiwan University grants NTU-FRG-98R0305 and NTU-CESRP-104R7602C4 (M.-S. Lee), and National Science Council grant NSC-99-1301-05-04-02 (H.-Y. Shyu).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 7, 2014; revised March 10, 2015; accepted April 15, 2015; published OnlineFirst May 27, 2015.

**References**


Androgen-Induced TMPRSS2 Activates Matriptase and Promotes Extracellular Matrix Degradation, Prostate Cancer Cell Invasion, Tumor Growth, and Metastasis


Cancer Res 2015;75:2949-2960. Published OnlineFirst May 27, 2015.