Novel Tumor Suppressive Mechanisms of Estrogen Receptor β in Prostate Cancer

by

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Dedicated to my mom, who recently became paralyzed physically and mentally due to a stroke. Sadly, I won't be able to share my success with her anymore.

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ABSTRACT

Estrogen receptor β (ERβ) was first identified in the rodent prostate and is abundantly expressed in human and rodent prostate epithelium, stroma, immune cells, and endothelium of the blood vessels. Genomic deletion of ERβ led to hyperplasia of prostate epithelium as well as upregulation of androgen receptor (AR) regulated genes. ERβ has been shown to inhibit proliferation and induce apoptosis in prostate cancer cells; however, role of ERβ in regulating AR activity in prostate cancer has not been studied in detail. Additionally, the role of ERβ in PI3K/Akt/PTEN pathway, which is one of the most altered in prostate cancer, is not known.

Chapter 2 of this dissertation describes the role of ERβ in regulating PI3K/Akt/PTEN pathway. ERβ upregulated INPP4B in prostate cancer cells, PC3, as well as non-malignant cells BPH-1. Upregulation of INPP4B inhibited Akt activity measured by phosphorylation of Ser473 and its downstream target GSK3β. Further, we show that ERβ inhibited migration of PC3 cells by upregulating INPP4B in wound healing assays. This regulation may indicate a role for ERβ in metastasis suppression.

Androgen receptor is the main driver of primary as well as metastatic prostate cancer. Chapter 3 describes the role of ERβ in regulating AR expression and activity in prostate cancer cells LNCaP. Using global transcriptomic analysis of ERβ-expressing LNCaP cells, we found AR-signaling is the most downregulated effect after ERβ activation. We validated this regulation independently by transcript and protein measurement of established AR target genes FKBP5, CaMKK2, TBC1D4, as well as by luciferase reporter assay. We further demonstrated that downregulation of

CaMKK2 inhibits activity of AMPK, a major energy sensing mechanism in cells. Taken together, these findings support tumor suppressive effects of ERβ in prostate cancer through novel mechanisms and indicate possibilities for therapeutic intervention.

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ADT	Androgen deprivation therapy
AF1	Activation function 1
AF2	Activation function 2
AMPK	5' adenosine monophosphate-activated protein kinase
AR	Androgen receptor
BPH	Benign prostatic hyperplasia
CaMKK2	Calcium/calmodulin-dependent kinase kinase 2
CBP	CREB binding protein
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation sequencing
CRPC	Castration resistant prostate cancer
DBD	DNA binding domain
DHEA	Dehydroepiandrosterone
DHT	Dihydrotesterone
E2	17β-Estradiol
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transitions
ERE	Estrogen response elements
ERG	ETS-related gene
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ETS	Erythroblast transformation specific

FKBP5	FK506 binding protein 5
GPER1	G-protein coupled estrogen receptor 1
GSEA	Gene set enrichment analysis
GSK3β	Glycogen synthase kinase β
GWAS	Genome wide association studies
HIF	Hypoxia inducible factor
HRE	Hormone response element
INPP4B	Inositol polyphosphate 4-phosphatase
LBD	Ligand binding domain
LHRH	Luteinizing hormone-releasing hormone
NCOA3	Nuclear receptor coactivator 3
NFkB	Nuclear factor kappa B
NR3A1	Nuclear receptor subfamily 3, group A, member 1
NR3A2	Nuclear receptor subfamily 3, group A, member 2
NR3C4	Nuclear receptor subfamily 3, group C, member 4
NTD	N-terminal domain
PCa	Prostate cancer
PELP-1	Proline-, glutamic acid-, leucine-rich protein
PI(3,4)P2	Phosphatidylinositol 3,4-bisphosphate
PI(3,4,5)P3	Phosphatidylinositol 3,4,5-trisphosphate
PI3K	Phosphoinositide 3-kinase
PIN	Prostatic intraepithelial neoplasia
PCR	Polymerase chain reaction

PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homologue
SHBG	Steroid hormone binding globulin
SNP	Single nucleotide polymorphism
SRC-1	Steroid receptor coactivator
TBC1D4	TBC1 domain family member 4
TMPRSS2	Transmembrane serine protease 2
TRAMP	Transgenic adenocarcinoma of the mouse prostate
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor

Chapter 1

General Introduction

1.1 Prostate Cancer

Prostate cancer (PCa) is the most common cancer in US men with an estimated 174,000 new cases and is the second leading cause of cancer related mortality with an estimated 31,600 deaths. These numbers represent approximately 20% incidence and 10% mortality attributable to all cases of cancer in US men. Prostate cancer is an indolent disease with a 5-year survival rate of 100% for those with localized or primary tumors; however, it is only 30% for metastatic cases [1].

The main risk factors for prostate cancer include age, race, family history, hormone levels, and diet. Prostate cancer is a disease mainly of old age as 99% of cases occur in males over the age of 50 [2]. Prostate cancer incidence varies greatly across different races. Highest incidence is observed in African-American (82/100,000) followed by White-American (62/100,000) in the United States. For a comparison, prostate cancer incidence is only 1/100,000 in Chinese population [3]. African-Americans are also at higher risk of dying from prostate cancer than White-Americans [4]. Various hypotheses have been advanced to explain the disparity in prostate cancer incidence among different races. A two-fold increase in prostate cancer risk is seen in a person who has family history of prostate cancer [5]. Genetic risk is also supported by many studies using single nucleotide polymorphism (SNP) arrays and genome-wide association studies (GWAS) where variants have been identified that might increase prostate cancer risk [6,7].

Dietary factors are implicated in higher incidence of prostate cancer in the western world. This is supported by the studies of prostate cancer incidence in the Asians living in Asia and those who have moved to the West. A higher incidence of prostate cancer was seen in the Asians living in the Western countries compared to those living in Asia. This observation is attributed to higher content of soy-rich diets containing isoflavones in the Asians [8].

The prostate is an androgen-responsive organ and hormones are the well-known risk factors for prostate cancer. The most important hormonal risk factors are testosterone (T) and dihydrotestosterone (DHT) which are ligands for androgen receptor (AR). Androgen receptor regulates growth and development of prostate both under the normal physiological conditions and in malignancy. AR plays an important role in prostate cancer as a strong driver of proliferation, and as such is the primary target for treatment of prostate cancer [9].

1.2 Prostate Cancer Diagnosis

A screening for prostate cancer starts with the blood test for prostate specific antigen (PSA) and a digital rectal exam in men 40 years or older. Elevated PSA levels can occur in prostate cancer as well as in non-malignant conditions such as benign prostatic hyperplasia (BPH) and prostatitis . Any abnormalities in these results are followed up with prostate biopsy. A case of primary prostate cancer is monitored using Gleason score of biopsy samples , lymph node involvement and PSA value. Metastatic prostate cancers are monitored with the measurement of blood

testosterone and PSA levels, computed tomography (CT) scan, whole body magnetic resonance imaging (MRI), and positron emission tomography (PET) [10,11].

1.3 Treatment Options for Prostate Cancer

Treatment strategies differ depending on factors such as the stage of the disease, the age and physical status of the patient, and previous treatments. A range of treatment options are available which include active surveillance, radiation, surgery, hormonal therapy, chemotherapy, and immunotherapy [11]. Primary prostate cancer is an indolent disease and except for periodic monitoring also called active surveillance, other interventions are generally not recommended for many people. For prostate cancers that appear to be growing and invading adjacent tissue, surgery is the first line of treatment where all of prostate tissue is removed [12]. Radiation therapy is generally used for localized tumors instead of surgery which has same cure rate as surgery. It is also used for tumors that have grown out of the prostate. Hormonal therapy is used for cancers that have invaded nearby tissues and includes androgen-deprivation therapy (ADT) to reduce circulating levels of testosterone. This is accomplished either by orchiectomy (surgical castration) or administration of luteinizing hormone-releasing hormone (LHRH) agonists (medical castration) [13]. The majority of the patients initially respond favorably but ADT eventually leads to resistant tumors in many patients. These tumors are called castration-resistant prostate cancer (CRPC) and show increased aggressiveness and enhanced metastatic potential. It is the metastatic CRPC which is responsible for the majority of

prostate cancer related deaths. Chemotherapy and immunotherapy are generally reserved for castration-resistant prostate cancer and metastatic lesions [14].

1.4 Molecular Lesions in Prostate Cancer.

Prostate cancer is a heterogeneous disease both in terms of disease manifestation and pathophysiology. High levels of histological heterogeneity is observed within the same prostate gland. As with all cancers, prostate cancer also involves multiple somatic gene mutations leading to either activation of oncogenes or inactivation of tumor suppressors [15]. The most common mutations occur in one or more of the following genes: PTEN, p53, PI3K, Akt, c-Myc, Rb, and AR. Most common of these is deletion of PTEN which occurs in 60% of primary and 100% of metastatic prostate cancers [16].

1.5 Nuclear Receptors

Nuclear receptors constitute a large class of transcription factors that regulate expression of a large number of genes upon stimulation with its ligands. The ligands for the majority of nuclear receptors are either steroid hormone, thyroid hormone, or retinoid but vitamin D, fatty acids, prostaglandins, cholesterol, xenobiotics, and some other small molecule metabolites are also known to activate nuclear receptors. Ligands for some nuclear receptors are not known and hence are called orphan nuclear receptors [17,18]. Nuclear receptors undergo conformational change upon ligand binding, translocate to the nucleus, bind to cognate DNA sequences and regulate the transcription of specific genes [19].

Nuclear receptors are found in all metazoans and in humans there are 48 members of this receptor superfamily. The genes regulated by nuclear receptors are indispensable for the development, metabolism, and homeostasis of an organism. Although nuclear receptors are diverse, they can be grouped into three classes: (i) Steroid receptors, (ii) thyroid and retinoid receptors, and (iii) orphan receptors [20].

Nuclear receptors are modular proteins with six identifiable regions named A, B, C, D, E, and F. Three major functional domains can be assigned: amino-terminal transactivation domain (NTD), central DNA-binding domain (DBD), and carboxy-terminal ligand binding domain (LBD). N-terminal domain includes A/B regions and has a weak activation function (AF1) domain. It is the least conserved domain among nuclear receptors and may activate transcription in the absence of ligands. The DNA binding domain (DBD), as its name suggests, binds specific DNA sequences called hormone response elements (HRE). This domain is highly conserved and has two zinc fingers which are responsible for receptor dimerization and DNA binding. D domain is also called the hinge region as it serves to connect DBD with the ligand binding domain. This region is required for intracellular trafficking and subcellular localization of nuclear receptors. LBD is responsible for ligand binding and is the main trans-activator of a nuclear receptor. It contains second transactivation domain (AF2). This domain is less conserved in amino acid sequence which determines ligand specificity of individual receptor. LBD is also required for receptor dimerization and recruitment of co-factors for optimum function [20, 21].

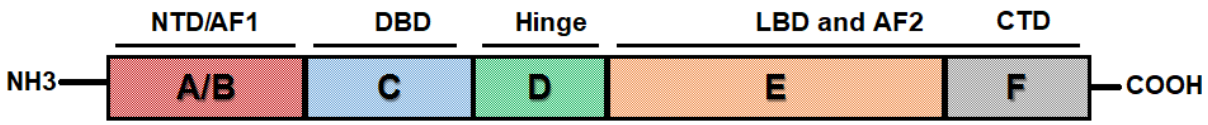


Figure 1.1 Modular structure of a nuclear receptor. Schematic of a nuclear receptor showing organization of different domains. NTD, N-terminal domain; DBD, DNA binding domain; LBD, ligand binding domain; CTD, C-terminal domain.

1.6 Androgens and Androgen Receptor in the Prostate

Androgens are steroid hormones responsible for the development and maintenance of male sex organs and secondary sexual features. Androgens are synthesized in the testes, the ovaries, and the adrenal glands. Testosterone is the main androgen found in men which is produced by testes whereas dehydroepiandrosterone (DHEA), androstenedione, and androstenediol are synthesized in the adrenal cortex [22,23]. The synthesis of testosterone is regulated by a feedback mechanism involving the hypothalamic-pituitary-gonadal axis. Steroid hormone binding globulin (SHBG) and albumin bind most of the testosterone in circulation and regulate its availability. Dihydrotesterone (DHT), a more potent agonist of androgen receptor is synthesized from testosterone in peripheral tissues by the enzyme 5α-reductase [24]. Androgens are ligands for the androgen receptor (AR) which is a nuclear receptor expressed in epithelial cells, stromal cells, and smooth muscle cells of many organs [25].

Prostate is a highly androgen responsive organ and is dependent on androgen for its development, maturation, maintenance, and function [26]. In the prostate, testosterone is converted irreversibly to the more potent DHT by the enzyme 5α-reductase [24]. Repression of androgens during embryogenesis of mice causes irreversible damage to the growing prostate. Suppression of circulating androgen leads to androgen-withdrawal-induced apoptosis in luminal prostate epithelium [27].

Androgen receptor (AR) also known as NR3C4 (nuclear receptor subfamily 3, group C, member 4) is a transcription factor of the nuclear receptor superfamily [23]. In humans, the AR gene is located on the X chromosome at Xq11-12 and encodes for a full length 110 kDa protein [25]. Androgen binding leads to conformational changes in the receptor, dissociation from heat-shock proteins, translocation to the nucleus, receptor dimerization, and DNA binding. The output of this AR genomic signaling is regulation of target gene expression [28]. On the other hand, non-genomic androgen signaling causes rapid changes in cell function independent of changes in gene transcription through interaction with other signaling proteins [22, 29].

1.7 Role of Androgen Receptor in Prostate Cancer

AR is important for growth and survival of normal and malignant prostate tissue and its role is demonstrated to be pro-proliferation, pro-differentiation, and pro-survival. Prostate develops from urogenital sinus under the influence of androgens through AR [27, 31]. Studies of genetically modified mice suggested a role for AR in initiation of prostate cancer. Transgenic mice overexpressing AR driven by probasin promoter developed dysplastic lesions and prostatic intraepithelial neoplasia (PIN), but cancer did not develop. However, mice expressing a mutant AR (E231G), which is constitutively active, developed adenocarcinoma in the ventral prostate and metastases [32,33]. Recently, it was shown that androgen signaling is essential for prostate cancer tumorigenesis from prostatic basal cells [34].

Studies of AR function demonstrate gain-of-function alterations in prostate cancer which is proposed to be due to a ‘molecular switch’. This molecular switch is the transition of AR from regulating differentiation to driving proliferation in the luminal epithelial cells [35]. One possible mechanism of altered behavior of AR depends on its binding to novel genes which is facilitated by FOXA1. In an AR chip-seq study comparing normal and malignant prostate samples, the consensus AR-binding site is the most significantly enriched motif in normal tissues, while FOXA1, HOXB13, and AR binding sites were most significantly enriched in tumor tissues [36]. In advanced prostate cancer, altered mRNA splicing events lead to production of proteins with truncated AR isoforms. These isoforms lack the ligand binding domain, and function as constitutively active, ligand-independent transcription factors. These truncated isoforms can regulate androgen-independent expression of AR target genes, at the same time exhibit resistance to androgen depletion therapy [37,38].

In a subset of castration-resistant prostate cancer (CRPC), there is increased expression of AR either through genomic amplification of AR locus or upregulation of AR protein level. Increased AR expression can be activated with sub-physiological level of androgens and show resistance to antiandrogens such as bicalutamide [39,40]. Moreover, prostate cancer cells overexpressing AR are capable of binding to a larger number of sites on chromatin, some of which are novel [36].

Somatic AR mutations occur in a subset of prostate cancer patients, the frequency of which increases in CRPC. Majority of these mutations are point mutations and occur in LBD which confer hypersensitivity to androgens and broaden AR ligand specificity. These AR LBD mutants were demonstrated to be activated by estrogen, progesterone, and glucocorticoids [41, 42].

In the absence or sub-physiological level of androgen, non-canonical pathways that include numerous growth factors, cytokines, and other hormones have been implicated in AR activation [43]. Androgen receptor can be activated through phosphorylation by hyperactive PI3K/Akt pathway [44]. Additionally, in the absence of androgens, IL-6 was shown to induce association of Stat3 with AR which led to the increased expression of AR target genes [45].

Expression of fusion gene product TMPRSS2: ERG is frequently observed in aggressive prostate cancers [41]. TMPRSS2 (transmembrane serine protease 2) is an enzyme which is highly expressed in luminal prostate epithelium and is upregulated by androgen receptor. ERG (ETS-related gene) is a member of the ETS (erythroblast transformation specific) family of transcription factors implicated in the development of different tissues as well as cancer progression. It is considered as a proto-oncogene. Chromosomal translocation of TMPRSS2 to ERG leads to excess production of this fusion protein and contributes to castration-resistant prostate cancer [46,47].

A key downstream target of the androgen receptor is CaMKK2 which regulates metabolic activity of prostate cancer cells and is emerging as a therapeutic target for controlling metastatic prostate cancer [48]. This protein kinase also affects bone remodeling and macrophage function which is relevant for preventing ADT-induced bone loss [49].

1.8 Tumor Metastasis

Metastasis is the spread of tumor cells to distant organs and development of a tumor in that organ. Tumor cells can disseminate into blood or lymphatics from the primary tumor and settle into a new tissue where they form metastases. Metastasis is the last stage (stage IV) of a cancer and is the main cause of cancer related mortality [50]. It is also genetically regulated and is a step wise process. Metastatic cancer cells maintain many of the molecular markers of primary cancers. If left untreated, almost all cancers will form metastatic lesion. Different cancers have different metastatic potential; while basal cell carcinoma rarely metastasize, pancreatic cancer is highly metastatic [51,52]. Prostate cancer tends to be less metastatic, but castration resistance leads to enhanced metastatic potential [53].

The first step in metastasis is the ability of tumor cells to break away from the primary tumor which is facilitated by the process of EMT [50]. To be able to leave the tumor, cancer cells also need to degrade extracellular matrix which surrounds the tumor itself and is accomplished by secreting proteases such as MMP-2, MMP-9, uPA

(urokinase-type plasminogen activator), and cathepsins [54,55]. The cancer cells also develop motility which is generally not a property of mature epithelial cells. Three different kinds of motility is exhibited by human cells: amoeboid movement, mesenchymal-type movement, and collective movement. Physiological cell motility is necessary for embryonic morphogenesis, wound healing, and immune cell trafficking [55].

To be able to move, a cell needs to modify its shape and stiffness, and hold on to a substrate which is the extracellular matrix (ECM). The sequential processes of cell movement include polarization and elongation of the cell, pseudopod formation by the extension of the leading edge, attachment to the ECM substrate, and finally contraction of the entire cell body [56]. Cell migration results from continuous cycle of these steps which involve generation of signals at the leading edge and remodeling of actin cytoskeleton. Tumor cells can be disseminated as individual cells which is called “individual cell migration” or expand in solid sheets, strands or clusters of cells termed “collective migration”. Collective cell migration is frequently observed in locally advancing tumors [57].

Prostate cancer related deaths occur mainly in patients with relapse or metastasis [2]. Tumor angiogenesis is a critical process in growth, and metastasis of many cancers. Upregulation of VEGF secretion, which is one of the most essential angiogenic factor, exerts its mitogenic effect on endothelial cells to induce tumor angiogenesis in prostate cancer [58]. Angiogenesis is an attractive target in cancer

therapy because it supplies oxygen and nutrients for the growth and survival of tumor cells. But cancers cells also use angiogenesis as a route for escape and metastasis to distant organs [59].

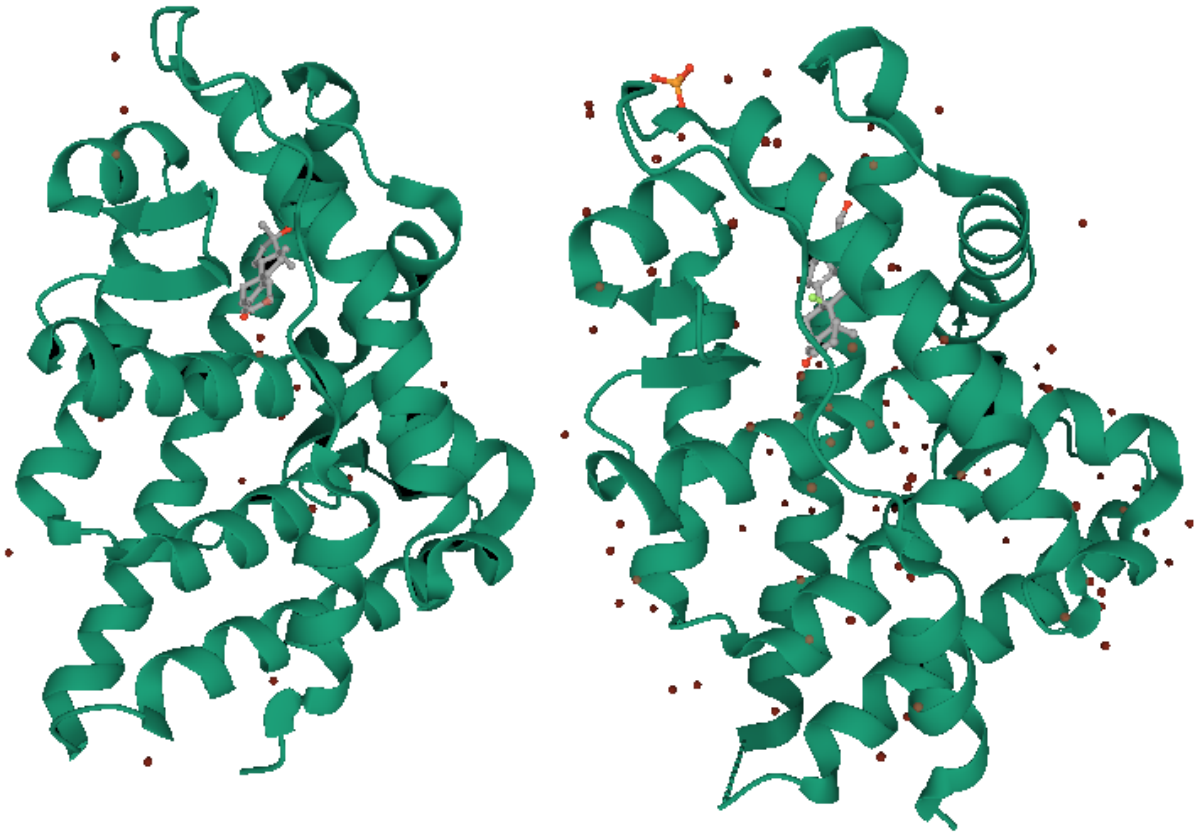


Figure 1.2 Crystal structure of androgen receptor ligand binding domain. Left in complex with R1881 and right in complex with a glucocorticoid. Uniprot accession ID P10275. Downloaded from https://www.ebi.ac.uk/pdbe/entry

1.9 Epithelial to Mesenchymal Transition (EMT)

Epithelial cells are characterized by an apical-basal polarity, tight junctions, and expression of cell-cell adhesion molecules such as E-cadherin and form stationary structures such as epithelium. On the other hand, mesenchymal cells are multipolar, spindle-shaped, motile, and express markers such as N-cadherin, vimentin, fibronectin, Twist, and Snail and are generally found in stromal compartment. Epithelial to mesenchymal transition (EMT) is a reversible biological process whereby epithelial cells lose their polarity, cell-cell adhesion and become migratory. EMT is essential for embryogenesis and also occurs during wound healing, fibrosis and metastasis of cancers [60,61].

Epithelial cells are held together in sheets with many cell junction proteins such as E-cadherin, loss of which is widely observed in advanced cancers and a fundamental event in EMT. Several transcription factors which promote EMT include Snail, Slug, Zeb1, KLF8 that repress cell-cell junction proteins such as E-cadherin, claudins, desmosomes, and upregulate mesenchymal markers such as vimentin. Many signaling pathways are also implicated in EMT and include PI3K/Akt, TGFβ/ Smad4, Wnt/ β-catenin, and Hif signaling. A reverse process called mesenchymal to epithelial transition (MET) is believed to facilitate the establishment and colonization of metastases [60,61].

1.10 Estrogen and Estrogen Receptors in the Prostate

Estrogen is the primary female sex hormone; however, low level is found in males as well [62]. In female, estrogen is responsible for the development and maintenance of female sex organs and secondary sexual characteristics [63]. In males, various effects of estrogen have been described including sperm maturation and function, bone health, immune regulation, and neuronal organization. Estrogen is synthesized from testosterone by the enzyme aromatase [62]. Out of the three naturally occurring estrogens, 17β-estradiol (E2) is the most potent and abundant. In addition to androgen, estrogen is also required for normal growth and differentiation of the prostate in mice [64].

Estrogen has been implicated in prostate cancer development in mice model [65]. Chronic treatment of mice with pharmacological dose of estrogen led to squamous metaplasia, keratinization, and development of prostatic intraepithelial neoplasia (PIN) lesions [64]. Moreover, incidence of prostate cancer in aromatase knockout mice was significantly higher when treated with testosterone and E2 simultaneously than either hormone [66].

Estrogens work by binding to estrogen receptors which include estrogen receptor α (ERα), estrogen receptor β (ERβ), and membrane estrogen receptors such as G-protein coupled estrogen receptor 1 (GPER1). All three estrogen receptors are

expressed in the prostrate but ERα is expressed mainly in stroma, whereas ERβ is expressed in both basal and luminal epithelial cells [67-69].

ERα and ERβ are members of the nuclear receptor superfamily and function as ligand activated transcription factors [70]. Upon ligand binding, these receptors dimerize and directly bind to specific DNA sequences called estrogen response elements (EREs), or tether to other transcription factors and regulate gene expression [71]. The two nuclear receptors are product of two separate genes located on different chromosomes. ERα, also called NR3A1 (nuclear receptor subfamily 3, group A, member 1), is transcribed from *ESR1* gene located on chromosome 6q25.1. ERα was discovered in 1956 by Elwood V. Jensen and was not cloned until 1985. ERα encodes a protein which is 595 amino acid long and has a molecular weight of 66 kDa. There are three known splice variants of ERα designated ERαΔ3, ERα36, and ERα46 [72].

ESR2 gene is located on chromosome 14q23.2 and encodes for ERβ or NR3A2 (nuclear receptor subfamily 3, group A, member 2). ERβ was discovered and cloned from rat prostate in 1996 by Jan-Åke Gustafsson and his team at Karolinska Institute. ERβ protein is 530 amino acid in length and 59 kDa in molecular weight [73,74]. Genomic studies have revealed at least four splice variants of ERβ namely ERβ2 or ERβcx, ERβ3, ERβ4, and ERβ5. The ERβ splice variants have shortened C-terminal

sequences which significantly compromise their ability to bind ligands and regulate different repertoire of genes than wild type [75,76].

The two nuclear receptors ERα and ERβ are structurally similar and share 97%, 59%, and 16% similarity in their DBD, LBD, and NTD respectively [74,75]. While the two estrogen receptors bind 17β-estradiol with somewhat similar affinity, unique repertoire of ligands have been described which bind specifically to one receptor only. These receptors also have divergent function; ERα mainly promotes cell growth and proliferation, whereas ERβ inhibits proliferation, induces apoptosis and maintains differentiation. ERα is a well-known oncogene in many cancers where as ERβ is established as a tumor suppressor [77-79].

The two nuclear estrogen receptors have large difference in amino acid sequences of their ligand binding domain which may explain differential binding of some ligands and in turn different outcomes [74]. Several ERβ agonists have been described that selectively bind ERβ over ERα and may be useful in studying ERβ function. Some well-known selective ERβ agonists are 5α-androstane-3β,17β-diol (3β-Adiol), the phytoestrogens Apigenin, Daidzenin, Genistein, Liquiritgenin, and the synthetic agonists Ly3201, Ly5003007, Diarylpropionitrile (DPN), ERB-196 (WAY-2021960), and 8β-VE2 [80,81]. DHT metabolite 3β-Adiol has higher affinity for ERβ than ERα and has been proposed as an endogenous ERβ agonist in prostate [82]. Additionally,

genistein and other phytoestrogens demonstrate higher binding affinity towards ERβ than ERα [83].

The regulation of transcription by ERβ depends on its ability to bind to genomic DNA sequences directly or indirectly and recruit transcriptional activators or repressors. Well known ERβ interacting partners are AP-1, SP-1, NFkB, cofactors steroid receptor coactivator (SRC-1), proline-, glutamic acid-, leucine-rich protein (PELP-1), CREB binding protein (CBP), p300, nuclear receptor coactivator 3 (NCOA3) as well as corepressor SMRT [84-86].

Genomic and transcriptomic studies have shed light on ERβ regulated genes and their mechanism of regulation. Although ERβ binds to estrogen response elements (EREs) with high affinity, it binds to non-ERE sites as well by tethering to transcription factors such as activating protein-1 (AP-1), stimulating protein-1 (SP-1), and nuclear factor kappa B (NFkB) among others [84].

Estrogen signaling is manifested in two distinct response times; rapid response occurring in minutes after treatment with estrogen or slow response evident in hours to days. The slower response is due to genomic effect of estrogen mediated through nuclear estrogen receptors leading to transcriptional regulation of target genes. However, rapid response is due to non-genomic effect mediated by membrane estrogen receptors such as GPER1 also known as G-protein coupled receptor 30 (GPR30) [87]. GPER1 mediates most of the non-genomic estrogen-induced rapid

responses and is preferentially localized to the plasma membrane and endoplasmic reticulum. Estrogen binding to GPER1 results in intracellular calcium mobilization and synthesis of phosphatidylinositol (3,4,5) triphosphate [88]. Both *in vitro* and *in vivo* studies have shown that GPER1 can promote cell proliferation in normal and malignant cells by activating PI3K/AKT signaling [89].

1.11 Role of ERβ in Prostate Cancer

ERβ is expressed in both basal and luminal cells of the prostate [90, 91]. Genomic deletion of ERβ in mouse prostate led to epithelial hyperplasia, as well as increase in the number of intermediate luminal cells . Additionally, older ERβ knock-out mice developed prostatic intraepithelial neoplasia (PIN) lesions. This observation suggested that ERβ regulates both differentiation of prostatic stem cells and proliferation of luminal epithelial cells [91]. Treatment with prostate specific ERβ ligand 3β-Adiol inhibited cell proliferation in wild-type but not in ERβ knock-out mice [92]. Furthermore, treatment of TRAMP (transgenic adenocarcinoma of the mouse prostate) mice with genistein, a dietary phytoestrogen and potent ERβ agonist, decreased the incidence of prostate cancer in those mice [93]. Transcriptional profiling of ERβ knock-out mice ventral prostate revealed a role of ERβ in regulation of AR activity. ERβ increased the expression of the AR co-repressor DACH1/2 and decreased the AR driver RORc [94].

Studies of ERβ function in cancer cell lines have demonstrated an anti-proliferative and pro-apoptotic function. Expression of ERβ in PC3 and 22Rv1 cells was found to significantly decrease proliferation as measured using MTT assay, BrdU incorporation, and cell counting. The anti-proliferative effect of ERβ was due to down regulation of oncogenic factors c-Myc, p45 Skp2, and cyclin E and up regulation of p21 and p27 KIP protein. In mouse xenograft studies, ERβ expressing PC3 cells formed smaller tumors as compared to controls [95].

Treatment of LNCaP cells with 3β-Adiol induced apoptosis whereas overexpression of ERβ caused G1 cell-cycle arrest [96,]. Treatment of prostate cancer cell lines PC3, LNCaP, and DU145 with DNA demethylating agent 5-AZAC and the HDAC inhibitor TSA increased the expression of ERβ followed by increase in caspase activity and apoptosis. Adenoviral mediated overexpression of ERβ in DU145 cells resulted in upregulation of proapoptotic factor, Bax, followed by increased PARP cleavage, increased caspase3 activity and apoptosis [97].

ERβ agonist 8β-VE2 induced apoptosis in prostate basal/ stem cells and caused cystic atrophy of prostate tissue. The treatment caused depletion of p63 positive basal cells at the site of cystic atrophy implying that basal cells are required for prostate regeneration post-ADT. After two rounds of treatment with 8β-VE2 and recovery of castrated wild-type mice, regeneration capability of prostate was significantly inhibited. This finding is relevant for castration resistant prostate cancer

[98]. It was suggested that androgen deprivation therapy fails after initial response because basal epithelial cells of the prostate harbor stem/ progenitor cells which are AR negative. Upon androgen deprivation, AR dependent luminal cells undergo apoptosis whereas stem/ progenitor cells regenerate [33].

In another study, activation of ERβ was found to induce apoptosis in both luminal and basal prostate cells via an extrinsic pathway that involves caspase 8. This effect was observed in both wild-type and aromatase knock-out mice and was suggested to involve tumor necrosis factor alpha (TNFα) as it was not observed in TNFα knock-out mice. Upon activation by ERβ, TNFα activates caspase 8 which further activates caspase 3 and leads to apoptosis [99].

Our lab previously published that ERβ induced apoptosis in prostate cancer cell lines PC3, LNCaP, and 22RV1 upon treatment with ERβ specific ligands 3β-Adiol, 8β-VE2, and DPN. It was shown that ERβ induced apoptosis in these cell lines by transcriptionally upregulating FOXO3a which in turn upregulated PUMA, a direct target of FOXO3a. PUMA is a potent pro-apoptotic factor which can induce apoptosis in p53-dependent or -independent manner. The regulation of FOXO3a was also observed in mice models where ERβ knock-out mice lacked FOXO3a expression in prostate epithelium. The expression of FOXO3a also correlated with expression of ERβ in prostate cancer. In higher Gleason grade prostate cancer, which shows a progressive decrease in ERβ expression, FOXO3a expression was lost [100].

Hypoxic signaling is a survival pathway extensively exploited by tumor cells to survive and proliferate in a tumor microenvironment which is generally hypoxic due to imbalance between angiogenesis and rapidly proliferating cells [101]. Hypoxia inducible factor (Hif) signaling involves activation or upregulation of several survival pathways such as energy metabolism, mTOR, and angiogenesis. In normoxia, prolyl hydroxylases (PHDs) mediate hydroxylation of Hif-1a which is then targeted by von Hippel-Lindau (VHL), a component of E3 ubiquitin-ligase complex, and is rapidly degraded [102]. ERβ regulates Hlf-1α stability by transcriptionally upregulating PHD2, an isoform of PHD which causes degradation of Hif-1α [103].

1.12 Role of ERβ in Metastasis and EMT

Various studies have demonstrated an anti-metastatic potential of ERβ using *in vitro* and *in vivo* experiments. Adenoviral-mediated expression of ERβ in DU145 cells showed a strong decrease in invasiveness [97]. Another study used stable ERβ expressing DU145 cells to make xenograft in immunocompromised mice and showed that treatment with 3β-Adiol blocked metastasis of prostate cancer cells which was due to upregulation of E-cadherin [104]. Our lab previously reported the down regulation of bone metastasis factors Runx2 and Dickkopf homolog 1 and the EMT factors Slug and β-Catenin in PC3 and 22Rv1 cells stably expressing ERβ [95].

Mak et al., 2010 reported inhibition of EMT in prostate cancer cells after treatment with 3β-Adiol in both AR positive and negative cells. The observation was correlated with high Gleason grade prostate tumors that show decreased expression of ERβ, loss of E-cadherin and gain of N-cadherin / vimentin expression. They suggested that EMT promoting pathways TGFβ and hypoxia reduces ERβ expression and promotes migration and invasion [103].

Grubisha et al., 2012 reported that 3β-Adiol treatment prevented migration and motility of DU145 cells by upregulating E-cadherin. They further showed that anti-migratory effect of ERβ was abrogated due to reactive oxygen species (ROS) produced by TGFβ signaling [105].

1.13 ERβ as a Potential Drug Target for Prevention and Treatment of Prostate Cancer

Epidemiological studies have suggested the low incidence of prostate cancer in Asian men to be due to high consumption of soy-based food. Soy food as well as some other vegetable products contain phytoestrogens such as daidzein, genistein, biochanin A, and coumestrol [5]. Phytoestrogens work by binding to estrogen receptors. Genistein, which is highly rich in soy foods, has more than 20-fold higher affinity for ERβ than ERα. Activation of ERβ by genistein leads to decrease in AR and increase in p21, which has been proposed as a mechanism by which soy foods may prevent prostate cancer [106].

1.14 Phosphoinositides

Phosphoinositides or inositides (PIP) are phosphorylated forms of a lipid called phosphatidylinositol (PI) and constitute major intracellular signaling molecules involved in cell signaling and membrane trafficking. Phosphatidylinositol is made up of a glycerol backbone, two non-polar fatty-acid chains, and a polar head of phosphate group substituted with inositol. Because of their amphiphilic nature phosphoinositides are easily targeted to inner side of the plasma membrane and on surfaces of cellular organelles. Kinases phosphorylate the 3, 4, and 5 hydroxyl groups of the inositol ring in different combinations producing seven different phosphoinositides as listed below [107-109].

Phosphatidylinositol Monophosphates

Phosphatidylinositol 3-phosphate (PI(3)P)

Phosphatidylinositol 4-phosphate (PI(4)P)

Phosphatidylinositol 5-phosphate (PI(5)P)

Phosphatidylinositol Bisphosphates

Phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2)

Phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2)

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2)

Phosphatidylinositol Trisphosphates

Phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3)

1.15 Phosphatidylinositol Kinases

Phosphatidylinositol are phosphorylated by specific kinases in response to various stimuli. Generally, phosphorylation of phosphatidylinositol is an activation signal which is transmitted into the interior of the cell through many proteins that recognize and bind PIPs and subsequently activated. There are more than 100 phosphatidylinositol kinases in human genome. These kinases work in sequential manner to produce phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) [110, 111]. Termination of signals conveyed by activated PIPs are accomplished by phosphatases which remove phosphate groups. Like kinases there are many phosphatases which work in sequence to convert PI(3,4,5)P3 to phosphoinositides. Of the many kinases that phosphorylate phosphatidylinositol, phosphoinositide 3-kinase (PI3K) is the most important and widely studied which phosphorylate only the 3-position of inositol ring. Members of the PI3K are shown to be involved in cell growth and proliferation, survival, motility, differentiation, and intracellular trafficking. PI3Ks constitute a family of enzymes grouped into 4 classes: Class I, II, III, and IV, of which class I is widely studied [112-114].

Only class I PI3Ks are involved in lipid phosphorylation in response to growth stimuli. Class I PI3Ks mainly phosphorylate phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) to phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) [112]. Additionally, class I PI3Ks may also convert phosphatidylinositol (PI) to phosphatidylinositol 3-phosphate (PI(3)P) and phosphatidylinositol 4-phosphate

(PI(4)P) to phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2). Class II PI3Ks catalyze the conversion of phosphatidylinositol (PI) to phosphatidylinositol 3-phosphate (PI(3)P) and phosphatidylinositol 4-phosphate (PI(4)P) to phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2). Class III only catalyzes the production of phosphatidylinositol 3-phosphate (PI(3)P) from phosphatidylinositol (PI). Numerous proteins have been found to bind phosphoinositides through some conserved motifs. Well described motifs are PX, PH, and FYVE [113, 114].

1.16 Protein Kinase B or Akt

Protein kinase B (PKB) or Akt is a serine/threonine-specific protein kinase that is involved in many vital cellular processes such as glucose metabolism, cell survival, proliferation, and migration. There are three isoforms of Akt: Akt1, Akt2, and Akt3 which are ubiquitously expressed. Akt has been found to phosphorylate as many as 100 different proteins affecting a large number of processes in the cell. Akt1 has been found to inhibit apoptosis and promote survival as well as induce protein synthesis. Akt2 is important for insulin signaling and is involved in glucose transport [115, 116].

PI3-kinases are activated by G-protein-coupled receptors (GPCR) and receptor tyrosine kinases (RTK) upon stimulation with growth signals. Activated PI3-kinases in turn phosphorylates phosphoinositides to produce PI(3,4)P2 and PI(3,4,5)P3. Akt binds phosphoinositides PI(3,4,5)P3 and PI(3,4)P2 with high affinity through the PH domain (pleckstrin homology domain). Binding of Akt to PIP3 brings it in the proximity

of PDPK1 (phosphoinositide dependent kinase 1) which phosphorylates Akt at threonine 308. Full activation of Akt occurs when it is phosphorylated at serine 473 by mTORC2. Activated Akt then phosphorylates myriad of substrates such as FOXO, GSK3β, and mTORC. Activity of PI3K/ Akt is regulated by many phosphatases the most important and widely studied is PTEN [117,118].

Overactivation of Akt has been seen in many cancers where it is implicated in tumor cell survival, proliferation, and invasiveness. Akt inhibits apoptosis and promotes survival mediated by growth factor signaling. It phosphorylates BAD on Ser136 which is a pro-apoptotic factor. Phosphorylation of BAD causes its dissociation from Bcl-2/Bcl-X complex which no longer can induce apoptosis. Akt also promotes transcription of pro-survival genes via regulating the activity of IkB kinase which in turn activates NFkB transcription factor. Hyperactivation of Akt has been shown to overcome cell cycle arrest in G1 and G2 phases caused by various factors [119,120]. Akt2 is also required for glucose up-take by cells and glycogen synthesis. Insulin-induced translocation of glucose transporter 4 (GLUT4) to the plasma membrane is mediated by activated Akt. It also inhibits glycogen synthase kinase (GSK) by phosphorylation which in turn leads to activation of glycogen synthase [121].

1.17 PI3K/ Akt / PTEN Signaling Pathway in Prostate Cancer

PI3K/Akt/PTEN signaling is one of the most commonly altered pathways in primary and metastatic prostate cancer. The alterations in this pathway generally involves

one or more of the following: deletion of PTEN, activating mutation of PIK3CA and/or reduced expression of PHLPP gene. Recent reports have described frequent decrease in INPP4B expression in prostate cancers and is regarded as a tumor suppressor [122, 123].

Phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is frequently activated in prostate cancer due to the loss of PTEN which is a negative regulator of PI(3,4,5)P3 [124]. INPP4B, another negative regulator of PI3K is also frequently reduced in advanced cancer. Overactivation of PI3K/Akt pathway plays a critical role in prostate cancer initiation and progression. PTEN terminates PI3K/AKt signaling by dephosphorylating PI(3,4,5)P3 on the 3-position phosphate inositol ring. It has been estimated that one or more components of the PI3K/Akt/PTEN signaling pathway are altered in 42% of localized and 100% of metastatic prostate carcinoma [125]. These cancers also have frequent loss of expression of PHLPP, a protein phosphatase that dephosphorylates the hydrophobic motif on Akt as well as other AGC kinases [126].
Role of PTEN in prostate cancer development and progression has been genetically confirmed using mice models with different levels of PTEN expression. Mice with progressive loss of PTEN results in progression from prostate hyperplasia to high grade prostate intraepithelial neoplasia (PIN) to finally prostate adenocarcinoma [127,128]. In various murine models, prostate cancer progression is a consequence of increased Akt and mTORC1 activity due to PTEN loss. But loss of only one allele

of PTEN is not sufficient for the development of prostate cancer from PIN lesions indicating that other alterations are necessary [129,130].

1.18 Inositol Polyphosphate 4-Phosphatases

Inositol polyphosphate 4-phasphatases (INPP4) are enzymes belonging to the PI3K signaling pathway which are involved in the metabolism of phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), inositol 3,4-bisphosphate (Ins(3,4)P2) and inositol 1,3,4-triphosphate (Ins(3,4,5)P3) [131]. Specifically, it catalyzes the hydrolysis of the 4-phosphate of the inositol ring producing phosphatidylinositol 3-phosphate (PI(3)P), inositol 1,3-bisphosphate and inositol 1-phosphate. Activity of INPP4 for (PI(3,4)P2) is 900-fold and 120-fold higher than for IP3 and IP2, respectively [132,133]. This enzyme neither requires Mg2+ for its activity nor is inhibited by lithium ions but is inactivated by calpain-mediated proteolysis [134].

Evidence for the presence of a 4-phosphatase enzyme came from the study of the metabolism of Ins(3,4,5)P3 in calf brain lysate and the enzyme was subsequently purified [132]. The enzyme was eventually cloned from rat and human brain samples [135]. Subsequent investigations revealed presence of another enzyme with the same function. So, the original enzyme was designated as INPP4 type I (INPP4A) and the later one as type II (INPP4B). The two enzymes share only 37% of sequences but both have a conserved motif CKSAKDRT that contains a consensus active site Cys-Xaa5-found in many Mg2+ independent phosphatases [136].

1.19 Tumor Suppressive Roles of INPP4B in Cancers

Study into biological role of INPP4B unraveled its role in PKB/Akt inhibition and tumor suppression. Barnache et al., 2006 first described the silencing of INPP4B gene in malignant proerythroblast. These cells showed enhanced Akt activity which could be reduced by stably expressing INPP4B [137]. Deletion of a genomic region, comprising 6 genes including INPP4B, was described to occur frequently in basal-like breast cancers and breast cancer cell lines [138]. Additionally, an RNAi screen to detect genes restraining transformation of human mammary epithelial cells (HMEC) identified INPP4B as a candidate tumor suppressor [139]. Similarly, knock-down of INPP4B expression in mammary epithelial cell line HMEC resulted in anchorage-independent growth, increased cell migration, and enhanced Akt activation. This same study also showed that stable expression of INPP4B in SUM149 mouse xenografts suppressed tumor growth [140].

INPP4B was found to be expressed only in non-proliferative ERα positive cells in normal breast epithelium and ERα positive breast cancer cell lines. shRNA mediated knock-down of INPP4B in MCF-7 cells resulted in increased Akt activation, cell proliferation and xenograft tumor growth. Conversely stable expression of INPP4B in ERα negative MDA-MB-231 cells resulted in reduced Akt activation, blocked cell cycle in G1 phase and inhibited anchorage-independent growth [141]. Furthermore, INPP4B expression was found to be lost most commonly in basal-like breast cancers as well as in some primary breast cancers with high clinical grade associated with

loss of hormone receptors. The study also reported frequent loss of INPP4B in PTEN negative tumors. These results indicate that INPP4B negatively regulates normal and malignant breast epithelial cell proliferation [141].

A tumor suppressive role of INPP4B in ovarian teratomas was suggested based upon observation that insertion of transgene Tgkd in 3' of INPP4B resulted in decreased expression of this phosphatase leading to increase in PI3K/Akt activity. It was hypothesized that fully mature oocytes that were unable to ovulate in Tgkd hemizygous mice were prone to developing into teratomas [142].

Decreased expression of INPP4B was found to correlate with melanoma progression and was suggested as a marker for disease progression and treatment outcome. In cell lines, ectopic expression of INPP4B led to decrease in Akt activity affecting proliferation, migration, and tumorigenicity of melanoma cells. An opposite effect was observed after depletion of endogenous INPP4B in melanoma cells [143].

In a study of 180 cases of non-small cell lung cancer, alterations in INPP4B was found to associate with squamous cell carcinoma. Nineteen percent cases had loss of copy number and 47% lacked expression of INPP4B whereas 67% had increased pAkt level [144].

In nasopharyngeal carcinoma (NPC), expression of INPP4B was found to be downregulated in 49% (32/65) of cases while it was consistently expressed in normal

nasopharyngeal epithelial cells. The down regulation of INPP4B in NPC was due to hypermethylation of 5'CpG island in its promoter. In five EBV-positive tumor cell lines established from nasopharyngeal carcinoma INPP4B was not expressed but treatment with the demethylation agent (5-aza-2'deoxycytidine) caused re-expression of INPP4B in NPC C666-1 cells leading to suppression of Akt activity [145].

In a *Pten* heterozygous mouse model of follicular-like thyroid cancer (FTC), complete or partial loss of INPP4B caused progression of benign thyroid adenoma into metastatic and lethal cancer. In thyroid cancer cell lines, INPP4B was enriched in the early endosomes where it selectively inhibited Akt2 activity suppressing tumor proliferation and anchorage independent growth. The authors proposed that PTEN and INPP4B co-operate to suppress tumorigenesis in thyroid [146, 147].

1.20 Role of INPP4B in Prostate Cancer

Tumor suppressive role of INPP4B in prostate cancer was first reported by Hodgson et al., 2011. INPP4B was shown to be direct AR target gene which was induced by androgen receptor in prostate cancer cells and reduced Akt activation. Additionally, knock-down of INPP4B in those cells led to increased Akt activation and cell proliferation. In VCaP cells that has wild-type PTEN, knock-down of INPP4B indeed caused increase in Akt activity. Micro-array comparison of normal and malignant prostate showed decreased expression of INPP4B in tumors which was associated with reduced time to biochemical recurrence. Also, in immunohistochemical staining,

while strong expression of INPP4B in adjacent normal tissue was observed, it was decreased or absent in infiltrating tumors. The authors argued that androgen ablation can compromise its efficacy through downregulation of INPP4B and activation of Akt [148].

Metastatic prostate cancer patients who are treated with androgen ablation therapy invariably relapse and develop castration resistant disease which is incurable. Transcriptomic analysis of 218 prostate cancer tumors revealed decrease in INPP4B expression in 50% of metastatic prostate cancer while it was reduced in <10% of non-invasive cases. Using androgen-responsive, INPP4B-positive human prostate cancer xenograft LTL-418 which was derived from high-grade prostate adenocarcinoma, it was shown that INPP4B expression significantly decreased after castration. It was suggested that androgen ablation therapy might contribute to cancer progression by reducing INPP4B expression and activating Akt [149].

Exogeneous expression of INPP4B in PC3 cells inhibited cell invasion in trans-well assays and when inoculated on chicken chorioallantoic membrane. Transcriptomic analysis of PC3 cells overexpressing INPP4B resulted in differential expression of genes associated with cell adhesion, ECM and the cytoskeleton. However, the study concluded that INPP4B may inhibit tumor invasion by inhibiting PKC-IL8-Cox2-BIRC5 axis, through dephosphorylating PI(4,5)P2 [150].

In a cohort of prostate cancer patient, loss of INPP4B expression was associated with tumor stage. Expression of INPP4B was highest (56%) in stage I and lowest (28.6%) in stage IV prostate cancer. INPP4B inhibited angiogenesis by reducing the secretion of VEGF. Specifically, conditioned medium from PC3 and DU145 cells expressing INPP4B inhibited the proliferation of human umbilical vein endothelial cells (HUVECs) compared with control cells. *In vivo*, xenograft of DU145 cells expressing INPP4B in nude mice led to significant decrease in microvascular density (MVD) of tumors compared with controls. Also, expression of INPP4B in PC3 and DU145 cells inhibited migration and invasion of these cells [151].

1.21 Tumor Promoting Role of INPP4B

Interestingly, INPP4B has been shown to promote malignancy in breast, pancreas, colon and acute myeloid leukemia (AML). In a subset of breast cancer, INPP4B mediated oncogenic effects of mutated PIK3CA by activating SGK3 which is activated by PI3P, a product of INPP4B [152].

An oncogenic role for INPP4B was reported in AML. AML patients with high levels of INPP4B had poor response to induction therapy and shorter overall survival. INPP4B overexpression in AML cell lines increased proliferation and colony formation, as well as enhanced chemotherapy resistance which was independent of Akt [153]. In a separate study using mass spectrometry-based protein profiling, INPP4B was found to be overexpressed in AML patients. The effects were enhanced chemoresistance,

early relapse, and poor overall survival. Ectopic expression of INPP4B in AML cell lines conferred chemo-resistance. Accordingly, expression of a catalytically inactive INPP4B (C842A) did not sensitize the chemotherapy resistant cells but siRNA mediated depletion of endogenous INPP4B did [154].

Chapter 2

Estrogen Receptor β regulates AKT activity through up-regulation of INPP4B and inhibits migration of prostate cancer cell line PC-3.

2.1 Introduction

Prostate cancer is the most common cancer in US men and second leading cause of cancer related death [1]. Despite decades of intensive research, the mechanism behind prostate cancer development and progression is not fully understood. While primary prostate cancer is indolent in nature, metastatic cancer is a fatal disease [10].

Hyperactive PI3K/Akt signaling pathway has been implicated in a host of activities including cell growth and survival, epithelial-mesenchymal transition (EMT), angiogenesis, metastasis, and development of chemoresistance in a wide range of tumors [156]. Loss of the tumor suppressor PTEN leads to overactive PI3K/AKT signaling and complete loss of PTEN is associated with increased metastasis and androgen independence in prostate cancer [157,158]. Loss of PTEN occurs at high frequency in prostate cancer where up to 60% is monoallelic loss in primary cancer and 100% in metastatic cancer [159]. Another phosphatase, inositol-polyphosphate 4-phosphatase type II (INPP4B) regulates AKT activity and can partially compensate for loss of PTEN [160]. Similar to the loss of PTEN, loss of INPP4B is also associated with increased aggressiveness and metastasis of prostate cancer [161].

PI3K generates lipid second messenger PI(3,4,5)P3 upon activation by growth factors and other mitogenic signals [162]. When Akt binds to PI(3,4,5)P3 and PI(3,4)P2 with its PH domain on plasma membrane it gets phosphorylated by PDPK1 on threonine 308 and this leads to partial activation [163]. Full activation of Akt occurs when Ser473 is phosphorylated by mTORC2 complex. Fully activated Akt can then phosphorylate many downstream proteins resulting in enhanced survival,

proliferation, metabolism, and migration [164]. PTEN, by dephosphorylating 3' position of PI(3,4,5) is the major negative regulator of this pathway. But in the absence of functional PTEN, there is accumulation of PI(3,4,5)P3 leading to increased Akt activity [165]. PI(3,4)P2 is generated from the activity of a group of phosphatases called SHIPs (SH2-containing inositol 5'-Phosphatases) which remove a phosphate group from 5' position of PI(3,4,5)P3 [166]. Loss of INPP4B expression leads to accumulation of PI(3,4)P2 further increasing Akt activity [167].

The estrogen receptor β (ERβ) was discovered and cloned in 1996 [73]. It has been shown to act as a tumor suppressor in various cancers [168-170]. Multiple tumor suppressive mechanisms of ERβ have been described. It inhibits cell proliferation by upregulating p21, p27 and/or downregulating p45 SKp2 [95]. ERβ was shown to induce apoptosis in prostate cancer cell lines through upregulation of PUMA and FOXO3a [100]. It was also shown to impede epithelial to mesenchymal transition (EMT) in TNBC cells by suppressing EGFR signaling [172] and decreased the invasiveness of TNBC breast cancer cells by inhibiting mutant p53 function [171].

Numerous studies have described anti-proliferative and pro-apoptotic effects of ERβ in various cancers, however, the role of ERβ in metastasis is not well studied. While there are some studies indicating that ERβ inhibits metastasis through regulating EMT in breast cancer [172], no such study has been done in prostate cancer. Expression of ERβ usually declines as tumors progress and becomes undetectable after Gleason grade III in prostate cancer [173]. The fact that ERβ is lost in advanced

and metastatic cancer poses a major limitation for studying its role in metastasis suppression.

Dysregulation of PI3K/Akt/PTEN signaling is one of the most common characteristics of prostate cancer [157,158]. The role of ERβ in regulating this pathway in prostate cancer is not known. Additionally, the potential of ERβ to inhibit metastasis in prostate cancer has not been studied. In this study using stably expressing PC3-ERβ1 cells, we investigated regulation of PI3K/Akt activity by ERβ. We also performed wound healing assay to investigate the ability of ERβ to inhibit migration of PC3 cells *in vitro*. We found that ERβ inhibited PC3 cell migration by inhibiting Akt activity through INPP4B upregulation. Further, using ChIP-seq assays, we found that ERβ binds to two enhancers in INPP4B gene. We propose that INPP4B is a direct ERβ target gene.

2.2 Results

2.2.1 Stable Expression of Estrogen Receptor β1 in prostate cancer cell lines PC3 and BPH1

Expression of ERβ1 is gradually reduced during progression of prostate cancer and cell lines established from metastatic disease express very low levels of ERβ1 [100,173]. In the present study, we used prostate cancer cell line PC3 and BPH1 to study the effect of ERβ1. PC3 cell line was established from bone metastasis of a stage IV (advanced) prostate cancer patient and are highly metastatic in xenograft animal models and exhibit high motility in cell culture [174]. As previously reported

and also observed from our RNA-seq data these cells do not express androgen receptor but express very low levels of ERβ1. When examined by electron microscopy, these cells show many characteristics common to neoplastic cells of epithelial origin including abnormal nuclei, and nucleoli, abnormal mitochondria, numerous microvilli, annulate lamellae, and lipoidal bodies. It was suggested that PC3 cells represent poorly differentiated adenocarcinoma and have high metastatic potential when compared to LNCaP cells [174]. BPH1 cells are immortalized benign prostate cell line of basal type. These cells express Δp63 as well as cytokeratin 5 and 14 [175].

We expressed ERb1 in PC3 and BPH1 cells using lentivirus mediated gene delivery and integration and selected for at least two weeks before doing any experiments. Stable expression of ERβ1 was detected using RT-qPCR, Western blotting and immunofluorescence. Our results show low level of ERβ expression in PC3 cells by immunoblot and some 70-80% cells being nuclear positive in immunofluorescence (Figure 2.1).

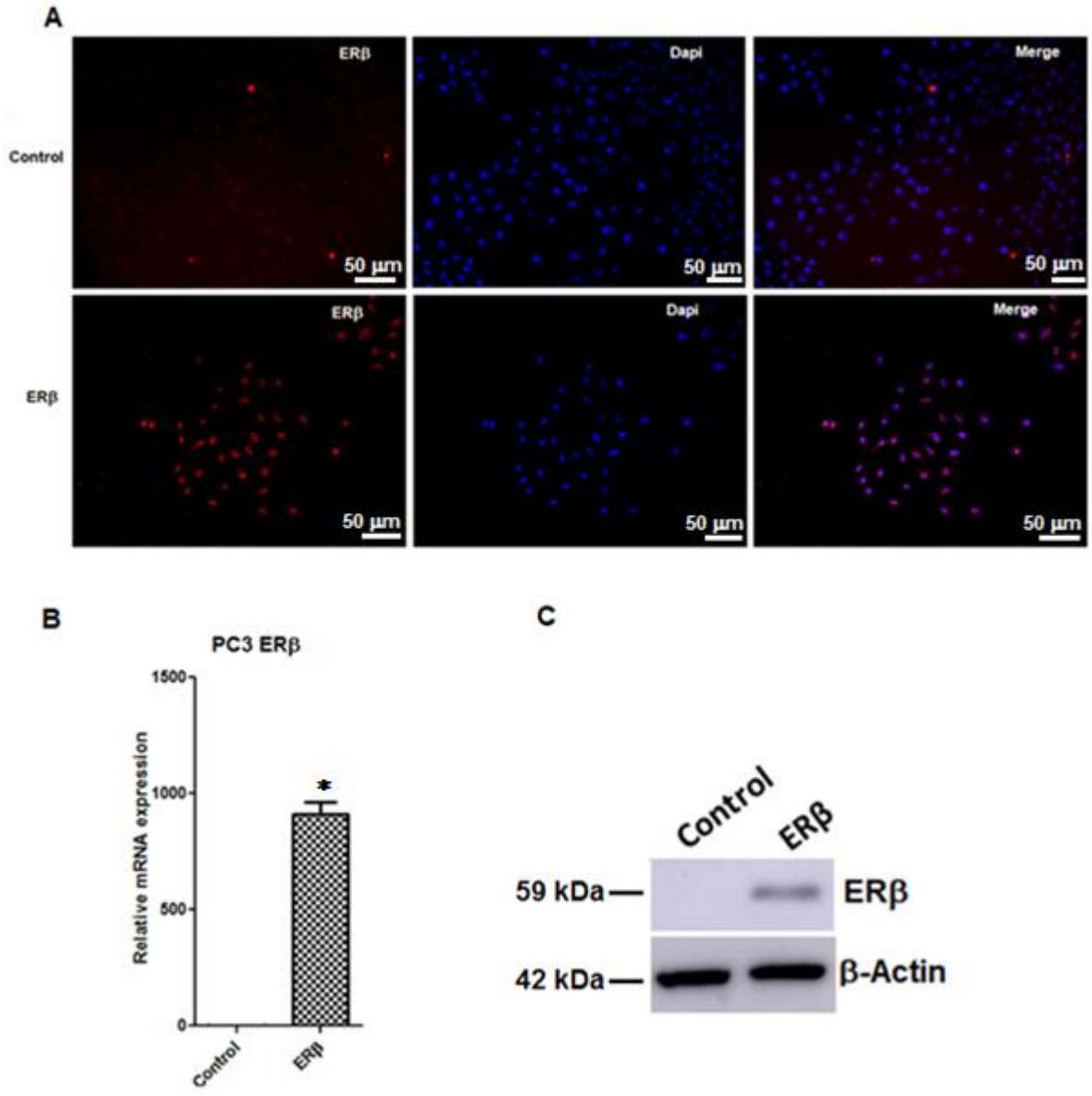


Figure 2.1 Expression of ERβ1 in PC3 cells. Early passage of PC3 cells were infected with the lentivirus Lenti6-TOPO-V5-D empty or containing cDNA for human ERβ1 at 2 M.O.I. and selected with 5 µg/ml blasticidin for two weeks. (A) Immunofluorescence staining of ERβ in PC3 cells showing mostly nuclear staining, top control PC3 cells, bottom ERβ PC3 cells. (B) ERβ1 mRNA expression in PC3-ERβ cells compared to control cells and presented as fold difference. Values represent mean ± SEM of three independent experiments; *p<0.05. (C) ERβ1 protein expression detected by western blot in PC3 control and PC3-ERβ1 cells .

2.2.2 Effect of ERβ1 expression on PC3 and BPH1 cells.

As previously reported [95], we also observed growth inhibition upon exogenous expression of ERβ1 in PC3 cells. We performed colony formation assay of stable cells in regular culture medium and found that colonies of ERβ1 expressing cells were smaller than those of control cells. In addition, ERβ1 expressing cells formed compact cobblestone colonies while control cells formed loose colonies (Figure 2.2).

2.2.3 ERβ1 upregulates INPP4B in PC3 and BPH1 cells

We performed RNA-seq of PC3 cells expressing ERβ1 or not (control) after treatment with vehicle control (DMSO) or ERβ specific ligand LY3201. Comparison of the transcriptome revealed 3.5-fold upregulation of INPP4B in ERβ1 expressing cells treated with Ly3201 (data not shown). We validated INPP4B upregulation in qPCR and western blot after treatment with ERb specific ligands Ly3201, DPN as well as estradiol (E2). Cells infected with empty vector which express antibiotic resistance gene but no ERβ1 were used as control in all experiments. While there was very little INPP4B expressed in control cells detectable upon longer exposure of WB membranes, it was readily detected after expression of ERβ1. The expression was also ligand dependent showing highest induction after ERβ specific ligand Ly3201 treatment. ERβ also upregulated INPP4B in BPH1 cells both at transcript and protein level (Figure 2.3).

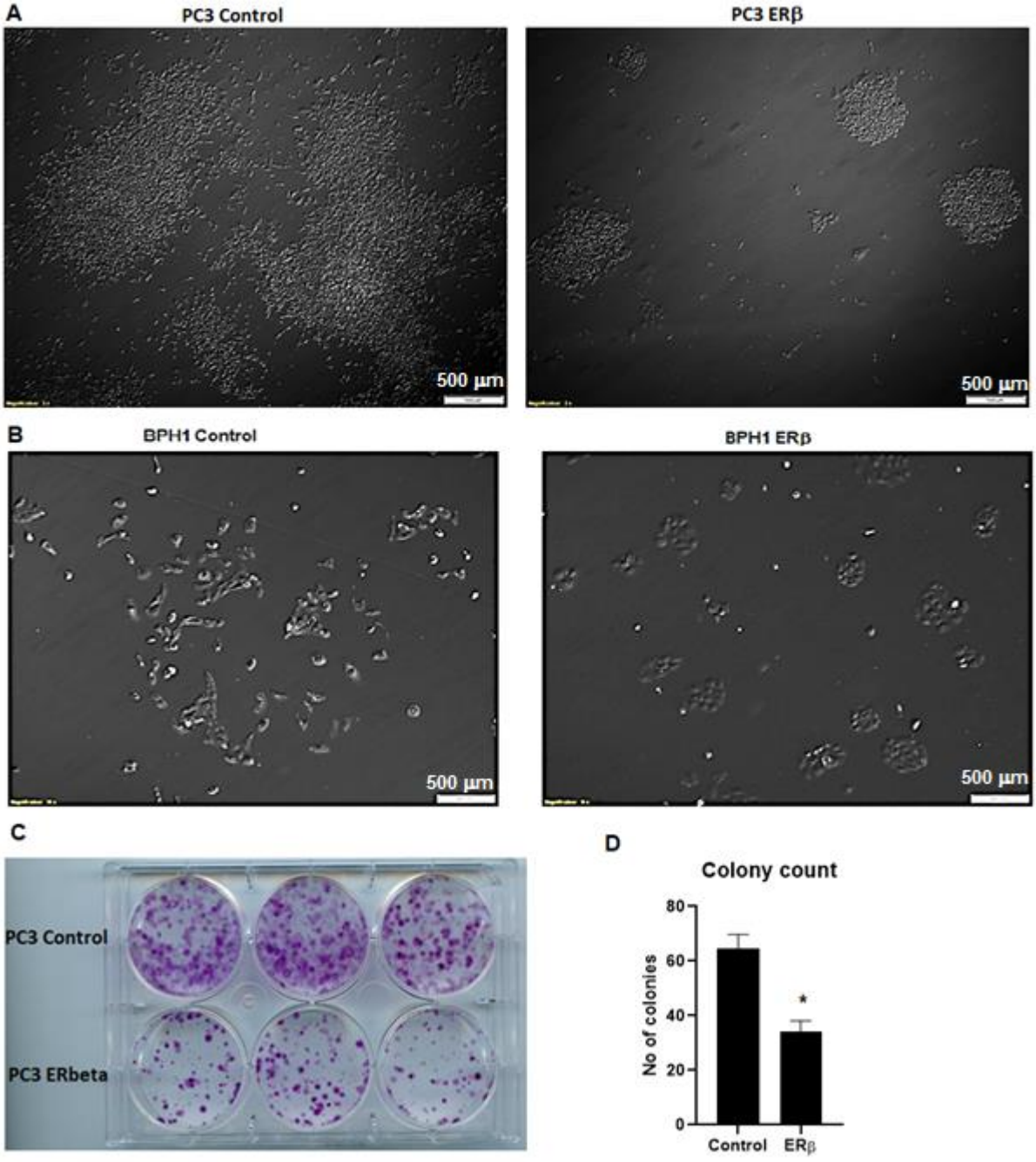
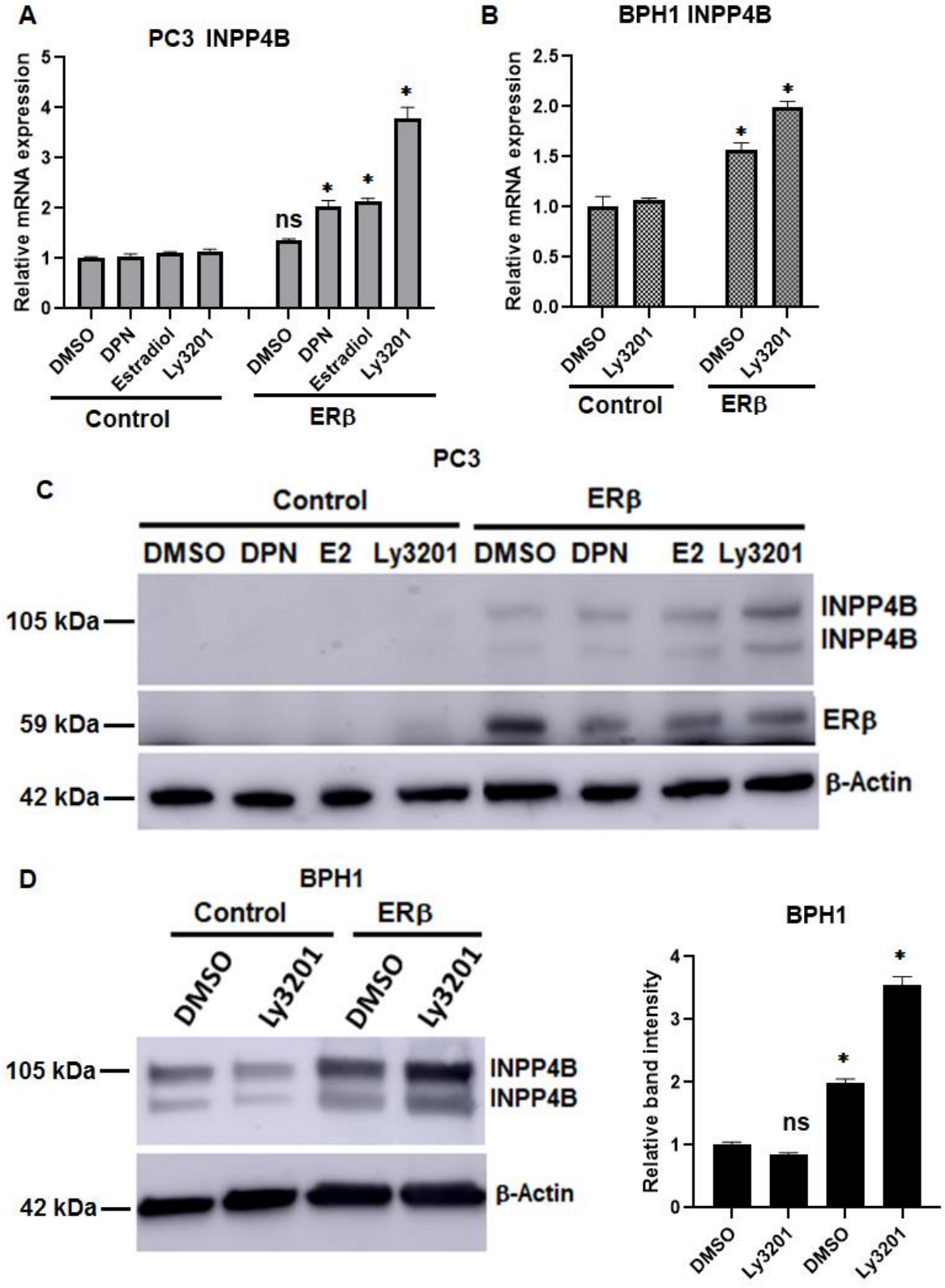


Figure 2.2 Effect of ERβ expression on colony formation. Stable PC3 and BPH1 cells were seeded into 6-well plate at 1000 cells /well and grown for 10 days in 10% FBS RPMI-1640. (A) colonies formed by PC3 control (left) and ERβ (right) cells. (B) colonies formed by BPH1 control (left) and ERβ (right) cells. (C) Crystal violet stained colonies of PC3 control (top) and ERβ (bottom) cells. (D) Quantitation of colonies from (C), values represent mean ± SEM of three independent experiments (*$p<0.05$).

Figure 2.3 ERβ1 induces expression of INPP4B in PC3 and BPH1 cells. Stable PC3 and BPH1 cells were grown to 60% confluency and treated with ligands in 10% DCC-FBS for 24 hrs, RNA and protein extracted and analyzed for expression of INPP4B. (A) Expression of INPP4B mRNA in PC3 control and ERβ1 cells. (B) Expression of INPP4B mRNA in BPH1 control and ERβ1 cells. Values presented as fold difference compared with control DMSO and represent mean ± SEM of three independent experiments (*$p < 0.05$). (C) Expression of INPP4B protein in PC3 control and ERβ1 cells. (D) Western blot of INPP4B protein in BPH1 control and ERβ1 cells (left), quantification of band intensity (right).


A
PC3 INPP4B
Relative mRNA expression
ns
DMSO
DPN
Estradiol
Ly3201
Control
ERβ
B
BPH1 INPP4B
Relative mRNA expression
C
PC3
Control
ERβ
DMSO DPN E2 Ly3201 DMSO DPN E2 Ly3201
105 kDa
59 kDa
42 kDa
INPP4B
INPP4B
ERβ
β-Actin
D
BPH1
Control
ERβ
DMSO
Ly3201
105 kDa
42 kDa
INPP4B
INPP4B
β-Actin
BPH1
Relative band intensity
ns

2.2.4 ERβ inhibits Akt activity in PC3 cells

Since INPP4B is known to dephosphorylate PI(3,4)P2 producing PI(3)P and inhibit Akt activity [140,176], we hypothesized that ERβ inhibits Akt activity through INPP4B. ERβ1 mediated induction of INPP4B significantly reduced Akt phosphorylation on Ser473 in PC3 cells (Figure 2.4). On the other-hand, Akt phosphorylation on T308 was not detectable in either control or ERβ expressing cells. We also observed that the level of pGSK3β, a substrate of Akt [177] used to measure its activity in cells, decreased along with pAkt Ser473. Although ERβ upregulated INPP4B in BPH1 cells in a ligand dependent manner, we could not detect pAkt Ser 473 or pAkt Thr308 in these cells.

2.2.5 ERβ regulated Akt activity is dependent on INPP4B upregulation

To confirm that inhibition of Akt activity by ERβ is mediated through INPP4B, we depleted INPP4B expression in ERβ expressing PC3 cells using siRNA and found that the level of pAkt Ser473 increased while total Akt did not change (Figure 2.5). The level of pGSK3β did not follow the level of pAkt Ser 473 (data not shown). We do not know any explanation for this anomalous observation.

Figure 2.4 ERβ inhibits Akt activity in PC3 cells. Stable PC3 cells were grown to 60-70% confluency, treated in 10% DCC-FBS for 24 hrs with DMSO or Ly3201 and analyzed for indicated proteins. (A) Representative western blot for INPP4B, pAkt S473, Total Akt, ERβ, pGSK3β, β-Actin, and GAPDH. (B) Band intensity for INPP4B, pAktS473 and pGSK3β. GAPDH was used as normalizing control. Values presented as fold difference compared with control DMSO and representing mean ± SEM of three independent experiments (*p< 0.05).

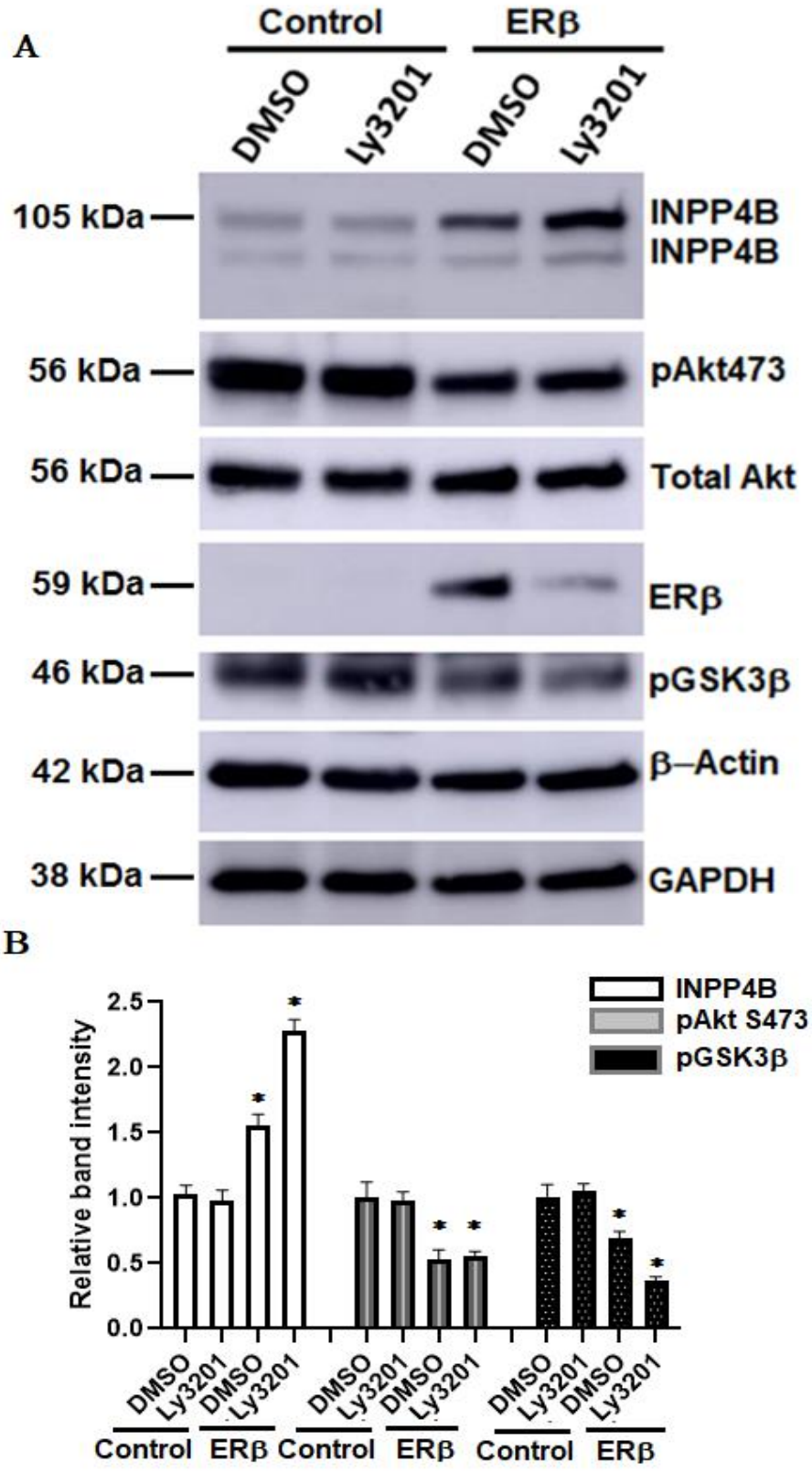
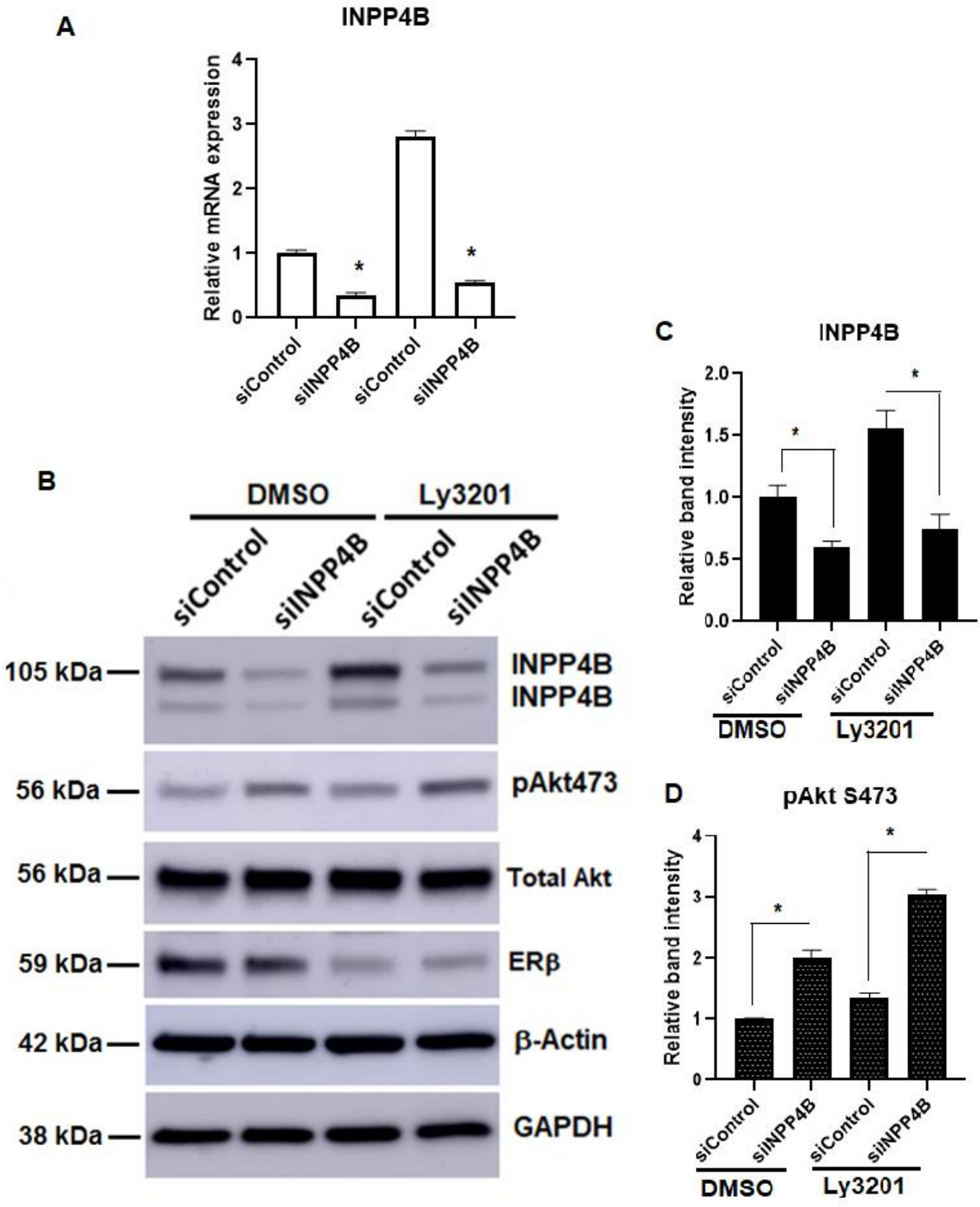

A
Control
ERβ
DMSO
Ly3201
DMSO
Ly3201
105 kDa
INPP4B
INPP4B
56 kDa
pAkt473
56 kDa
Total Akt
59 kDa
ERβ
46 kDa
pGSK3β
42 kDa
β–Actin
38 kDa
GAPDH
B
INPP4B
pAkt S473
pGSK3β
Relative band intensity
2.5
2.0
1.5
1.0
0.5
0.0
DMSO
Ly3201
Control
ERβ
Control
ERβ
Control
ERβ

Figure 2.5 Knock-down of INPP4B increases Akt activity in PC3 ERβ cells. PC3 ERβ cells were transfected with siRNA for INPP4B or negative control for 48hrs and treated with DMSO or Ly3201 in 10% DCC-FBS for 24 hrs. (A) Relative mRNA expression of INPP4B in siControl and siINPP4B PC3 ERβ cells. (B) Protein expression of INPP4B, pAkt S473, Total Akt, ERβ, β-Actin, and GAPDH in siControl and siINPP4B PC3 ERβ cells. (C) and (D) Band intensities of INPP4B and pAkt S473 of siControl and siINPP4B PC3 ERβ cells. Values presented as fold difference compared with control DMSO and representing mean ± SEM of three independent experiments (*$p < 0.05$).

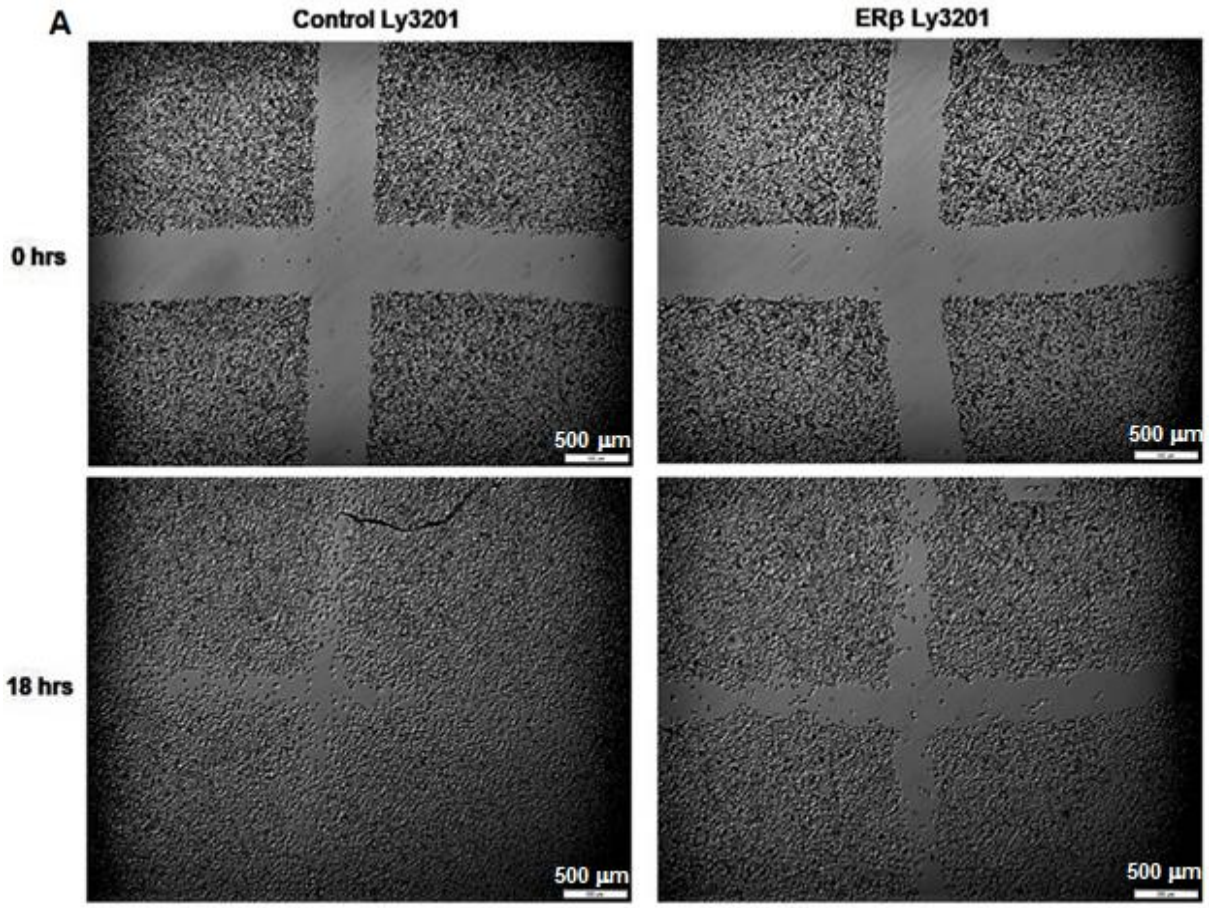

A
INPP4B
Relative mRNA expression
siControl
siINPP4B
siControl
siINPP4B
B
DMSO
Ly3201
siControl
siINPP4B
siControl
siINPP4B
105 kDa
INPP4B
INPP4B
56 kDa
pAkt473
56 kDa
Total Akt
59 kDa
ERβ
42 kDa
β-Actin
38 kDa
GAPDH
C
INPP4B
Relative band intensity
siControl
siINPP4B
siControl
siINPP4B
DMSO
Ly3201
D
pAkt S473
Relative band intensity
siControl
siINPP4B
siControl
siINPP4B
DMSO
Ly3201

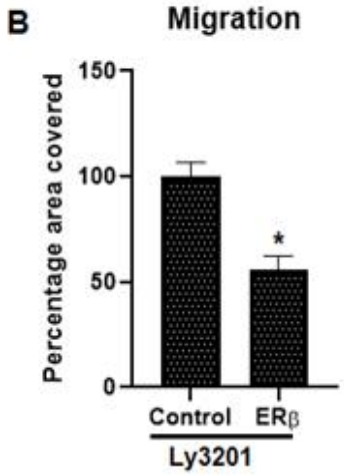
2.2.6 ERβ1 inhibits migration of PC3 cells

Wound healing assays are a commonly used technique to measure collective cell movement on a two-dimensional substrate [178]. A wound is made on a confluent sheet of monolayer cells, washed of the floating cells, image of the wound captured and incubated in desired condition for a certain period to allow migration of cells into the empty area. At the end, image is taken again and the gap in the wound is compared with control. Wound healing assays are frequently used because they are easy to perform, inexpensive, and fast. If at least 10-cell wide scratch is made and measured within cell doubling time, effect of cell proliferation is minimized [189].

We performed standard wound healing assay to measure the effect of ERβ expression on PC3 cell migration. Cells were treated with Ly3201 for 18hrs in phenol red-free RPMI supplemented with 10% DCC-FBS. Images of the wound were taken before and after incubation and analyzed with Image J software. We found that ERβ1 expressing cells migrated 45% slower than control cells (Figure 2.6).

Figure 2.6 ERβ1 inhibits migration of PC3 cells. (A)PC3 control and ERβ1stable cells were grown to confluency in 6-well plate and a wound was made with a pipette tip. Cells were incubated with Ly3201 for 18 hrs in 10% DCC-FBS. Images were taken initially (0 hrs) and after the end of incubation (18 hrs). (B) Quantification of cell migration; empty spaces in all images were delineated and measured with Image J software. Total area covered in control cells at the end of incubation (18 hrs) was set as 100%. Empty area from initial (0 hrs) was used for normalization.

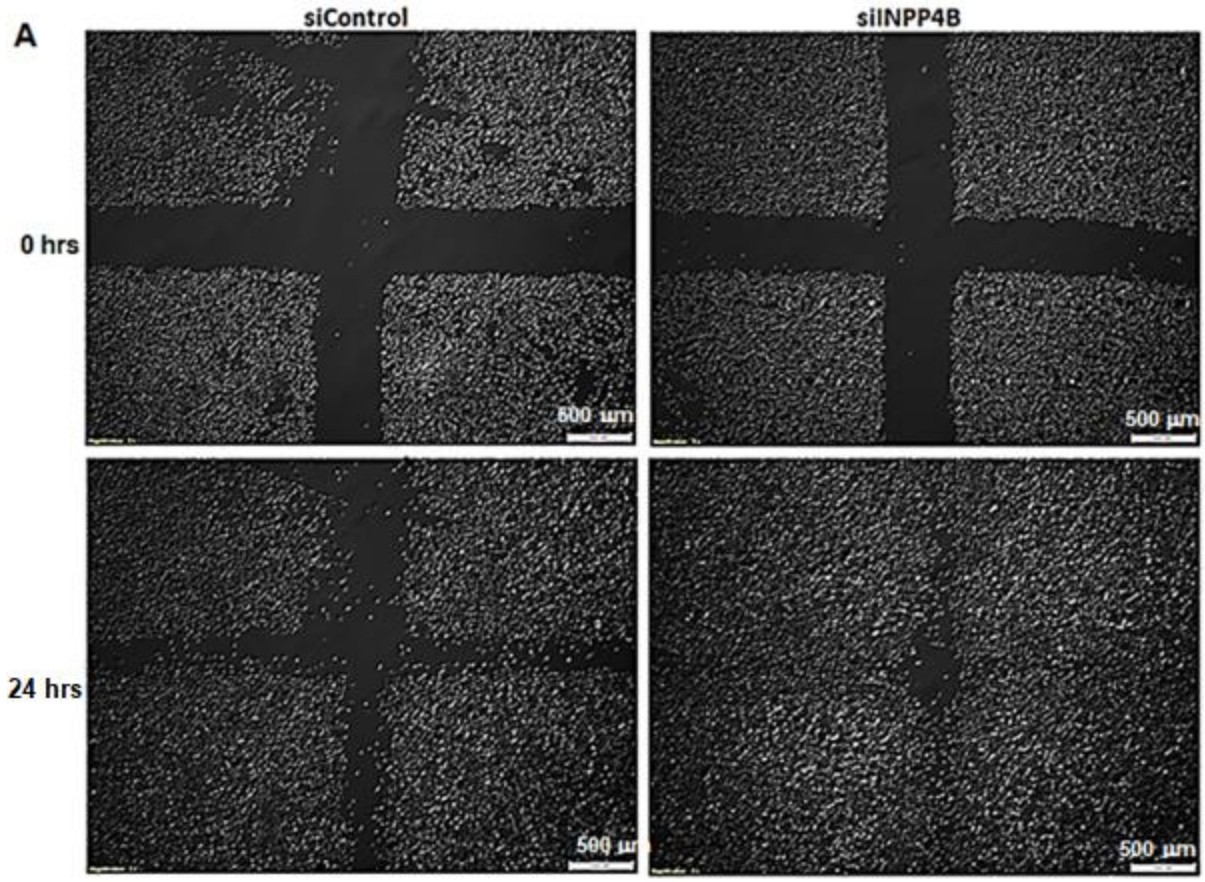

A
Control Ly3201
ERβ Ly3201
0 hrs
18 hrs
500 µm
500 µm
500 µm
500 µm

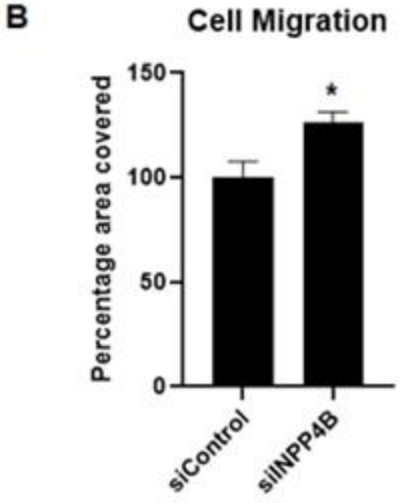

B
Migration
Percentage area covered
150
100
50
0
*
Control
ERβ
Ly3201

2.2.7 Depletion of INPP4B abrogates effect of ERβ1 on migration.

PI3K/Akt activity is implicated in cell survival, proliferation, and migration. Hyperactive Akt activity is strongly associated with EMT and tumor metastasis [119]. We tested whether ERβ1 inhibited PC3 cell migration is regulated through INPP4B. We observed that PC3 ERβ1 cells migrated faster in wound healing assay when INPP4B level was reduced using siRNA (Figure 2.7). The effect was modest but statistically significant. We believe that partial reversal of migration is due to presence of still significant amount of INPP4B protein in siRNA transfected PC3 ERβ1 cells.

Figure 2.7 ERβ1 inhibited migration is dependent on level of INPP4B. (A) PC3 ERβ1stable cells were grown to 50-60% confluency in 6-well plate and transfected with siRNA for negative control (siControl) or INPP4B. After 48 hrs a wound was made with a pipette tip. Cells were incubated with Ly3201 for 24 hrs in 10% DCC-FBS. Images were taken initially (0 hrs) and after the end of incubation (18 hrs). (B) Quantification of cell migration; empty spaces in all images were delineated and measured with Image J software. Total area covered in siControl cells at the end of incubation (18 hrs) was set as 100%. Empty area from initial (0 hrs) was used for normalization.


A
siControl
siINPP4B
0 hrs
24 hrs
500 µm
500 µm
500 µm
500 µm


B
Cell Migration
Percentage area covered
150
100
50
0
*
siControl
siINPP4B

2.2.8 INPPP4B is a direct target gene of ERβ1

To further elucidate the mechanism of INPP4B upregulation by ERβ1, we performed global ChIP-sequencing (data not shown). We found that ERβ1 bound to two intronic sequences in INPP4B gene which we named Enhancer 1 (Enh1) and Enhancer 2 (Enh2) relative to the promoter of the gene (Figure 2.8). We also analyzed 400 bp stretch of DNA from the enhancers using Position Specific Scoring Matrices (Possum) [180] for the enriched cis-elements. The Possum analysis resulted in common and uncommon motifs being enriched in these enhancers (Figure 2.9). For Enhancer 1, which is smaller and closer from the promoter, two EREs, two AP1, and two GATA motifs were found. Out of the two EREs, one is perfect and the other imperfect ERE. Some uncommon motifs CCAAT, SRF, LSF, Ets, NF-1, and TATA were also enriched. CCAAT motif is located adjacent to the prefect ERE which raises the possibility of interaction between ERβ and C/EBPs (CCAAT enhancer binding proteins). Largest score was for perfect ERE followed by AP1 and CCAAT. For Enhancer 2, which appeared to have larger peak, the cis-elements found were ½ EREs, Sp1, Tef, Mef-2, LSF, CRE, and CCAAT. Interestingly, no AP1 elements were enriched. Again, largest score was for ½ ERE followed by Sp1 and LSF.

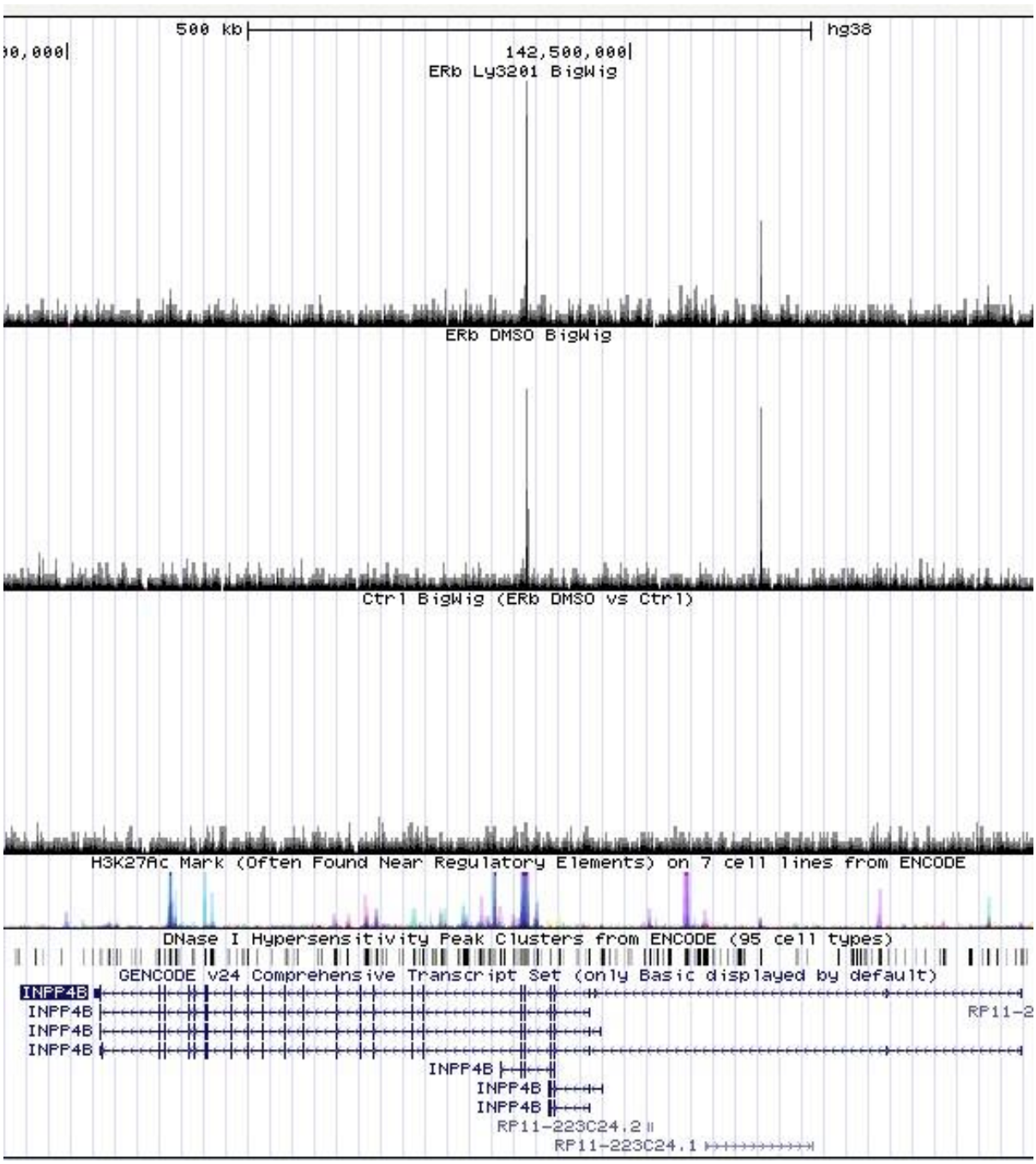


Figure 2.8 ERβ1 binds to intron of INPP4B gene. A screen shot of ERβ binding to UCSC genome browser extending INPP4B gene.

Figure 2.9 Analysis of motifs enriched in ERβ binding regions in INPP4B. A DNA stretch of 400 bp centered around Enhancer 1 and Enhancer 2 was extracted and analyzed for cis-elements enrichment using Possum software. (A, B) Positioning of motifs in the Enhancer 1 (A) and Enhancer 2 (B). (C, D) List of motifs found in Enhancer 1 (C) and Enhancer 2 (D). (E, F) Sequences of Enhancer 1 (E) and Enhancer 2 (F), EREs are highlighted in green and yellow color.

Possum Results

Possum home | Gene Regulation Hub

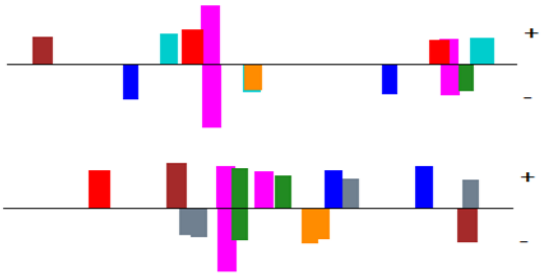
Color key: TATA Sp1 CRE ERE NF-1 E2F Mef-2 Myf CCAAT AP-1 Ets Myc GATA LSF SRF
= protein-coding

A **Enhancer 1**

hg38_dna range=chr4:142615346-142615745
400 bp

B **Enhancer 2**

hg38_dna range=chr4:142408155-142408554
400 bp



C **Enhancer 1**

hg38_dna range=chr4:142615346-142615745

motif	position	strand	sequence	score
LSF	21 - 35	+	acaggttataggtgg	5.68
AP-1	92 - 102	-	ttggagtcagc	7.27
GATA	121 - 133	+	ttaagataaggaa	6.21
CCAAT	138 - 153	+	gttaaccaataaacta	7.22
ERE	153 - 166	+	aggtcagagtgacc	12.3
ERE	154 - 167	-	ggtcagagtgacct	13.2
GATA	186 - 198	-	gcccttgtctggc	5.80
SRF	187 - 199	-	cccttgtctggca	5.43
AP-1	295 - 305	-	tgcgagtcagt	6.22
TATA	332 - 346	+	gtataacaaggtacg	5.05
ERE	340 - 353	+	aggtacgtctgacc	5.07
ERE	341 - 354	-	ggtacgtctgacct	6.54
Ets	355 - 365	-	cccttcccatc	5.51
NF-1	364 - 381	+	tcatggctgggaattcag	5.29

D **Enhancer 2**

hg38_dna range=chr4:142408155-142408554

motif	position	strand	sequence	score
CCAAT	68 - 83	+	agcagccaataaagaa	6.96
LSF	129 - 143	+	ggtggcttatgcctg	8.43
Tef	139 - 150	-	gcctggaatccc	5.06
Tef	148 - 159	-	cccagcaatttg	5.40
ERE	168 - 181	+	aggccaggatgacc	7.82
ERE	169 - 182	-	ggccaggatgacct	11.8
CRE	180 - 191	+	cctgacatcagg	7.41
CRE	180 - 191	-	cctgacatcagg	6.06
ERE	198 - 211	+	agaccagcctggcc	6.72
CRE	214 - 225	+	catgacgaaacc	5.96
Mef-2	235 - 246	-	ttaaaaatacaa	6.55
Mef-2	244 - 255	-	caaaaattagct	5.85
Sp1	253 - 265	+	gctgggcgtggtg	7.03
Tef	267 - 278	+	cacactcctgta	5.41
Sp1	324 - 336	+	caggggcggaggt	7.72
LSF	357 - 371	-	ccactgcactccagc	6.35
Tef	361 - 372	+	tgcactccagcc	5.12

E

Enhancer 1

>hg38_dna range=chr4:142615346-142615745
ATCCAAAAAGATAGATATCTACAGGTTATAGGTGGATTAAGAGATTCCTT
AATTTGCAACTGGTTAAAGGAGTAAAGCTTTGTCTACAAATTTGGAGTCA
GCAGAAAGGAATGTTTTAAGTTAAGATAAGGAAGTCTGTTAACCAATAAA
CTAGGTCAGAGTGACCTGTAGGGATGTGTGAGTTAGCCCTTGTCTGGCAT
GGCCTTAGGTCCTATTTGTGATTCAGTATCTTACTGTCACGAAGAGTCCA
CCTAGTTAGTCTCATGATCTCTATTTTTATTAATTTTTTATTAATGCGAG
TCAGTTGTGCCTAAACTCCAAAAGGGAAAGGGTATAACAAGGTACgtcTG
ACCTCCCTTCCCATCATGGCTGGGAATTCAGTTTTTAAGGTTTTTCTGAG

F

Enhancer 2

>hg38_dna range=chr4:142408155-142408554
TTGGATTGTTTTGTGAAGATATTACTCATATGATCACTATAAAATCTAAT
CCGTATGAGAATTATTTAGCAGCCAATAAAGAATCCTGAAAAATCTGTTT
TCAGGAAAAAAAAAAAAGTGAATGGGTGAGGTGGCTTATGCCTGGAATCCC
AGCAATTTGAGAGGCCGAGGCCAggaTGACCTGACATCAGGAATTCCAGA
CCAGCCTGGCCAACATGACGAAACCCCATCTCTATTAAAAATACAAAAAT
TAGCTGGGCGTGGTGGCACACTCCTGTAGTGCCAGCTACTTGGGAGGCTG
AGGCACAAGAATTGCTTGAACCCCAGGGGCGGAGGTTGCAGTGAGCTGAG
ATCACACCACTGCACTCCAGCCTGGGTGACAGAATGAGACTCTGTCTCAT

2.2.9 ERβ regulates transcription from the INPP4B enhancers

Next, we evaluated the ability of ERβ1 to induce transcription from these two sites (Enhancer 1 and Enhancer 2) using luciferase reporter assay. A 500 bp stretch of DNA was PCR amplified from genomic DNA containing enhancers and cloned into a pGL3-promoter luciferase reporter. PC3 cells were transfected with one of these constructs with or without ERβ expression vector and treated with Ly3201 for 24 hrs in 10%DCC-FBS. We found that ERβ could induce robust expression of luciferase from these enhancers which was also ligand dependent. Additionally, Enhancer 1 showed higher induction with ligand than the Enhancer 2 (Figure 2.9).

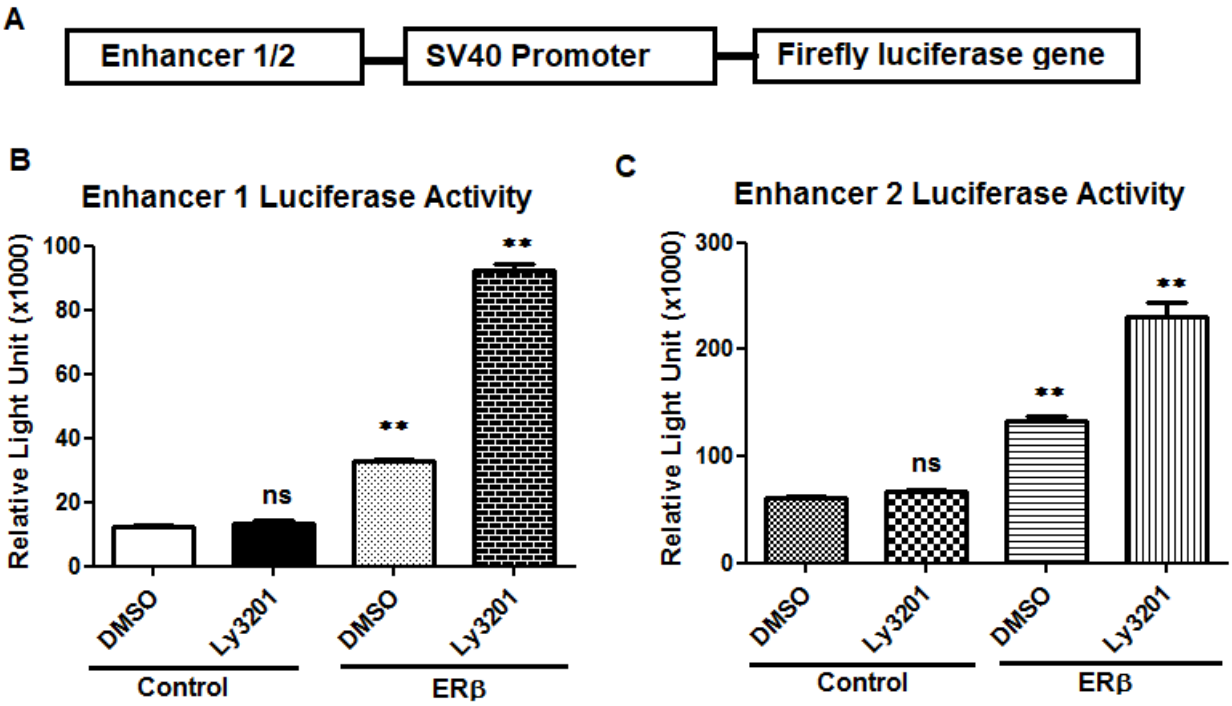


Figure 2.10 ERβ1 transcriptional activity from INPP4B enhancers. PCR amplified product of Enhancer 1 and Enhancer 2 was cloned into pGL3 promoter luciferase vector and transfected into PC3 cells with or without ERβ1 expression plasmid. Luciferase activity was measured after treatment with DMSO or Ly3201 for 18 hrs. (A) Schematic of the reporter construct. (B) ERβ1 activity from Enhancer 1 and (C) from Enhancer 2.

2.3 Discussion

Discovery of estrogen receptor β expanded the domain of estrogen signaling. While ERα has been implicated in various endocrine dependent cancers, ERβ emerged as a tumor suppressor. Various mechanisms of tumor suppressor activity are assigned to ERβ including inhibition of proliferation [95], inhibition of epithelial to mesenchymal transitions [172], and induction of apoptosis [100].

The role of ERβ in regulating PI3K/Akt pathway in prostate cancer is not known. Additionally, the potential of ERβ to inhibit metastasis in prostate cancer has not been studied. PTEN is deleted in PC3 cells and they express very low levels of INPP4B which may contribute to high AKT activity in these cells [181]. In this regard, PC3 cells with high metastatic potential [174] and highly migratory dynamics appear to be a good *in vitro* model for studying intervention strategies for treatment of metastatic prostate cancer and PI3K/Akt signaling. In the present study, we investigated the role of ERβ in cell migration and PI3K/Akt oncogenic signaling pathway in PC3 cells.

Since cancer cell lines established from metastatic prostate cancer express very little ERβ [182], we exogenously expressed ERβ1 in PC3 cells using lentivirus at MOI of 2. Initially, we observed low level of ERβ expression by western blot and immunofluorescence. But ERβ protein expression decreased to a low level in two-weeks. So, we were forced to generate multiple batches of stable cells. As yet, we do not know the exact mechanism behind ERβ loss, but it has been reported that Mdm2

and CREB-binding protein (CBP) co-ordinate the degradation of ERβ in response to growth signals [183].

To better understand the tumor suppressive effects of ERβ, we used RNA-seq to compare the differences in gene expression between PC3 cells stably expressing ERβ and control cells. We discovered that ERβ upregulated INPP4B > 3.5-fold when activated with specific agonist Ly3201. We validated this finding using RT-PCR in different RNA preparations and western blot. INPP4B has been previously reported to be a direct target of androgen receptor in LNCaP cells and is proposed to be direct AR target in normal prostate [150]. Additionally, INPP4B has been also reported to be expressed in ERα positive, non-dividing normal breast luminal cells. Despite these observations, treatment of ERα positive MCF-7 breast cancer cells with estradiol did not regulate INPP4B expression [141]. Interestingly, glioblastoma cells stably expressing ERβ, when treated with Ly5003007 induced expression of INPP4B in an RNA-seq study [184]. To examine whether ERβ can regulate INPP4B in other cell lines, we made stable ERβ expressing cells of BPH1 and DU145. BPH1 cells are immortalized prostate epithelial cells that express Δp63, cytokeratin 5 and 14 but not androgen receptor, hence are considered prostate basal cells [175]. On the other hand, DU145 cells are prostate cancer cells established from brain metastasis [185]. We found ERβ robustly induced INPP4B expression in BPH1 cells but not in DU145 cells.

INPP4B is a phosphatase belonging to PI3K/Akt pathway where it negatively regulates PI3K signaling by removing 4-phosphate group from PI(3,4)P2 [186]. In

normal cells PTEN is the major regulator of PI3K/Akt, but it is frequently lost in prostate cancer leading to overactivated Akt [159]. We tested whether expression of ERβ in PC3 cells and BPH1 cells affected Akt activity. Akt activity is regulated by phosphorylation at Thr308 and Ser473. In PC3 cells pAkt Thr308 could not be detected but pAkt Ser473 level was decreased by approximately 50%. BPH1 cells express wild type PTEN [175] and no pAkt could be detected. We hypothesized that decrease in Akt activity is due to increase in INPP4B. To test this hypothesis, we partially decreased INPP4B level in PC3-ERβ cells using siRNA and observed that pAkt Ser473 level increased. This leads to the conclusion that ERβ regulates Akt activity through upregulation of INPP4B. ERβ inhibited Akt activity in MCF-7 and T47D breast cancer cells through upregulation of PTEN and down regulation of Her2 [187]. Also, in glioma cells treatment with liquiritgenin, a novel ERβ ligand, inhibited Akt activity [188].

Akt activity has been implicated in many biological processes such as survival, proliferation, and migration of cells. Overactive Akt activity is widely associated with aggressive tumor and enhanced metastasis [189]. To understand the biological effect of Akt inhibition, we performed wound healing assay with stable PC3 cells. Wound healing assay is an easy, inexpensive and fast *in vitro* assay to test the migratory behavior of cells. We observed that PC3-ERβ cells migrated significantly slower than control cells. To confirm that this effect is mediated through INPP4B, we performed scratch assay on PC3-ERβ cells after knock-down of INPP4B with siRNA. As expected siINPP4B transfected cells migrated faster than siControl transfected

cells. Several reports have described inhibitory effect of INPP4B expression on cell migration [148,151, 190]. Exogeneous expression of INPP4B in PC3 and DU145 cells significantly inhibited migration in trans-well assays and this was found to be mediated through inhibition of Akt activity [151]. However, Hodgson et al., 2014 overexpressed INPP4B in PC3 cells and found inhibition of cell invasion but not migration in trans-well chambers [148].

To understand the mechanism of INPP4B upregulation by ERβ, we analyzed global ERβ binding in PC3 cells. ChIP-seq revealed two locations in INPP4B gene where ERβ was bound. We further analyzed DNA sequences in 400 bp stretch of those two ERβ binding enhancers in INPP4B. Possum analysis revealed >15 cis-elements including ERE, ½ ERE, AP1, and SP1 among others being enriched in small region. These motifs may indicate possible co-operation of different transcription factors involved in INPP4B regulation by ERβ. Androgen receptor was shown to bind near INPP4B gene [150] but both ERβ binding loci are different from AR binding region.

INPP4B has been shown to function as tumor suppressor in prostate cancer and is lost in advanced stages of the disease. It was shown to be regulated by androgen receptor in LNCaP cells and inhibit Akt activity [150]. This indicates that androgen ablation therapy may increase Akt activity because of loss of INPP4B expression. Our findings suggest that treatment with ERβ specific agonist can benefit patients with PTEN loss and overactive Akt signaling.

2.4. Materials and Methods

2.4.1 Reagents and Cell Culture

The PC3 and BPH1 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were cultured in RPMI-1640 (Invitrogen Inc., Carlsbad, CA) medium supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), and Antibiotic-Antimycotic (Invitrogen Inc., Carlsbad, CA) at 37°C and 5% CO_2. Phenol red-free medium supplemented with 10% dextran coated charcoal (DCC) treated FBS was used for ligand treatment. All experiments used cells below passage 30. Ly3201 was provided by Eli Lily company. 17β-estradiol and 4OH-tamoxifen were purchased from Millipore Sigma (St. Louis, MO).

2.4.2 Stable expression of ERβ in PC3 and BPH1 cells

Early passage of PC3 and BPH1 cells were infected with the lentivirus Lenti6-TOPO-V5-D empty or containing cDNA for human ERβ1 at 2 M.O.I. (multiplicity of infection). Cells were selected with 5 µg/ml blasticidin for at least two weeks. The cells infected with empty virus vector were used as control in all experiments.

2.4.3 RNA extraction and real-time PCR

RNA extraction was performed with Qiagen mRNA extraction kit according to manufacturer's protocol from cells grown in 6-well plate. cDNA was synthesized from 1 µg of total RNA with iScript first strand cDNA synthesis kit according to manufacturer's protocol (Bio-Rad Laboratories, Inc. CA). Real-time PCR was performed with iTaq Universal SYBR Green supermix (Bio-Rad Laboratories, Inc.

CA) on a 7500 Fast Real-Time PCR System (Applied Biosystems) using optimized conditions for SYBR Green I dye: 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s. Optimum primer concentration was determined in preliminary experiments, and amplification specificity confirmed by melt curve. Relative gene expression was calculated using ΔΔCt using GAPDH as normalization control. Primer sequences for the real-time PCR are listed in table 1. All primers were designed using NCBI primer-BLAST, and ordered from IDT (Coralville, IA).

2.4.4 RNA sequencing and transcriptomic analysis

Libraries for RNA sequencing were prepared with KAPA stranded RNA-seq kit. The workflow consisted of mRNA enrichment, cDNA generation, and end repair to generate blunt ends, A-tailing, adaptor ligation and PCR amplification. Sequencing was performed on Illumina HiSeq 3000 for a single read of 50 bp. The reads were mapped to the latest UCSC transcript set using Bowtie2 version 2.1.0 [192] and the gene expression level was estimated using RSEM v1.2.15 [193]. TMM (trimmed mean of M-values) was used to normalize the gene expression across samples. Differentially expressed genes were identified using edgeR program [194]. Genes showing altered expression with $p<0.05$ and more than 1.5-fold changes were considered differentially expressed.

2.4.5 Protein extract preparation

To prepare whole-cell extracts, cells were washed twice with cold PBS, scraped with a rubber policeman in PBS and cell pellet collected by centrifuging at 3000 g for 5 min. Cells were suspended in 10 times packed cell volume of RIPA lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS and 140 mM NaCl) supplemented with protease inhibitor cocktail, and PhosStop (Roche, Indianapolis, IN), syringed 10 times through narrow needle and then centrifuged at 14,000 rpm for 10 min. Clear supernatant was transferred to a new centrifuge tube and protein concentration measured using Pierce 660 nm protein assay kit (Thermo Fisher Scientific, Waltham, MA). Samples were prepared by boiling with 4X loading dye and β-mercaptoethanol.

2.4.6 Western blotting

Thirty-five micrograms of protein were loaded on an SDS-PAGE 4-20% Bis-Tris gel with Tris running buffer and transferred to a PVDF membrane after electrophoretic separation. Membranes were blocked with 5% non-fat powdered milk in 0.1% TBST buffer and probed with anti-ERb (PPZ0506, Invitrogen), anti-INPP4B (D9K1B), pAkt Thr308 (D25E6), pAkt Ser473 (D9E), panAkt (C67E7), pGSK3b (D85E12), (Cell Signaling Technology), GAPDH-HRP (sc-47724) (Santa Cruz Biotechnology), β-Actin (A19780) (Millipore Sigma, St. Louis, MO). Primary antibodies were used at 1:200–1000 dilutions, and secondary antibody was used at 1:10,000. The western blot experiments were repeated at least three times.

2.4.7 Chromatin Immunoprecipitation (ChIP)

ChIP assay was modified from a previous report [195]. Sub-confluent PC3 cells (90%) were grown in a 100 mm dish and treated with indicated ligand for 24 hrs in 10% charcoal stripped serum. Cells were fixed with 1.5% formaldehyde by adding directly into the media on the plates for 10 min at RT, quenched with 0.125 M glycine, washed 2X with cold PBS, scraped in PBS and cells collected by centrifugation. Cell pellets were suspended in 500 µl ChIP buffer (TrisHCl 20 mM, NaCl 150 mM, EDTA 2 mM, TritonX 100 1%, SDS 0.1% and protease inhibitor), sonicated with Diagenode UCD200 in ice-cold water for 60 cycles (30 s on/ 30 s off), and lysates were cleared by centrifugation at 14000 rpm for 5 min. Following sonication, 25 µl samples were mixed with 75 µl elution buffer (TrisHcl 50 mM, EDTA 10 mM, 1% SDS) incubated at 65°C for >6 hrs and purified using PCR purification kit (Qiagen) for input DNA. Rest of the lysate was divided equally into two Eppendorf tubes, 10 µl ERβ-LBD antibody (homemade) or 10 µl hyperimmune rabbit IgG was added and incubated at 4°C O/N on slow rotation. Next day 20 µl protein G beads (Dynabeads, Invitrogen) added to IP samples and rotated at RT for 2 hrs. Beads were washed twice with 1 ml each of wash buffer II (HEPES 50 mM, NaCl 500 mM, EDTA 1 mM, Sodium Deoxycholate 0.1%, , TritonX 100 1%) , III (TrisHcl 10 mM, LiCl 250 mM, EDTA 1 mM, Sodium Deoxycholate 0.5%, NP40 0.5%), & IV (Tris HCl 10 mM, EDTA 1 mM) for 2 min on a slow rotation at RT, then suspended in 100 µl elution buffer and incubated at 65^{o}C for >6 hrs, and finally purified using PCR purification kit (Qiagen). Purified DNA was used for qPCR and sequencing. Fold enrichment was calculated using ΔΔCt method.

2.4.8 ChIP-seq Analysis

ChIP DNA samples were analyzed for quality and integrity on a Qbit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA). Nextera DNA Flex Library prep kit was used for library preparation as per manufacturer's recommendation and DNA was sequenced on an Illumina NextSeq 500. The reads were mapped to the latest UCSC genome build using Bowtie2 version 2.1.0. From the aligned reads, peaks were called and annotated using MACS2 [196]. This methodology drew pairwise comparisons between each ChIP sample and control.

2.4.9 siRNA transfection

siRNA for human INPP4B (Catalogue# 4392420) and negative control (Catalogue# 4390843) were purchased from Thermo Fisher Scientific. PC3 ERβ cells were grown to 50% confluency in complete medium and transfected with siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) at the final concentration of 50 nM of each (control or INPP4B). After 48 hrs, cells were treated with DMSO (vehicle control) or Ly3201 1 μM in 10% DCC-FBS for 24 hrs, and RNA and protein extracted. For scratch assay sub-confluent (70-80%) cells were transfected as above and after 48 hrs scratch assay performed.

2.4.10 Wound healing assay

PC3 cells expressing either ERβ or empty vector (control cells) were grown to confluency in 12-well plate in complete medium, then wounded with a 200 μl pipette tip washed with PBS, added 10% DCC with indicated treatment and image taken with

an Olympus inverted microscope with Axiocam software. Cells were incubated at 37°C with 5% CO_2 and 100% humidity for 18 hrs or 24 hrs. Images were taken again after the end of incubation. For wound healing assay comparing between control cells and ERβ cells incubation was for 18 hrs and for wound healing assay after siRNA transfection, incubation was for 24 hrs.

2.4.11 Luciferase assay

A 500 bp sequence surrounding each of the ERbeta binding enhancer sequences identified from ChIP-seq were amplified using PCR and cloned into pGL3 luciferase reporter in front of a minimal promoter. PC3 cells were grown to 50% confluency in 6-well plate and transfected with one microgram of the luciferase constructs along with or without 0.5 ug ERβ expression vector in 10% DCC medium. 24 hrs post transfection, cells were treated with DMSO (vehicle control) or Ly3201 10 nM in fresh 10% DCC for 24 hrs more. Next day media aspirated and 200 µl lysis buffer added to cells in each well and collected lysates were centrifuged at 14000 rpm for 10 min. Fifty µl of cleared lysate was used to measure luminescence on a Perkin-Elmer (Waltham, MA) Victor x luminescence plate reader using luciferase kit from BioVision (Milpitas, CA).

2.4.12 Statistical analysis

Statistical analysis was performed with Prism GraphPad version software. Student's t-test and one-way ANOVA were used for statistical analysis. Results were considered significant with p-value ≤ 0.05.

Chapter 3

Estrogen Receptor β exerts tumor suppressive effects in prostate cancer through repression of androgen receptor activity

3.1 Introduction

Prostate cancer is the most common cancer in men worldwide (excluding non-melanoma skin cancer) and is the cause for at least 3,66,000 cancer related deaths annually. Despite many recent advances in diagnosis and treatment, prostate cancer is still a significant medical problem for the affected men. While multiple therapeutic approaches are available for cure of localized prostate cancer, metastatic castration resistant prostate cancer is incurable by any means [197].

The prostate is a highly androgen responsive organ and is dependent on androgen for its development, maturation, maintenance, and function [27,35]. Androgens regulate the activity of androgen receptor (AR) both under normal physiological conditions and in malignancy. AR plays an important role in prostate cancer as a strong driver of proliferation, and as such is the primary target for treatment of prostate cancer. A common treatment option for advanced prostate cancer is androgen deprivation therapy (ADT) or castration which targets androgen receptor. Upon depletion of androgen, prostate cancer regresses due to an effect called androgen withdrawal-induced apoptosis [31]. Despite initial favorable outcome, many patients relapse leading to a prostate cancer called castration resistant prostate cancer (CRPC). CRPC is highly aggressive and shows enhanced metastatic potential leading to most of the deaths [10].

Again, androgen receptor is implicated in the development of CRPC and various mechanisms are attributed. The transcriptional activity of AR is regulated by many co-activators and co-repressors. Loss of expression of co-repressors or increased

expression of co-activators are frequently observed in CRPC [198]. There are also reports of mutation in the AR ligand binding domain which sensitizes it to low levels of androgen [199] or expands the ligand specificity such that AR can be activated by glucocorticoids, progesterone, or estrogens [42]. Genomic amplification of AR loci, increased expression of AR [39,40], and synthesis of isoforms that lack ligand binding domain rendering constitutively active receptor are all implicated in the development of CRPC [200]. Importantly, a constitutively active variant, AR-V7, is being intensively studied and is implicated in CRPC. Specifically, it was found to change the metabolic activity of LNCaP cells by enhancing citrate utilization and, increasing dependence on glutaminolysis and reductive carboxylation [37].

Estrogen receptor β (ERβ) was discovered and cloned from the rodent prostate [73] and is abundantly expressed in human and rodent prostate epithelium, stroma, immune cells, and endothelium of the blood vessels [74]. Knock-out of ERβ led to epithelial hyperplasia in mouse prostate which was attributed to its anti-proliferative effect [91]. Transcriptomic analysis of ventral prostate of ERβ knock-out mice revealed increased expression of androgen receptor (AR) regulated genes, most of which are also upregulated in prostate cancer [94].

ERβ functions as a tumor suppressor in several types of cancer [95, 201-203]. ERβ is abundantly expressed in normal and benign prostatic hyperplasia however, it is gradually lost in prostate cancer [90,204,205]. The loss of ERβ in advanced prostate cancer suggests a role in opposing androgen signaling. The overall effect of ERβ regulated gene networks in prostate cancer is not known. In the present study, we

investigated the role of ERβ in an AR expressing prostate cancer cell line to better understand its tumor suppressive effects in prostate cancer. Transcriptomic analysis of ERβ expressing LNCaP cells revealed the downregulation of androgen signaling by ERβ as one of the most significant effects. The present study, which is the first transcriptomic study of ERβ in an AR positive cell line, suggests a key role for ERβ in regulating AR expression and activity in prostate cancer.

3.2 Results

3.2.1 ERβ regulated transcriptome in AR-positive LNCaP cells

To understand the role of ERβ in AR-positive prostate cancer we stably expressed ERβ in LNCaP cells using lentiviral-mediated gene delivery and integration. After selection, we observed robust expression of ERβ in these cells by immunofluorescence, RT-qPCR, and western blot (Figure 3.1). Then we performed RNA-seq of LNCaP cells stably expressing ERβ1 or empty vector after treatment with DMSO (vehicle), estradiol (E2) or LY3201 plus AR synthetic ligand R1881. Since prostate cancer is an androgen regulated disease, we sought to understand ERβ function in the presence of fully activated AR. Differential gene expression analysis included comparing ERβ expressing vs non-expressing as well as DMSO treated vs ligand treated. Exogeneous expression of ERβ had very little impact on LNCaP transcriptome representing just over 1% of total transcript change (Figure 3.2A, heat map and Table 3.1). Closer examination of data showed a majority of DMSO regulated transcripts were also regulated by ERβ ligands in the same direction suggesting an effect of residual estrogenic activity in DCC-FBS.

E2 and Ly3201 treatments resulted in 4185 and 3456 differentially expressed transcripts (adjusted p-value < 0.05), respectively in ERβ expressing cells (Table 3.1). The proportion of upregulated (60%) and downregulated (40%) genes between the two ligands was similar. With the upregulated genes, there was strong overlap (63%). However, there was only a modest overlap in the downregulated genes (46%) (Figure 3.2B). In both upregulated and downregulated categories, there were fewer Ly3201 regulated transcripts that were unique.

Table 3.1. Summary of numbers of differentially expressed genes identified in RNA-seq study.

	Differentially Expressed Genes*		
Treatment	***Up-regulated***	***Down-regulated***	***Total***
DMSO	29	14	43
E2	2354	1831	4185
LY	2062	1394	3456

*Defined as those with FDR-adjusted p<0.05.

Table 1 showing differentially expressed genes up-regulated and down-regulated after treatment with E2 or LY3201.

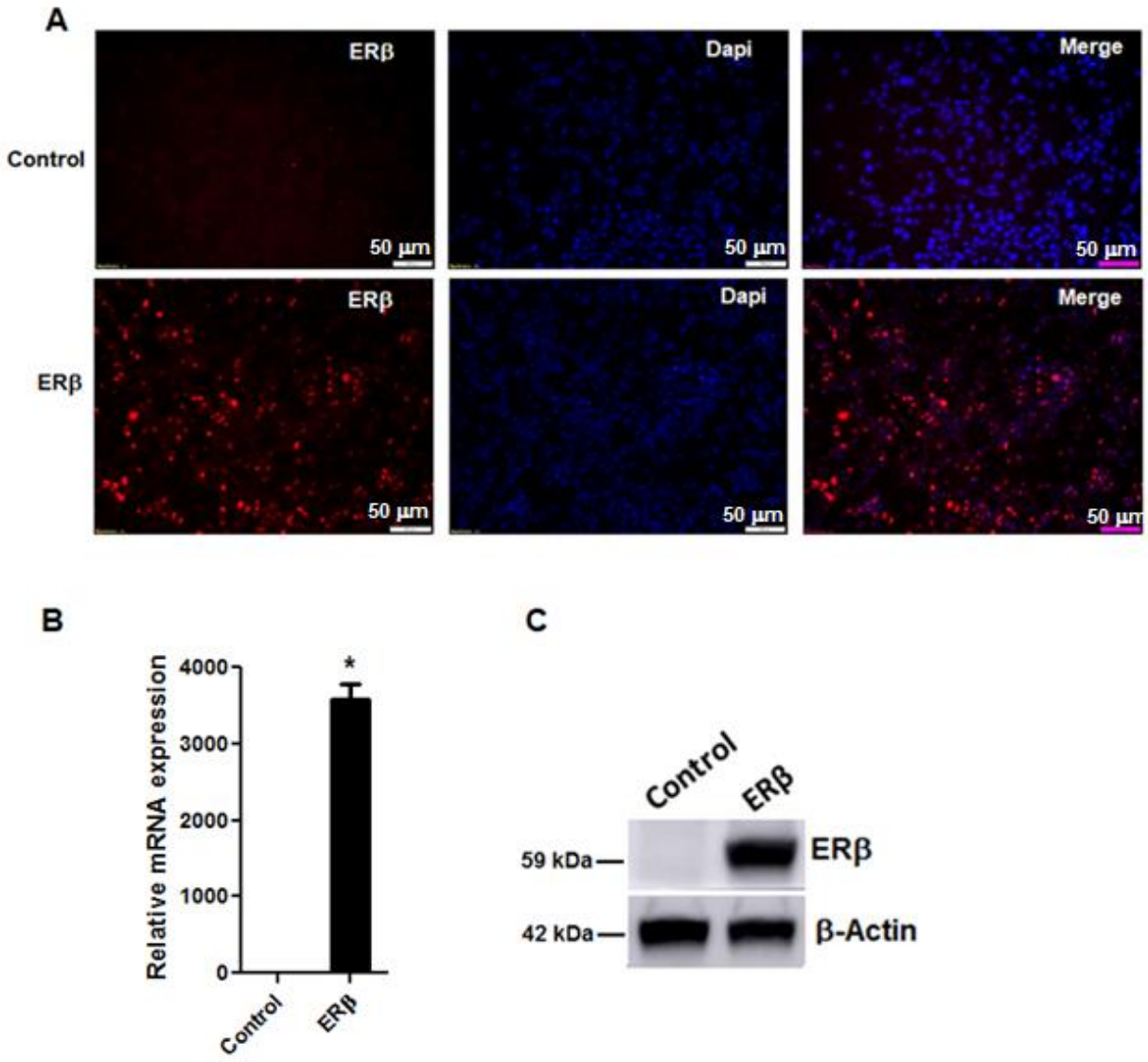
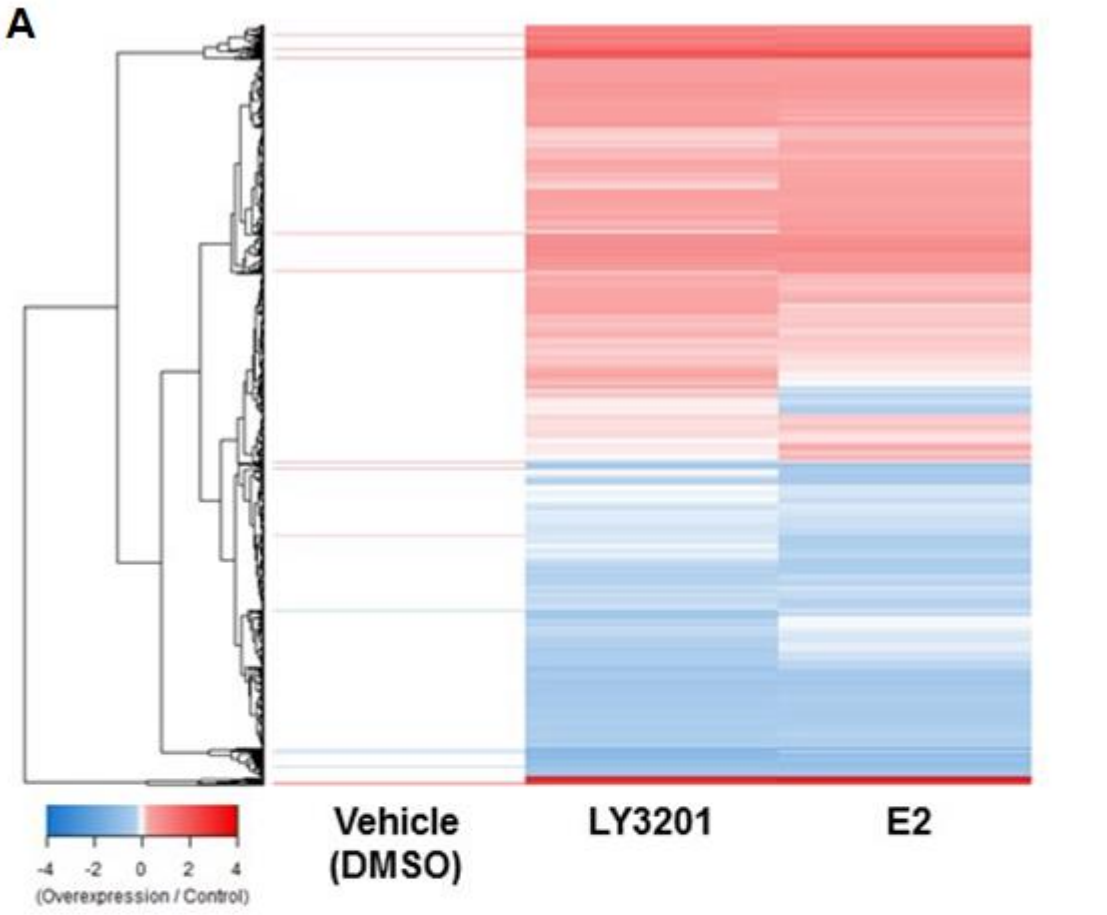


Figure 3.1 Expression of ERβ in LNCaP cells. Early passage of LNCaP cells were infected with the lentivirus Lenti6-TOPO-V5-D empty vector or containing cDNA for human ERβ1 at 2 M.O.I. and selected with 5 µg/ml blasticidin for two weeks. (A) Immunofluorescence staining of ERβ in LNCaP cells showing strong nuclear staining, top control LNCaP cells, bottom ERβ LNCaP cells. (B) ERβ1 mRNA expression in LNCaP-ERβ cells compared to control cells and presented as fold difference. Values represent mean ± SEM of three independent experiments; *$p<0.05$. (C) ERβ1 protein expression detected by western blot in LNCaP control and LNCaP-ERβ1 cells.

Figure 3.2 RNA-seq analysis identified differentially expressed genes which respond to ERβ activation. A, Gene expression profiles of responsive genes in cells treated with vehicle, E2, or LY3201 are presented in a heatmap of log2-transformed fold-change values. Hierarchical clustering of expression profiles and resulting dendrogram of grouped genes based on their similarities in response to ligand treatment. B, Venn diagrams show the proportion of responsive genes which are common and specific to each of the two ERβ ligands used in this study.


A
-4 -2 0 2 4
(Overexpression / Control)
Vehicle (DMSO)
LY3201
E2
B
E2
LY
649
1705
357
Up-regulated
E2
LY
821
1010
384
Down-regulated

3.2.2 Gene set enrichment analysis (GSEA) of ERβ regulated transcriptome in LNCaP cells

Pre-ranked gene set enrichment analysis (GSEA) was used to identify pathways regulated by ERβ in LNCaP cells which may be relevant to ERβ expression and activation in prostate cancer. Treatment with LY3201 was chosen for enrichment analysis. The most positively enriched set in the LY3201-treated cells were genes responsive to estrogen; other highly enriched sets contained genes involved in MTORC1 signaling and the unfolded protein response. GSEA also revealed downregulated genes involved in several pathways associated with cancer hallmarks such as hypoxia and glycolysis (Table 3.2). The most negatively enriched set contained genes involved in response to androgens—a central driver in androgen-sensitive as well as androgen refractory prostate cancer (Figure 3.3). These androgen-responsive genes were investigated further due to the well-established importance of androgen signaling in prostate cancer.

Table 3.2. Summary of top hits from Gene Set Enrichment Analysis (GSEA) of ERβ regulated differentially expressed genes identified in RNA-seq study.

Gene Set	NES	FDR q-value*
Positive Enrichment Score		
HALLMARK_ESTROGEN_RESPONSE_EARLY	7.2	0
HALLMARK_ESTROGEN_RESPONSE_LATE	6.0	0
HALLMARK_MTORC1_SIGNALING	4.3	0
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	3.7	0
HALLMARK_E2F_TARGETS	3.7	0
HALLMARK_G2M_CHECKPOINT	3.2	0
HALLMARK_PROTEIN_SECRETION	3.2	0
HALLMARK_UV_RESPONSE_UP	3.1	0
HALLMARK_OXIDATIVE_PHOSPHORYLATION	2.7	0
HALLMARK_MITOTIC_SPINDLE	2.5	3.9E-04
Negative Enrichment Score		
HALLMARK_ANDROGEN_RESPONSE	-3.4	0
HALLMARK_HYPOXIA	-2.5	9.5E-04
HALLMARK_P53_PATHWAY	-2.4	0.002
HALLMARK_GLYCOLYSIS	-2.2	0.007
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	-2.0	0.01
HALLMARK_KRAS_SIGNALING_UP	-2.0	0.02
HALLMARK_UV_RESPONSE_DN	-2.0	0.02
HALLMARK_WNT_BETA_CATENIN_SIGNALING	-1.9	0.02

*FDR q<0.05 NES Normalized Enrichment Score and FDR q-value False Discovery Rate

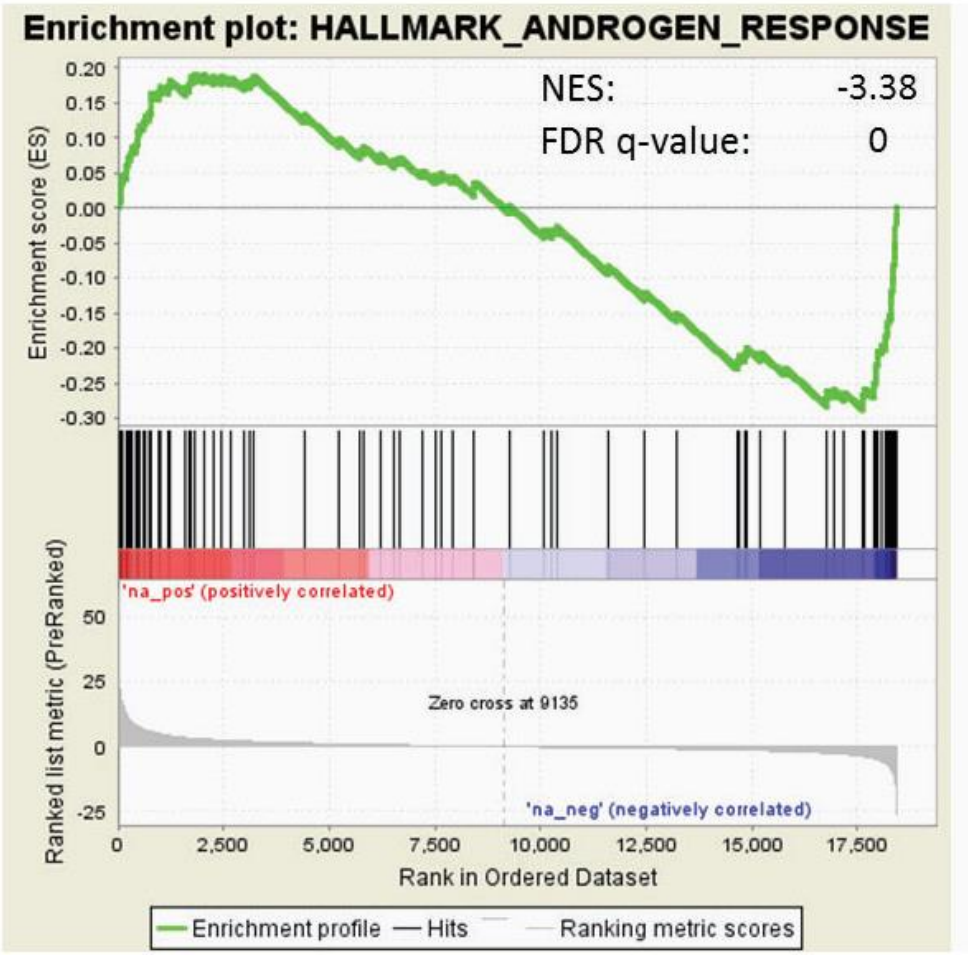


Figure 3.3. Effect of ERβ activation on androgen response. Gene Set Enrichment Analysis (GSEA) showing ERβ activation downregulated an enrichment of genes involved in androgen response. (NES: Normalized Enrichment Score and FDR q-value: False Discovery Rate).

Table 3.3 List of androgen-responsive genes differentially regulated by ligand-activated ERβ in prostate cancer cells.

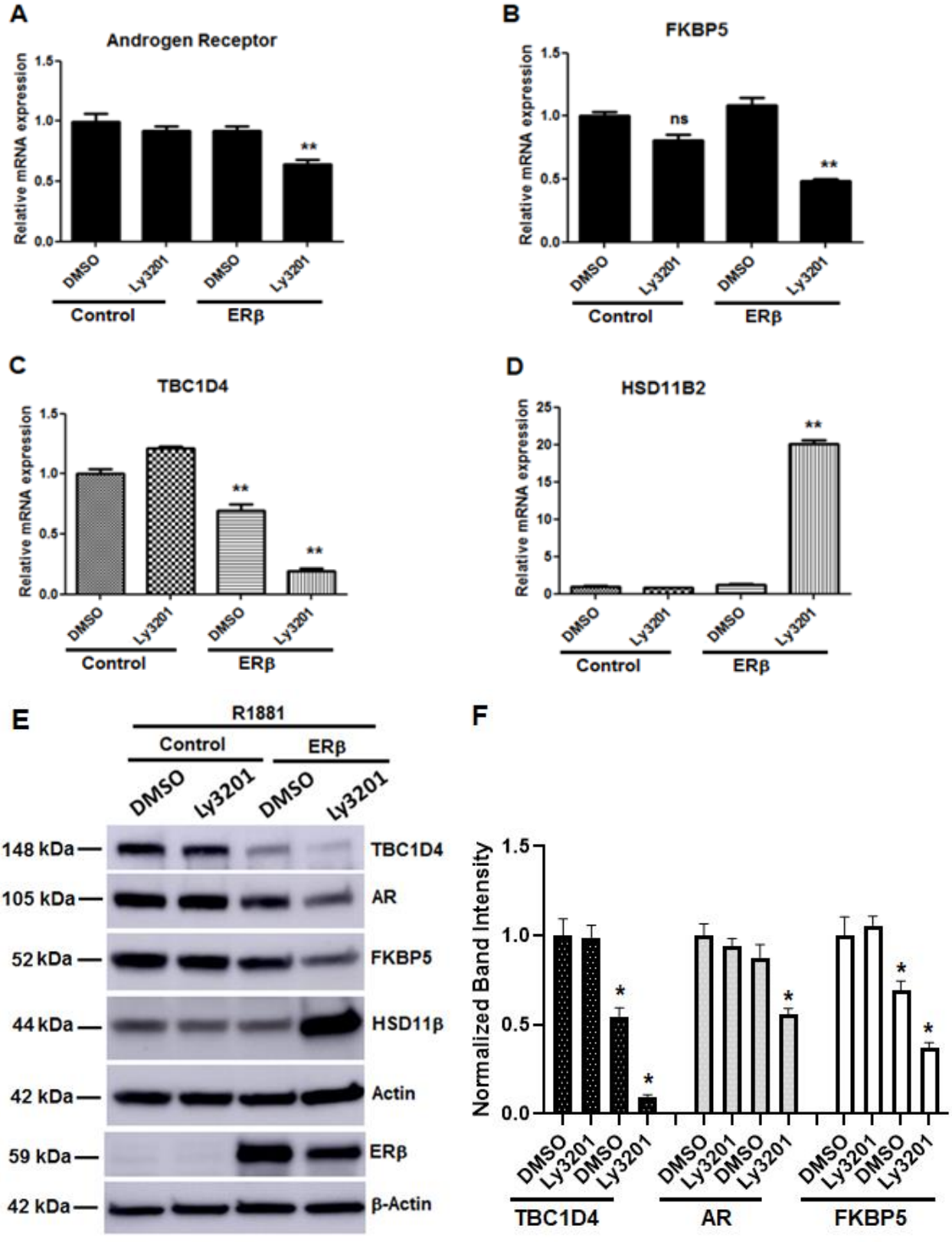
Up-regulated			*Down-regulated*		
Symbol	**log_2 FC**	**p-value***	**Symbol**	**log_2 FC**	**p-value***
FADS1	2.4	0	AKAP12	-1.7	1.6E-18
KRT19	1.9	1.8E-57	STEAP4	-1.2	7.8E-05
HOMER2	1.3	2.6E-80	MAF	-1.2	1.3E-57
SCD	1.0	2.2E-97	ADAMTS1	-1.1	6.6E-42
TARP	0.9	2.0E-08	CAMKK2	-1.0	3.6E-80
ACTN1	0.9	2.3E-73	NDRG1	-0.9	1.2E-09
CENPN	0.8	1.6E-33	PTPN21	-0.9	3.0E-28
ALDH1A3	0.7	7.2E-20	ZMIZ1	-0.8	3.0E-30
ELOVL5	0.7	5.2E-32	PMEPA1	-0.8	5.0E-48
CCND1	0.6	3.6E-29	ELK4	-0.8	2.7E-35
B4GALT1	0.6	6.6E-23	HERC3	-0.7	0.002
SPCS3	0.5	4.4E-13	ARID5B	-0.7	1.6E-24
SLC38A2	0.4	7.8E-14	UAP1	-0.7	3.1E-41
DBI	0.4	8.8E-08	FKBP5	-0.7	6.4E-06
H1F0	0.4	2.0E-12	IQGAP2	-0.7	1.9E-15
ABHD2	0.4	1.2E-14	RAB4A	-0.6	1.4E-23
ELL2	0.4	5.6E-06	ZBTB10	-0.6	1.7E-19
INSIG1	0.3	7.1E-08	ABCC4	-0.5	3.3E-18
HMGCS1	0.3	8.1E-11	ACSL3	-0.5	6.5E-11
LMAN1	0.3	6.1E-10	APPBP2	-0.5	5.7E-16
MYL12A	0.3	6.8E-08	TNFAIP8	-0.5	8.1E-06
SEC24D	0.3	1.3E-05	STK39	-0.5	1.6E-11
IDI1	0.2	0.0001	CDC14B	-0.4	0.001
SRF	0.2	0.003	NKX3-1	-0.4	4.1E-15
TMEM50 A	0.2	0.02	INPP4B	-0.4	7.3E-05
KLK3	0.2	0.0002	TSC22D1	-0.4	5.0E-10
RRP12	0.2	0.005	TPD52	-0.4	1.4E-05
PDLIM5	0.2	0.009	SPDEF	-0.4	4.5E-07
PTK2B	0.2	0.03	HMGCR	-0.3	2.8E-09
NCOA4	0.2	0.006	TMPRSS2	-0.3	2.0E-05
			SLC26A2	-0.2	0.001
			DHCR24	-0.2	3.4E-05
			PIAS1	-0.2	0.05
			MAP7	-0.2	0.04
			UBE2J1	-0.2	0.01
			ANKH	-0.1	0.05

*FDR adjusted p≤0.05.

3.2.3 ERβ activation decreases AR expression and transcriptional activity

Since androgen signaling was found to be down-regulated, we investigated AR expression and some of the known AR target genes by RT-PCR and western blot which were also changed in RNA-seq. We found a modest but statistically significant decrease in AR transcript (30%) and protein (50%) level after Ly3201 treatment. Informed by RNA-seq data and previous reports as AR downstream targets, we also investigated the expression of FKBP5, TBC1D4, and HSD11β2 by RT-PCR and western blot. Expression of TBC1D4 reduced by 70% for mRNA and 90% for protein. Expression of FKBP5 was decreased by 45% for mRNA and 60% for protein. HSD11β2 expression increased by 22-fold for mRNA and 5-fold for protein. (Figure 3.4) We also found many established AR target genes being upregulated or downregulated by ERβ (Table 3.3)

Figure 3.4 ERβ activation decreases AR expression and transcriptional activity. Effect of ERβ activation on androgen receptor and some of its downstream target genes in LNCaP cells. RT-PCR results of (A) AR, (B) FKBP5, (C) TBC1D4, and (D) HSD11β2; (E) Western blot image showing protein levels of TBC1D4, AR, FKBP5, HSD11β2 and ERβ in control and ERβ expressing cells. β -Actin was used as loading control. (F) densitometric analysis of bands in (E) normalized to β -Actin. Values represent mean ± SEM of three independent experiments; **p<0.05.


A
Androgen Receptor
Relative mRNA expression
1.5
1.0
0.5
0.0
**
DMSO
Ly3201
DMSO
Ly3201
Control
ERβ
B
FKBP5
Relative mRNA expression
ns
**
C
TBC1D4
**
**
D
HSD11B2
25
20
15
10
5
0
**
E
R1881
Control
ERβ
DMSO
Ly3201
DMSO
Ly3201
148 kDa
TBC1D4
105 kDa
AR
52 kDa
FKBP5
44 kDa
HSD11β
42 kDa
Actin
59 kDa
ERβ
42 kDa
β-Actin
F
Normalized Band Intensity
1.5
1.0
0.5
0.0
*
TBC1D4
AR
FKBP5

3.2.4 ERβ inhibits AR transcriptional activity in a reporter assay

To further corroborate our finding of androgen signaling inhibition by ERβ, we used AR responsive reporter assays in LNCaP and 22RV1 cells. We first validated the regulation of luciferase construct p(ARR)2 in LNCaP and 22RV1 cells by AR synthetic ligand R1881 and found to it be induced approximately by 800 and 250-fold, respectively. Then cells were transfected with luciferase construct with or without ERβ expression plasmid and treated with DMSO, E2, and Ly3201 plus R1881, and after 18 hrs luciferase activity was measured. In LNCaP cells AR transcriptional activity decreased by 40% and 60% after treatment with DMSO or E2, respectively. In 22RV1 cells it decreased by 30%, 85%, and 80% after treatment with DMSO, E2 or Ly3201, respectively (Figure 3.5).

3.2.5 ERβ down-regulates CaMKK2 and inhibits phosphorylation of AMPK.

CaMKK2 is a well-known target of AR which is involved in regulating AMPK activity in cells [20]. We found ERβ repressed CaMKK2 transcript by 60% and protein level by 90%. The effect of CaMKK2 repression is evident as reduction in pAMPK level while total AMPK did not change.

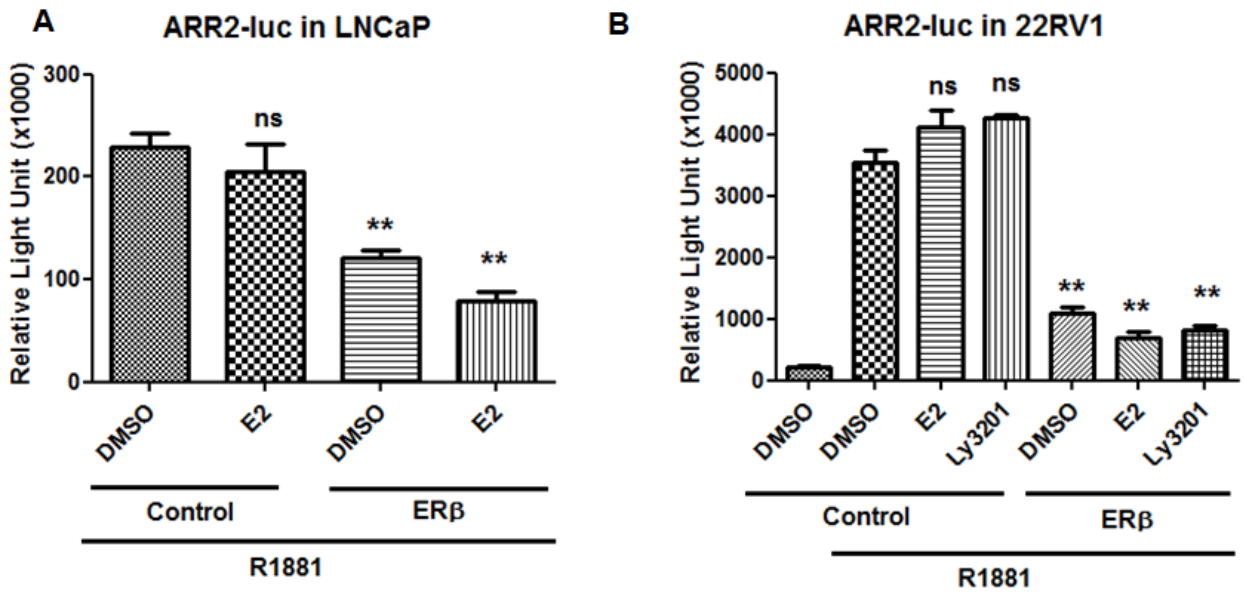


Figure 3.5 ERβ inhibits AR transcriptional activity in a reporter assay. LNCaP and 22RV1 cells were transfected with AR responsive luciferase construct p(ARR)2 PB-LUC with or without ERβ1 expression plasmid. Luciferase activity was measured after treatment with DMSO, Estradiol or Ly3201 plus R1881 for 18 hrs. AR activity in (A) LNCaP and (B) 22RV1 cells. Values presented are normalized RLU of mean ± SEM of three independent experiments (**p< 0.05, ns: not significant).

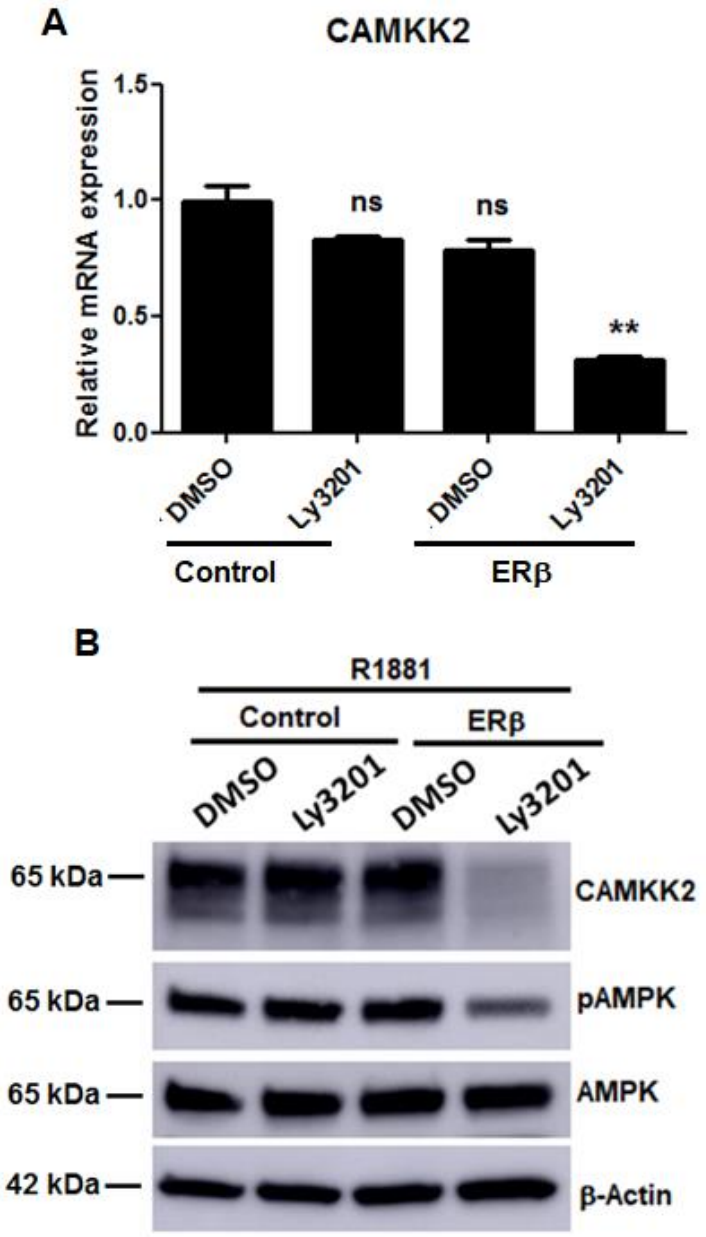


Figure 3.6 ERβ mediated down-regulation of CaMKK2 inhibits phosphorylation of AMPK. (A) AR target gene CAMKK2 transcript levels were reduced by ERβ activation. Values represent mean ± SEM of three independent experiments; **p<0.05. (B) CaMKK2 protein expression and activity (measured by the level of pAMPK).

3.3 Discussion

Anti-proliferative role of ERβ in prostate is well established from various studies. Studies of prostate from mice with genomic deletion of ERβ showed hyperplasia of luminal epithelium and increase in intermediary luminal cells [91]. Additionally, ERβ was found to reduce AR activity by increasing the expression of co-repressor DACH1/2 and decreasing the AR driver RORc [94]. Previously, we reported anti-proliferative effect of ERβ in prostate cancer cell lines that was mediated by down regulation of Skp2 and upregulation of p27 KIP1 protein [95].

In the present study, we specifically investigated the effect of ERβ on AR activity in AR-positive LNCaP cells. We stably expressed ERβ in LNCaP cells and performed RNA-seq after ligand treatment. AR was activated by synthetic ligand R1881 whereas E2 and Ly3201 were used to activate ERβ. Estradiol is a common ligand for ERα, ERβ, and GPER1 [206] whereas Ly3201 is known to bind only ERβ. Overexpression of ERβ had very little impact on LNCaP transcriptome representing just over 1% of total transcript change. Closer examination of data showed majority of DMSO regulated transcripts were also regulated by ERβ ligands in the same direction suggesting an effect of residual estrogenic activity in DCC-FBS. But treatment with E2 or Ly3201 resulted in altered expression of thousands of genes in ERβ expressing cells .

Next, we performed Gene Set Enrichment Analysis (GSEA) of differentially expressed transcripts from RNA-seq to identify class of genes that were over-represented in our data. As expected, GSEA results revealed highest positive

enrichment score for ER-response genes. The other pathways with significant positive enrichment score were mTORC1 signaling, unfolded protein response, E2F targets, G2M checkpoint, protein secretion, UV-response up, oxidative phosphorylation and mitotic spindle. The pathways in negative enrichment score were androgen response, hypoxia, p53 pathway, glycolysis, epithelial to mesenchymal transition (EMT), KRAS signaling up, UV response down, and Wnt/ β-catenin. Topmost negative enrichment score was for androgen signaling which is of particular interest regarding major role of AR in prostate cancer.

We independently validated some of the genes in androgen signaling pathway using RT-PCR and western blot. Androgen receptor was downregulated both at mRNA and protein level, as were AR targets such as FKBP5, CaMKK2, and TBC1D4 indicating inhibition of AR activity. We further confirmed the inhibition of AR activity by ERβ in reporter assays in LNCP and 22RV1 cells. GSEA results and follow-up studies on the expression and activity of AR and target genes provide evidence that ERβ exerts tumor suppressive effect in prostate cancer through the inhibition of androgen signaling.

TBC1D4 is the most ERβ downregulated gene which is a Rab GTPase-activating protein involved in the membrane localization of Glut4 in response to insulin stimulation [207]. It is an androgen upregulated gene and the major regulator of glucose uptake in prostate [208]. Another AR upregulated gene CaMKK2 which activates AMPK activity by phosphorylation [209] was also significantly down regulated by ERβ. AMPK is important for regulating cellular energy homeostasis by

activating glucose and fatty acid uptake and oxidation [210]. CaMKK2 was reported as a key downstream target of AR in coordinating prostate cancer cell growth through cellular metabolism [211]. Thus, our results indicate that ERβ may interfere with the glucose metabolism of prostate cancer. The two most ERβ upregulated genes FADS1 and KRT19 are also upregulated by AR indicating overlap in gene regulation.

In addition to regulating AR, GSEA and additional gene ontology analysis of RNA-seq data revealed an enrichment of genes involved in cancer-related processes, including apoptosis, response to hypoxia, KRAS signaling, and key metabolic pathways. Another pathway that can drive prostate cancer is CYP epoxygenases which catalyze the formation of epoxyeicosatrienoic acids (EETs) from arachidonic acid. The CYPS involved belong to family 4A, as well as 2U2 and 2J2 [212, 213]. CYP2U2 catalyzes the conversion of arachidonic acid into two bioactive compounds, the 19- and 20-HETE. Fatty acid epoxides are short-lived because they are hydrolyzed to less active or inactive dihydroxyeicosatrienoic acids by soluble epoxide hydrolases [214]. Thus, the activity of these epoxides are also dependent upon the expression levels of epoxide hydroxylases which are being regulated by ERβ .

Yet another pathway factor associated with increased risk of prostate cancer is reduction in vitamin D. The CYP involved in the first step of the activation of vitamin D, is the 25-hydroxylase (CYP 2R1). In addition to being the precursor of the active hormone, 1, 25(OH)2D3, 25-hydroxy vitamin D has actions of its own in the prostate. It is involved in keeping metabolism in the prostate in the normal mode i.e., oxidative

phosphorylation predominating over glycolysis. A reduction of oxidative phosphorylation occurs when prostate cells become malignant [215].

Another interesting finding is cytochromes P-450, involved in the formation of epoxides from fatty acids and the synthesis of 25-hydroxy vitamin D [216], which was reduced to 50 % and the lysophosphatidic acid receptor LPAR3 to 25% of levels in untreated cells. These findings reveal that even in malignant cells, introduction of ERβ down regulates AR signaling as well as other possible drivers of prostate cancer such as fatty acid epoxygenases, (lysophosphatidic/ GPR pathway) and vitamin D synthesis. Whether the effects of ERβ on these genes are the consequences of its interaction with AR or through independent mechanisms remain to be determined. Nonetheless, these findings reveal that re-expression and activation of ERβ can suppress oncogenic mechanisms in androgen-responsive cancer cells. These findings reveal possible early therapeutic interventions in androgen-responsive prostate cancer through activation of ERβ.

3.4 Materials and Methods

3.4.1 Reagents and Cell Culture

The LNCaP and 22RV1 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI-1640 (Invitrogen Inc., Carlsbad, CA) medium supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), and Antibiotic-Antimycotic (Invitrogen Inc., Carlsbad, CA) at 37 °C and 5% CO_2. Phenol red-free medium supplemented with 10% dextran coated charcoal

(DCC) treated fetal bovine serum (DCC-FBS) was used for ligand treatment. All experiments used cells below passage 30. Ly3201 was provided by Eli Lily company. R1881, 17β-estradiol, DHT, and 4OH-tamoxifen were purchased from Millipore Sigma (St. Louis, MO).

3.4.2 Stable expression of ERβ in LNCaP cells

Early passage of LNCaP cells were infected with the lentivirus Lenti6-TOPO-V5-D empty or containing cDNA for human ERβ1 at 2 M.O.I. (multiplicity of infection). Cells were selected with 5 μg/ml blasticidin for at least two weeks. The cells infected with empty virus vector were used as control in all experiments.

3.4.3 RNA extraction and real-time PCR

RNA extraction was performed with Qiagen mRNA extraction kit according to manufacturer's protocol from cells grown in 6-well plate. cDNA was synthesized from 1 μg of total RNA with iScript first strand cDNA synthesis kit according to manufacturer's protocol (Bio-Rad Laboratories, Inc. CA). Real-time PCR was performed with iTaq Universal SYBR Green supermix (Bio-Rad Laboratories, Inc. CA) on a 7500 Fast Real-Time PCR System (Applied Biosystems) using optimized conditions for SYBR Green I dye: 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Optimum primer concentration was determined in preliminary experiments, and amplification specificity confirmed by melt curve. Relative gene expression was calculated using ΔΔCt using GAPDH as normalization control. Primer sequences for the real-time PCR are listed in appendix

A. All primers were designed using NCBI primer-BLAST, and ordered from IDT (Coralville, IA).

3.4.4 RNA sequencing

Poly(A) mRNA was isolated using NEBNext poly(A) mRNA Magnetic Isolation Module. Libraries were prepared using NEBNext Ultra II RNA Library Prep Kit for Illumina. Sequencing was performed on NovaSeq 6000 with 150 bp paired-end reads. Treatments include three independent replicates.

3.4.5 Transcriptome Analysis

Reads were aligned to reference genome (GRCh38) indexes using STAR (v2.5) [217]. HTSeq21 (v0.6.1) [218] was used for mapped gene count quantification. Differential expression analysis was performed using DESeq2 (1.24.0) [219]. The resulting p-values were adjusted using the Benjamini and Hochberg's method. Genes with an adjusted p-value <0.05 found by DESeq2 were assigned as differentially expressed. Venn diagrams and heat map were prepared in R. Gene set enrichment analysis (GSEA) was performed using rankings based on the test statistic from differential expression analysis and the hallmarks gene set (h.all.v7.0.symbols.gmt) [220]. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the cluster Profiler R package, in which gene length bias was corrected. GO terms with corrected p-values less than 0.05 were considered significantly enriched by differentially expressed genes.

3.4.6 Protein extract preparation

To prepare whole-cell extracts, cells were washed twice with cold PBS, scraped with a rubber policeman in PBS and cell pellet collected by centrifuging at 3000 g for 5 min. Cells were suspended in 10 times packed cell volume of RIPA lysis buffer [10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS and 140 mM NaCl] supplemented with protease inhibitor cocktail, and PhosStop (Roche, Indianapolis, IN)], syringed 10 times through narrow needle and then centrifuged at 14,000 rpm for 10 min. Clear supernatant was transferred to a new centrifuge tube and protein concentration measured using Pierce 660 nm protein assay kit (Thermo Fisher Scientific, Waltham, MA). Samples were prepared by boiling with 4X loading dye and β-mercaptoethanol.

3.4.7 Western blotting

Fifty micrograms of protein were loaded on an SDS-PAGE 4-20% Bis-Tris gel with Tris running buffer and transferred to a PVDF membrane after electrophoretic separation. Membranes were blocked with 5% non-fat powdered milk in 0.1% TBST buffer and probed with anti-AR (sc-816), HSD11B2 (sc-365529), GAPDH-HRP (sc-47724), p57 KIP2 (sc-1037), E-cadherin (sc-7870), Cytokeratin 19 (sc-6278), TGFBR3 (sc-74511) (Santa Cruz Biotechnology,Inc., Santa Cruz, CA), Jagged 1 (70109T), RhoB (63876S), AMPK (2532S), pAMPK (2535S), FKBP5 (122105) (Cell Signaling Technology Danvers, MA), CAMKK2 (H00010645-M01) (Abnova Taipei, Taiwan), β-Actin (A1978) (Sigma Millipore Sigma, St. Louis, MO). Primary antibodies

were used at 1:200–1000 dilutions, and secondary antibody was used at 1:10,000. The western blot experiments were repeated at least three times.

3.4.8 Luciferase assay

LNCaP and 22RV1 cells were grown to 70% confluency in 6-well plate and transfected with one microgram of the ARR2 luciferase construct along with or without 0.5 µg ERβ expression vector in 10% DCC-FBS medium. 24 hrs post transfection, cells were treated with R1881 1 nM plus DMSO (vehicle control) or Ly3201 10 nM in fresh 10% DCC for 24 hrs more. Next day media aspirated and 200 µl lysis buffer was added to cells in each well and collected lysates were centrifuged at 14000 rpm for 10 min. Fifty µl of cleared lysate was used to measure luminescence on a Perkin-Elmer (Waltham, MA) Victor X luminescence plate reader using luciferase kit from BioVision (Milpitas, CA).

3.4.9 Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 (v 8.3.0) software. Student's t-test and one-way ANOVA were used for statistical analysis. Results were considered significant with p-value $\leq$ 0.05.

Chapter 4

Key Findings and Conclusion

The estrogen receptor β (ERβ) was discovered and cloned in 1996 from rat prostate [73]. ERβ is established as a tumor suppressor and inhibits proliferation, induces apoptosis and maintains differentiation in the prostate [91,95,100]. Genomic deletion of ERβ in mouse prostate led to epithelial hyperplasia, as well as increase in the number of intermediate luminal cells . Additionally, older ERβ knock-out mice developed prostatic intraepithelial neoplasia (PIN) lesions [91]. Expression of ERβ in PC3 and 22Rv1 cells was found to significantly decrease proliferation by down regulation of oncogenic factors c-Myc, p45 Skp2, and cyclin E and up regulation of p21, and p27 KIP protein [95]. Our lab previously reported that ERβ transcriptionally up-regulated FOXO3a and induced apoptosis in prostate cancer cell lines PC3, LNCaP, and 22RV1 upon treatment with ERβ specific ligands 3β-Adiol, 8β-VE2, and DPN [100].

To further understand ERβ regulated pathways in prostate cancer, we investigated the role of ERβ in prostate cancer cells PC3, BPH1, and LNCaP. Using lentivirus, we made these cells to stably express ERβ1 and found that ERβ induced expression of INPP4B in PC3 and BPH1 cells. We also found that ERβ inhibited Akt activity in PC3 cells by inhibiting phosphorylation of Ser473. INPP4B is known to regulate Akt activity and to establish a cause and effect relationship between INPP4B induction and pAkt inhibition, we reduced the level of INPP4B in ERβ expressing cells using siRNA and found increased Akt phosphorylation at Ser473. Akt activity is known to

affect myriad of biological activities including survival, proliferation and migration. We observed that ERβ expressing PC3 cells migrated slower than control cells in wound healing assay. Again, using siRNA against INPP4B, we showed that this effect is mediated through INPP4B and Akt activity. To understand the mechanism of INPP4B induction by ERβ, we performed ChIP-seq of stable PC3 cells and found two ERβ binding regions in INPP4B intron which we cloned into reporter vector and performed luciferase assay. ERβ regulated transcription from each enhancer in a ligand dependent manner. We also performed Possum analysis of 500 bp sequence of these enhancers and found EREs and other motifs being enriched.

Androgen receptor is the main driver of prostate cancer both in primary and metastatic cases [35]. By using RNA-seq, we analyzed the transcriptome of LNCaP cells stably expressing ERβ and found thousands of genes being differentially regulated. We performed gene set enrichment analysis (GSEA) of differentially expressed genes and found androgen receptor signaling to be the most downregulated pathway. We independently validated some AR downstream targets using both RT-PCR and western blot in LNCaP cells. While androgen receptor was modestly repressed at mRNA and protein level, its downstream targets FKBP5, TBC1D4 and CaMKK2 were highly reduced both at mRNA and protein level. CaMKK2 is an upstream kinase for AMPK which regulates cellular glucose and fatty acid uptake and oxidation. We demonstrated that ERβ mediated suppression of CaMKK2-inhibited AMPK activation.

Androgen receptor regulates growth and maintenance of normal prostate tissue but is also responsible for initiation, progression and castration resistance of prostate cancer [35]. Prostate cancer related deaths occur in patients following castration resistance and metastasis [1]. In prostate cancer, AR function is altered due to a 'molecular switch' which causes gain-of-function alterations in the receptor. As a consequence, AR function changes from regulating differentiation to driving proliferation in the luminal epithelial cells [36]. In a subset of castration-resistant prostate cancer (CRPC), AR expression is increased which can be activated with sub-physiological level of androgens and show resistance to antiandrogens such as bicalutamide [39,40]. Our results show that ERβ can decrease the expression of AR in prostate cancer and may enhance the efficacy of antiandrogens in CRPC.

ERβ is expressed in both basal and luminal cells of the prostate [90, 91] and is known to regulate AR activity through upregulation of DACH1/2 (a co-repressor of AR), and downregulation of RORc (a driver of AR) in mouse prostate [94]. Although our results did not overlap with studies in ERβ knock-out mice, we did show that ERβ inhibited AR activity. We assume that non-overlap in ERβ function in a normal mouse prostate and tumor cells is due to altered AR function in prostate cancer. Activated ERβ down-regulated well-known AR target genes FKBP5, CaMKK2, and TBC1D4 in LNCaP cells. CaMKK2, a key downstream target of androgen receptor, is emerging as a therapeutic target for controlling metastatic prostate cancer [48]. CaMKK2 regulates metabolic activity of prostate cancer cells by regulating the function of

AMPK. We showed that ERβ-inhibited phosphorylation of AMPK, a central energy sensing pathway in all types of cells.

AR can be activated through phosphorylation by hyperactive PI3K/Akt pathway in prostate cancer [44]. In Chapter 2 of this dissertation, we showed that ERβ suppressed hyperactive Akt in PC3 cells. This may be another mechanism whereby ERβ may restrain AR function in prostate cancer.

Our results also show that expression and activation of ERβ in LNCaP cells not only opposes AR function but also overlaps in regulating some target genes. FADS1 and KRT19 are the two most ERβ upregulated genes which are also upregulated by AR. The up-regulation of KRT19 by ERβ, a luminal differentiation marker, may help explain the observation of increased intermediary luminal cells in ventral prostate of ERβ knock-out mice [91].

Through multiple studies we demonstrated that ERβ functions as a tumor suppressor in prostate cancer cells which may indicate potential mechanisms of ERβ activity in prostate cancer itself. We showed that ERβ inhibits Akt activity as well as AR signaling which are the two most oncogenic signals in prostate cancer development and progression. We suggest that activation of ERβ in prostate cancer may be a potential therapeutic target.

Appendix A: List of Primers Used for RT-qPCR

Target	Forward	Reverse
GAPDH	5’-TGACAACTTTGGTATCGTGGAAGG-3’	5’-AGGCAGGGATGATGTTCTGGAGAG-3’
panERβ	5’-AGAGTCCCTGGTGTGAAGCAA-3’	5’-GACAGCGCAGAAGTGAGCATC-3’
ERβ1	5’-GTCAGGCATGCGAGTAACAA-3’	5’-GGGAGCCCTCTTTGCTTTTA-3’
INPP4B	5’-AGAGCTTTAGATTGCATGAGAAGAGA-3’	5’-CCTCCTGCATTTGATATTCTTCAGT-3’
AR	5’-TCACCAAGCTCCTGGACTCC-3’	5’-CGCTCACCATGTGTGACTTGA-3’
FKBP5	5’-ATTGGAGCAGGCTGCCATTGTC-3’	5’-CCTGCATGTATTTGCCTCCCTTG-3’
CAMKK2	5’-TCCAGACCAGCCCCGACATAG-3’	5’-CAGGGGTGCAGCTTGATTTC-3’
HSD11B2	5’-GGCCAAGGTTTCCCAGTGA-3’	5’-CAGGGTGTTTGGGCTCATGA-3’
TBC1D4	5’-CTTTGATATCCCGGGTGCGT-3’	5’-CCGTCCAGACTGCTTGCTTA-3’

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