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Mechanism Of Action And Regulation Of Membrane Serine Protease Prostasin In The Prostate And Prostate Cancer

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MECHANISM OF ACTION AND REGULATION OF MEMBRANE SERINE PROTEASE PROSTASIN IN THE PROSTATE AND PROSTATE CANCER

by

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ABSTRACT

The glycosylphosphatidylinositol (GPI)-anchored serine protease prostasin (PRSS8) is expressed at the apical membrane surface of epithelial cells and acts as a suppressor of tumor invasion when re-expressed in highly invasive human prostate and breast cancer cell lines. To better understand the molecular mechanisms underlying the anti-invasion phenotype associated with prostasin re-expression in prostate cancer cells, we expressed wild-type human prostasin or a serine active-site mutant prostasin in the PC-3 human prostate carcinoma cells. Molecular changes were measured at the mRNA and the protein levels. The expression of several invasion-promoting molecules is regulated by prostasin re-expression, mediated by a protein-level down-regulation of the epidermal growth factor receptor (EGFR). As a result, the cellular response to EGF was reduced as shown by the down-regulation of EGF-stimulated Erk1/2 phosphorylation. The expression of Slug, urokinase-type plasminogen activator (uPA), urokinase-type plasminogen activator receptor (uPAR), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and granulocyte-macrophage colony stimulating factor (GM-CSF) was also down-regulated by prostasin re-expression in the PC-3 cells.

Co-expression of prostasin and its activating protease matriptase with EGFR in FT-293 cells induces an apparent proteolytic cleavage of the EGFR in the extracellular domain at two specific sites, generating two N-terminally truncated EGFR fragments, named EGFR135 and EGFR110. The EGFR110 is constitutively tyrosine-phosphorylated, and in its presence the phosphorylation of downstream signaling molecules including Erk1/2 and Akt is increased under serum-free conditions. Neither EGFR135 nor EGFR110 is responsive to EGF stimulation. Deletions of

the EGFR extracellular domain (ECD) were generated to map the matriptase-prostasin cleavage sites. Two candidate sites were localized to regions AA1-273 and AA273-410. These data support a mechanism of action for the matriptase-prostasin epithelial extracellular serine protease activation cascade by proteolytically modulating the EGF-EGFR signaling.

Prostasin gene expression is down-regulated in high-grade and hormone-refractory prostate cancers. We investigated the mechanisms by which androgens regulate prostasin expression in the prostate and prostate cancer. We treated the LNCaP human prostate cancer cells with dihydrotestosterone (DHT) and measured the mRNA expression of prostasin and potential transcription regulators of prostasin predicted by interrogation of the prostasin gene promoter sequence. Prostasin mRNA expression in the LNCaP cells was not responsive to DHT treatment. DHT marginally up-regulated mRNA expression of SREBP-1c, SREBP-2, and SNAIL, but not SREBP-1a, while dramatically increased SLUG mRNA expression, in a dose-dependent manner. Co-transfection of a prostasin promoter-reporter and SREBP cDNA in HEK-293 cells resulted in stimulation of the promoter activity at ~2 fold by SREBP-1c, and up to 6 fold by SREBP-2; while co-transfection with SNAIL or SLUG cDNA resulted in repression of the promoter activity to 43% or 59%, respectively. Co-transfection of the SLUG cDNA negated SREBP-2's stimulation of the prostasin promoter in a dose-dependent manner. Transfection of an SREBP-2 cDNA in HEK-293 and DU-145 cells resulted in up-regulation of the endogenously expressed prostasin while transfection of a SLUG cDNA in the LNCaP cells repressed prostasin expression. Multiple SREBP-2 binding sites, known as sterol regulatory elements (SRE's), were identified at positions -897, -538, +8, +71, and +98 (named SRE-897,

SRE-538, SRE+8, SRE+71, and SRE+98) in the human prostasin gene promoter. Mutagenesis of the five SRE's was carried out to evaluate their roles in SREBP-2 up-regulation of prostasin. SRE+98, a novel functional sterol regulatory element was found to be the major site for the stimulatory response of prostasin gene expression to SREBP-2.

CONCLUSIONS: Prostasin regulates the expression of several invasion-promoting molecules in prostate cancer cells by down-modulating the EGF-EGFR signaling pathway. Active prostasin induces proteolytic cleavage in the EGFR ECD at two specific sites. One of the N-terminally truncated EGFR, the EGFR110 is auto-phosphorylated along with increased phosphorylation of downstream signaling molecules. The effect of the androgen DHT on prostasin expression in prostate cells is mediated via SREBP's, which stimulate the promoter, and Slug, which represses the promoter. Slug is up-regulated by DHT and EGF, providing a molecular mechanism by which epithelial cell-specific genes are silenced during prostate cancer development and progression.

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CHAPTER ONE GENERAL REVIEW

1.1 Introduction of Prostate Cancer Today

Cancer has been a major public health problem that is responsible for about one fourth of all deaths in the world today (Jemal et al., 2007). It is a group of diseases characterized by uncontrolled division and spread of dysregulated cells. There are many types of cancers which are usually named from the tissues presumed to be the origin of the tumor: *Carcinoma* refers to the malignant tumors derived from epithelial tissues, such as the breast, prostate, lung, and colon. *Leukemia* refers to the malignant tumors derived from blood and bone marrow cells. *Sarcoma* and *Glioma* refer to the malignant tumors derived from mesenchymal cells and glia cells, and *Germinoma* and *Choriocarcinoma* are the malignant tumors derived from the germ cells and placenta, respectively.

Currently, prostate cancer is the most frequently diagnosed cancer (with an estimated 218,890 new cases in 2007) and second leading cause of cancer-related deaths in men in the US (with an estimated 27,050 new cases in 2007, American Cancer Society. *Cancer Facts and Figures* 2007). Patients with prostate cancer usually have no symptoms in the early stages. With progression of prostate cancer, patients may experience difficulty controlling urine flow, frequent nocturnal urination, voiding pain, or blood in the urine. Severe pain in the lower back, pelvis, or upper thighs may indicate metastasis, caused by the more malignant and dangerous cancer cells which are highly invasive and migrate to other tissue sites.

The PSA (prostate-specific antigen) blood test and the digital rectal exam (DRE) are two major screening tests for early-stage prostate cancer. A biopsy is indicated if the PSA test and the DRE produce inconclusive findings. A small sample of the prostate tissue is removed for pathological examination to assist in diagnosis. Prostate cancer cells present a different histology from normal prostate epithelial cells and the degree of difference is used to determine the cancer grade. The Gleason grading system consists of five distinct patterns that prostate cancer cells differ from normal prostate epithelial cells. The scale runs from 1 to 5, where 1 represents cells that are very similar to normal prostate cells and well differentiated; while 5 represents cells that are poorly differentiated (Figure 1-1A). The Gleason score is determined by adding the Gleason grade to the most common pattern of the biopsy sample and the Gleason grade to the second most common pattern. The higher the Gleason score is, the more aggressive the cancer cells tend to be. Prostate cancer is clinically diagnosed by staging, based on the tumor size, characteristics of the cells, and the extent of metastasis. Two systems are commonly used for staging prostate cancer: the Jewett-Whitmore system and the TNM (Tumor, Node, Metastases) system (Figure 1-1B). Early-stage prostate cancers are confined in the prostate, but late-stage prostate cancers have invaded into the surrounding tissues or to distant sites, most frequently, the skeletal bones. Early-stage prostate cancers are considered curable while the prognosis of late-stage prostate cancers is still discouraging.

Current options for treatment of prostate cancer include prostatectomy (surgery to remove all or part of the prostate), radiation therapy (killing cancer cells and surrounding tissue cells with radioactive exposure), hormone/androgen-deprivation therapy (preventing hormone to act on

prostate cancer cells), and chemotherapy (using chemicals to kill or inhibit cancer cells). Patients with early-stage prostate cancer which is confined to the prostate usually undergo radical prostatectomy. Radiation therapy is often used as an initial treatment for local and regional prostate cancer, and the cure rate has been improved in recent years (Speight and Roach, III, 2007). Hormone therapy has been controversial for decades because androgen-deprivation therapy usually initiates several biological changes that may induce the development of hormone refractory prostate cancers. But it still remains in practice as an adjuvant therapy to fight recurrent and advanced prostate cancers. Chemotherapy has been used with great success in the treatment of advanced or metastatic prostate cancers. A major drawback, however, is that it does not distinguish between tumor cells and normal cells. Developing molecularly targeted therapies that specifically kill the tumor cells but have minimal effects on normal cells, therefore, is the main challenge to prostate cancer care and management today. Understanding the molecular mechanisms of prostate cancer is critical to developing specific therapies.

In prostate cancer research, several immortalized and malignant adult human prostatic epithelial cell lines have been developed, such as the LNCaP, DU-145, and PC-3 (Webber et al., 1996; Webber et al., 1997a; Webber et al., 1997b). The LNCaP cell line was isolated in 1977 from a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male with confirmed diagnosis of metastatic prostate carcinoma. It expresses both the androgen receptor (AR) and estrogen receptors (ER) and is responsive to 5-alpha-dihydrotestosterone (DHT) (Horoszewicz et al., 1980). The LNCaP cells are tumorigenic in nude mice; the tumor take and growth in male mice are significantly higher than that in female mice. The DU-145

cell line was isolated from the brain lesion of a patient with metastatic carcinoma of the prostate and a history of lymphocytic leukemia (Stone et al., 1978). Cytogenetic analysis reveals a hypotriploid phenotype with an abnormal Y chromosome and a single copy of X chromosome. DU-145 cells are poorly sensitive to hormones and do not express the prostate-specific antigen (Papsidero et al., 1981). The PC-3 cell line was established from the bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian (Kaighn et al., 1979). The cell line has a greatly reduced dependence on androgens, glucocorticoids, or growth factors when compared to normal prostate epithelial cells, and presents a near-triploid phenotype with a modal number of 60 chromosomes but a loss of Y chromosome (Chen, 1993). Both DU-145 and PC-3 cells are highly tumorigenic and invasive (Mickey et al., 1980).

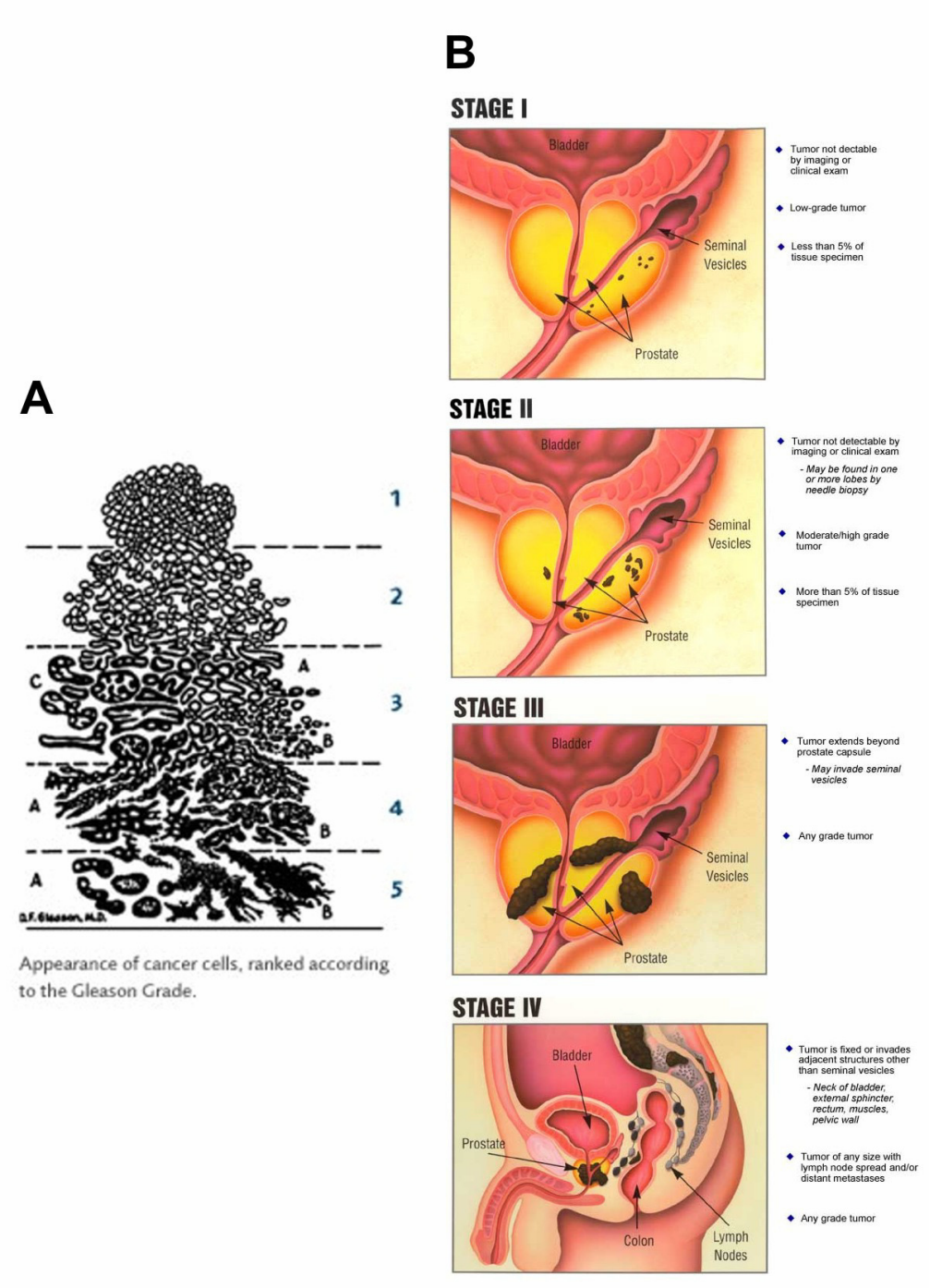


Figure 1-1 Schematic representation of Gleason grade (A) and prostate cancer staging (B).

Cited from Prostate Cancer Foundation website (http://www.prostatecancerfoundation.org)

1.2 Prostasin: a Multi-functional GPI-anchored Serine Protease on the Epithelial Cell Surface

1.2.1 Prostasin gene structure, expression pattern, and cellular localization

Prostasin/PRSS8, also named CAP-1 (Channel Activated Protease-1), was originally purified from human seminal fluid as a 40-kD active trypsin-like serine protease (Yu et al., 1994). It is abundantly expressed in the bronchus, kidney, lung, prostate and placenta in mice and humans. Low-level expression of prostasin can also be found in other tissues including the bladder, colon, heart, liver, mammary gland, pancreas, salivary gland and thyroid gland (Yu et al., 1995; Vuagniaux et al., 2000a; Fan et al., 2005). By immunohistochemical staining of prostasin in mouse prostate, lung and bladder tissues, protein expression of prostasin was localized mainly to the epithelial cells in the tissues (Verghese et al., 2004b; Chen et al., 2006k).

The human prostasin gene (PRSS8) and the mouse prostasin gene (mCAP-1) are both single-copy genes located on human chromosome 16p11.2 and mouse chromosome 7, respectively. The human and mouse prostasin genes share the six-exon and five-intron genomic organization, wherein the catalytic triad His-Asp-Ser residues are encoded by different exons (Verghese et al., 2004b; Yu et al., 1996). The human prostasin gene encodes a protein of 343 amino acids, including a 32-amino acid signal peptide and a 311-amino acid pro-prostasin. The pro-prostasin is further cleaved between Arg12 and Ile13 to generate the two-chain mature form of prostasin. The physiological substrate of prostasin, however, still remains unidentified.

In human prostate and mouse bladder tissue sections, prostasin expression was detected in the cytoplasm and at the apical membrane of the epithelial cells (Chen et al., 2006k; Chen et al.,

2001a). Epithelial cell lines derived from airway epithelia (Tong et al., 2004) and the cortical collecting duct (Vuagniaux et al., 2000a) also express prostasin. Prostasin localizes at the apical surface through a glycosylphosphatidylinositol (GPI) anchor (Chen et al., 2001b) and can be secreted from the surface through shedding off by GPI-specific phospholipase D1 (Verghese et al., 2006). All currently-known biological functions of prostasin are dependent on its membrane-anchorage with the GPI moiety; the physiological function of the secreted prostasin is unclear.

1.2.2 Functions of prostasin in epithelial sodium channel activation

The epithelial sodium channel (ENaC) plays a critical role in sodium balance at the apical surface of epithelial cells lining the cortical and collecting ducts of the kidney, the distal colon, the ducts of secretory glands such as prostate and salivary glands, and the respiratory airways (Varez de la et al., 2000). ENaC mediates Na^+ absorption with high selectivity (permeability to sodium/permeability to potassium > 20), high electric resistance/low unitary conductance (4–5 picosiemens in the presence of sodium), and high sensitivity to amiloride (inhibition constant: 0.1 μM) (Rossier, 2004). ENaC is made of three homologous subunits: α, β, and γ (Canessa et al., 1994) and the most common form of ENaC is a heteromultimeric protein consisting of two α subunits, one β subunit and one γ subunit (Eskandari et al., 1999). Each subunit of ENaC contains two transmembrane domains, a large extracellular loop with multiple N-glycosylation sites, and short cytoplasmic amino- and carboxyl- regions.

ENaC activity is mainly regulated by two hormones: aldosterone and vasopressin. Aldosterone is a steroid hormone synthesized in the adrenal cortex and controls Na^+ and K^+ balance in the blood. Aldosterone acts on the mineralocorticoid receptor to stimulate sodium resorption by the epithelial cells lining the cortical collecting ducts and distal colon by activating the ENaC expressed in the apical membrane and the Na^+/K^+ATPase pump expressed in the basolateral membrane (Varez de la et al., 2000). Vasopressin, also known as antidiuretic hormone (ADH), is a peptide hormone synthesized in the hypothalamus and transported to the posterior pituitary. Vasopressin is released when the body is low on water, and causes the kidney to retain water by stimulating the apical absorption of sodium, leading to concentration of urine and increase of blood pressure. Vasopressin increases the ENaC activity through a cAMP-dependent mechanism. Whether the modulation of the ENaC activity is mediated by increase of the open probability (P_o) or an increased number (N) of the channels on the apical membrane is still controversial (Rossier, 2002).

In addition to the classic hormone pathways that regulate ENaC activities, ENaC can also be regulated by several other molecules in completely different pathways, including the Channel Activated Proteases (CAPs) (Rossier, 2004), the ubiquitin ligase Nedd4 (Staub et al., 1996), and the ABC transporter CFTR (Kunzelmann et al., 1997). The ENaC-activation activity of CAPs was first identified in 1997 from the data that the amiloride-sensitive Na+ transport in a *Xenopus* kidney epithelial cell line (A6) was inhibited by the exposure of the apical membrane to the serine proteinase inhibitor aprotinin (Vallet et al., 1997). *Xenopus* CAP (xCAP-1) was then cloned using A6 cells and a functional complementation assay, and its mammalian orthologue

was identified to be prostasin. Co-expression of *Xenopus* prostasin (xCAP-1), mouse prostasin (mCAP-1) or rat prostasin with the α-, β-, and γ-subunits of ENaC in Xenopus oocytes showed that prostasin significantly increased the ENaC-mediated amiloride-sensitive sodium current (Adachi et al., 2001; Vuagniaux et al., 2000b; Vallet et al., 1997). The serine protease activity and the GPI-anchorage but not the N-glycosylation of Xenopus prostasin (xCAP-1) were shown to be required for its ENaC activation role (Vallet et al., 2002). Two additional membrane serine proteases that regulate ENaC activity were further identified in a mouse cortical collecting duct (CCD) cell line: mCAP-2 and mCAP-3 (Vuagniaux et al., 2002). mCAP-2 is the mouse homologue of TMPRSS3 and mCAP-3 is the mouse homologue of matriptase/MT-SP1/ST14 found in humans. Unlike xCAP-1, the catalytic activity of mCAP-1 is not required for it role in the ENaC activation (Andreasen et al., 2006).

The precise molecular mechanism by which prostasin activates ENaC remains unclear until recently. Bruns JB et al. reported that prostasin activates ENaC by inducing a cleavage of the γ-subunit at a site distal to the furin cleavage site (Bruns et al., 2007). The ENaC with a double-cleaved γ-subunit presents higher open probability and the 43-amino acid peptide released by the cleavage serves as an inhibitor of ENaC activity as a feedback regulation. The serine active-site mutant prostasin could also induce the γ-subunit cleavage, consistent with the previous finding that the catalytic activity of mCAP-1 is not required for ENaC activation (Andreasen et al., 2006). Prostasin does not activate a mutant ENaC that lacks the furin-dependent cleavage site in the γ-subunit, suggesting that its activation of ENaC requires pre-processing of ENaC by furin.

1.2.3 Functions of prostasin in tumor biology

Prostasin belongs to a unique subfamily of trypsin-like membrane serine proteases tethered to the outer-surface of the plasma membrane through a GPI linkage. Several new family members have been identified by homology cloning (Netzel-Arnett et al., 2003), but only prostasin and testisin (Nakamura et al., 2003; Hooper et al., 2000) have been investigated for potential functions. Both prostasin and testisin are lost in expression in advanced cancers and capable of suppressing tumor invasion or growth (Hooper et al., 1999; Manton et al., 2005b; Chen et al., 2001a; Chen and Chai, 2002). In immunohistochemical analysis of human prostate sections from patients with prostate cancer, the protein expression of prostasin was shown to be positive in 89% of benign areas and 93% of the low-grade prostate tumors, while percentages of the prostasin-positive staining decreased in high-grade prostate tumors, with 44% in Gleason grade 3 and 15% in Gleason grade 4-5 tumors, respectively (Chen et al., 2001a). Down-regulation of human prostasin gene expression is associated with the hormone-refractory phenotype in prostate cancer patients (Takahashi et al., 2003). In highly invasive human prostate and breast cancer lines DU-145, PC-3, MDA-MB-231, and MDA-MB-435, the expression level of prostasin is much lower than in the non-invasive cell lines LNCaP or MCF-7; prostasin re-expression significantly reduces *in vitro* invasion of these malignant cancer cells (Chen et al., 2001a; Chen and Chai, 2002). The down-regulation of prostasin expression in the malignant cancer cell lines is partly due to hypermethylation at the 5'-flanking region of the prostasin gene, but the prostasin gene is not completely silenced by these epigenetic events because it can be up-regulated by nerve growth factor (NGF) (Chen and Chai, 2002; Chen et al., 2004). At the post-translational level, several serine protease inhibitors of prostasin have been identified: protease nexin-1 (PN-1)

and hepatocyte growth factor activator inhibitor-1B (HAI-1B) (Chen et al., 2004; Fan et al., 2005). PN-1 belongs to the serpin family and it forms a SDS/heat-resistant complex with prostasin through a covalent linkage. HAI-1B is a membrane-associated extracellular Kunitz-type serine protease inhibitor. Highly up-regulated expression of these prostasin inhibitors has been reported in breast, prostate and pancreatic cancers (Parr and Jiang, 2006; Nagakawa et al., 2006; Yekebas et al., 2006), suggesting an imbalance of proteases and inhibitors in these malignancies. The mechanisms by which prostasin suppresses cancer cell invasion and is down-regulated in poor-prognosis tumors have not been investigated.

1.2.4 Other functions of prostasin in epithelial cells

Prostasin also plays several other important roles in the epithelia of different tissues in addition to acting as an ENaC activator and tumor invasion suppressor.

(1) *Prostasin is required for epidermal permeability barrier function* (Leyvraz et al., 2005). Conditional knockout mice lacking prostasin expression in the skin died 60 hours after birth due to aberrant skin development which lead to impaired skin barrier function and fatal dehydration. Prostasin-deficient skin cells present disturbed the stratum corneum (SC) lipid composition, abnormal corneocyte morphogenesis, and incomplete processing of profilaggrin. Loss of prostasin expression is also associated with an absence of occludin, a key tight-junction protein, suggesting that prostasin is required for the tight junction formation.

(2) *Prostasin attenuates inflammation-responsive gene expression in the bladder epithelium* (Chen et al., 2006k). Prostasin is expressed in the normal epithelium of the mouse bladder, and its expression is down-regulated during inflammation induced by lipopolysaccharide (LPS). With forced prostasin expression, the LPS-induced up-regulation of the inducible nitric oxide synthase (iNOS) gene was significantly reduced.

(3) *Prostasin may be involved in endometrial epithelial morphogenesis, tissue remodeling, and trophoblastic invasion during early pregnancy* (Lin et al., 2006). Prostasin expression was shown to be spatially and temporally regulated during the progress of pregnancy in rhesus monkey. Prostasin is abundantly expressed in the glandular epithelium on Day 12 and 18, followed by a significant decrease in expression. Expression of prostasin was detected in the placental villi, trophoblastic column, trophoblastic shell, and fetal-maternal border on Day 18 and further enhanced on Day 26 of pregnancy. The expression level of PN-1, the serpin-class inhibitor of prostasin, is rather low during these early stages of pregnancy, potentially creating an environment favorable for prostasin's catalytically-related functions during embryo implantation and trophoblastic invasion.

(4) Prostasin regulates epithelial monolayer function in cortical collecting duct cells (Verghese et al., 2006). In addition to activating amiloride-sensitive sodium transport, expressing prostasin in the M-1 mouse cortical collecting duct cell line regulates transepithelial resistance, current and paracellular permeability, requiring its GPI-anchor and proteolytic activity.

1.3 Targeting of Epidermal Growth Factor Receptor (EGFR) Signaling Pathways in Cancers

1.3.1 The epidermal growth factor receptor (EGFR) family

The epidermal growth factor receptor (EGFR), also named (HER1), is a member of the HER family of membrane receptor tyrosine kinases (RTK's), which also includes HER2 (also named ErbB2 or HER2/*neu*), HER3 (also named ErbB3), and HER4 (also named ErbB4). All four RTK's share similar molecular structures with an N-terminal extracellular ligand-binding domain, a single alpha-helix transmembrane domain, and an intracellular tyrosine kinase domain in the C-terminal tail (Wells, 1999). Under physiologic conditions, epidermal growth factor (EGF), transforming growth factor α (TGFα), and amphiregulin bind exclusively to EGFR. Heparin-binding EGF-like growth factor (HB-EGF), β-cellulin and epiregulin bind to both EGFR and HER4, and heregulin binds to HER3 and HER4 (Ono and Kuwano, 2006). Ligand binding leads to EGFR dimerization as a homodimer or a heterodimer with other HER proteins (Yarden and Sliwkowski, 2001), further inducing autophosphorylation at specific tyrosine residues inside the intracellular domain and activating downstream signaling pathways. No high-affinity ligand has been identified for HER2, but HER2 can form heterodimers with other HER family RTK's. HER2 is the preferred heterodimerization partner of EGFR as well as other ErbB receptors after ligand-induced activation (Graus-Porta et al., 1997), and it is an important signaling partner for EGFR. In a mammary epithelial cell system, the EGFR/HER2 heterodimer was shown more stable and recycling more rapidly to the cell surface than the EGFR/EGFR homodimer, suggesting that HER2 serves as an EGFR signaling amplifier during cancer progression (Worthylake et al., 1999).

1.3.2 Ligand-induced, receptor mediated dimerization and activation of EGFR

The full-length human EGFR gene consists of 110-kbp of DNA with 26 exons (Haley et al., 1987), and encodes a 1210-amino acid precursor protein with a 24-amino acid signal peptide (Ullrich et al., 1984). The 621-amino acid extracellular region of EGFR contains four subdomains, designated domains I, II, III, and IV (Bajaj et al., 1987). Domains I and III (also termed L1 and L2) are members of the leucine rich repeat (LRR) family while Domains II and IV (also termed CR1 and CR2) contain multiple small disulfide-bonded modules(Burgess et al., 2003; Abe et al., 1998). The crystal structure of EGFR showed that the L1 and L2 domains form a six-turn right-hander β helix which is capped at each end by an α helix and a disulfide bond, and the CR1 and CR2 domains adopt laminin-like folds (Burgess et al., 2003).

It has been established that two EGFR ligands are bound in the ligand-induced dimer, forming a 2:2 complex with the extracellular regions of EGFR (Domagala et al., 2000). It was previously speculated that EGF induces EGFR dimerization by crosslinking two EGFR molecules, however, the crystal structures of ligand-bound sEGFR (extracellular region of EGFR) showed that the dimerization is entirely receptor mediated (Garrett et al., 2002; Ogiso et al., 2002). The two ligand molecules in the ligand-EGFR complex could hardly form the dimer interface, and the dimer interface is mainly provided by Domain II. The crystal structure of non-activated sEGFR showed that the relationship between Domains II and III dramatically differs from that in the activated form (Ferguson et al., 2003). The intramolecular interaction between the two cysteine-rich Domains II and IV restrains the relationship between II and III in the non-activated sEGFR and also buries the dimerization arm of Domain II. The current consensus model of

ligand-induced EGFR dimerization is shown in Figure 1-2A. Without ligands, EGFR is mainly presented in a tethered monomer configuration which can only form low-affinity interaction with ligands and can not dimerize. When ligands are present, the ligand binding induces a 130^{o} rotation of Domains I and II, traps the extended configuration, and reveals the dimerization arms in Domain II for the dimerization.

1.3.3 Tyrosine kinase domain and C-terminal regulatory domain of EGFR, where multiple signaling pathways are originated and integrated.

The intracellular part of EGFR consists of three major subdomains: the juxtamembrane region, the tyrosine kinase domain and a C-terminal regulatory domain (Figure 1-2B). EGFR signaling is initiated by ligand binding, followed by homo- or hetero- dimerization of the receptor and subsequent autophosphorylation by its kinase domain. The phosphorylated tyrosine residues in the cytoplasmic domain of EGFR serve as docking sites for downstream signaling pathways. Unlike many receptor tyrosine kinases (RTKs), the tyrosine kinase domain of EGFR lacks the autoinhibitory intracellular interactions (Burgess et al., 2003). Crystal structure showed that EGFR activation loop adopts the conformation normally seen in the activated tyrosine kinases even when the center tyrosine residue Y845 is not phosphorylated (Stamos et al., 2002). Mutation of the analogous tyrosine in other tyrosine kinase receptor reduces the catalytic activity. In contrast, the catalytic activity of EGFR was not affected when the tyrosine residue at position 485 was substituted by phenylalanine. The currently accepted model of EGFR activation is that the kinase domain of EGFR is constitutively active and the ligand-induced dimerization of

EGFR delivers substrates, such as the dimer partner's C-terminal regulatory region, to the kinase active site for the trans-autophosphorylation.

Multiple tyrosine residues of the EGFR cytoplasmic domain are autophosphorylated upon EGFR dimerization and further recruit various downstream signaling molecules (Figure 1-2B). The Grb2 (Growth factor receptor-binding protein 2) adaptor protein binds to pY1068 and pY1086, the Dok-R adaptor protein binds to pY1086 and pY1148, and the Shc adaptor protein binds to pY1148 and pY1173. The phosphatase PTB-1B can interact with pY992 and pY1148 and the SHP-1 phosphatase binds pY1173. The phospholipase PLC-γ is recruited to pY992 and pY1173 while the Abl tyrosine kinase binds to pY1086. The ubiquitin ligase c-Cbl is recruited to pY1045 (Sebastian et al., 2006). Upon binding to these phosphorylated tyrosine residues, these molecules further recruit or activate downstream targets to trigger the signaling pathways. For example, an extensively studied EGFR signaling pathway is the mitogen-activated protein kinase (MAPK) pathway. The adaptor protein Grb2 is recruited to the phosphorylated tyrosine residues of EGFR through its SH2 domain. Translocation of the Grb2/Sos complex to the plasma membrane further facilitates the Sos-mediated activation of Ras proteins (Lowenstein et al., 1992). The activation of Ras sequentially induces the activation of Raf family kinases, the mitogen activated extracellular singal regulated kinases (MEKs), and finally extracellular signal regulated kinases (Erk1/2). Once activated, Erk1 and Erk2 activate major transcription factors associated with cell proliferation to stimulate cell growth (Murphy et al., 2002). In addition to the classic EGFR/Ras/Raf/MEK/ERK pathway, several other signaling pathways are also triggered by ligand-activation of EGFR: the EGFR/PI3K/PDK1/Akt survival pathway to prevent

programmed cell death, promoting sustained proliferation; the EGFR/JAK/STAT pathway to up-regulate expression of genes contributing to cancer cell survival; the PLC-γ dependent pathway to reorganize actin cytoskeleton; and signaling pathways involved in angiogenesis and metastasis (Sebastian et al., 2006).

1.3.4 Vesicular trafficking of EGFR: internalization, degradation, recycling and nuclear translocation.

The function of membrane signaling receptors is tightly regulated through post-translational modifications, including internalization, degradation and dephosphorylation. As for EGFR, receptor activation not only triggers multiple positive signaling pathways but also pathways to shut down the signaling. Once the ligands bind to EGFR at the cell surface and activate the receptor, the ligand-receptor complexes are internalized through clathrin-dependent or clathrin-independent pathways (Sigismund et al., 2005; Gorden et al., 1978). The clathrin-dependent internalization begins with recruitment of soluble clathrin molecules to the plasma membrane region where activated EGFR is located, and formation of clathrin-coated pits (CCP). Following EGF binding, activated EGFR recruits the E3 ubiquitin ligase, c-Cbl, to the intracellular domain either through direct interaction between phosphorylated tyrosine 1045 or via indirect binding through the adaptor protein Grb2 (Huang and Sorkin, 2005). This leads to phosphorylation and a conformational change of c-Cbl, resulting in enhancement of its interaction with the Cbl-interacting protein of 85k (CIN85). CIN85 further recruits disabled2 (DAB2) and endophilin to drive clathrin assembly and budding (Soubeyran et al., 2002). The ubiquitinated EGFR also serves as docking sites for Esp15 (EGFR pathway substrate 15) and

epsin, which in turn recruit clathrin interacting proteins such as AP2 and dynamin-2 to facilitate clathrin-coated vesicle (CCV) budding and release. Following CCV internalization, the receptor-ligand complexes are transferred to early endosomes for recycling and degradation. The endocytosed EGFRs for degradation remain ubiquitinated and are sorted to multi-vesicular bodies (MVBs) which mature to form late endosomes and further fuse with pre-existing lysosomes for degradation (Miller et al., 1986). In addition to the ubiquitination by c-Cbl, recent studies have also shown that the dileucine (LL)-motif (679-LL) of EGFR is required for EGF-EGFR endosome retention as well as lysosome sorting (Tsacoumangos et al., 2005). The mild acidic environment in the early endosome facilitates dissociation of the ligand-receptor complex and recycling of EGFR back to the plasma membrane (Xie et al., 2004).

EGF signaling can be directly transmitted into the nucleus through EGFR nuclear transport (Lo and Hung, 2007; Carpenter, 2003). Ligand stimulation induces accumulation of EGFR in the nucleus and detection of EGF and TGF-α in the nucleus of proliferating cells (Raper et al., 1987). Interestingly, some of EGFR exists in the nucleus as intact protein (Lin et al., 2001) but the mechanism for nuclear transport of the free receptors still remains elusive. The EGFR nuclear-cytoplasmic trafficking is mediated by the importins α1/β1 and exportin CRM1 (Chromosome Region Maintenance -1), dependent on the putative NLS (nuclear localization sequence) with three clusters (underlined) of basic amino acids (^{645}RRRHIVRKRTLRR^{657}) in the juxtamembrane region of EGFR (Lo et al., 2006b; Hsu and Hung, 2007). Nuclear EGFR was recently found to function as a transcription co-activator for the cyclin D1 gene and the iNOS gene (Lin et al., 2001; Lo et al., 2005). Cyclin D1 is a key positive regulator of cell

proliferation, while up-regulation of the iNOS gene expression has been associated with tumor growth and angiogenesis. Activation of the iNOS gene by nuclear EGFR is dependent on the physical interaction between EGFR and STAT3 (signal transducer and activator of transcription-3) and EGFR-mediated phosphorylation of STAT3 (Lo et al., 2005).

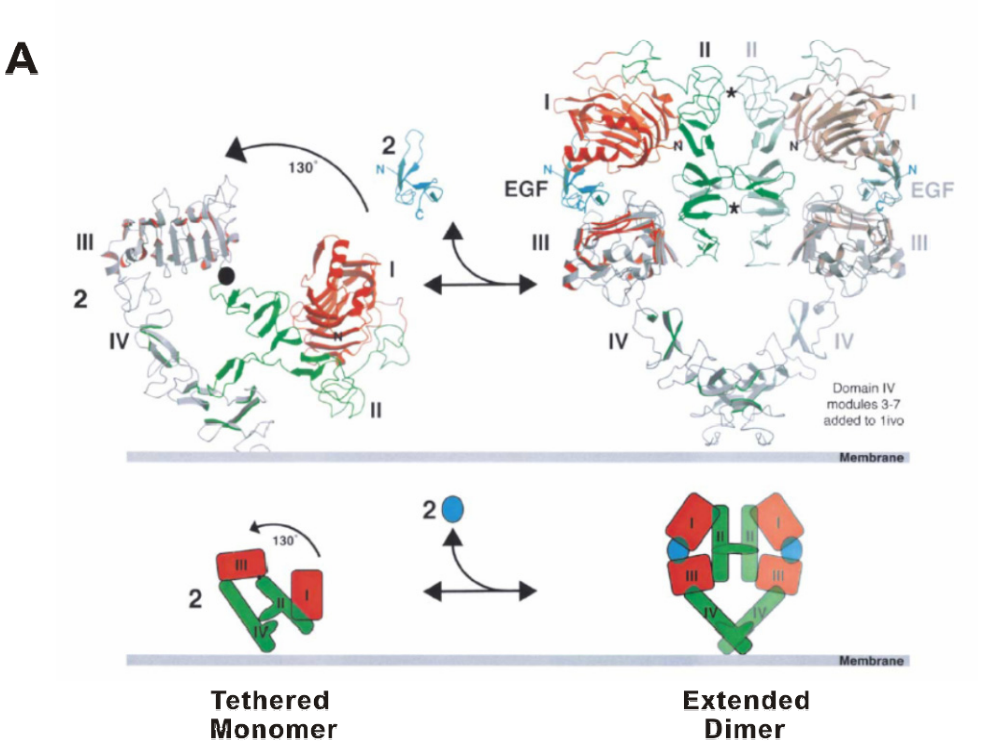
1.3.5 EGFR signaling in cancers and current therapeutic methods targeting EGFR

Under normal physiological conditions, EGFR is essential for epithelial cell functions. Knock-out mutations of EGFR results in developmental defects in the epithelial structures of epidermis, mammary gland, lung, pancreas, intestine, and central nerve system (Miettinen et al., 1995). On the other hand, dys-regulation of EGFR signaling is associated with many types of cancers including prostate cancer (Bellezza et al., 2006; Lo et al., 2006a; Normanno et al., 2006; Ahmed and Salgia, 2006). By overproducing EGFR ligands like EGF and TGF-α, tumor cells stimulate their proliferation through an autocrine loop (Sato, 1999). Especially for hormone-resistant breast and prostate cancers, this autocrine signaling allows the tumor cells to grow independently of hormones. By overexpression of EGFR, tumor cells produce intense signal generation and amplification and activation of downstream signaling pathways, resulting in more aggressive cell phenotypes. In prostate cancers, EGFR is weakly expressed in the benign tissues and in areas of low-grade PIN (prostatic intraepithelial neoplasia) while it is abundantly expressed in high-grade PIN and neoplastic cells (Harper et al., 1998). EGFR signaling functions can be potentiated and enhanced by dys-regulated expression and activation of other synergistic signaling molecules including HER2, G-protein coupled receptors, tyrosine kinase Src, and hormone receptors (Yarden and Sliwkowski, 2001). Genetic alterations of

EGFR were also found in many cancers. The mutations are usually deletion and tandem duplication of the EGFR gene or site-mutations in the kinase domain (Normanno et al., 2006). The most frequent variant of EGFR is the EGFRvIII, characterized by the lack of amino acid residues 6-276 in the extracellular ligand-binding domain. EGFRvIII is a constitutively activated tyrosine kinase capable of stimulating cell proliferation without ligand interaction. Overexpression of the mutation has been found in many types of human cancers, especially in glioblastoma, breast and prostate cancer (Pedersen et al., 2001).

The ultimate goal of cancer therapy is to specifically target cancer cells to stop their proliferation and metastasis without affecting normal cells. Given its causative role in cancer and overexpression during cancer development, EGFR has been proposed for an anti-cancer target for many years. Currently, the most extensively studied drugs targeting EGFR are small molecules that act on the intracellular tyrosine kinase domain and monoclonal antibodies that target the extracellular domain of EGFR (Gschwind et al., 2004; Goel et al., 2002). The monoclonal antibody drugs targeting EGFR such as the ImCloneC225/Erbitux/Cetuximab exhibit higher specificity and lower working concentration than the small-molecule compounds such as the tyrosine kinase inhibitor ZD1839/Gefinitib, but fail to recognize several EGFR mutants, which miss part of the extracellular domain. Some cancer cells can also develop resistance to these drugs by constitutive activation of downstream signaling molecules to bypass the receptor (Ono and Kuwano, 2006). In cells without clearly identified constitutively activated downstream effectors, the mechanisms of resistance are unknown. Understanding not only the signal pathways activated by EGFR but also the regulation of the receptor expression,

post-translational modification, trafficking and turnover will provide more information on designing new anti-cancer therapy methods and improve our ability to predict patient response to anti-EGFR therapies.



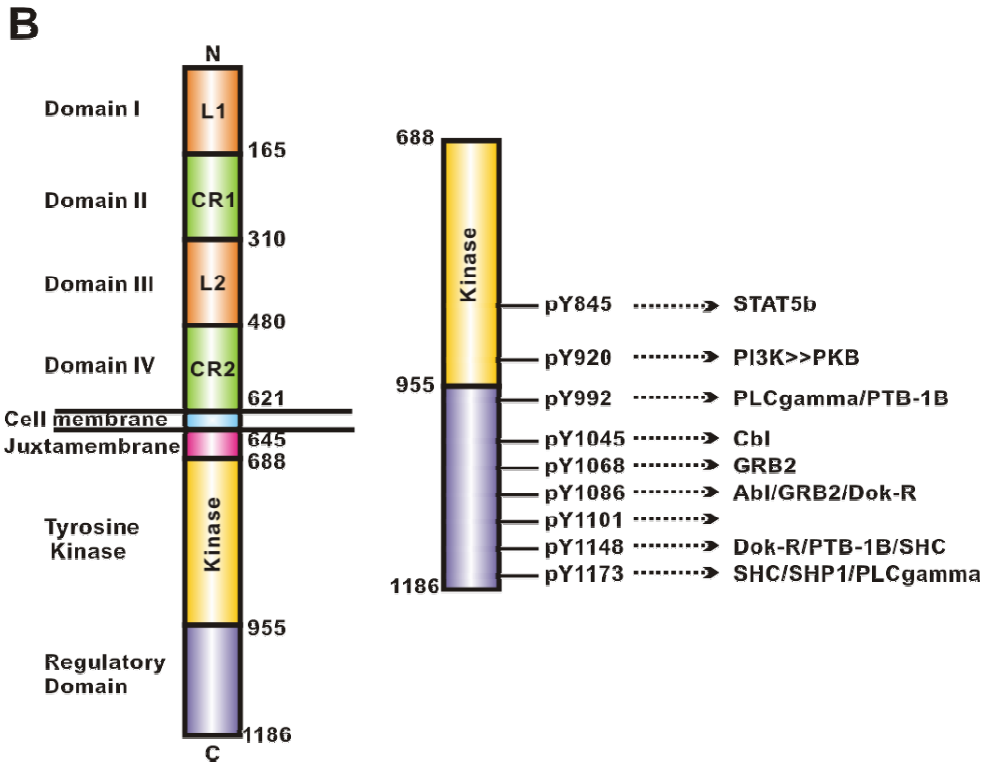


Figure 1-2 Schematic of domain organization of EGFR and ligand-Induce dimerization.

(A) *Schematic of Ligand-Induced conformational changes in sEGFR and dimerization* (cited from Burgess et al., 2003). The unactivated (tethered) sEGFR structure is shown on the left. A model of the EGF-induced dimer is shown on the right. This model uses the coordinates of L domains in the receptor (domains I and III) are colored red, and CR domains (domains II and IV) are green. Ligand is colored cyan. Domains I and III are distinguished from one another by the addition of gray to the outer surfaces of strands and helices. The two subunits in the dimer are distinguished by the fogging of the right-hand dimerization partner. The speculated position of the plasma membrane is depicted as a gray bar. EGF binding is proposed to induce a 130^{o} rotation of a rigid body containing domains I and II. This exposes the dimerization arm and allows dimerization of sEGFR, as depicted on the right.
(B) *Schematic representation of the Domain organization of EGFR.* The extracellular domain of EGFR consists of four subdomains: domain I and III (L1 and L2, in orange color) and domain II and IV (CR1 and CR2, in green color). The cytoplasmic region of EGFR includes a juxtamembrane domain (magenta), tyrosine kinase domain (yellow), and C-terminal regulatory domain (purple). Residue numbers for domain boundaries are marked. Major tyrosine residues which are phosphorylated after EGFR activation and the downstream signaling molecules are indicated in the right panel.

CHAPTER TWO PROSTASIN INDUCES PROTEASE-DEPENDENT AND INDEPENDENT MOLECULAR CHANGES IN THE HUMAN PROSTATE CARCINOMA CELL LINE PC-3

2.1 Introduction

Since its discovery more than a decade ago, prostasin, a glycosylphosphatidylinositol (GPI)-anchored extracellular serine protease has been shown to play important roles in epithelial physiology. These functional roles include suppression of invasion (Chen et al., 2001a; Chen and Chai, 2002), regulation of gene expression during inflammation (Chen et al., 2006j), and activation of the epithelial sodium channel (ENaC) (Bruns et al., 2007).

Activation of ENaC by prostasin is mechanistically the best defined function for prostasin, with very recent data consistent with a proteolytic cleavage of the gamma subunit of ENaC by prostasin (Bruns et al., 2007). The other in vitro and in vivo phenotypic changes induced by prostasin remain mechanistically undefined. We undertook the current study to probe into the potential molecular mechanisms by which prostasin impacts the cell's behavior. We used the PC-3 human prostate carcinoma cell line, which has a compromised prostasin expression due partly to promoter DNA hypermethylation (Chen et al., 2004). Upon prostasin re-expression the PC-3 cells displayed reduced in vitro invasion through a Matrigel barrier (Chen et al., 2001a). We focused on the extracellular tumor cell invasion molecular players that were previously established for this cell line, because prostasin is an active serine protease anchored to the outside of the plasma membrane (Chen et al., 2001b).

The epidermal growth factor receptor (EGFR) was the first member of the erbB family of receptor tyrosine kinases to be discovered and the first cell surface receptor to be shown with a role in cancer biology (de Larco and Todaro, 1978). EGFR overexpression is implicated for a causative role in ten different types of solid tumor (Nicholson et al., 2001). The cellular signals that are initiated by activation of this receptor tyrosine kinase confer cancer cells with advantages in angiogenesis, growth, and motility (Oliveira et al., 2006). The PC-3 cell line is a high expresser of EGFR (Ching et al., 1993), which is a major mediator of prostate cancer cell motility and invasiveness (Zhou et al., 2006). The urokinase-type plasminogen activator (uPA), a serine protease, through interactions with its membrane receptor, the GPI-anchored uPAR, also promotes prostate cancer invasiveness (Liu and Rabbani, 1995). The invasion promoting effect of the uPA-uPAR signaling route in the PC-3 cell line is modulated by an EGFR tyrosine kinase inhibitor (AG1478) (Skogseth et al., 2006), reducing uPA production (Skogseth et al., 2005). E-Cadherin, a cell-cell adhesion molecule and a tumor suppressor, is down-regulated in the PC-3 cell line (Morton et al., 1993), contributing to its invasive behavior (Davies et al., 2000). The PC-3 cell line also expresses the granulocyte-macrophage colony-stimulating factor (GM-CSF), which, acts in an autocrine loop to stimulate the tumor cells (Savarese et al., 1998). GM-CSF release is also regulated by the EGFR signaling pathway (Blanchet et al., 2004). These target genes and proteins were investigated in PC-3 cells expressing either a wild-type human prostasin or a serine active-site mutant.

The prostasin serine protease can be activated by the type-II transmembrane extracellular serine protease matriptase in vitro and in vivo (Netzel-Arnett et al., 2006c). Inhibition of matriptase

expression in the PC-3 cells resulted in suppression of invasion (Sanders et al., 2006). It is not contradictory to the prostasin invasion-suppression phenotype because the prostasin expression is almost silenced in PC-3 cells. Prostasin expression in the prostate is regulated at the transcription level partly by promoter DNA methylation (Chen et al., 2004), and partly by transcription factors such as the sterol-regulatory element-binding proteins (SREBP's), the SNAIL and the SLUG (Chen et al., 2006l). SLUG is a well-known E-cadherin expression repressor and an inducer of epithelial-mesenchymal transition (EMT) (Moreno-Bueno et al., 2006). The inflammation-associated expression of the inducible nitric oxide synthase (iNOS), but not of the cyclooxygenase-2 (COX-2) was attenuated in the mouse bladder by prostasin (Chen et al., 2006i). In PC-3 cells expressing the wild-type or the mutant prostasin, we also examined the expression states of several cancer cell invasion-related molecules, including these regulators of prostasin expression and genes that are regulated by prostasin, for the purpose of identifying the signaling pathways affected by prostasin.

2.2 Materials and Methods

2.2.1 Materials

The human prostate carcinoma cell line PC-3 (Passage 18) was obtained from the American Type Culture Collection (ATCC, Manassas, VA), and cultured in conditions previously described (Chen et al., 2001a). A polyclonal antibody against human prostasin was described previously (Chen et al., 2001b). Polyclonal antibodies to EGFR (sc-03), Erk1/2 (sc-94), and a monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-32233) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A monoclonal antibody to E-cadherin

(C20820) was purchased from BD Biosciences (San Jose, CA). A monoclonal antibody to human matriptase (M32) was described previously (Oberst et al., 2005b). A polyclonal antibody against phospho-Erk1/2 (V803A) was purchased from Promega (Madison, WI). A recombinant human EGF was purchased from Invitrogen (Carlsbad, CA).

2.2.2 Cell culture and transfection

PC-3 cells were transfected with episomal expression plasmids carrying a wild-type human prostasin cDNA, or an active-site mutant prostasin cDNA, using methods previously described (Chen et al., 2001a). Construction of the episomal expression plasmids and the mutant prostasin cDNA was described previously (Chen et al., 2001a; Chen et al., 2006h). Transfectants harboring the control plasmid, expressing the wild-type prostasin, or expressing the mutant prostasin were plated in 60-mm dishes and cultured to confluence for harvest of the culture medium, and the cells.

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

Confluent monolayers were lysed with the Trizol reagent (Invitrogen) for total cellular RNA isolation per supplier's protocol. Reverse transcription was carried out for each sample using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) per supplier's protocol. Real-time polymerase chain reaction was carried out for each sample using the iQ SYBR Green Supermix (Bio-Rad) per supplier's protocol. PCR programs and methods of quantification were described previously (Chen et al., 2006g). The message number of the GAPDH was used as the reference

for calculating specific gene messages. PCR primers for GAPDH and SLUG were described previously (Chen et al., 2006f; Chen et al., 2006l). PCR primers for the following human gene transcripts are listed in the order of forward and reverse:

COX-2: 5'- CCT CCT GTG CCT GAT GAT TG -3'

5'- ACT GAT GCG TGA AGT GCT G -3'

E-Cadherin: 5'- AGA ATG ACA ACA AGC CCG AAT -3'

5'- CGG CAT TGT AGG TGT TCA CA -3'

EGFR: 5'- CTG ACC AAA ATC ATC TGT GCC C -3'

5'- CGT GGC TTC GTC TCG GAA TT -3'

GM-CSF: 5'- AGC CAC TAC AAG CAG CAC -3'

5'- ACA AGC AGA AAG TCC TTC AG -3'

iNOS: 5'- ATC TCT GGT CAA GCT GGA TGC -3'

5'- GCC TTA TGG TGA AGT GTG TCT TG -3'

Matriptase: 5'- GTC CTG CTC ATC ACA CTG -3'

5'- GTC AAT GTT GGG TGG GTA G -3'

uPA: 5'- GAC ATT GCC TTG CTG AAG -3'

5'- CGG ATA GAG ATA GTC GGT AG -3'

uPAR: 5'- CAC TCA GAG AAG ACC AAC AG -3'

5'- GCA CAA GTC TAA CCC ACA C -3'

2.2.4 Enzyme-linked immunosorbant assay (ELISA)

A QuantikineTM human GM-CSF immunoassay kit (R&D Systems, Minneapolis, MN) was

used for determining the quantities of secreted GM-CSF in the medium collected from the PC-3 transfectant cultures. The medium was collected at 48 hours after a fresh medium change over sub-confluent cultures. The assay was performed per supplier's protocol. Quantities of GM-CSF were expressed as pg/ml after normalizing with the cell number in the culture dishes.

2.2.5 Western blot analysis

Total cell lysate harvested from each cell type at equal amounts for all cell types, was subjected to western blot analysis with appropriate antibodies as described previously (Chen et al., 2001b). Each membrane was re-blotted with a GAPDH antibody as a control for protein loading. For each sample, 40 μg of protein were loaded, except for the Erk-phospho-Erk blot for which 20 μg of protein per sample were loaded. The antibodies were used at the following dilution: prostasin at 1:5,000, GAPDH (sc-32233) at 1:5,000, EGFR (sc-03) at 1:4,000, phospho-Erk1/2 (V803A) and Erk1/2 (sc-94) at 1:5,000, matriptase (M32) at 1:2,500, and E-cadherin (C20820) at 1:2,000. Appropriate secondary antibodies conjugated to horse-radish peroxidase (HRP) were used at 1:10,000, and signals were developed by enhanced chemilluminescence (ECL). Specific target protein signals were digitally quantified and normalized against the quantity of the GAPDH signal.

2.2.6 EGF treatment of cells and analysis of EGF signaling pathway activation

Confluent PC-3 transfectants were placed under serum-free medium for overnight, and EGF treatment of the cultures was performed for 15 minutes at 50 ng/ml concentration of EGF, diluted

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

2.2.7 Statistical Analysis

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

2.3 Results

2.3.1 EGFR signaling pathway is down-regulated by prostasin

To understand the molecular mechanism by which re-expression of prostasin inhibits cancer cell's invasion, we expressed wild-type prostasin and a serine active-site mutant in the PC-3 cells and used Real-time RT-PCR and western blot to measure molecular changes induced by prostasin re-expression. The wild-type and the serine active-site mutant prostasin proteins were expressed in the PC-3 cells at equal quantities, as shown by the results of a western blot analysis (Figure 2-1).

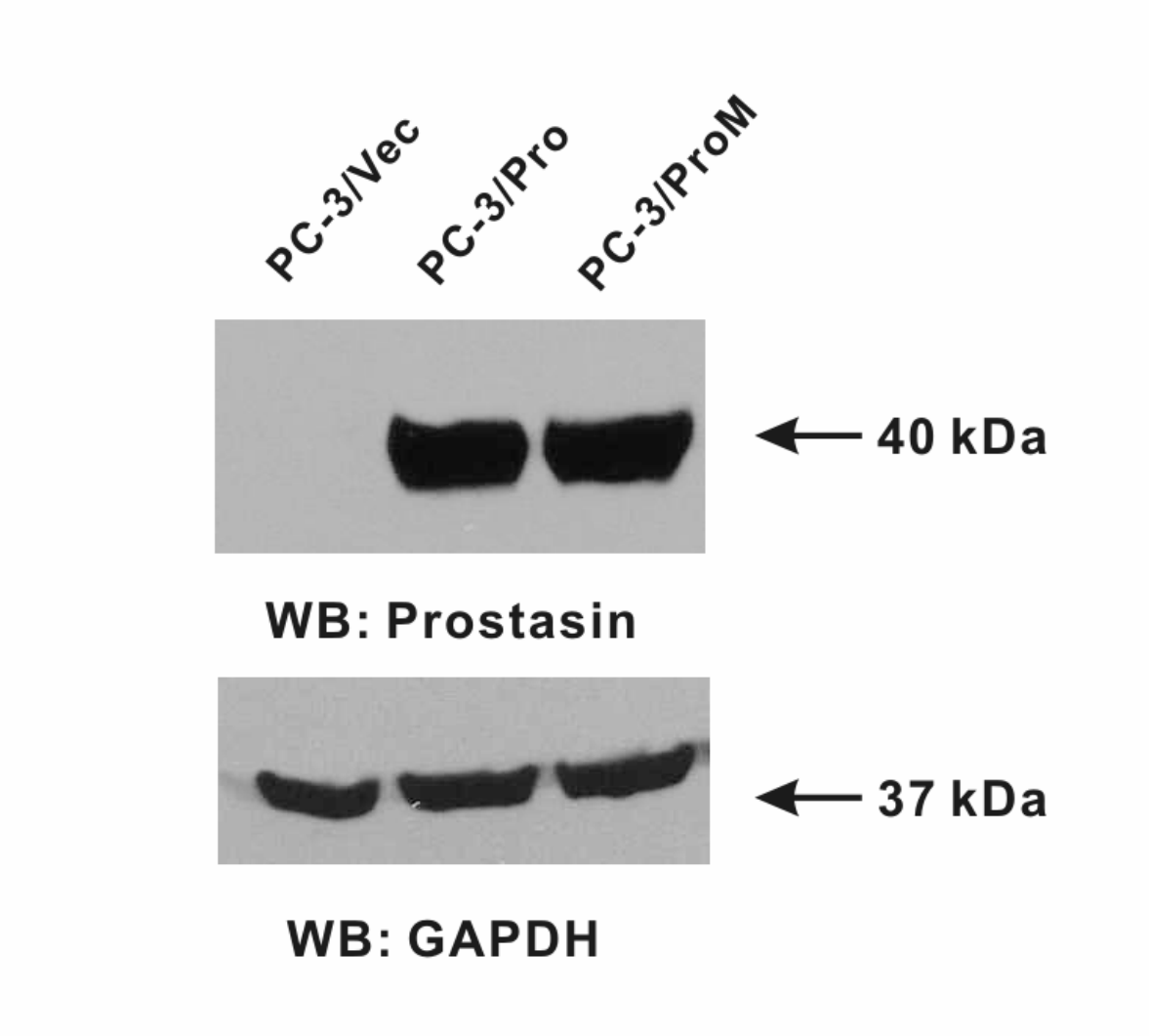


Figure 2-1 Expression of the wild-type and a serine active-site mutant prostasin in PC-3.

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

At the protein level, EGFR expression was down-regulated by the wild-type prostasin and the serine active-site mutant prostasin expressed in the PC-3 cells, by 66% and 48%, respectively, when compared to cells carrying the control plasmid (Figure 2-2A). The wild-type prostasin

expressed in the PC-3 cells also down-regulated EGFR mRNA expression by 34% when compared to cells carrying the control plasmid, while the mutant prostasin did not affect EGFR mRNA expression (Figure 2-2B, and Table 2-1, page 46). At the cellular function level, only the wild-type prostasin was able to inhibit EGF-EGFR signaling, with a 61% reduction of Erk1/2 phosphorylation following EGF stimulation, when compared to cells carrying the control plasmid (Figure 2-2C). The mutant prostasin did not have an effect on Erk1/2 phosphorylation after the EGF stimulation.

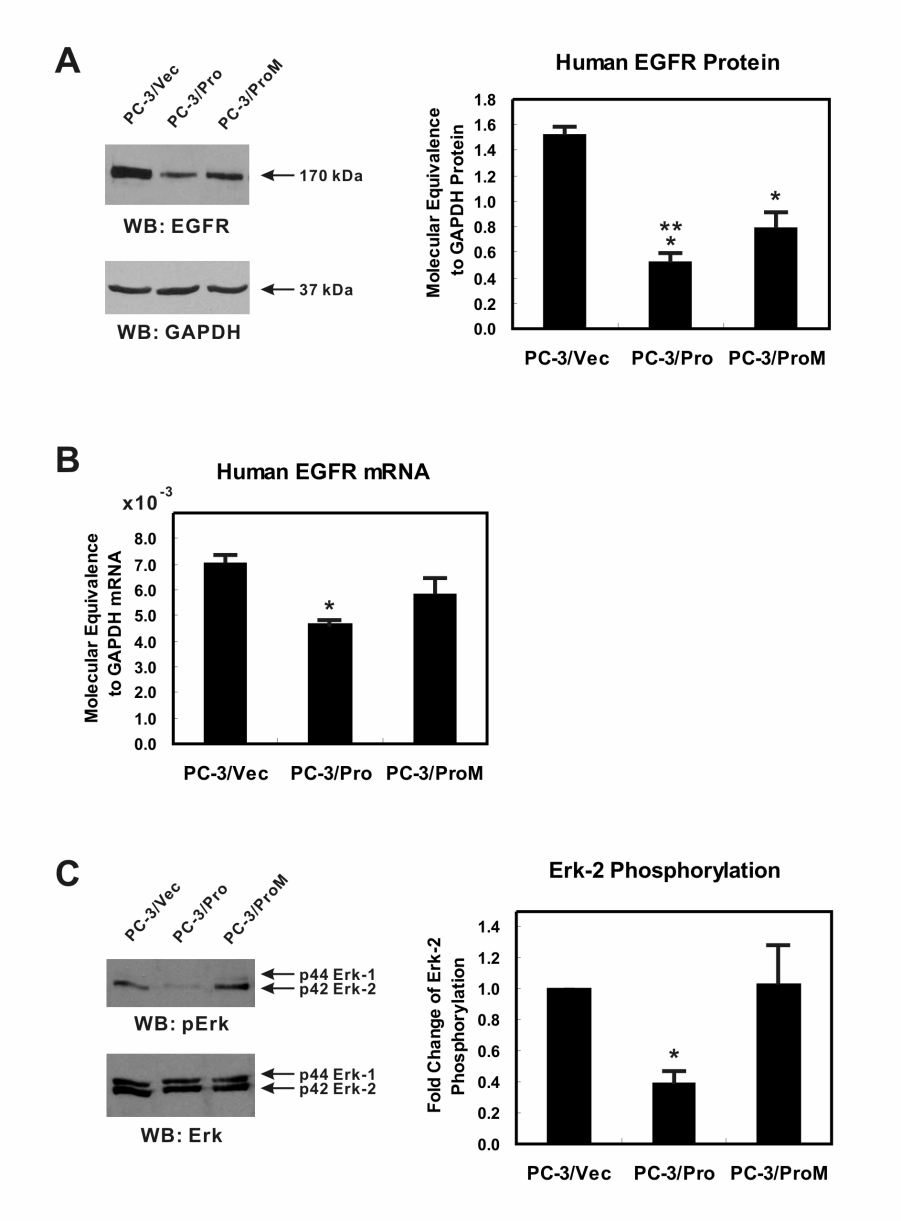


Figure 2-2 Regulation of EGFR expression and EGF-EGFR signaling in PC-3 by the wild-type and the mutant prostasin.

A. Western blot analysis of EGFR in PC-3 transfected with a vector control plasmid (PC-3/Vec), a wild-type human prostasin cDNA plasmid (PC-3/Pro), and a serine active-site mutant prostasin cDNA plasmid (PC-3/ProM). Relative EGFR expression levels between PC-3/Vec, PC-3/Pro, and PC-3/ProM were determined following densitometric measurement of the specific protein bands and normalization against the GAPDH signals, as shown in the bar graph to the right. Calculations were based on data from three independent experiments. The

single-asterisk indicates a statistical difference between the PC-3/Vec and the PC-3/Pro, or between the PC-3/Vec and the PC-3/ProM data groups ($p < 0.05$). The double-asterisk indicates a statistical difference between the PC-3/Pro and the PC-3/ProM data groups ($p < 0.05$).

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

C. Western blot analysis of Erk1/2 phosphorylation in response to EGF stimulation. Relative Erk1/2 phosphorylation levels between PC-3/Vec, PC-3/Pro, and PC-3/ProM were determined following densitometric measurement of the phospho-Erk2 bands and normalization against the total Erk2 bands, as shown in the bar graph to the right. Calculations were based on data from three independent experiments. The single-asterisk indicates a statistical difference between the PC-3/Vec and the PC-3/Pro data groups ($p < 0.05$).

Expression of SLUG, uPA, and COX-2 is down-regulated differentially by the wild-type and the mutant prostasin: At the mRNA level, expression of SLUG was down-regulated by the wild-type prostasin expressed in the PC-3 cells, by 71%, when compared to cells carrying the control plasmid, while the mutant prostasin had no effect on SLUG mRNA expression (Figures 2-3A, and Table 2-1, page 46).

For uPA or COX-2, however, both the wild-type and the mutant prostasin were able to down-regulate the mRNA expression, but with the wild-type prostasin displaying a more robust phenotype (i.e., statistically different from the mutant phenotype). The wild-type prostasin reduced uPA or COX-2 mRNA expression by 72.5% or 79%, respectively; while the mutant prostasin was only able to reduce uPA or COX-2 mRNA expression by 31% or 31%, respectively (Figures 2-3B and 2-3C, and Table 2-1, page 46).

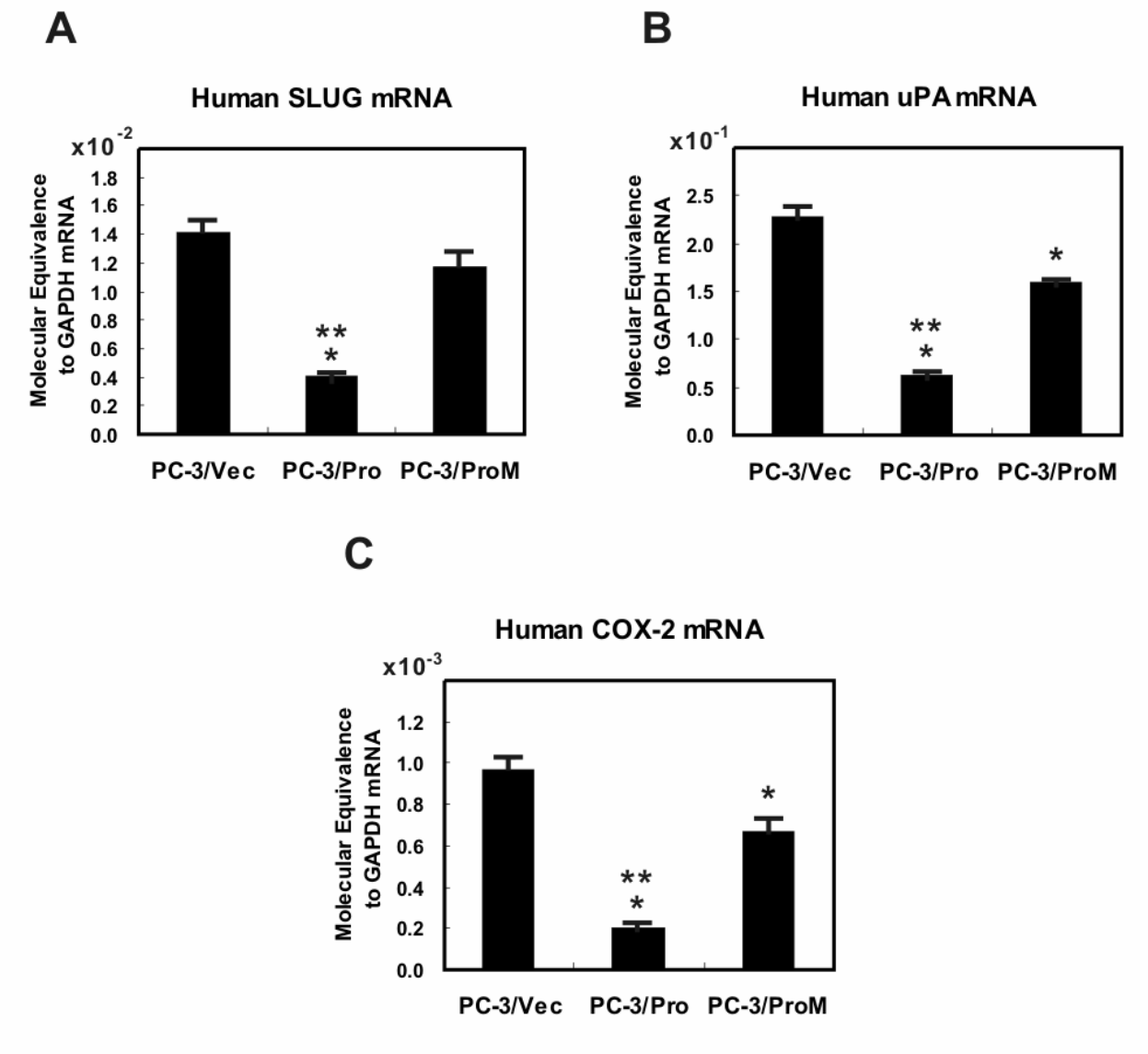


Figure 2-3 Real-time PCR analysis of reverse-transcribed cellular RNA for expression of SLUG (A), uPA (B), and COX-2 (C).

Experimental groups are as indicated under each data column, representing relative levels of expression normalized to the mRNA level of GAPDH. The single-asterisk indicates a statistical difference between the PC-3/Vec and the PC-3/Pro, or between the PC-3/Vec and the PC-3/ProM data groups ($p < 0.05$). The double-asterisk indicates a statistical difference between the PC-3/Pro and the PC-3/ProM data groups ($p < 0.05$). Calculations were based on data from four independent experiments.

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

At the mRNA level, expression of uPAR was down-regulated equally well, by 27%, by the wild-type prostasin, or the mutant prostasin expressed in the PC-3 cells, when compared to cells carrying the control plasmid (Figures 2-4A, and Table 2-1, page 46). For the iNOS mRNA, wild-type prostasin down-regulated its expression in the PC-3 cells by 70%, while the mutant prostasin down-regulated the iNOS mRNA by 68%, when compared to cells carrying the control plasmid (Figure 2-4B, and Table 2-1, page 46). There is no statistical difference in iNOS mRNA expression levels of the cells expressing the wild-type prostasin and those expressing the mutant.

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

At the mRNA level, GM-CSF expression was up-regulated by the mutant prostasin expressed in the PC-3 cells, to 2.66 fold, when compared to cells carrying the control plasmid (Figures 2-5A, and Table 2-1, page 46). On the other hand, wild-type prostasin expressed in the PC-3 cells produced a down-regulation effect on GM-CSF mRNA expression, reducing it to 79% of that in the control cells. The mRNA level changes of GM-CSF expression is reflected by the quantitative changes of the secreted GM-CSF in the culture medium (Figure 2-5B). The mutant prostasin increased the amount of secreted GM-CSF to 3.2 fold of that of the control cells, while the wild-type prostasin reduced the amount to 69%.

For the matriptase mRNA, only the mutant prostasin expressed in the PC-3 cells had an effect on its expression, increasing it to 16.8 fold of that in the control cells (Figure 2-5C, and Table 2-1, page 46). At the protein level, only in the mutant prostasin-expressing cells was the matriptase band detected in the western blot analysis (Figure 2-5D).

For the E-cadherin mRNA, both the wild-type and the mutant prostasin expressed in the PC-3 cells had an inducing effect on its expression, increasing it to 1.9 fold and 16.6 fold, respectively, of that in the control cells (Figure 2-5E, and Table 2-1, page 46). At the protein level, the changes corresponded to the mRNA changes, with the wild-type prostasin increasing the cellular E-cadherin protein to 2.1 fold, and the mutant prostasin, 7 fold, over the level of E-cadherin protein in the vector control cells (Figure 2-5F).

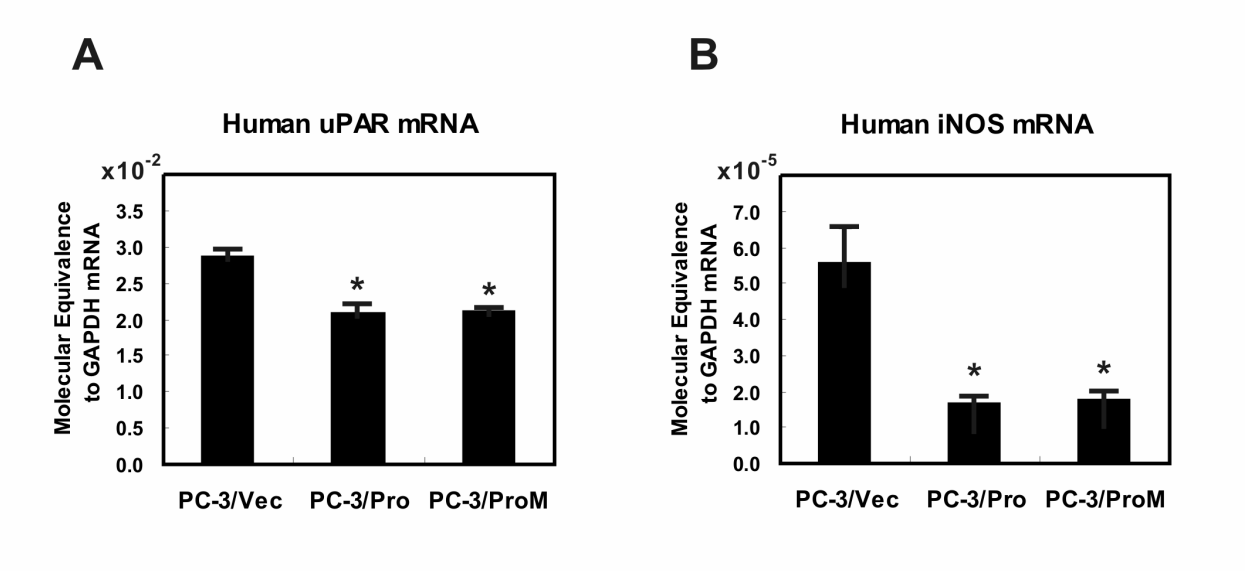


Figure 2-4 Real-time PCR analysis of reverse-transcribed cellular RNA for expression of uPAR (A) and iNOS (B).

Experimental groups are as indicated under each data column, representing relative levels of expression normalized to the mRNA level of GAPDH. The single-asterisk indicates a statistical difference between the PC-3/Vec and the PC-3/Pro, or between the PC-3/Vec and the PC-3/ProM data groups ($p < 0.05$). Calculations were based on data from four independent experiments.

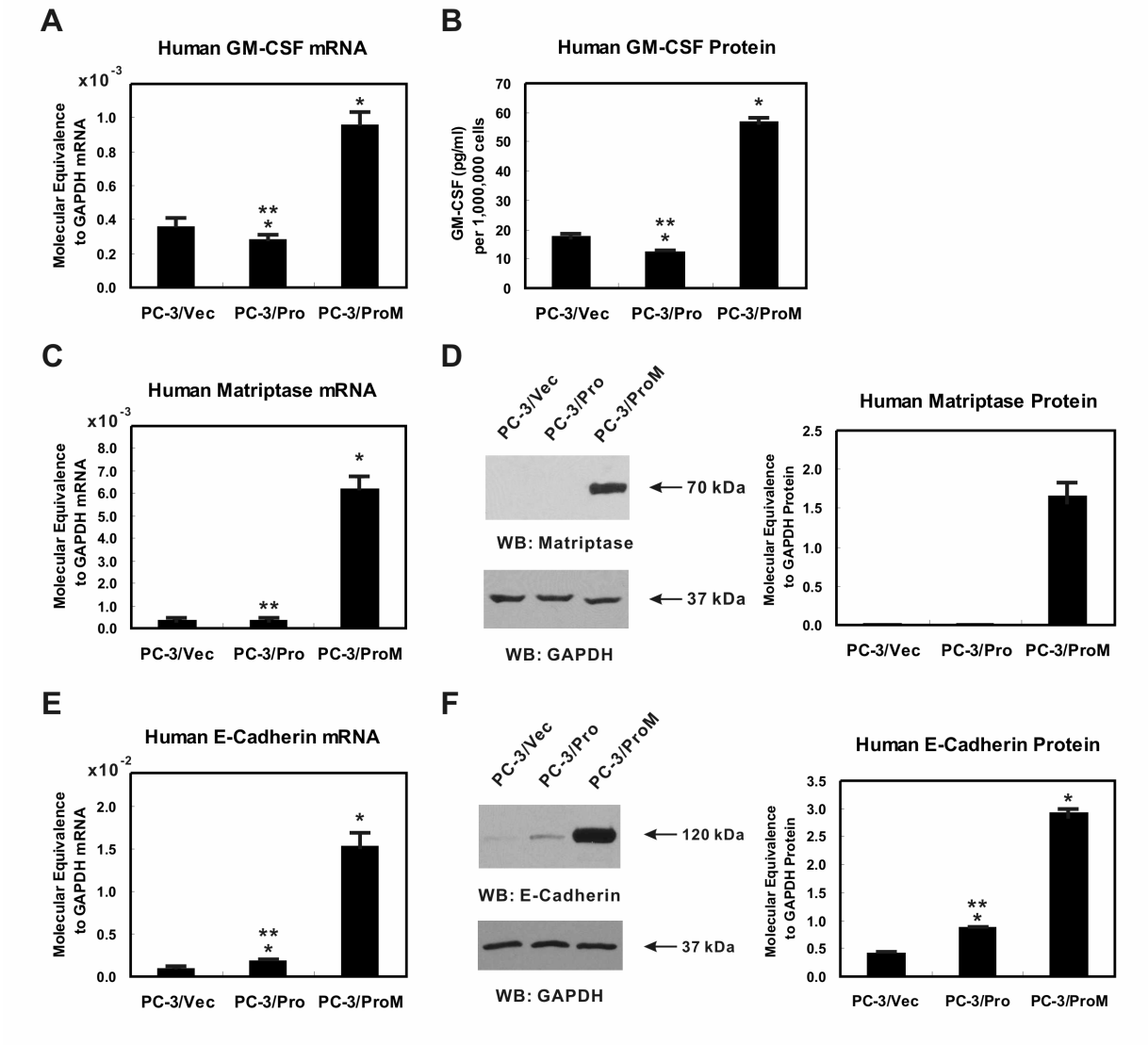


Figure 2-5 The serine active-site mutant prostasin induces the expression of GM-CSF (A and B), Matriptase (C and D), and E-Cadherin (E and F).

Experimental groups are as indicated under each data column, or over each sample, representing relative levels of expression normalized to the mRNA/protein level of GAPDH, whichever appropriate. The single-asterisk indicates a statistical difference between the PC-3/Vec and the PC-3/Pro, or between the PC-3/Vec and the PC-3/ProM data groups ($p < 0.05$). The double-asterisk indicates a statistical difference between the PC-3/Pro and the PC-3/ProM data groups ($p < 0.05$). Calculations of the mRNA expression levels were based on data from four independent experiments. Calculations of the protein expression levels were based on data from three independent experiments.

2.4 Discussion

In this chapter, we present our initial findings in the efforts of defining the molecular mechanisms by which prostasin serine protease, as an extracellular enzyme, impacts epithelial cell behavior. We have previously determined that in human prostate and breast cancer cells that lost prostasin expression, an anti-invasion phenotype was associated with the re-expression of prostasin, while cell proliferation in vitro or in vivo was not affected (Chen et al., 2001a; Chen and Chai, 2002). Here, we focused our attention on the molecules that play a role in the invasive behavior of a model cell line, the human prostate cancer cell line PC-3.

The PC-3 cells were shown to display active prostasin protease on the membrane upon re-expression (Chen et al., 2001b). This cell line also presents the prostasin-activating enzyme, matriptase (Sanders et al., 2006), and in our hands we confirmed an mRNA-level matriptase expression (Figure 2-5C, and Table 2-1, page 46). We then used our sublines expressing the wild-type or the active-site mutant prostasin to investigate the molecular mechanisms that are dependent or independent of prostasin's protease function.

The first target molecules that we examined are the EGFR and those regulated by the EGFR during tumor invasion. The EGFR was identified as a major player in prostate cancer invasion, in vivo, and in vitro in the model cell line PC-3. The EGF-EGFR signaling initiates on the outside of the cells, where prostasin serine protease is located. We first evaluated the potential of prostasin to regulate EGFR protein expression in the PC-3 cells following re-expression. The wild-type prostasin produced a 66% reduction of EGFR protein expression when compared

to the control cells (Figure 2-2A). At the mRNA level, a 34% reduction of EGFR expression was also observed in the cells expressing the wild-type prostasin (Figure 2-2B, and Table 2-1, page 46). Moreover, the wild-type prostasin but not the serine active-site mutant down-regulated EGF-EGFR signaling, as EGF-stimulated Erk1/2 phosphorylation was reduced by 61% (Figure 2-2C). It had been previously shown that EGF-EGFR signaling could regulate EGFR mRNA expression in prostate cancer cell lines, including the PC-3 (Seth et al., 1999). Our observation on EGFR expression regulation by prostasin is consistent with a model of EGFR protein down-regulation followed by EGFR mRNA down-regulation. In the PC-3 cells, activation of the EGF-EGFR signaling pathway does not stimulate cell proliferation (El Sheikh et al., 2004). The Erk1/2 phosphorylation down-regulation by the wild-type prostasin is consistent with its anti-invasion but not anti-proliferation phenotype (Chen et al., 2001a).

The serine active-site mutant prostasin also produced a protein-level EGFR down-regulation, at 48% (Figure 2-2A), but not as great as the down-regulation observed with the wild-type prostasin. The mutant prostasin, however, did not have an effect on the EGFR mRNA expression (Figure 2-2B, and Table 2-1, page 46) or EGF-stimulated Erk1/2 phosphorylation (Figure 2-2C). A possible explanation is that the extent of the mutant prostasin-associated EGFR protein down-regulation was not at a threshold point to affect the effective number of surface receptor presentation to impact receptor binding-activated signaling or EGFR mRNA expression.

The uPA mRNA was down-regulated by the wild-type prostasin in the PC-3 cells (Figure 2-3B, and Table 1, page 46), a result that is consistent with the invasion-suppressing role of prostasin (Chen et al., 2001a). This result is also consistent with an EGF-EGFR-stimulated uPA expression (Skogseth et al., 2005) being down-regulated by the wild-type prostasin. Both the wild-type and the mutant prostasin were shown to down-regulate the uPAR mRNA expression (Figure 2-4A, and Table 1, page 46), but the mechanism and the impact of this regulation are unclear. It is also difficult to speculate, at present, on the mechanism or impact of the uPA expression down-regulation by the mutant prostasin (Figure 2-3B, and Table 2-1, page 46).

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

Bruns et al. (Bruns et al., 2007) recently reported that the proteolytic activation of the gamma subunit of the ENaC is carried out by prostasin, but they also noted that the serine active-site mutant prostasin produced a similar proteolytic effect. A potential mechanism was offered to explain the apparent proteolytic effect of the serine active-site mutant prostasin on ENaCgamma, that it could have retained a residual serine protease activity. An alternative explanation was that the abundance of over-expressed prostasin, wild-type or mutant, could have exhausted the supply of serine protease inhibitors, allowing other proteases to take action. We have observed in the PC-3 cells, however, a dramatic inducing effect by the mutant prostasin, on the activating serine protease, matriptase. Matriptase and prostasin share a great deal in substrate preference, for example, cleaving the Gln-Ala-Arg-X type of substrates (Chen et al., 2004; Yamasaki et al., 2003). It is plausible that in the experiments performed to confirm prostasin cleavage of ENaCgamma, the mutant prostasin transfected into the cells also induced matriptase expression to cleave ENaCgamma.

The biological relevance of mutant prostasin-induced gene expression requires careful consideration as such is the case with the E-cadherin expression. The wild-type prostasin produced a moderate induction effect on E-cadherin expression, a result that is also consistent with prostasin's anti-invasion role. A robust induction of E-cadherin expression at the mRNA level, reflected at the protein level, however, was observed in cells expressing the mutant prostasin. This induction appears to correlate with that of the matriptase expression, suggesting that matriptase and prostasin may have an overlapping point in the mechanisms and pathways by which they modulate cell signaling.

We also investigated two inflammation-induced genes for potential regulation by prostasin, namely COX-2 and iNOS. Induction of bladder iNOS mRNA by inflammation was attenuated by prostasin, while induction of bladder COX-2 mRNA was not (Chen et al., 2006e). Prostasin did not regulate inflammation-induced COX-2 expression because it is not co-expressed in the COX-2 producing cells (Chen et al., 2006d). We tested the expression of these two genes in response to prostasin re-expression in the PC-3 cells, and found that both the wild-type prostasin and the mutant prostasin produced a down-regulation on both COX-2 and iNOS (Figures 2-3C and 2-4B, and Table 2-1, page 46). For COX-2 the down-regulation by the mutant prostasin was not as great as that by the wild-type prostasin, but for iNOS, the two versions of prostasin were equally effective. We could reason from these results that prostasin is also capable of regulating COX-2 expression, in cells where it is co-expressed with COX-2. We did not observe a down-regulation of iNOS expression by the mutant prostasin in the bladder inflammation model (Chen et al., 2006c), but an effect was seen in the PC-3 cells in the present study. It is also possible that an induction of other membrane-type extracellular proteases, such as matriptase, were responsible for the COX-2 or iNOS expression down-regulation in PC-3 cells expressing the mutant prostasin.

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

Table 2-1 Gene expression changes in PC-3 evaluated by RT-rtPCR.

Experiment Group	Gene Evaluated	Expression Level Normalized to GAPDH	Change *versus* Control (Fold)
PC-3/Vec	EGFR	$7.06 \pm 0.27 \times 10^{-3}$	1.0
PC-3/Pro	EGFR	$4.65 \pm 0.16 \times 10^{-3}$	0.66*
PC-3/ProM	EGFR	$5.84 \pm 0.65 \times 10^{-3}$	nsd
PC-3/Vec	SLUG	$1.42 \pm 0.08 \times 10^{-2}$	1.0
PC-3/Pro	SLUG	$0.41 \pm 0.02 \times 10^{-2}$	0.29*
PC-3/ProM	SLUG	$1.18 \pm 0.10 \times 10^{-2}$	nsd
PC-3/Vec	uPA	$2.29 \pm 0.10 \times 10^{-1}$	1.0
PC-3/Pro	uPA	$0.63 \pm 0.04 \times 10^{-1}$	0.275*$^{\phi}$
PC-3/ProM	uPA	$1.59 \pm 0.03 \times 10^{-1}$	0.69*
PC-3/Vec	uPAR	$2.87 \pm 0.10 \times 10^{-2}$	1.0
PC-3/Pro	uPAR	$2.09 \pm 0.12 \times 10^{-2}$	0.73*
PC-3/ProM	uPAR	$2.10 \pm 0.05 \times 10^{-2}$	0.73*
PC-3/Vec	COX-2	$9.69 \pm 0.51 \times 10^{-4}$	1.0
PC-3/Pro	COX-2	$1.99 \pm 0.23 \times 10^{-4}$	0.21*$^{\phi}$
PC-3/ProM	COX-2	$6.73 \pm 0.60 \times 10^{-4}$	0.69*
PC-3/Vec	iNOS	$5.55 \pm 0.97 \times 10^{-5}$	1.0
PC-3/Pro	iNOS	$1.65 \pm 0.22 \times 10^{-5}$	0.30*
PC-3/ProM	iNOS	$1.79 \pm 0.23 \times 10^{-5}$	0.32*
PC-3/Vec	GM-CSF	$3.60 \pm 0.21 \times 10^{-4}$	1.0
PC-3/Pro	GM-CSF	$2.84 \pm 0.12 \times 10^{-4}$	0.79*$^{\phi}$
PC-3/ProM	GM-CSF	$9.56 \pm 0.32 \times 10^{-4}$	2.66*
PC-3/Vec	Matriptase	$3.68 \pm 0.48 \times 10^{-4}$	1.0
PC-3/Pro	Matriptase	$3.53 \pm 0.45 \times 10^{-4}$	nsd
PC-3/ProM	Matriptase	$6.195 \pm 0.51 \times 10^{-3}$	16.8*
PC-3/Vec	E-Cadherin	$9.23 \pm 1.13 \times 10^{-4}$	1.0
PC-3/Pro	E-Cadherin	$1.79 \pm 0.10 \times 10^{-3}$	1.9*$^{\phi}$
PC-3/ProM	E-Cadherin	$1.53 \pm 0.18 \times 10^{-2}$	16.6*

Real-time PCR analysis of reverse-transcribed cellular RNA from PC-3 cells transfected with a control plasmid (PC-3/Vec), a wild-type human prostasin cDNA plasmid (PC-3/Pro), and a serine active-site mutant prostasin cDNA plasmid (PC-3/ProM). Experiments were performed at least four times. Calculations in the table were based on data from four representative experiments.

* Indicates a statistical difference ($p < 0.05$) between the PC-3/Vec and the PC-3/Pro, or between the PC-3/Vec and the PC-3/ProM data groups.

ϕ Indicates a statistical difference ($p < 0.05$) between the PC-3/Pro and the PC-3/ProM data groups.

nsd: No statistical difference, between the data group indicated and PC-3/Vec.

CHAPTER THREE THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) IS PROTEOLYTICALLY MODIFIED BY THE MATRIPTASE-PROSTASIN SERINE PROTEASE CASCADE

3.1 Introduction

In the previous chapter, we have shown that the expression of several invasion-promoting molecules is regulated by prostasin re-expression in the PC-3 cells, possibly via down-regulation of EGFR and EGF-EGFR signaling.

EGFR, a member of the ErbB (erythroblastic leukemia viral (v-erb-b) oncogene) family of membrane receptor tyrosine kinases (RTK's), is one of the most important membrane molecules that plays pivotal roles in many cellular responses ranging from proliferation to apoptosis, migration to adhesion, and differentiation to depolarization (Yarden and Sliwkowski, 2001; Wells, 1999). Dys-regulation of EGFR by overexpression or mutation resulting in constitutive activation is associated with many types of cancers (Bellezza et al., 2006; Lo et al., 2006a; Normanno et al., 2006; Ahmed and Salgia, 2006). The PC-3 cells express an abundance of EGFR and its ligands (Morris and Dodd, 1990; Ching et al., 1993), creating an autocrine signaling loop to confer the cells with highly invasive properties. Inhibition of EGFR signaling reduced the invasiveness of PC-3 cells either through down-regulation of uPA-uPAR signaling (Skogseth et al., 2006; Skogseth et al., 2005) or up-regulation of cell adhesion molecules such as the E-Cadherin (Yates et al., 2007). The PC-3 cell line is not dependent on EGFR signaling for proliferation (El Sheikh et al., 2004).

Given the extracellular localization of prostasin and the EGF-binding domains of the EGFR, we had speculated that a proteolytic modification of the EGFR in its extracellular domain (ECD) might be a mechanism for prostasin down-regulation of EGFR expression and signaling in the PC-3 cells. We present biochemical evidence that prostasin induces site-specific cleavages of the EGFR ECD following activation by matriptase in the HEK-293 Flp-In TRex (FT-293) cells, generating two N-terminally truncated EGFR fragments at 135 kD and 110 kD. The N-terminally truncated EGFR fragments are predicted to have lost the functional EGF-binding domain and are no longer responsive to EGF stimulation.

3.2 Materials and Methods

3.2.1 Materials

A full-length human EGFR cDNA (Clone No. PR1116_D04, NM_005228) was purchased from OriGene (Rockville, MD). The HEK-293 Flp-In TRex (FT-293) cells and a recombinant human EGF were obtained from Invitrogen (Carlsbad, CA). A polyclonal antibody to EGFR (sc-03), a monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-32233), and a polyclonal antibody to Erk1/2 (sc-94) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A monoclonal antibody to the HA tag (6E2), and a polyclonal antibodies to phospho-EGFR (Tyr1068, #2234) was purchased from Cell Signaling Technology, Inc. (Beverly, MA). A monoclonal antibody to phospho-tyrosine (PY-20, #03-7799) was purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Monoclonal antibodies to phospho-Akt (#550747) and Akt (#610860) were purchased from BD Biosciences (Franklin Lakes, NJ). A polyclonal antibody to phospho-Erk1/2 (V803A) was purchased from

Promega (Madison, WI). Tyrphostin AG1478 (#658548) was obtained from Calbiochem (San Diego, CA).

3.2.2 Construction of expression plasmids

The cDNA's of the wild-type human prostasin and the active-site mutant human prostasin (Chen et al., 2001a) were subcloned into the pcDNA3 plasmid (Invitrogen, Carlsbad, CA) to generate the prostasin expression plasmids pcDNA3-Pro and pcDNA3-ProM for transient expression in tissue-cultured cells. The full-length human EGFR cDNA clone from OriGene was also subcloned into pcDNA3, generating the EGFR expression plasmid pcDNA3-EGFR. A modified pcDNA3 plasmid, pcDNA3-HA, was generated by inserting a double-stranded oligonucleotide encoding the 9 amino-acid coding sequence of a hemagglutinin (HA) tag (YPYDVPDYA) into pcDNA3. A full-length human matriptase cDNA (Oberst et al., 2005a) was then subcloned into the pcDNA3-HA plasmid in frame to generate a C-terminally HA-tagged matriptase expression plasmid pcDNA3-Mat-HA. An active-site mutant of the HA-tagged matriptase expression plasmid, pcDNA3-MatM-HA was also generated by PCR-based site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

3.2.3 Cell culture, transfection and western blot

The FT-293 cells were cultured in the D-MEM (High Glucose) medium supplemented with 10% (v/v) FBS (fetal bovine serum). On day 1, cells were plated on a poly-L-lysine (PLL) coated

12-well plate at a density of 4 x 10^5 cells per well. On day 2, the appropriate expression plasmids (0.8μg of total DNA per transfection) were transfected into the cells using the Lipofectamine 2000 reagent (Invitrogen, 2μl per transfection) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were lysed in the RIPA lysis buffer supplemented with a protease inhibitor cocktail. Twenty μg of total protein lysate were subjected to western blot analysis with appropriate antibodies as describes previously (Chen et al., 2001b). Each membrane was also blotted with a GAPDH antibody as a control of protein loading.

3.2.4 PN-1 cell binding assay

Transfected FT-293 cells cultured to confluence were washed with 1x PBS once, scraped off in 1x PBS and collected by centrifugation. The cell pellet was then re-suspended in 100 μl of 25mM Tris-HCl, pH 7.6, and incubated with 5 μl of mouse seminal vesicle fluid at 37^{o}C for 2 hours as described previously (Chen et al., 2001b). Following the incubation, the cells were re-centrifuged and lysed in the RIPA lysis buffer supplemented with a protease inhibitor cocktail. The cell lysate was subjected to western blot analysis with a prostasin antibody. Active prostasin on the cell membrane are indicated by the appearance of a heat-stable SDS-resistant complex of prostasin and mouse PN-1.

3.2.5 EGF treatment of cells and analysis of EGF-EGFR signaling pathway activation

Transfected FT-293 cells cultured to confluence were washed with 1x PBS once and placed under serum-free medium for overnight. Serum-starved cell cultures were then treated with 10 ng/ml of EGF for 10 minutes, or left untreated. The cells were then immediately lysed and subjected to western blot analysis for phospho-EGFR and total EGFR, phospho-Erk1/2 and total Erk1/2, and phospho-Akt and total Akt. Each membrane was also blotted with a GAPDH antibody as a control of protein loading. For EGFR phosphorylation inhibition assays, FT-293 cells were transfected, serum-starved, and EGF-stimulated in the medium with 2 μM AG1478. An equal volume of DMSO was applied to control cultures.

3.2.6 Generation of deletion mutants of EGFR

To generate EGFR ECD deletion mutants to map the protease cleavage site, the EGFR-pcDNA3 construct plasmid except the specific deletion regions were re-amplified by polymerase chain reaction (PCR) using the Phusion high-fidelity DNA polymerase (New England Biolabs, Beverly, MA). The amplified fragments were then ligated using the T4 DNA ligase (Invitrogen) and transformed into the TOPP10 competent cells (Invitrogen). All deletion mutant constructs were verified by DNA sequencing before transfection-quality DNA was prepared using a QIAGEN kit (Valencia, CA). Listed below are the primers used for amplification of the EGFR ECD deletion cDNA:

EGFRΔ6-272-3: 5'- TTT CTT TTC CTC CAG AGC CCG ACT CGC C -3'

EGFRΔ6-272-5: 5' - AAT TAT GTG GTG ACA GAT CAC GGC TCG TGC GTC -3'

EGFRΔ272-409-3: 5'- GGG ACA CTT CTT CAC GCA GGT GGC AC -3'

EGFRΔ272-409-5: 5’- GGT CAG TTT TCT CTT GCA GTC GTC AG -3’

EGFRΔ6-409-3: 5'- TTT CTT TTC CTC CAG AGC CCG ACT CGC C -3'

EGFRΔ6-409-5: 5’- GGT CAG TTT TCT CTT GCA GTC GTC AG -3’

EGFRΔ6-612-3: 5’- TTT CTT TTC CTC CAG AGC CCG ACT CGC C -3’

EGFRΔ6-612-5: 5’- CCA ACG AAT GGG CCT AAG ATC CCG TCC -3’

3.3 Results

3.3.1 Prostasin activated by matriptase induces the cleavage of EGFR in FT-293 cells

In the previous chapter, a proteolytic mechanism of EGFR regulation by prostasin was suggested from the observation that re-expression of prostasin down-regulated the protein expression of EGFR in the PC-3 prostate carcinoma cells. In this chapter, to determine whether EGFR is a biological substrate of prostasin, we co-expressed EGFR, prostasin and an HA-tagged matriptase in FT-293 cells by transient transfection. FT-293 cells were derived from the human embryonic kidney cell line HEK-293 which does not express significant levels of EGFR, prostasin or matriptase (Stern et al., 2007; Chen et al., 2001b). First, we confirmed the serine protease activity of prostasin co-expressed with matriptase (HA-tagged, same hereon) by performing a binding assay with PN-1, the specific serpin-class inhibitor for prostasin. The PN-1 binding assay is a reliable method for evaluating prostasin’s serine protease activation state in vitro and in vivo (Chen et al., 2001b; Chen et al., 2004; Netzel-Arnett et al., 2006b). When expressed without matriptase, prostasin remains in the zymogen form and no prostasin-PN-1 complex was detected, indicating a lack of serine protease activity (Figure 3-1, lane 2). When co-expressed with matriptase, prostasin was shown to be cleaved, apparently by matriptase, to produce a

lower-molecular weight prostasin molecule, and at the same time a prostasin-PN-1 complex (Figure 3-1, lane 5). The serine active-site mutant prostasin was also cleaved by matriptase but no prostasin-PN-1 complex was observed (Figure 3-1, lane 6). The serine active-site mutant matriptase, expectedly, was unable to cleave and activate the prostasin zymogen (Figure 3-1, lane 7-9).

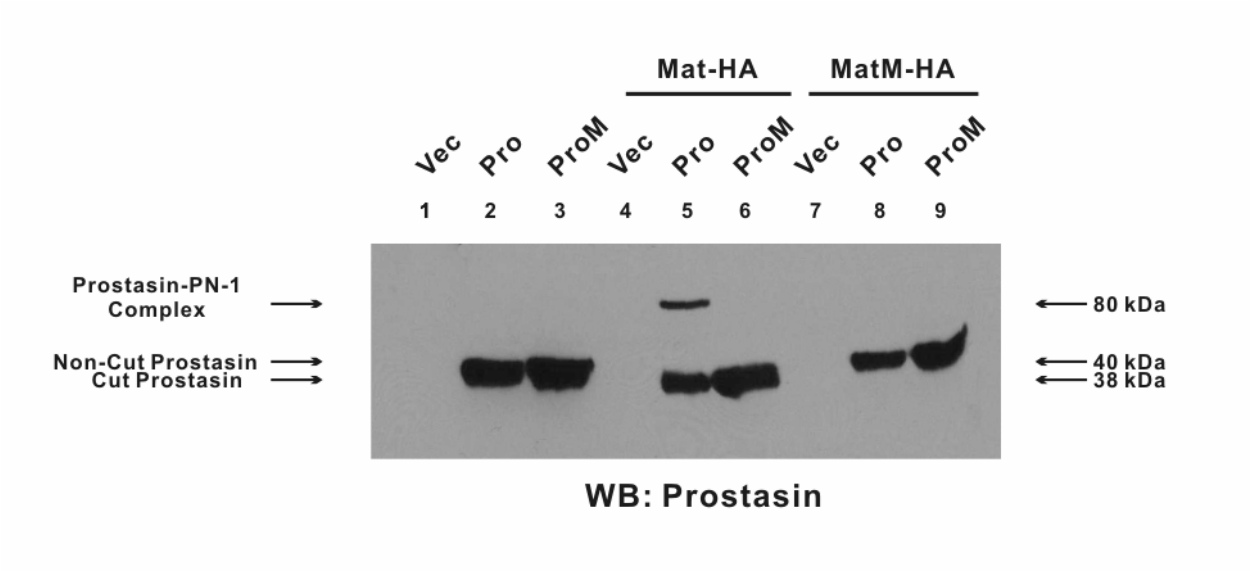


Figure 3-1 Prostasin is activated by HA-tagged matriptase in FT-293 cells.

FT-293 cells were transfected with cDNA's encoding EGFR (0.5 μg), wild-type prostasin (Pro, 0.2 μg), serine-active site mutant prostasin (ProM, 0.2 μg), HA-tagged matriptase (Mat-HA, 0.1 μg) or HA-tagged serine-active site mutant matriptase (MatM-HA, 0.1 μg), or in combinations as indicated in the figure. Each transfection was carried out with an equal amount of total plasmid DNA using the vector plasmid pcDNA3 as a substitute when appropriate. At 24 hours post transfection, cells were subjected to PN-1 binding assays with mouse seminal vesicle fluid (mSVF) followed by western blot analysis for prostasin under boiling and reducing conditions. The covalent prostasin-PN-1 complex, uncut prostasin, and cut prostasin are indicated by arrows. The results shown are representative of three independent experiments.

In a western blot analysis using a polyclonal antibody that specifically recognizes a carboxyl-terminal intracellular domain of human EGFR, two apparently amino-terminally truncated EGFR fragments were produced when matriptase was co-expressed (Figure 3-2, long exposure, lane 4). These apparent proteolytically modified fragments of EGFR were detected at approximately 135 kDa and 110 kDa, and were named EGFR135 and EGFR110, respectively. Quantities of the truncated EGFR, EGFR135 and EGFR110 were greatly increased when the wild-type prostasin was co-expressed with EGFR and matriptase. There was a decrease in quantities of EGFR135 and EGFR110 when the serine active-site mutant prostasin instead of the wild-type enzyme was co-expressed with EGFR and matriptase (Figure 3-2, lane 5 and 6). The amount of uncut full-length EGFR (the 170-kDa band) was also reduced with matriptase co-expression, and to a much greater extent with the addition of the wild-type prostasin. In a control experiment, we confirmed that the serine active-site mutant matriptase was unable to induce any cleavage of EGFR (Figure 3-2, lane 7-9).

To determine whether the membrane anchorage of prostasin is required for the EGFR cleavage, we generated a GPI anchor-free but protease competent mutant prostasin and co-expressed it with EGFR and matriptase. The GPI anchor-free mutant produced a similar pattern of EGFR cleavage as that seen with the wild-type when co-expressed with matriptase (Figure 3-3), suggesting that the GPI membrane anchorage is not required for the prostasin-induced EGFR cleavage.

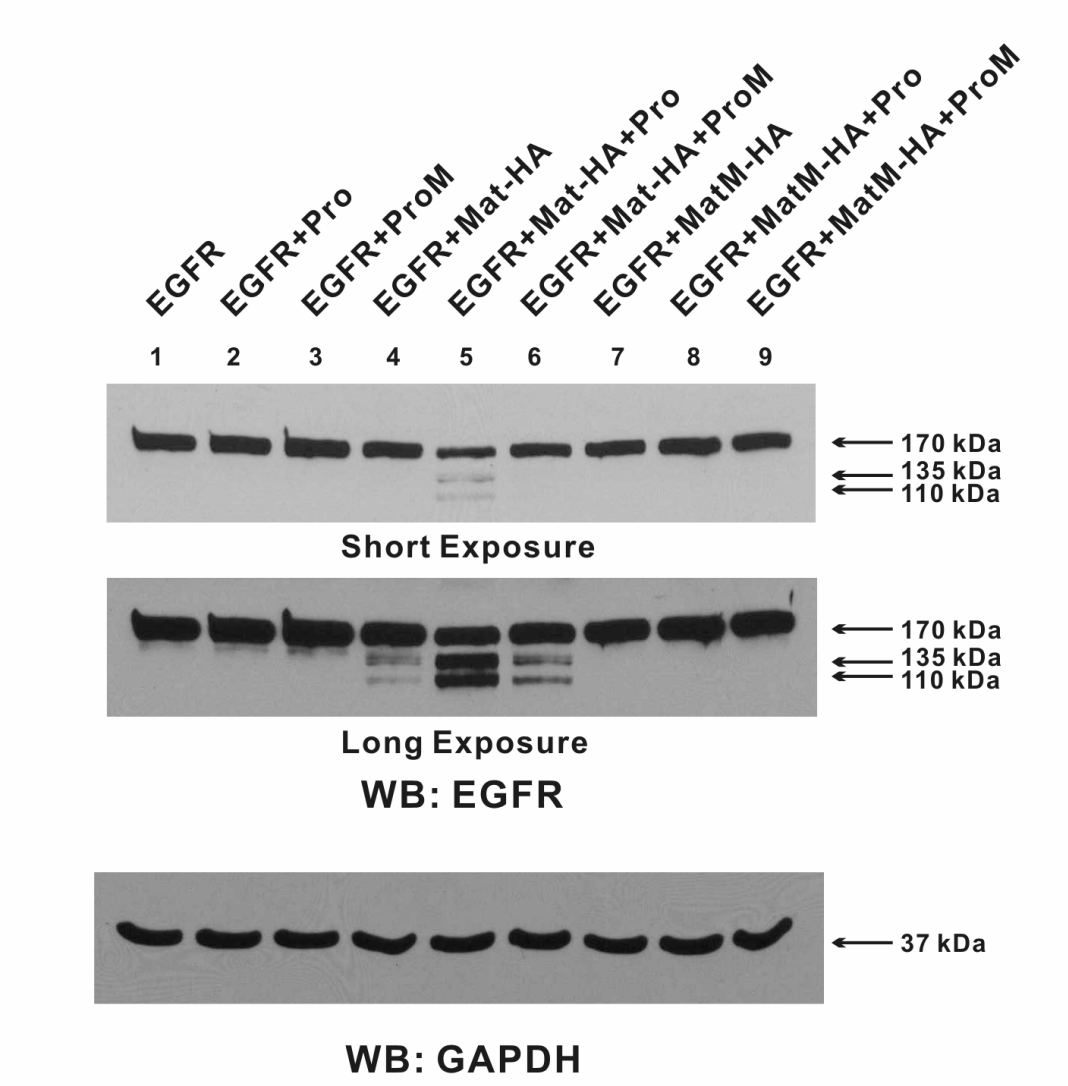


Figure 3-2 Prostasin and matriptase induce the cleavage of EGFR in FT-293 cells.

FT-293 cells were transfected with cDNA's encoding EGFR (0.5 μg), wild-type prostasin (Pro, 0.2 μg), serine-active site mutant prostasin (ProM, 0.2 μg), HA-tagged matriptase (Mat-HA, 0.1 μg) or HA-tagged serine-active site mutant matriptase (MatM-HA, 0.1 μg), or in combinations as indicated in the figure. Each transfection was carried out with an equal amount of total plasmid DNA using the vector plasmid pcDNA3 as a substitute when appropriate. At 24 hours post transfection, cells were assayed for EGFR protein expression by SDS-PAGE and western blot analysis with an anti-EGFR antibody that recognizes a C-terminal intracellular epitope of EGFR. The membrane was re-blotted with a GAPDH antibody as a sample loading control. The results shown are representative of three independent experiments.

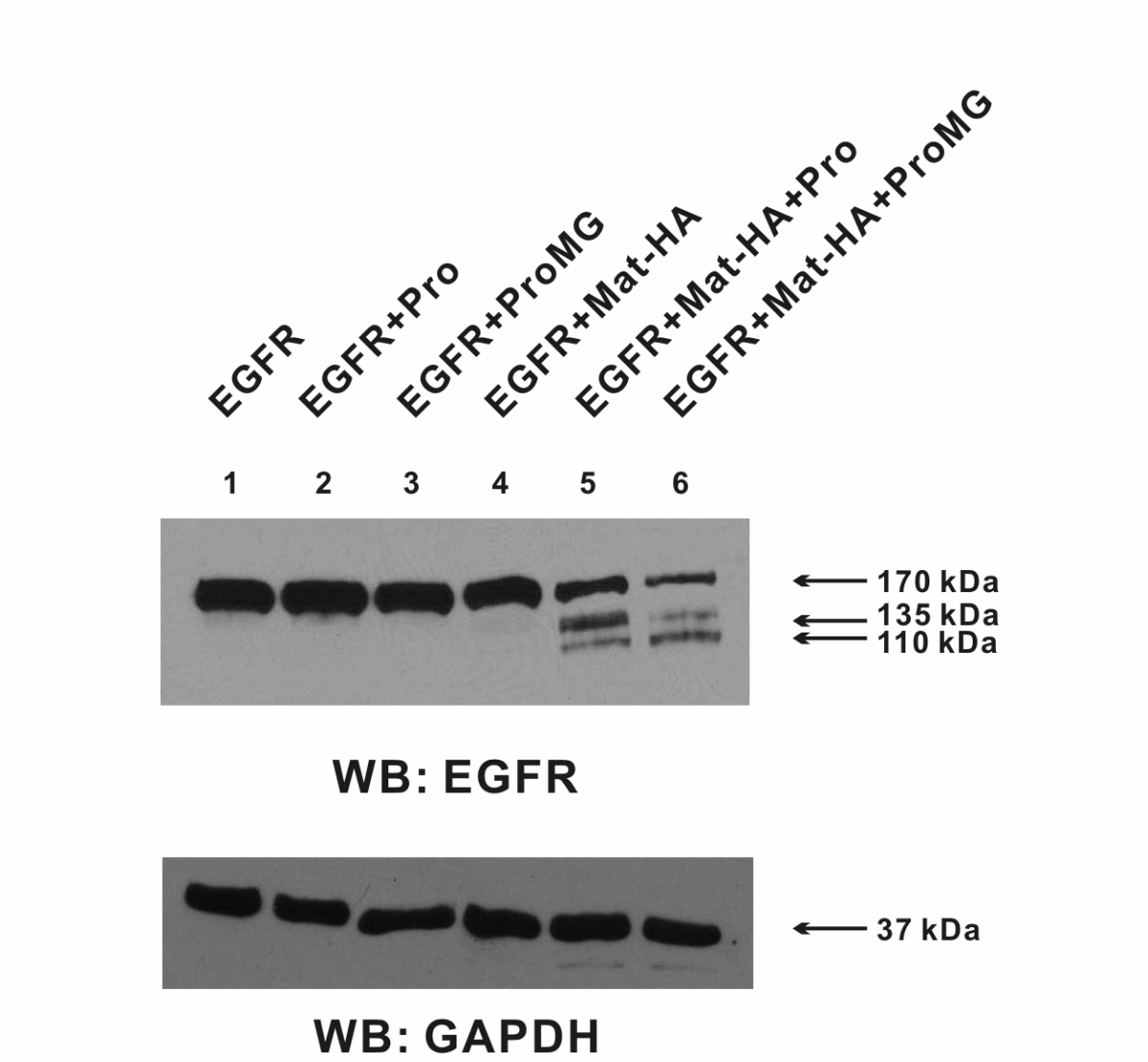


Figure 3-3 Prostasin-induced cleavage of EGFR is not dependent on its GPI-anchor in FT-293 cells.

FT-293 cells were transfected with cDNA's encoding EGFR (0.5 μg), wild-type prostasin (Pro, 0.2 μg), anchor-free mutant prostasin (ProMG, 0.2 μg), or HA-tagged matriptase (Mat-HA, 0.1 μg), or in combinations as indicated in the figure. Each transfection was carried out with an equal amount of total plasmid DNA using the vector plasmid pcDNA3 as a substitute when appropriate. At 24 hours post transfection, cells were assayed for EGFR protein expression by SDS-PAGE and western blot analysis as described in previous figure legend. The results shown are representative of three independent experiments.

3.3.2 Prostasin-induced EGFR ECD truncation activates EGFR and downstream signals

FT-293 cells presenting uncleaved or cleaved EGFR following EGFR and double protease (prostasin and matriptase) co-transfection were assayed for changes in EGF-EGFR signaling. Tyrosine phosphorylation of EGFR was detected via an antibody against phospho-tyrosine or an antibody recognizing the phosphorylated EGFR (at tyrosine-1068). The uncleaved wild-type EGFR (170 kDa) remains in the unphosphorylated state under the serum-free culture condition and becomes highly tyrosine phosphorylated once stimulated with EGF (Figure 3-4A). On the other hand, the EGFR110 fragment was shown to be tyrosine phosphorylated under serum-free culturing conditions, i.e., in the absence of an EGFR ligand (Figure 3-4A, lane 2), but the tyrosine phosphorylation of EGFR110 was not responsive to EGF stimulation (Figure 3-4A, lane 4). The EGFR135 fragment did not present detectable tyrosine phosphorylation under either serum-free or EGF-stimulated conditions. At the same time, we also examined the phosphorylation states of EGFR downstream signaling molecules Erk1/2 and Akt. In the presence of the cleaved EGFR, Erk1/2 and Akt phosphorylation was dramatically increased when compared with the cells with only the uncleaved wild-type EGFR (Figure 3-4B).

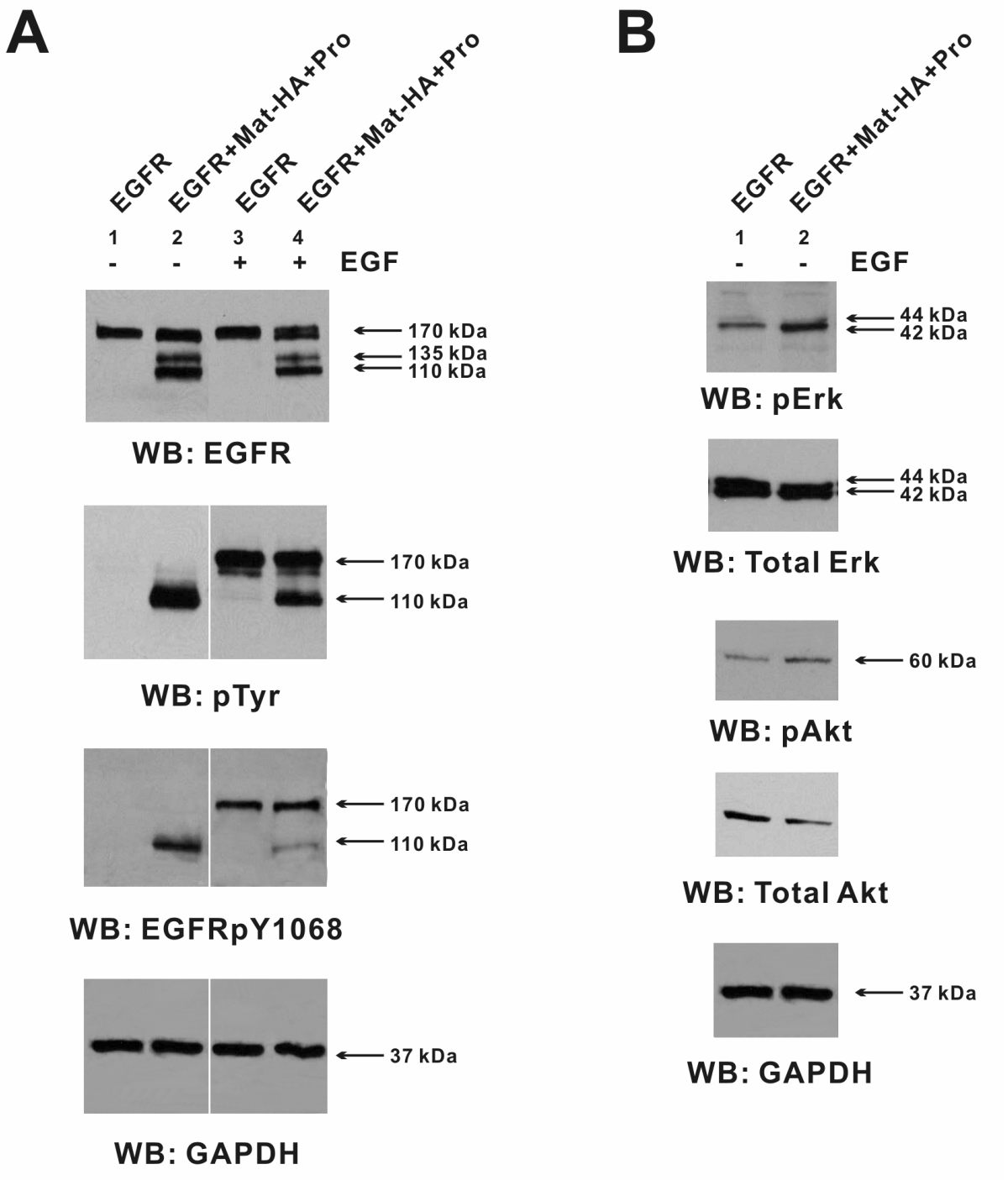


Figure 3-4 Phosphorylation changes of EGFR, Erk and Akt after the EGFR cleavage.

FT-293 cells were transfected with cDNA's encoding EGFR (0.05 μg for lane 1 and 3; 0.2 μg for lane 2 and 4),

wild-type prostasin (Pro, 0.2 μg), or HA-tagged matriptase (Mat-HA, 0.1 μg), or in combinations as indicated in the figure. Each transfection was carried out with an equal amount of total plasmid DNA using the vector plasmid pcDNA3 as substitute when appropriate. At 24 hours after transfection, cells were placed under serum-free medium for overnight and treated with 10ng/ml EGF for 10 minutes (lanes 1 and 2) or left untreated (lanes 3 and 4) before harvested for western blot analysis. (A) The phosphorylation state of EGFR tyrosine residues (pY20) and a specific phospho-tyrosine (pY1068) in FT-293 cells presenting wild-type uncleaved or cleaved EGFR under serum-free or EGF-stimulated conditions. (B) The phosphorylation state of Erk1/2 and Akt in FT-293 cells presenting wild-type uncleaved or cleaved EGFR under serum-free conditions. The results shown are representative of three independent experiments.

3.3.3 Prostasin-induced EGFR cleavage is not dependent on tyrosine-phosphorylation of EGFR

When EGFR is over-expressed in the FT-293 cells, a small portion of EGFR was shown to be tyrosine-phsophorylated (Figure 3-5, lane 1). To test whether this auto-phosphorylation of EGFR is required for the prostasin-induced cleavage, we performed similar experiments in the presence of AG1478, a potent inhibitor of EGFR kinase activity and auto-phosphorylation. Incubation with AG1478 completely eliminated not only the auto-phosphorylation and EGF-stimulated phosphorylation of wild-type EGFR but also the cleavage-induced phosphorylation of EGFR110 (Figure 3-5, lane 5-8). On the other hand, the cleavage of EGFR induced by prostasin was not affected by the AG1478 treatment (Figure 3-5, lane 5-8 compared with lane 1-4), suggesting that the prostasin-induced EGFR cleavage is independent on EGFR's tyrosine phosphorylation.

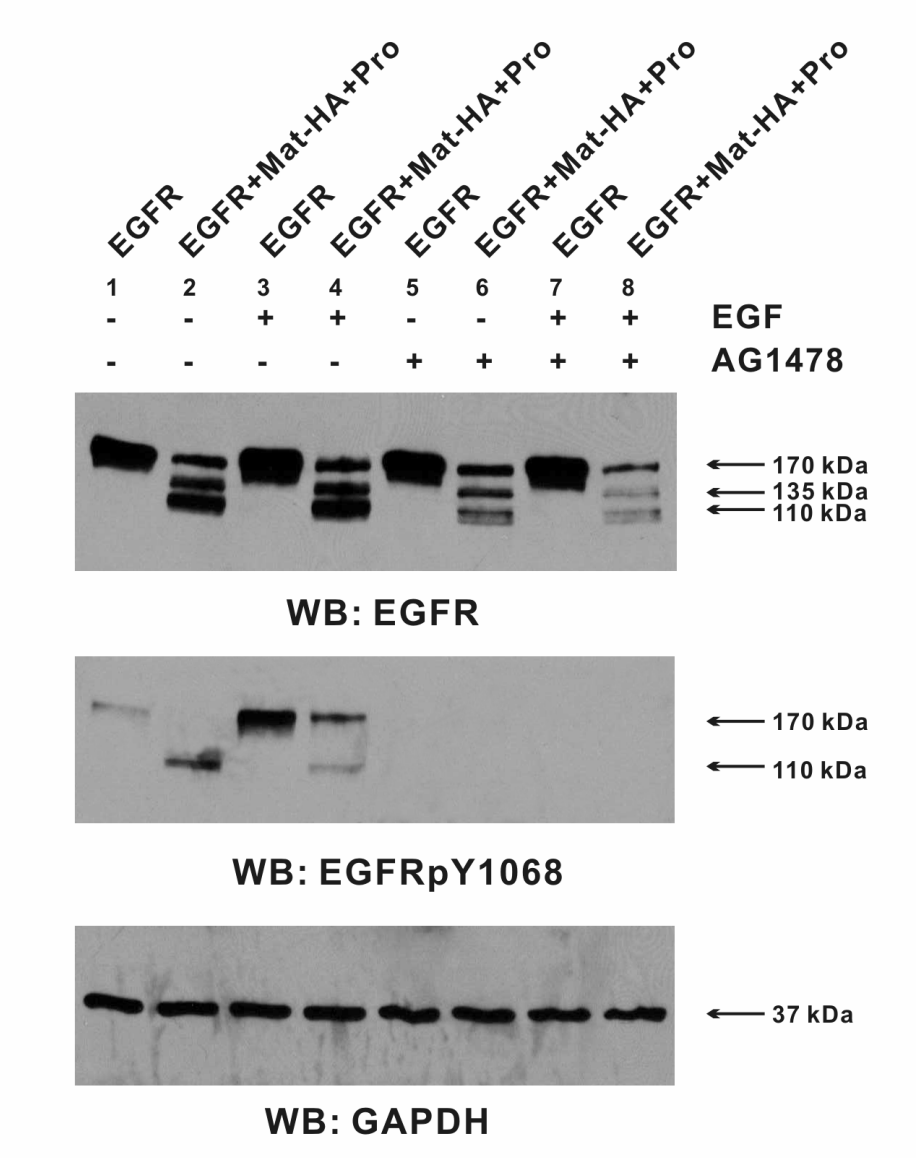


Figure 3-5 Prostasin-induced EGFR cleavage is independent of EGFR phosphorylation.

FT-293 cells were treated with AG1478 (2 μM, lane 5-8), or with DMSO (solvent control of AG1478, lane 1-4) during transfection, serum-starvation and EGF stimulation. Cells were transfected with cDNA's encoding EGFR (0.2 μg), wild-type prostasin (Pro, 0.2 μg), or HA-tagged matriptase (Mat-HA, 0.1 μg), or in combinations as indicated in the figure. Each transfection was carried out with an equal amount of total plasmid DNA using the vector plasmid pcDNA3 as a substitute when appropriate. At 24 hours after transfection, cells were placed under serum-free medium for overnight and treated with 10 ng/ml of EGF for 10 minutes (lanes 3, 4, 7 and 8) or left untreated (lanes 1, 2, 5 and 6) before harvested for western blot analysis. The results shown are representative of three independent experiments.

3.3.4 Mapping the protease cleavage sites in the EGFR ECD

In an attempt to map the cleavage sites for matriptase-prostasin in the EGFR ECD, we generated several deletion mutant constructs (EGFRΔ6-272-pcDNA3, EGFRΔ272-409-pcDNA3, EGFRΔ6-409-pcDNA3, EGFRΔ6-612-pcDNA3) and co-expressed them with the wild-type prostasin and matriptase, Apparent proteolytic cleavage patterns of these EGFR deletion mutants by matriptase and prostasin are show in Figure 3-6. The EGFR deletion mutants EGFRΔ6-272 or EGFRΔ272-409, without amino acid residues 6 to 272 or amino acid residues 272 to 409, respectively, could still be cleaved by the activated prostasin, but only generating one cleavage product, suggesting that each deletion region contains one cleavage site. The EGFR deletion mutants EGFRΔ6-409 or EGFRΔ6-612, without amino acid residues 6 to 409 or amino acid residues 6 to 612, respectively, was no longer cleaved by prostasin.

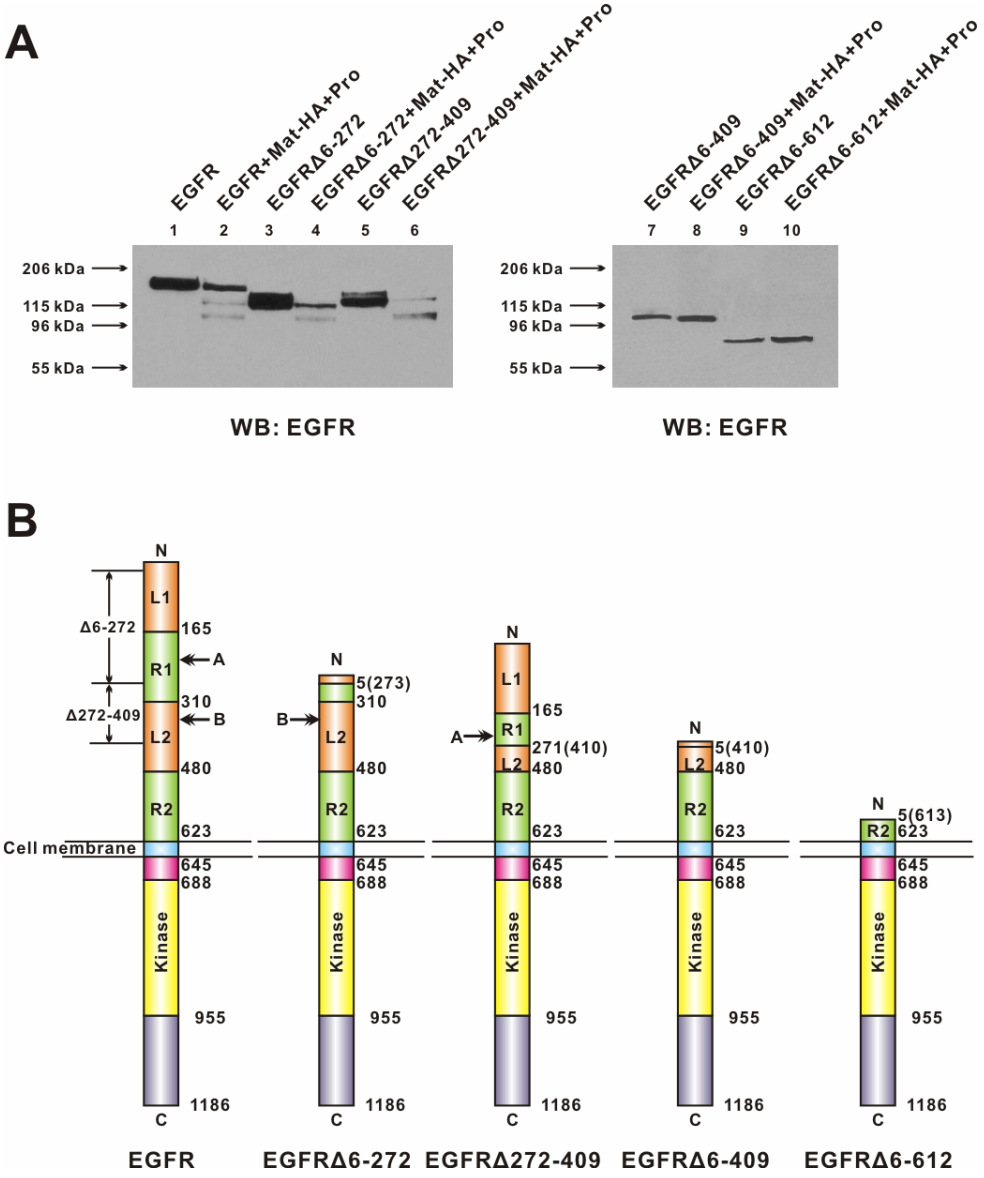


Figure 3-6 Mapping protease cleavage sites within the EGFR ECD using deletion mutants.

(A) FT-293 cells were transfected with cDNA's encoding appropriate EGFR fragments (EGFR, EGFRΔ6-272, EGFRΔ272-409, EGFRΔ6-409, or EGFRΔ6-612, 0.2 μg) with or without wild-type prostasin (Pro, 0.2 μg) and HA-tagged matriptase (Mat-HA, 0.1 μg) as indicated in the figure. Each transfection was carried out with an equal amount of total plasmid DNA using the vector plasmid pcDNA3 as a substitute when appropriate. At 24 hours post transfection, cells were assayed for EGFR protein expression by SDS-PAGE and western blot analysis. (B) Schematic illustration of the EGFR deletion mutants. Two potential cleavage sites (Site A and Site B) were indicated by arrows. Four structurally defined domains of the EGFR extracellular region are referred to as L1, CR1, L2, and CR2. Residue numbers for domain boundaries are also indicated.

3.4 Discussion

In this report, we present biochemical evidence that EGFR may be a biological substrate for matriptase and prostasin serine proteases in the epithelial cells. The actions of this serine protease activation cascade result in extracellular N-terminal truncation of EGFR, and phosphorylation of the truncated receptor, along with up-regulated downstream signals in the model cell line FT-293.

Prostasin as a GPI-anchored protein is routed to the apical membrane of the normally polarized epithelial cells (Chen et al., 2001b). EGFR and matriptase are normally routed to the basolateral sides of the cells (Hobert and Carlin, 1995; List et al., 2007a). Topologically distinct but coordinate expression of matriptase and prostasin is observed in many terminally differentiated epithelial tissues, implicating coordinate roles for these two serine proteases in normal epithelial physiology (List et al., 2007b). But during epithelial carcinogenesis expression of matriptase and prostasin begins to show divergent patterns of change, implicating diverging and independent roles for the two serine proteases in cancer. In the prostate and the breast, prostasin (PRSS8) is abundantly expressed in the normal tissue, but down-regulated in cancers, until its expression is lost in high-grade tumors (Chen et al., 2001a; Chen and Chai, 2002). An exception may be the ovarian cancer, which is marked by an up-regulation of prostasin (Mok et al., 2001). For matriptase (also known as MT-SP1), its expression is up-regulated in prostate and breast cancers (Jin et al., 2007; Kang et al., 2003), and its mechanistic role in cancer is believed to be activating the ligands of the Ron signaling pathway (Welm et al., 2007).

In terminally differentiated epithelia, EGFR is not expressed in abundance, but over-expression of EGFR is commonly associated with cancer. In the normal tissue, therefore, prostasin and matriptase are controlled by topological separation on the plasma membrane, if not also by differential expression, i.e., a high abundance of prostasin and a low abundance of matriptase. We may view prostasin as a sensor or surveillance agent for epithelial polarity and integrity. Once an insult, e.g., inflammation, results in injury of the normal epithelium, depolarization ensues and injury repair programs are mobilized. EGFR is a key player in epithelial injury repair (Repertinger et al., 2004) but at some point the cells need to initiate a "stop program" of EGFR signaling to allow epithelial differentiation and re-polarization. The executor of the "stop program" appears to be prostasin or related proteases, activated or re-expressed to initiate terminal differentiation. Prostasin's role in this regard is in complete agreement with the observation that in prostasin-knocked-out mouse skin, terminal differentiation of the skin epithelium is defective, marked by lack of tight junction formation and absence of occludin expression (Leyvraz et al., 2005). Also in agreement with this model is the observation that prostasin expression is down-regulated during inflammation while forced prostasin expression attenuates inflammation-induced gene expression (Chen et al., 2006b). Silencing of prostasin by epigenetic events or growth factors and cytokines (Chen and Chai, 2002; Chen et al., 2004; Chen et al., 2006a; Chen et al., 2006l), would be a pre-requisite for tumorigenesis and gain of aggressive properties such as invasion and metastasis.

The overall impact of the prostasin cleavage of EGFR on the cell is dependent on the rate of internalization for the cleaved receptor. In cells with normal or even accelerated turnover rate,

such as the PC-3 (Bonaccorsi et al., 2007), the net effect is inhibition of EGF-EGFR signaling due to reduced cell surface receptor presentation. We predict for cancer cells that are EGFR-dependent this will be the predominant phenotype as in these cells the EGF-EGFR signaling is tightly coupled with receptor internalization (Carpenter, 2000). In these cells, the rapid receptor internalization also prevented our detection of the cleaved forms of EGFR (Chen et al., 2007a). In cells with impeded receptor internalization, such as the HEK-293 and its derivatives (the FT-293) (Johannessen et al., 2001), the net effect is a constitutively activated and truncated EGFR remaining on the membrane, activating the downstream signals. Cells that are EGFR-over-expressing but are not EGFR-dependent would potentially present this phenotype.

The HA-tagged matriptase has previously been shown to retain the matriptase protease activity and substrate specificity. We have confirmed its ability to activate prostasin in the FT-293 cells when the two proteases are co-expressed from their full-length cDNA's (Figure 3-1). Matriptase is also capable of cleaving the EGFR at apparently the same sites cleaved by prostasin, but appeared to be a weaker enzyme in this role (Figure 3-2, compare Lanes 4 and 5). Its action on EGFR could be amplified, however, if an abundance of matriptase is expressed, as was seen with the serine active-site mutant prostasin transfected PC-3 cells (Chen et al., 2007b). In these cells, we now know that the mutant prostasin is incapable of cleaving EGFR (Figure 3-2, compare Lanes 5 and 6), but the robustly induced matriptase certainly could. Several of the molecular changes that were induced by both the wild-type and the serine active-site mutant prostasins, such as the down-regulation of EGFR (protein), uPA, uPAR, COX-2, and iNOS (mRNA) (Chen et al., 2007c), could be attributed to matriptase action. We must emphasize that

we attribute the EGFR ECD cleavages to matriptase and prostasin when they are co-expressed in the FT-293 cells because these cells express very little to no EGFR, matriptase, or prostasin. We do not at the moment have evidence for a direct interaction between matriptase and EGFR, or between activated prostasin and EGFR. The molecular landscape of the membrane serine proteases is still expanding (Netzel-Arnett et al., 2006a), we are limited by our knowledge of this new family of proteases to determine whether the EGFR ECD is cleaved by a downstream protease activated by prostasin. We did not observe a significant induction of matriptase expression in the FT-293 cells expressing prostasin (data not shown), ruling out a solo matriptase action on the EGFR and implicating prostasin in this signal modulation mechanism.

The Erk1/2 or Akt phosphorylation up-regulation observed in the FT-293 cells co-expressing EGFR, matriptase, and prostasin was attributed to the auto-tyrosine-phosphorylated EGFR110 N-terminally truncated fragment (Figure 3-4). We intended to generate a deletion mutant EGFR representing the EGFR110 by determining the precise location of the matriptase-prostasin cleavage. We had attempted N-terminal amino acid sequencing of the purified EGFR110, but this fragment was N-terminally blocked, preventing amino acid sequence determination. The N-terminal deletion/truncation experiments shown in Figure 3-6 have allowed us to confirm for two independent cleavages by the serine proteases, ruling out other potential mechanisms for the observed molecular weight differences between the wild-type EGFR, EGFR135, and EGFR110. Efforts are under way for a precise mapping of the two cleavage sites by site-directed mutagenesis and ultimately for generating a cDNA encoding the EGFR110 to fully evaluate its impact on cell signaling.

The EGFR ECD contains the ligand-binding domains that form the target of the monoclonal antibody (Mab) drug ImClone C225/cetuximab/Erbitux, approved for advanced head-and-neck and colorectal cancers as a third-line treatment option. The matriptase → prostasin → EGFR cascade can play a critical role in anti-EGFR therapies for cancer using Mab drugs targeting the ECD. The cleaved EGFR, EGFR135 and EGFR110, are no longer responsive to EGF stimulation (Figure 3-4A), presumably due to the loss of the ligand-binding domains, all or partial. By this reasoning, Erbitux or similar Mab drugs targeting the ligand-binding domains of EGFR would no longer be effective. If the cleaved EGFR is retained on the membrane and activates downstream signaling, as we have observed in the FT-293 cells in this study, we will expect to see lower sensitivity to the Mab drugs, i.e., requiring higher doses for growth inhibition. In this scenario, protease inhibitors specific for the matriptase-prostasin cascade may be considered as an appropriate adjuvant. If the proteolytic cleavage of EGFR results in its rapid internalization and turnover, as we have reported for the PC-3 cell line (Chen et al., 2007d), EGFR-mediated signaling is expected to be reduced and drug sensitivity to the Mab's should increase, i.e., showing growth inhibition at a lower dose. In this scenario, prostasin, pre-activated or activated upon reaching the cancer cells by their endogenously over-expressed matriptase or related proteases, may be used as an adjuvant. We have shown that the EGFR ECD cleavage role of prostasin is not dependent on its membrane anchorage via the GPI (Figure 3-3), making it a suitable candidate for development of a systemically deliverable agent to treat certain cancers that are dependent on over-expressed EGFR for growth and survival signals.

CONCLUSIONS: We have identified EGFR as a biological substrate for the epithelial extracellular serine protease activation cascade involving matriptase-prostasin. Cleavage in the EGFR ECD induces changes in receptor tyrosine kinase activation and cell signaling changes depending on the cell type with regard to receptor internalization rate. The novel protease-activated EGFR signal modulation mechanism may have clinical implications in therapies for treating cancers by targeting the EGFR ECD.

CHAPTER FOUR ANDROGEN REGULATION OF PROSTASIN GENE EXPRESSION IS MEDIATED BY STEROL REGULATORY ELEMENT BINDING PROTEINS AND SLUG

4.1 Introduction

Prostasin belongs to a unique group of serine proteases that also include testisin (Manton et al., 2005a) and NES1 (Goyal et al., 1998), which are downregulated in advanced cancers and capable of suppressing tumor growth or invasion. Loss of prostasin expression in human prostate and breast cancer cell lines is due partly to DNA methylation in the 5'-flanking region and exon 1 of the prostasin gene (Chen and Chai, 2002; Chen et al., 2004).

In human prostate cancers, the loss of prostasin expression is associated with the hormone-refractory phenotype (Takahashi et al., 2003). We set out to investigate whether androgen regulates prostasin gene expression. We examined the 5'-flanking region sequence of the prostasin gene but did not identify any direct repeats of the consensus androgen response element (ARE) half-site TGTTCT (Claessens et al., 2001), or its variants. Yu et al., (Yu et al., 1996) had identified a sterol regulatory element (SRE) at position -897 of the prostasin gene by sequence homology with a published SRE (Osborne et al., 1988). The SRE's are the binding sites of mature sterol-regulatory element-binding proteins, or, the SREBP's, and the SRE's may provide an alternative mechanism for androgen regulation of gene expression (Swinnen et al., 1997). First, androgens up-regulate mRNA expression of SREBP-1c and SREBP-2 (Heemers et al., 2001c); and second, androgens also significantly increase the expression of

sterol-regulatory element-binding protein cleavage-activating protein (SCAP), resulting in an increase of mature SREBP's (Heemers et al., 2001b).

In this chapter, we examined prostasin expression changes in the human prostate cancer cell line LNCaP in response to the androgen dihydrotestosterone (DHT). DHT upregulated SREBP-1c and SREBP-2 mRNA expression in the LNCaP cells, but prostasin mRNA expression was not affected in response to DHT in this cell line. The SREBP's (SREBP-1c and SREBP-2) were shown to be capable of up-regulating prostasin's promoter activity when co-transfected in the HEK-293 cells, prompting the search for a potential negative regulator of the prostasin gene under DHT treatment. The Snail family of zinc-finger transcription factors, including Snail and Slug, play a key role in epithelial-to-mesenchymal transition (EMT) during embryonic development and tumor progression (Hemavathy et al., 2000a; Nieto, 2002b). Recent research has shown that the expression of these factors is dramatically induced during the progression of hepatocellular carcinoma (Sugimachi et al., 2003), esophageal squamous cell carcinoma (Uchikado et al., 2005), breast cancer (Blanco et al., 2002; Hajra et al., 2002c) and ovarian cancer (Kurrey et al., 2005). Snail and Slug are responsible for the down-regulation of E-cadherin expression in tumor cells (Hajra et al., 2002b; Batlle et al., 2000a), resulting in loss of cell-cell adhesion, increased motility and invasiveness. The Snail/Slug repression of E-cadherin transcription is mediated by their interaction with a CACCTG sequence (named E-Box) in the promoter region (Batlle et al., 2000b; Bolos et al., 2003a) and further recruitment of histone deacetylase 1 (HDAC1)/HDAC2 (Peinado et al., 2004). In addition to down-regulating the expression of adheren junction components, Snail represses the expression

of several differentiation markers including Na/K-ATPase (Espineda et al., 2004), cytokeratin 18, and MUC1 (Guaita et al., 2002b) but activates several mesenchymal genes such as ZEB1 and LEF-1 (Guaita et al., 2002a). Furthermore, Snail family transcription factors up-regulate the expression of matrix metalloproteases (MMP's) (Miyoshi et al., 2004) and confer upon cells anti-apoptotic properties (Vega et al., 2004; Tribulo et al., 2004). A candidate E-box sequence is located in the prostasin promoter, providing a potential mechanism for expression regulation by these transcription factors. Indeed, we found that Snail and Slug repress prostasin expression, while the Slug mRNA was up-regulated dramatically by DHT in the LNCaP cells. Androgen's effect on the transcription of the prostasin gene is apparently mediated by two opposing classes of transcription factors, i.e., the SREBP's and the Slug.

4.2 Materials and Methods

4.2.1 Cell lines and maintenance

The PrEC normal prostate epithelial cells were obtained from Clonetics (San Diego, CA). The LNCaP, DU-145, and PC-3 cells were from the American Type Culture Collection (ATCC, Manassas, VA). The HEK-293-EBNA (hereon referred to as HEK- 293) cells were from Invitrogen (Carlsbad, CA). Cell culturing and maintenance procedures were as described previously (Chen et al., 2001b; Chen et al., 2001a).

4.2.2 Treatment of cells with DHT or EGF

The LNCaP cells were seeded in RPMI-1640/10% FBS (fetal bovine serum) at 50% confluence in 25-cm2 tissue culture flasks for 48 hours. Cells were washed twice in OPTI-MEM I medium (Invitrogen), and placed under fresh OPTI-MEM I medium for 24 hours. DHT was dissolved in 95% ethanol but diluted to a working stock solution of 10 μM in 0.95% ethanol before being added to the culture media. A solution of 0.95% ethanol was used as the solvent control. Cells were treated for 24 hours in 1 nM, 10 nM, or 100 nM DHT added to the OPTI-MEM I before RNA preparation.

DU-145 cells receiving EGF treatment were seeded in RPMI-1640/10% FBS at 50% confluence in 25-cm2 tissue culture flasks for 48 hours and washed twice with RPMI- 1640 medium before continued culturing under RPMI-1640/0.1% BSA (bovine serum albumin) for 24 hours. Recombinant human EGF (Invitrogen) was diluted in 1640/0.1% BSA at a concentration of 10 ng/ml and used to treat the cells. Cells were harvested for RNA preparation at 6, 12, or 24 hours after EGF treatment.

4.2.3 RNA isolation, reverse transcription, and real-time PCR analysis

Total RNA of cultured cells was prepared using the Trizol reagent (Invitrogen). Reverse transcription was carried out using random primers of the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocols.

Primers used for the real-time PCR analysis of reverse-transcribed mRNA's were designed with the aid of the Beacon Designer 4.0 software (PREMIER Biosoft International, Palo Alto, CA), and synthesized by Integrated DNA Technologies (Coralville, IA). Real-time PCR was performed on a Bio-Rad MyiQ system using the iQ SYBR Green Supermix reagents, following the manufacturer's protocols. The thermal cycling program starts with an initial denaturation step at 95°C for 3 minutes, and is followed by 40 cycles between 15 seconds at 95°C and 1 minute at 60°C. The relative quantities of gene-specific mRNA expression were determined by the comparative CT method: wherein CT refers to the "threshold cycle", and is determined for each experiment with the aid of the MyiQ software. Amplification of the 18S rRNA was performed for each reverse-transcribed sample as an endogenous quantification standard. ΔCT = (Gene-specific CT – 18S rRNA CT); ΔΔCT = (ΔCT of experimental conditions - ΔCT of control conditions). The fold-difference in gene expression was determined by 2-(ΔΔCT). The primers used for real-time PCR are as follows, in the order of forward and reverse:

18S rRNA:	5'- GTA ACC CGT TGA ACC CCA TT -3'
	5'- CCA TCC AAT CGG TAG TAG CG -3'
Prostasin:	5'- ATC TTG GAT TAC TCC GGT CGG -3'
	5'- ACA CAT GGA CGC CTT CAT AGG -3'
PSA:	5'- TAT TGT AGT AAA CTT GGA ACC TTG -3'
	5'- TTA CAC CAT TTA AGA AAC ACT CTG -3'
SREBP-1a forward:	5'- ATG GAC GAG CCA CCC TTC A -3'
SREBP-1c forward:	5'- GGA GCC ATG GAT TGC ACT TTC -3'

SREBP-1 reverse for 1a and 1c: 5'- GAA GTC ACT GTC TTG GTT GTT G -3'

SREBP-2: 5’- GCT GCA ACA ACA GAC GGT AAT G -3’

5’- CTG GTA TAT CAA AGG CTG CTG GAT -3’

SNAIL: 5'- TCC CTC TTC CTC TCC ATA CC -3'

5'- TGG CAG TGA GAA GGA TGT G -3'

SLUG: 5’- GAG CAT ACA GCC CCA TCA CTG -3’

5’- AGG AGG TGT CAG ATG GAG GAG -3’

4.2.4 Cloning of the 5’-flanking region of the human prostasin gene

An 1,848-bp fragment corresponding to nucleotides 391 to 1838 (5’-flanking region and exon 1) of the human prostasin gene (U33446) was amplified using genomic DNA of the LNCaP cells and the Advantage-GC Genomic PCR kit (Clontech, Palo Alto, CA). The forward primer is 5’-AAT GCC AGC CTT TGC CAG GCT GTG GTG TGC -3’, and the reverse primer is 5’-CTG TCC CCG ACC GGA GTA ATC CAA GAT AGA -3’. The amplified fragment was subcloned into the pGEM-T Easy vector (Promega, Madison, WI), and named pProPro-TA.

4.2.5 Plasmid construction and molecular cloning

The prostasin promoter-luciferase reporter plasmid pProPro1031-GL3 was constructed by subcloning a 1,263-bp EcoR I-BamH I fragment from the pProPro-TA. The sticky ends were filled with the Klenow enzyme (Invitrogen) and ligated into Kpn I / Hind III digested,

Klenow-filled pGL3-Basic vector (Promega). This 1,263-bp prostasin promoter fragment contains 1,031 bp of the 5'-flanking region and 232 bp of exon 1.

The cDNA fragments encoding the mature forms of human SREBP-1c (amino acids 1 to 436) and SREBP-2 (amino acids 1 to 481), and full-length human SNAIL and SLUG were generated by RT-PCR using the total RNA of LNCaP cells and the SuperScriptTM III RNase H- reverse transcriptase (Invitrogen). The amplified fragments were subcloned into the plasmid pcDNA3 (Invitrogen) for transient expression in tissue cultured cells. The following primers, listed in the order of forward and reverse, were used for amplification of the specific cDNA's:

SREBP-1c: 5'- GAATTC GCC ATG GAT TGC ACT TTC GA -3'

5'- TCA GTC AGG CTC CGA GTC ACT GCC A -3'

SREBP-2: 5'- GGG CGA TGG ACG ACA GCG GCG AGC T -3'

5'- TCA CCG TGA GCG GTC TAC CAT GCC -3'

SNAIL: 5'- GAATTC ACT ATG CCG CGC TCT TTC CTC -3'

5'- TCA GCG GGG ACA TCC TGA GCA GCC GGA -3'

SLUG: 5'- GAATTC AAG ATG CCG CGC TCC TTC CTG -3'

5'- TCA GTG TGC TAC ACA GCA GCC AGA TTC -3'

The underlined sequences are restriction site linkers used for subcloning.

4.2.6 Promoter activity assays

On day 1, HEK-293 cells were plated on a poly-L-lysine-coated 12-well plate at the density of 4 x 10^5 cells per well. On day 2, the luciferase reporter plasmid (pProPro1031-GL3, 1.3 μg), an

pSV-β-Galactosidase reference plasmid (Promega, 0.2 μg) and an appropriate transcription factor expression plasmid (pcDNA3-based, 0.1 μg) were transfected into the cells using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocols. Twenty-four hours after transfection, the cells were lysed with the Reporter Lysis Buffer (Promega). Cell lysate samples were assayed for luciferase activity using a Luciferase Assay Kit (Promega). The β- galactosidase activity was measured using a β-Galactosidase Enzyme Assay Kit (Promega) and used to normalize for transfection efficiency.

4.2.7 Transfection of cell lines and analysis of prostasin expression

Transfection of the HEK-293 cells (4 x 10^5 cells) with the SREBP-2 cDNA plasmid (2 μg) was carried out using the Lipofectamine 2000 reagent. Transfection of the DU-145 or the LNCaP human prostate cancer cells was carried out on a BTX ECM-600 electroporator (Harvard Apparatus, Holliston, MA). Cells were cultured in regular medium to confluence, harvested, and re-suspended in OPTI-MEM I medium at a density of 2 x 10^7/ml. Six million cells were then mixed with 20 μg of cDNA plasmid (SREBP-2 for DU-145, or SLUG for LNCaP) and electroporated in 4-mm BTX cuvettes, at 305 V and 700 μF, with 360 ohms resistance.

All transfected cells were then cultured for 48 hours in regular medium before total RNA isolation with Trizol, or total protein extraction as described before (Chen et al., 2001a). Total RNA samples were subjected to RT/real-time PCR analysis as described above, and total cell lysates were subjected to western blot analysis with a prostasin specific polyclonal antibody as described previously (Chen et al., 2001b). Lysate protein concentration was determined with a

DC Protein Assay Kit (Bio-Rad). An immunoblot with a β–tubulin antibody (Sigma- Aldrich, St. Louis, MO; used at 1:4,000) was performed as a control for protein loading.

4.2.8 Statistical analysis

The Student t-Test (one-tailed, equal variance) was performed for the real-time PCR data and the promoter activity data, for which $p < 0.05$ was used to define statistically significant difference.

4.3 Results

4.3.1 Prostasin mRNA expression is unaffected by DHT in the LNCaP cells

We have previously shown that prostasin is expressed in normal prostate epithelial cells (PrEC) and the androgen-responsive LNCaP prostate cancer cells, but down-regulated due to prostasin promoter DNA hypermethylation in the DU-145 and PC-3 prostate cancer cells (Chen et al., 2001a; Chen et al., 2004), which are not responsive to androgen. In this chapter, we used the quantitative method of reverse-transcription/real-time PCR to determine the relative expression levels of prostasin in these four cell types. In Figure 4-1A, we show that the prostasin mRNA in the LNCaP cell line is expressed at approximately 0.82 (82%) of that in the PrEC cells. The DU-145 and PC-3 cells express very small amounts of the prostasin mRNA, at 0.041 (4.1%) and 0.095 (9.5%) of that in the PrEC cells.

To determine the effect of androgen on prostasin expression, we treated the LNCaP cells with 1 nM, 10 nM, or 100 nM DHT for 24 hours as described in Materials and Methods. Prostasin

mRNA expression was measured by reverse-transcription/real-time PCR using the 18S rRNA as the reference. As shown in Figure 4-1B, prostasin mRNA expression in the LNCaP cells was unaffected by DHT under these conditions. The same samples were also applied to a real-time PCR analysis of the mRNA encoding the human prostate-specific antigen (PSA) to validate the DHT treatment. PSA mRNA was found to be up-regulated to 4.2 fold at 1 nM, 7.5 fold at 10 nM, and 8.5 fold at 100 nM (Figure 4-1C), consistent with previously reported findings by others that DHT upregulates PSA expression in the LNCaP cell line (Zhu et al., 2003).

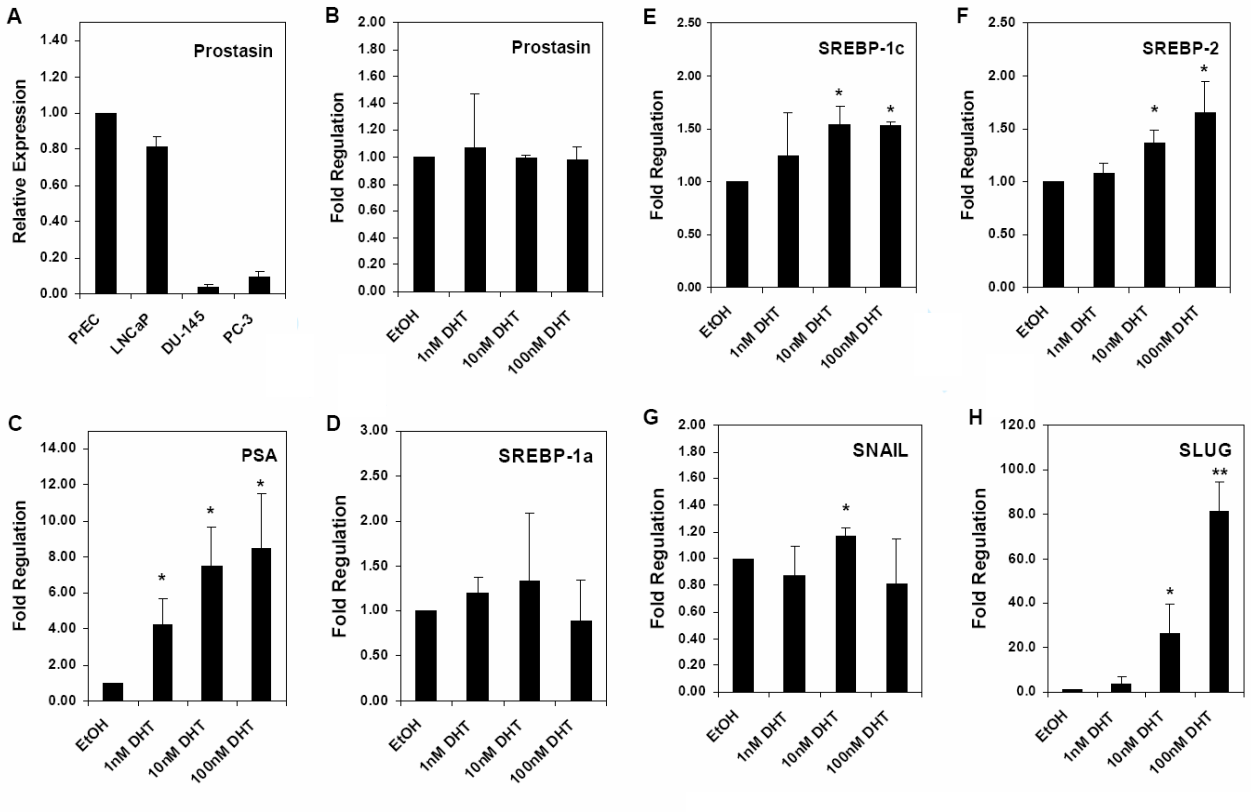


Figure 4-1 Prostasin expression in prostate cells and effect of DHT on expression of prostasin, PSA, SREBP's, SNAIL, and SLUG mRNA in the LNCaP cells.

Relative prostasin mRNA expression in the PrEC, LNCaP, DU-145, and PC-3 cells is shown in (A). LNCaP cells

were treated with 1 nM, 10 nM, or 100 nM DHT for 24 hours followed by RNA extraction. The total RNA samples of vehicle- or DHT-treated LNCaP cells were subjected to RT/real-time PCR analysis to quantify the mRNA expression level of Prostasin (B), PSA (C), SREBP-1a (D), SREBP-1c (E), SREBP-2 (F), SNAIL (G), and SLUG (H). The data are expressed as an x-fold of stimulation or repression of the mRNA expression level in the DHT-treated cells as compared to the vehicle-treated cells. The results represent the Mean ± S.D. of three independent experiments (two for Panel A). The asterisk (*) denotes a statistically significant difference ($p<0.05$) in the mRNA expression level for the indicated gene between the DHT-treated and vehicletreated LNCaP cells. The double asterisk (**) denotes that the SLUG mRNA expression level in the LNCaP cells treated with 100 nM DHT is statistically different from that in the cells treated with 10 nM DHT.

4.3.2 DHT up-regulates the expression of transcription factors SREBP-1c, SREBP-2, Snail, and Slug in the LNCaP cells

While PSA expression is responding expectedly to the DHT treatment of the LNCaP cells, but the prostasin expression did not, we turned to investigate the role of potential alternative regulators of prostasin expression. Based on our sequence analysis in the prostasin promoter region, we have identified candidate regulatory sites for the SREBP's and the Snail family transcription factors. A candidate SRE was previously identified by Yu et al. at position -897 of the prostasin promoter (Yu et al., 1996). We have also identified a candidate E-box sequence, CACCTG, at -574 of the prostasin promoter, a potential site for regulation by the Snail family transcription factors. This candidate site is identical to the one that confers repression of the E-cadherin gene (Batlle et al., 2000c; Bolos et al., 2003b). Realtime PCR analysis of the DHT-treated LNCaP cells following reverse transcription showed that the mRNA expression of SREBP-1c, SREBP-2, Snail, and Slug was upregulated by DHT, at the 10 nM or the 100 nM dose, but not at the 1 nM dose. The change of SREBP-1a mRNA expression under DHT treatment was not statistically different at either 10 nM or 100 nM (Figure 4-1D). The

SREBP-1c mRNA was upregulated to 1.5 fold at either 10 nM or 100 nM DHT (Figure 4-1E). The SREBP-2 mRNA was up-regulated to 1.37 fold at 10 nM DHT, and 1.66 fold at 100 nM DHT (Figure 4-1F). The Snail mRNA was marginally up-regulated to 1.17 fold at 10 nM DHT, but its change at 100 nM DHT was not statistically different (Figure 4-1G). The up-regulation of Snail might be random statistical noise although the p-value was calculated less than 0.05. In contrast, expression of the Slug mRNA was up-regulated dramatically by DHT in a dose dependent manner, to 26 fold at 10nM, and 81 fold at 100 nM (Figure 4-1H).

4.3.3 The prostasin gene promoter is regulated by SREBP's, Snail, and Slug

Next we set out to evaluate the direct impact of the SREBP's and the Snail family transcription factors on the prostasin promoter, and the impact of combinations of these factors. We chose the HEK-293 cells for the evaluation of the prostasin promoter because this cell line yields high and consistent transfection efficiency, especially for multiple plasmids in one cell (e.g., a promoter-reporter, two transcription factor expression plasmids, and a β-galactosidase expression plasmid). The HEK-293 cell line is of human kidney epithelial origin where prostasin expression is relatively abundant in the native tissue (Verghese et al., 2004a; Yu et al., 1995), making this cell line relevant to analysis of prostasin expression.

A prostasin promoter-reporter DNA construct, pProPro1031-GL3, was co-transfected with plasmids carrying the cDNA's coding for the transcription factors SREBP-1c (mature peptide), SREBP-2 (mature peptide), Snail, or Slug into the HEK-293 cells. As a result, the prostasin promoter's activity was up-regulated to 1.88 fold and 6.7 fold, respectively, by SREBP-1c and

SREBP-2, but down-regulated to 43% by Snail and to 59% by Slug (Figure 4-2A). The previously reported SRE in the multiple cloning site of the pGL3-Basic vector (Annicotte et al., 2001) was removed during the construction of pProPro1031-GL3. The effect of prostasin promoter activity up-regulation by the SREBP's can be attributed to the prostasin promoter.

In the next set of experiments, we tested the effects of co-transfecting SREBP-2 and SLUG on the activity of the prostasin promoter to mimic the effect of DHT on the LNCaP cells, i.e., a small up-regulation of a strong positive regulator SREBP-2 but a dramatic up-regulation of a moderate repressor SLUG. We maintained a constant level of the SREBP-2 plasmid while gradually increased the amount of the SLUG plasmid in this series of experiments. The results showed that SLUG co-transfection was able to negate SREBP-2's positive impact on the prostasin promoter in a dose-dependent manner (Figure 4-2B). SREBP-2 up-regulation of the prostasin promoter was reduced by 38% at 1:1 molar ratio of SREBP-2/SLUG co-transfection, further reduced by 51% at 1:2 molar ratio, and by 59% at 1:4 molar ratio.

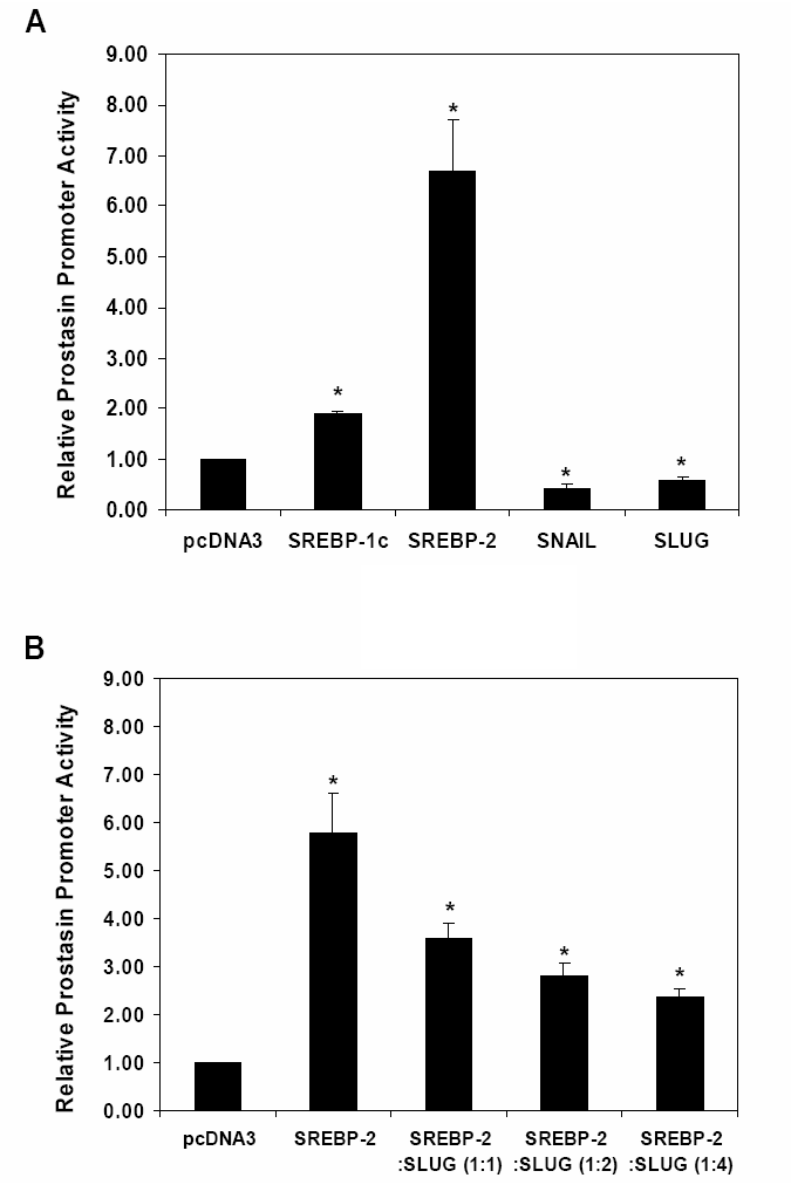


Figure 4-2 Prostasin promoter activity is regulated by transcription factors SREBP-1c, SREBP-2, SNAIL, and SLUG in 293-HEK cells.

(A) HEK-293 cells were transiently transfected with 1.3μg of the pProPro1031-GL3 plasmid containing the luciferase reporter gene under the control of the –1031/+232 fragment of the human prostasin gene promoter-exon 1 region, 0.2μg of the pSV-β-Galactosidase reference plasmid, and 0.1μg of an appropriate transcription factor expression plasmid (SREBP-1c-pcDNA3, SREBP-2-pcDNA3, SNAIL-pcDNA3, or SLUG–pcDNA3). The empty pcDNA3 was used as the control. (B) Cells were transfected with the pProPro1031-GL3, pSV-β- Galactosidase, and a mixture of SREBP-2-pcDNA3 and SLUG-pcDNA3 at the indicated molar ratios. Luciferase activities were determined 24 hours after transfection and normalized with β-galactosidase activities. The data are expressed as an

x-fold of stimulation or repression of the prostasin promoter activity in cells co-transfected with the indicated transcription factor expression plasmid, relative to the control cells cotransfected with the pcDNA3 empty vector. The results represent the Mean ± S.D. of three independent experiments. The asterisk (*) denotes a statistically significant difference ($p<0.05$) in the promoter activity between the transcription factor expressing cells and control cells.

4.3.4 SREBP-2 or Slug transfection regulates endogenous prostasin expression

In order to show that SREBP-2 alone can regulate endogenous prostasin expression, we transfected the SREBP-2 cDNA into the HEK-293 cells, which do not express prostasin (Chen et al., 2001b). The HEK-293 cells responded to the SREBP-2 transfection with an increase of endogenous prostasin mRNA expression to 46 fold (Figure 4-3A), and the expressed prostasin protein product was readily detected by a western blot (Figure 4-3B). Transfection of SREBP-2 in the DU-145 cell line resulted in an increase of prostasin mRNA expression to 4.68 fold (Figure 4-3C). Again, the increased expression of the prostasin protein product was detected by a western blot (Figure 4-3D).

Similarly, we tested if Slug alone can regulate endogenous prostasin expression by transfecting a Slug cDNA into the LNCaP cells. The results showed that a transient transfection of Slug cDNA reduced prostasin mRNA expression in the LNCaP cells by 33% (Figure 4-3E). The prostasin protein expression was also reduced (Figure 4-3F). The transfection efficiency for the HEK-293, DU-145, or LNCaP cells using the appropriate methods was evaluated in parallel experiments using the pEGFP-N1 plasmid (BD Biosciences, Palo Alto, CA). Conditions chosen for experiments yielded >90% transfection for the HEK-293 cells, and >50% transfection for the DU-145 and LNCaP cells (data not shown).

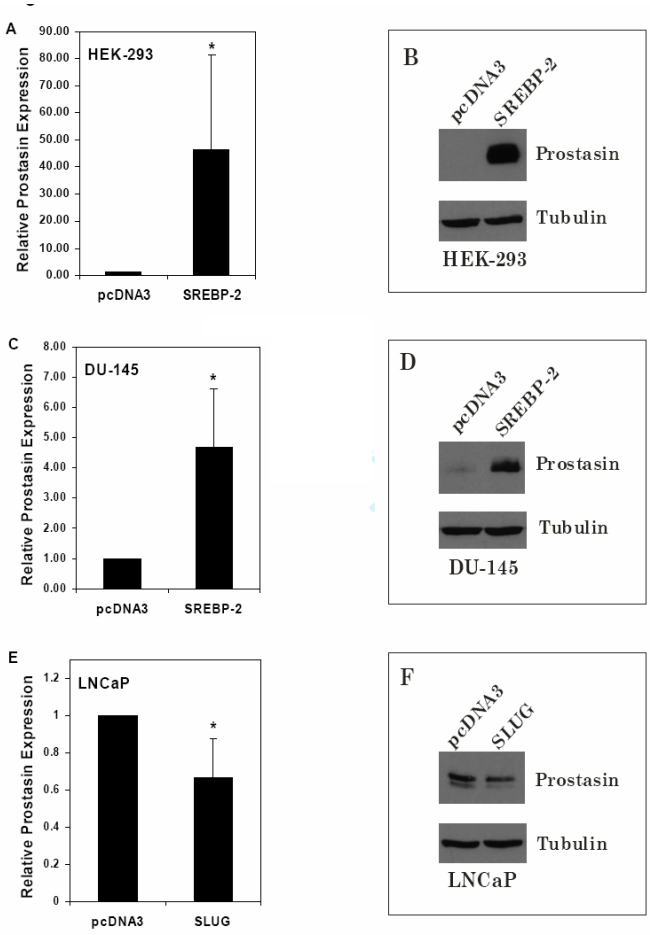


Figure 4-3 Prostasin promoter activity is regulated by transcription factors SREBP-1c, SREBP-2, SNAIL, and SLUG in 293-HEK cells.

(A, B) HEK- 293 cells were transiently transfected with pcDNA3 or SREBP-2-pcDNA3 (shown as SREBP-2) using Lipofectamine 2000 reagent. (C, D) DU-145 cells were transiently transfected with pcDNA3 or SREBP-2-pcDNA3 (shown as SREBP-2) via electroporation. (E, F) LNCaP cells were transiently transfected with pcDNA3 or SLUGpcDNA3 (shown as SLUG) via electroporation. Total RNA samples (A, C, E) and whole cell lysates (B, D, F) were prepared 48 hours after transfection, RT/real-time PCR (A, C, E) and western blotting (B, D, F) were performed to determine the levels of endogenous prostasin expression. The RT/real-time PCR results represent the Mean ± S.D. of three independent experiments. The asterisk (*) denotes a statistically significant difference ($p<0.05$) in the prostasin mRNA expression level between the transcription factor expressing cells and control cells. The western blot results are representative of duplicate experiments showing the regulation of prostasin protein expression by SREBP-2 or SLUG. For the prostasin western blots, 50 μg of total protein were loaded for each HEK-293 sample (without β–mercaptoethanol), 45 μg were loaded for each DU-145 sample (without β–mercaptoethanol); and 30 μg were loaded for each LNCaP sample (with β–mercaptoethanol). For the β–tubulin western blots, 40 μg of total protein were loaded for each sample (with β–mercaptoethanol).

4.3.5 EGF up-regulates the expression of Snail and Slug in the DU-145 cells

Because hormone-refractory prostate cancers are marked by their active growth factor autocrine signaling (Hofer et al., 1991), we tested if epidermal growth factor (EGF) can affect the expression of the transcription factors Snail and Slug as a potential mechanism of prostasin down-regulation. Real-time PCR analysis of the EGF-treated DU-145 cells following reverse transcription showed that the Snail mRNA expression was upregulated by EGF to 1.55 fold at 24 hours of treatment, while the changes of Snail mRNA level at 6 or 12 hours were not statistically different (Figure 4-4A). The Slug mRNA expression was up-regulated at 6 and 12 hours after EGF treatment, to 2.26 and 2.91 fold, respectively; while the changes of Slug mRNA level at 24 hours were not statistically different (Figure 4B).

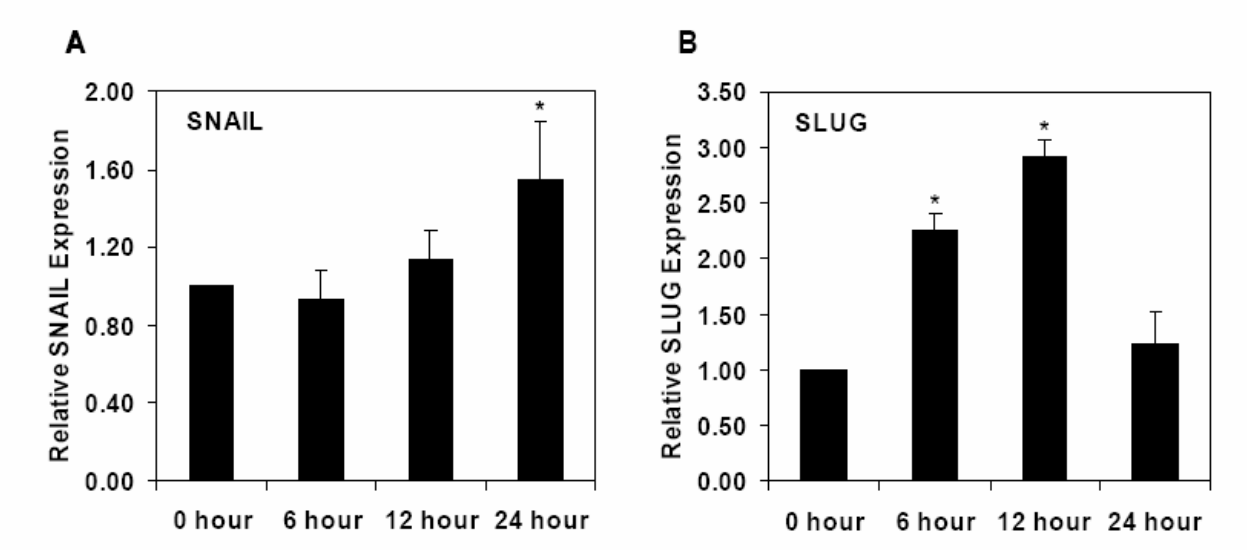


Figure 4-4 EGF up-regulates mRNA expression of SNAIL and SLUG in DU-145 cells.

DU- 145 cells were treated with EGF (10 ng/ml) for the indicated times. The total RNA samples were subjected to RT/real-time PCR analysis to quantify the mRNA expression levels of (A) SNAIL and (B) SLUG. The results represent the Mean ± S.D. of three independent experiments. The asterisk (*) denotes a statistically significant difference ($p<0.05$) in the mRNA expression level between the EGF-treated cells and untreated cells.

4.4 Discussion

The down-regulation of prostasin serine protease, an invasion suppressor, in the hormone-refractory prostate cancers (HRPC) suggests a potential cause of the enhanced malignance of the HRPC. The purpose of this chapter's study was to probe for the molecular basis of this down-regulation. We have previously investigated prostasin down-regulation in two androgen-independent, or non-responsive human prostate cancer cell lines, the DU-145 and PC-3, and showed that promoter DNA methylation is partly responsible for prostasin gene silencing, while nerve growth factor alone could up-regulate prostasin mRNA in these cell lines (Chen et al., 2004). The promoter sequence of the prostasin gene does not contain any consensus ARE's to confer transcription regulation by androgen directly. This is further demonstrated in the hormone-responsive LNCaP human prostate cancer cells that prostasin expression did not respond to DHT treatment, while the classic androgen-regulated serine protease PSA showed the expected response (Figure 4-1B and 4-1C).

We were prompted, by the previously identified SRE in the prostasin promoter (Yu et al., 1996), to investigate the impact of the sterol-regulatory element-binding proteins on prostasin expression in response to androgen. We found that both SREBP-1c and SREBP-2 are capable of up-regulating the prostasin gene promoter. In the case of SREBP-2 the upregulation was quite dramatic to 6.7 fold. It has been shown previously by others using northern blot analysis that androgen up-regulates SREBP-1c and SREBP-2, but not SREBP-1a in the LNCaP cells (Heemers et al., 2001a), a result confirmed by us in this study using RT/realtime PCR. We did not pursue the role of the SREBP-1a protein because it is unlikely to be a major mediator of

androgen's effect on prostasin gene expression in the LNCaP cells. We showed that SREBP-2 expression alone was able to induce a >4-fold increase of prostasin mRNA expression, and a corresponding prostasin protein upregulation in the DU-145 cells (Figure 4-3C and 4-3D). A similar effect was observed in the embryonic kidney cells HEK-293 transfected with an SREBP-2 cDNA (Figures 4-3A and 4-3B). It is evident that an SREBP-2 up-regulation is sufficient for up-regulation of prostasin expression. It is then expected that treatment of a hormone-responsive cell line like the LNCaP with DHT would result in an increase of prostasin mRNA expression because the SREBP's would be up-regulated and sufficient to up-regulate the prostasin gene. The apparent lack of response of the prostasin gene to the DHT treatment at either the 10 nM or the 100 nM dose (Figure 4-1B) indicates that there is a negative regulatory factor under these conditions. Our finding that the transcription repressor SLUG is dramatically up-regulated by DHT in the LNCaP cells provides the basis of the expected negative regulation of the prostasin gene upon androgen stimulation. The Slug was shown to repress the prostasin promoter in a promoter activity assay (Figure 4-2A), and its transfection into the LNCaP cells was sufficient to repress the endogenous prostasin mRNA and protein expression (Figures 4-3E and 4-3F). More important, Slug, at increasing levels, was able to reduce the up-regulation of prostasin promoter by SREBP-2 in a dose-dependent manner (Figure 4-2B).

What then, may be the cause of the prostasin down-regulation associated with the HRPC? Clearly, promoter DNA methylation can provide only part of the answer because the DU-145 cell line, despite its rather extensive methylation in the prostasin promoter-exon 1 region (Chen et al., 2004), is readily responsive to SREBP-2 up-regulation of prostasin expression (Figures 4-3C and

4-3D). A lower level of SREBP's in the hormone-independent cell lines would be a potential molecular mechanism of prostasin down-regulation, in addition to the effect of promoter DNA methylation. Indeed, it has been reported previously by others that the DU-145 and PC-3 human prostate cancer cell lines have lower levels of SREBP-2 expression than normal prostate epithelial cells (Chen and Hughes-Fulford, 2001). The fact that Slug itself is highly responsive to androgen stimulation (Figure 4-1H) would suggest that in the absence of androgen the prostasin promoter is less repressed. In the HRPC, however, functional cross-talks between the AR and growth factor-dependent transmembrane signaling pathways is a molecular mechanism for prostate cancer cells to gain growth advantage in the absence of androgen (Culig, 2004). These cross-talks result in activation of the AR by phosphorylation, through the actions of various kinases. It is reasonable to postulate that in the HRPC, AR activation by the various ligand independent mechanisms may be the molecular basis of sustained up-regulation of Slug expression. Moreover, growth factor signaling may also affect the Slug levels directly, and independently of the AR. In the DU-145 cells, which lack the AR (Culig et al., 1993), EGF was able to up-regulate SNAIL mRNA expression to 1.55 fold at 24 hours, and to upregulate Slug mRNA expression to 2.91 fold at 12 hours (Figure 4-4), confirming that growth factors can also act to sustain an elevated expression of transcription repressors by AR independent mechanisms. On the other hand, EGF has also been shown to upregulate SREBP-1c but not SREBP-1a mRNA, nor SREBP-2 mRNA (Swinnen et al., 2000; Yang et al., 2003). In the HRPC, we could reason that the conditions favor the expression of a weak positive regulator of prostasin expression, SREBP-1c, but not a strong positive regulator, SREBP-2, while these same conditions favor a strong up-regulation of a moderate negative regulator, Slug.

The Snail and Slug transcription repressors are involved in the process of epithelialto-mesenchymal transition (EMT) (Hemavathy et al., 2000b; Nieto, 2002a). And the result of aberrant expression of the Snail family transcription repressors is change of cell-cell adhesion, associated with change of motility and invasiveness. For example, Slug is responsible for E-cadherin down-regulation in breast cancer (Hajra et al., 2002a). In light of the fact that prostasin is an invasion suppressor and its expression is repressed by Slug, it will be worthwhile to evaluate expression of the Snail family transcription repressors in human prostate cancer.

CONCLUSIONS: We have unveiled a new molecular mechanism by which prostasin expression may be down-regulated in hormone-refractory prostate cancers, through the increased expression of the transcription repressor Slug. Expression of Slug was found to be highly responsive to androgen stimulation, and also responsive to EGF stimulation, and should be investigated as a potential causal factor of prostate cancer development and progression.

CHAPTER FIVE MECHANISMS OF STEROL REGULATORY ELEMENT BINDING PROTEIN-2 (SREBP-2) REGULATION OF HUMAN PROSTASIN GENE EXPRESSION

5.1 Introduction

In the previous chapter, we found that androgen treatment regulates human prostasin gene expression through up-regulation of transcription factors SREBP-2 and Slug (Chen et al., 2006l). Activated SREBP-2 up-regulates either human prostasin gene promoter activity, or endogenous prostasin expression at both the mRNA and protein levels in HEK-293 and DU-145 cells. In the LNCaP cells, the stimulatory effects of SREBP-2 were negated by the transcription repressor Slug, which is up-regulated dramatically by the androgen dihydrotestosterone (DHT).

Sterol regulatory element-binding protein-2 (SREBP-2) belongs to the SREBP family of transcription factors that play a pivotal role in cellular lipid homeostasis (Horton, 2002; Rawson, 2003). Two different genes, SREBP-1 and SREBP-2, encode three SREBP isoforms (SREBP-1a, SREBP-1c, and SREBP-2) in mammalian cells. SREBPs are synthesized as precursor proteins that are retained in the endoplasmic reticulum when the cellular sterol level is high. When the cellular sterol level drops, the N-terminal domain of an SREBP is released as the mature form from the membrane by a two-step proteolytic cleavage. The mature SREBP then enters the nucleus, and binds the sterol regulatory elements (SREs) or E-box sequences to enhance the transcription of target genes (Horton J.D. et al., 2002; Osborne, 2000; Shimano, 2001). SREBP-1a and SREBP-1c are mainly involved in fatty acid metabolism, while

SREBP-2 mainly activates genes related to cholesterogenesis and lipogenesis (Horton et al., 2003).

In this chapter, we investigated the mechanisms of up-regulation of the human prostasin gene by SREBP-2. We defined an SREBP-2-response region in the 5'-flanking region of the human prostasin gene. Various SRE sites in the prostasin gene promoter were analyzed for their roles in the SREBP-2-mediated up-regulation of human prostasin gene expression.

5.2 Materials and Methods

5.2.1 Construction of human prostasin promoter-luciferase reporter plasmids

The promoter-luciferase reporter plasmid pProPro1031-pGL3 was generated as described previously (Chen et al., 2006l). The construct contains the human prostasin promoter sequence located between positions -1031 to +232 relative to the transcription initiation site, and is referred to as p-1031-pGL3 in this paper. DNA fragments extending from –719 to +232, -271 to +232, -17 to +232, and –1031 to +9 were produced by PCR amplification using p-1031-pGL3 as the template, and subcloned into the luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI). The new promoter-reporter constructs were designated as p-719-pGL3, p-271-pGL3, p-17-pGL3, and p-1031/+9-pGL3.

5.2.2 Site-directed mutagenesis

Mutations at different SRE sites were introduced into prostasin promoter-luciferase reporter constructs by PCR-based site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The wild-type promoter-reporter constructs were used as the amplification template, and were then digested by the restriction enzyme *Dpn I* after PCR amplification. The *Dpn I*-treated PCR products were transformed into super-competent XL-1 Blue *E. coli* cells and all mutant constructs were verified by DNA sequencing. Listed below are the primers (upper strand shown) used for mutagenesis of appropriate SREs.

SRE-897M: 5'- GCCCAGGCTGGAGTGCAGGAATTCGATCATAGCTCAATGC –3'

SRE-538M: 5'- CACTTAGGAAATGTCTGGAATTCGATTGGTGCTGCTCCAC –3'

SRE+8M: 5'- GGACTCATGACTTTGTCTTAAAGAGGAGCTGGCGGAGCCC –3'

SRE+71M: 5'- GGCGGGCAGGTAGGTGCATAAGATCCTGGGAGGACCCTGC -3'

SRE+98M: 5'- CTGGGAGGACCCTGCTCTTACAGACGGTGCTGGTGACTCG –3'

5.2.3 Cell culture, cell transfection and promoter activity assay

HEK-293 (EBNA) cells were obtained from Invitrogen (Carlsbad, CA) and cultured in D-MEM (High Glucose) medium supplemented with 10% (v/v) FBS (fetal bovine serum). On day 1, cells were plated on a poly-L-lysine-coated 12-well plate at a density of 4 x 10^5 cells per well. On day 2, the appropriate prostasin promoter-luciferase reporter plasmid (1.3 μg), an SV-β-galactosidase reference plasmid (pSV-β-gal, Promega, 0.2 μg) and an SREBP-2 expression plasmid (pcDNA3-SREBP-2, 0.1 μg) (Chen et al., 2006l), or a control plasmid (pcDNA3,

Invitrogen, 0.1 μg) were transfected into the cells using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were lysed with the Reporter Lysis Buffer (Promega). One hundred micro-liters of cell lysate were assayed for luciferase activity using the Bright-Glo Luciferase Assay System (Promega). The β-galactosidase activity was measured by using a β-Galactosidase Enzyme Assay Kit (Promega) and used to normalize for transfection efficiency. The data were expressed as the relative prostasin promoter activity and an x-fold of increase of the promoter activity in the cells co-transfected with the SREBP-2 expression plasmid (pcDNA3-SREBP-2) compared with the cells co-transfected with the empty pcDNA3 vector.

5.2.4 Purification of recombinant SREBP-2

The cDNA fragment encoding the mature form of SREBP-2 (amino acids 1 to 481) was cloned previously (Chen et al., 2006l) and subcloned into the GST-fusion protein expression vector pGEX-6P-1 (GE Healthcare Life Sciences, Piscataway, NJ). A GST-SREBP-2 fusion protein was expressed in *E. coli* (XL-1 Blue) cells and purified. Briefly, an exponential bacterial culture harboring the GST-SREBP-2 expression plasmid was grown in 250 ml of Luria broth (LB) at 37°C until A_{600} reached 0.8 before induction with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 90 minutes. The bacterial cells were pelleted, re-suspended in ice-cold 1x PBS, and sonicated on ice. Triton X-100 was added to a final concentration of 1% to aid the solubilization of fusion proteins. After gentle mixing for 30 minutes, cell lysate was centrifuged at 12,000 x *g* for 10 minutes at 4°C. The supernatant was

then mixed with glutathione-Sepharose beads (GE Healthcare Life Sciences) for 1 hour at room temperature on a rotator. After incubation, the beads were washed with 1x PBS and then incubated with the PreScission Protease (GE Healthcare Life Sciences) in the PreScission cleavage buffer (50 mM Tris-HCl, pH7.0, 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol) for 4 hours at 4°C. Following the incubation, the supernatant containing free SREBP-2 without GST was collected and used for the electrophoretic mobility shift assay.

5.2.5 Electrophoretic mobility shift assay

The digoxigenin (DIG) Gel Shift Kit (2nd Generation) from Roche Applied Science (Indianapolis, IN) was used to perform the electrophoretic mobility shift assay for testing DNA-protein binding. The complementary oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA), annealed to generate the double-stranded oligonucleotides, and labeled with digoxigenin-11-ddUTP according to the manufacturer's instructions. The DNA-protein binding reaction mixture containing 0.8 ng DIG-labeled oligonucleotides and 100 ng purified recombinant SREBP-2 was incubated at room temperature for 15 minutes with or without 0.1 μg (125-fold excess) unlabeled wild-type or mutant oligonucleotides. For the super-shift assay, purified recombinant SREBP-2 was pre-incubated with 1 μl of a polyclonal goat anti-SREBP-2 antibody (2 μg/μl, Santa Cruz Biotechnology, #sc-8151x) or a polyclonal rabbit anti-SREBP-1 antibody (2 μg/μl, Santa Cruz Biotechnology, #sc-8984x) for 15 minutes at 4°C. The DIG-labeled oligonucleotides were then added in the mixture and incubated at room temperature for an additional 15 minutes. Native polyacrylamide gels (4-6%) in 0.5x TBE (Tris-borate-EDTA buffer)

were used for electrophoresis in the EMSA. After electrophoresis, the separated oligonucleotides were electroblotted onto positively charged Immobilon-Ny$^+$ nylon membranes (Millipore, Bedford, MA) in 0.5x TBE buffer and fixed by cross-linking at 120 mJ in a UV Stratalinker (Stratagene). DIG-labeled oligonucleotides on the membrane were detected by an anti-DIG antibody conjugated with alkaline phosphatase (AP) and visualized by a chemiluminescent reaction using the CDP-Star substrate (Roche).

5.2.6 Statistical Analysis

The Student t-Test (one-tailed, equal variance) was performed for the promoter activity assay data, for which $p<0.05$ was used to define statistical difference.

5.3 Results and Discussion

5.3.1 Two classic SREs are present in the human prostasin promoter

In previous chapter, we showed that SREBP-2 up-regulates human prostasin gene expression. Besides the SRE site at position -897 which was previously identified by Yu *et al.* (Yu et al., 1996), we identified another SRE site, located at position –538 in the 5'-flanking region of the human prostasin gene (Figure 5-1A) following an extensive interrogation of the DNA sequence. Both SRE-897 and SRE-538 are 90% identical to the classic SRE sequence (Shimano, 2001) (5'-ATCACCCCAC-3') found in the promoter region of the human low-density lipoprotein (LDL) receptor gene (Briggs et al., 1993).

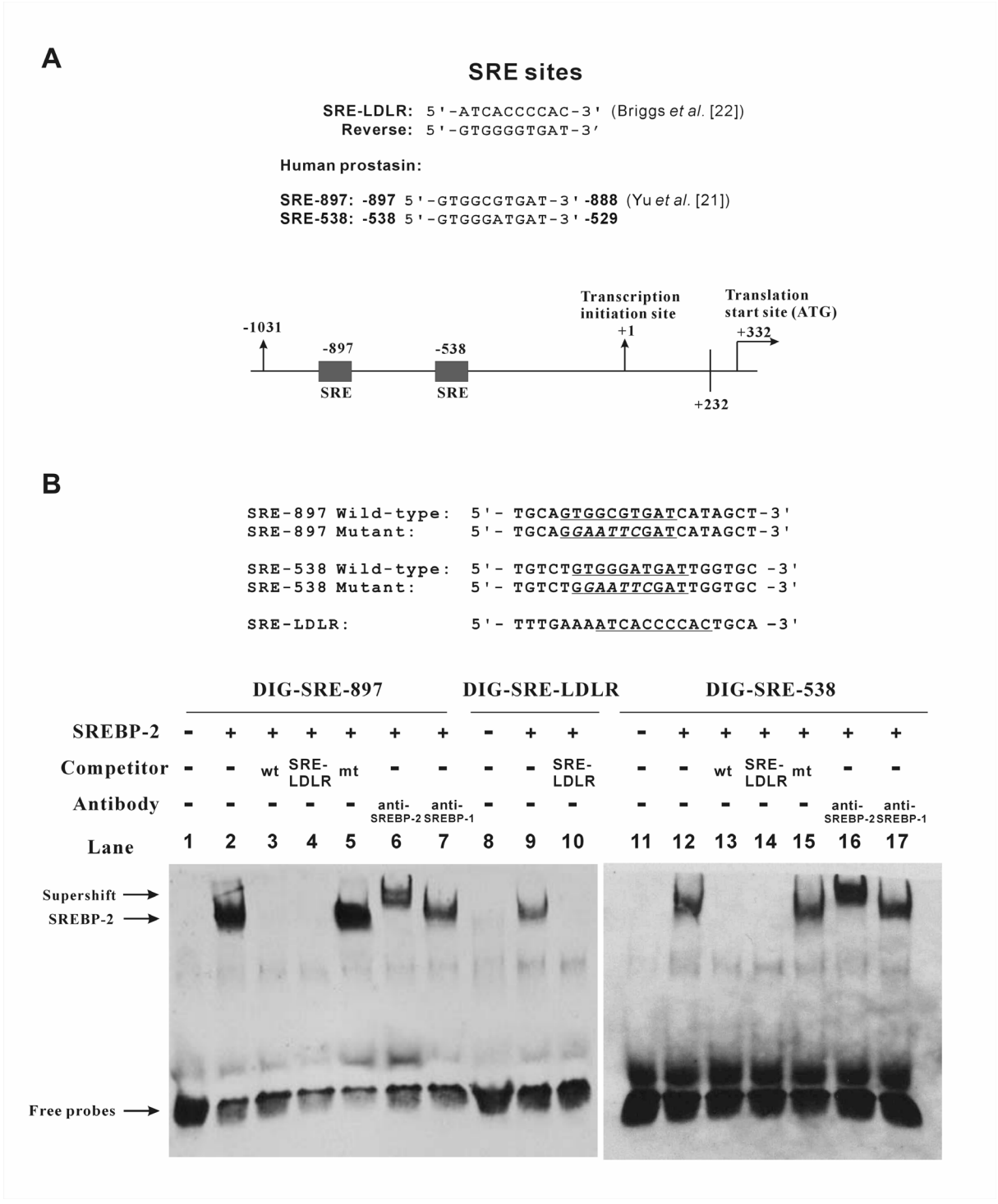


Figure 5-1 Electrophoretic mobility shift assays of SRE-897 and SRE-538 probes with recombinant SREBP-2.

(A) The nucleotide sequences and locations of SRE-897 and SRE-538 in the human prostasin gene promoter.

SRE's are indicated by filled boxes. SRE-LDLR: the SRE in the human LDL receptor gene promoter. (B) EMSA performed with SRE-897 and SRE-538 probes. The SRE sequences are underlined and mutated nucleotides are presented in italic. In each binding reaction, 0.8 ng of each DIG-labeled probe was incubated with 100 ng of recombinant SREBP-2. An excess of unlabeled probes (wt: wild-type, mt: mutant, or SRE-LDLR, in 125-fold excess) was used as competitors. Antibodies specific for SREBP-1 (anti-SREBP-1) and SREBP-2 (anti-SREBP-2) were used for the super-shift assays. Free DIG-probes, SREBP-2 shifted probes, and super-shifted probes are indicated by the arrows.

To test whether SREBP-2 is able to bind SRE-897 and SRE-538 in the human prostasin gene promoter, electrophoretic mobility shift assays (EMSAs) were performed using digoxigenin (DIG)-labeled double-stranded oligonucleotide probes (shown in Figure 5-1B). Recombinant SREBP-2 in the EMSA shifted the DIG-labeled SRE-897 (Figure 5-1B, Lane 2) and SRE-538 (Figure 5-1B, Lane 12) as indicated by the arrow. Addition of excess unlabeled SRE-897 and SRE-538 probes abolished the band shift (Figure 5-1B, Lanes 3 and 13), but excess unlabeled mutant SRE-897 and SRE-538 probes could not compete off the specific binding (Figure 5-1B, Lane 5 and 15). Addition of specific antibodies against human SREBP-2 produced a further super-shifted band (Figure 5-1B, Lane 6 and 16), while addition of antibodies against SREBP-1 had no effect (Figure 5-1B, Lane 7 and 17). A probe containing the classic SRE from the human LDL receptor gene was used as a control (Figure 5-1B, lane 8-10). An excess of unlabeled SRE-LDLR abolished formation of the specific DNA-protein complex between SREBP-2 and SRE-897 or SRE-538 (Figure 5-1B, lane 4 and 14).

5.3.2 Mapping of the SREBP-2-response region in the 5'-flanking sequence of the human prostasin gene

To test whether SRE-897 and SRE-538 are functionally regulated by SREBP-2 in the human prostasin gene promoter, we used the following two approaches: a serial deletion analysis of the prostasin promoter and, a mutagenesis analysis with the full-length promoter. We generated four different luciferase reporter constructs which contain unidirectional deletion sequences of the human prostasin gene 5'-flanking region (Figure 5-2A). The p-1031-pGL3 construct contains both SRE-897 and SRE-538 while the p-719-pGL3 only contains SRE-538. The p-271-pGL3 and p-17-pGL3 plasmids do not contain any of the two SRE sites. These four serial-deleted promoter-reporter constructs presented similar uninduced promoter activities in HEK-293 cells (Figure 5-2A). The promoter activity of the construct with a deletion of +10 to +232 (p-1031/+9-pGL3), however, was completely abolished (Figure 5-2A). It may be suggested that the region from -17 to +232 is required for the uninduced transcriptional activity of the human prostasin gene. Next, we co-transfected these promoter-reporter constructs with an SREBP-2 expression plasmid for evaluation of their responses to SREBP-2 (Figure 5-2B). SREBP-2 up-regulated the promoter activities of all four promoter-reporter constructs (p-1031-pGL3, p-719-pGL3, p-271-pGL3 and p-17-pGL3), the responses of these four different constructs to SREBP-2 were not statistically different.

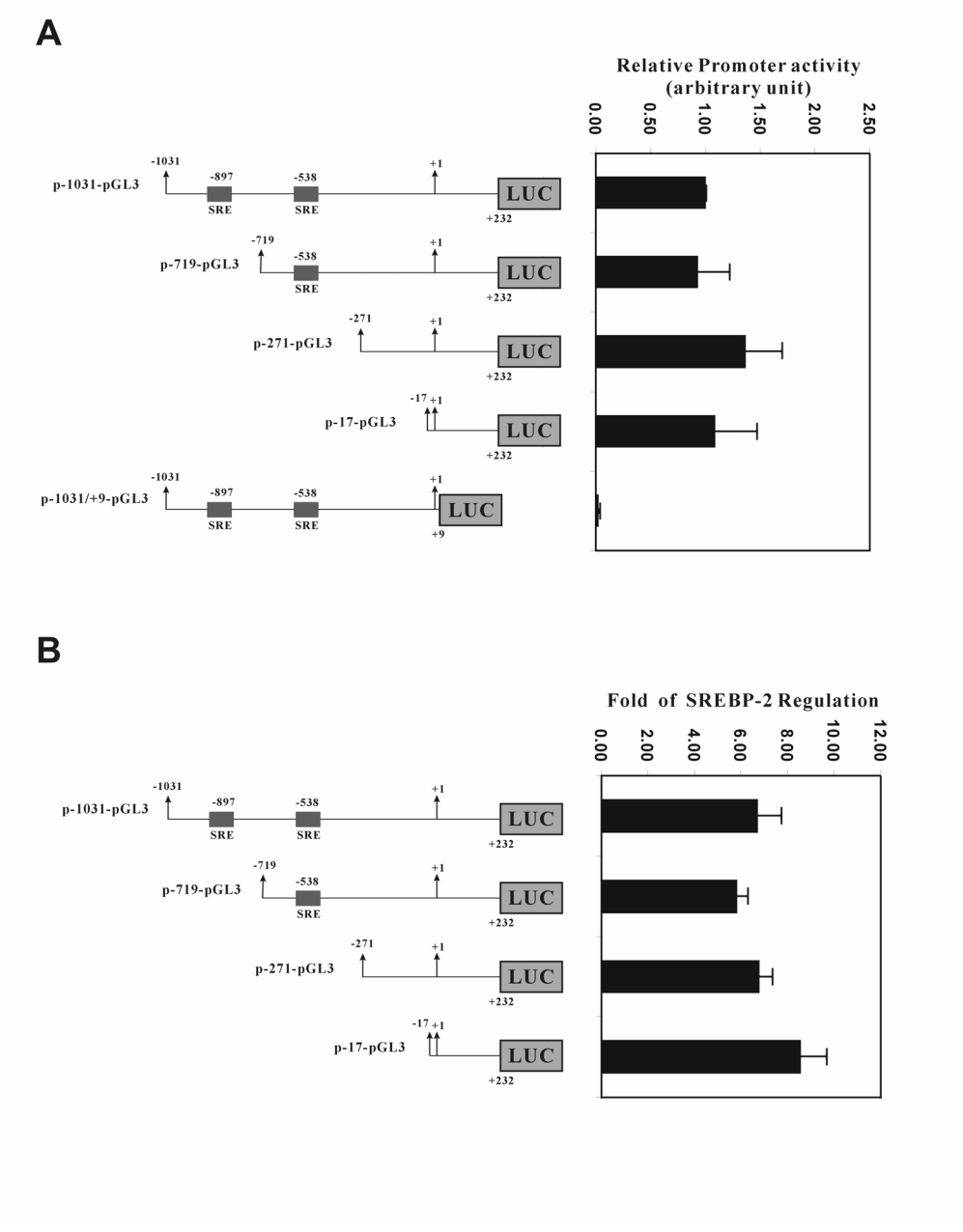


Figure 5-2 Promoter activity assays of serial-deleted human prostasin promoter constructs.

Schematic diagrams of serial-deleted human prostasin promoter-luciferase reporter constructs were shown on the left. The numbers represent the positions relative to the transcription initiation site. (A) HEK-293 cells were transiently transfected with 1.4 μg of the appropriate serial-deleted promoter-reporter plasmid and 0.2 μg of the SV40-β-galactosidase reference plasmid. Luciferase activities were determined at 24 hours after transfection and normalized with the β-galactosidase activities. The data are expressed as relative promoter activity (per serial

deletion construct versus the full-length construct p-1031-pGL3 for which the promoter activity was set as 1 arbitrary unit). The results represent the Mean ± S.D. of three independent experiments. (B) HEK-293 cells were transiently transfected with 1.3 μg of the appropriate serial-deleted promoter-reporter plasmid, 0.2 μg of the SV40-β-galactosidase reference plasmid, and 0.1 μg of the SREBP-2 expression plasmid or the empty pcDNA3 vector. Luciferase activities were determined at 24 hours after transfection and normalized with β-galactosidase activities. The data are expressed as an x-fold of increase of the promoter activity in the cells co-transfected with the SREBP-2 expression plasmid compared with the cells co-transfected with the empty pcDNA3 vector. The results represent the Mean ± S.D. of three independent experiments. SRE's are indicated by filled boxes. The grey box with "LUC" indicates the firefly luciferase gene in the pGL3 Basic vector.

Mutagenesis was then performed to inactivate these two classic SREs in the full-length human prostasin promoter-reporter construct p-1031-pGL3 and the mutant constructs were evaluated for their responses to SREBP-2 (Figure 5-3). Mutations in SRE-897 (p-1031-SRE-897M-pGL3) or SRE-538 (p-1031-SRE-538M-pGL3) did not reduce the full-length promoter's response to SREBP-2. The promoter construct p-1031-SRE-897M/-538M-pGL3 with mutations in both SRE-897 and SRE-538 was shown to be further up-regulated by SREBP-2, by 14%, in this set of experiments.

It may be suggested from these observations that SREBP-2 regulation of the prostasin gene promoter is mediated by the region defined in -17 to +232, and SRE-897 and SRE-538 are not necessary for this regulation.

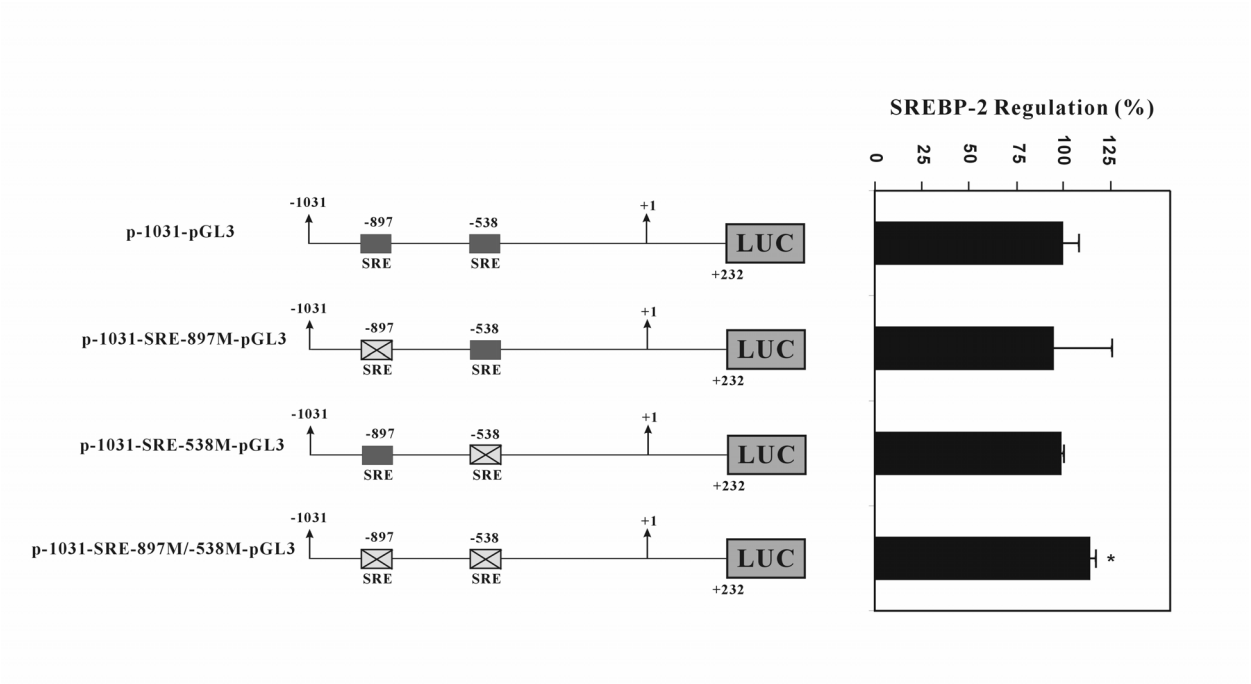


Figure 5-3 Evaluation of SRE-897 and SRE-538 in the full-length human prostasin promoter-reporter construct.

HEK-293 cells were transiently transfected with 1.3 μg of the wild-type (p-1031-pGL3) or a mutant (p-1031-SRE-897M-pGL3, p-1031-SRE-538M-pGL3, or p-1031-SRE-897M/-538M -pGL3) promoter-reporter plasmid, 0.2 μg of the SV40-β-galactosidase reference plasmid, and 0.1 μg of the SREBP-2 expression plasmid or the empty pcDNA3 vector. Luciferase activities were determined at 24 hours after transfection and normalized with β-galactosidase activities. The data are expressed as an x-fold of increase of the promoter activity in the cells co-transfected with the SREBP-2 expression plasmid compared with the cells co-transfected with the empty pcDNA3 vector. The level of increase for the wild-type construct was arbitrarily set as 100%. The results represent the Mean ± S.D. of two independent experiments (p-1031-SRE-538M-pGL3 and p-1031-SRE-897M/-538M-pGL3) or three independent experiments (p-1031-pGL3 and p-1031-SRE-897M-pGL3). The asterisk (*) denotes a statistical difference ($p<0.05$) in SREBP-2 responses between the mutant and the wild-type promoter constructs. The filled box represents the wild-type SRE while the open box with "X" represents the mutated SRE. The grey box with "LUC" indicates the firefly luciferase gene in the pGL3 Basic vector.

5.3.3 Identification of SREBP-2 binding sites in the SREBP-2-response region of the human prostasin promoter

We identified three potential novel SREBP binding sites at positions +8, +71, and +98, sharing 50%~60% identity with the classic SRE, in the SREBP-2-response region (-17 to +232) of the human prostasin promoter (Figure 5-4A). Electrophoretic mobility shift assays were performed to test whether SREBP-2 binds these putative SRE-like sites. Single-shifted DNA-protein bands were observed when recombinant SREBP-2 was incubated with the DIG-labeled probes containing SRE+8, SRE+71, or SRE+98 (Figure 5-4B). The specific DNA-protein band-shift could be competed off by an excess of unlabeled wild-type probes but not mutant probes.

5.3.4 Evaluation of SRE+8, SRE+71 and SRE+98 in SREBP-2 regulation of the human prostasin promoter

To determine whether SRE+8, SRE+71, and SRE+98 are functional for SREBP-2 regulation, mutagenesis of these sites was performed in the human prostasin promoter-reporter construct p-17-pGL3. A mutation in SRE+8 (construct p-17-SRE+8M-pGL3) did not reduce the up-regulation by SREBP-2 (Figure 5-5), disqualifying this SRE as a potential functional site for SREBP-2 regulation of prostasin expression. A mutation in SRE+71 (construct p-17-SRE+71M-pGL3), however, decreased the SREBP-2 up-regulation by 34% when compared with the wild-type construct, for which the extent of SREBP-2 up-regulation was arbitrarily set as 100%. A mutation in SRE+98 (construct p-17-SRE+98M-pGL3) decreased the SREBP-2 up-regulation by 72% when compared with the wild-type construct. It appears that SRE+98 is the major site required for SREBP-2 regulation of the human prostasin promoter, while the reduction of SREBP-2 regulation following the mutagenesis of SRE+71 was not as dramatic.

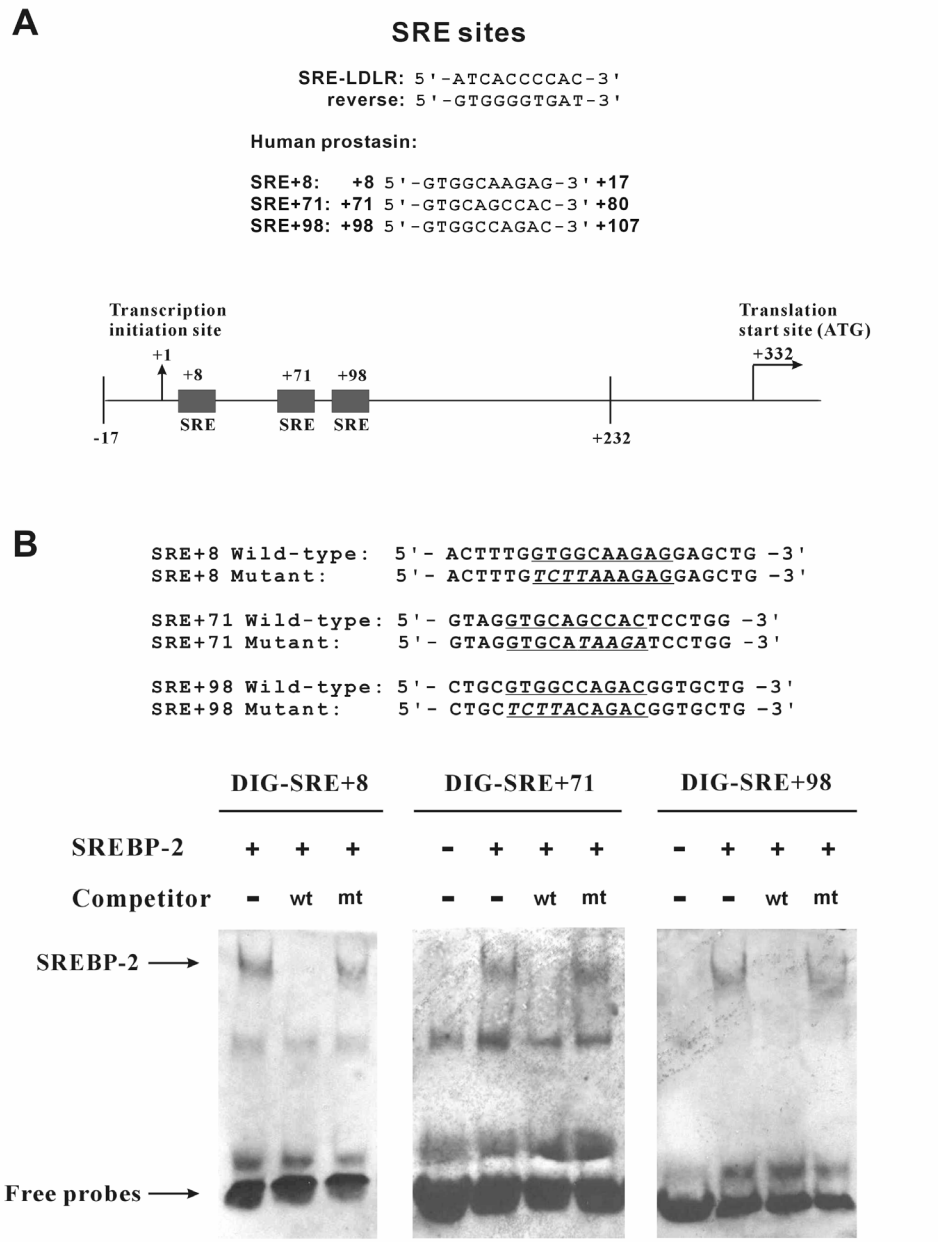


Figure 5-4 Electrophoretic mobility shift assay of SRE+8, SRE+71 and SRE+98 probes with recombinant SREBP-2.

(A) The nucleotide sequences and locations of putative SREs in the SREBP-2-response region (from -17 to +232) of the human prostasin gene promoter. SRE's are indicated by filled boxes. (B) EMSA performed with SRE+8, SRE+71 and SRE+98 probes (The SRE sequences are underlined and mutant sequences are presented in italic). In

each binding reaction, 0.8 ng of each DIG-labeled probe was incubated with 100 ng of recombinant SREBP-2. An excess of unlabeled probes (wt: wild-type or mt: mutant, in 125-fold excess) was used as competitors. Free DIG-probes and SREBP-2 shifted probes were indicated by the arrows.

Mutations in both SRE+71 and SRE+98 (construct p-17-SRE+71M/+98M-pGL3) decreased the SREBP-2 up-regulation by 58%. The double-mutation construct produced more reduction of SREBP-2's stimulatory effects when compared with the SRE+71 single-mutation construct p-17-SRE+71M-pGL3, but less when compared with the SRE+98 single-mutation construct p-17-SRE+98M-pGL3. We presently have no explanation as to why the double-mutation had less impact on SREBP-2 regulation than the SRE+98 single mutation.

To evaluate the importance of SRE+98 in the full-length prostasin promoter for SREBP-2 regulation, the p-1031-SRE+98M-pGL3 mutant construct was generated. Mutation of SRE+98 in the full-length prostasin promoter also significantly reduced the up-regulation by SREBP-2, by 73%, when compared with the wild-type construct, for which the extent of SREBP-2 up-regulation was arbitrarily set as 100% (Figure 5-6). The novel sterol regulatory element identified at position +98, GTGGCCAGAC (SRE+98) is, therefore, the major site for the human prostasin gene to respond to SREBP-2-mediated up-regulation. The 14% increase of SREBP-2 up-regulation observed with the full-length promoter construct containing mutations in both SRE-897 and SRE-538 (Figure 5-3) may be the result of a surplus of SREBP-2 for the SRE+98 and SRE+71 sites because the two classic SRE sites are no longer available to bind the transcription factor.

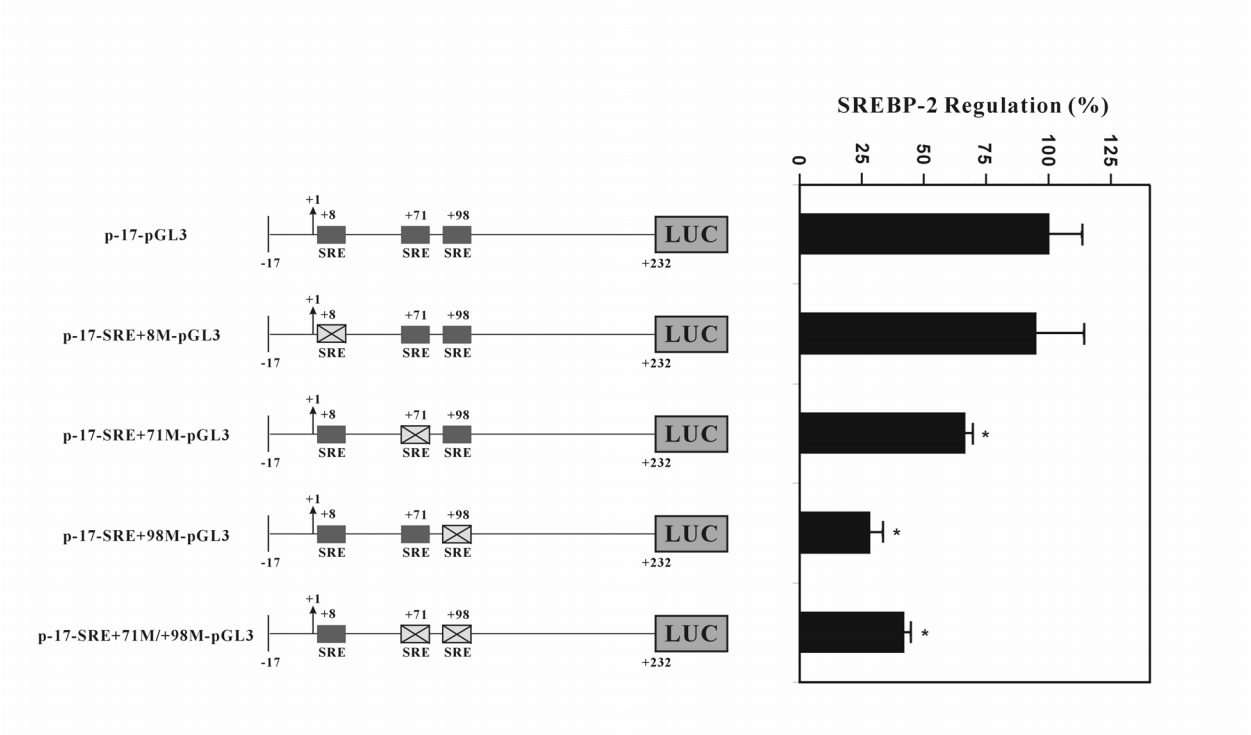


Figure 5-5 Evaluation of SRE+8, SRE+71 and SRE+98 in the SREBP-2-response region of the human prostasin promoter.

HEK-293 cells were transiently transfected with 1.3 μg of the wild-type (p-17-pGL3) or a mutant (p-17-SRE+8M-pGL3, p-17-SRE+71M-pGL3, p-17-SRE+98M-pGL3 or p-17-SRE+71M/+98M -pGL3) promoter-reporter plasmid, 0.2 μg of the SV40-β-galactosidase reference plasmid, and 0.1 μg of the SREBP-2 expression plasmid or the empty pcDNA3 vector. Luciferase activities were determined at 24 hours after transfection and normalized with β-galactosidase activities. The data are expressed as an x-fold of increase of the promoter activity in the cells co-transfected with the SREBP-2 expression plasmid compared with the cells co-transfected with the empty pcDNA3 vector. The level of increase for the wild-type construct was arbitrarily set as 100%. The results represent the Mean ± S.D. of three independent experiments. The asterisk (*) denotes a statistical difference ($p<0.05$) in the SREBP-2 responses between the mutant and the wild-type promoter constructs. The filled box represents the wild-type SRE while the open box with "X" represents the mutated SRE. The grey box with "LUC" indicates the firefly luciferase gene in the pGL3 Basic vector.

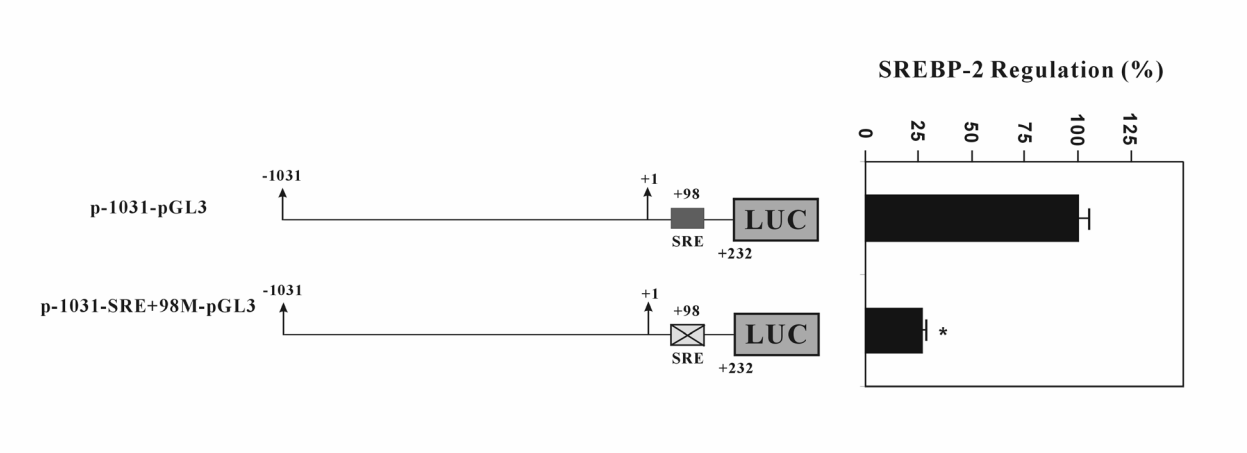


Figure 5-6 Evaluation of SRE+98 in the full-length human prostasin promoter-reporter construct.

HEK-293 cells were transiently transfected with 1.3 μg of the wild-type (p-1031-pGL3) or the mutant (p-1031-SRE+98M-pGL3) promoter-reporter plasmid, 0.2 μg of the SV40-β-galactosidase reference plasmid, and 0.1 μg of the SREBP-2 expression plasmid or the empty pcDNA3 vector. Luciferase activities were determined at 24 hours after transfection and normalized with β-galactosidase activities. The data are expressed as an x-fold of increase of the promoter activity in the cells co-transfected with the SREBP-2 expression plasmid compared with the cells co-transfected with the empty pcDNA3 vector. The level of increase for the wild-type construct was arbitrarily set as 100%. The results represent the Mean ± S.D. of three independent experiments. The asterisk (*) denotes a statistical difference ($p<0.05$) in the SREBP-2 responses between the mutant and the wild-type promoter constructs. The filled box represents the wild-type SRE while the open box with "X" represents the mutated SRE. The grey box with "LUC" indicates the firefly luciferase gene in the pGL3 Basic vector.

CONCLUSIONS: SREBP-2 activates human prostasin gene expression through the 5'-flanking region, specifically, by interacting mainly with a novel sterol-regulatory element, SRE+98. Prostasin, as a member of SREBP-2 responsive genes, might also be suggested to play a potential role in lipid homeostasis in human cells.

APPENDIX
EXPERIMENTAL PROCEDURES

Mini-Prep of Plasmids (Phenol/$CHCl_3$)

1. Inoculate single colonies to 2ml LB media with appropriate antibiotics. Grow the bacteria in 37°C incubator at speed of 180~200 rpm for O/N (16~18 hours).
2. Transfer 2 x 600µl bacteria cultures into 1.5 ml tubes → 6,000 rpm x 5 min at room temperature.
3. Discard supernatant → Aspirate the leftover supernatant → Suspend bacteria in 120µl of Sol-I mix by pipetting (100µl Sol I, 10µl 10mg/mlRNase A, 10µl 50mg/ml lysozyme; Preparing master mix in advance). Incubate at room temperature for 15 min or longer.
4. Add 200 µl of Sol II. Mix gently. Incubate on ice for exactly 5 min.
5. Add 150 µl ice-cold Sol III, cap the lid immediately and invert several times to mix the solution thoroughly. Spin at 14,000 rpm for 10 min at room temperature.
6. Transfer the supernatant to a new 1.5 ml tube. Add 450µl of "25:24:1" solution.
7. Shake 50 times to mix well. Spin at 8,000 rpm for 5 min at room temperature.
8. Transfer the top layer to another new 1.5 ml tube. Add same volume of chloroform.
9. Shake 50 times to mix well. Spin at 8,000 rpm for 1 min at room temperature.
10. Transfer the top layer to another new 1.5 ml tube. Add same volume of iso-propanol.
11. Mix thoroughly by inverting. Spin at 4°C, 14,000 rpm for 10 min.
12. Carefully decant the supernatant. Add 700µl of cold 70% ethanol. Rotate tubes to wash the DNA pellet, decant ethanol (If the pellet is detached from the bottom, re-spin at 9,500 rpm for 5 min), and air-dry in culture hood for 5 min.
13. Dissolve the DNA pellet in 50µl sterile ddH_2O. Take 3~5µl of DNA solution for restriction enzyme digestion.

Sol I: 50mM glucose, 25mM Tris-HCl, pH8.0 10mM EDTA

(500ml) 4.505g Glucose (Dextrose); 12.5ml 1M Tris (8.0); 10ml 0.5M EDTA (8.0)

Autoclave Fluid/15min (no longer!)

Sol II: 1%SDS and 0.15 M NaOH

(400ml) 20ml 20% SDS; 6ml 10N NaOH

Sol III: 3M potassium, 5M acetate, pH 4.8

(500ml) 147.23g KAc dissolved in 400ml H2O → Add in 57.5ml Glacial Acetic Acid → Adjust to 500ml.

1xTE: 10mM Tris-HCl, pH8.0, 1mM EDTA

"25:24:1":

- Thaw the frozen phenol (stored at –20^{o}C near centrifuge) at 50~55^{o}C water bath for 20 ~ 30 min (until thaw).
- After thawed, add 25 ml sterile ddH_2O (mix, invert 50 times)
- Spin at top speed for 5 min at room temperature.
- Remove top layer with plastic transfer pipette. (In sink with cold water on)
- Add 5xTE Buffer up to 50 ml, mix, wait till phase separate, remove top layer.
- Add 1xTE Buffer up to 50 ml, mix, wait till phase separate, remove top layer.
- Adjust the volume of saturated phenol to 25ml. Transfer the extra phenol to the Φ storage tube (stored at -20^{o}C).
- Add 24 ml of chloroform and 1ml Iso-amyl alcohol → Mix.
- Spin at top speed for 1 min or put in 4^{o}C to let phase separate overnight.

Midi-Prep of Plasmids (Phenol/$CHCl_3$)

1. Inoculate a single colony or 50µl of bacteria culture to 25ml LB media. Grow the bacteria in 37°C incubator at speed of 180~200 rpm for O/N (16~18 hours).
2. Pour the bacteria culture into polycarbonate tubes (on the top of microwave). Spin at top speed (Clinical centrifuge in the corner) for 10 min, Decant supernatant.
3. Add 2.5 ml Sol I, 100µl RNase A, 100µl lysozyme to each tube, suspend the pellet completely by pipetting and vortexing. Incubate at room temperature for 20 min.
4. Add 5.0 ml Sol II to each tube, mix completely by gentle swirling, incubate on ice for 5 min.
5. Add 3.75 ml Sol III to each tube, mix thoroughly by swirling, incubate on ice for 15 min.
6. Spin at 12,000 rpm for 20 min @ 4°C. (**Precise balance needed!**).
7. Transfer supernatant into 50ml conical organic resistant tubes (red caps). Add 10.5-11 ml "25:24:1". Shake 50 times and spin at top speed for 10 min at room temperature.
8. Transfer top layer into new tubes. (leave some interface)
9. Add 10.5ml chloroform. Shake 50 times and spin at top speed for 1 min at room temp.
10. Transfer top layer to DNA tubes (~30ml, near autoclaved glass-tubes). Add 8ml iso-propanol. Balance. Spin at 12,000 rpm for 20 min at 4°C. Decant supernatant carefully.
11. Add 8ml -20°C 70% ethanol (DNA side up). Turn vertically to wash entire tube. (If DNA pellet breaks, must re-spin)
12. Dry (DNA side up) for 5 min in culture hood. (until white DNA becomes clear)
13. Dissolve in 0.5~1ml sterilized ddH2O. (No need to measure DNA concentration b/c high amount of RNA)

Mini-Prep of Plasmid by Qiagen Kit

(Cat. No. 27104)

1. Inoculate single colonies to 2ml LB media with appropriate antibiotics. Grow the bacteria in 37^{o}C incubator at speed of 180~200 rpm for O/N (16~18 hours).
2. Transfer 1.5ml bacteria culture into 1.5 ml tubes → 8,000 rpm x 3 min at room temperature.
3. Discard supernatant → Aspirate the leftover supernatant → Suspend bacteria in 250μl **Buffer P1** (stored at 4^{o}C) and mix thoroughly by pipetting.
4. Add 250 μl **Buffer P2**. Mix thoroughly by gently inverting 10 times.
5. Add 350 μl **Buffer P3**. Mix thoroughly by gently inverting 10 times.
6. Spin at 13,000 rpm for 10 min at room temperature.
7. Apply the supernatant to the **QIAprep spin column**. Centrifuge for 30~60 seconds (From now on, all centrifuges are performed at 13,000 rpm, room temperature.)
8. Discard the flow-through → Wash the column by 0.5ml **Buffer PB** → Centrifuge for 30~60s.
9. Discard the flow-through →Wash the column by 0.75ml **Buffer PE**→ Centrifuge for 30~60s.
10. Discard the flow-through →Centrifuge for additional 1 min to remove residual wash buffer.
11. Place the column in a clean 1.5ml tube→ Add 50 μl sterile ddH_2O to the center of column→ Incubate at room temp for 1 min → Centrifuge for 1 min (Cut the cap to fit centrifuge).
12. Transfer to a clean 1.5 tube. DNA samples are ready for measurement of concentration and further cell transfection experiments.

Midi-Prep of Plasmid by Qiagen Kit

(Cat. No. 12143)

1. Inoculate a single colony to two flasks of 25ml LB media. 37°C, 200 rpm, O/N (~16 hours).
2. Pour the bacteria culture into polycarbonate tubes (on the top of microwave). Spin at top speed (old machine) for 10 min. Decant supernatant.
3. Add 4.0 ml of **Buffer P1** (stored at 4°C). Suspend the pellet completely.
4. Add 4.0 ml of **Buffer P2**, mix gently but thoroughly by swirling the tube and pipetting without vortexing. Incubate at room temperature for no more than 5 min.
5. Add 4.0 ml of **pre-chilled Buffer P3**, mix thoroughly and incubate on ice for 15 min.
6. Spin at 12,000 rpm for 20 min @ 4°C. (New machine, need precise balance).
7. Equilibrate a **Qiagen-tip 100** by applying 4ml of **Buffer QBT**, and allow the column to empty by gravity flow.
8. Apply the supernatant from step 6 to the Qiagen-tip through a double layer cheese cloth (with a plastic transfer pipette). Wait till all samples flow through.
9. Wash the Qiagen-tip with 10ml of **Buffer QC** twice → Elute DNA with 5ml of **Buffer QF**.
10. Repeat the procedure from step 7 to step 10. Elute DNA in the same tube.
11. Precipitate DNA by adding 7.0 ml of iso-propanol to the eluted DNA (10ml). Mix, balance and centrifuge immediately at 12,000 rpm for 20 min @ 4°C. Decant supernatant carefully.
12. Add 5 ml of 70% ethanol (stored at –20°C) with DNA side up. Turn vertically to wash entire tube. (If DNA pellet breaks, must re-spin)
13. Air-dry DNA for 5 min in Bio-safety Cabinet. And dissolve in 0.3~0.5ml of sterilized ddH_2O.

Reverse-Transcription and Polymerase Chain Reaction

Reverse-Transcription (Super-Script III RT, invitrogen)

(Wear new gloves and do not make skin contacts!)

1. In one sterile 0.5ml tube mix the following in the order given:

Reagents	Volume
DEPC-H_2O	μl
2mM dNTP	5 μl
3'-primer (**2μM**)	1 μl
RNA (total 1μg)	μl
Total	14 μl

2. Cap the tube, and then heat the mixture on MJ Research thermocycler at 65°C for 5 minutes.
3. Place the heated tube on ice for 1 minute. → Spin down the content.
4. Add the following in the order given:

Reagents	Volume
5x First-strand Buffer	4 μl
0.1 M DTT	1 μl
SS-III RT	1 μl

5. Cap the tube and mix the content by finger tapping → spin down the content
6. Run program "RT55" (1 hour at 55°C followed by 15 minutes at 70°C) on the MJ Reserch thermocycler (Check the temperature, when reaches 55°C, then place the tube on it).
7. When the RT step is over → short spin → mix → re-spin.
8. Use 2μl as cDNA template for a 50μl PCR to amplify of the target gene.

PCR using the Taq polymerase

In a thin-walled 0.5ml tube, mix the following in the order given:

Reagents	Volume
Sterile-ddH_2O	34 µl
2mM dNTP	5 µl
10x PCR Buffer	5 µl
5'-primer (25µM)	1 µl
3'-primer (25µM)	1 µl
Template	2 µl
Taq DNA Pol (05/01/02)	2 µl
Total	50 µl

PCR using the Phusion polymerase

(Cat. No. F-530S, New England) Blunt amplified product.

- Mix the following in the order given:

Reagents	Volume	
Sterile-ddH_2O	31.5	µl
2mM dNTP	5	µl
5x HF/GC Buffer	10	µl
5'-primer (25µM)	1	µl
3'-primer (25µM)	1	µl
Template (10ng/µl)	1	µl
Phusion Pol	0.5	µl
Total	50	µl

Program: "MaxPCR"
98°C, 1min 30sec
↓
98°C, 10sec
60°C, 30sec
72°C, 25sec/kb extension
↓ 30 cycles
72°C, 10 min

- Mix content well by gentle vortexing, cover with 3 drops of light mineral oil. → Short spin.
- Run with appropriate PCR program on MJ Reserch thermocycler.
- After reaction, add in 30µl CHCl3 and 10µl 5xTAE dye. →Mix by vortexing. → Centrifuge at 8,000 rpm for 1 min.
- Load supernatant to agarose gel for electrophoresis and purification.

Agarose Gel Electrophoresis and Gel Purification

Large agarose gel (2x14-well)

1 Dilute 20 ml 50xTAE to 1000 ml 1xTAE
2 Weigh 1g of agarose. Put into a 500ml Erlenmeyer.
3 Pour in 125ml 1xTAE. (No shake) Weigh the Erlenmeyer.
4 Microwave for about 2 minutes. When boiled, take the Erlenmeyer out. Shake. Put it back. Until completely dissolved (no particle seen).
5 Weigh the Erlenmeyer. Adjust to the previous weight by ddH_2O.
6 Add 25μl EB (1mg/ml), Shake at the speed 3~4 for about 15 minutes to the temperature at which your hand won't feel hot.
7 Seal the tray, insert the comb, and pour in the gel solution. When gel cools to solid (turn white), take off the tape, put the tray in tank (contain 1xTAE) and pull out the comb, add 1xTAE buffer to sink the gel.
8 Mix samples with 5xTAE dye. Loading samples and run at 90mA (constant) for 50~60 min.

Small agarose gel (8-well)

1 Dilute 10 ml 50xTAE to 500 ml 1xTAE
2 Weigh 0.4g of agarose. Put into a 250ml Erlenmeyer
3 Pour in 50ml 1xTAE. (No shake) Weigh the Erlenmeyer.
4 Microwave (**70% power**) for about 2 minutes. When boiled, take the Erlenmeyer out. Shake. Put it back. Until completely dissolved (no particle seen).
5 Weigh the Erlenmeyer. Adjust to the previous weight by ddH_2O.
6 Add 10μl EB(1mg/ml), Shake at the speed 3~4 for about 5 minutes to the temperature at which your hand won't feel hot.
7 Seal the tray (not need to fold back), insert the comb (standing free), pour in the gel solution.
8 When gel cools to solid (turn white). Comb→ Tape→ Sink in tank (The buffer solution's surface should be no more than 2mm higher than gel's surface)
9 Mix samples with 5xTAE dye. Loading samples at 50mA (constant) for 50~60 min.

50 x TAE:

242g Tris Base; 100ml 0.5M EDTA (8.0); 57ml Glacial Acetic Acid.

Dissolve and add water to 1 liter. Not necessary to adjust pH.

DNA Extraction from Agarose Gels

(QIAEX^{R}II Gel Extraction Kit, Qiagen, Cat. No. 20051)

*All centrifugation steps are at speed 13,000 rpm for 30 sec.

1. Excise the DNA band from the agarose gel with a clean sharp scalpel. Transfer to a 1.5ml tube.
2. Add 500~750µl **Buffer QX1** to the gel. Dissolve the gel at 50^{o}C for about 5 minutes.
3. Add in 15µl~25µl of resuspended **QIAEX II beads** (10µl beads is enough for 2µg DNA binding). Incubate at 50^{o}C for 10 minutes. Mix by inverting tube several times every 2 min to keep the beads in suspension.
4. Centrifuge and remove the supernatant with a pipet.
5. Wash the pellet with 500µl of **Buffer QX1**. (Suspend the pellet by pipetting → Centrifuge → Remove the supernatant with a pipet.)
6. Wash the pellet **twice** with 500µl of **Buffer PE**.
7. Centrifuge and remove the left supernatant as much as possible.
8. Air-dry the pellet at room temperature for 5 minutes with cap open.
9. Add 20~30µl ddH_2O and resuspend the pellet by gentle pipetting. Incubate as following: (DNA <4kb: room temp. for 5min; DNA 4-10kb: 50^{o}C for 5 min; DNA >10kb: 50^{o}C for 10 min)
10. Centrifuge. Carefully transfer the supernatant to a clean tube.

Measure DNA and RNA concentration by Bio-Spectrophotometer

1. Dilute DNA or RNA samples at ratio 1:100 in 1x TE buffer. (5µl samples + 495µl 1xTE)
2. Turn on the Bio-Spec. Pre-warm.
3. Select DNA mode (press 2). Make sure A_1=260nm & A_2=280nm.
4. Take cuvettes out carefully (not touch the glass sides). Add 500µl of 1xTE to each cuvette. Wipe the cuvette with Kim-wipes if necessary. Put cuvettes in machine (letters face out)
5. Close the lid, press "auto zero".
6. Take out the sample cuvette (close lid), aspirate the solution inside. Add samples (500µl). Put it back to the machine.
7. Press "Start". Write down A1, A2 & A1/A2.
8. Wash the cuvette 3 times by ddH_2O. Put it back to the box. (lean it and close the box cover lightly)
9. Press "Return" and "Mode". Close the machine.
10. Calculate the DNA concentration (A_1 x 5µg/µl for dsDNA; A_1 x 3.3µg/µl for ssDNA; A_1 x 4µg/µl for RNA; For the oligo-nucleotide synthesized by IDT, use the specific µg/OD260 number they provide).

Bacterial Transformation

Preparing Competent Cells

1. Pick one colony from (TOPP10/XL1-Blue) plate into 2ml LB media. Grow the bacteria in 37^{o}C incubator at speed of 180~200 rpm for O/N (16~18 hours).
2. Add 1ml bacteria culture into 25ml LB media. Grow the bacteria in 37^{o}C incubator at speed of 200 rpm for 2 hours.
3. Prepare 25ml 50mM $CaCl_2$. (1.25ml 1M $CaCl_2$). Pre-chill on ice.
4. Transfer bacteria culture (grown to mid-log phase) to a 50ml conical tube. Spin at top speed (7) for 10 min at room temp.
5. Decant supernatant. Add 20ml pre-chilled $CaCl_2$ (50mM).
6. Resuspend cell pellet completely by vortexing mildly. (make solution swirling)
7. Incubate on ice for more than 30min.
8. Spin at Speed (5) at room temp for 5 min. Decant supernatant.
9. Pour in the rest of 50mM $CaCl_2$ (about 5ml). Resuspend cells by gentle swirling (no vortex) until no visible clumps.
10. Store on ice for more than 1 hour and the competent cells are ready for transformation.

Transformation

* For one LB plate: 125µl Amp (20mg/ml); 100µl 2%X-gal/DMF; 100µl IPTG (100mM).

Plate the appropriate solutions before plating the transformation reactions.

1. Aliquot 200µl of competent cells to a 1.5ml tube on ice.
2. Add DNA in (plasmid: 0.5µg; ligation: all reaction solution). Mix well by finger tapping.
3. Incubate on ice for 30 min (No shaking).
4. Heat shock at 42oC for 45 sec (No shaking).
5. Put tubes back to ice (No shaking). Incubate on ice for more than 2 min.
6. Mix the reaction and spread it on LB plate (plasmid: half amount; ligation: all of it)
7. Incubate plates at 37^{o}C (with bottom up) for overnight (Topp10: 16 hours; XL1-Blue: 18 hours).

2% x-gal/DMF:

Weigh 0.20g X-gal powder (-20^{o}C top shelf in the middle)

Dissolved in 10ml DMF (N'-N' dimethyl formaid)

20mg/ml Ampicillin:

Weigh 0.20g of Ampicillin (stored at 4^{o}C refrigerator near centrifuge). Pour in a 15ml tube. Add 10ml of sterile dH_2O and one drop of 10N NaOH. Mix solution by vortex. Check the pH after dissolving. (pH 7~8)

Prepare LB plates

1. Weigh 20 g of LB powder in flask (placed under the sink in Room 322). Add 500ml ddH2O in the flask. Swirl flask to mix the solution by hand.
2. Weigh 17g Agar (in a blue tank). Pour in flask. Add second 500ml ddH2O to wash off the agar on the side surface. Do not shake flask.
3. Autoclave for 20 min (Fluid).
4. Shake flask gently to mix the solution. (Do this step on bench. The solution is really hot.)
5. Agitate at speed 3~4 at room temperature for 45~60 min to let the solution cool down.
6. The LB plates are stored in Room 346. Before pour the solution. Clean bench by alcohol.
7. Pour media in about half amount of the plate. 5 plates per stack.
8. Next day, get off the condensation on the top of plates. Pile the plates up. The top two plates of each stack are piled together, marked No. 1 and No. 2, used at first.
9. 4~5 days after pouring, check the contamination. Pack plates in bags and store them at 4^{o}C (left top shelf of beer cooler).

Molecular Cloning Methods and Reactions

Phenol/CHCl3 extraction:

1. Add Sterile ddH_2O to ~400 µl volume of solution.
2. Add same volume of "25:24:1"
3. Shake 50 times to mix well. Spin at 8,000 rpm for 5 min
4. Transfer the top layer to new tube. Add same volume of chloroform
5. Shake 50 times to mix well. Spin at 8,000 rpm for 1 min.
6. Transfer the top layer to new tube→ **Add 1/10 volume of 3M NaAc** → Add same volume of iso-propanol.
7. Mix thoroughly. Spin at 4°C, 14,000 rpm for 10 min.
8. Carefully decant the supernatant. Add 700µl of cold 70% ethanol. Wash, decant ethanol, air-dry, dissolve pellet in water. (Keep eye on DNA pellet)

Preparation of DNA marker

1. Perform midi-prep to obtain enough amount of pREP8 plasmid.
2. 400 µl pREP8 digested by Bgl I, 37°C overnight.
3. 352 µl DNA (midi-prep) + 40µl Reaction buffer (2) + 4µl Bgl I + 4µl RNase (10mg/ml).
4. 200 µl pREP8 digested by BamH I, 37°C overnight.
5. 176 µl DNA (midi-prep) + 20µl Reaction buffer (3) + 2µl BamH I + 2µl RNase (10mg/ml).
6. After overnight digestion, Phenol/Chloroform extraction to get rid of the enzyme (skip the precipitation step.
7. Mix two extracted solutions together and DNA marker is ready for use.
8. Stored at the –20°C freezer near the cell culture incubator.
9. Use 5µl marker (with 1.25µl 5x TAE Dye) for a well.

Fragment Size of each band (From top to bottom)

11,800 → 4,565 → 2,738 → 1,249 → 1,093 → 659 → 534 → 195

Restrictive enzyme digestion (Mini-prep)

10 x reaction buffer:	1	μl
Restriction enzyme:	0.5	μl
Sterile ddH_2O:	5.5	μl
Mini-prep:	3	μl
Total volume	10	μl

Note: Make master solution→ aliquot → add in different mini-prep solution.

37°C water bath for 1 hour→ 65°C, 15min (inactivate enzyme) → electrophoresis

Klenow reaction

10 x Reaction Buffer (2):	5 μl
Large Fragment Polymerase (4~5u/μl):	0.2 μl
dNTP (2mM):	2 μl
DNA:	43 μl
Total volume	50 μl

Reaction: 37°C, 15min → Phenol/$CHCl_3$ extraction

Dephosphorylation with CIP alkaline phosphatase

10 x NEB Buffer(3):	5 μl
CIP (10u/μl):	1 μl
DNA:	44 μl
Total volume	50 μl

Reaction: 37°C, 1 hour → Phenol/$CHCl_3$ extraction

(One unit of CIP can dephosphorylate 2μg DNA.)

T vector ligation (Promega)

2 x ligation buffer:	5	μl
PCR product (0.05~0.5μg):		μl
T easy vector (50ng/μl):	0.5	μl
T4 DNA ligase:	1	μl
Sterile ddH_2O:		μl
Total volume	10	μl

Reaction: room temp for more than 2 hours or 4^oC for overnight

Use all reaction to transformation.

Ligation with T4 ligase (Invitrogen)

5 x ligation buffer:	2	μl
Insert (0.1~0.5μg):		μl
Vector (3:1 insert:vector):		μl
Ligase:	0.5	μl
Sterile ddH_2O:		μl
Total volume	10	μl

The 5x ligation buffer should be thawed on ice!

Ligase: HC (high concentration) for blunt-end DNA fragment ligation

LC (low concentration) for sticky-end DNA fragment ligation

Reaction: room temp for more than 3 hours or 4^oC overnight

Use all reaction to transformation.

RNA Extraction with Trizol Reagent

1. Aspirate culture media carefully. Add appropriate amount of Trizol Reagent (Stored at 4^{o}C, no need to warm up). ~1ml for a 35mm dish or 6-well or 60mm dish.

2. Rock for 5 min at room temperature. (Until complete cell dissociation)

3. Transfer everything to a sterile 1.5ml centrifuge tube.

4. Centrifuge at 12,000xg (12,500 rpm) at 4^{o}C for 10~15 minutes. (For cell culture: 10min; For tissue extraction: 15min.)

5. Transfer the supernatant to a new 1.5 centrifuge tube.

6. Add 0.2ml Chloroform. Shake tubes vigorously for 15~30 seconds. Leave at room temperature for 2-3 minutes.

7. Centrifuge at 12,000xg (12,500 rpm) at 4^{o}C for 15 minutes.

8. Transfer the top aqueous phase to a new 1.5ml centrifuge tube. (~0.6ml)

9. Centrifuge at 12,000xg (12,500 rpm) at 4^{o}C for 5~10 minutes. (Optional)

10. Transfer the supernatant to a new 1.5 centrifuge tube.

11. Add 0.5ml Iso-propanol. Mix by inverting for 20 times. Leave at room temperature for 10 minutes.

12. Centrifuge at 12,000xg (12,500 rpm) at 4^{o}C for 10 minutes.

13. Carefully discard supernatant. Wash the RNA pellet with 1ml 75% EtOH (DEPC). Mix by vortexing (make sure pellet suspended).

14. Centrifuge at 7,500xg (9,500 rpm) at 4^{o}C for 5 minutes.

15. Discard supernatant. Dry RNA pellet in the culture hood.

16. Dissolve RNA in appropriate amount of DEPC-water. (Slowly pipetting up and down) (For confluent 6-well, dissolve RNA in 70ul. Final concentration will be around 0.5ug/ul)

17. Incubate in 55~60^{o}C water bath for 10 minutes for fully dissolving.

Gently vortex to mix. 14,000rpm x 1min at room temp. Measurement of RNA concentration. (Diluted in 1x TE; conc.=OD_{260}x4; A_1/A_2 should >1.8)

QuikChange Site-Directed Mutagenesis Kit

(QuikChange Site-Directed Mutagenesis Kit, Stratagene, Cat. No. 200519)

---- Design the primers following the manuscript's protocol.

----Prepare the stuff for the PCR reaction.

Primers: diluted to 100ng/µl by ddH_2O prior experiment.

Template: the DNA should be supercoiled plasmid. Template DNA is diluted to 10ng/µl prior experiment. Ensure the template DNA is from a dam^+ *E.coli* strain (including XL1-Blue and TOPP10).

dNTP: we can use the 2mM dNTP in our lab instead of the one come from the Kit. Aliquot the Kit's dNTP mix after first usage. Thaw the dNTP on ice.

----Mutant Strand Synthesis Reaction (PCR)

*Using the thin-wall tubes.

*Recipe:

Sterile ddH_2O:	39.5	µl
10x reaction buffer:	5	µl
Template (10ng/µl):	1	µl
Primer #1 (100ng/µl):	1.25	µl
Primer #2 (100ng/µl):	1.25	µl
dNTP (2mM):	2	µl

⇓

Mix gentle by vortexing → Add 1ul of *PfuTurbo* DNA polymerase (2.5U/ul).

⇓

Mix gently by vortexing → Add 30ul of mineral oil → Short spin.

⇓

Running the "MUTAGEN" program:

95^oC, 30 sec

⇓

95^oC, 30 sec.
55^oC, 1 min. → 18 cycles
68^oC, 1min/kb of plasmid

Following temperature cycling, place the reaction on ice for 2 min.
Take out 10ul of PCR product for gel electrophoresis if needed.
(Using the p10-tip: aerosol-resistant)

----*Dpn I* Digestion of the Amplified Products

1. Add 1µl of the *Dpn I* restriction enzyme (10U/µl, Kit) directly to the left PCR reaction (~40µl) below the mineral oil overlay using p10 pipet tip.
2. Gently and thoroughly mix the reaction by pipetting the solution up and down (using p10 tip, p20 pipetman).
3. Spin down the reaction mixture at 8,000 rpm x 1 min, room temperature.
4. Immediately incubate the reaction at 37^{o}C water bath for 1 hour.

---- Transformation of XL1-Blue Supercompetent Cells.

1. Gently thaw the XL1-Blue Supercompetent cells (Kit) on ice. For each transformation, aliquot 50µl of the supercompetent cells to a prechilled 1.5ml microcentrifuge tubes.
2. Transfer 3µl of the Dpn I-digested DNA to the supercompetent cells. (Use p10 tips, wipe away the mineral oil on the tip with Kimwipes papers before adding the DNA into supercompetent cells.)
3. Mix the transformation reactions gently by finger tapping. Incubate on ice for 30 minutes.
4. Take out the LB-plates stored at 4^{o}C. Warm the plates at room temperature.
5. Prepare NZY^{+} broth (0.5 ml for each transformation reaction)
   Recipe: 1ml LB + 12.5µl 1M $MgCl_2$ + 12.5µl 1M $MgSO_4$ + 20µl 1M Glucose
   Mix in 5ml 75 x 12mm tubes. Preheat the broth in 42^{o}C
6. Heat Pulse the transformation reactions for 45 seconds at 42^{o}C and then place the reactions on ice for 2 minutes.
7. Add 0.5 ml of preheated NZY^{+} broth and incubate the transformation reactions at 37^{o}C for 1 hour with shaking at 225~250 rpm.
8. Prepare the plates 30 minutes prior to plating:
   Amp: 125ul of 20mg/ml Ampicilin;
   X-Gal: 100µl of 2% X-gal in DMF (For color-screening)
   IPTG: 100µl of 10mM IPTG (For color-screening)
9. Plate 250µl of transformation reaction on the plates. Incubate at 37^{o}C for > 16 hours. (For plasmid transformation, use 30ul of transformation reaction + 220µl NZY^{+} broth.)

Purification of GST fusion protein (PreScission Cleavage)

1. Inoculate a single colony containing recombinant pGEX plasmid to 5ml of 2XYT medium with Amp. 37^{o}C overnight.
2. Dilute 2ml bacteria culture into 25ml of fresh LB medium with 62.5µl Amp. Grow at 37^{o}C with shaking (~220) for about 3.5~4 hours.
3. Prepare 1liter LB culture media, 2 flask, 500ml/flask, autoclave. (Fluid 20min)
4. Dilute 13ml bacteria culture into 500ml of fresh LB medium (with 1.25ml Amp). Grow at 37^{o}C with shaking (~220, power on, lamp on) for 3 ~3.5 hours. (loosen the foil cap)
5. Measure A_{600}. Between 0.8 is optimal.
6. Cool bacteria culture down in cold room for about 5~10 min.
7. Add 1ml 100mM IPTG (-20^{o}C) to each flask. (Final concentration of 0.2mM)
8. Induction for 60~90 min with shaking (power off, lamp off, door open). Bacteria culture can be stored at 4 ^{o}C overnight.
9. Spin down cells in 500ml container at 3,800 rpm x 20 min at 4^{o}C. (Balance)
10. Decant the supernatant in sink. Dry the pellet. Wipe inside of container with Kimwipe carefully. (Not touching pellet)
11. Add 40ml ice-cold 1X PBS. Suspend bacteria pellet by gentle swirling (no frothing)
12. Pour all suspensions into 50ml conical tubes. 3,800 rpm x 20min, 4^{o}C
13. Decant supernatant. Resuspend pellet in 25ml cold 1xPBS by vortexing.
14. Sonication: (At Dr. Naser's Lab)
    - ❖ Install the large probe
    - ❖ Turn on the machine. Press "Tune". Tuning from 0 to 10. (For small probe, only tune 0~5)
    - ❖ Press "Program" to start sonication. (tune to the scale between 7 and 8 for large probe)
    - ❖ The probe should not touch the bottom of the tube during sonication. The value of output should be 20~30. If over 30, means too hot, tune lower.
    - ❖ Remember to wash the probe by ddH_2O (using plastic cup).
    - ❖ Put the tube back on ice. (5~10 minutes to cool down)

15. Aspirate the foam on the top of solution by the vacuum at sink. (Remember clean the vacuum tube after done.)
16. Add 1.25 ml 20% Triton X-100 (in 1xPBS) to a final concentration of 1%.
17. Rotate tubes at room temperature for 1 hour. (Facilitate solubilization of the fusion protein.)
18. Transfer to polycarbonate tubes. Centrifuge at 12,000 rpm for 10 min at 4^{o}C.
19. Transfer the supernatant to 50ml conical tubes (combine 2 together).
20. Prepare Glutathione Sepharose 4B beads (For 500ml bacteria culture, 250µl bed volume is needed):
21. Mix beads slurry gently by rotating bottle. (1ml bed volume= 1.33ml 75% slurry.
22. Add 0.67ml 75% slurry in 10ml 1x PBS in 15ml tube. (For 1L bacteria culture)
23. Invert tube several times
24. Spin at speed 5 for 5 min.
25. Carefully decant supernatant. Add washed beads to sonication supernatant.
26. Use some sonication supernatant to wash the beads tube and pour back.
27. Rotating at room temperature for 1 hour.
28. Pour supernatant/beads mixture into 10ml column.
29. After all supernatant flow through, Wash by 10ml 1 x PBS for 3 times. (For the first wash, pour PBS in mixture tube first and then load to column)
30. Wash by PreScission cleavage buffer once.
31. Prepare PreScission Protease mixture. (Protease: Cleavage buffer = 1:24)
32. Cap the column. Add the same volume of Protease mixture as the bed volume. Cap the top end (with parafilm wrapping plug) Make sure no leakage.
33. Agitation at 4^{o}C for 4 hours. Collect flow-through in 1.5ml tube.
34. Wash beads by the bed volume of Cleavage buffer, collect the flow-through.
35. Repeat step 34 twice.
36. Add 7~8ml 1xPBS. Cap ends. Store the beads at 4^{o}C.
37. The protein flow-through is stored at –20^{o}C.

Cell Culture Media

HEK-293/FT-239 cells:

DEME (High Glucose) / 10% FBS (Fetal Bovine Serum)

293-FT (For lentivirus generation):

DMEM (High Glucose) / 10% FBS/ 0.1mM NEAA/ 2mM L-Gln/ 1mM Sodium Pyruvate with 500ug/ml G418 (100ul 50mg/ml stock in 10ml media)

PC-3 cells: F12K/ 10% FBS

DU-145/LNCaP cells: RPMI-1640/ 10%FBS

MDA-MB-231/MDA-MB-435 cells:

MEM / 5% FBS/ 0.1mM NEAA/ 2mM L-Gln/ 1mM Sodium Pyruvate/ Vitamin

Drug starting concentration:

Zeocin: 100ug/ml (5ul of 100mg/ml stock in 5ml)

Blasticidine: 15ug/ml (7.5ul of 10mg/ml stock in 5ml)

Hygromycine: 100ug/ml (5ul of 100mg/ml stock in 5ml)

G418: 500ug/ml (50ul of 50mg/ml stock in 5ml)

Histidinol: 5mM (0.5ml of 50mM stock in 5ml)

Tetracycline: 1ug/ml (5ul of 1mg/ml stock in 5ml)

Freezing and thawing tissue-cultured cells

Freezing cells:

(1) Grow cells to confluent in a T75 flask.

(2) Trypsinize cells → Add culture media to neutralize trypsinization → Spin down cells.

(3) Resuspend the cell pellet in 5ml culturing media with 7.5% DMSO

(4) Aliquot 1ml to Nalgene cryogenic vials

(5) Put the vials into the Nalgene freezing container with 250ml Iso-propanal.

(6) Place at -80^{o}C for 24 hours and next day take the frozen vials out and stored at designated boxes.

Thawing cells:

(1) Remove the frozen vial from -80oC freezer, hold the cap portion by hand and submerge the entire cell portion of the vial in 37^{o}C water bath.

(2) Gently shake the vial around to facilitate thawing, the entire content must be thawed before removing the vial from water (about 2 minutes).

(3) Dry off the water and clean with paper towel with 70% ethanol.

(4) Add 14mls of pre-warmed culture media into a T-75 flask

(5) Carefully remove the cap off the vial and transfer the entire cell suspension content into the 14 ml culture media in the T75.

(6) Mix the cells and media, culture in 37^{o}C incubator for 24 hours.

(7) Remove the medium with DMSO, change with fresh media and continue with culturing and passaging for experiments.

Cell Staining for β-gal Activity

1. For 35mm dish or 6-well, rinse cells with 2ml 1x PBS.
2. Add in 1ml fixative solution. (1ml PBS + 50µl Formalin + 2µl glutaraldehyde)
3. Room temperature for 5 mins.
4. Rinse cells with 2 ml PBS x 2 times.
5. Staining cells with 1ml staining solution. (1ml staining buffer, stored at 4^oC) + 50µl 2% X-gal/DMF)
6. Incubate at 37^oC for more than 2 hours. Rinse cells with PBS. Observation.

Formalin: 100% (37% v/v Formaldehyde solution)

Staining Buffer: 5mM K Ferri-cyanide; 5mM K Ferro-cyanide; 2mM $MgCl_2$; in 1x PBS.

Glutaraldehyde: 25% aqueous (Sigma)

X-gal: 2% in dimethyl formamide. (Stored at -20oC)

DMF: N,N,-Dimethyl Formamide

To store stained cell plates, fix each well with 1ml of 10% formalin in 1xPBS for 10 min at room temperature. → Rinse with 1xPBS and store in 1xPBS at 4^oC.

Gel Preparation for Tris/Glycine SDS-PAGE

Separating Gel:

7.5%	1ml	5ml	10ml	15ml	20ml
ddH_2O	0.49	2.43	4.85	7.28	9.70
1.5M Tris (8.8)	0.25	1.25	2.50	3.75	5.00
30% Acryl. Mix	0.25	1.25	2.50	3.75	5.00
10% SDS	0.01	0.05	0.10	0.15	0.20
10% APS	0.005	0.025	0.05	0.075	0.10
TEMED	0.0005	0.0025	0.005	0.0075	0.01

10%	1ml	5ml	10ml	15ml	20ml
ddH_2O	0.40	2.01	4.02	6.03	8.04
1.5M Tris (8.8)	0.25	1.25	2.50	3.75	5.00
30% Acryl. Mix	0.33	1.67	3.30	5.00	6.66
10% SDS	0.01	0.05	0.10	0.15	0.20
10% APS	0.005	0.025	0.05	0.075	0.10
TEMED	0.0005	0.0025	0.005	0.0075	0.01

12%	1ml	5ml	10ml	15ml	20ml
ddH_2O	0.34	1.68	3.35	5.03	6.70
1.5M Tris (8.8)	0.25	1.25	2.50	3.75	5.00
30% Acryl. Mix	0.40	2.00	4.00	6.00	8.00
10% SDS	0.01	0.05	0.10	0.15	0.20
10% APS	0.005	0.025	0.05	0.075	0.10
TEMED	0.0005	0.0025	0.005	0.0075	0.01

Stacking Gel:

	1ml	2ml	3ml	5ml	8ml
ddH_2O	0.61	1.22	1.83	3.05	4.88
1.5M Tris (8.8)	0.25	0.50	0.75	1.25	2.00
30% Acryl. Mix	0.13	0.26	0.39	0.67	1.06
10% SDS	0.01	0.02	0.03	0.05	0.08
10% APS	0.005	0.01	0.015	0.025	0.04
TEMED	0.001	0.002	0.003	0.005	0.008

If necessary, the amount of 10%APS can be increased to 150% to facilitate gel polymerization.

Western Blot Analysis

Cell lysate preparation:

1) Wash cell by 1x PBS twice. (All steps should be performed on ice)
2) Add optimal amount of ice-colded RIPA Lysis Buffer. (The protein concentration of lysate should be around 1ug/ul.)
   (For 100% confluent cells in 6-well plate:
   293-HEK: 0.6ml; PC-3: 0.3ml; DU-145: 0.3ml; LNCap: 0.3ml)
   RIPA lysis buffer (stored at 4^oC) + inhibitors (for 1ml lysis buffer)
   2ul Aprotinin (1mg/ml); 2ul Leupeptin (1mg/ml)
   4ul EDTA (0.5M); 10ul PMSF (100mM); 10ul Na_3VO_4 (100mM)
   20ul NaF (0.5M); 5ul SBTI (10mg/ml)
3) Gently rock plates on the agitator at 4^oC for 30min.
4) Scrape cells down by scrapers (if needed). Transfer all to 1.5ml tubes.
5) Vortex for several seconds.
6) Centrifuge tubes at 12,000 x g (13,000 rpm) for 10min at 4^oC.
7) Transfer the supernatant (cell lysate) to another new 1.5ml tube.
8) DC-protein assay to measure the protein concentration.

Electrophoresis and Blotting:

1) Load 40~60μg whole cell lysate to each well on SDS-PAGE gel. (with 10x SDS loading buffer B-ME; boiling for 5 mins.)
2) Electrophoresis (200V, 40min) in 1x SDS Running buffer. (MWM: 2ul)
3) Transfer proteins from the gel to nitrocellulose membrane (100V, 1 hour or 20V overnight).
4) Stain the membrane by ink. (3~5 drops in Washing buffer) for 10~15min.
5) Rinse membrane by washing buffer. Block the membrane by Blocking solution for 1 hour.
6) Change to primary antibody solution (antibodies in blocking solution). Room temperature agitation for 1~2 hours. (Or overnight at 4^oC)

7) Wash membrane by washing buffer for 5~10 min x 3 times.
8) Change to secondary antibody solution (antibodies in blocking solution). Room temperature agitation for 1 hour.
9) Wash membrane by washing buffer for 5~10min x 3 times
10) Add 2~3ml ECL solution to membrane surface. Room temperature for 3 min.
11) Wrap membrane with plastic membrane. Expose to the film for 1~2 min.
12) Develop the film.

RIPA stock solution (4^oC refrigerator near 37^oC incubator)

10mM Tris, pH 8.0; 150mM NaCl; 1%NP40; 0.1% SDS; 0.5% Na deoxycholate.

10X PBS (7.2): 1.44g KH_2PO_4 + 90g NaCl + 4.2g Na_2HPO_4 → 1L solution → Autoclave.

10X SDS Running Buffer: 144.4g Glycine + 30.3g Tris-Base + 10g SDS → 1L solution.

Transferring buffer: 100ml 10xRunning buffer + 200ml Methanol + ddH_2O to 1L.

Block solution: 5%-7% milk in washing buffer.

Washing buffer: 1x TBST (20mM Tris pH7.6; 140mM NaCl; 0.1% Tween-20)

10 x TBST: 200ml 1M Tris + 280ml 5M NaCl + 10ml Tween-20 → 1L solution.

Silver Staining of SDS-PAGE gel

Note: Wearing gloves all the time! Do not use any metal stuff to touch the gel!

Before staining:
Get the 100mm glass dish ready. (in the drawer under the pH meter)
Pre-warm the Development Accelerator Solution (Stored at 4°C) at room temperature.
All other solutions are stored on the shelf near ventilation hood at room temperature.

1) Prepare 20ml fixative solution in the 100mm dish. (For one gel)

Methanol	10ml
Acetic Acid	2ml
Fixative Enhancer Concentrate	2ml
Sterile ddH_2O	6ml

Mix by agitation.

2) Fix the gel for 20 mins at room temperature with agitation (speed 3~4)

3) Decant the fixative solution. Rinse the gel with sterile ddH_2O for 10mins.

4) Repeat the step (3) 3~4times.

5) Prepare the staining solution (20ml) Decant the water before preparing the staining solution.

Add 7ml sterile ddH_2O into a 100ml beaker.
↓
Add following reagents in the order of:
1ml Silver Complex Solution
1ml Reduction moderator Solution
1ml Image Development Reagent
Swirl the beaker to mix when adding the solution.
↓
QUICKLY add 10ml Development Accelerator Solution all at once with swirling.

6) Add the staining solution immediately to the rinsed gel. Stain for 20min at room temperature with agitation.

7) Change to 40ml stop solution (5% acetic acid) to stop the staining for >15mins. Change to sterile ddH2O

Promoter Activity Assay

Cell transfection

(1) Day 1, HEK-293 cells were plated on a poly-L-lysine-coated (0.5 ml of 20ug/ml PLL, room temperature for more than half hour) 12-well plate at a density of 4 x 10^5 cells per well.

(2) Day 2, the appropriate prostasin promoter-luciferase reporter plasmid (1.3 μg), an SV-β-galactosidase reference plasmid (pSV-β-gal, Promega, 0.2 μg) and an appropriate transcription factor expression plasmid (pcDNA3-based, 0.1 μg) or a control plasmid (pcDNA3, Invitrogen, 0.1 μg) were transfected into the cells using the Lipofectamine 2000 reagent (1.6 ug total DNA in 100ul OPTI + 4ul lipofectamine 2000 in 100ul OPTI) according to the manufacturer's protocol.

(3) Day 3, twenty-four hours after transfection, the cells were lysed with the Reporter Lysis Buffer (Promega). One hundred micro-liters of cell lysate were assayed for luciferase activity using the Bright-Glo Luciferase Assay System (Promega). The β-galactosidase activity was measured by using a β-Galactosidase Enzyme Assay Kit (Promega) and used to normalize for transfection efficiency.

Prepare cell lysate with Report Lysis Buffer (RLB)

(1) Add 4 volumes of water to 1 volume of 5x RLB (stored at 4°C) to produce 1x RLB

(2) Remove the growth media from cells. Wash cells with 1x PBS twice.

(3) Add a sufficient volume of 1x RLB to cover cells (250μl per well).

(4) Rock the dish at room temperature for 15 minutes to completely lyse the cells.

(5) Scrape all areas of the plate surface. Transfer all the cell lysate to a microcentrifuge tube and place the samples on ice.

(6) Vortex the tube for 10-15 seconds. Centrifuge at top speed for 2 minutes at 4°C.

(7) Transfer the supernatant to a fresh tube. For measure the protein concentration, use the 5mg/ml BSA in RLB to prepare the standard curve.

(8) The lysates may be assayed directly or stored at –80°C for at least 2 month.

β-galactosidase activity assay:

(1) Transfer 20µl ~ 50µl RLB lysates to 96 well plate.

(2) Add 50µl 2x Assay Buffer + 30~0 µl 1x RLB (Total volume in the well will be 100µl) to each well. Mix the solution by pipetting.

(3) Incubate the plate at 37°C for 60 minutes.

(4) Terminate the reaction by adding 150µl stop solution (1M Na_2CO_3).

(5) Read the absorbance at ~420nm of each sample by plate reader. (Use 405nm filter)

2x β-gal Assay Buffer (stored at -20°C):

200mM Sodum Phsphate Buffer (pH 7.3); 2mM $MgCl_2$; 100mM β-ME; 1.33mg/ml ONPG

For preparation of 10ml:

2.22ml of 6mg/ml ONPG in 200mM Sodum Phsphate Buffer (pH 7.3)

0.02ml of 1M $MgCl_2$

0.07ml β-ME

7.7ml 200mM Sodum Phsphate Buffer (pH 7.3)

Luciferase activity assay

(1) Pre-equilibrate the Bright-Glo Luciferase Assay Buffer (Promega, Cat.# E2620, stored at -70°C) by placing the tube in a water bath at room temperature.

(2) Pre-warm the luminometer at Dr. Cole's lab.

(3) Add 100µl RLB lysate to 96-well assay plate (Corning, Cat.# 3603).

(4) Add 100µl Bright-Glo Luciferase Assay Buffer to each well.

(5) Take the plate immediately to Dr. Cole'lab for measuring the luminescence value (try to finish reading in 5 minutes although the luminescence should be stable in half hour.)
Starting reading time: 1~5 seconds
P-injection: off
M-injection: off

Gelatinase Assay

Solutions:

2.5% Triton X-100:
Slowly add 12.5ml Triton X-100 into 500ml ddH_2O with constant stirring.

Tris Reaction Buffer:
50mM Tris-HCl (pH7.6), 200mM NaCl, 1μM $ZnCl_2$, 5mM $CaCl_2$
500ml Solution:
25ml 1M Tris-HCl (pH7.6) + 20ml 5M NaCl + 50μl 10mM $ZnCl_2$ + 2.5ml 1M $CaCl_2$
Mix in 452.5ml ddH_2O

Glycine Reaction Buffer:
0.1M Glycine/NaOH (pH 8.3) (Glycine M.W.: 75.07)
3.754g Glycine in 500ml ddH_2O → Adjust pH to 8.3 by 10N NaOH.

10% Gelatin Gel:

Separating gel:	**5ml**	**10ml**
30% Acrylamide (29:1)	1.67ml	3.33ml
1.5M Tris (8.8)	1.25ml	2.5ml
1% Gelatin	0.5ml	1ml
10% SDS	50μl	100μl
10%APS (w/v)	25μl	50μl
TEMED	2.5μl	5μl
Sterile ddH_2O	1.51ml	3.02ml

Stacking gel: Same as normal SDS-PAGE stacking gel.

1% Gelatin: (From Sigma Company, on shelf)
Weigh 0.4g Gelatin→ Pour gelatin powder into ~20ml ddH_2O (in a 50ml beaker) → Swirl beaker very gently→ Microwave for 10 sec. → Take beaker out, swirl gently → Microwave 5sec, Take out, Microwave 5 sec… until solution is clear. No boiling!! → Pour the solution into a 50ml conical tube→ Adjust final volume to 40ml.

Destaining Solution:
40% Methanol; 10% Acetic Acid
400ml methanol + 100ml acetic acid (Done in the hood) → Add water to 1000ml (Use bottle's scale)→ Mix solution well.

Coomassie Blue Staining buffer:
0.2% Coomassie Blue R250; 40% Methanol; 10% Acetic Acid.

Procedure:

Seed ~500,000 cells on 60mm dish

↓

Grow cells to 70%-80% confluency in regular culture media

↓

Change to serum-free media or OPTI-MEM. Culture cells at 37^{o}C for 24 hours

↓

Load 35µl conditioned media (with 10X SDS loading buffer no β-ME) to 10%Acryl (0.1%gelatin) SDS-PAGE gel

↓

Electrophoresis at 200 V, 50 min in pre-cold (4^{o}C) 1X SDS Running buffer

↓

Rinse gel by ddH_2O briefly, 3 x 1min

↓

Rinse by 40ml 2.5%Triton, 2 x 30min (RT)

↓

Deplete Triton solution. Rinse gel by ddH_2O briefly, 3 x 1min

↓

Add in 40ml Tris or Glycine reaction buffer. Wrap the container with plastic wrap (prevent evaporation)

↓

Incubate at 37^{o}C for 24 hours

↓

Stain gel by Coomassie–blue staining solution, 2 hours

↓

Destain gel by Destaining solution, 1hour.

↓

Store gel in water.

Preparation of conditioned media:

1ml of serum-free culture media→ 14,000 rpm x 1 min at 4^{o}C → Transfer supernatant to another tube → Stored at -80^{o}C.

Prepare Nuclear Extract

Nuclear Extract Kit from Active Motif Cat. No. 40010

Prepare prechilled solutions:

PBS/Phosphatase inhibitors: (8ml for 1 dish)
6.8ml ddH2O + 0.8ml 10xPBS +0.4 ml Phosphatase inhibitor (stored at 4°C, warm up in 50°C for 10 min to make solution clear)

1X Hypotonic Buffer: (500µl for 1 dish)
450µl H2O + 50µl 10 x Hyptonic buffer (stored at 4°C)

Complete Lysis Buffer: (50µl for 1 dish)
For 100µl buffer: 89µl Lysis Buffer AM1 (4°C) + 10µl 10mM DTT (-20 °C) + 1.0 µl protease inhibitor cocktail (-20 °C)

Procedures:

1. Wash cells (100mm dish of confluent) with 5 ml ice-cold PBS/Phosphatase Inhibitors. Aspirate solution out and add 3 ml ice-cold PBS/Phosphatase Inhibitors.

2. Remove cells from dish by gently scraping with cell lifter. Transfer cells to a pre-chilled 15 ml conical tube. Centrifuge cell suspension for 5 minutes at 500 rpm at 4ºC.

4. Discard supernatant. Gently resuspend cells in 500 µl 1X Hypotonic Buffer by pipetting up and down several times. Transfer to a pre-chilled 1.5ml tube.

5. Incubate for 15 minutes on ice.

6. Add 25 µl Detergent (4°C) and vortex 10 seconds at highest setting.

7. Centrifuge suspension for 30 seconds at 14,000 x g at 4ºC.

8. Transfer supernatant (cytoplasmic fraction) into a pre-chilled tube. Use the pellet for nuclear fraction collection.

9. Resuspend nuclear pellet in 50 µl Complete Lysis Buffer by pipetting up and down. Vortex 10 seconds at highest setting.

10. Incubate suspension for 30 minutes on ice on a rocking platform set at 150 rpm. (put the tube in a box full of ice, agitate at 4°C)

11. Vortex 30 seconds at highest setting. Centrifuge for 10 minutes at 14,000 x g (13,000 rpm) at 4ºC. Transfer supernatant (nuclear fraction) into a pre-chilled microcentrifuge tube.

13. Aliquot (10µl each tube) and store at –80ºC. Avoid freeze/thaw cycles.

DIG Shift Assay

(DIG Gel Shift Kit, Roche Applied Science, Cat. No. 3 353 591)

Recipe for solutions:

20xSSC: (1L)

(3M NaCl; 0.3 M Na-Citrate, PH 7.0)

- Dissolve 175.3g of NaCl and 88.2g of sodium citrate in 800ml of distilled H_2O.
- Adjust the pH to 7.0 with a few drops of 1M HCl.
- Adjust the volume to 1L with additional distilled H_2O.
- Sterilize by autoclaving.

10x TBE*:* (1L)

108g Tris base + 55g Boric acid + 9.4g EDTA (or 40ml 0.5 M EDTA 8.0)

TEN buffer: **(30ml)**

10mM Tris, 1mM EDTA, 0.1M NaCl, PH 8.0

29ml ddH2O + 300µl 1M Tris (8.0) + 600µl 5M NaCl + 60µl 0.5M EDTA (8.0)

0.5 M EDTA: (200ml)

29.23g EDTA dissolve in ~200ml H_2O. Adjust PH to 8.0 by NaOH.

Maleic Acid Buffer: (1L)

0.1M Maleic Acid, 0.15M NaCl, PH 7.5 (maleic acid pKa1=1~2; pKa2=6.07)

- Dissolve 11.61g Maleic Acid and 30ml 5M NaCl in 800ml of distilled H_2O.
- Adjust the pH to 7.5 with more than 6g solid NaOH.
- Adjust the volume to 1L with additional distilled H_2O.
- Sterilize by autoclaving.

10x Detection Buffer**: (200ml)**

1M Tris-HCl, 1M NaCl, pH 9.5

24.23g Tris + 40ml 5M NaCl +~200ml H_2O → Adjust pH to 9.5 by 12N HCl → autoclaving.

***Washing Buffer*:**

Maleic Acid Buffer with 0.3% (v/v) Tween-20.

(Add Tween-20 to previously sterilized solutions)

***10x Blocking solution*:**

Dissolve Blocking reagent (bottle 12) 10% (w/v) in Maleic acid buffer under constantly stirring on a heating block (65^{o}C).

Autoclave → Aliquot solution (15ml) → Store at -20^{o}C (same refrigerator to store the restriction enzymes). → Thawed solutions can be stored at 4^{o}C for 4 weeks if keep sterile.

For 1x Blocking solution: dilute 10x solution in Maleic acid buffer.

***4-6% native PAGE gel*:** (For two gels, 3ml 6% at bottom, 4% fill up)

	6% (7ml)	**4% (5ml)**
Sterile ddH_2O	4.87ml	3.79ml
30% Acrylamide (29:1)	1.4ml	0.667ml
10X TBE	0.35ml	0.25ml
50% Glycerol	0.35ml	0.25ml
10%APS (w/v)	52.5µl	37.5µl
TEMED	3.5µl	2.5µl

***5x Binding Buffer*:**

100mM Hepes, pH 7.6, 5mM EDTA, 50mM $(NH_4)_2SO_4$, 5mM DTT, 1% Tween-20, 150mM KCl

40ml solution:

Sterile ddH_2O, 25.2 ml + 0.26g $(NH_4)_2SO_4$ + 0.4ml Tween-20 + 0.4ml 0.5M EDTA (8.0) + 8ml 0.5M Hepes/KOH (pH 7.6) + 6ml 1M KCl

Prepare 1ml aliquots.

Add 5µl 1M DTT in each tube to a final concentration of 5mM.

Annealing oligonucleotides

1. Dissolve DNA: Briefly centrifuge to spin down the lyophilized DNA powders → Dissolve DNA in ddH_2O (final concentration around 1μg/μl). If original cap doesn't have the black ring inside of it, change to another sterilized cap in the beaker) → Finger tapping to mix, Room temperature for 10 min → Vortex 10 sec, short spin.
2. Measure the concentration: If the value of μg/OD260 is not provided, use 33μg/OD260.
3. Anneal DNA: Mix equal amount of both complementary ssDNA together in TEN buffer. 95^{o}C, 10 min → slowly cool down to 25^{o}C (in 45 mins) (PCR program "RAMP95")

DIG labeling

1. For each reaction, add 100ng ds oligonucleotide and sterile double distilled water to a final volume of 10μl to a reaction tube. (use vial 6 to do control reaction)
2. Add the following on ice:

Reagent	Volume	Final conc.
5x Labeling buffer (vial 1)	4 μl	1x
$CoCl_2$-solution (vial 2)	4 μl	5 mM
DIG-ddUTP solution (vial 3)	1 μl	0.05 mM
Terminal transferase (400 U) (vial 4)	1 μl	20 U/μl

3. Mix and spin briefly.
4. Incubate at 37^{o}C for 15 min, then place on ice.
5. Stop the reaction by adding 2μl 0.2M EDTA (pH 8.0).
6. Add 3μl double dist. Water to a final volume of 25ml. (Final concentration: 4ng/μl)

DIG shift assay

1. Perform DNA shift assay on ice (total volume: 15ul). Reaction at RT for 15min
2. Put tubes back on ice and add 3.75ul 5x loading buffer (w/o dye).
3. During reaction time, pre-run the 4-6% PAGE gel in 0.5x TBE (stored in 4^{o}C) for 150V, 10~15 min. After pre-run, use plastic pipette to clean wells.
4. Load samples to the gel. Run gel at 150V for 45 min in 0.5X TBE.
5. Electroblotting (nylon positive-charged membrane, 8.5cm x 7.5cm) 400mA for 30 min. (Transferring buffer: 0.5x TBE)
6. Air-Dry membrane between 2~3 layers of filter paper at Room temperature for more than 30 min (can leave overnight).
7. UV-linking by Stratalinker at 120mJ (autolink)
8. Rinse membrane in Washing buffer for 1~5 min. Prepare 1x Blocking buffer.
9. Block membrane in 20ml 1x Blocking buffer at RT for 30 min.
10. Change to 7.5 ml Antibody solution (anti-DIG-AP 1:7,500 dilution in 1x Blocking buffer), RT agitation for 30min.
11. Wash membrane by 20ml washing buffer 15min x 2 times.
12. Equilibrate membrane in 20ml 1x Detection buffer for 3~5 min.
13. Add 1ml reaction solution (Dilute substrate 1:100 in Detection buffer) onto membrane. Cover membrane immediately with plastic wrap. RT, 5min. (keep surface flat.)
14. Take the membrane out. Put it on another clean plastic membrane. Wrap the membrane.
15. For CSPD substrate, incubate membrane at 37^{o}C for 10 min to increase the intensity. For CDP-Star, skip this step.
16. Expose to X-ray film for 20min. Develop the film.

Deglycosylation Assay

(Using Enzymatic Protein Deglycosylation Kit, Sigma # E-DEGLY)

(1) Lyse the cells in the lysis buffer with 1% TX-100 (20mM Tris-HCl, pH 8.0; 150mM NaCl). Make the final concentration of lysate to be more than 1.0 μg/μl.

(2) Add 8μl **5x Reaction Buffer** and 2μl of **Denaturation Solution**. Mix gently.

(3) Prepare the master solution for following reagent:

Sterile ddH_2O:	3.6 μl
15% TX-100:	0.4 μl
PNGase F:	0.4 μl
O-Glycosidase:	0.4 μl
Neuraminidase:	0.4 μl
Galactosidase:	0.4 μl
Acetylglucosaminidase:	0.4 μl

Aliquot 6 μl of mixture solution to each reaction sample. Mix gently.

(4) Incubate for 72 hours (3 days) at 37^{o}C

(5) Regular western blot analysis.

Generation of pENTR-Gene Constructs

1. **Design the primers for cloning into the pENTR/D-TOPO vector**

2. **PCR amplification of the gene of interest**
   (Using the Fusion Polymerase from New England, stored at -20°C, the same floor as restriction enzymes)

Total reaction volume: 50ul		Program: "Max PCR"
Sterile ddH_2O	31.5 ul	98°C, 1min 30sec
5x HF Buffer	10ul	↓
2mM dNTP	5ul	98 °C, 10sec
ENTR5 primer (25mM)	1ul	60 °C, 30sec
ENTR3 primer (25mM)	1ul	72 °C, 45sec (15~30sec/kb extension)
Template (10ng/ul)	1ul	↓ 30 cycles
Fusion Polymerase	0.5ul	72 °C, 10min

3. **Gel purification of the amplified fragment by QIAEX II Agarose Gel Extraction Kit (Qiagen)**

4. **Measure the DNA concentration of the purified DNA fragment**

5. **TOPO Cloning Reaction**
   The Kit is stored at -20°C, second floor from bottom.
   Total volume: 6ul

PCR product (diluted to 10ng/ul)	1ul
Salt solution	1ul
TOPO vector	1ul
Sterile ddH_2O	3ul

   Room temperature incubation: 15min

6. **Put back to ice. Take 1ul to transform the One-Shot Topp10 competent cells (stored at -80°C) (Use half amount of the competent cells, heat-shock 30sec)**

7. **Plate all transformed cell suspension to <u>Kanamycin</u> plate (125ul of 20mg/ml <u>Kanamycin</u> per plate)**

8. **For mini-prep, culture in LB with Kanamycin 50ug/ml. (5ul stock solution in 2ml). 37°C 200~250rpm for 16 hours.**

9. **Sequencing primers for pENTR plasmids (M13 Forward and M13 Reverse primers.**

10. **Alternative way to clone target genes into pENTR vector:**
pENTR-Pro digested by Not I and EcoR I→Generate pENTR/Not I + EcoR I

Generation of pENTR-shRNA Constructs

1. **Design and synthesize complementary DNA oligos containing the overhangs from IDT company. (Use the RNAi Designer software on the website www.invitrogen.com/rani)**

2. **Dissolve the lyophilized oligos in appropriate amount of sterile ddH_2O to a final concentration of 200uM. (The quantity accuracy of the oligo synthesized from IDT is good enough to skip the step of measuring DNA concentration.)**

*** Annealing at concentration lower than 50uM can significantly reduce the efficiency. Note that the annealing step is not 100% efficient.

Total reaction volume: 20ul

Top strand oligo (200uM)	5 ul
Bottom strand oligo (200uM)	5 ul
10X Oligo Annealing Buffer	2 ul
DNAse/RNase free Water	8 ul

3. **Incubate the reaction at 95 oC for 4 minutes. (Use the PCR machine)**

4. **Remove the tube to your laboratory bench and let the reaction cool down to the room temperature. (About 15 minutes) Short spin. (Final concentration of the annealed ds Oligo is 50uM)**

5. **Add 1ul of 50uM ds oligo into 99ul DNAse/RNase free Water. (diluted to 500nM). And dilute the 500nM ds Oligo 100-fold into 1x Anealing Buffer. (Final conc. is 5nM): Store the 5nM ds oligo on ice!!**

500nM ds Oligo	1ul
10x Oligo Annealing Buffer	10ul
DNAse/RNase free Water	89ul

6. **Ligation reaction: (Thaw the reagent on ice)**

Total reaction volume: 10ul

5x Ligation Buffer	2 ul
pENTR/H1/TO (0.75ng/ul)	1 ul
ds oligo (5nM)	2.5 ul
DNAse/RNase free Water	4 ul
T4 DNA Ligase (1U/ul)	0.5 ul

Mix reaction well by pipetting up and down. (Do not vortex!!) Incubate for 5 minutes at room temperature for 5 minutes.

7. **Put tube on ice. Take 1ul to transform the One-Shot Topp10 competent cells (stored at -80^{o}C) (Use half amount of the competent cells, heat-shock 30sec)**

8. **Mini-prep digested by BamH I to check the insert. Sequencing primers for pENTR plasmids (H1 Forward and M13 Reverse).**

Generation of pLenti-DEST vector by LR recombination

**Miniprep DNA is ready to be performed for the LR recombination reaction. But correct estimation of DNA concentration is required.
**Thaw LR Clonase II enzyme mix from -20^{o}C on ice

1. **Add following components in 0.5 ml microcentrifuge tubes and mix.**

pENTR mini-prep (50-150ng/reaction) : usually 0.5 ul is good for the reaction
pLenti vector (150ng/ul) : 1ul
(pLenti4/TO/V5-DEST or pLenti4/BLOCKiT-DEST)
TE buffer (pH8.0): 6.5ul

2. **Vortex the LR Clonase II enzyme mix briefly twice (2 seconds each time).**

3. **Add 2ul of LR Clonase II enzyme mix. Mix well by pipetting up and down. (Return the Clonase mix to -20^{o}C immediately after use)**

4. **Incubate the reaction at 25^{o}C for more than 2 hours (PCR machine, Program "LR25")**

5. **Add 1ul of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37^{o}C.**

6. **Put back to ice. Take 1.25 ul to transform the One-Shot Stbl3 competent cells (stored at -80^{o}C) (Use half amount of the competent cells, heat-shock 30sec)**

7. **Plate all transformed cell suspension to Ampicilin plate (200ul of 20mg/ml Ampicilin per plate)**

8. **For mini-prep, culture in LB with Ampicilin 100ug/ml. (10ul stock solution in 2ml). 37^{o}C 200~250rpm for 20 hours.**

9. **Sequencing primers for pLenti plasmids**

pLenti4/TO/V5-DEST: pCMV Forward or V5 Reverse primers.
pLenti4/BLOCKiT-DEST: H1 Forward or V5 Reverse primers.

Producing Lentivirus in 293FT Cells

293FT Culture media:
D-MEM (HG)/ 10% FBS / 0.1mM NEAA/ 2mM L-Gln/ 1mM Sodium Pyruvate
with 500ug/ml G418 (100ul 50mg/ml stock in 10ml media)

About 3 x 10^7 cells when cells are grown confluent.
Passage: 3x10^6 cells per T75 flask, 3 days later, ready for next round experiment.
(One T75 flask of confluent 293FT cells is enough for two transfections.)
Do not over grow the cells!
Do not use cells which have been cultured more than 20 passages!

Procedure:

(1) Add 1.5ml OPTI-MEM media to four 5ml (75x12mm) tubes.
(2) Add appropriate amount of DNA or Lipofectamine 2000 into the OPTI media

3ug of pLenti-based DNA + 9ug of the ViraPower Packaging Mix in 1.5 ml OPTI

36ul of Lipofectamine 2000 in 1.5ml OPTI, mix gently and incubate for 5min at R.T.

(3) During the 5minute incubation time, Wash the 293FT cells with PBS once.
(4) Add 1.3ml Trypsin/EDTA to trypsinize cells.
(5) Combine the diluted DNA and Lipofectamine solutions together. Incubate at room temperature for 20 minutes.
(6) Stop trypsinization by adding regular culture media. Spin down the cells.
(7) Resuspend cells in 10ml regular media. Count cell number (dilute 3 times)
(8) Prepare 10~11ml of 293FT cell suspension (2x10^6 cells/ml, 5ml total will be 1x 10^7)
(9) When DNA-lipid 20 minute incubation is over, add the 3ml mixture to a 100mm dish containing 5ml fresh culture medium. (Do not include antibiotics!)
(10) Add 5ml of the prepared 293FT cell suspension to the plate. Mix gently by rocking the plate back and forth several times
(11) Incubate the cells overnight at 37^{o}C. (usually 18 hours)
(12) The next day. Change the media with 10ml fresh culture media.
(13) Incubate the cells at 37^{o}C for 48~72 hours.
(14) Collect the virus-containing media into 15ml sterile tubes and centrifuge at 3000 rpm for 5 minutes at 4^{o}C (Autoclaving room)
(15) Aliquot the supernatant into 2ml red-cap-tubes (1ml/tube) (the same shelf as 10xPBS for culture). Store the viral stocks at -80^{o}C.

Titering the Lentiviral Stock Notes

** Viral titers can decrease as much as 10% with each freeze/thaw cycle. So avoid multiple freeze/thaw cycles.

** All reagent stock aliquots are stored at -20^{o}C (same refrigerator as restriction enzymes, the second floor from bottom)
Zeocin (100mg/ml): light sensitive
Blasticidine (10mg/ml): After thawed, store at 4^{o}C (tissue culture refrigerator, bottom floor, good for 2 weeks, label the tube with date after thawed.)
Polybrene (hexadimethrine bromide, 6mg/ml): After thawed, store at 4^{o}C (tissue culture refrigerator, bottom floor, good for 2 weeks, label the tube with date after thawed.)

** For Zeocin selection, make sure cells are not greater than 50% confluent.

Drug Concentration for cell selection:

	Zeo Conc.	Vol. of Stock	Blast Conc.	Vol. of Stock
HEK-293	100ug/ml	5ul for 5ml	15 ug/ml	7.5ul for 5ml
MDA-231	100ug/ml	5ul for 5ml	15 ug/ml	7.5ul for 5ml
MDA-435	100ug/ml	5ul for 5ml	15 ug/ml	7.5ul for 5ml
DU-145	100ug/ml	5ul for 5ml	15 ug/ml	7.5ul for 5ml
PC-3	50ug/ml	2.5ul for 5ml	20 ug/ml	10ul for 5ml
A549				

Generating a ViraPower T-REX Host Cell Line

(1) Plate cells into a 100mm dish in complete growth media as appropriate (~50% confluency).

(2) On the day of transduction (Day 1), thaw the Lenti6/TR lentiviral stock and dilute (if necessary) the appropriate amount of virus into fresh complete medium (If single colony picking is wanted, use 10ul of virus; otherwise, use all 1ml virus solution.) Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency (5ml is good enough for 100mm dish culture). **DO NOT vortex.**

(3) Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.

(4) Add 5ul of Polybrene stock solution to a final concentration of 6 μg/ml (1:1000 dilution). Swirl the plate gently to mix. Incubate at 37°C overnight.

(5) The following day (Day 2), remove the medium containing virus and replace with fresh, complete culture medium.

(6) The following day (Day 3), remove the medium and replace with fresh, complete medium containing the appropriate amount of Blasticidin to select for stably transduced cells.

(7) Replace medium with fresh medium containing Blasticidin every 2-3 days until Blasticidin-resistant colonies can be identified (generally 10-12 days after selection).

(8) Note: Transducing cells with Lenti6/TR lentivirus at a high MOI should result in most of the cells being Blasticidin-resistant. In this case, you may not be able to see distinct Blasticidin-resistant colonies when performing stable selection. You may also not see many non-transduced cells (i.e. dead cells).

(9) Pick at least 10 Blasticidin-resistant colonies and expand each clone to assay for Tet induction abilities. Alternatively, you may pool the heterogeneous population of Blasticidin-resistant cells.

Generating a pLenti-GENE Cell Line

(1) Plate cells into a 35mm dish in complete growth media as appropriate (~50% confluency).

(2) On the day of transduction (Day 1), thaw the pLenti4/TO/GENE or pLenti4/BlockiT/RNAi lentiviral stock and dilute (if necessary) the appropriate amount of virus into fresh complete medium (1ml virus solution + 1ml culture media + 2µl Polybrene). Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency (**2.5ml is good enough for 60 mm dish culture or T25**). **DO NOT vortex.**

(3) Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.

(4) Add 2.5ul of Polybrene stock solution to a final concentration of 6 µg/ml (1:1000 dilution). Swirl the plate gently to mix. Incubate at 37°C overnight.

(5) The following day (Day 2), remove the medium containing virus and replace with fresh, complete culture medium with Blasticidine.

(6) The following day (Day 3), Trypsinize all cells in 0.2 ml of Typsin/EDTA and stop the digestion by 1.5ml of culture media. Add 1ml of cell suspension to a 100mm dish (or T75) containing 9ml of fresh media with appropriate Zeocin. (1:9 dilution seeding)

(7) Replace medium with fresh medium containing Zeocin every 2-3 days until Zeocin-resistant colonies can be identified (generally 10-12 days after selection).

(8) Pick Zeocin-resistant colonies and expand each clone to assay for Tet-induced expression of target genes. Alternatively, you may pool the heterogeneous population of Zeocin-resistant cells.

Selection of single clone cells

(1) When the visible cell colonies are grown to 2-3mm diameter size, they are ready to be trypsinized and expanded.
(2) Wash the cells with 10ml 1x PBS.
(3) Apply 40ul of Trypsin/EDTA to digest the cell colony. Scratch and pipet at same time to get all cells detached from the dish.
(4) Transfer all cells to 12-well plate containing 1ml fresh media.
(5) Next day, Remove the culture media and change with media containing drug.
(6) Culture cells in 37^{o}C for about 7 days (change media every 2-3 days).
(7) When the cells are reaching confluent. Wash with PBS and digested in 100ul of Trypsin/EDTA. Stop trypsinization by 1ml fresh media. Transfer the cell suspension to 6-well plate containing 2ml fresh media.
(8) Next day, Change with media containing drug.
(9) When cells are reaching confluent. Passage to T25 and perform further experiments.

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