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碩士論文

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Master Thesis

第二型膜上絲胺酸蛋白酶 II 在攝護腺癌細胞扮演的角色

Role of TMPRSS2 in the progression of prostate cancer cells

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中文摘要

攝護腺癌是西方男性最常發生的癌症之一。在台灣，因為生活飲食習慣逐漸地西化，攝護腺癌有逐年上升的趨勢，其死亡率在癌症死亡中排名第七位。目前對於人類攝護腺癌在轉變成為較惡化或具高度轉移的分子機制，尚未十分明瞭。近年來，有相關報告提出細胞表層蛋白分解反應的失調被認為與癌症的侵襲及轉移有直接的相關性。

因此，在本篇研究中，我們想探討可受雄性荷爾蒙調控的嵌膜絲胺酸蛋白酶:第二型膜上絲胺酸蛋白酶 II (TMPRSS2)，在攝護腺癌細胞演進過程中所扮演的角色。首先我們將可表現此蛋白酶膜外區域的 DNA 片段建構在一個帶有組胺酸標記的哺乳動物分泌載體內，利用 HEK293T 細胞株進行蛋白表達並純化此蛋白做為抗原，以製作抗體。與林陳鏞博士合作下，我們成功地獲得一株可專一辨認第二型膜上絲胺酸蛋白酶 II 的單株抗體。在三株雄性荷爾蒙非依賴型細胞株 (DU145、PC-3 及 LNCaP C-81) 及一株雄性荷爾蒙依賴型細胞株 LNCaP C-33 中，第二型膜上絲胺酸蛋白酶 II 主要表現在荷爾蒙依賴型的細胞，其表現量會隨著細胞對雄性賀爾蒙非依賴的程度及高度侵襲能力的上升而下降。當使用核糖核酸干擾技術專一地將第二型膜上絲胺酸蛋白酶 II 的表現抑制時，會提升細胞生長的能力，但卻會使雄性荷爾蒙接收體以及攝護腺特異性抗原 (PSA) 的表現量減低。同時，我們也發現第二型膜上絲胺酸蛋白酶 II 對雄性荷爾蒙所調控的間質蛋白酶 (matriptase)

活化、攝護腺特異抗原的產生以及攝護腺癌細胞的生長，都扮演著重要的角色。另一方面，在攝護腺癌細胞演進的過程中，當第二型膜上絲胺酸蛋白酶 II 表現受到抑制時，會促使上皮細胞生長因子接受體表現上升，進而增強細胞對上皮細胞生長因子的敏感性，易於反應生長刺激。總歸上述，本篇研究結果顯示當減少或失去第二型膜上絲胺酸蛋白酶 II 的表現時，會促進細胞生長以及對上皮細胞生長因子的敏感度，進而在攝護腺癌細胞演進的過程中扮演一個重要的角色。

關鍵詞: 第二型膜上絲胺酸蛋白酶 II；攝護腺癌；雄性荷爾蒙接受體；上皮細胞生長因子接受體；間質蛋白酶；前列腺蛋白酶。

ABSTRACT

Prostate cancer is one of the most common cancers among men in the western countries. In Taiwan, the incidence of prostate cancer has been rising to the seventh leading cause of cancer-related death, partly due to westernized life style and diets. Human prostate cancer usually undergoes several alterations to progress to advanced stages with androgen-independence and/or high metastasis. The molecular mechanisms for the progression are still not well understood. Recently, it has been proposed that deregulation of cell surface proteolysis is strongly involved in cancer cell invasion and metastasis.

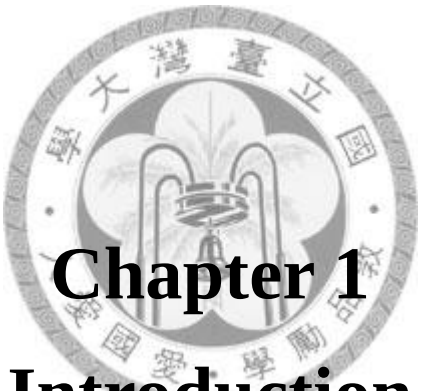
In this study, we are interested in addressing the role of an androgen-regulated, membrane-anchored serine protease TMPRSS2 in the progression of prostate cancer cells, since a decreased expression of TMPRSS2 was shown in advanced prostate cancer. To explore the role of TMPRSS2 in prostate cancer, a monoclonal antibody (AL-20) against the protease was successfully generated by constructing the cDNA fragment encoding the extracellular region of TMPRSS2 inserted into a mammalian secretory vector with a His tag and purifying the fusion proteins as an antigen. With three androgen-independent prostate cancer cells (DU145, PC-3 and LNCaP C-81 cells) and an androgen-sensitive LNCaP C-33 cell, our data showed that the protein levels of

TMPRSS2 were decreased in those cells with androgen-independence and highly invasive potentials. Moreover, a reduction of TMPRSS2 expression by shRNA approaches increased prostate cancer cell proliferation, but decreased the expression of androgen receptor (AR) and prostate-specific antigen (PSA). Furthermore, our data showed that TMPRSS2 was important for Dihydrotestosterone (DHT) -induced matriptase activation, PSA production and prostate cancer cell growth. On the other hand, down regulation of TMPRSS2 during the progression of prostate cancer cells decreased matriptase activation and increased the protein level of EGFR, leading to enhancement of EGF sensitivity for growth stimulation. Taken together, this study indicates that a decrease/loss of TMPRSS2 expression may play a role in the progression of prostate cancer cells, at least in part *via* increasing cell growth and EGF sensitivity.

Key words: TMPRSS2; prostate cancer; androgen receptor; EGFR; matriptase; prostasin.

CONTENTS

LIST OF FIGURES



Chapter 1
Introduction

1. Introduction

1.1 Prostate cancer.

Prostate cancer is the most frequent male malignancy in many western countries [1]. In Taiwan, prostate cancer becomes the seventh cancer lesion and its incidence arises annually [2]. Prostate cancer mainly derives from prostate epithelia with a progression from early to advanced stages. Early-stage prostate cancer requires androgens for survival and growth. During the progression of prostate cancer, cancers cells gradually lose their androgen requirement and become an androgen-independent phenotype [3]. Alternatively, androgen-independent prostate cancer cells (AIPC) also can come from hormone-refractory prostate cancer after androgen ablation therapy [4]. AIPC is a lethal form of prostate cancer that is malignant with poor prognosis [5]. Currently, there is no effective therapy for this type of prostate cancer. Therefore, to understand the molecular mechanisms that reduce androgen requirement for prostate cancer is an important issue and will provide more information for developing a novel therapeutic approach for the cancer.

1.2 Type II transmembrane serine protease.

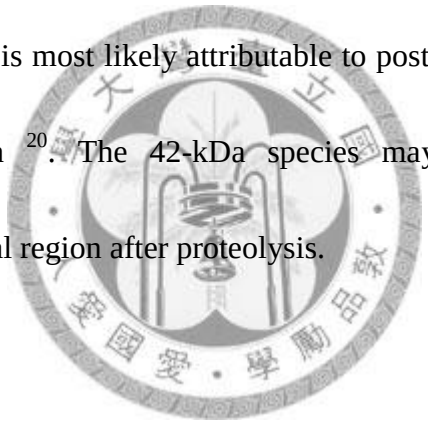
Type II transmembrane serine proteases (TTSPs) belong to a family of membrane anchored serine proteases. In the past few years, at least 17 members of this family were indentified from human and mouse genome sequences or EST databases [6, 7]. The TTSPs family has a common structure with a short cytoplasmic domain and transmembrane domain in the amino terminus and an extracellular serine protease domain in the carboxyl terminus. The transmembrane and protease domains are linked by a stem region that is composed of several structural domains and serves as regulatory and/or binding domains for the protease [7]. TTSPs have specific tissue distribution and been proposed to exhibit specific biological functions in tissues, with aberrant roles in diseases and tumorigenesis [8]. For example, matriptase has a physiological role in epithelial integrity and junction formation [9]. When matriptase is dysregulated, it will promote tumor formation and cancer cell invasion [10, 11]. However, although several TTSPs have been identified and reported recently [12, 13], there is little information to describe their physiological functions, proteolytic cascade and regulation.

1.3 Type II transmembrane protease, serine 2 (TMPRSS2)

TMPRSS2 has been reported as an androgen responsive gene that locates at human

chromosome 21 and is able to express a 3.8-kb *TMPRSS2* transcript [14]. TMPRSS2 protein has a calculated molecular mass of approximately 55-kDa, and contains a short cytoplasmic domain, a transmembrane domain, a stem region and protease domain. There are two structural domains in the stem region including a low density lipoprotein receptor class A (LDLRA) domain and a scavenger receptor (SR) domain [15]. TMPRSS2 has been reported to be highly expressed in prostate tissues and prostate cancer cells [16]. In addition, TMPRSS2 expression is also observed in pancreatic, stomach, colon tissues and related cancer [16-18]. Moreover, it has been reported that TMPRSS2 was down-regulated in androgen-independent prostate cancer xenograft tissue from a bone metastasis [16]. Thus, the expression level of TMPRSS2 has been suggested to be down-regulated in advanced prostate cancer cells [19, 20]. Recently, the studies pay more attentions on the TMPRSS2 gene fusion with several members of the ETS family. The gene fusions of TMPRSS2 with transcription factors ERG or ETV often occur during the progression of prostate cancer [21], and those fusions may promote the formation of neoplasms. For example, *TMPRSS2-ERG* can activate c-myc or abrogate prostate epithelial differentiation for tumor growth [22]. In contrast, a previous report showed that TMPRSS2 was correlated with high-grade cancers and its subcellular localization was mislocalized [19]. Thus, the exact role of TMPRSS2 that plays in cancer progression is

still unclear and needs to be clarified. However, the functional study of TMPRSS2 was few published, due to lacks of a useful antibody and cell models. Thus, in order to delineate the possible role of TMPRSS2 in prostate cancer, a monoclonal anti-TMPRSS2 antibody, AL-20, was successfully generated and useful to detect TMPRSS2 protein with two sizes of 58 and 42 kDa in western blots [20]. The 58-kDa TMPRSS2 appears to be the full-length form of the protein and this size is close to the calculated molecular mass of 53,859 Da. The difference between the calculated mass and actual molecular mass is most likely attributable to posttranslational modifications, especially N-glycosylation [20]. The 42-kDa species may be a cleaved fragment containing the NH_2-terminal region after proteolysis.



1.4 Matriptase

Matriptase is a member of the TTSPs family and mainly expressed in epithelia [8]. Matriptase is often co-expressed with its cognate inhibitor, hepatocyte growth factor activator inhibitor-1 (HAI-1). In transgenic mice, matriptase has been proposed as an oncogene to promote tumorigenesis and carcinogen-induced tumor formation [23]. In human cancers, dysregulation of matriptase in favor of proteolysis is correlated with histologic stages including ovarian, breast and prostate cancers, and related with cancer

cell invasion [24, 25]. Thus, matriptase has been implicated in carcinoma onset and malignant cancer progression. several substrates have been identified for matriptase, including pro-HGF [26], uPA, PAR2 [27] and prostasin [28]. Those substrates have been reported to be involved in the neoplastic progression. Matriptase-activated HGF can promote tumor cell motility through its receptor c-Met signal pathway [26]. Moreover, PAR-2 was reported to have a function in the stimulation of cell growth and motility [29, 30]. uPA protease cascade functions as a strong proteolytic system for extracellular matrix degradation. Prostasin, a GPI-linked serine protease, plays a role in processing the epidermal growth factor receptor (EGFR), and enhancing the activity of EGFR by proteolytic truncation [13]. Thus, dysregulation of matriptase can promote tumorigenesis and cancer progression *via* activating those substrates. In mammalian epithelial cells, sphingosin 1-phosphate and suramin can make matriptase relocate to form activation foci for the protease activation [31] [32]. In androgen-sensitive prostate cancer cells, androgens are *via* androgen receptor (AR) to regulate transcription and translation, leading to induction of matriptase activation [33]. However, the molecular mechanisms for sphingosine 1-phosphate, suramin or androgens to regulate matriptase activation are still not well understood.

1.5 Prostasin (PRSS8)

Prostasin is a glycosylphosphatidylinnositol (GPI)-anchored extracellular serine protease and ubiquitously expressed in epithelial tissues including bladder, colon, kidney, lung, prostate, breast, skin, and placenta [34]. It has been reported that prostasin plays an important role in epithelial physiology such as suppression of cell invasion [35, 36], regulation of the inflammation gene expression [37], and activation of the epithelial sodium channel ENaC by proteolytic cleavage on the gamma subunit of the channel [38]. A recent report showed that prostasin was able to be cleavaged and activated by matriptase [28], which can further proteolytically modify the extracellular domain of EGFR to generate two truncated fragments (EGFR135 and EGFR110) with molecular masses of 135 and 110-kDa [13]. The physiological function of matriptase/prostasin on EGFR processing is still unknown.

1.6 Epidermal growth factor receptor (EGFR)

EGFR is a receptor tyrosine kinase (RTK) and belongs to the ErbB family [39, 40]. EGFR plays key roles in many diverse cellular responses and also affect several tumor development and progression [41-43], due to its oncogenic effects on several signalings [44, 45]. In prostate cancer, it has been reported that EGFR over-expression may cause drug

resistance in a subset of advanced prostate cancer [46]. This is because EGF signaling can increase the AR transcriptional activity, by turning on the gene expression of AR coactivators such as TIF2/GRIP1 in a low androgen condition [47]. Thus, understanding the regulation of EGFR becomes important for delineating the role of EGFR signaling in hormone sensitivity and the cancer progression.

1.7 The purpose for this study

The progression of prostate cancer to an androgen-independent, metastatic stage is a big issue and the molecular mechanisms involved in this process remain largely unclear. Since our preliminary data showed that down regulation of androgen-regulated, membrane-anchored serine protease TMPRSS2 was correlated with the progression of prostate cancer cells, in this study, we would like to explore the role of TMPRSS2 in androgen sensitivity, proteolytic cascade and the progression of this cancer. Specific Aims were going to be addressed as follows: 1) To analyze the role of TMPRSS2 in prostate cancer cell growth and migration, 2) To examine the role of TMPRSS2 in androgen signaling for PSA production, AR transcription activity and protein stability, 3) To test the role of TMPRSS2 in a proteolytic cascade for androgen induction of matriptase activation, 4) To delineate the molecular mechanisms in which down

regulation of TMPRSS2 resulted in the progression of prostate cancer cells. The long-term goals for the study are going to understand more mechanisms for the prostate cancer progression and to provide more information to develop a useful therapeutic approach for the cancer lesion.

Chapter 2
Material & Methods

2.1 Materials

2.1.1 Cell lines

LNCaP C-33 and C-81 cells were a gift from Dr. Ming-Fong Lin, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, USA (Igawa et al., 2002). DU145, PC-3, and HEK293T cells were originally obtained from American Type Culture Collection.

2.1.2 Antibodies

(1) anti-AR Ab: sc-816, Santa Cruz, CA, USA

(2) anti-AKT 1/2/3 Ab: sc-8312, Santa Cruz, CA, USA

(3) anti-phospho-AKT1/2/3 (Ser473) Ab: sc-7985-R, Santa Cruz, CA, USA

(4) anti-β-actin Ab: AC-15, Sigma, MO, USA

(5) anti-ERK2 Ab: sc-153, Santa Cruz, CA, USA

(6) anti-EGFR Ab: sc-03, Santa Cruz, CA, USA

(7) anti-phospho-EGF receptor (Tyr1068) Ab: #3777, Cell Signaling, MA, USA

(8) anti-Matriptase Ab: monoclonal M32 (total matriptase) and M69 (activated matriptase), were a gift from Dr. Chen-Yong Lin, Greenebaum Comprehensive

Cancer Center, Deartment of Biochemistry and Molecular Biology, University of Maryland, Baltimore, MD 21201((Benaud et al., 2001)).

(9) anti-phospho-p44/p42 MAPK (Thr202/Tyr204) Ab: #4370, Cell Signaling, MA, USA

(10) anti-PSA Ab: sc-7316, sc-7638, Santa Cruz, CA, USA

(11) anti-TMPRSS2 Ab: monoclonal AL-20 were a gift from Dr. Chen-Yong Lin, Greenebaum Cancer Center, Deartment of Biochemistry and Molecular Biology, University of Maryland, Baltimore, MD 21201[20]

(12) anti-α-tubulin Ab: #2125, Cell Signaling, MA, USA

2.1.3 Enzymes

(1) SuperScriptTM Ⅲ Reverse Transcriptase: Invitrogen, CA, USA

(2) RNase: Invitrogen, CA, USA

2.1.4 Reagents

(1) DMEM medium: Gibco, CA, USA

(2) ECL (enhanced chemilumunescence): Millipore, USA

(3) FBS: Gibco, CA, USA

(4) Glutamine: Sigma-Aldrich, MO, USA

(5) Lipofectamine 2000: Invitrogen, CA, USA

(6) Matrigel: BD Biosciences, NJ, USA

(7) OPTI-MEM: Invitrogen, CA, USA

(8) Penicillin/Streptomycin: Gibco, CA, USA

(9) PBS: Gibco, CA, USA

(10) RPMI 1640 medium: Gibco, CA, USA

(11) Triton X-100: J.T.Baker, NJ, USA

(12) Trizol: Invitrogen, CA, USA

(13) Trypsin-EDTA (0.25%): Gibco, CA, USA

(14) Tween-20: Riedel-deHaeu, UK

2.1.5 Organic solvents

(1) Chloroform: Sigma-Aldrich, MO, USA

(2) Ethanol: Sigma-Aldrich, MO, USA

(3) Isopropanol: Sigma-Aldrich, MO, USA

(4) β-Mercaptoethanol: Sigma-Aldrich, MO, USA

(5) Methanol: Riedel-deHaeu, UK

(6) TEMED: J.T.Baker, NJ, USA

2.2. Methods

2.2.1 Cell Culture

Androgen-responsive human prostate carcinoma LNCaP cells was originally obtained from American Type Culture Collection (Rockville, MD, USA), which was isolated from lymph node metastasis [48]. The cells were maintained in a regular culture medium (RPMI 1640 medium with 5% FBS, 2 mM L-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin) in a 5% CO_2 incubator at 37^{o}C. LNCaP cells were subcultured once every 6 days and the medium was refreshed every other day. Two subpopulations of LNCaP cells, LNCaP C-33 and C-81 cells, were a gift from Dr. Ming-Fong Lin, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, USA [49]. LNCaP cells with passage number below 33 were designated as C-33 LNCaP cells, and those cells with passage number over 80 as C-81 LNCaP cells. LNCaP C-33 cells are androgen-sensitive while LNCaP C-81 cells become androgen-independent and highly tumorgenic [41, 49, 50]. These two LNCaP cells have been shown to be a useful tool for studying the progression of human prostate cancer cells.

For lentiviral production, HEK293T cells were cultured in DMEM medium with 5% FBS, 2 mM L-glutamine and 1% penicillin-streptomycin and incubated in a humidified,

5% CO_2 incubator at 37^{o}C. HEK293T cells were subcultured once every 4 days and the medium was refreshed every two days.

2.2.2 DHT treatment of cells

For DHT treatment, cells were grown in a regular RPMI 1640 medium for 48 h and then were steroid-starved in a phenol red-free RPMI 1640 medium with 3% charcoal/dextran-stripped fetal bovine serum (CFBS), 2 mM L-glutamine and 1% penicillin-streptomycin for 2 days. Starved cells were treated with or without 10 nM DHT in the presence or absence of an anti-androgen, bicalutamide (Casodex, 10 μM) for 24 h. Control cells were added with an equal amount of solvent ethanol during the treatment.

2.2.3 Transfection

One day before transfection, cells were seeded at a density of 1X10^{6} cells per 60-mm culture dish with 3 mL regular culture medium. Transfection reagents were prepared according to the commercial protocol (Invitrogen, USA). Ten μg of plasmids were diluted with 250 μL OPTI-MEM medium in a tube. Then, 15 μL Lipofectamine^{TM} 2000 were mixed gently with 250 μL OPTI-MEM in another tube. Both solutions were kept

in a lamina flow hood at RT for 5 min. Then, the solution with plasmid DNA was mixed well with the Lipofectamine^{TM} 2000 solution and incubated at RT for 30 min to generate DNA-liposome complexes. The mixtures were dropped slowly onto cell culture dish and then cells were incubated in a humidified, 5% CO_2 incubator at 37^{o}C for 4-5 h. After incubation, the medium was discarded and refilled with a regular cell culture medium.

2.2.4 Lentiviral particle preparation and infection

Small hairpin RNAs to knock down TMPRSS2 (shTMPRSS2, clone ID TRCN0000000265, TRCN0000000266) were constructed in a lentiviral pLKO.1-puro vector and obtained from the National RNAi Core Facility of Academia Sinica, Taiwan. A shRNA for knockdown of luciferase (shLuc) was used as a control for experiments. Lentivirus was produced from HEK293T cells by co-transfecting pCMVdR8.91, pMDG, and pLKO.1-puro shRNA plasmids into cells (9: 1: 10) using Lipofectamine^{TM} 2000 (DNA: liposome = 10 μg: 15 μL), according to the recommended protocol (Invitrogen, USA). Four hours after incubation with the transfection solution, the medium was refreshed with a regular culture medium contain 1% BSA. The conditioned media of the transfected cells containing lentiviral particles were collected at 24 h and 48 h after

medium refreshment. For infection, LNCaP cells were seeded with a density of 1 X 10^6 cells per 60-mm dish and incubated for 24 h up to 60-70% confluence. Lentiviral infection was performed by adding 50% (v/v) of lentivirus-containing medium in a regular culture medium for 24 h, and the infected cells were selected by 2 mg/mL puromycin for a week. After selection, cells were grown in a regular RPMI 1640 medium and subcultured every 6 days.

2.2.5 RT-PCR

Total RNA isolated from cells was prepared with the TRIZOL reagent (Invitrogen, USA). The reaction of reverse transcription was performed by SuperScript™ III Reverse Transcriptase. The procedures were carried out according to the manufacturer's instructions (Invitrogen, USA).

RNA extraction:

Cells cultured in a 60-mm dish were lysed with 1 mL of Trizol reagent at RT for 5 min. 0.2 mL of chloroform was used to extract RNA into an aqueous phase and the aqueous solution was taken out into a new tube after centrifugation (12,500 rpm, 4°C, 5 min). RNA was precipitated by mixing 0.5 mL isopropanol with the aqueous solution. After centrifugation at 12,500 rpm for 10 min at 4°C, the supernatant was discarded and RNA

pellets were washed with 1 mL of 75% ethanol and then briefly air-dried at RT for 5-10 min.

Reverse transcription:

Total RNA (5 μg) was reversely transcribed using 50 μM of oligo $(dT)_{15}$, 10 mM dNTP mixture, 0.1 M dithiothreitol (DTT), First-Strand Buffer and 200U of SuperScrip^{TM} III Reverse Transcriptase at 50^{o}C for 50 min. RNase was then added to remove excess RNA.

PCR:

PCR was performed in a thermocycler (PC320, Astec, Japan). The primers used for PCR were listed as follows:

name	primers	size
Slug	5'-GCCTCCAAAAAGCCAAACTAC-3' 5'-GTGTGCTACACAGAAGCC-3'	767 bp
PSA	5'-TTGTCTTCCTCACCCTGTCC-3' 5'-TCACGCTTTTGTTCCTGATG-3'	203 bp
GAPDH	5'-AAAGGATCCACTGGCGTCTTCACCACC-3' 5'-GAATTCGTCATGGATGACCTTGGCCAG-3'	200 bp

The PCR program was briefly described. After an initial denaturation step at 94^{o}C for 1 min, DNA fragment amplification was performed in 23 cycles including 30 sec denaturation at 94^{o}C, 30 sec primer annealing at 51^{o}C and 40 sec DNA extension at

72°C. A final cycle of DNA elongation was performed at 72°C for 3 min to complete the reaction. PCR products were separated by 1% agarose gel electrophoresis, stained by ethidium bromide and visualized by a UVP gel imager (GDS-7900 system, USA).

2.2.6 Immunoblotting

After washed with cold PBS twice, cells were harvested and lyzed in an ice-cold lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100, 10% glycerol, 1 mM PMSF.) with a fresh protease inhibitor cocktail (Roche) on ice for 10 min. Cells were then scraped with a rubber scraper and the lysate was taken out into an eppendorf. The lysate was centrifugated at 13,000 rpm for 20 min at 4°C and the supernatant was collected. The protein concentration was determined using BioRad Protein Assay. Samples were prepared by mixing cell lysates with an SDS loading buffer (10 mM Tris, pH 6.8, 2% SDS, 5% glycerol, 0.01% bromophenol blue, 0.35 M β-mercaptoenthanol) with boiling for 15 min. Sixty μg of each sample were loaded into a well on a 8%, 10% or 12% SDS-PAGE gel. After electrophoresis, the proteins in gel were transferred to a nitrocellulose membrane with Towbin transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol). The membrance was blocked in 5% skim

milk in Tris-buffered saline Tween-20 (TBST: 50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween 20) for 1 h at RT, and incubated overnight with an appropriate primary antibody at 4 ^{o}C in 5% skim milk TBST. After incubation with a primary antibody, the membrane was washed three times with TBST (10 min each time), incubated with a 1:10,000 diluted secondary antibody conjugated with a horse radish peroxidase at RT for 1 h and then washed three times with TBST. Proteins were visualized with an ECL detection kit, and detected by a luminescent image analyzer with a cool CCD camera (LAS-4000, Fujifilm, Japan). Equivalent protein loading in each well was confirmed by loading controls using antibodies to detect β-actin (1:2000) or α-tubulin (1:2,000).

2.2.7 Cell growth assay

Cells were plated at a density of 3 X 10^4 cells per well with 500 μL regular medium in a 24-well plate. After 48 h, the medium was refed with a steroid-free medium. After 48-h steroid starvation, cells were refreshed with a medium and/or received treatments as indicated in legends. Cell growth was measured at each time point using MTT assay. 0.5 mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, USA) in a serum-free medium were added to each well and incubated with cells for 3 h. After the medium was removed, the formazan dye trapped in cells

was dissolved in Dimethyl sulfoxide (DMSO) and the absorbance at 540 nm for the DMSO-extracted solution was measured in a spectrophotometer.

2.2.8 Migration assay/Invasion assay

Cells were seeded at a density of 1 X 10^6 cells per 60-mm dishes for 48 h and starved with phenol red- and serum-free RPMI 1640 medium for 36 h. After trypsinization, 3 X 10^5 cells were seeded with serum-free RPMI 1640 medium with or without phenol red in the upper well of Boyden chambers, and the lower chambers were added with 5% FBS RPMI 1640 medium or 5% charcoal/dextran-stripped FBS RPMI 1640 medium without phenol red as chemoattractants. For invasion assay, 1 μL of Matrigel (10 μg/μL) diluted in 100 μL serum-free cell culture medium was added on the top of each filter and air-dried overnight before seeding. After incubation for 48 h, the cells penetrating to the lower surfaces of the filters were fixed in methanol for 10 min and stained with giemsa dye (Sigma-Aldrich, USA) for 30 min. After washing with 1X PBS, the non-penetrating cells on the upper surfaces of chambers were wiped off by cotton swabs. The penetrating cells were photographed by a light microscope (100X), counted and statistically calculated.

Chapter 3
Results

3. Results

The mechanisms of prostate cancer progression from hormone dependence to independence remain unclear. The aim of the present study was to investigate the role of androgen-regulated, membrane-anchored serine protease TMPRSS2 in prostate cancer progression by clarifying its involvement in androgen sensitivity and the molecular mechanisms for the protease to play in the cancer progression.

3.1 Generation of a TMPRSS2 monoclonal antibody and examination of TMPRSS2 expression in human prostate cancer cells.

In order to explore the role of TMPRSS2 in prostate cancer, we first generated an antibody against this protease, by constructing the cDNA fragment encoding the extracellular region of TMPRSS2 into a mammalian secretory vector with a His tag in the carboxyl terminus and a Flag tag in the amino terminus of the fusion protein, and purifying the fusion proteins as an antigen. A useful monoclonal antibody (AL-20) was successfully obtained for TMPRSS2 detection [20]. We utilized this antibody to detect the protein levels of TMPRSS2 in three most commonly used prostate cancer cell lines, DU145 [51], LNCaP [52] and PC-3 cells [53]. DU145 and PC-3 cells are androgen-independent (AR-negative) cells while LNCaP cells are androgen-sensitive (AR-positive) cells. As

shown in Fig. 1, the data showed that the level of TMPRSS2 protein was higher in LNCaP cells than that in PC-3 cells, with no detection in DU145 cells. Thus, TMPRSS2 expression was decreased in androgen-independence prostate cancer cells and inversely correlated with androgen sensitivity. The monoclonal anti-TMPRSS2 antibody was going to be a useful tool for the following study.

3.2 Cell migration and invasion of different human prostate cancer LNCaP cells and the expression levels of TMPRSS2 in these two cells.

To further explore the role of TMPRSS2 in the progression of human prostate cancer, a progression model of human prostate cancer cells including LNCaP C-33 and C-81 cells were used. LNCaP C-33 cells are androgen-sensitive while C-81 cells represent a subgroup of hormone-refractory cancer cells with high tumorgenicity [49]. We further characterized the migratory and invasive capabilities of the two cells by migration and invasion assays. In a regular culture medium (FBS) or charcoal-stripped FBS (CFBS) condition, the migration and invasion potentials were increased in androgen-independent, highly tumorigenic LNCaP C-81 cells, compared to androgen-sensitive LNCaP C-33 cells (Figs. 2A & 2B). With AL-20 antibody, the immunoblot results showed that the protein levels of TMPRSS2 were reduced in androgen-independent

LNCaP C-81 cells, compared to androgen-sensitive LNCaP C-33 cells (Fig. 2C). To further analyze the transcriptional activity of androgen receptor (AR) in these two cells, we examined the gene expression level of prostate-specific antigen (PSA), a well-known AR-regulated gene, by RT-PCR. The mRNA levels of PSA were significantly reduced in LNCaP C-81 cells (Fig. 2D), suggesting that the transcription activity of AR was decreased in LNCaP C-81 cells and may serve as one of the factors to reduce the level of TMPRSS2 in LNCaP C-81 cells. The results taken together indicated that the expression level of TMPRSS2 was down-regulated during the progression of prostate cancer cells and inversely correlated with the abilities of prostate cancer cell proliferation, tumorigenesis, migration and invasion.

3.3 Role of TMPRSS2 in prostate cancer cell growth.

To further examine the involvement of TMPRSS2 in the cell growth of human prostate cancer cells, we knocked down TMPRSS2 in LNCaP C-33 cells using shRNA-TMPRSS2 and selected the stable pools of transfectants by puromycin for two weeks. As shown in figure 3A, the results from Western blot showed that shTMPRSS2 successfully reduced the protein levels of TMPRSS2 in LNCaP C-33 cells, compared to control cells (shLuc). Interestingly, decreased TMPRSS2 expression reduced the protein

levels of AR and PSA. We further analyzed the effect of TMPRSS2 knockdown on the cell growth by MTT assay. As shown in figure 3B, knockdown of TMPRSS2 in LNCaP C-33 cells enhanced their cell growth. Thus, a decrease of TMPRSS2 expression in prostate cancer cells increased their cell growth and simultaneously reduced the protein levels of AR and PSA.

3.4 Effect of TMPRSS2 knockdown on prostate cancer cell migration.

To delineate the role of TMPRSS2 in prostate cancer cell migration, we examined the cell migration abilities of TMPRSS2 knockdown cells by migration assay in a regular culture medium or steroid-reduced condition. As a result in figure 4B, TMPRSS2 knockdown suppressed the LNCaP cell migration in a regular culture condition, with no significant effect in the steroid-reduced condition. This no effect in the steroid-depleted condition may be due to the nature of androgen-regulated TMPRSS2 gene; in fact, the expression level of TMPRSS2 in shLuc cells was decreased down to shTMPRSS2-knockdown cells in this condition. Thus, downregulation of TMPRSS2 reduced prostate cancer cell migration. The data indicated that the migratory ability of prostate cancer cells was related with TMPRSS2 expression.

3.5 Effect of DHT on TMPRSS2 expression.

It had been reported that TMPRSS2 expression was regulated by androgens [14, 15, 54]. To further elucidate the effect of androgens on the expression of TMPRSS2 in prostate cancer cells, we examined TMPRSS2 proteins by western blot in LNCaP C-33 and C-81 cells after treatment with dihydrotestosterone (DHT, 10 nM) or Bicalutamide (Casodex, 10 μM), a non-steroidal competitive AR antagonist. As shown in figure 5, in a steroid-reduced condition, the expression of TMPRSS2 protein level was subsided and ably induced by DHT in LNCaP C-33 cells up to 4 folds, with a less induction fold seen in LNCaP C-81 cells. The induction of TMPRSS2 expression by DHT was ably blocked by Casodex in these cells, suggesting AR was important for DHT-induced TMPRSS2 expression in human prostate cancer cells. Since PSA is a classic androgen-regulated gene (Young, 1991), we further analyzed the protein levels of PSA in these two cells upon these treatments. In androgen-sensitive prostate cancer cells, DHT was able to significantly induce PSA protein expression and Casodex reversed the DHT effect on this protein (Figs. 5A & 5C). However, in androgen-independent LNCaP C-81 cells, DHT had no significant effect on PSA production. These results indicated that TMPRSS2 protein expression was significantly regulated by androgens in androgen-sensitive prostate cancer cells.

3.6 Role of TMPRSS2 in AR transcriptional activity and protein stability.

Since TMPRSS2 knockdown would reduce the protein level of androgen receptor (AR), leading to decreasing the expression of PSA, to further investigate whether TMPRSS2 may play a role in regulating AR function, we first examined the protein levels of AR and PSA in shLuc and shTMPRSS2 LNCaP cells, in response to serum treatment. The cells were starved for two days and then maintained with a regular culture medium for different time points. The protein levels of TMPRSS2, AR and PSA were analyzed by Western blots. As shown in figure 6, we observed that the protein levels of TMPRSS2, AR and PSA raised in shLuc cells after the cells grew in a regular culture medium treatment, while in TMPRSS2 knockdown cells, the serum lost its induction effect on AR and PSA. Thus, TMPRSS2 was involved in serum-induced the protein expression of AR and PSA.

Since TMPRSS2 is an androgen-regulated gene [19], to further analyze the role of TMPRSS2 in androgen-induced the transcription activity and protein stability of AR, we treated shLuc and shTMPRSS2 LNCaP cells with or without DHT. In shLuc LNCaP cells, the Western blot results showed that DHT was able to induce AR transcription activity, indicated by an increase of PSA protein expression, and to increase the protein levels of AR, approximately by 2 folds. The up regulation of AR protein by DHT was

similar to the effect of serum on increasing AR protein (Fig. 6). When TMPRSS2 was knocked down, DHT lost its effect on PSA production and increasing the level of AR proteins. Taken together, the results indicated that TMPRSS2 was important for DHT-induced AR transcription activity. Since TMPRSS2 knockdown can reduce the protein level of AR (Figs. 3A, 6A & 7A), we further analyzed the role of TMPRSS2 in the protein stability of AR, by treating shLuc and shTMPRSS2 LNCaP cells with cycloheximide (10 ng/mL) and detecting the AR protein level at different time points. The results showed that the protein degradation rate of AR was increased about 1.7 fold (the slope from 0.0095 to 0.016, Fig. 8B) in the TMPRSS2 knockdown cells, compared to control cells (shLuc). This suggested that a decrease of AR protein stability by down regulation of TMPRSS2 was one of the reasons to reduce the protein level and transcriptional activity of AR.

3.7 Role of TMPRSS2 in DHT-induced prostate cancer cell growth.

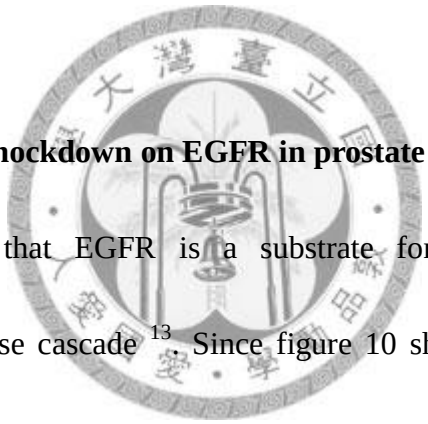
Since androgen have been shown to ably induce both prostate cancer cell growth [49] and TMPRSS2 expression (Fig.5, [19]), we further examined whether TMPRSS2 played a role in DHT-induced prostate cancer cell growth by MTT assay. As shown in figure 9, the cell growth of shLuc LNCaP C-33 cells was stimulated by DHT. When TMPRSS2

was knocked down, the stimulation effect of DHT on cell growth was blocked. It indicated that TMPRSS2 was required for DHT-induced prostate cancer cell growth.

3.8 Role of TMPRSS2 in activation of matriptase and prostasin zymogens.

It had been reported that the transcription activity of AR is required for androgens to induce matriptase activation in prostate cancer cells [33]. Since our previous data showed that androgens were able to induce TMPRSS2 expression and TMPRSS2 knockdown reduced the AR transcription activity. To further examine whether TMPRSS2 was involved in androgen-induced matriptase activation in prostate cancer cells, we first analyzed the total and activation form of matriptase by western blot with anti-total matriptase (M32) and anti-activated matriptase (M69) monoclonal antibodies in shLuc and shTMPRSS2 LNCaP cells. As shown in figure 10B, the activated levels of matriptase were reduced in the TMPRSS2 knockdown cells, compared to control cells. We further determined an *in vivo* substrate of matriptase, prostasin, in these two cells by western blots [28]. As shown in figure 10C, TMPRSS2 knockdown also increased the level of prostasin zymogen, suggesting that TMPRSS2 was required for matriptase activation in prostate cancer cells. To further address the role of TMPRSS2 in androgen-induced matriptase activation, we treated shLuc and shTMPRSS2 LNCaP

cells with or without DHT in the presence or absence of Casodex for 24 h, and analyzed the status of prostasin zymogens by immunoblotting. As shown in figure 11, DHT induced prostasin maturation from its zymogens. Casodex reversed the DHT effect on prostasin activation, indicating AR was involved in this biological event. When TMPRSS2 was knocked down, DHT lost its effect on prostasin maturation. Thus, the data indicated that TMPRSS2 was important for DHT-induced matriptase and prostasin activation.



3.9 Effect of TMPRSS2 knockdown on EGFR in prostate cancer cells.

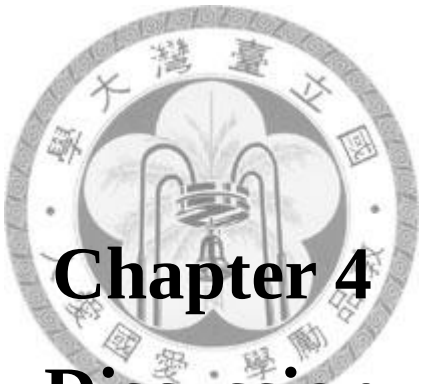
It had been reported that EGFR is a substrate for the membrane-anchored matriptase/prostasin protease cascade [13]. Since figure 10 showed that TMPRSS2 was required for matriptase and prostasin activation, we further elucidated the effect of TMPRSS2 knockdown in EGFR processing, by analyzing the total protein and activated levels of EGFR by western blots with anti-EGFR and anti-phospo-EGFR (Tyr-1068) antibodies in TMPRSS2 knockdown LNCaP cells. In figure 12, we found that knocking down TMPRSS2 decreased the proteolytic modification on EGFR, as shown an increased level of mature EGFR (170 kDa), and simultaneously increased EGFR activation, indicated by its tyrosine phosphorylation and the activities of ERKs and Akt,

two main downstream molecules of EGFR. To further investigate whether an increased level of EGFR by TMPRSS2 down regulation will alter cells to response EGF growth stimulation, we treated these two cells with or without EGF (10 ng/mL) in the presence and absence of an EGFR inhibitor, AG1478 (10 μM) and analyzed cell growth by MTT assay. As shown in figure 13, TMPRSS2 knockdown cells had more growth stimulation by EGF than shLuc control cells. AG1478 reduced the EGF effect on growth stimulation. Thus, down regulation of TMPRSS2 enhanced the EGFR protein level, leading to an increased sensitivity to EGF growth stimulation. The results may explain why TMPRSS2 knockdown cells had better cell growth than control cells in regular cell culture condition (Fig. 3B).

3.10 Role of TMPRSS2 as a mediator between the signal pathways of androgens and EGF in prostate cancer cells.

It has been reported that slug was also ably up-regulated by DHT and EGF [55]. To further determine whether TMPRSS2 knockdown will affect the gene expression of slug, we cultured cells with a regular culture medium and charcoal-stripped medium with or without DHT after hormone starvation. Total RNAs were prepared to detect the gene expression levels of slug and PSA. As shown in figure 14, in the regular culture medium,

the slug gene expression was increased by approximately 1.7 folds but the expression of PSA was reduced approximately by 25% in TMPRSS2 knockdown cells, compared to control cells. In the steroid-starved condition, there was no significant difference of slug expression between those two cells. Upon DHT treatment, the slug gene expression was significantly up-regulated in shLuc LNCaP cells. In TMPRSS2 knockdown cells, DHT had less effect on inducing the gene expression of slug. The data taken together from figure 12 & 14 indicated that TMPRSS2 down regulation may increase the sensitivity of EGFR to ligands by enhancing the EGFR protein level due to an inactivation of the matriptase/prostasin cascade and a decrease of AR protein level. Normally, TMPRSS2 are important for DHT to regulate AR transcription activity and cell growth as well as to induce the gene expression of PSA and slug. Taken together, the data suggested a modulatory role for TMPRSS2 between androgens and growth factor-induced signal pathways.



Chapter 4
Discussion

4.1 TMPRSS2 in androgen sensitivity, cell growth and migration.

In order to explore the role of TMPRSS2 in prostate cancer progression, we generated a specific monoclonal antibody, AL-20, for TMPRSS2 detection [20] and established stable TMPRSS2 knockdown LNCaP cells for this study. In prostate cancer cells, TMPRSS2 expression was correlated with androgen sensitivity (Fig.1), similar to a previous report that *TMPRSS2* exhibited a higher expression in LNCaP cells than in PC-3 cells but not in DU145 cells [16].

With an LNCaP progression model, TMPRSS2 expression was inversely correlated with cell proliferation, tumorigenicity, migration, invasion and androgen receptor transcription activity. Knocking down TMPRSS2 reduced cell migration and AR protein level. A decrease of AR protein by TMPRSS2 knockdown may cause a reduction of prostate cancer cell migration, since it has been shown that AR is required for androgen-induced prostate cancer cell invasion [56]. A decreased protein level of AR in TMPRSS2 knockdown cells was at least in part due to the role of TMPRSS2 in AR protein stability. Moreover, we also observed that reduced TMPRSS2 expression increased prostate cancer cell growth. The result further support the fact that the level of TMPRSS2 expression was inversely correlated with the cell growth of human prostate cancer cells (Fig.2, [49]). Thus, TMPRSS2 may serve as a negative regulator for cell

growth. However, in the steroid-reduced condition, upon DHT treatment, TMPRSS2 was important for androgen-induced prostate cancer cell growth and PSA production. It suggested that androgens needed TMPRSS2 gene expression, to facilitate AR function in androgen-sensitive prostate cancer cells. Thus, our data suggested a differential role of TMPRSS2 in steroid hormone function and in the prostate cancer progression.

4.2 TMPRSS2 in androgens-induced matriptase activation and EGFR processing.

Kiyomiya *et al*. reported that androgens can induce matriptase activation in human prostate cancer cells and AR transcription activity is important for androgen-induced matriptase activation [33]. An androgen-regulated gene was suggested to be required for androgens to induce matriptase activation. In this study, we further identified TMPRSS2 as an androgen-induced gene for matriptase activation. This was because DHT lost its stimulation effect on matriptase activation when TMPRSS2 was knocked down. Thus, TMPRSS2 expression induced by androgens was essential for the hormone to promote matriptase activation. Since androgens have been reported as an inducer for prostate cancer cell invasion [57], the protease cascade of TMPRSS2/Matriptase may be employed by androgens to execute their function in cell invasion. However, how androgen-induced TMPRSS2 activates matriptase activation and where the matriptase

activation by TMPRSS2 occurs are still unclear.

The LNCaP cell progression model was established by a regular cell culture. With increasing passage numbers, C-81 cells acquire an androgen-independent phenotype, while their parental LNCaP cells (C-33 cells) remain an androgen sensitivity. Thus, this model can recapitulate the progression of human prostate cancer with increases of androgen-independent cell growth, PSA secretion and tumorigenicity [49]. We further characterized this model and found that the migration and invasion of these cells also increased following the progression, which was concomitant with a decrease of TMPRSS2 expression. Our result further showed that reduced TMPRSS2 expression promoted prostate cancer cell growth but decreased cell migration. This observation may explain the fact that down regulation of TMPRSS2 expression is correlated with the prostate tumor formation in xenographed mice [16]. Thus, the LNCaP cell progression model can serve as a useful tool for studying the progression of human prostate cancer. Within using this cancer progression model, the results suggested that reduced TMPRSS2 protein expression by down regulation or gene recombination may contribute to prostate tumorigenesis and progression.

Membrane-anchored matriptase/prostasin protease cascade has been identified as a proteolytic modifier for EGFR in epithelial cells [13, 58]. In this study, we further showed

TMPRSS2 was involved matriptase activation because TMPRSS2 knockdown in LNCaP cells decreased the activation of matriptase/prostasin zymogens and the proteolytic modification on EGFR. Since a previous study showed that EGFR expression was correlated with disease relapse and progression to androgen-independence in human prostate cancer [59, 60], the increase of EGFR protein by down regulation of TMPRSS2 may be an explanation for TMPRSS2's role in the cancer progression.

4.3 TMPRSS2 in the EGF sensitivity of prostate cancer cells and in the cancer progression.

Several lines of evidence have shown that aberrant androgen/AR signaling and/or the growth factor signal pathways are implicated in the progression of prostate cancer to advanced stages [46, 61, 62]. During the progression of prostate cancer cells, TMPRSS2 expression was decreased and concurrent with increased cell growth. We further explored the role of decreased TMPRSS2 expression in promoting the cancer progression. In fact, in TMPRSS2 knockdown cells, there were increases of cell growth, EGFR protein level and activity, due to inactivation of matriptase/prostasin. The high level of mature EGFR in TMPRSS2-knockdown cells enhanced their sensitivity to EGF

growth stimulation. Thus, our data suggested that down regulation of TMPRSS2 during the progression of prostate cancer cells promoted EGFR signaling, leading to cancer cell growth and obtaining a hormone-independent phenotype. On the other hand, our result also showed that TMPRSS2 was required for androgen signaling for growth stimulation. It has been also shown that TMPRSS2 expression is correlated with clinical stages of human prostate cancer [14, 19, 63]. It postulates that overexpression of TMPRSS2 in a subgroup of prostate cancer patients may enhance AR signaling without ligand stimulation. More experiments are required to examine this possibility.

4.4 TMPRSS2 as a mediator between androgen signaling and EGFR pathway.

In androgen-sensitive prostate cancer cells, TMPRSS2 expression was dependent on androgen stimulation and essential for the steroid hormone to enhance cell growth, PSA production and matriptase activation. Thus, TMPRSS2 played an important role in androgen function. In androgen-independent prostate cancer cells, down regulated TMPRSS2 decreased the activation of matriptase/prostasin zymogens and AR transcription activity, leading to increasing EGFR protein and EGF sensitivity. A high EGF sensitivity may make cancer cells survive and further progress independent of androgens. This may provide an explanation why TMPRSS2 was shown a down

regulation in androgen-independent prostate tumors in xenograft mice [16]. We also examined the role of TMPRSS2 in slug expression, which was regulated by EGF and androgens [55]. The results showed that down regulation of TMPRSS2 enhanced the slug gene expression, possibly due to increased EGFR signaling. Knocking down TMPRSS2 blocked androgens to induce slug expression. Thus, TMPRSS2 may serve as a mediator between AR signaling and EGFR signal pathway. Dysregulation of TMPRSS2 function may contribute to prostate cancer progression to advanced stages. Thus, the results suggest some thoughts for prostate cancer therapy that drug combination to target both AR signaling and EGFR signal pathway may further enhance therapeutic efficiency.

Chapter 5
Figures

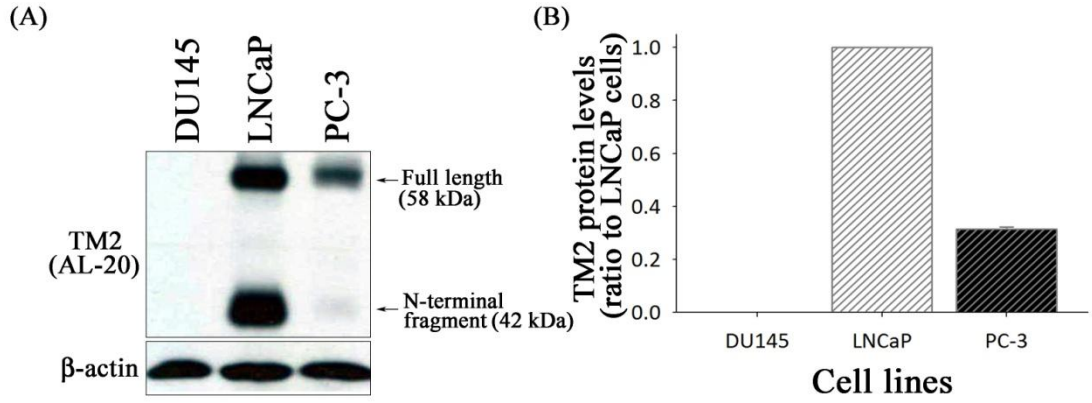


Figure 1. Protein levels of TMPRSS2 in prostate cancer cell lines.

(A) Prostate cancer cell lines, DU145, LNCaP and PC-3 cells, were respectively seeded at a density of 1 X 10^6 cells in a 60-mm dish. Two days after plating, cells were steroid starved in phenol red-free RPMI 1640 medium with 3% charcoal/dextran-stripped FBS for 48 h. Cells were refreshed with a regular culture medium for 24 h and then harvested for Western blot with an anti-TMPRSS2 antibody (AL-20). A loading control was analyzed with an anti-β-actin monoclonal antibody (AC-15, Sigma). (B) TMPRSS2 protein levels in three cell lines were measured by a densitometer and statistically normalized to control β-actin. Values are mean ± S.D. of three independent experiments.

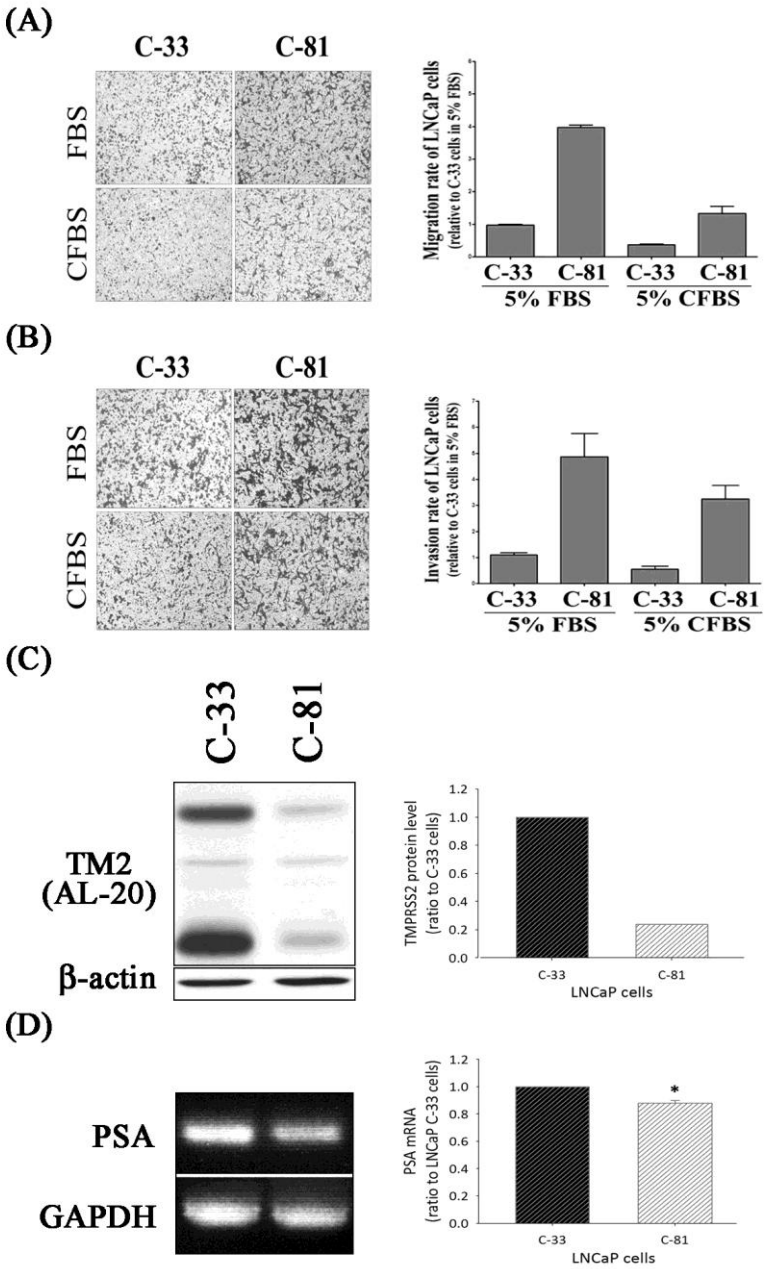


Figure 2. Expression of TMPRSS2 in different LNCaP cells.

LNCaP C-33 and C-81 cells were seeded at a density of 1 X 10^6 cells per 60-mm dish. Two days after seeding, (A) cells were starved with phenol red and serum free RPMI 1640 medium for 36 h. After trypsinization, 3 X 10^5 cells were seeded with serum-free RPMI 1640 medium with or without phenol red in the upper chamber, and the lower chambers were added with 5% FBS RPMI 1640 medium (FBS) or 5% charcoal/dextran-stripped FBS RPMI 1640 medium without phenol red (CFBS) as

chemoattractants. Transwell migration assay was carried out for 24 h. Migratory cells were fixed in methanol and stained with giemsa stain, and images were captured by a light microscope (100X). (B) For the transwell invasion assay, each filter of insert was coated with 30 μg/cm^2 matrigel, and the experiment procedures followed the migration assay, as described in (A). Migration and invasion rates were statistically measured and normalized to control cells. (C) Cells were starved in phenol red-free RPMI 1640 medium with 3% charcoal/dextran-stripped FBS. After 48-h steroid depletion, cells were refresh with a regular medium for 24 h and harvested for Western blot with a monoclonal anti-TMPRSS2 (AL-20) antibody. Loading control was analyzed with an anti-β-actin monoclonal antibody (AC-15, Sigma). TMPRSS2 protein levels in the cells were measured by a densitometer and statistically normalized to their individual β-actin. The intensity value of TMPRSS2 in LNCaP C-33 cells was indicated as 1. (D) Total RNA was isolated by using TRIZOL reagent (Invitrogen, USA). PSA mRNA levels were analyzed by RT-PCR amplification with primers (5'-TTGTCTTCCTCACC CTGTCC-3' and 5'-TCACGCTTTTGTTCCTGATG-3'). PCR products were separated by 1% agarose gel electrophoresis, stained by ethidium bromide and visualized by a UVP imager (GDS-7900 system, USA). The band intensities were measured by a densitometer and calculated with normalization to their respective GAPDH. The intensity of PSA mRNA in LNCaP C-33 cells was indicated as 1. Data from the Ming-Shyue Lee's lab.

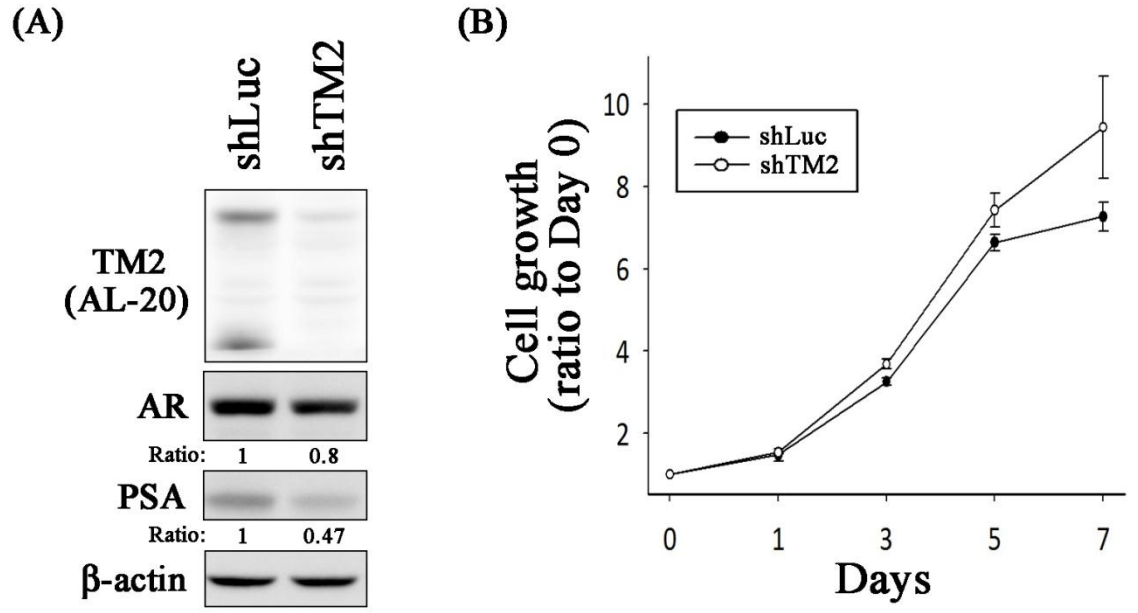


Figure 3. Role of TMPRSS2 in prostate cancer cell growth.

LNCaP C-33 cells were seeded at a density of 1 X 10^6 cells per 60-mm dish. One day after seeding, cells were infected with lentiviral particles containing TMPRSS2 shRNA and maintained for 24 h. Control cells were infected with lentiviral particles containing luciferase shRNA. A week after a selection with 2 mg/ml puromycin, cells were grown in a regular RPMI 1640 medium and subcultured every 6 days. (A) Stable pools of shTMPRSS2 and shLuc LNCaP C-33 cells were seeded at a density of 1 X 10^6 cells per 60-mm dish. Two days after seeding, cells were starved in phenol red-free RPMI 1640 medium with 3% charcoal/dextran-stripped serum for 2 days and then refreshed with a regular medium for 24 h. Cell lysates were collected with and analyzed by Western blots with anti-TMPRSS2 (AL-20), anti-PSA (sc-7638, Santa Cruz) and anti-AR (sc-816, Santa Cruz) antibodies. A loading control was analyzed with an anti-β-actin monoclonal antibody (AC-15, Sigma). (B) Cells were plated at a density of 3 X 10^4 cells per well in a 24-well plate with 500 μL regular medium. After 48h, the cell was starved with a steroid-free medium for two days. Cells were then refreshed with a regular cell culture media every two days and cell growth at each time point was measured using MTT assay. Results are shown as mean ± S.D of triplicate data obtained from single experiment. Similar results were observed in three separate experiments.

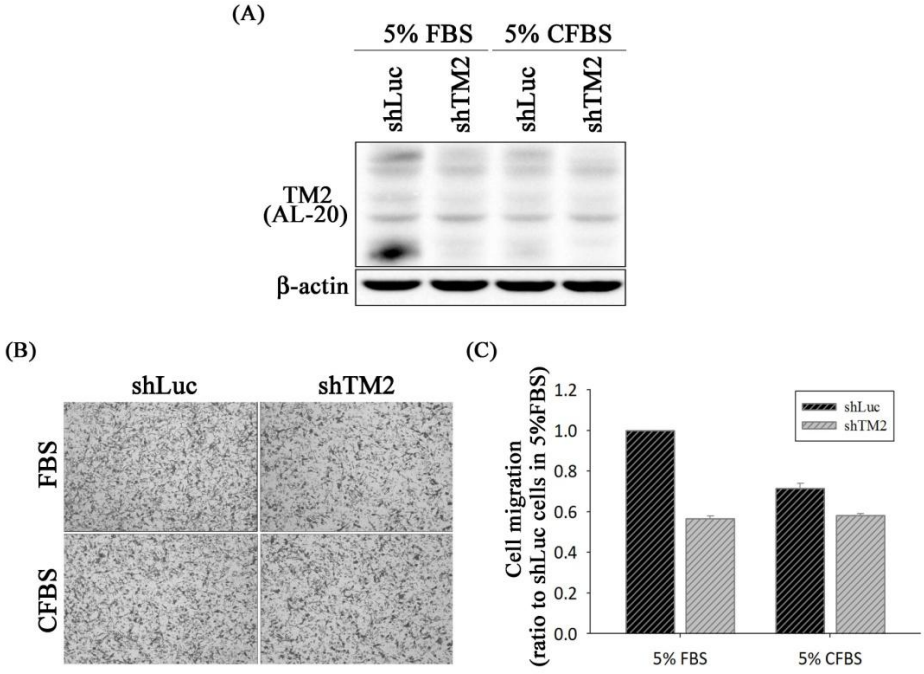


Figure 4. Effects of TMPRSS2 on prostate cancer cell migration.

shTMPRSS2 and shLuc LNCaP C-33 cells were seeded at a density of 1 X 10^6 cells per 60-mm dish for 48 h. (A) Cells were starved in phenol red-free RPMI 1640 medium with 3% charcoal/dextran-stripped FBS. After 48-h steroid depletion, cells were refreshed with a regular culture medium for 24 h and then harvested for Western blot analysis with a monoclonal anti-TMPRSS2 (AL-20) antibody. Loading control was analyzed with an anti-β-actin monoclonal antibody (AC-15, Sigma). (B) Cells were starved with phenol red and serum free RPMI 1640 medium for 36 h. After trypsinization, 3 X 10^5 cells were seeded with serum-free RPMI 1640 medium with or without phenol red in the upper chamber, and the lower chambers were added with 5% FBS RPMI 1640 medium or 5% charcoal/dextran stripped FBS RPMI 1640 medium without phenol red as chemoattractants. Transwell migration assay was carried out for 24 h. Migratory cells were fixed in methanol and stained with giemsa stain, and images were captured by a light microscope (100X). (C) Migration rate was statistically calculated with normalization to shLuc cells culture in 5% FBS medium. Values are mean ± S.D. of three independent experiments.

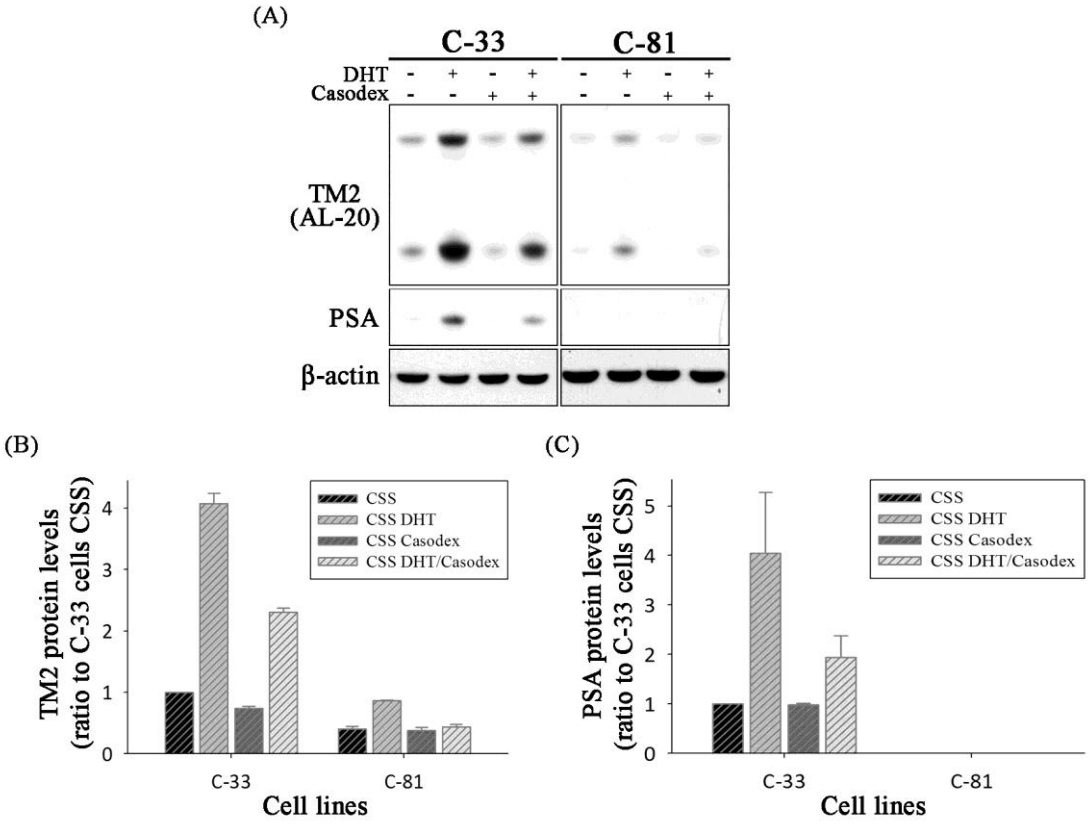


Figure 5. DHT effect on TMPRSS2 expression in LNCaP C-33 and C-81 cells.

LNCaP C-33 and C-81 cells were seeded at a density of 5 X 10^5 cells per well in a 6-well plate. Two days after plating, cells were starved in phenol red free RPMI 1640 medium with 3% charcoal/dextran-stripped serum for 48 h. Cells were treated with or without DHT (10 nM) in the presence or absence of Casodex (10 μM) for 24 h. Cell lysates were collected and used for immunoblottings to detect the TMPRSS2 and PSA with anti-TMPRSS2 (AL-20) and anti-PSA (sc-7316, Santa Cruz) antibodies. A loading control was analyzed with an anti-β-actin monoclonal antibody (AC-15, Sigma). TMPRSS2 (B) and PSA (C) protein levels were measured by a densitometer and statistically normalized to their individual β-actin. The intensities of TMPRSS2 and PSA proteins in LNCaP C-33 cells with CSS treatment were indicated as 1. Values are mean ± S.D. of three independent experiments.

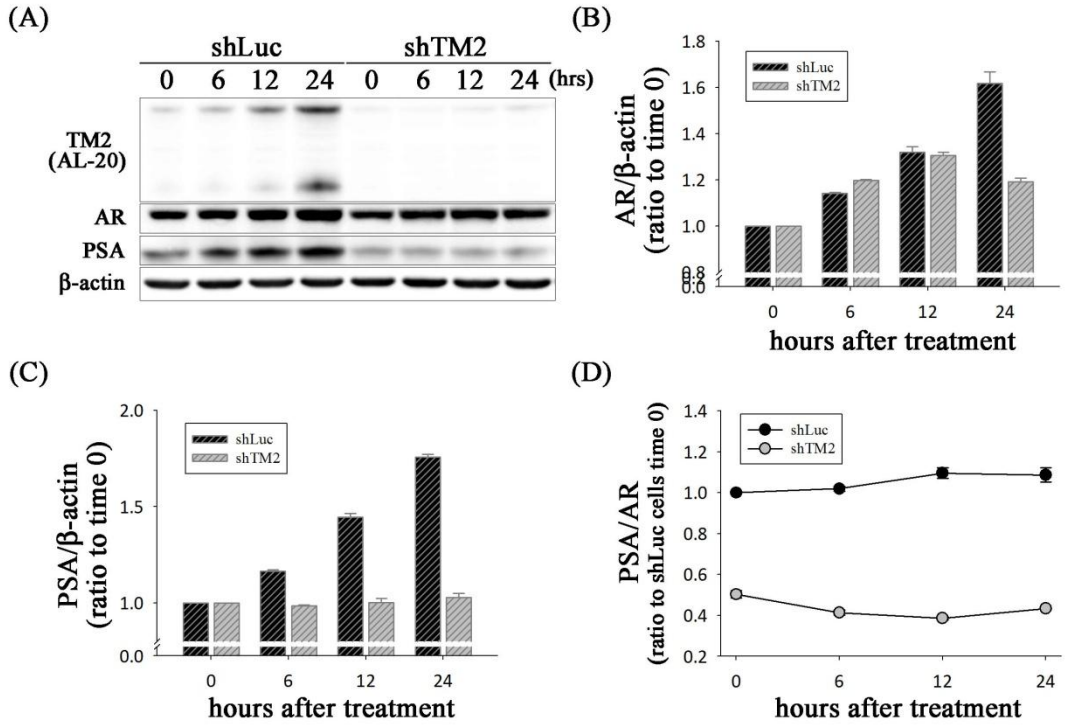


Figure 6. Role of TMPRSS2 in serum-induced AR and PSA protein expression of prostate cancer cells.

shTMPRSS2 and shLuc LNCaP C-33 cells were seeded at a density of 1 X 10^6 cells per 60-mm dish. Two days after seeding, cells were starved in phenol red free RPMI 1640 medium with 3% charcoal/dextran-stripped serum for 48 h. cells were refreshed with a regular culture medium and then harvested at each point for Western blot. (A) Cell lysates were collected and used for immunoblottings to detect the TMPRSS2, PSA and AR with anti-TMPRSS2 (AL-20), anti-PSA (sc-7683, Santa Cruz) and anti-AR (sc-816, Santa Cruz) antibodies. A loading control was analyzed with an anti-β-actin monoclonal antibody (AC-15, Sigma). AR (B) and PSA (C) protein levels were measured by a densitometer and statistically normalized to their individual β-actin. The intensities of AR and PSA proteins at 0 h were indicated as 1. (D) AR transcriptional activity was evaluated by the ratios of PSA to AR protein with a ratio value of shLuc cells at time 0 as 1. Values are mean ± S.D. of three independent experiments.

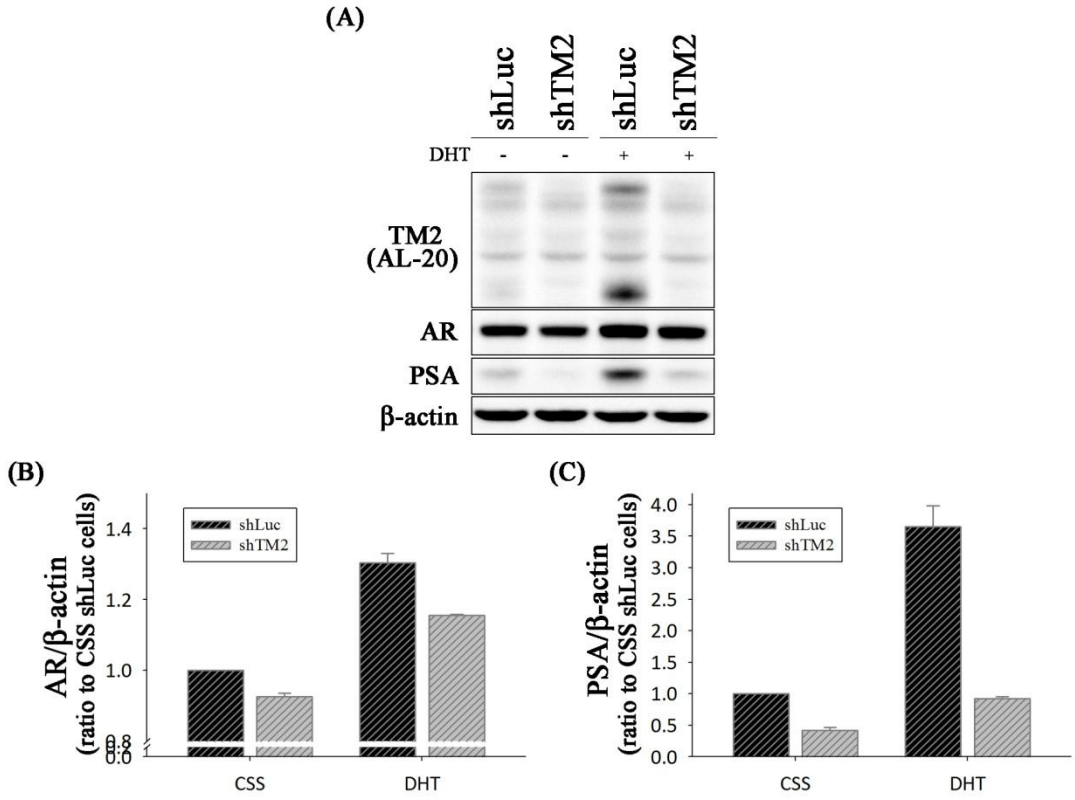


Figure 7. Role of TMPRSS2 in DHT-induced AR and PSA protein expression in prostate cancer cells.

(A) shTMPRSS2 and shLuc LNCaP C-33 cells were seeded at a density of 1 X 10^6 cells per 60-mm dish. Two days after seeding, cells were starved in phenol red free RPMI 1640 medium with 3% charcoal/dextran-stripped FBS for 48 h. Cells were treated with or without DHT (10 nM) for 24 h. Cell lysates were collected and used for Western blot analyses to detect TMPRSS2, PSA and AR with anti-TMPRSS2 (AL-20), anti-PSA (sc-7683, Santa Cruz) and anti-AR (sc-816, Santa Cruz) antibodies. A loading control was analyzed with an anti-β-actin monoclonal antibody (AC-15, Sigma). AR (B) and PSA (C) protein expression levels were analyzed by a densitometer and calculated with a normalization to their respective β-actin. The intensities of AR and PSA proteins at CSS shLuc was indicated as 1. Values are mean ± S.D. of three independent experiments.

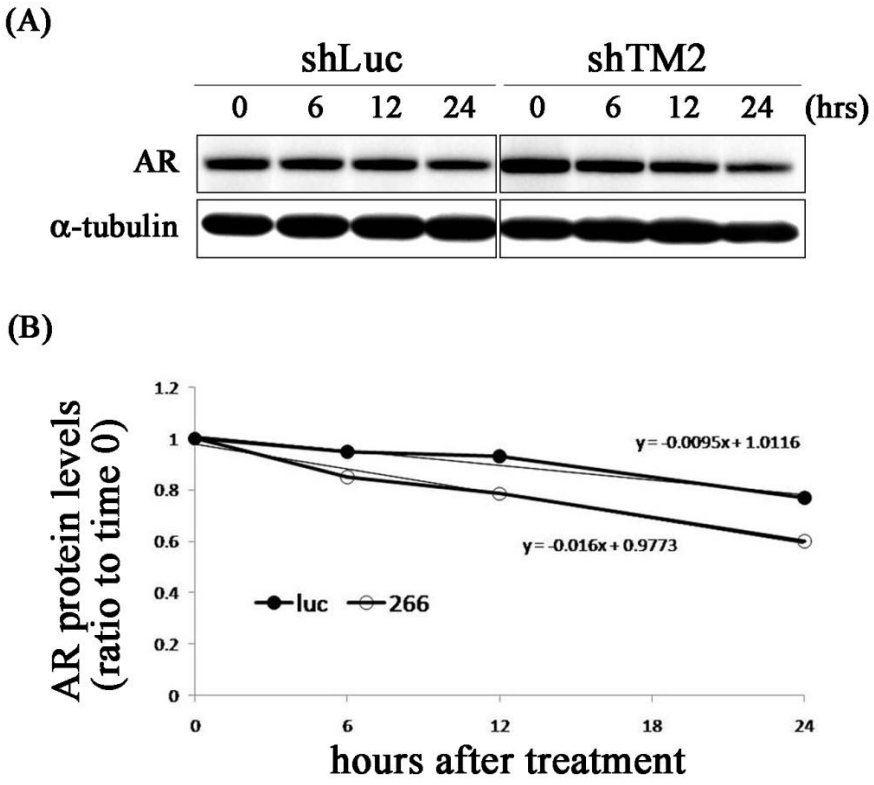


Figure 8. Role of TMPRSS2 in the protein stability of androgen receptor.

shTMPRSS2 and shLuc LNCaP C-33 cells were seeded at a density of 1 X 10^6 cells per 60mm dish for 48 h. (A) Cells were cultivated medium containing 3% charcoal/dextran-stripped FBS with or without cycloheximide (10 ng/mL). Cell lysates were collected at different time points and then immunoblotted for androgen receptor (AR) with an anti-AR (sc-816, Santa Cruz) antibody. A loading control was analyzed with an anti-α-tubulin (#2125, Cell Signaling) antibody. (B) AR protein levels at different times were measured by a densitometer, normalized to their individual α-tubulin with a value of AR protein level at 0 h as 1. Values are mean on two independent experiments.

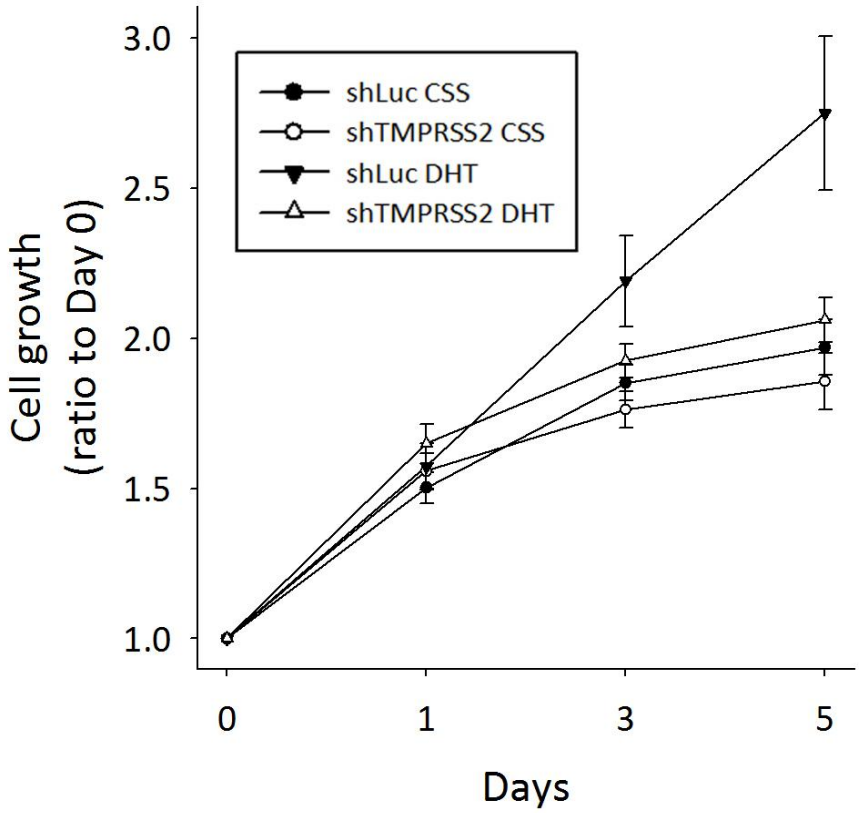


Figure 9. Role of TMPRSS2 in DHT-induced cell growth.

shTMPRSS2 and shLuc LNCaP C-33 cells were plated at a density of 3 X 10^4 cells per well in 24-well plates with 500 μL regular medium. After 48 h, cells was starved with a steroid-free medium for 48 h. Cells were then treated with or without DHT (10 nM) in a steroid-reduced medium for different times. Cells growth was measured at each time point using MTT assay. Cell growth rates were statistically calculated and normalized to Day 0. Results are shown as mean ± S.D of triplicate data obtained from single experiment. Similar results were observed in three separate experiments.

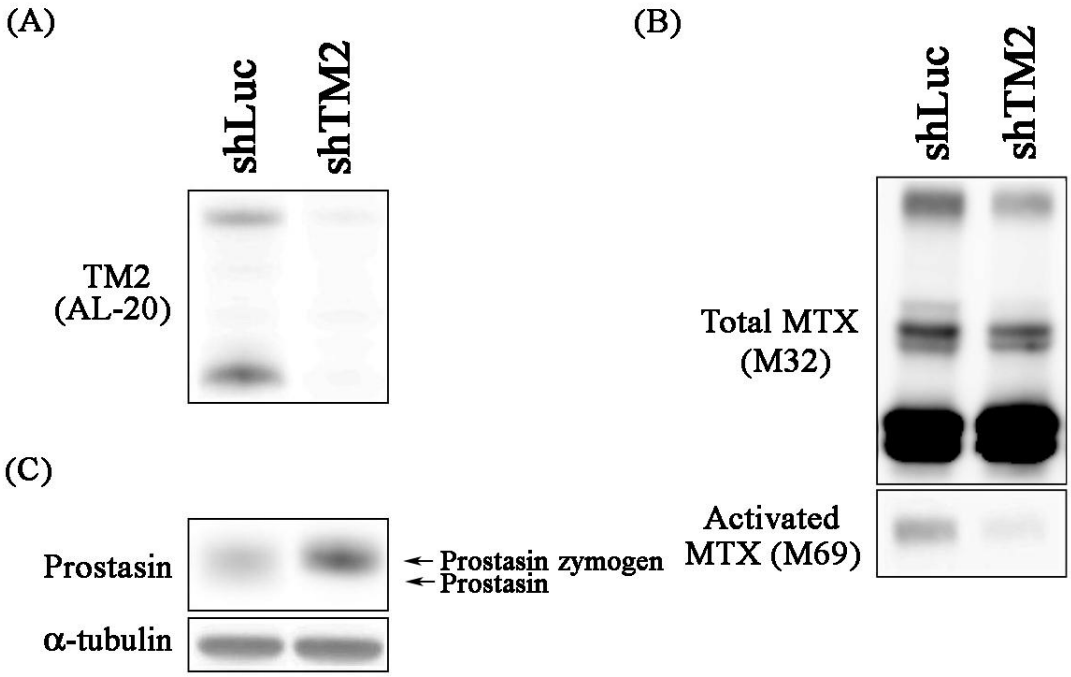


Figure 10. Role of TMPRSS2 in matriptase-prostasin proteolytic cascade.

shTMPRSS2 and shLuc LNCaP C-33 cells were seeded at a density of 1 X 10^6 cells per 60-mm dish for 48 h. Cells were then starved with a steroid-free medium for two days and refreshed with a regular medium for 24 h, cell lysates were then harvested for Western blot analyses. (A) TMPRSS2 protein levels were analyzed by immunoblotting with anti-TMPRSS2 (AL-20) antibody. (B) The levels of total matriptase and activated matriptase were detected with anti-total matriptase (M32) and anti-activated matriptase (M69) monoclonal antibodies, under a non-reduced and non-boiled condition. (C) The protein levels of prostasin were examined by Western blot with anti-prostasin (612172, BD Biosciences) antibody. A loading control was analyzed with an anti-α-tubulin (#2125, Cell Signaling) antibody.

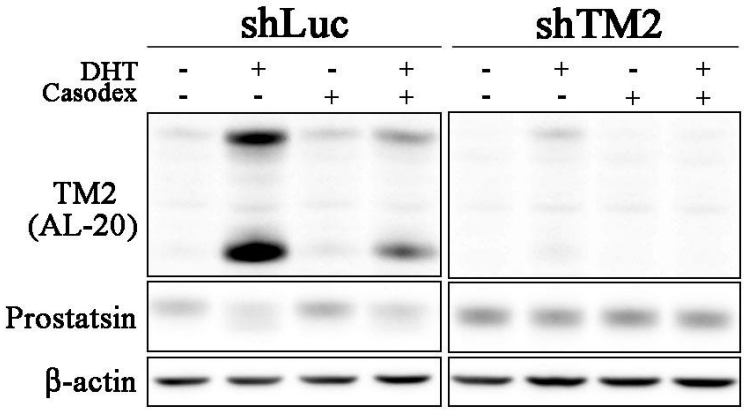


Figure 11. Effect of DHT on TMPRSS2 and prostasin in prostate cancer cells.

shTMPRSS2 and shLuc LNCaP cells were seeded at a density of 1 X 10^6 cells per 60-mm dish. Two days after seeding, cells were starved in phenol red free RPMI 1640 medium with 3% charcoal/dextran-stripped FBS for 48 h. Cells were treated with or without DHT (10 nM) in the presence or absence of Casodex (10 μM) for 24 h. Cell lysates were collected and used for immunoblottings to detect TMPRSS2 and prostasin with anti-TMPRSS2 (AL-20) and anti-prostasin (612712, BD Biosciences) antibodies. A loading control was analyzed with an anti-β-actin monoclonal antibody (AC-15, Sigma).

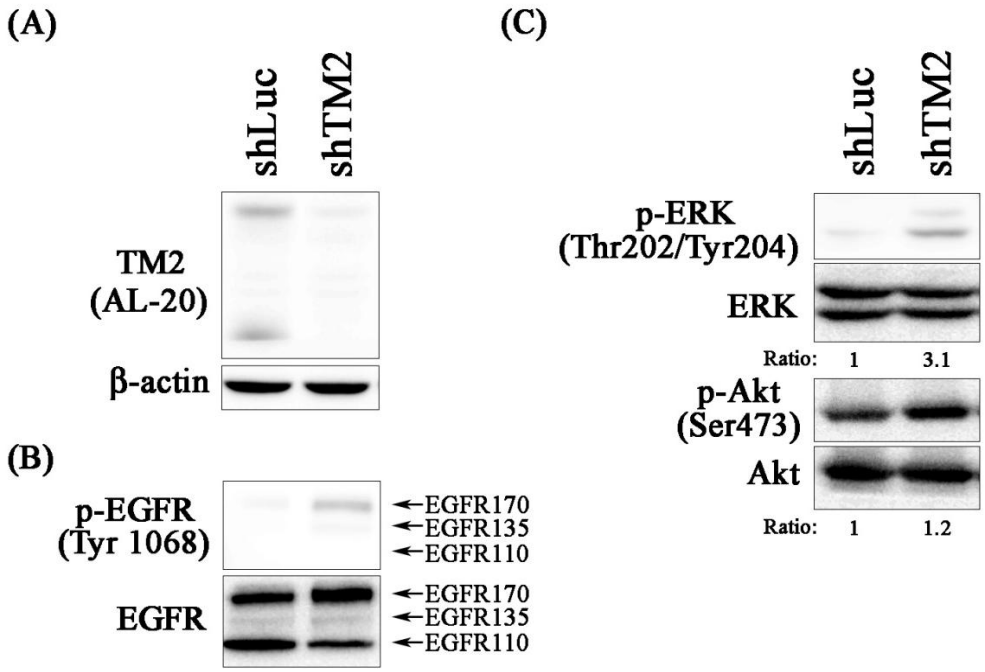


Figure 12. Effect of TMPRSS2 knockdown in EGFR of prostate cancer cells.

shTMPRSS2 and shLuc LNCaP C-33 cells were seeded at a density of 1 X 10^6 cells per 60mm dish for 48 h. Cells were starved with a steroid-free medium for two days. Cells were refreshed with a regular medium for 24 h and then harvested for Western blot. (A) The protein level of TMPRSS2 was detected by an anti-TMPRSS2 antibody (AL-20), and the phosphorylation and protein levels of EGFR (B), ERK (C) and Akt (C) were detected by anti-phospho-EGFR (Tyr1068) (#3777, Cell Signaling), anti-EGFR (sc-03, Santa Cruz), anti-phospho-ERK (Thr202/Tyr204) (#4370, Cell Signaling), anti-ERK (sc-153, Santa Cruz), anti-phospho-Akt (sc-7985-R, Santa Cruz) and anti-Akt (sc-8312, Santa Cruz) antibodies. A loading control was analyzed with an anti-β-actin monoclonal antibody (AC-15, Sigma).

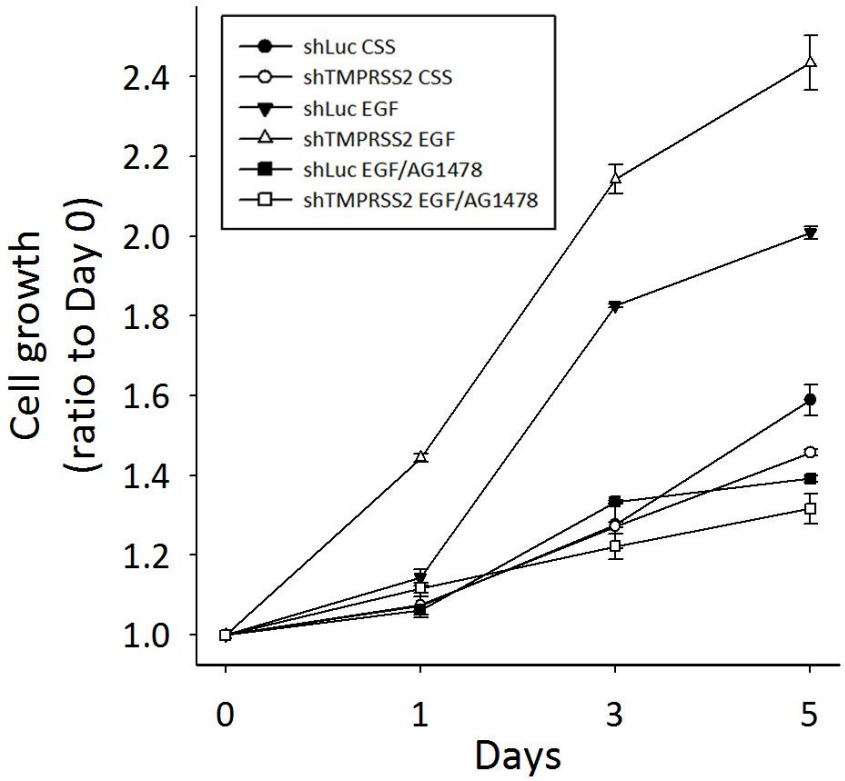


Figure 13. Cell growth of TMPRSS2-knockdown cells upon EGF treatment.

shTMPRSS2 and shLuc LNCaP C-33 cells were plated at a density of 3 X 10^4 cells per well in 500 L of regular medium in a 24-well plate. After two days, cells were starved with a steroid-free medium for 48 h. Cells were then treated with or without EGF (10 ng/mL) in the presence or absence of AG1478 (10 μM). Cell growth was measured at different time points using MTT assay. Cell growth rates were statistically calculated and normalized to Day 0. Results are shown as mean ± S.D of triplicate data obtained from single experiment. Similar results were observed in three separate experiments.

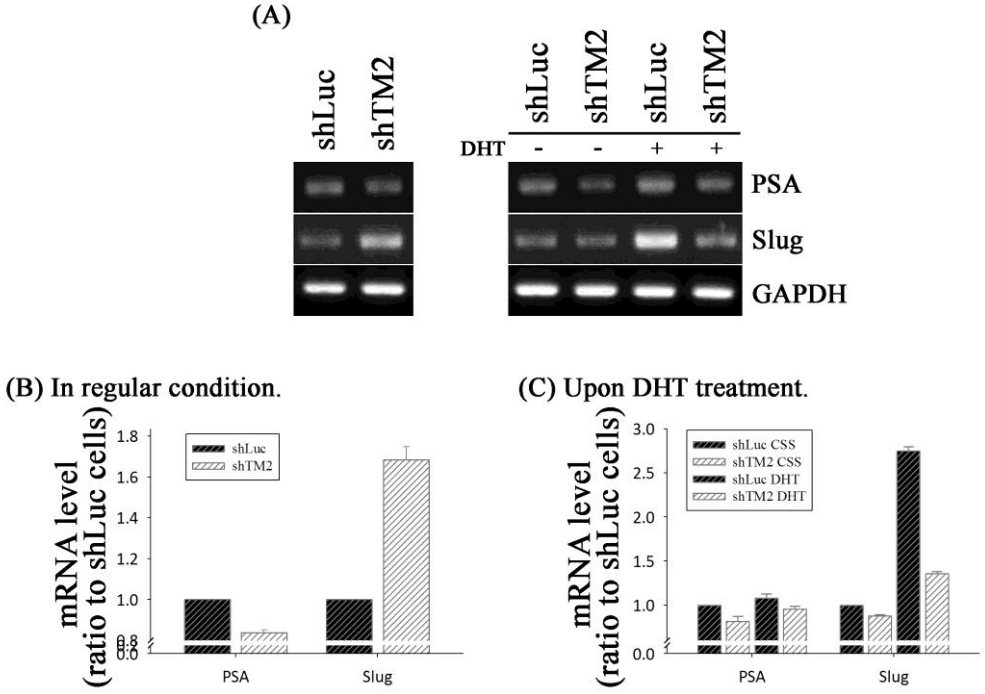


Figure 14. Role of TMPRSS2 in PSA and slug expression in prostate cancer cells.

(A) shTMPRSS2 and shLuc LNCaP C-33 cells were seeded at a density of 1 X 10^6 cells per 60-mm dish. Two days after seeding, cells were starved in phenol red-free RPMI 1640 medium with 3% charcoal/dextran-stripped FBS for 48 h. Cells were refed with a regular culture medium (FBS, left lane 1 and 2) or charcoal-stripped FBS medium with (CSS, right, lane 1 and 2) or without (DHT, right, lane 3 and 4) DHT (10 nM) for 24 h. Total RNA was isolated using TRIZOL reagent (Invitrogen, USA). The mRNA levels of PSA and slug were analyzed by RT-PCR amplification with their individual primer pairs (PSA: 5'-TTGTCTTCCTCACCCTGTCC-3' and 5'-TCACGCTTTTGTTCCTGATG-3', slug: 5'-GCCTCCAAAAAGCCAAACTAC-3' and 5'-GTGTGCTACACAGAAGCC -3'). PCR products were separated by 1% agarose gel electrophoresis, stained by ethidium bromide and visualized by a UVP imager (GDS-7900 systerm, USA). The band intensities of PSA and slug were measured by a densitometer and calculated with normalization to their respective GAPDH in a regular culture condition (B) or upon DHT treatment (C). Values are mean ± S.D. of three independent experiments.

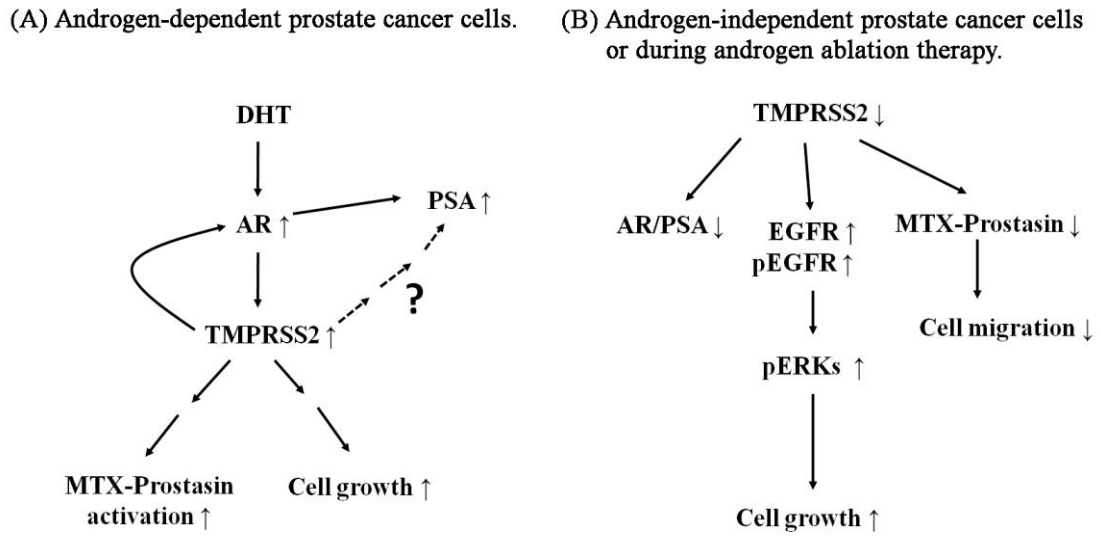
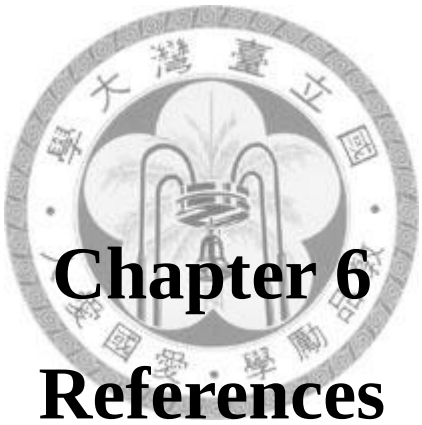


Figure 15. Proposed model for the roles of TMPRSS2 in prostate cancer.

(A) In androgen-dependent prostate cancer cells, TMPRSS2 expression was ably regulated by androgens and essential for the steroid hormone to enhance cell growth, PSA production and matriptase activation. Thus, TMPRSS2 played an important role in delivering androgen function. (B) In androgen-independent prostate cancer cells or during androgen ablation therapy, down-regulated TMPRSS2 reduced the activation of matriptase/prostasin zymogens and AR transcription activity, leading to increasing cell growth, EGFR protein and EGF sensitivity.



Chapter 6
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