TUMOR-PROMOTING EFFECTS OF GENISTEIN AND ERβ IN PROSTATE CANCER

by

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Abstract

Genistein is an isoflavone found in soy, and its chemotherapeutic effects have been well established from *in vitro* studies. Recently, however, its therapeutic actions *in vivo* have been questioned due to contradictory reports from animal studies, which rely on rodent models or implantation of cell lines into animals. Using patient-derived prostate cancer xenograft models, in which clinical prostatectomy samples were grafted into immune deficient mice, this study showed that genistein promoted metastatic progression *in vivo*. To test if the metatstasis-promoting effects of genistein may be mediated via ERβ activation, we treated the xenografted mice with genistein, an anti-estrogen compound (i.e. ICI 182 780) or a combination of both. The results showed that anti-estrogen treatment significantly decreased metastatic spread compared to genistein, which promoted lung metastasis in a dose-dependent manner. Gene expression analyses showed that genistein and anti-estrogen treatments targeted the same signaling pathway but different molecules, producing opposite effects on tumour biology. Genistein stimulated expression of upstream molecules that reside in the Focal Adhesion Kinase (FAK) pathway, while anti-estrogen down-regulated downstream molecules within the same pathway.

Further analysis of the microarray data revealed a unique set of genes that were up-regulated by genistein and also were down-regulated by ICI 182,780. Five out of the six genes identified from this comparison belonged to the metallothionein (*MT*) gene family. Using qRT-PCR, the changes in expression levels were validated in metastatic and non-metastatic tumour lines of LTL313b, both of which had been derived from the same PCa patient, indicating a strong association between *MT* gene expression and prostate cancer metastasis.

In summary, genistein-activated-ERβ promotes metastasis in two ways; genomic and non-genomic pathways. In the non-genomic pathway, ERβ stimulates kinase signaling pathways,

leading to cell survival and increased motility. In the genomic pathway, ERβ increases *MT* and/or other metastasis-associated gene expression, which can be inhibited by anti-estrogen treatment.

This study has demonstrated that genistein elicits cancer promoting effects *in vivo* and that ERβ is important in metastatic progression of human PCa. The significant inhibition of metastasis by anti-estrogen treatment shown here potentiates a promising new selective estrogen receptor modulator treatment for metastatic patients.

Preface

All experiments were conducted by H. Nakamura except for the gene expression microarray and the HPLC-MS, which were performed by A. Haegerts and H. Adomat from the Vancouver Prostate Centre, respectively. For tumour xenografting, technical assistance was provided by Y. Wang and H. Xue of the Dr. Y. Wang lab. Dr. M. Romanish provided assistance in the vector construction, using reagents obtained from the Dr. D. Mager lab.

Animal care and experiments were carried out in accordance with the guidelines of the Canadian Council of Animal Care (CCAC), and the use of animals for our experiments was examined and approved by the Animal Care Committee of University of British Columbia (permit #: A10-0100).

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List of Abbreviations

- AR: androgen receptor
- ABC: avidin-biotin complex
- BCA: bicinchoninic acid
- ER: estrogen receptor
- PSA: prostate-specific antigens
- LHRH luteinizing hormone-releasing hormone
- FSH: follicle-stimulating hormone
- GnRH: gonadotropin hormone–releasing hormone
- CRH: adrenocorticotropin-releasing hormone
- ACTH: adrenocorticotropic hormone
- SHBG: sex-hormone-binding globulin
- DHT: 5-α-dihydrotestosterone
- ARE: androgen response element
- PCa: prostate cancer
- BPH: benign prostatic hyperplasia
- PIN: prostatic intraepithelial neoplasia
- AI: androgen-insensitive
- VACURG: Veterans Administration Cooperative Urological Research Group
- *GSTP1*: glutathione S-transferase gene
- PIP_3: phosphatidylinositol 3,4,5-trisphosphate
- PIP_2: phosphatidylinositol 4,5-biphosphate
- PSCA: prostate stem cell antigen
- ETS: erythroblast transformation–specific
- ECM: extracellular matrix
- FAK: focal adhesion kinase
- PTK2: protein kinase 2
- ITG: integrin
- VN: vitronectin
- EMT: epithelial to mesenchymal transition
- EGFR: epidermal growth factor receptor
- TGFα: transforming growth factor α
- μPA: urokinase plasminogen activator
- BCa: breast cancer
- Src: non-receptor tyrosine kinase
- ADT: androgen ablation therapy
- CRPC: castration-resistant prostate cancer
- CYP17: cytochrome P450 enzyme
- DES: diethylstilbestrol

- ARKO: aromatase-knockout
- SERM: selective estrogen receptor modulators
- TRAMP: transgenic mouse model for PCa
- RAL: raloxifene
- GR: glucocorticoid receptor
- MR: mineralocorticoid receptor
- PR: progesterone receptor
- DBD: DNA binding domain
- LBD: ligand-binding domain
- ERE: estrogen response element
- SRC-1: steroid receptor co-activator 1
- GRIP1: glucocorticoid receptor interacting protein 1
- AP1: activator protein 1
- SP1: specificity protein 1
- ERKO: estrogen receptor knockout
- K1 cells: carcinogen-induced accessory sex gland carcinoma cells
- NOD-SCID: non-obese diabetic, severe combined immune-deficient
- RWPE: non-cancerous prostate epithelial cell line
- PTK: protein tyrosine kinases
- ERK: extracellular signal regulated kinase
- SCC: squamous cell carcinoma
- MT: metallothionein
- IAP: inhibitor of apoptosis protein
- BIR: baculoviral IAP repeat
- MMP: matrix metalloproteinase
- MAPK: mitogen-activated protein kinase

Acknowledgements

In the journey to completing my PhD degree, I feel very fortunate to be given this opportunity and privilege of higher learning at one of the well-respected Canadian academic institutions.

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I appreciate all the technical support of the lab members and friends at BCCRC and the Prostate Centre for making the lab a fun place to work at. I give special thanks to Dr. Mark Romanish for his support and encouragement throughout my PhD program. His encouraging words have made me realize the importance of my study and given me confidence to carry on and to never give up. This accomplishment is, therefore, the result of love and support of my mentors, friends and family. Without you, this was not possible, so thank you all very much.

Dedication

The very reason for who I am today and for what I have achieved in my life, I owe it all to my loving parents. I dedicate this highest achievement of my life to you, お父さん&お母さん. And to all the people in the world who have been touched by cancer and their family members, I dedicate to you all.

Chapter 1: INTRODUCTION

Cancer is a devastating disease that afflicts an increasing number of individuals, families and communities at the global level and contributes to worldwide mortality. Since the identification of this disease, an immeasurable amount of effort has been placed on research and public education to combat this pandemic.

Cancer results from accumulation of multiple insults placed upon or within genetic materials over many years. Such damage creates genome instability by inducing mutations in critical genes involved in DNA repair, generating heterogeneity among cancer cells that is one of the unique characteristics of this disease. According to the classic hallmarks of cancer proposed by Hanahan and Weinberg, normal cells acquire neoplasticity through the following eight properties; prolonged proliferation, evading growth suppression, apoptosis inhibition, unlimited replication potential, angiogenesis, invasion and metastasis, altered energy metabolism, and immune destruction resistance [1]. Carcinogenesis is, therefore, not a simple process of uncontrolled cell growth but an elaborate acquisition of events that occur locally within and between cells, whose actions are supported by the tumour microenvironment. Metastasis is systemic progression of the disease in which neoplastic cells acquire the ability to invade, migrate and to establish themselves in other parts of the body. It is at this stage of progression that cancer claims the lives of the majority of patients.

According to the National Cancer Institute, there are more than 100 different types of cancer that exist in humans, and they are categorized according to the cellular or tissue origin as summarized below. Carcinoma: epithelial cell origin. Adenocarcinoma: glandular epithelium tissue origin. Sarcoma: connective tissue origin including bone, fat, muscle, cartilage and other

soft tissues. Leukemia: white blood cell or bone marrow origin. Lymphoma: lymphoid cell origin. Other cancers include those which arise from the central nervous system such as the brain and spinal cord.

There are a number of risk factors associated with human cancers such as genetic, viral, and environmental (chemical exposure, diet and physical activity) factors. With the exception of childhood cancers such as leukemia and lymphoma, cancer is, for the most part, considered as an age-related disease, thus the rise in incidence and mortality rates is expected to continue as the population age of developed nations increases. Prostate cancer (PCa) is one of the many forms of cancer that are on the rising trend, and it is the second leading cause of cancer-related deaths in the Western men [2,3]. Therefore, much needed improvements in therapeutics have been the focus of current research.

1.1 The prostate

The prostate is a fibromuscular exocrine gland of the male reproductive system. It is located underneath the bladder and functions to secrete an alkaline, proteolytic solution that forms part of the seminal fluid and is important for the survival of sperm in the vaginal environment [4,5]. The adult prostate normally reaches a walnut-size and is divided into anatomically recognizable zones, known as the periurethral transition zone (TZ), the peripheral zone (PZ), the central zone (CZ) and the anterior fibro-muscular zone (or stroma) [6].

The epithelial compartment of the prostate is composed of three cell types: basal, luminal and neuroendocrine [5,7]. In the normal human prostate, cuboidal basal cells form a continual layer on the basement membrane, which separates the epithelium from stroma [5,7]. Luminal cells make up the majority of prostatic epithelium and surround a glandular lumen into which they secrete prostate-specific proteins such as prostate-specific antigen (PSA) and prostatic acid phosphatase [5,7,8]. The luminal cells respond to androgen stimuli and express the androgen receptor (AR), cytokeratin 8 and 18 and a distinct set of markers such as Nkx3.1 and prostate-specific-secretory proteins [9,10]. In comparison, basal cells weakly express AR and mainly express cytokeratin (K14) and p63 [3] [11,12]. The function of basal cells is to maintain the glandular/ductal structure, survival and function of luminal cells [7]. The neuroendocrine cells form a minor constituent of the prostatic epithelium, and their origin is being debated. Some argue endoderm origin, while others believe basal cell origin [13]. Their function remains unclear but is speculated to be involved in the growth, differentiation and secretory function of the prostate [13]. The stroma is composed of a mixture of cells: fibroblasts, smooth muscle cells, endothelial cells, nerves and lymphocytes [14]. Proper growth and development of the normal prostate relies heavily on paracrine interactions between the stroma and epithelium [14].

Testosterone, the male steroid sex hormone (androgen), and its receptor are critical factors in the stroma-epithelial interaction necessary for growth and function of the prostate [14]. Testosterone is produced by Leydig cells in the testes, and its production is closely regulated by the brain, hypothalamus and pituitary, and the hormones released by these organs [15]. When luteinizing hormone-releasing hormone (LHRH) is secreted by the hypothalamus, it stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland, which in turn stimulate the testes to produce androgens [15,16]. 90-95% of testosterone in the human body is produced by the testes, and the remaining small proportion is synthesized by the adrenal glands [15]. In the circulation, testosterone is bound to serum proteins such as albumin and sex-hormone-binding globulin (SHBG), and only a small fraction is present in the free form [14]. When testosterone reaches target (prostate) cells, 90% is converted by cell-associated 5-α reductase to 5-α-dihydrotestosterone (DHT), the active form of testosterone with a higher affinity for AR [17]. Binding of testosterone or DHT to AR induces androgen-responsive gene transcription [14,15], which regulates a variety of male-specific, physiological responses [17].

The AR was first discovered as a specific androgen-binding protein by several groups including Mainwarning, Liao and Baulieu in late 1960's [18-20] and cloned from human testis and rat prostate cDNA libraries by Chang, Lubahn and Trapman in 1988 [21-23]. The *AR* gene, which is composed of eight exons, is located at Xq11.2-12 [24].

This steroid receptor protein has two independent transcription-activating domains, AF-1 and AF-5 at the amino-terminus, which also act to bind ligand-binding domain [24-26]. This amino- and carboxy-interaction is necessary for stabilization of the AR dimer and makes the complex in-accessible for degradation [27,28]. The carboxy-terminus harbours a ligand-binding

domain (AF-2), which induces transcription of androgen-specific genes [24,29]. Upon hormone binding to the hydrophobic core, the amino acid helix 12 folds or orients to create a binding site for coactivators [24,29]. In between these amino- and carboxy-transcription-activation domains exist a zinc-finger-containing DNA-binding domain and hinge region, which are responsible for DNA-binding and nuclear localization, respectively [14].

In its inactive state, the AR is tightly bound to heat-shock proteins which prevent nuclear translocation of the receptor [14]. Testosterone or DHT binding induces a conformational change to AR, which leads to dissociation of the heat-shock proteins and translocation to the nucleus where dimerized receptors can bind to a specific DNA region, called androgen response element (ARE), in the promoter region of target genes [14]. Depending on co-regulators that bind to the transcriptional machinery of the AR complex, transcription of the target gene is suppressed or stimulated [14]. Androgens and androgen receptor are responsible for development and differentiation of Wolffian duct-origin structures including epididymides and seminal vesicles as well as urogenital synus-origin structures such as prostate, urethra and penis during development [24].

As androgens are responsible for sex differentiation and spermatogenesis in males, their ability to stimulate cell proliferation and inhibit apoptosis are exceedingly important factors in the development of prostate cancer [7]. As observed during androgen ablation, castration induces regression of the prostate, which is characterized by loss of luminal cells, reduced secretory protein production and ductal size [7]. Despite loss of the majority of the luminal cells, the regressed prostate is enriched in p63-positive basal cells and maintains structural integrity [7]. However, proper restoration of prostatic glands occurs only after re-introduction of androgens.

This precise regression and regeneration mechanism of the prostate is regulated by the paracrine interaction between stroma and epithelium.

Although androgen is required for cell proliferation in the developing and regenerating prostate, it is questionable whether stimulation of cell proliferation is the sole function of androgens in the adult prostate, as epithelial cells of developing or regenerating prostates become quiescent upon reaching a certain size [7]. The role of androgen may shift more toward the differentiation and maintenance of the prostate after puberty or adulthood.

1.2 Prostate cancer (PCa)

The adult prostate is divided into separate zones, TZ, PZ, CZ, and stroma, as mentioned in the above section [6]. Depending on the prostate zone, the risks for developing benign and malignant diseases differ [8]. For example, benign prostatic hyperplasia (BPH), which can be seen in > 70% of men over the age of 60 is found in the TZ, where it is closer to the urethra [8,30]. A small proportion of carcinomas (~20%) found in the TZ is usually of low grade [8]. In contrast, most (70-80%) premalignant and malignant lesions are found in the PZ, which is located on the posterior/lateral orientation of the prostate [8].

Although there is no direct link established, PCa is believed to progress through multiple stages: prostatic intraepithelial neoplasia (PIN), high grade PIN, invasive cancer, and finally the castration-resistance state [31-34]. The cancer stem cell theory hypothesizes that the emergence of castration resistant cancer originates from the presence of 'seeding' cells that are androgen-insensitive (A.I.) within the primary tumour, and as most cells undergo cell death during androgen ablation, A.I. cells survive and proliferate [35,36]. Another theory hypothesizes that

A.I. cells are acquired through mutations occurring during androgen ablation, and that only those cells survive and undergo *de novo* clonal expansion [35-37].

The biological mechanisms of castration resistance acquisition by prostate cancer are well described by Feldman and others:

1) hypersensitivity of AR: PCa cells become more sensitive to low levels of androgen via AR amplification or increased 5-α reductase activity.

2) promiscuity: AR mutations allowing multiple ligands to bind and activate the receptor.

3) the outlaw pathway: ligand-independent activation of AR by which receptor tyrosine kinases such as EGFR and MAPK can phosphorylate and activate the receptor in the absence of androgen.

4) the bypass pathway: AR-independent pathway in which PCa cells proliferate and avoid apoptosis by up-regulating pro-survival genes or anti-apoptotic genes.

5) the lurker cell theory: androgen-insensitive PCa cells exist prior to androgen ablation therapy and will be clonally selected after the hormonal treatment [14,38].

6) cytokines: IL-6 is released after androgen ablation and promotes cell proliferation and inhibits apoptosis via Stat 3 pathway [39]. IL-8 has been shown to transform androgen-dependent PCa cells into castration-resistant cells [40].

7) neuroendocrine (NE) cells: an increased number of neuroendocrine cells are reported in CRPC [41]. Enrichment of NE cells in CRPC may result from elimination of cancer cells that are sensitive to androgen ablation, leaving AR-null NE cells within tumour. These cells secrete neuropeptides such as bombesin (gastrin-releasing peptides and neuromedin B), which stimulates cancer cell growth [41,42].

8) intracellular androgen biosynthesis: CRPC cells have higher levels of metabolic enzymes such as HSD3B and AKR1C3 which can synthesize androgens from cholesterol or other precursors [41].

9) AR isoforms: splice variants of AR which the lack ligand-binding domain but retain transactivation and DNA binding domains are known to exist in CRPC and promote proliferation in androgen deprived conditions [43].

1.2.1 Stages of PCa

The degree of PCa progression can be assessed based on clinical and histological staging or score. The TNM staging system assesses the clinical state of PCa at a given time, evaluating primary tumour (T), the lymph node status (N) and distant metastasis (M). It is used to assist in prognosis and appropriate treatment options for the patient [44]. For PCa, it was first developed in the late 1960s and early 1970s; however, it was not till the early 1990s that it became internationally established [45,46].

TNM Score	Clinical Description
T0	No evidence of tumour
T1	Clinically no obvious tumour (cannot be detected by digital rectal exam)
T2	Tumour confined within prostate gland
T3	Tumour spreads through peri-prostatic tissue but does not invade into other organs
T4	Tumour invasion into adjacent/other organs
N0	No spread to lymph nodes
N1	Evidence of cancer in lymph nodes
M0	No distance metastasis
M1	M1a: spread to lymph node. M1b: to bone. M1c: to other secondary organs.

Table 1. TNM staging system of prostate cancer, describing clinical assessment of prostate cancer.

The Gleason grading system was developed by a pathologist, Dr. Donald F Gleason, and a panel of the Veterans Administration Cooperative Urological Research Group (VACURG) in the 1970s [47]. Using five basic histological patterns, a score between two to ten is given. Because PCa is thought to be heterogeneous, PCa patients often present with various degrees of histopathological lesions within a tumour [47]. In fact, 14-18% of PCa patients have been reported to have more than two grades of cancer and 3% having four histologically different patterns [47-49]. A primary score from a dominant histological pattern is added to the secondary score from the second common pattern within the biopsied sample to give the total Gleason score to reflect the whole tumour [47]. If only one type of histology is viewed, then the primary score is multiplied by two [47].

Gleason Score	Histopathological Characteristics	Gland size
1	Nodular, well-defined, oval, uniform, closely packed but separate glands.	medium
2	Less-defined, oval, separate but non-uniform size and shape of glands	medium
3	Glands with ill-defined edges, more variation in size and shape with papillary and cribriform epithelium	small to medium
4	Infiltrative fused glands	small to medium
5	Papillary, cribriform or anaplastic with small glands	variable, but usually small

Table 2. Gleason scoring of prostate cancer.

The five histological patterns are described above. A total score representing the whole tumour or biopsy sample is given by adding two scores from two common histological patterns.

1.2.2 Risk factors

Prostate cancer is considered an age-associated malignancy as its incidence and mortality rates increase exponentially after the age of 50 [50]. While autopsy studies found the presence of histologically-identified carcinoma in 15 ~ 30 % of men aged 50 years old, the number increased to 60 ~ 70% in men who were 80 years old or older [51-53].

In addition to age, there are other risk factors associated with PCa. For example, 5-10% of all PCa is thought to have genetic predispositions [54], and having a family history increases the risk by two to three fold [55]. Race is another risk factor for this disease. Carter *et al.* found that, in a comparison of African American men and Caucasians of similar socioeconomic status, the African Americans had a higher incidence at all age groups, more advanced disease and worse survival rates than Caucasians or their counterparts living in Africa [56-58]. While the highest rates are observed in African Americans, people of Asian descent living in the USA or in their native countries have the lowest risk [59,60]. Interestingly, however, there is a geographical difference in risk among Asians, as migrant studies show that Asians living in the Western countries tend to have a higher risk than their counterparts living in their native countries [31,32]. This highly suggests that environmental factors such as lifestyles and diet play significant roles in prostate carcinogenesis. For example, diets high in vitamin D and E, lycopene, and selenium are known to have protective roles, whereas high-fat and -red meat consumption is correlated with PCa risk [61-65].

1.2.3 Genes associated with PCa

The pathogenesis of PCa requires a number of somatic genetic changes such as point mutations, deletions, amplifications and translocations [66]. Moreover, epigenetic alterations such as histone modifications and DNA methylation, which affect gene expression without directly targeting the sequence, are frequently observed in PCa [66]. Such epigenetic modifications are thought to occur early and frequently in prostate carcinogenesis [66]. For example, DNA methylation of *GSTP1* occurs more consistently at an earlier stage of PCa development [67,68]. This gene encodes glutathione S-transferase, which is a detoxifying enzyme and protects cells from carcinogens [66]. Loss of this protein function would make cells susceptible to genetic damage. Hypermethylation of *GSTP1* is in fact reported in more than 90% of PCa cases [67,68]. Other genes silenced by hypermethylation in PCa include *APC, RASSF1a, PTGS2* and *MDR1* [69].

Some chromosomal alterations are also considered to take place early in prostate oncogenesis. Loss of 8p, for instance, is frequently reported in high-grade PIN lesions, which are thought to be precursors of PCa [66]. Among the many genes located on 8p, *NKX3.1* is thought to be a putative tumour suppressor gene [66] because it encodes a prostate-specific homeobox protein, which is involved in regulating normal prostate development, and exhibits reduced expression in PIN and PCa tissues relative to normal prostatic tissues [9,70].

Another commonly altered chromosome in PCa is 10q. A well-known tumour suppressor gene, *PTEN*, is located on 10q23 and is often mutated or deleted in PCa [66,71]. PTEN is a phosphatase, which negatively regulates the PKB/Akt pathway by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate (PIP_3) to generate phosphatidylinositol 4,5-biphosphate (PIP_2), the inactive form [66]. Loss of PTEN function results in increased cell proliferation and

reduction of apoptosis [72]. Other tumour suppressors such as *p53, RB* and *p16* are often lost relatively later during PCa progression as such losses are reported mainly in metastatic tumours [66].

Similar to chromosomal losses, gains in 7p, 7q, 8q and Xq are frequently observed in PCa [66]. Examples of such gains include amplifications of *MYC* and *PSCA* (prostate stem cell antigen) oncogenes located on 8q, as well as *AR* on Xq [14,73]. These gene amplifications are correlated with aggressive PCa phenotypes and the development of castration-resistant disease [66].

Besides losses and gains, chromosomes can also undergo re-arrangements. The most frequently observed gene fusion reported in PCa is *TMPRSS2* and *ERG* on chromosome 21q 22.2 [66]. The 5' untranslated region (UTR) of the androgen-regulated *TMPRSS2* gene is fused with the coding region of *ERG,* which encodes the erythroblast transformation–specific (ETS) transcription factor [74]. This fusion occurs in approximately 50% (ranging from 40% ~ 70% [74-77]) of all prostate cancers [74]. Understanding such molecular changes, whether genetic or epigenetic, could lead to the development of effective diagnostic and/or prognostic markers.

1.3 Metastasis

Metastasis is the process by which cancer cells with disseminating potential spread from a primary tumour to distant organs in the body [78]. Although many studies actively investigate the molecular signatures that are associated with metastasis, its underlying mechanisms remain largely unknown. In order for cancer cells to acquire invasive abilities, they must first detach from surrounding cells and the extracellular matrix (ECM), break through the basement

membrane, travel through and survive in blood or lymph and finally invade into and establish at a distant site [79].

Extracellular signaling of ECM received from surrounding cells is important in initiating the metastatic process and must be properly received by cell surface receptors such as integrins in order to propagate invasion signals [80]. It has been shown that ECM proteins such as fibronectin and vitronectin bind to integrins located on the cell membrane. This ECM protein-integrin binding activates a series of metastasis-associated molecules such as FAK, Src, Akt and Rho, leading to cytoskeletal rearrangement and increased matrix metalloproteinase expression, which are necessary for acquiring invasive ability [80-85].

1.3.1 Metastasis-linked molecules: focal adhesion kinase (FAK)

Focal adhesions are large protein complexes that connect the cytosol to the extracellular matrix and play an important role in cellular anchorage [86]. Focal adhesion kinase, also known as protein kinase 2 (PTK2), is a 125kDa non-receptor tyrosine kinase whose primary role is to regulate cell adhesion and cytoskeletal rearrangement [87,88]. It is concentrated in the region of cell attachment to the ECM along with other focal adhesion macromolecules such as actin, filamin, vinculin, talin, paxillin and tensin [89].

FAK has both extracellular and intracellular activation mechanisms. In PCa, FAK is known to be extracellularly activated via binding to integrins, growth factors, IL-8 and urokinase plasminogen activator (uPA), and invasion-associated molecules [88,90,91]. Intracellularly, FAK is activated via binding to Src; this association is said to be ‘reciprocal’, meaning that they can transactivate each other [88]. Once activated, FAK transduces signals intracellularly to distort cell adhesion and promote migration. Besides its primary role in cell motility, FAK is thought to

function in angiogenesis by increasing transcription of VEGF [88,92]. FAK is negatively regulated by PTEN, a well-known tumor suppressor with a phosphatase function, which is lost in advanced PCa [93].

The evidence from early studies suggests that FAK activation plays a central role in cell adhesion and migration. For instance, high phosphorylation/activation of FAK is observed in early embryonic development, and the cells isolated from FAK-deficient embryos have reduced motility *in vitro* [94]. In addition, mutation at tyrosine 397 (Y397), an autophosphorylation site of FAK, reduced cell migration of Chinese hamster ovarian cells, while overexpression of FAK protein led to increased migration [95]. In PCa, Y397-autophosphorylation is believed to be adhesion-dependent and not Src-dependent (ie, not intracelluarly activated), and is important in integrin-mediated cell motility in carcinoma of the prostate [88,94,96,97].

1.3.2 Integrin and its ligand, vitronectin

It is known that ligand-binding of the integrin heterodimer, ITGα5β3, induces autophosphorylation of FAK at Y397, which increases its kinase activity [98,99]. The cytoplasmic amino and carboxyl regions of β3-integrin are critical domains for binding and phosphorylating FAK [100,101]. This FAK-associated ITGα5β3 assists in metastatic progression as this complex mediates angiogenesis as well as cell adhesion and migration processes [85]. During angiogenesis, endothelial cells express this integrin subunit heterodimer, which binds to ECM proteins facilitating invasion of endothelial cells into the tumor microenvironment for *de novo* vessel synthesis [102-104].

Vitronectin (VN) is an adhesive glycoprotein found in serum and extracellular matrix, commonly expressed in skin, mature bone and stroma of wounded tissue [105,106]. It is also a

known ligand for ITGα5β3 [105,106]. The interaction of VN and ITGα5β3 is very important in cell migration. As demonstrated in a study by Zheng *et al.*, cultured human prostate cancer PC3 cells and isolated clinical PCa cells, which expressed ITGα5β3, adhered to VN and showed enhanced migration abilities, whereas ITGα5β3 -ve LNCaP and benign cells did not show mobility in a migration assay [105]. Interestingly, transfection of ITGα5β3 into LNCaP cells allowed the low-invasive cells to gain a migratory phenotype via FAK activation [105].

1.3.3 Integrin expression

In human cancers, aberrant expression of integrins has been reported [34]. Normally, an ITGα5β3 heterodimer complex is not expressed in benign epithelial cells; however, it is reported to be expressed in bone-metastasized breast cancer (BCa) cells, metastatic melanoma and ovarian cancers [107-109]. Although ITGα5β3 is highly expressed in the PC3 cell line, compared to a less aggressive cell line such as LNCaP [279], its expression level in clinical PCa remains to be determined due to difficulties in collecting metastatic specimens. It can be hypothesized, however, that this integrin heterodimer plays a significant role in PCa progression as ~90% of PCa metastases have been found in bone where VN, the integrin-ligand protein, is found [105,110,111].

1.3.4 Epithelial to mesenchymal transition (EMT) and ITGα5β1

Epithelial to Mesenchymal Transition (EMT) is a hallmark of metastatic progression and is characterized by a change in cell adhesion, migratory and invasive abilities [112]. Changes in integrin expression patterns are among the many EMT phenotypes observed in human cancers. For example, ITGα5β1, another form of integrin heterodimer, is up-regulated in late stage

cancers, which exhibit more invasive phenotypes than early stage cancers [113]. During wound healing, $\alpha5\beta_1$ has been reported to interact with plasma fibronectin through a PHSRN sequence to stimulate cell invasion [114]. In PCa, Zeng *et al.* have shown that this peptide sequence is indeed required for the ITG$\alpha5\beta_1$-fibronectin association and important in cancer cell invasion [115]. Similar to ITG$\alpha5\beta_3$ inducing autophosphorylation of FAK, Zeng *et al.* have shown that $\alpha5\beta_1$ binding to PHSRN sequence of fibronectin induces tyrosine phosphorylation of FAK, which in turn activates other protein kinases, increasing the migratory ability of DU145 [115]. This group and others have shown that gene silencing techniques, such as siRNA or with the use of small-molecule inhibitors specifically targeting FAK, or Y397- inhibition, reduced cell adhesion and migration in PCa [115-119].

Not only is the phosphorylation level of FAK important in PCa, but its expression level is also well correlated with metastatic progression as it is highly expressed in more aggressive cell lines such as PC3, compared to low-metastatic LNCaP cells [97,105]. The FAK expression pattern was validated using clinical specimens; higher expression of FAK was observed in metastatic tumours than in normal tissues, BPH or localized PCa [34,120]. In other cancers such as colon, breast cancers and sarcomas, elevated expression and increased activity of FAK is also associated with aggressive phenotypes [121]. All this evidence supports the notion that FAK and its associated protein complexes play a central role in providing cancer cells invasive abilities.

1.3.5 Epidermal growth factor receptor (EGFR)

EGFR is a membrane-associated tyrosine kinase and belongs to the ErbB family of receptors comprised of four members: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4) [122].

EGFR activity is regulated by ligand-binding and by interactions with ErbB members or other receptors [123]. Upon binding of a ligand such as epidermal growth factor (EGF) or transforming growth factor α (TGFα), the kinase undergoes homodimerization or heterodimerizes with another ErbB member [124,125]. This dimerization induces autophosphorylation of tyrosine residues at the C-terminus of this kinase [126]. Once this molecule is phosphorylated, the activated signal is passed down through signaling cascades, activating numerous downstream molecules via their SH2 domains, which recognize and bind phosphorylated tyrosine residues [127,128]. The result of such signal transduction ultimately affects cell proliferation and migration [123].

Abnormal signaling in EGFR-related pathways leads to uncontrolled cell proliferation and has been reported to be elevated in many solid tumours such as prostate, breast, colorectal, head and neck and pancreatic cancers [123,129]. Several lines of evidence indicate that EGFR plays a role in metastatic progression [130-132]. In PCa, elevated EGFR expression is correlated with higher Gleason scores, disease recurrence and castration-resistant cancer progression [129,133].

Similarly, Src is a member of a non-receptor tyrosine kinase located in the cytoplasm and is one of the downstream molecules of EGFR [134,135]. Dysregulation of Src has been linked to oncogenesis in many cancers [136] and is also known to play a role in metastasis by modulating cell motility and invasive abilities in skin, breast and colon cancers [137].

1.3.6 Metallothionein

Metallothioneins are low-molecular weight, metal-binding proteins, which are normally located in the nucleus and cytosol [138]. They are known to bind both essential metals (zinc and copper) and toxic metals (cadmium and mercury) in two distinct clusters at N- and C-terminal domains [138,139]. In humans, there are at least ten functional isoforms of MT (1A, 1B, 1E, 1F, 1G, 1H, 1X, 2A, 3 and 4) expressed in various tissues and organs [140].The major ones, MT-1 and MT-2, are known to mainly bind zinc and cadmium [138,140]. Due to this metal-chelating property, it is suggested that they may provide protection against DNA damage and oxidative stress and also may function as a reservoir for essential metals, which can be donated to transcription factors and metallo-enzymes [140].

There are many enzymes and proteins that require zinc for their functions and play important roles in cancer. For example, DNA/RNA polymerases and many transcription factors have protruding structures called zinc-fingers (named so because they consist of many Zn ions), which are responsible for recognition and binding of specific DNA sequences [141]. Inhibitor of apoptosis protein (IAP) families also have zinc-binding motifs in the baculoviral IAP repeat (BIR) domains, and it is this motif that exerts anti-apoptotic effects via protein-protein interaction with caspases [141]. In addition, other enzymes such as matrix metalloproteinases (MMPs) also require zinc for their catalytic reaction or ECM-degrading function that is important in invasion and migration [142]. Zinc and a zinc-provider are, therefore, very important in nucleic acid and protein synthesis, cell proliferation, apoptosis inhibition and invasion processes, which are all hallmarks of cancer. With the proposed potential to store and donate zinc, metallothinein may be the driving force for both processes of carcinogenesis and metastasis.

During growth and development, *MT* expression increases as the demand for zinc and copper reaches a peak in the human fetus; however, once reaching adulthood, its expression level declines unless it is stimulated by metal exposure [143]. In cancer, increased *MT* expression has been reported in a large number of malignancies, including breast, colon, kidney, prostate, ovary and lung cancers [140].

1.4 Treatment of PCa

In North America, PCa is the most commonly diagnosed noncutaneous malignancy [2]. In 2010, the Canadian Cancer Society estimated 24,600 new cases of PCa and 4,300 deaths were reported in Canada, ranking PCa highest in cancer mortality after lung and colorectal cancers.

Since the introduction of PSA as a serum biomarker for PCa, the incidence has increased dramatically [44]. With this increase, over-diagnosis and unnecessary treatments for low-risk PCa have been debated for many years. Treatment of early stage PCa usually includes active surveillance, radiation and surgery (radical prostatectomy) [144]. When patients present with a clinically localized tumour, active surveillance is a reasonable treatment option, which includes physical examination, PSA measurements and a one-year follow-up biopsy [144]. Although about 60~70% of all men diagnosed with PCa per year have low-risk cancer, more than 90% of patients are reported to have undergone surgical or radiation therapies, which may have been unnecessary [145-148]. While such treatments may provide psychological benefits to the patients, since they receive therapy instead of active surveillance, the majority of them experience adverse side effects such as erectile dysfunction and urinary problems, which greatly affect their quality of life [144].

In order to spare those patients who do not require treatments and to lessen the burden on the health care system, it is important to distinguish patients who would benefit from therapies from those who will not die of the disease. Active surveillance would preclude adverse effects in patients and provide an opportunity for oncologists to closely monitor disease progression and apply therapy only when needed [144].

Patients who undergo initial treatments will be faced with recurrence risk when their PSA levels start to incline [144]. Once the disease progresses after failure of the local treatment, the cancer is usually treated with surgical castration or chemical/hormonal manipulation [149], and those patients with recurrent disease will eventually die of metastatic disease [144].

1.4.1 Androgen ablation therapy

Androgen ablation is a hormone-manipulating therapy in which the production of androgens is suppressed or inhibited. Since the introduction of androgen ablation therapy (ADT) by Huggins and Hodges in the 1940s, it has been used worldwide as the main therapy for metastatic PCa or advanced PCa cases without metastasis but very high PSA levels [15,150]. Most of PCa cases treated with androgen ablation therapy respond well initially; however, they will all eventually succumb to therapy-resistant metastatic disease, of which 80~90% takes place in bone and 10~20% in soft tissues such as liver, lung and lymph nodes [110,111]. The progression to castration-resistant prostate cancer (CRPC) with high propensity to metastasize, usually takes about 2 to 3 years of androgen ablation therapy [151].

To deprive endogenous androgen, surgical or medical castration (using estrogens, LHRH agonists or antagonists) is performed [152]. Alternatively, androgen action can be blocked at the target cell level by using anti-androgens and 5-α reductase inhibitors (figure 1) [152]. Medical or

surgical castration significantly reduces the testosterone levels in the circulation; however, 20-30% of testosterone still remains in the prostate [153,154].

Recent clinical evidence indicates that combined anti-androgen blockade is a more effective means of controlling PCa than castration alone. This may be because testosterone can be produced from precursors such as DHEA and androstenedione released by adrenal glands and/or via *de novo* synthesis from cholesterol within PCa cells [155]. Therefore, to maximize the effect of hormone manipulation, AR antagonists are administered at the same time or given as a secondary hormone therapy when ADT fails and the disease progresses to castration-resistant PCa [156].

There are several anti-androgens or AR antagonists that are currently used in management of CRPC: bicalutamide, flutamide, nilutamide and cyproterone acetate [17,152]. Bicalutamide is reported to be more effective due to its higher specificity for AR and to have fewer adverse effects (such as breast pain and gastrointestinal discomfort) than the other two and thus is clinically preferred [157-160]. Nilutamide and flutamide have been tested as anti-androgen agents in clinical trials in the 1990s; however, the results from various studies are inconsistent [161].

MDV3100 and ARN-509 are novel AR antagonists that are currently in clinical trials [17,152]. MDV3100 is known to bind to the ligand-binding domain of AR and have a much higher affinity than bicalutamide [162,163]. Once it binds to AR, it abrogates DNA binding and co-regulator recruitment [162,163]. The results of clinical trials are promising as more than 50% of the patients claim stabilized bone disease and a 50% decline in PSA serum levels [111]. Overall survival and disease progression of a phase III clinical trial is currently being evaluated [162,163]. Since all currently available anti-androgen therapies target the LBD at the C-terminus,

new peptides and small molecules such as EP1-100 or sintokamide peptides are emerging as potential anti-androgen drugs for CRPC that target the transactivational domain at the N-terminus [17,152].

It has been shown that expression of steroidogenic enzymes is enhanced in CRPC, supporting the evidence of *de novo* biosynthesis of testosterone intratumorally [164,165]. In addition to AR antagonists, inhibitors that block androgen biosysnthesis are used for CRPC treatment; ketoconazole, abiraterone acetate, TOK-001 and TOK-700. The latter two molecules are currently tested in clinical trials [17,152]. These inhibitors block enzymatic action of CYP17A1 to reduce steroid hormone production [154] (figure 2). CYP17 is a cytochrome P450 enzyme, which is involved in steroid synthesis. It is expressed in the testes, ovaries, prostate and adrenal glands and localized in the endoplasmic reticulum [166,167]. It catalyzes conversion of the steroid hormone, pregnenolone, to 17OH-hydroxypregnenolone (precursor of DHEA and testosterone) (figure 2) [166]. CYP17 -/-mice are embryonic lethal; the cause of this lethality remains unknown [168]. In humans, CYP17 deficiency is characterized by lack of sex organ development, delayed puberty and hypertension [169]. Even though inhibition of CYP17 reduces androgens and estradiol levels in the circulation, the level of corticosterol/glucocorticoid (an adrenal steroid hormone that regulates blood glucose) is maintained [166].

The results of early clinical trials with abiraterone have shown that patients who had failed hormonal therapies had suppressed levels of both testosterone and estradiol as well as reduced levels of PSA [167]. The results of phase III clinical trials show improved survival and longer time to disease progression compared to patients who received placebos [162,163]. These promising results have led to an approval of this drug by the Food and Drug Administration as a CRPC therapy in April 2011.

Although androgen ablation therapy has been proven effective in reducing PSA and shrinking initial tumour sizes by an average 30 to 40% [152], it is associated with adverse effects, including weight gain, insulin insensitivity and lipid metabolism abnormalities, reduced reproductive functions and disease progression [170]. Despite initial tumour regression following androgen ablation, some PCa cells may survive the therapy by having intrinsic or by acquiring castration-resistance [14]. Once the disease progresses, there is currently no effective treatment available [171]. Therefore, enormous efforts have been placed on developing more effective, less toxic therapeutic agents.

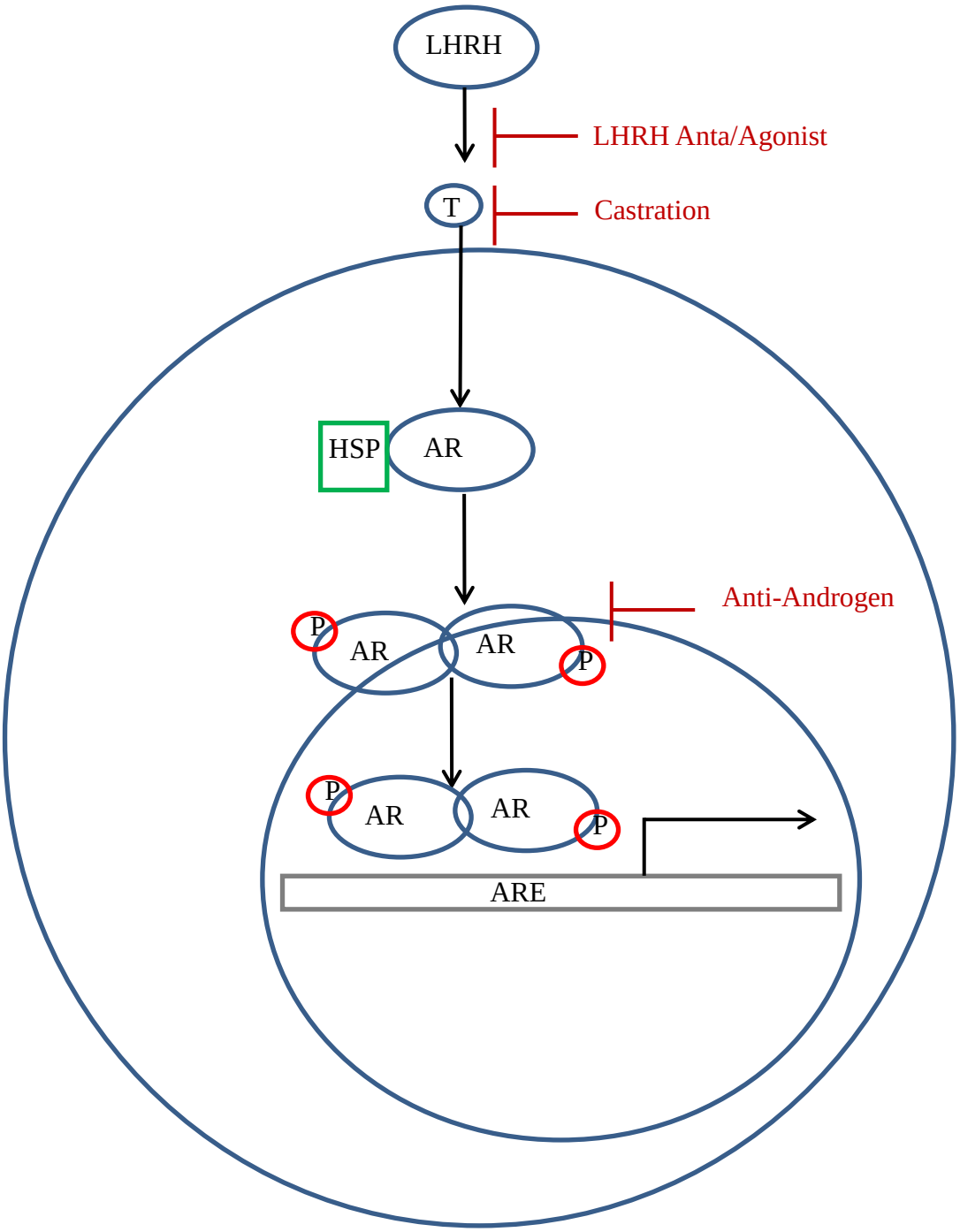


Figure 1. The mechanism of androgen ablation therapy at the cellular level.

There are a number of ways to target AR or production of androgen. LHRH Anta/agonist: luteinizing hormone-releasing hormone anta/agonist uses the hypothalamus-pituitary axis to control production of androgen. T: testosterone production can be blocked by surgical or chemical castration. AR: androgen receptor. Anti-androgen binds to AR and inhibits its transcriptional activity. HSP: heat-shock-protein. ARE: androgen-responsive element.

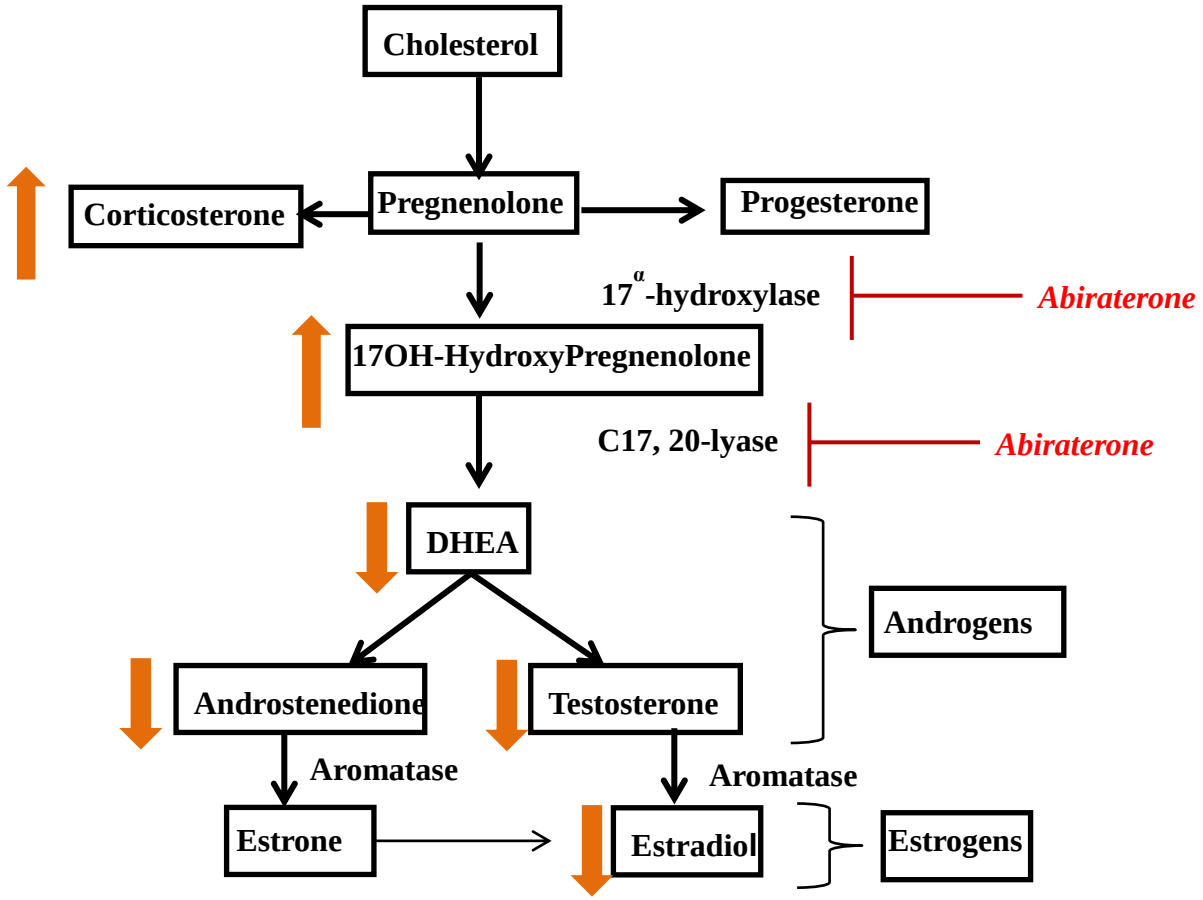


Figure 2. The mechanism of abiraterone therapy.

Abiraterone inhibits CYP17 enzyme, which converts cholesterol into DHEA, a precursor of androgens, resulting in reduction of both testosterone and estradiol. Orange arrows indicate changes in steroid hormone levels induced by abiraterone. (Adopted from Ang et al. Br J. Cancer. 2009)

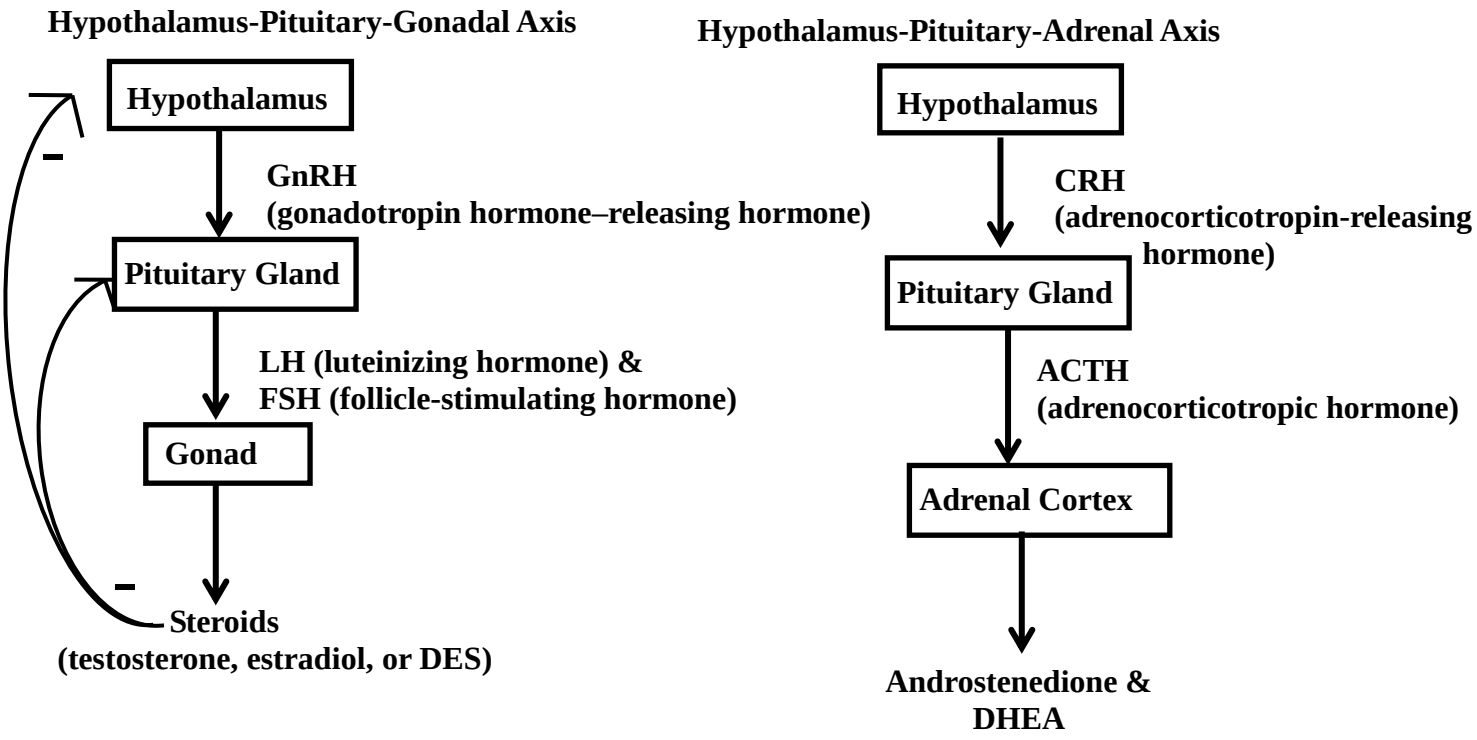


Figure 3. Hypothalamus-pituitary axis controlling sex- and adrenal hormone production.

GnRH: gonadotropin hormone-releasing hormone and CRH: adrenocorticotropin-releasing hormone are secreted by hypothalamus, reaching pituitary gland, which produce LH, FSH and ACTH. LH and FSH stimulate gonads for production of sex hormones. ACTH stimulates Adrenal cortex to release androstenedione and DHEA. (Modified from Kennedy et al. Clinical Pharmacology & Therapeutics. 2008)

1.5 Estrogen: castration agent

Historically, estrogen has been viewed and used partially as a therapeutic or chemopreventive agent agaist PCa as shown in a study using Lobund-Wistar rat model, which prevented onset of prostate adenocarcinoma [172]. Since Huggins and Hodges introduced the concept of androgen ablation in the 1940s, hormone manipulation has been used as the main therapy for advanced PCa [149]. Originally, the xenoestrogen or diethylstilbestrol (DES) was used as a chemical castration agent to treat patients [150]. Although DES was effective in controlling PCa growth as it suppressed production of gonadotropin in the hypothalamus-pituitary-gonadal axis (figure 3) and decreased testosterone production by the testes [173], its clinical usage was discontinued due to severe adverse cardiac effects [174,175]. Currently, instead of using synthetic estrogen, LHRH-anta/agonists are used to decrease the levels of androgen via the hypothalamus-pituitary axis. Whether traditional or current methods are used, the main purpose of the hormonal manipulation is to decrease the levels of circulating and tissue levels of androgen and minimize its effects on tumour growth.

1.5.1 Controversy regarding estrogen treatment of PCa

Controversy regarding the use of estrogens for PCa therapy stems from the adverse effects of estrogen on the cardiovascular system and its potential growth-stimulatory effects [174-178]. Despite an initial reduction in tumour growth which mainly results from reduced bioavailability of androgens in the body, previous studies have indicated that estrogen has certain growth- and metastasis- stimulatory effects [176-178].

In 1986, de la Monte *et al.* examined histopathology of metastatic PCa patients after autopsy and discovered confounding clinical evidence for estrogen's role in prostate cancer

progression [179]. They found that patients who received estrogen treatment had increased tumor burden, significantly greater number of metastases and greater number of deaths caused by metastasis compared to patients who did not receive estrogen therapy [179]. Those patients on estrogen treatment showed a significantly higher frequency of metastatic spread to the liver, bone, lymph nodes, lungs, adrenal glands, intestine and central nervous system (CNS) [179].

1.5.2 Role of estrogen in prostate carcinogenesis

Even though the prostate is androgen-dependent for its growth and development, estrogen is also a significant player in the development of normal and malignant prostates. As demonstrated in a study performed with beagles, estradiol treatment induced dose-dependent prostate growth in castrated dogs, implying the significance of estrogen on the growth of prostatic epithelium when compared to intact or castrated dogs not treated with estrogen [180].

In a classic study using Noble rats, combination treatment of androgen and estrogen induced carcinoma in dorsal prostate in 50% of ten rats [181]. Ho *et al.* found that combined exposure to androgen and 17 beta-estradiol induced dysplastic lesions and adenocarcinomas in dorsolateral prostate regions [176,182]. Moreover, testosterone alone (without estrogen) does not induce onset of prostate carcinoma as demonstrated in aromatase-knockout (ARKO) mice, which have increased levels of androgen, since the conversion of androgen into estrogen is inhibited due to lack of aromatase (figure 2) [178].

Abnormal expression and activity of aromatase is linked to carcinogenesis and malignant progression of breast cancer [183]. In the normal prostate, aromatase is not expressed in the epithelium but is present in the stroma [184]. Aberrant aromatase expression has been reported in PCa; in the normal prostate, its expression is confined to the stromal compartment; however, it

was detected in the epithelium of prostate tumor cells [184]. Ellem *et al.* investigated the expression and activity levels of aromatase in primary tumour cells and in a panel of PCa cell lines. They found that aromatase was expressed in LNCaP, PC3, DU145 and in primary tumour samples but was not expressed or inactive in benign epithelial cells [184]. Although expression and activity levels vary among samples, all PCa cell lines and primary tumours showed significantly higher aromatase activity than non-malignant or benign control cells [184]. While more aggressive cells such as PC3 and DU145 showed the highest aromatase activity in their study, less aggressive LNCaP cells had the lowest level, which was, however, still significantly higher than the control [184]. Their results provide additional support for the role that estrogen plays in prostate carcinogenesis and progression.

The importance of estrogen in prostate carcinogenesis is also evident from epidemiological data: African American males with a higher serum estrogen level are at a higher risk of developing PCa than Caucasians, while these men have similar testosterone levels. Likewise, Japanese men who are known to have a low PCa risk have much lower estrogen levels than men in the Western countries [185-187]. All these lines of evidence point at the importance and complexity of hormone interactions in prostatic carcinogenesis. Understanding such interactions and the mechanisms by which hormone receptor anta/agonists modulate gene transcription and signal transduction is the key in developing effective steroid receptor-based therapies against hormone-dependent cancers.

1.5.3 Estrogen deprivation therapy

As evidenced by early rodent studies [176,178,182], the development of PCa is critically dependent on the action of both testosterone and estrogen, and thus one could expect estrogen deprivation along with castration to be an effective strategy for therapy of PCa.

1.5.3.1 Aromatase inhibitors

In the early 1980s and 2000s, several aromatase inhibitors were developed for application of estrogen deprivation therapy in clinical trials of patients with advanced PCa [188,189] [190,191]. However, the studies were inconclusive due to varying effectiveness of the inhibitors and inconsistent results among the trials. For instance, treatment of castrate resistant-metastatic PCa (CRPC) patients with aminoglutethimide (an aromatase inhibitor used in the early 1980s) led to relief of some pain and a reduction in testosterone levels [188,189]. In contrast, newer aromatase inhibitors, such as anastrozole and letrozole, did not produce favorable results as the disease progressed in some of the patients [190,191]. The reason for the disparity in the efficacies of the aromatase inhibitors remains to be elucidated.

1.5.4 SERMs: selective estrogen receptor modulators

Although little is known about the exact role that estrogen plays in PCa development and progression, many compounds that mimic estrogen or anti-estrogens have been proposed as promising therapeutic agents. Such Selective Estrogen Receptor Modulators, or SERMs, include phytoestrogens, synthetic estrogens and anti-estrogens, [192], which exert estrogenic or anti-estrogenic actions by direct binding to estrogen receptors in target tissues. As shown in figure 4, SERM binding to ER causes conformational changes in the receptor in a way that reduces co-

regulator affinity, thus preventing or reducing transcription [193]. The effects of SERMs depend on the SERM-ER complex conformational changes, dimerization, co-factor recruitment and ER subtype tissue expression [194].

1.5.4.1 Tamoxifen and toremifene

The anticancer effects of SERMs are variable. Preclinical studies showed promising results for tamoxifen and toremifene; the former inhibited growth of xenografts of androgen-dependent PCa cell lines [195], and the latter, toremifene, prevented development of PIN lesions and delayed the onset of adenocarcinoma in the TRAMP model (transgenic mouse model for PCa) [196]. In a phase II clinical trial, toremifene prevented PCa development in high-grade PIN patients [197]. Although these anti-estrogenic agents may exhibit chemopreventive properties in the animal model as well as in men presenting with premalignant lesions[197], they may not be as effective against late-stage cancers as reported in phase II trial studies performed by Stein *et al* and Bergan *et al*.[198,199].

1.5.4.2 Raloxifene

Raloxifene (RAL) is another SERM which targets both types of estrogen receptors, but with a higher affinity for ERα than for ERβ, and has been shown to be effective against tamoxifen-resistant breast cancer (BCa) [193,200,201]. RAL has a partial agonistic function in bone, thus preventing bone loss/osteoporosis and has been shown to inhibit progression of invasive BCa in postmenopausal women [202].

In vitro studies investigating the efficacy of RAL in PCa indicated that it induced apoptosis in androgen-sensitive LNCaP cells as well as androgen-insensitive PC3 and DU145

cells, all of which express ERβ, whereas only PC3 cells express both types of ER [203]. Another *in vitro* study conducted by Rossi *et al.* investigated if different expression levels of ERα and β would affect the efficacy of RAL in two prostate cell lines: the EPN cell line is an androgen-dependent cell line derived from normal prostate tissue which expresses both ERs; the CPEC cell line is derived from a prostate cancer specimen which expresses low levels of ERβ and not ERα [204]. They reported that RAL inhibited cell cycling and induced apoptosis in the normal prostate epithelial cell line; however, in the CPEC cancer cell line, there was very little apoptosis but an increase in c-myc transcription and sustained phosphorylation of ERK 1/2 was observed [204]. Moreover, at high RAL concentrations, cell proliferation was enhanced in the CPEC cells [204]. These studies imply that effectiveness of RAL may depend on cell types, the expression levels of ERs, and presence or absence and types of co-regulators that interact with ERs within target cells.

There are a very few *in vivo* and clinical data on the efficacy of treatment with RAL. Agus *et al*. studied the *in vivo* effects of RAL on proliferation and apoptosis of androgen-sensitive and -insensitive PCa cell lines, CWR22 and CWRSA9, both of which express ERβ but lack ERα, [205]. They showed that although regression was not observed in CWR22 and CWRSA9 xenografts, RAL significantly inhibited tumour growth of both cell types [205]. In the same study, Agus *et al.* conducted a phase II clinical trial with androgen-insensitive PCa patients. Despite some limitations such as low patient recruitment (21 patients were enrolled but only 18 remained in the study) and few outcome parameters (ie, only PSA was used to monitor disease progression), they reported stabilization of the disease with minimum side effects [205]. The results from such a study could lead to the development of effective SERM therapy for patients who failed other hormone therapies.

1.5.4.3 Anti-estrogen: ICI 182,780

Due to its well-established efficacy in clinical trials, tamoxifen is known as the most widely used SERM against BCa [206]. However, despite its antagonistic activity in mammary glands, it was found to have a partial agonistic function in the endometrial tissue, increasing the risk of endometrial carcinoma [206,207]. In view of this, ICI 182, 780 was developed as a novel anti-estrogen without agonistic effects [206]. Its 'pure' anti-estrogenic activities; ie, no agonistic effects, were demonstrated in various pre-clinical models and clinical trials. Studies with rodent models indicated that this novel compound had no stimulatory effect on the growth of the uterus as observed with tamoxifen [208-210], and it even blocked the uterotrophic effect of tamoxifen [209,210]. In an *in vitro* study, tamoxifen-resistant BCa cell lines were found to be sensitive to ICI 182, 780 [211].

Such antagonistic effects on the endometrial tissues were also reported in clinical trials, as ICI 182, 780 inhibited the thickening of the uteral wall in patients compared to controls [212]. In randomized clinical studies, ICI 182, 780 reduced the expression of ER and proliferation activity in premenopausal women with benign gynecological diseases as well as in postmenopausal BCa patients [213,214]. In phase II trials, ICI 182, 780 produced favorable results (37% partial response and 32% stable disease) in advanced BCa patients who experienced disease progression after tamoxifen treatment [215]. These early studies indicate promising results on usage of ICI 182, 780 as a therapeutic compound for BCa. However, there are possible adverse effects associated with this compound such as changes in serum lipid profiles and bone loss [206]. These unfavorable effects need to be considered before approval of this drug as first line therapy for BCa.

Compared to the aforementioned cancers, data on ICI 182, 780 usage in PCa are rather limited. An early *in vitro* study showed that ICI 182, 780 inhibited the growth of androgen-independent cell lines, DU145 (ERβ-positive) and PC3 (ERβ- and α-positive)[216] . However, as recently reported by Nakajima *et al.*, ICI 182, 780 did not inhibit the growth of the same cell lines [217]. However, when ICI 182, 780 was used in a soft agar assay, it reduced cell colony formation in DU145 and PC3, while treatment with estrogen increased the number of colonies, suggesting that ICI 182, 780 can exert an inhibitory effect when PCa cells are not anchored [217]. Furthermore, when such cells were subcutaneously injected into nude mice, treatment with ICI 182, 780 resulted in the development of smaller tumours in comparison with estradiol-treatment [217].

Most of the clinical trials to test the efficacy of ICI 182, 780 have been carried out with BCa patients, and such information is not available for PCa with one exception of a recently published single-patient case report. In 2010, Blesa *et al.* reported that ICI 182,780 decreased the PSA levels in a 79-year old metastatic PCa patient without any significant adverse effects[218]. While no data are available on the survival outcome of this patient, the reduction in PSA levels indicates promising potential of this compound as a therapeutic agent for metastatic PCa.

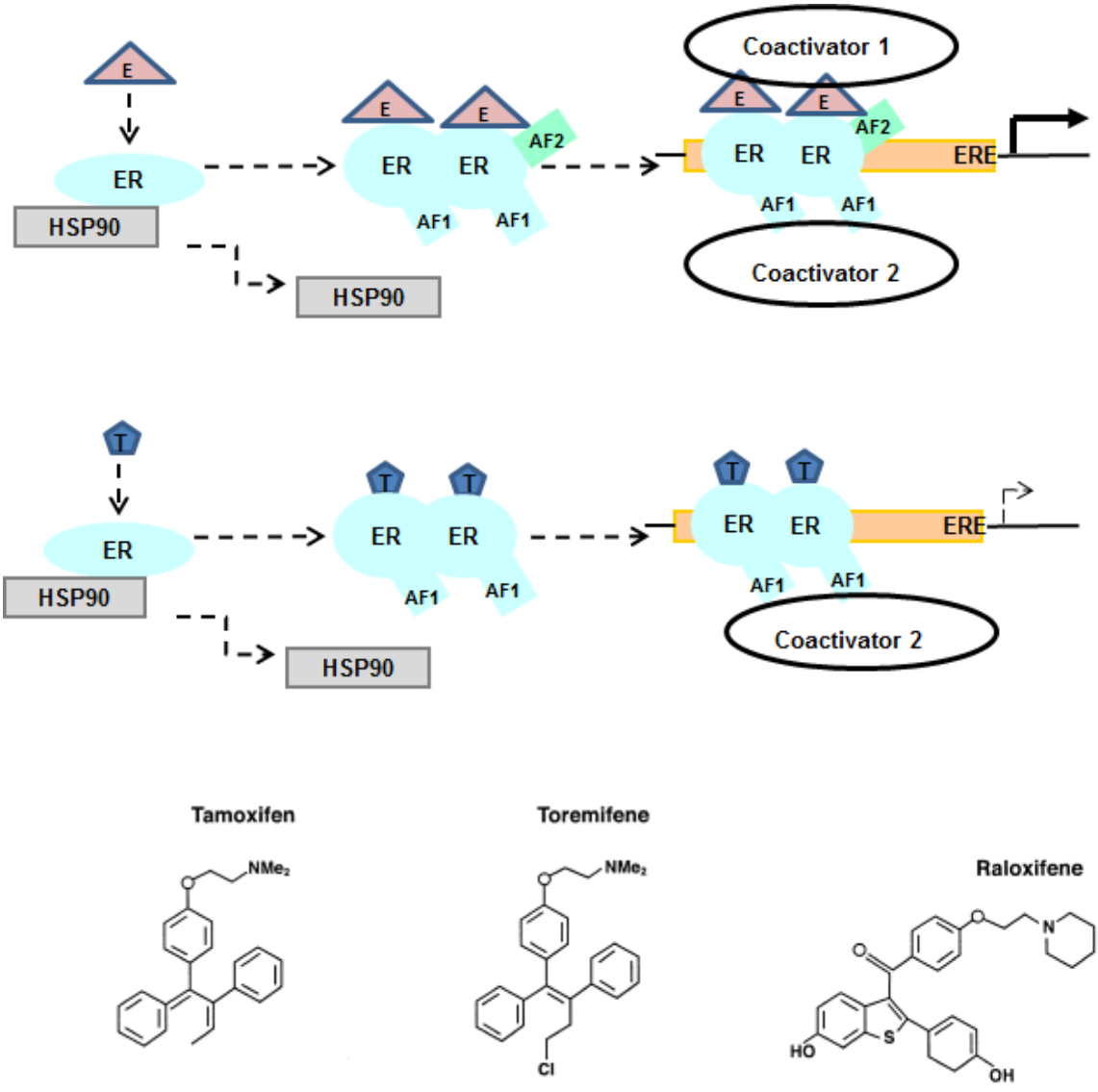


Figure 4. The chemical structures and mechanism of estrogen-responsive gene transcription and partial antagonistic effects of SERMs.

E: estradiol. When E binds to the ligand-binding domain of the receptor, it activates AF-2 domain and allows ligand-dependent transcription. T: tamoxifen. When tamoxifen binds to ER, it inactivates AF-2 domain. However, AF-1 domain is still active, which could explain partial agonistic activity in certain tissues. (modified from Howell *et al. Cancer*. 2000)

1.6 Estrogen receptors

Similar to testosterone, estrogen is a sex hormone, whose function is vital in growth, development and maintenance of female reproductory organs, and its activity is mediated via binding to estrogen receptors [219,220].

Similar to the androgen receptor (AR), the estrogen receptor (ER) and other steroid receptor family members, such as glucocorticoid (GR), mineralocorticoid (MR), and progesterone (PR) receptors are activated by ligand-binding, which allows for hormone-responsive gene transcription [219]. The expression and functions of steroid receptors are cell- and tissue- specific. Unlike AR, bound or unbound ERs are normally found in the nucleus [221]. Mutations and disruptions of receptor functions can often lead to cancer development in the breast, ovaries, prostate, and lungs [219].

There are two types of estrogen receptors, ERα and ERβ, which are mainly comprised of three major functional domains [222]. The activation function (AF-1) domain at the N-terminus is ligand-independent and is responsible for constitutive transcriptional activation of genes [219,220]. The DNA binding domain (DBD) is the site, at which ER binds to a specific region of DNA termed, estrogen response element (ERE). ERα and ERβ share highly homologous DBDs (96% homology), indicating that both receptors bind to the same sequence of DNA [223]. The ligand-binding domain (LBD) is also known as AF-2 domain. It is a site for co-regulator binding and receptor dimerization, and upon ligand binding activates target gene expression [223,224]. The homology between the AF-2 domains of ERα and ERβ is 53% [223]. This low conservation of LBDs between the two receptors may be crucial for generating distinct functional outcomes in various tissues. Figure 5 depicts the functional domains of ERβ.

According to Kuiper *et al.*, estradiol has a similar affinity for both receptor types;

however, a certain estrogenic compound such as genistein has a much higher affinity for ERβ (87%) than for ERα (4%) [225]. Such difference in affinity between estradiol and genistein may lead to differential expression of target genes induced by distinct ligand-binding. For example, in the nucleus, a discrete ligand-bound ER can bind to ERE in the promoter of a target gene and activate its transcription by recruiting distinct sets of co-activators [226]. In other words, different co-regulators may interact with LBDs of a specific ligand bound-ERα or -ERβ to induce different and sometimes opposite effects on transcription [206].

To date, several co-activators have been identified to interact with ERs, including steroid receptor co-activator 1 (SRC-1), glucocorticoid receptor interacting protein 1 (GRIP1) and the histone acetyltransferases p300/cAMP response element binding protein [194,227].

ERs are best characterized as transcription factors, whose functions are to bind to a specific region of DNA upon ligand activation and to induce estrogen-responsive gene transcription. Besides their direct genomic function, ERs can also function as co-activators themselves; they can bind to other transcription factors such as activator protein 1 (AP1) or specificity protein 1 (SP1) and indirectly activate gene transcription without binding to ERE [228,229]. Moreover, ERs are known to have non- genomic functions in which the receptors bind to activate protein tyrosine kinases and modulate their signaling pathways, leading to increased proliferation, cell survival and invasion [230].

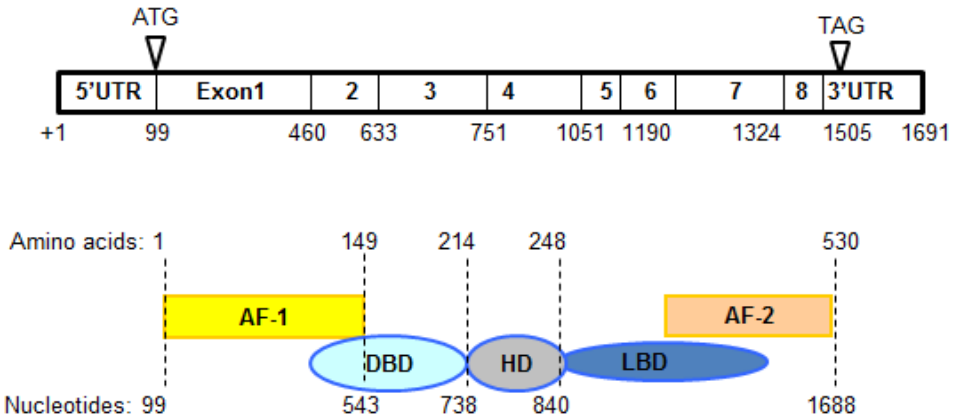


Figure 5. The mRNA structure (full length) and protein domains of ERβ.

There are eight exons in ERβ, and the corresponding numbers of amino acid sequence and nucleotides are indicated above and below, respectively. AF-1: ligand-independent transactivation domain. DBD: DNA-binding domain. HD: hinge region. LBD: ligand-binding domain. AF-2: ligand-dependent transactivation domain. (adapted from Herynk *et al. Endocr. Rev.* 2004*)*

1.6.1 ER expression in the prostate and PCa

The two ER types are also different in their expression patterns. For example, ERα is expressed primarily in the uterus and mammary glands, while ERβ is predominantly expressed in the brain, bladder, ovary and prostate epithelial cells [231-234]; and both receptors are expressed in bone and cardiovascular systems [235-238].

In the normal prostate, ERβ is predominantly expressed in both basal and luminal epithelial cells, while ERα is expressed mainly in stroma [239]. The expression of ERβ varies among cell lines. Lau *et al.* have demonstrated differential expression of ERs in PCa cell lines. They showed that only ERβ transcript (and not ERα) was expressed in LNCaP, DU145 and normal prostate epithelial cells isolated from biopsy samples; while PC3 expressed both ERα and ERβ transcripts [216]. These differential ER expression patterns may explain the differential responses to SERMs of the cell lines in their study. They found that both estrogen (ie, estradiol and DES) and anti-estrogen exhibited anti-proliferative effects in PC3 cells (ERα and β-positive); while only anti-estrogen, ICI 182 780, was effective in inhibiting cell proliferation in DU145 cells, which exclusively express ERβ [216]. It can be implied from their data that the response to SERMs is dependent on the ER subtypes that cancer cells express.

The expression of ERβ in PCa development and progression is very unique and stage-dependent. IHC analyses of clinical specimens show loss of ERβ in high-grade dysplasia, an increase in expression in Gleason grade-3 PCa, low expression or absence of ERβ in grade 4/5 and intense staining reappearance in almost all bone metastases [240,241]. This expression variation in disease progression is believed to be caused by epigenetic silencing or DNA methylation, not by mutation [242].

Horvath *et al.* have reported that more than 75% of 159 clinical localized PCa specimens

had low or no expression of ERβ; however, patients who retained ERβ (the remaining 25%) had an increased risk of recurrence and poorer prognosis compared to ERβ-negative patients [243]. Taken together, the animal studies and clinical reports indicate the possible dual roles of ERβ: it may function to prevent development of localized PCa; however, once cancer forms, ERβ may promote metastatic progression.

1.6.2 Functions of ERα vs ERβ in the prostate

To study the functions of ERs, mice lacking either receptor have been constructed [244,245]. Using such knockout (ERKO) mice and tissue recombination techniques, Risbridger *et al*. showed that administration of synthetic estrogen, diethylstilbestrol (DES), led to prostate squamous metaplasia (multi-layering of basal cells) in wild type and βERKO mice, whereas αERKO mice were unaffected [246]. They concluded that ERα is important in cell proliferation of basal cells and suggested that estrogen acts via an indirect paracrine mechanism mediated by both stroma and epithelial ERα to stimulate cell proliferation [246]. This conclusion, however, needs to be validated as their βERKO mice were not completely devoid of the β-receptor as shown in their immunostaining data.

In 2001, Weihua *et al.* demonstrated that ERβ knockout mice developed hyperplasia in the ventral prostate at 5 months of age, and that these mice later progressed to develop neoplasia, which morphologically resembled prostatic intraepithelial neoplasia (PIN) lesions. These studies provided support for the distinct differences in the functions of the two types of receptor, suggesting a proliferative function for ERα vs a protective (anti-proliferative) role for ERβ [244,247]. However, these results were challenged by subsequent studies using identical or different βERKO mice, which showed that there were no abnormalities in the prostates of

βERKO mice [245,248-250]. Due to the inconsistency and inadequacy of βERKO mouse models, the function of ERβ in prostate carcinogenesis still remains unresolved.

1.7 Genistein

Compared to the USA, the incidence and mortality rates of prostate cancer are considerably lower in Asia [251-253]. Such geographical differences in PCa risk may be partially attributed to regional or cultural differences in diet [254,255].

Studies have shown that Asians, who adopted the Western diet after immigration to the USA, had a significantly higher prostate cancer incidence than their counterparts in Asia [254,255]. Among many dietary differences between North America and Asia, the difference in soy consumption is exceptional. It is estimated that Asians consume 20-50 times more soy-based foods per capita compared to North Americans [256]. Epidemiological reports have indicated an inverse correlation between soy consumption and PCa risk [257].

Soy contains a large amount of isoflavones, which are comprised of tricyclic polyphenols [258] and known to contribute to a variety of health benefits such as reduction in cholesterol levels, protection from osteoporosis and chemopreventative effects against cancer [259-261]. The primary isoflavone contained in soy is called genistein (4,5,7-trihydroxyisoflavone) [262-265] (figure 6). Because genistein has estrogen-like properties and preferentially binds to ERβ, it has been well studied for its beneficial effects on health, in particular, for its anti-cancer effects [262-265], which will be discussed in the following section.

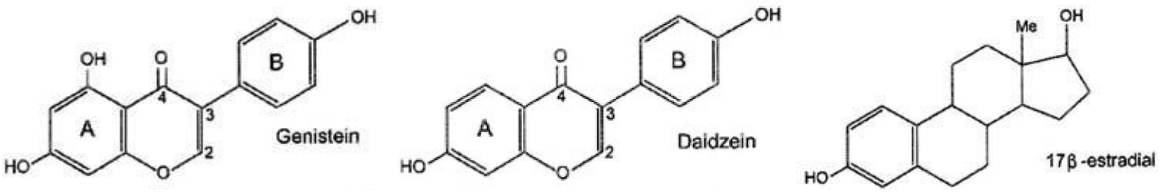


Figure 6. Molecular structures of genistein, daidzein (another soy isoflavone) and 17β-estradiol.

(adapted from Sarkar and Li. *Cancer and Met Reviews*. 2002)

1.7.1 Metabolism of genistein

Asian diets containing high amounts of soy are thought to provide a sufficient amount of isoflavone to instigate physiologic effects in humans. The amount of genistein that can be obtained from raw soy can range from 0.4 to 2.4mg/g [266,267]. It is reported that Japanese people consume about 30~50mg of isoflavone daily from their diet, reaching peak plasma levels of 0.4~4 μM [263,268,269]. Upon consumption of soy, genistein is metabolized to mono- or di-glucuronide conjugates in the liver and the intestines, and only a small amount is circulated as aglycone, the active unbound form, and as sulfate conjugates in the blood [262-265,270-272].

Rodent studies have shown that oral administration of genistein leads to accumulation of this compound in various tissues [273,274]. As shown by acid hydrolysis analysis, high amounts of aglycone were detected in the liver, kidney and uterus of female rats; in male animals, the highest concentrations were detected in the kidney and prostate [274]. At the subcellular level, both conjugated and aglycone were found in the cytosol and nucleus; 55-60% in the cytosol and 10-13% in the nucleus and some in microsomes [274]. Since the binding affinity for ER of genistein in the conjugated form is 10- to 40-fold lower than that of aglycone, conjugated genistein has to be metabolized by a glucuronidase to aglycone (the active form) in order to elicit physiological effects [275].

In clinical trials of soy extract supplementation, Guy *et al.* evaluated the amounts and composition of isoflavone metabolites that accumulated in prostatic tissues of men with BPH [276]. It was found that the major metabolite of genistein in the benign prostatic tissues was glucuronide conjugate; only small amounts of aglycone and sulfates were present [276]. In view of a report that glucuronidase (the hydrolytic enzyme) has a higher activity in tumour tissues than in benign prostatic tissues [277], it may be expected that the concentration of aglycone in PCa tissues is higher than in BPH or benign prostatic tissues. Studies investigating the levels of genistein in PCa patients who received soy supplements revealed a higher concentration of genistein in the prostate tissue than in serum, indicating that this cancerous tissue may have a greater ability to accumulate isoflavones than other tissues or organs [278,279]. This could be explained partially by predominant ERβ expression in the prostate epithelium and genistein's preferential binding to ERβ, causing an accumulation of ERβ-bound genistein in epithelial cells.

1.7.2 *In vitro* effects of genistein

The anticancer effects of genistein have been well studied *in vitro* and reported for several cancer cell lines, including leukemia, lung, neuroblastoma, melanoma, prostate and breast [260,280-284]. Data from early studies demonstrate that genistein has a wide spectrum of biological effects, including: induction of cell differentiation and apoptosis [260,281,282,285], inhibition of cell proliferation [282,286-289], and abrogation of signal transduction pathways [290-292]. Genistein has, therefore, attracted a considerable amount of interest in cancer research, especially for its inhibitory action on protein tyrosine kinase (PTK) activities important in cell survival and proliferation [290-292].

PTKs play a central role in regulating cellular functions such as proliferation, apoptosis, differentiation and cell survival [293]. PTKs are the fundamental driving force in signal transduction pathways associated with cellular receptors such as epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor. The phophorylation activity of PTKs is, thus, of major importance in processes such as carcinogenesis and metastatic progression.

In 1987, Akiyama *et al.* first identified the PTK inhibitory action of genistein *in vitro,* using human epidermal cancer cells. They showed that tyrosine phosphorylation of EGFR was reduced in genistein-treated cells. They claimed that genistein prevented phosphorylation by blocking ATP binding to the kinase. Because ATP and genistein do not share structural similarities, it was postulated that genistein does not compete for the same binding site but may bind to multiple sites and induce a conformational change in the kinase, making it non-functional [290].

Additional anti-cancer mechanisms of genistein that have been reported *in vitro* include: down-regulation of AR expression [294,295], inhibition of topoisomerase I and II [296] by stabilizing the DNA-topoisomerase complex [297], which in cancer cells, leads to double and single strand breaks and cell death [298], inhibition of 5α-reductase activity [299] and antioxidant effects by decreasing production of reactive oxygen species [300,301].

1.7.3 *In vivo* effects of genistein

Despite promising *in vitro* anticancer data, recent *in vivo* studies have reported contradictory results regarding the effects of genistein on metastasis. In a study using a rat tumour model, in which K1 cells (carcinogen-induced accessory sex gland carcinoma cells) were subcutaneously injected, Schleicher *et al*. demonstrated that treatment with genistein reduced tumour growth [302]. The genistein-treated animals showed reduction in serum testosterone levels and subsequently smaller testes than the control group, which is indicative of estrogenic effects and negative feedback by genistein on the hypothalamic-pituitary axis [302]. Other groups showed that genistein inhibited PC3 bone tumour growth in SCID mice [303], and another showed that genistein decreased lung metastasis in androgen receptor-negative PC3-M implanted mice, which were fed genistein-enriched chow [304].

In contrast to the early promising *in vivo* data, Raffoul *et al*. found that genistein ingestion led to an increase in lymph node metastases in their PC3 implanted animal model [305]. In 2009, Touny and Banerjee demonstrated biphasic effects of genistein using the TRAMP mouse model. Genistein inhibited poorly differentiated PCa in these mice when incorporated in their diet before tumour initiation (ie, when the mice were fed genistein-diet between 4~12 weeks of age). However, if the genistein-diet was given to older TRAMP mice at ages of 12~20 weeks, when prostatic intraepithelial neoplasia was already present, it promoted PCa progression, inducing lymph node metastases [306]. From such data, it can be implied that life-time moderate consumption of (or early exposure to) genistein is important in prevention of the disease, but that genistein may not exhibit chemotherapeutic effects *in vivo* once PCa has already been established or progressed to an advanced stage.

1.7.4 Clinical data

With epidemiological and early preclinical data as a foundation, clinical trials have been carried out in the last few years to investigate the safety and efficacy of genistein in PCa patients. Results of phase I trials, which measured the pharmacokinetics, found that genistein was well tolerated by most PCa patients who showed no sign of toxicity, with exception of a few cases reporting minor side effects such as rash and changes in breast morphology [271,307-309].

As proposed by *in vitro* studies, which suggested inhibitory effects of genistein on topoisomerase and potentially causing DNA damage and cell death, one phase II trial investigated the potential genotoxic effects of genistein. All twenty PCa patients recruited in this study, however, showed no DNA or chromosomal damage [309]. Other studies provided evidence of increased apoptosis in the tumours of the treated patients [310], stable disease or decreased PSA serum levels (Table 3) [311,312].

Although genistein has generated promising clinical data, there have also been negative reports. While dietary genistein decreased serum PSA level in six PCa patients of a phase II study investigated by Pendleton *et al.*, the level increased in the remaining 13 patients over the course of the study [313]. Similarly, White *et al.* reported disease progression in 67% of PCa patients treated with genistein and only 17% partial response and 0% complete response [314].

Author/ Year	Phase	# of Patients	Duration	Supplement Composition	Dose	Outcome
Takimoto *et al.*, '03	I	13	8 months	Genistein (90%), daidzein (9%) & glyceitein (1%)	3 different sequential doses/pt. (2, 4, 8mg/kg)	Most cases: no toxicity. 1 case: rash
Busby *et al.*, '02	I	30	30 days	Genistein (90%), daidzein (9%) & glyceitein (1%)	1, 2, 4, 8, or16 mg/kg	No/little toxicity (eg, increase in serum lipase activity)
Fischer *et al.*, '04	I	20	3 months	Genistein & daidzein	300mg or 600mg/day	Only minor side effects: 1) breast change. 2) decreased serum dihydroepiandrosterone
Miltyk *et al.*, '03	I	20	28 days of 300mg followed by 56 days of 600mg	Genistein (139.5mg/capsule), daidzein (74mg/cap) & glyceitein (11mg/cap)	300mg of gen/day followed by 600mg/day	No toxicity (DNA/chromosome damage)
Jarred *et al.,* '02	II	38 PCa & 18 control	6 weeks	Dietary Isoflavones (containing genistein and daidzein)	160mg/day	Apoptosis in prostatectomy samples who were treated with isoflavones compared to control
Hussain *et al.*, '03	II	41	3-6 months	Soy isoflavones 2 x 100mg/day	2 x 100mg/day	No complete or partial response but some report stable disease.
Pendleton *et al.*, '08	II	20	12 months	Soy milk	3 x 47mg of isoflavone/8oz/ day	PSA level decreased in 6 pts but increased in the remaining 13 pts.
Dalais *et al.,* '04	II	20	23 days	Ground soy	117mg of soy	Decreased PSA
DeVere White *et al.,* '03	II	52	6 months	Genistein-rich extracts containing genistein & other aglycone isoflavones	450mg gen/day	0%: complete regression & 17%: partial response 15%: stable PSA. 15%: decreased PSA. 67%: PSA progressed.

Table 3. List of phase I and II clinical trials of supplementation of soy isoflavones to PCa patients.

1.8 Hypothesis and objectives

The anticancer effects of genistein *in vitro* have been well established. However, recent *in vivo* studies report contradictory results regarding its effects on metastasis. These reports in combination with inconclusive preliminary results from phase II clinical trials [308,313] suggest a need for closer examination of the effects of genistein *in vivo* using models that are more clinically relevant than conventional *in vivo* models that rely on usage of cell lines.

To resolve the controversy of the effects of genistein *in vivo* and to investigate the role of the estrogen receptor in PCa progression, we have developed clinically relevant xenograft models generated from patient prostatectomy specimens. The resulting ERβ-positive prostate cancer tissue lines, LTL163a and LTL313h, have been derived from tumours of two distinct patients and have been passaged in immune-deficient mice. The advantage of using these models is that the xenograft tumours have retained the original histopathological and genotypical characteristics of the patient tumour samples, closely resembling the clinical setting [315,316].

Hypothesis:

In contrast to its *in vitro* growth-inhibitory effects reported, genistein may have different *in vivo* effects and may promote cancer progression in the patient-derived PCa tissue xenograft model. Because genistein is known as a phytoestrogen and possesses estrogen-like properties, it may affect tumour biology (ie, cell proliferation, apoptosis and migration ability) and promote metastasis by activating the estrogen receptor and regulating transcription of genes such as caspases, integrin, PTEN, and other genes involved in cancer progression (figure 7).

Objectives:

1. To investigate *in vivo* effects of genistein in advanced prostate cancer using patient-derived PCa tissue xenograft models.
2. To examine if the tumor -promoting effects are due to the estrogenic property of genistein.
3. To characterize estrogen-receptor regulated genes, which are important in PCa metastasis.

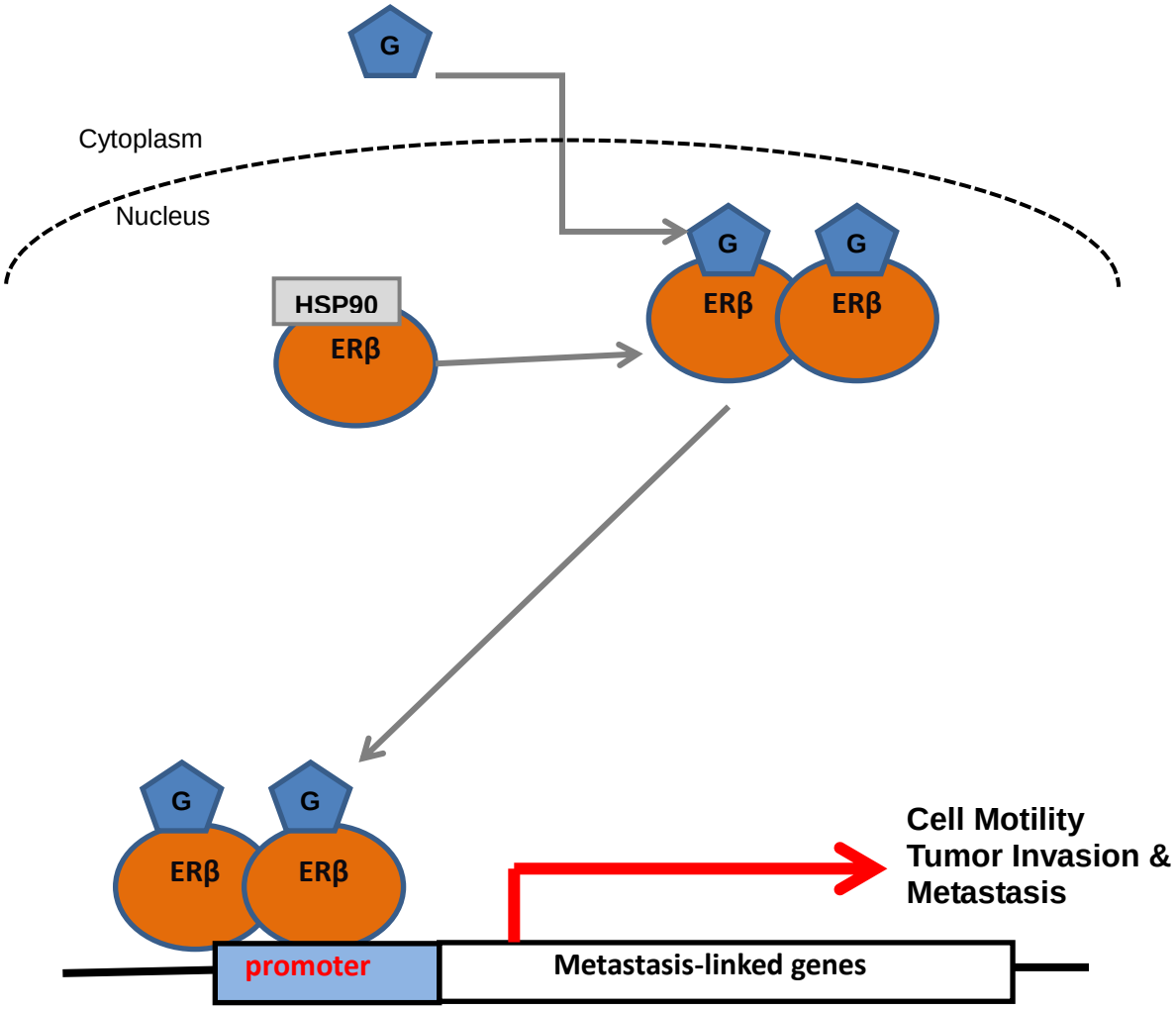


Figure 7. Model depicting the hypothesis.

G:genistein. ERβ: estrogen receptor beta. HSP90: heat-shock protein. Genistein may promote PCa progression via activation of ERβ and inducing estrogen-responsive genes that are linked to metastasis.

Chapter 2: METHODOLOGY AND RESULTS

2.1 Investigation of the *in vivo* effects of genistein on human prostate cancer

Genistein is a primary isoflavone and the major biologically active component of soy. Its chemopreventive effects have been well established in *in vitro* studies. Recently, however, the *in vivo* therapeutic actions of genistein have been questioned due to contradictory reports from animal studies based on rodent models or implantation of human cancer cell lines into immuno-deficient mice. To elucidate the *in vivo* effects of genistein in advanced prostate cancer patients, we developed a patient-derived prostate cancer xenograft model that more closely resembles the clinical situation, i.e. retaining tumour heterogeneity and micro-environment of prostate cancers. To this end, *tissue* from a patient's prostatectomy specimen was grafted under the kidney capsules of immuno-deficient mice and serially transplanted.

2.1.1 Methods

2.1.1.1 Materials and animals

Chemicals, stains, solvents and solutions were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada), unless otherwise indicated. High-grade prostate cancer samples were obtained from two patients at the Vancouver General Hospital. Written consent was obtained from the patients. Ethics approval was obtained from the University of British Columbia-British Columbia Cancer Agency Research Ethics Board (UBC BCCA REB), Vancouver, Canada. Male NOD-SCID mice 6 to 8-weeks old were bred by the BC Cancer Research Centre Animal Resource Centre (BC Cancer Agency, Vancouver, Canada). They were housed in groups of three in microisolators with free access to food (PicoLab Rodent Diet 20) and autoclaved drinking water, and their health was monitored daily. Animal care and experiments were carried out in accordance with the guidelines of the Canadian Council of Animal Care (CCAC), and the use of animals for experiments was examined and approved by the Animal Care Committee of the University of British Columbia (permit No.: A10-0100).

2.1.1.2 Establishment of transplantable, metastatic prostate cancer tissue xenograft lines

To establish transplantable cancer tissue xenograft lines from the two patient-derived prostate cancer biopsy specimens, fresh tissue was cut uniformly into multiple small pieces, 1x3x3 mm and grafted under the renal capsules (two pieces per kidney) of 6-8 week old NOD-SCID male mice [315,316]. The host mice were supplemented with testosterone pellets (s.c.; 10 mg/mouse), which were prepared with a PARR pellet press (PARR Instrument Co., Moline, IL) and implanted subcutaneously to promote tumour growth. The grafts were grown in the renal site for 60~90 days, harvested and portions of the grafts were fixed for histopathological analysis.

Tumour grafts exhibiting rapid growth were cut into small pieces and re-grafted under the kidney capsules of new hosts. After three serial transplantations, swollen lymph nodes in the hosts were noted, which were dissected and histologically examined for metastasis. When metastasis was confirmed, fresh lymph node tissues containing metastatic deposits were re-grafted into the kidneys of new mice to develop a metastatic tumour line. Using this protocol, a metastatic prostate cancer tissue line was developed from each of the two prostate cancer specimens. The two lines were designated LTL163a and LTL313h (www.livingtumorlab.com); their metastatic ability was confirmed via orthotopic grafting and histological examination of secondary tissues, as described below.

2.1.1.3 Treatment with genistein

Mice bearing xenografts of the metastatic LTL163a and LTL313h tumour lines were used to investigate the pharmacological effects of genistein on metastasis of human prostate cancer. To this end, well-established xenografts were cut into 1x3x3 mm pieces and grafted under the renal capsules (1 graft per kidney) into 18 NOD-SCID male mice (2 grafts/mouse). Testosterone pellets (10 mg) were implanted subcutaneously, as described above, in all animals at the time of grafting to maintain adequate serum testosterone levels since treatment with genistein has been shown to decrease serum testosterone levels via the hypothalamic/pituitary/gonadal axis [317]. When the grafts reached a size of approximately 50 mm^3 (1 week for LTL163a and 6 weeks for LTL313b tumours), the animals were randomly (unbiased distribution) distributed into three groups: control (untreated), low-dose and high-dose of genistein (purity >99%, LC Laboratories, Woburn, MA). The genistein dosages used are presented in Table 4. For the low-and high-dose groups, genistein was dissolved in peanut oil

and administered to the mice by gavage. Control mice received an oral dosage of 0.1 ml of oil only. All mice were fed the same rodent diet (PicoLab Rodent Diet 20), which may contain a minute amount of soy. The treatment was given 7 days per week for a period of four weeks. At the end of the 4^{th} week, animals were sacrificed and blood samples and organs (kidneys with tumour grafts, liver, lung, spleen and lymph nodes) were collected for analyses.

Tumour Line	Untreated Control	Low-dose genistein	High-dose genistein
LTL163a	0.1 ml of Oil-only	2 mg/day (80 mg/kg body weight/day)	10 mg/day (400 mg/kg/day)
LTL313h	0.1 ml of Oil-only	5 mg/day (200 mg/kg body weight/day)	10 mg/day (400 mg/kg/day)

Table 4. Genistein dosages used for treatment of LTL163a and LTL313h xenografts.

Genistein was dissolved in peanut oil and administered orally. Control groups were given oil-only. The treatment was given 7 days/week for a four week period.

2.1.1.4 Measurement of serum genistein levels.

Liquid chromatography-mass spectrometry (LC-MS) was used to measure the serum levels of genistein. First, frozen serum samples were thawed on ice and 5 µl of luteolin (1 µg/ml in ethanol) was added to 25 µl of serum as an internal standard (IS). Extraction was carried out by vortexing with 80 µl of acetonitrile and 5 µl of 0.2 M HCl. The extract was clarified by centrifugation for 5 minutes at 20,000 x g, and the supernatant collected, diluted 1:1 with distilled water and centrifuged for another 5 minutes. The resultant supernatants were analyzed. An Acquity UPLC with a 2.1x100 mm BEH 1.7 µM C18 column coupled to a PDA detector in line with a Quattro Premier XE (Waters, Milford, MA) was used with water and acetonitrile containing 0.1% formic acid as mobile phases. A 25–100% acetonitrile gradient from 0.2–2.0 minutes was used, followed by re-equilibration to starting conditions for a total run time of 4.5 minutes. The MS was run at unit resolution with 3 kV capillary, 120 ºC and 350 ºC source and desolvation temperatures, 50 and 1000 L/hr cone and desolvation N_2 gas flows and Ar collision

gas set to $5.0e^{-3}$ mbar. M/z 287>89 and 287>153 were used to detect luteolin IS, and m/z 271>91 and 271>153 were used for genistein with cone voltage and collision energies optimized for each. OD data from 210-600 nm was collected with the PDA detector and OD260 was also used as an endpoint. A 5 point linear calibration curve from 1-800 ng/ml with 218 ng/ml IS was used for quantitation (R2 > 0.99; bias < 10%). Genistein-glucoronide was presumed to be the most common form of conjugate, and the levels were estimated from OD260 data with the assumption that the extinction coefficient is similar to that of genistein.

2.1.1.5 Histopathology and immunohistochemistry

Harvested tumour grafts were cut in half at their thickest point. One half was fixed in 10% neutral buffered formalin for histological and immunohistological (IHC) analyses. The other half was snap frozen in liquid nitrogen for protein analysis. The fixed tissue was processed to paraffin and embedded with the midline cut surface facing down in the paraffin block, so that sections began at the original cut midline surface. The kidney grafts contained tumours grown at the surface of the kidney as well as locally invaded cancer cells into the organ. The magnitude of local invasion into the kidney varied among treatment groups. The whole kidneys with grafts were processed.

Two or three sections were mounted per slide and stained with hematoxylin and eosin (H&E) stain for histological analysis. For immunohistochemistry, slides were deparaffinized in xylene, treated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity and washed in phosphate buffered saline (PBS, pH 7.4). To block non-specific binding, a blocking solution (Clear Vision™/Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK) was applied to the sections for one hour at room temperature. The slides were then incubated

with primary antibodies overnight at 4 ^{o}C. Following this, the slides were washed in PBS and incubated for 30 minutes with biotinylated secondary anti-mouse or anti-rabbit IgG antibodies diluted to a concentration of 1:200 with blocking solution. The sections were then washed with PBS and treated for 30 minutes at room temperature with avidin-biotin complex (ABC) purchased from Vector Laboratories (Foster city, CA), followed by washing in PBS. Immunoreacitivity was visualized after the final treatment in 3,3'-diaminobenzidine tetrahydrochloride (DAB), PBS and 3% hydrogen peroxide solution.

The primary antibodies used in this study were Ki67 (Dako, Carpinteria, CA) and caspase-3 (Cell Signaling Technology, Danvers, MA). All tissue sections were counter-stained with 5% (w/v) Harris hematoxylin and coverslipped with Permount (Fisher/Thermo Scientific, Waltham, MA).

2.1.1.6 Local tissue invasion and metastasis analyses

To examine metastatic incidence, lung, liver, renal, pancreatic, lumbar and thoracic lymph nodes were collected from mice bearing LTL163a or LTL313h tumours at the subrenal capsule site. Each of these organs was fixed and processed as above for IHC analysis. Sections were stained with an anti-human-specific mitochondria antibody (Millipore, Billerica, MA) and screened for the presence of human cells. Images of IHC-stained sections from each organ were captured using an AxioCam HR CCD mounted on an Axioplan 2 microscope and Axiovision 3.1 software (Carl Zeiss, Toronto, ON), with final magnifications of x400. Percentages of animals containing human cancer cells in either lumbar or thoracic lymph nodes were calculated. (In renal and pancreatic lymph nodes, all animals in the three groups showed presence of human cells-roughly same proportion of invading cells-regardless of treatment and hence were omitted in the calculation.) For lung and liver, the number of positively stained cells per specimen was counted within randomly selected microscopic fields.

2.1.1.7 Cell proliferation and apoptosis analyses

The proliferation index (PI) was assessed by IHC using a human-specific proliferation marker, Ki67. Images of IHC-stained sections from each graft were captured as above. The mid section of the graft with the most densely populated human cancer cells was selected for imaging for all tumours. The numbers of Ki67-positive and -negative human cells were counted within the entire microscopic field for all grafts and averaged. Based on the averaged cell count for each treatment group, the PI was calculated as follows: PI (%) = (Ki67-positive cell count/total human cell count) x 100.

Similarly, the Apoptotic Index (AI) was calculated from cleaved-caspase-3-stained IHC

sections of tumour grafts. The number of apoptotic cells per 10,000 human cells was counted for each tumour graft and averaged. The AI was calculated as described for the PI above.

2.1.2 Serum levels of genistein

To ensure that genistein-treated mice received a pharmacological dosage of the compound, the serum concentrations of genistein were measured using high-performance liquid chromatography-mass spectrometry (HPLC-MS). The HPLC-MS analysis showed that the ingested genistein formed glucuronide conjugates in the treated mice, and only a small portion of genistein remained as free aglycones in the blood. In LTL163a-grafted mice, the serum levels of free genistein were 0.48 μM and 2.46 μM for low- and high-dose-treated animals, respectively (figure 8). In LTL313h tumour-carrying mice, the levels were 1.61 μM and 2.06 μM for low and high-doses, respectively. The average genistein serum level in the low-dose LTL313h group was higher than that of the LTL163a group because the low-dose LTL313h group received 5 mg of genistein per day compared to the 2 mg dosage used for the low-dose LTL163a group (The difference in the dosages used for the low-genistein groups between the two studies are explained in the method section 2.2.)

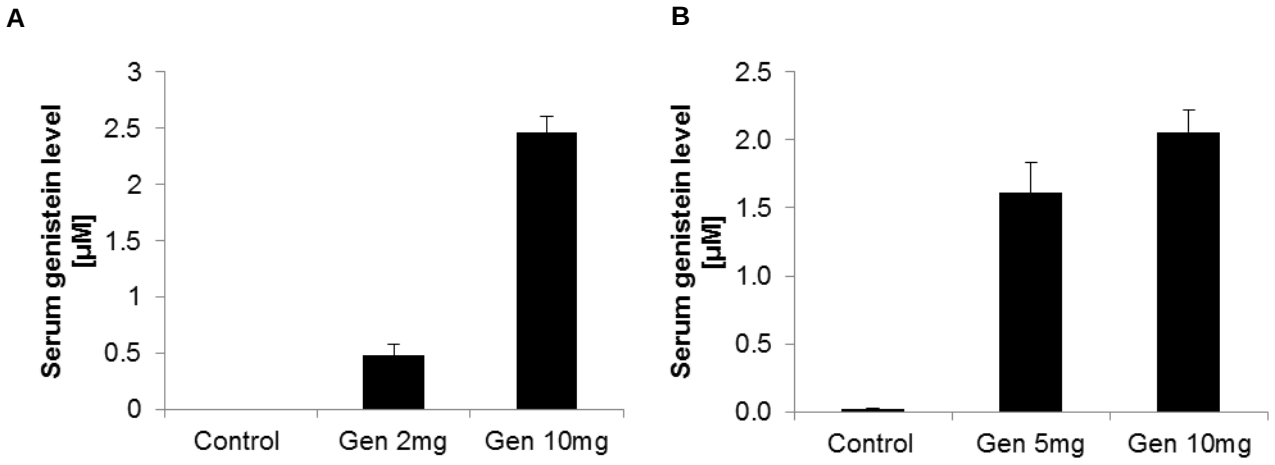


Figure 8. Serum levels of unconjugated genistein measured by LC-MS.

Blood was collected at the end of the treatment. Serum genistein levels in: A: LTL163a xenografted mice. For treatment of the low-dose group, animals received genistein dissolved in peanut oil by gavage at 2 mg/day (80 mg/kg body weight/day). The high-dose group was given 10 mg/day (400 mg/kg/day). B: LTL313h xenografted mice. For the low-dose group of mice, 5 mg/day (200 mg/kg/day) was administered, 10 mg/day (400 mg/kg/day) for the high-dose group. Control mice in both studies received 0.1 ml of vehicle only. *Columns*: mean serum concentration of free genistein ± SD. n=6/group for LTL163a. n=5/group for LTL313b.

2.1.3 Effects of genistein on tumour growth

Pieces of tissue from LTL163a and LTL313h patient-derived prostate cancer xenografts (1x3x3 mm) were grafted under the kidney capsules of NOD-SCID mice. Following establishment of the grafts (average size about 50 mm^3), the host animals were randomly distributed into three groups: untreated (control), low-dose and high-dose genistein. Genistein or oil (for the control group) was administered orally for four weeks. At the time of harvest, enlarged kidneys with well-grown tumours were noted for all groups. The average volumes of LTL163a tumours were 650 mm^3, 655 mm^3 and 670 mm^3 for control, low-dose and high-dose groups, respectively. Those of the LTL313b group were 157 mm^3, 177 mm^3, and 242 mm^3 (figure 9). Because of the faster-growing/more aggressive nature of the LTL163a line, its overall final tumour size was much larger than that of the LTL313h line. Although the data show a slight increase in the average size of the genistein-treated LTL313h tumours, the effect of genistein on tumour growth was not statistically significant.

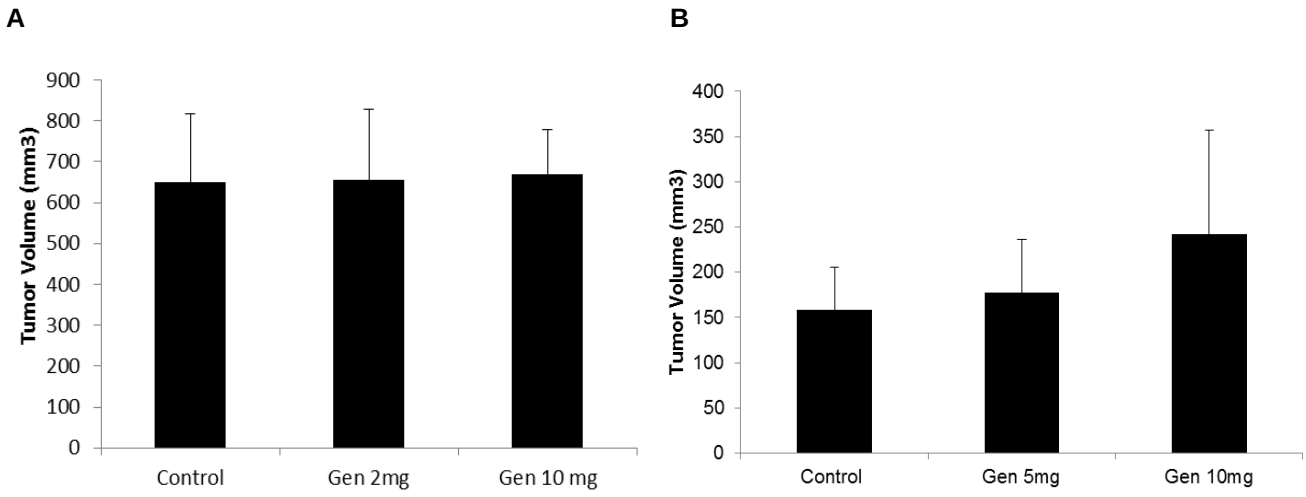


Figure 9. Tumour volumes of LTL163a (A) and LTL313h (B) xenografts at harvest.

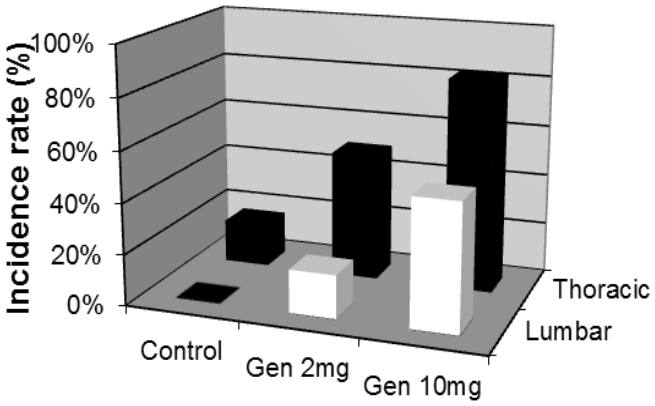
After four weeks of treatment with genistein, tumour grafts were surgically removed from the renal graft sites. For each animal, height, width and length of the tumours were measured using calipers. *Columns*: mean tumour volumes (mm^3) ± SD. n=6/group for LTL163a. n=5/group for LTL313h. Unpaired ANOVA was used for statistical analysis.

2.1.4 Effect of genistein on metastasis

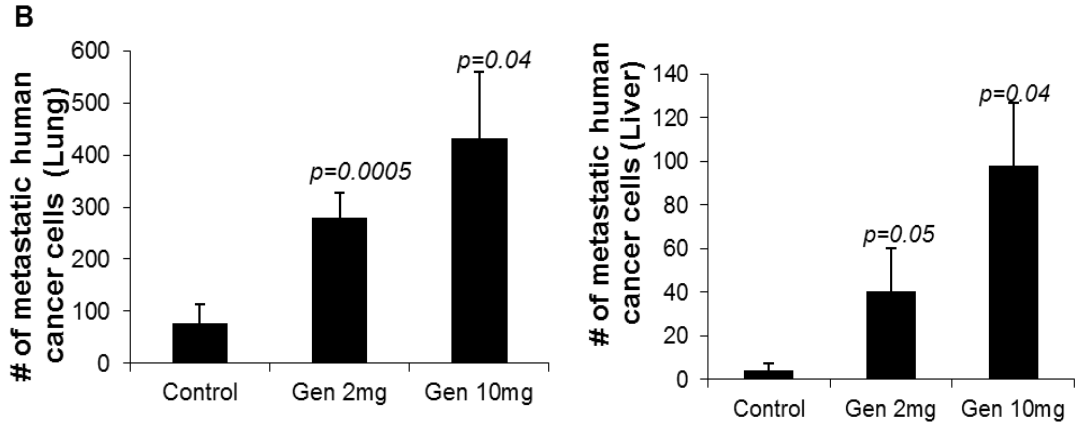
To assess the distant spread of tumour cells, pancreatic, thoracic, lumbar and renal lymph nodes as well as internal organs (i.e. lung and liver) were collected and analyzed using an anti-human-specific anti-mitochondria antibody. All three groups of the LTL163a-tumour line showed evidence of human cells in the renal and pancreatic lymph nodes regardless of treatment. However, in the lumbar and thoracic nodes, the LTL163a-control group had no or low incidence of metastasis (0% in lumbar and 17% in thoracic nodes), whereas the mice treated with both low- and high-dose genistein dosages showed markedly higher metastatic incidence in these two types of nodes (figure 10a) (17% for the low-dose and 50% for the high-dose in the lumbar nodes; 50% for the low-dose and 83% for the high-dose in the thoracic nodes). Similarly, the numbers of metastatic cancer cells in the lungs and livers of the LTL163a tumour-bearing mice were significantly higher in the groups treated with genistein than in the control (figure 10b).

Although scattered cancer cells were observed in the lungs and livers of all mice (figure 10c), only genistein-treated mice showed aggregation of cancer cells (>75 cell aggregate) in those organs to form islands/micrometastases, as shown in figure 10d. Thus, by several criteria, genistein had a dose-dependent metastasis-promoting effect on lymph nodes, lung and liver in the LTL163a prostatic cancer model.

A


Incidence rate (%)
100%
80%
60%
40%
20%
0%
Control
Gen 2mg
Gen 10mg
Thoracic
Lumbar

B


# of metastatic human cancer cells (Lung)
600
500
400
300
200
100
0
p=0.0005
p=0.04
Control
Gen 2mg
Gen 10mg
# of metastatic human cancer cells (Liver)
140
120
100
80
60
40
20
0
p=0.05
p=0.04
Control
Gen 2mg
Gen 10mg

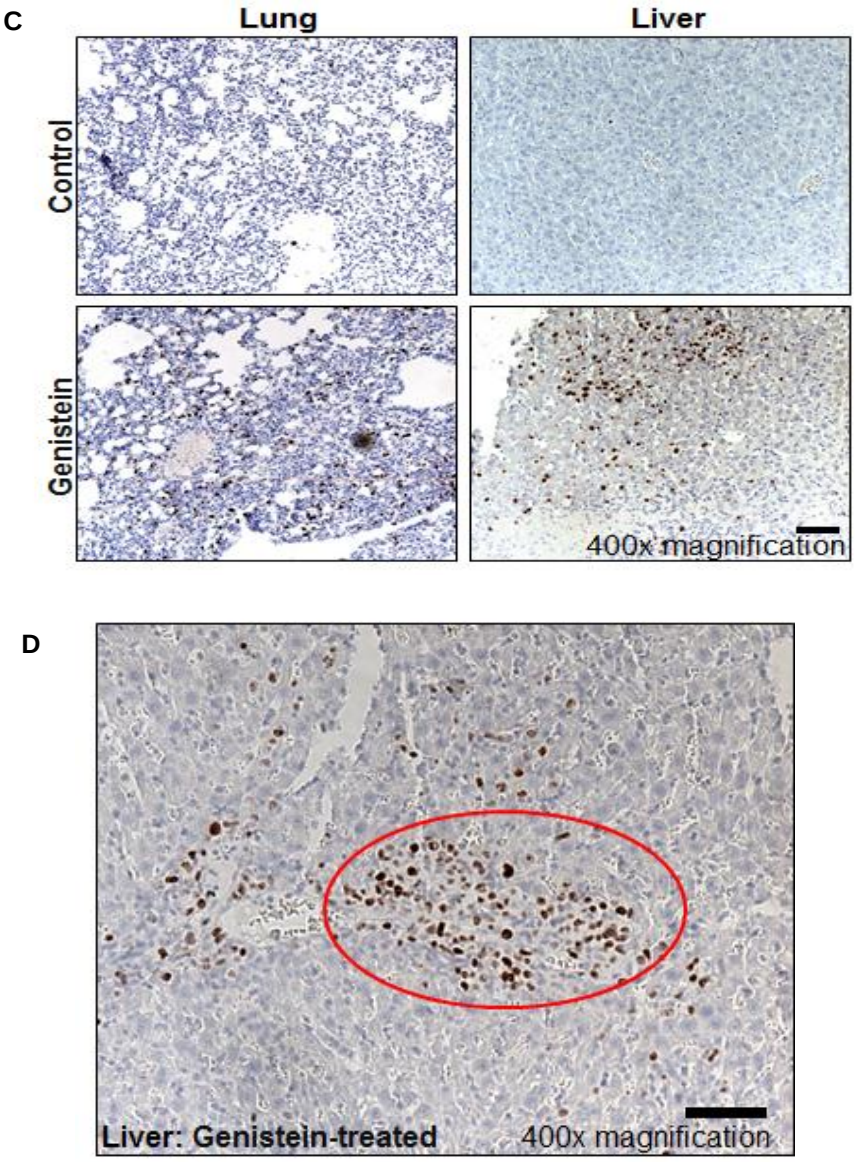


Figure 10. Genistein increases lymph node and secondary organ metastasis in LTL163a xenograft-bearing mice.

At the time of harvest, lung, liver and four lymph nodes, including renal, pancreatic, lumbar and thoracic nodes, were collected from all animals. Each of these organs was fixed, processed, stained with a human-specific anti-mitochondria antibody and screened for presence of human cells. Images of IHC-stained sections from each organ were captured using an AxioCam HR CCD mounted on an Axioplan 2 microscope with final magnifications of x400. Black scale bar:50µm.

A. Percentages of animals containing human metastatic cells in either lumbar or thoracic lymph nodes were calculated. In renal and pancreatic lymph nodes, all animals in the three groups showed ample evidence of human cancer cells regardless of treatment (data not shown) and hence were omitted in the calculations. However, genistein-treated groups showed evidence of increased incidence of metastasis in lumbar and thoracic nodes compared to the untreated control group. The metastatic spread into lumbar and thoracic lymph nodes is presented as mean percentage in mice carrying LTL163A tumour grafts from three groups (untreated-control, low-dose and high-dose genistein). *Black columns: metastatic incidence of thoracic lymph nodes. (SD:0.47, 0.5, and 0.37 for control, low and high genistein groups.) White columns:*

metastatic incidence of lumbar lymph nodes. (SD:0.4, 0.37, and 0.5 for control, low and high genistein groups.)

B. For lung and liver, the number of positively stained cells was counted within randomly-selected microscopic fields. *Columns:* mean number of invading cancer cells per microscopic field observed in lung and liver of the three groups ± SD. Results were statistically analyzed by student t-test (comparing between control and treatment) at the 95% confidence level.
C. Ki67-IHC staining of representative sections of secondary organs (liver and lung) from untreated control and genistein-treated mice. Ki67 antibody used in this study is human-specific. All magnifications are X400.
D. Ki67-IHC staining of liver sections from a genistein-treated (high-dose) animal showing aggregation of invading cancer cells. Red circle indicates a small island/micrometastasis focus containing >75 human cancer cells, a phenomenon that was observed only in genistein-treated mice. Magnification is X400.

Compared to the LTL163a xenografts, the LTL313h xenografts have a lower growth rate in NOD-SCID mice and metastasize at a lower rate. At the time of harvest, the lymph nodes were considerably smaller than in the case of the LTL163a tumour-bearing mice and hence were not collected for analysis. The presence of malignant cells was detected in the lungs but not in the liver of mice grafted with this tumour line. Although the lungs of all animals showed cancer cell infiltration regardless of treatment, the genistein-treated mice had higher counts of invading cells (low-dose: average 21 invading cells and high-dose: 33 cells) compared to control (13 invading cells). As found with the LTL163a tumour-bearing mice, the invasive effect of genistein in the LTL313h was dose-dependent, although only in the case of the high-dose genistein treatment was the effect statistically significant (p=0.008) as compared to the control (figure 11). The data generated with both tumour lines indicate that genistein's metastasis-promoting effect is not patient-specific.

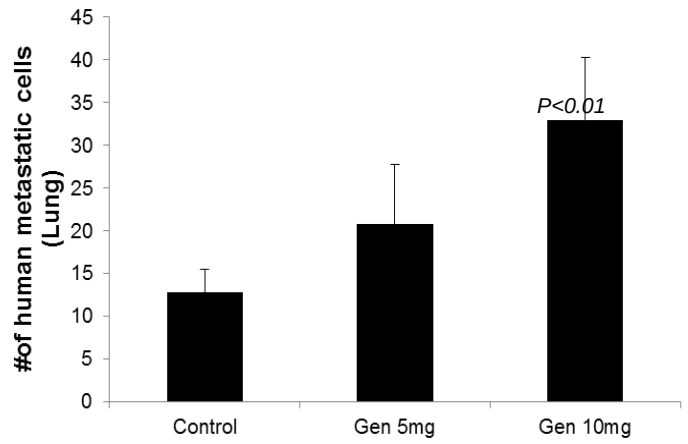


Figure 11. Genistein increases metastasis in the lungs of LTL313h tumour-carrying mice.

At the time of harvest, secondary organs (lung and liver) were collected from all animals. Each of the organs was fixed, processed, stained with a human-specific anti-mitochondria antibody and screened for presence of human cells. Images of IHC-stained sections from each organ were captured using an AxioCam HR CCD mounted on an Axioplan 2 microscope with final magnifications of x400. No invading cells were detected in the liver. The number of positively stained cells in the lungs was counted within randomly-selected microscopic fields. *Columns:* mean number of invading cancer cells per microscopic field observed in the lungs of the three groups ± SD. Results were statistically analyzed by student t-test/unpaired ANOVA at the 95% confidence level. (comparing between control and treatment)

2.1.5 Effect of genistein on cell proliferation and apoptosis

Genistein promoted the spread of human cancer cells in our human PCa tumour models. To investigate if the genistein-induced increase in metastasis of the LTL163a and LTL313h tumour lines was due to altered cell proliferation, immuno-histochemistry was performed using a human-specific Ki67 antibody. The results showed strong positive staining within the tumour grafts of all groups as well as in locally invaded regions of the mouse kidney. The Proliferation index (PI) was measured from the mid-sections of the tumour graft areas. A comparison of the two tumour lines confirmed that the more aggressive LTL163a tumours had a much higher proliferation rate than the LTL313h tumours. Despite the difference in aggressiveness of the two lines, the genistein-treated xenografts in both studies showed significantly higher PIs than their untreated controls (figure 12).

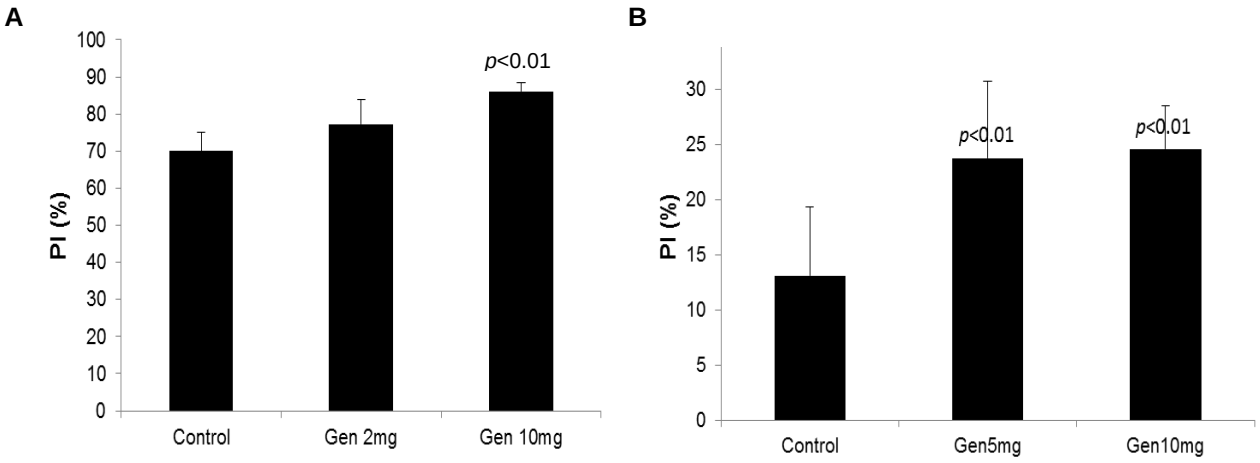


Figure 12. Genistein stimulates tumour cell proliferation.

Proliferation index (PI) was assessed by IHC using a human-specific proliferation marker, Ki67. The numbers of Ki67-positive and –negative human cells were counted within randomly selected microscopic fields for every tumour graft and averaged. Proliferation Index (%): (Ki67- positive cell counts/total human cell counts) x 100. Proliferation Index: A. LTL163a tumours, B. LTL313h tumours. Results were statistically analyzed by student t-test/unpaired ANOVA at the 95% confidence level. (comparing between control and treatment)

Next, the rates of programmed cell death, apoptosis, were investigated. Immunohistochemical analysis using an anti-caspase 3 antibody revealed that tumours in genistein-treated mice had fewer caspase-3-positive cells than those in the control group in both studies (figure 13). The lower Apoptotic Index (AI) values observed in the genistein-treated groups, relative to controls, indicate that genistein had an apoptosis-inhibitory effect.

Treatment of mice carrying xenografts with genistein resulted in increased metastases to lymph nodes and secondary organs compared to untreated controls. Interestingly, tissue-invasive malignant cells aggregated in the secondary organs to form micrometastases only in the genistein-treated mice. Such *in vivo* cancer-promoting effects of genistein were also observed in another tumour tissue line derived from a different patient, indicating that this effect was not patient-specific.

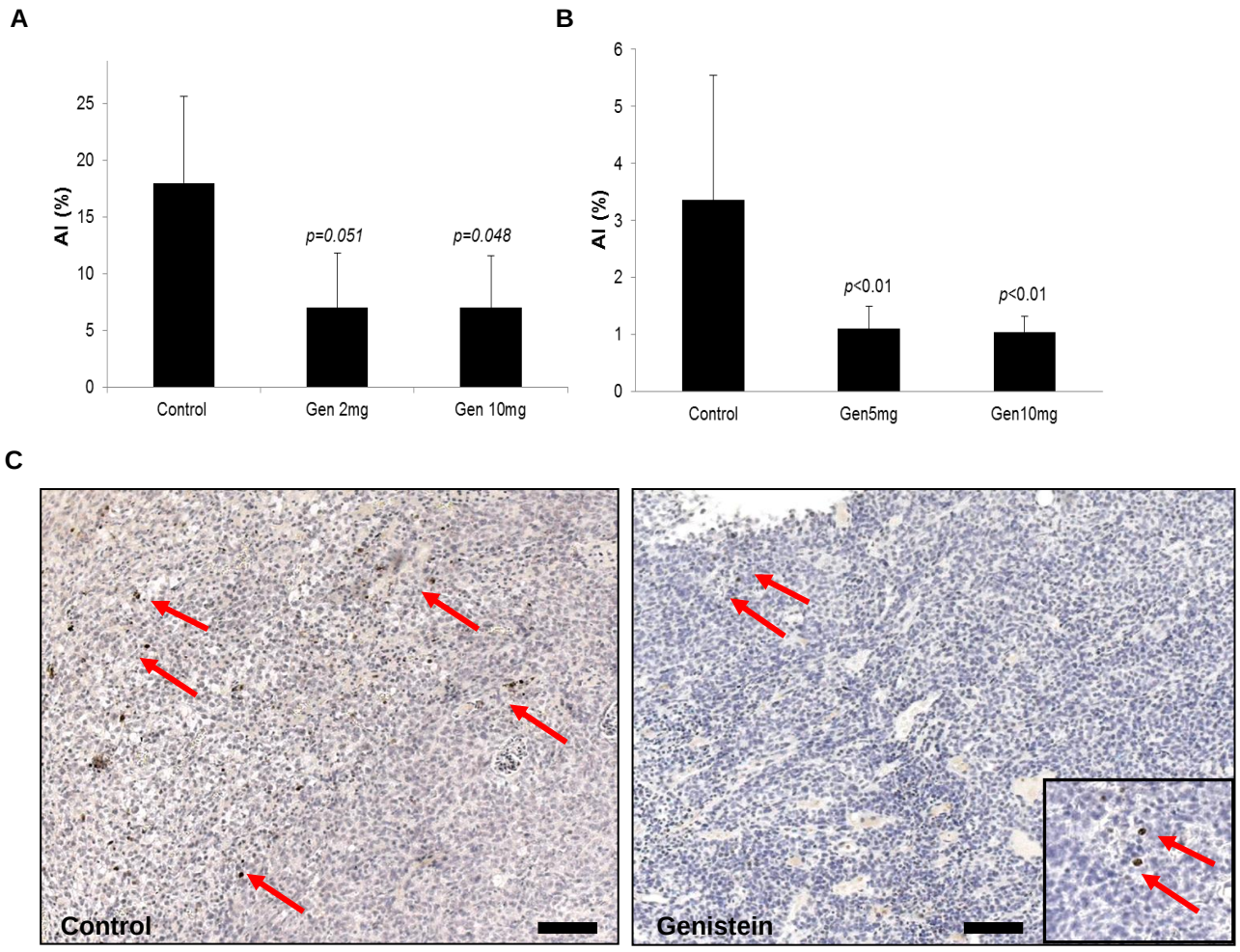


Figure 13. Genistein affects apoptosis.

The Apoptotic Indices (AI) were calculated from caspase-3-stained IHC sections of tumour grafts from all mice. The number of apoptotic cells per 10,000 human cells was counted for each tumour graft and averaged. Apoptotic Index (%) = (Caspase-3-positive cell counts/total human cell counts) x 100.

A. Apoptotic Index of LTL163a tumours.
B. Apoptotic Index of LTL313h tumours.
C. Caspase-3 immunohistological staining of tumour grafts from untreated and high-dose genistein-treated LTL163 animals. Red arrows indicate positive apoptotic cells. All magnifications are X200. Scale Bar: 50µm. Inset is a magnified image of positively stained cells.

2.2 Estrogenic effects of genistein

Due to its similar molecular structure to estradiol, genistein exhibits weak estrogenic activity by binding to the estrogen receptor (ER) and thus can modulate estrogen-regulated gene transcription in target organs [318,319]. Here, genistein's estrogenic transcription activity was tested using an estrogen response element (ERE) reporter assay.

2.2.1 Methods

2.2.1.1 Cell cultures

Freshly isolated LTL163a tumour tissue was rinsed with calcium and magnesium-free Hanks' Balanced Salt solution (Stem Cell Technologies, Vancouver, BC), cut into small pieces and digested with 0.5% type I collagenase (Gibco/Invitrogen, Burlington, ON) for 45 minutes at 37^{o}C on a shaker. The cell mixture was filtered using 100µm-filters and centrifuged at 800 x g for 10 minutes, after which the supernatant was discarded. The dispersed LTL163a cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 ^{o}C and maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL), 100 IU/ml penicillin G, and 100 µg/ml streptomycin (Stem Cell Technologies).

2.2.1.2 Vector construction

In collaboration with Dr. D. Mager's laboratory (British Columbia Cancer Research Centre, Vancouver, Canada), a luciferase reporter gene vector was constructed in which an estrogen response element (ERE) was inserted upstream of the luciferase gene. While several variations of ERE binding motifs are known to exist in the human genome [320], a consensus ERE sequence, EREc38, was selected that was recently reported to induce high transcriptional

activity upon human ERβ binding [320]. The following two oligonucleotides complementary to the consensus sequence (underlined) were obtained from Invitrogen (Burlington, ON).

ERE-F: 5′-CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3′

ERE-R: 5′-GGTCCAGTCTCACTGGACTCGATTTTATTGTGTAAGTC-3′

Briefly, the ERE-encoding oligos were resuspended in water to 10 uM and equal amounts of each were mixed. Next, the oligoucleotide mixture was placed in a boiling water bath for 5 minutes then allowed to cool to room temperature. Subsequently, the hybridized oligos were cleaved by the restriction enzymes, SacI and XhoI (Invitrogen), followed by purification using a PCR purification kit (Qiagen, Toronto, ON). The insert was ligated into the pGL4 Basic luciferase vector (Promega, Madison, WI), which was digested using the same enzymes as used for the insert and also purified in the same manner.

The derived pGL4-ERE vector was amplified in DH5a *Eschirichia coli* and purified using a published mini-prep protocol [321]. The isolated pGL4-ERE plasmids were sequenced at the McGill Sequencing Centre to verify their fidelity, prior to final amplification/purification using the Invitrogen maxi-prep kit.

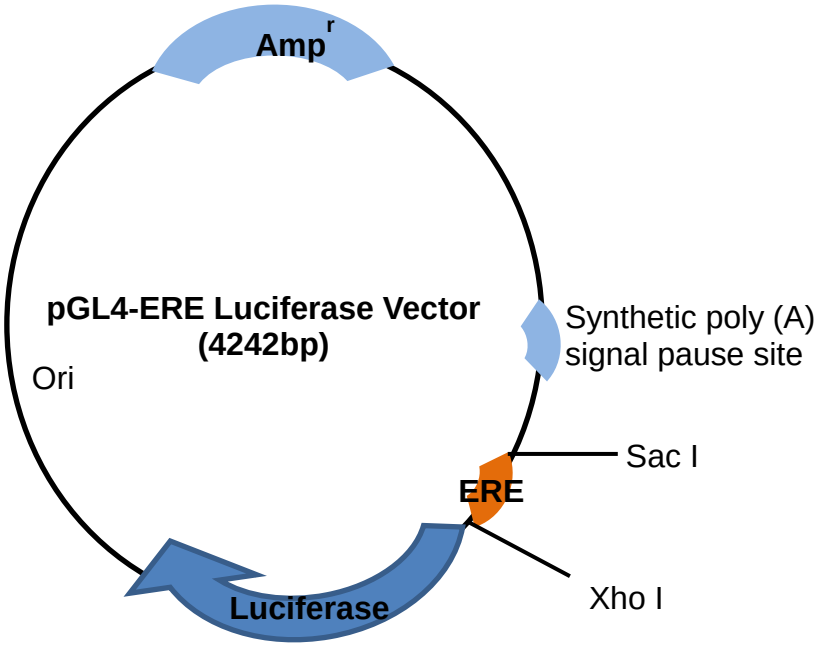


Figure 14. Map of the constructed ERE luciferase reporter gene vector.

Oligos containing an ERE consensus sequence, CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG, were hybridized, cleaved by restriction enzymes, SACI and XhoI, and inserted upstream of the luciferase gene.

2.2.1.3 Luciferase assay

To investigate whether genistein has estrogenic transcriptional activity, primary cultured LTL163a cells were transiently transfected with a reporter construct containing an estrogen response element in a promoter region upstream of a luciferase gene. The pGL4-ERE-Luciferase reporter and ARR3TK-Luciferase plasmid (a gift from P. Renny, The Vancouver Prostate Centre, Vancouver, Canada) were used for the assay.

LNCaP cells and primary cultured cells of the LTL163a tumour were seeded in 24-well plates for 24 hours prior to transfection in phenol red-free RPMI 1640 media supplemented with 5% charcoal-stripped FBS (Hyclone/ Thermo Scientific, Waltham, MA). After a 24 hour-transfection with lipofectamin (Invitrogen), cells were incubated with genistein (50 μM), ICI 182,780 (200 nM), 17β-estradiol (100nM), R1881 (1, 10, and 50nM) or vehicle for an additional 24 hours. The luciferase activity was measured using a Steady Glo Luciferase assay kit

(Promega) with a luminometer (Montreal Biotech, Kirkland, PQ). Bicinchoninic acid (BCA) quantification kit (Pierce/Thermo Scientific, Waltham, MA) was used to measure protein concentrations for normalizing the luciferase activity.

2.2.1.4 Treatments with genistein and ICI 182,780

The objectives of the following study were to determine if (1) genistein promotes prostate cancer progression via estrogen receptor activation *in vivo* and (2) ICI 182,780 could block the estrogenic activity of genistein and inhibit tumour growth and/or metastatic progression.

LTL313h tumour-bearing mice were treated with genistein, ICI 182,780 (anti-E_2) and a combination of both. Grafting of LTL313h tumour tissue into NOD-SCID mice was accomplished as described in section 2.1.1., with supplementation of testosterone to maintain tumour growth and circumvent reduction of endogenous androgen levels resulting from phytoestrogen action via the hypothalamic/pituitary/gonadal axis. The animals were randomly distributed into 5 groups: control (untreated), low-dose and high-dose of genistein, ICI 182,780 (AstraZeneca, Mississauga, ON) and a combination of genistein and ICI 182,780. Each group consisted of 5 mice except for the ICI 182,780 group, which contained 4 mice. The LTL313h tumour line grows more slowly than other prostate cancer tissue lines in our laboratory, thus requiring a longer incubation time in the renal site before treatment could be initiated. After the tumours had reached a size of approximately 50 mm^3 (~6 weeks after grafting), genistein, dissolved in peanut oil, was administered to the mice in the low-dose genistein group by gavage at 5 mg of genistein/day (200 mg/kg body weight/day). Mice in the high-dose group received 10 mg/day (400 mg/kg/day). Mice in the control and ICI 182,780-only groups received 0.1 ml of oil-vehicle daily, while the combination group received 5 mg of genistein/day.

Previously, Yellayi *et al.* demonstrated that the estrogenic effects of genistein at a 5 mg/day-dosage on uterus and vaginal weights were inhibited by administration of ICI 182,780 at 5 mg/mouse/week [322]. Therefore, the same dosage (5 mg/week) of ICI 182,780 was administered to the mice in the ICI-only and combination groups as a single subcutaneous injection in this study. The treatments were carried out for four weeks and at the end of the fourth week, animals were sacrificed and blood samples and organs (kidney with tumour grafts, liver, lung, and spleen) were collected for analyses.

2.2.1.5 Histopathology and immunohistochemistry

Immunohistological analyses were carried as described in section 2.1.1. The primary antibodies used in this study were human-specific anti-ERα and PCNA (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERβ (Abcam, Cambridge, MA) and anti-AR (Upstate-Millipore, Billerica, MA). All tissue sections were counter stained with 5% (w/v) Harris hematoxylin and coverslipped with Permount (Fisher/Thermo Scientific).

2.2.1.6 Western blot analysis

The remaining half of the harvested tumour tissues was snap frozen for protein analysis. The frozen tissues were homogenized using a mortar and pestle in NP-40 lysis buffer (150 mM sodium chloride, 1.0% NP40, and 50 mM Tris-HCl, pH 8.0) on ice, centrifuged at 12,000 rpm for 20 minutes at 4 ˚C in a microcentrifuge, and their supernatants were collected. The bicinchoninic acid (BCA) assay (Thermo Scientific) was performed according to the company's instructions to measure the protein concentration of each tumor sample via colorimetric detection and absorbance at 562 nm. For protein separation via gel electrophoresis, protein samples were

boiled for 5 minutes in hot water bath, and 20 µg of protein was subjected to SDS-PAGE (polyacrylamide gel electrophoresis) and electrotransferred to a PVDF membrane. The proteins on the membrane were blocked with 5% bovine serum albumin (Sigma-Aldrich) or 5% non fat milk in Tris-Buffered Saline (pH 7.4) containing 0.1% Tween 20 (TBST) and incubated with primary antibodies to ERα (Santa Cruz Biotechnology), ERβ (Abcam) and actin (Sigma-Aldrich). Following this, the membranes were washed with TBST and probed with appropriate HRP-conjugated secondary anti-mouse (Pierce) or anti-rabbit (Fisher/Thermo Scientific) antibodies. For the detection of proteins of interest, an enhanced chemiluminescence Western Blotting kit was used (Pierce).

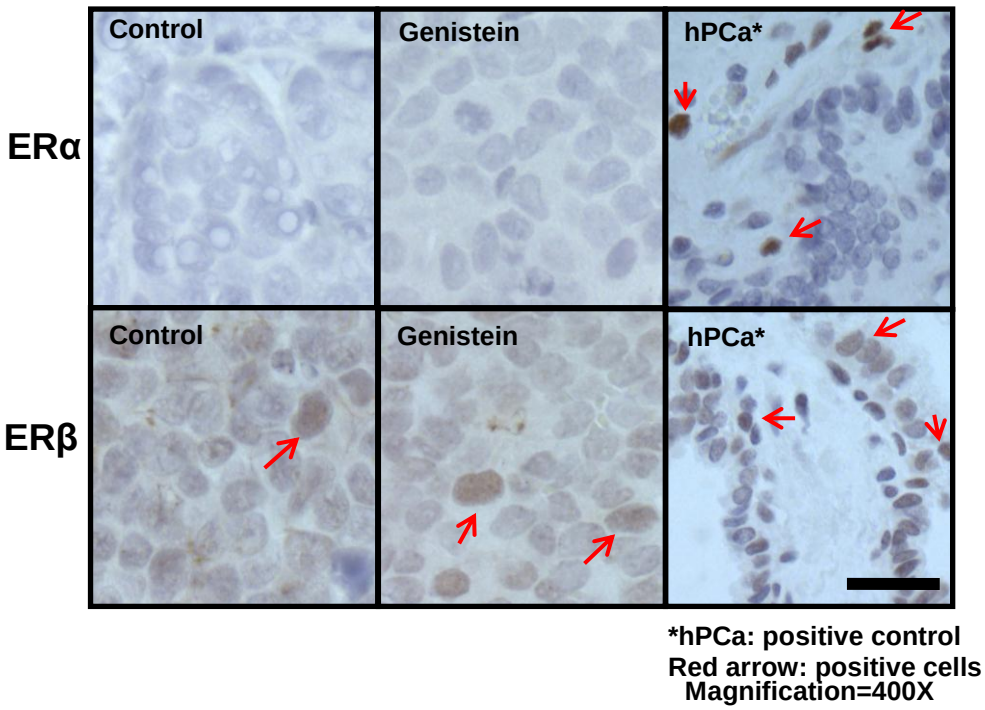
2.2.2 Estrogen receptor β (ERβ): a dominantly expressed ER in LTL163a and LTL313h tumour lines.

Previous studies have shown that genistein binds preferentially to ERβ with a 30-fold higher affinity compared to ERα [225,323]. Therefore, the estrogenic actions of genistein likely depend upon the relative expression levels of ERβ by its target cells/tissues.

To determine the relative protein expression levels of ERα and β in LTL163a and LTL313h tumour lines, IHC and western blot analyses were conducted in both genistein-treated and control (untreated) tumours. As can be seen from the IHC sections below (figure 15), ERα expression in both tumour lines was undetectable via IHC analysis. In contrast, weak to moderate nuclear staining of ERβ was observed in most of the cancer cells, with some cells showing intense positive staining. Although there was no apparent difference in ERβ staining patterns between the two tumour lines, the LTL163a tumours had more intense staining than the

LTL313h tumours, which are less aggressive in nature (i.e. grow and metastasize more slowly in mice).

A



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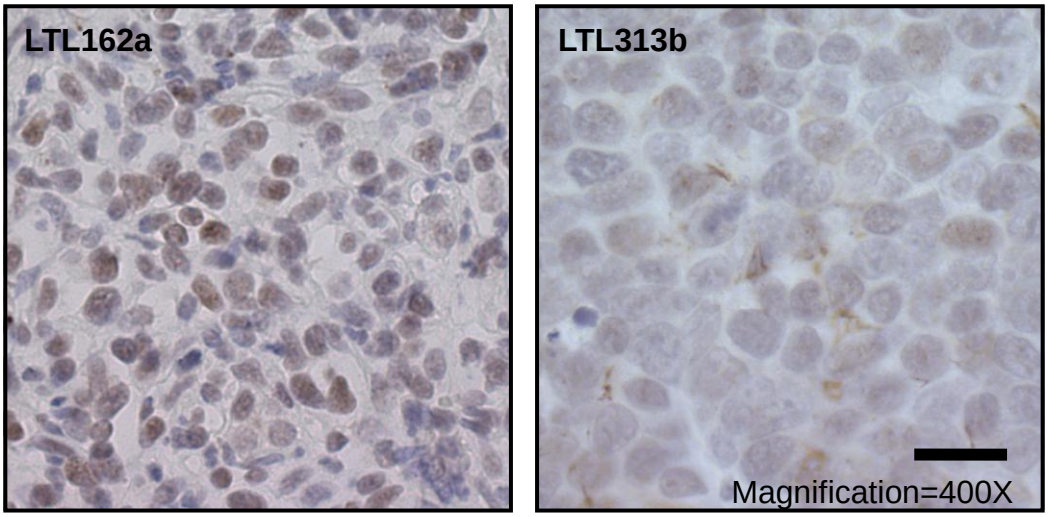


Figure 15. Estrogen receptor expression in LTL163a and LTL313h tumour lines.

A. ERα and ERβ-IHC staining of representative sections of LTL313h xenograft tumours from untreated-control and genistein-treated mice. Right panels: human prostate cancer tissues as positive controls. All magnifications are X400.
B. ERβ-IHC staining of LTL163a and LTL313h tumours in untreated mice. Magnification is X400. Scale bar: 50μm.

To investigate whether ICI 182,780 has an effect on ER expression levels, we performed an immunoblotting analysis. Due to hemoglobin contamination in almost all of the protein lysates which was caused by heavy red blood cell infiltration of the xenografts, band intensity was normalized using actin and quantified using densitometry. As can be seen in the Western blot image below (figure 16), ERα expression was very minimal with only very faint bands visible in a few tumours of genistein-treated and control mice. There was a slight increase in ERβ expression in genistein-treated tumours when compared to controls, but this increase was not statistically significant. ICI 182,780, on the other hand, significantly inhibited the protein expression of ERβ in all mice.

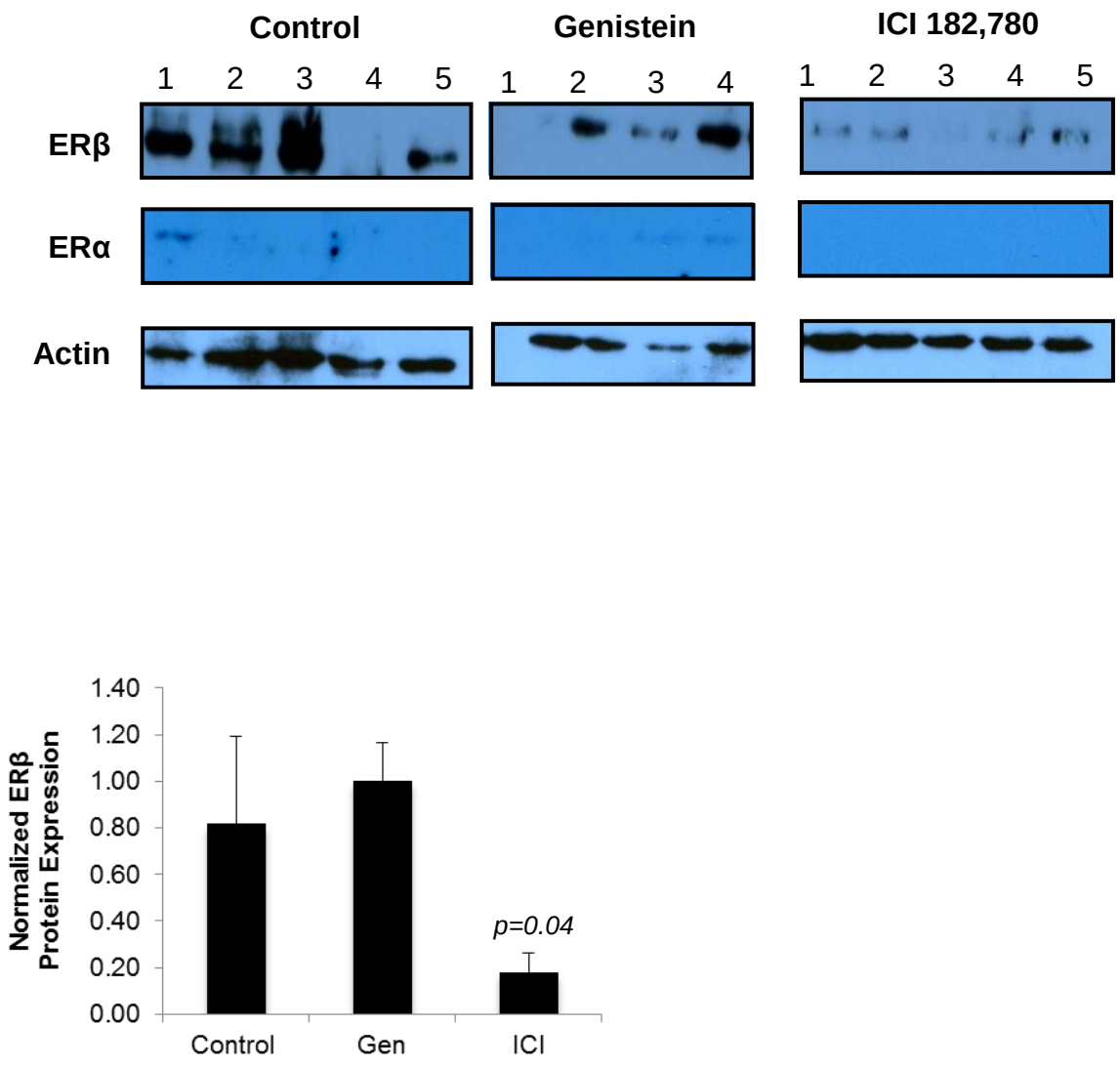


Figure 16. Western blot analysis of ERα and β expression in LTL313h tumours of untreated, genistein- or ICI 182,780-treated mice.

Expression of ERβ, as exhibited by control and genistein-treated mice, was significantly inhibited by ICI 182,780. Each lane represents protein lysate harvested from tumours of individual mouse. Below is the quantification of band intensity for ERβ from protein lysates of 5 untreated control and 4 genistein-treated tumours. *Columns*: mean ratio of ERβ/actin band intensity± SD.

2.2.3 Genistein activates estrogen-responsive gene transcription

Since the LTL163a and LTL313b tumour lines predominantly express ERβ, we hypothesized that genistein modulated its estrogenic effects on these tumours via ERβ. To investigate if genistein can bind to ERβ and induce estrogen-responsive gene transcription, a luciferase reporter assay was carried out using a plasmid, in which an estrogen-responsive element is inserted upstream of a luciferase-coding region.

48 hours after transfection of LTL163a primary culture cells and subsequent 24 hour-incubation with genistein, the genistein-treated cells showed an almost 20-fold increase in luciferase activity compared to the vehicle control. This genistein-induced luciferase activity was inhibited by addition of ICI 182,780 (figure 17). To rule out the possibility that the genistein's effects are mediated by the androgen receptor, LNCaP cells (AR- and ERβ-positive) were transfected with an Androgen Response Element (ARE)-reporter construct. As shown in the figure below (figure18), genistein did not activate AR or induce androgen- responsive gene transcription. Estradiol treatment, in comparison, increased ARE-luciferase activity slightly. These data demonstrate the ability of genistein to bind to and activate ERβ (not ERα or AR), and to induce transcription of estrogen-responsive genes, which is inhibited by an estrogen antagonist, ICI 182,780.

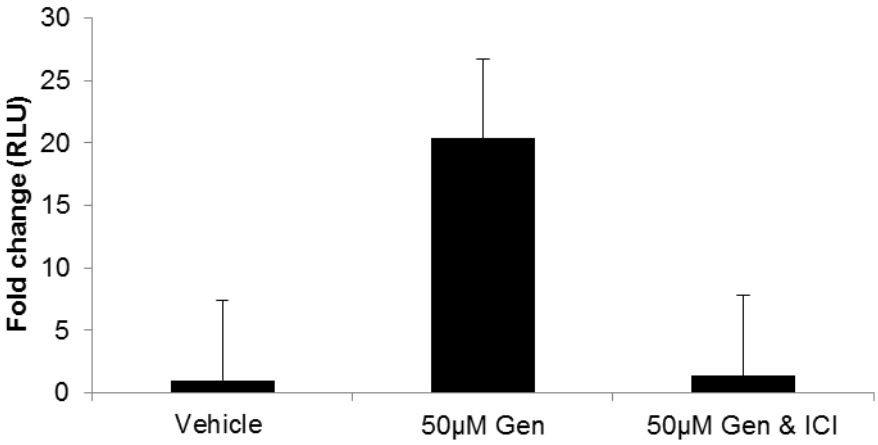


Figure 17. Genistein activates ER transcriptional activity.

Primary culture cells of LTL163a were transfected with pGL4B-ERE luciferase reporter for 24 hours and incubated with genistein and ICI 182,780 for an additional 24 hours. Genistein treatment induced luciferase transcription, while addition of ICI inhibited such activity. *Columns*: mean of fold change in relative light unit ± SE. Unpaired ANOVA was used for statistical analysis.

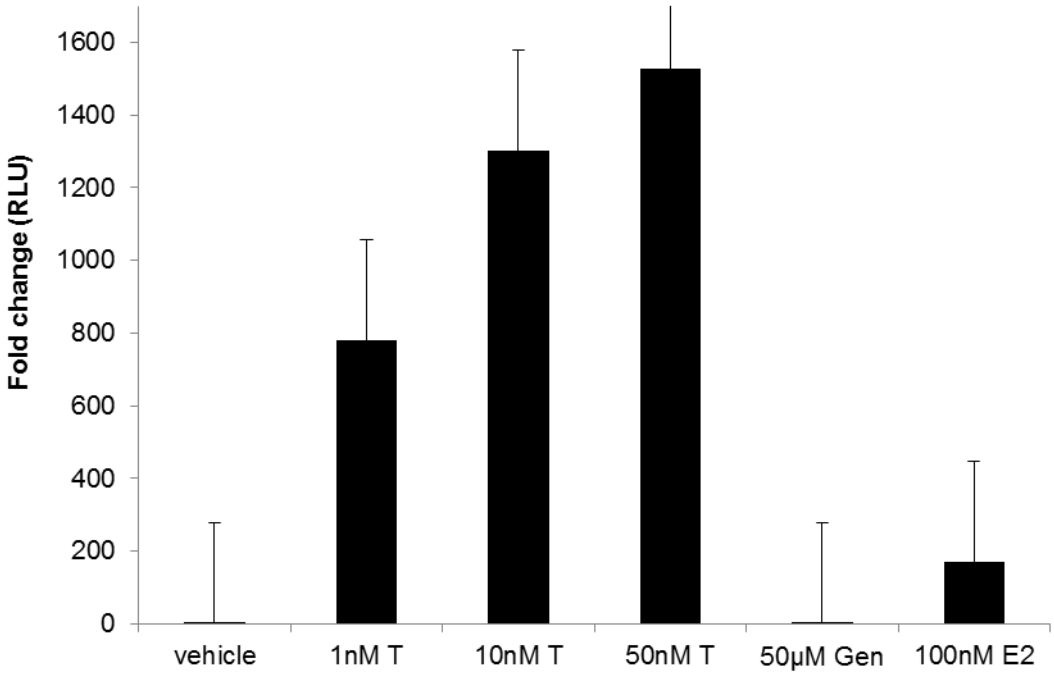


Figure 18. An ARE-luciferase reporter assay was conducted to test genistein's AR-activation and ARE-inductive abilities.

LNCaP cells were transfected with an ARR3TK luciferase vector for 24 hours and incubated with various drugs for an additional 48 hours. T: R1881-androgen. While testosterone showed transcriptional activity, genistein has no effect. Gen: genistein. E2: 17β-estradiol. *Columns*: mean of fold change in relative light units (RLU) ± SE. Unpaired ANOVA was used for statistical analysis.

2.2.4 Effects of the anti-estrogen, ICI 182,780, on metastasis

The results from earlier sections showed the estrogenic property of genistein and the predominant ERβ expression in the LTL163a and LTL313b tumour lines. From these observations, it was hypothesized that the estrogenic activity of genistein would promote metastasis in the prostate cancer model. To test this hypothesis, the mice carrying LTL313b tumour grafts (average size about 50 mm^3) were treated with genistein, ICI 182,780, and a combination of both. To this end, the mice were distributed into five groups: control (untreated), low-dose and high-dose of genistein, ICI 182,780, and combination (5mg-genistein plus ICI 182,780). Each group had 5 mice except for the ICI 182,780 group, which had 4 mice. Genistein was given orally as described before and ICI 182,780 was subcutaneously injected once per week. Treatment continued for four weeks.

Although there was no significant difference in tumour size among the various groups, differences in metastatic incidence in the lungs were substantial: the genistein-treated mice showed higher numbers of lung-invading cells (low-dose: average 21 invading cells and high-dose: 33 cells) compared to controls (13 invading cells) or ICI 182,780-treated mice (average 3 cells). The tissue-invasive effect of genistein was dose-dependent in the LTL313b prostate cancer model, although only the effect with the high-dose genistein treatment was found statistically significant (p=0.008) when compared to controls. Interestingly, ICI 182,780-only group showed a significant reduction in the number of lung-invading cells, indicating an inhibitory effect of the anti-estrogen against metastasis in this model (figure 19).

Using an estrogen-responsive element reporter assay, genistein induced estrogenic gene transcription in primary cultures of the LTL163a tumour line, providing evidence for an estrogenic function of genistein in the patient-derived prostate cancer models. From these data it

was hypothesized that the metastasis-promoting effect of genistein observed in the tumour models could be due to its estrogenic properties. To test this hypothesis, LTL313h tumour-bearing mice were treated with genistein, ICI 182,780 (an anti-estrogen compound) and a combination of both. The results showed increased metastasis in the genistein-treated mice. Although no difference in the combination group was observed, the anti-estrogen treatment alone significantly inhibited cancer spread.

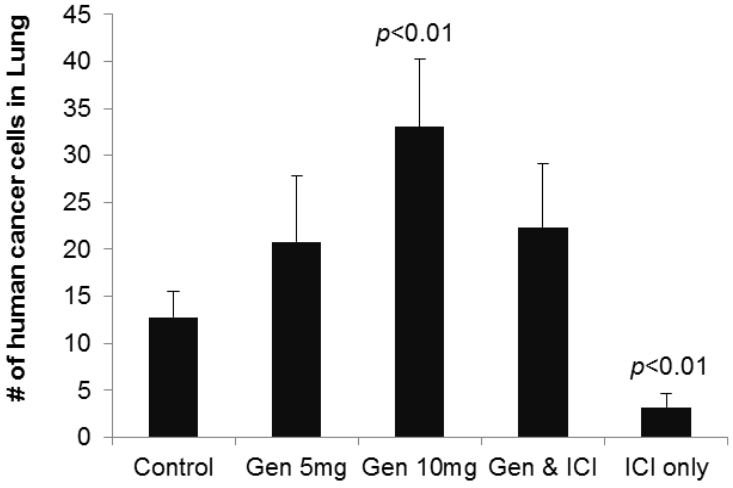


Figure 19. Lung metastatic incidence after treatment of LTL313h tumour-carrying mice with genistein and anti-estrogen (ICI 182,780).

The number of positively stained cells was counted within randomly-selected microscopic fields. Genistein promoted metastasis; on the other hand, ICI treated mice showed reduced number of invading cells in the lung. *Columns:* mean number of invading cancer cells per microscopic field observed in the lungs of the five groups ± SD. Results were statistically analyzed by unpaired ANOVA at the 95% confidence level.

2.3 Gene expression profiles of genistein-treated and anti-estrogen-treated tumours

In contrast to earlier findings in *in vitro* studies [260,280-282], we found that genistein promoted prostate cancer metastasis *in vivo*, and that the anti-estrogen, ICI 182,780, effectively inhibited metastasis. To study genes involved in genistein-induced cancer progression and anti-estrogen-mediated inhibition, whole-genome expression array analysis using messenger RNA extracted from the treated tumours was performed. The microarray data were analyzed using Ingenuity Pathway Software. The results revealed that the majority of genes regulated by both genistein and ICI 182,780 were categorized in “Cancer” and “Genetic Disorder” groups (p<0.002).

2.3.1 Methods

2.3.1.1 Agilent gene expression microarray

Total RNA was extracted from snap frozen LTL313b tumours that were untreated (control), treated with genistein or ICI 182,780 using the RNeasy mini kit by following the manufacturer's instructions (Qiagen). The quality of RNA was analyzed using an Agilent Bioanalyzer (Agilent), and total RNA samples (1 μg) were submitted to the Vancouver Prostate Centre, Microarray Core Facility for gene expression microarray analysis. Using a reverse transcriptase, total RNA was converted into cDNA, which was used to generate cyanine-3-labeled cRNA in accordance with the Agilent protocol (Agilent). After quantification, fluorescently labeled cRNA (1.65 μg) was hybridized on an Agilent Human GE 4X44K v2 Microarray, which targeted 34,127 human genes. After hybridization of the microarray at 65 ^{o}C for 17 hours, the slides were washed and scanned with an Agilent DNA Microarray Scanner (Agilent), and the data were processed using the Agilent Feature Extraction Software (Agilent). To normalize expression data, each measurement was divided by the median expression of all genes on the array, provided that the data followed a normal distribution. Functional and Canonical Pathway Analysis was carried out using Ingenuity Pathway Analysis Software (IPA 8.7).

2.3.1.2 Statistical analysis.

For microarray data analysis, Fisher's Exact Test was performed with a confidence interval of 95%. Differences were considered statistically significant if *p* values were smaller than 0.05.

2.3.2 Analysis of the gene expression microarray data

In order to delineate differential gene expression levels that underlie the mechanism behind the metastasis-promoting effect of genistein or protective effect of ICI 182,780, a whole-genome gene expression array analysis was performed using an Agilent 4x44K platform. Of all 34,127 human genes screened, only the genes which exhibited ≥1.5-fold differences in expression between control and genistein-treated and between control and ICI 182,780-treated tumours were considered in this differential gene expression classification. Figure 20 shows that genistein stimulated 458 genes, and that treatment with ICI 182,780 up-regulated 533 genes. Thirty one genes were identified as commonly regulated by both genistein and ICI 182,780 (figure 20). Similarly, genes down-regulated by these two treatments were classified according to the symmetrical cut-off of fold change<0.66. There were 275 genes down-regulated in the genistein treated tumours, while the treatment with ICI 182,780 lowered expression of 145 genes. A comparison of the two groups led to identification of 44 common genes.

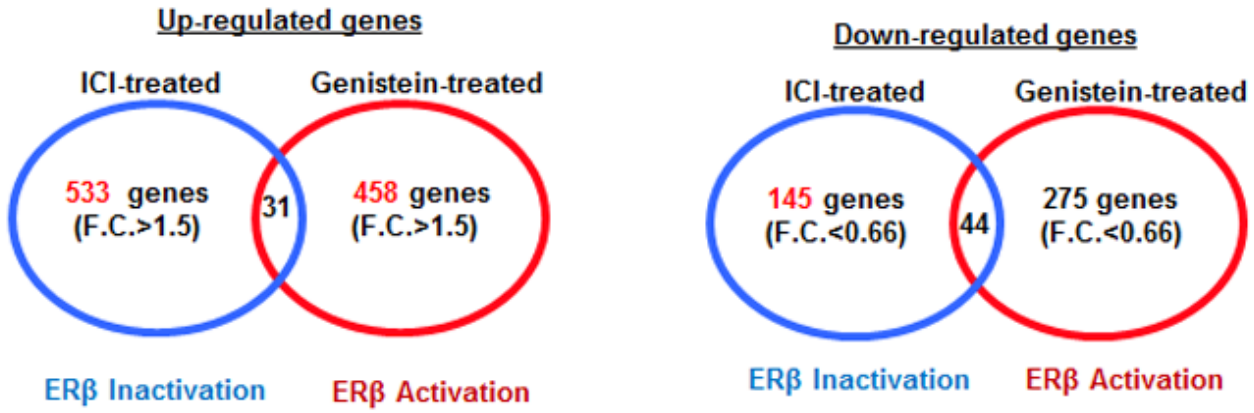
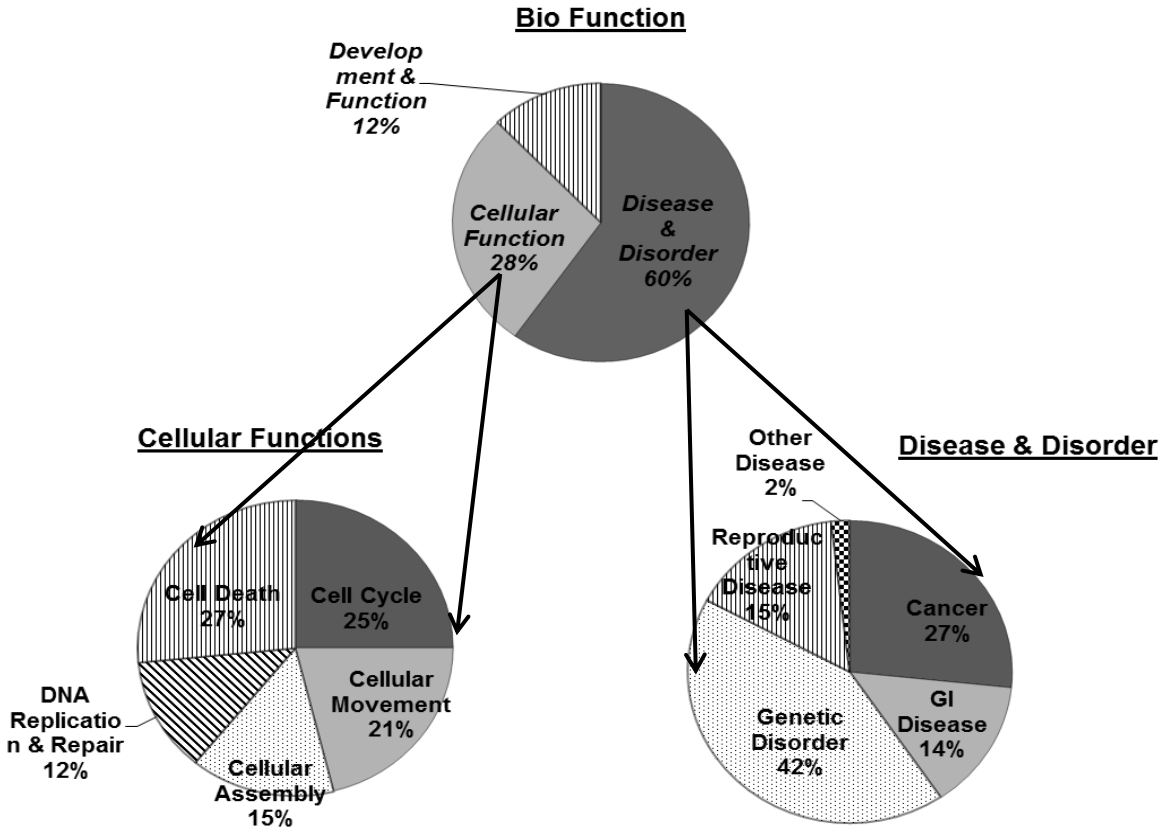


Figure 20. Genistein- and ICI 182,780-regulated genes analyzed by an expression array.

RNA samples extracted from tumours of three mice each from untreated, high-dose genistein and ICI 182,780-treated groups were used for the analysis. Agilent Human GE 4X44K v2 Microarray with 34,127 human specific probes was chosen as a platform. Genes which exhibited at least 1.5-fold increase or 0.66-fold decrease were categorized as up-regulated and down-regulated genes, respectively.

The genes identified by microarray data were further evaluated and categorized by biological functions and mapped to canonical pathways using Ingenuity Pathway Analysis (IPA) software. This program is a powerful database and analysis system for identifying the top biological functions of the genes modulated in the treatment groups, based on p-value, expression levels and the number of molecules that appear in the network. Figure 21 shows significant biological functions associated with up-regulated genes in the genistein and ICI treatment groups, respectively. The top biological functions in genistein-regulated genes included “Cell Cycle”, “Cellular Movement” and “Cell Death”, and in the disease category, the majority of genes stimulated by genistein fell into “Cancer” (27%) and “Genetic Disorder” (42%) (p<0.002). The top cellular functions in the ICI 182,780-modulated genes were “Cell Morphology”, “Cell Development” and “Small Molecule Biochemistry”, and in the disease category, 38% of the genes fell into the “Genetic disorder” group and 19% into the “Cancer” category (p<0.002).

A


Bio Function
Develop ment & Function 12%
Cellular Function 28%
Disease & Disorder 60%
Cellular Functions
Cell Death 27%
Cell Cycle 25%
Cellular Movement 21%
Cellular Assembly 15%
DNA Replicatio n & Repair 12%
Disease & Disorder
Other Disease 2%
Reproduc tive Disease 15%
Cancer 27%
GI Disease 14%
Genetic Disorder 42%

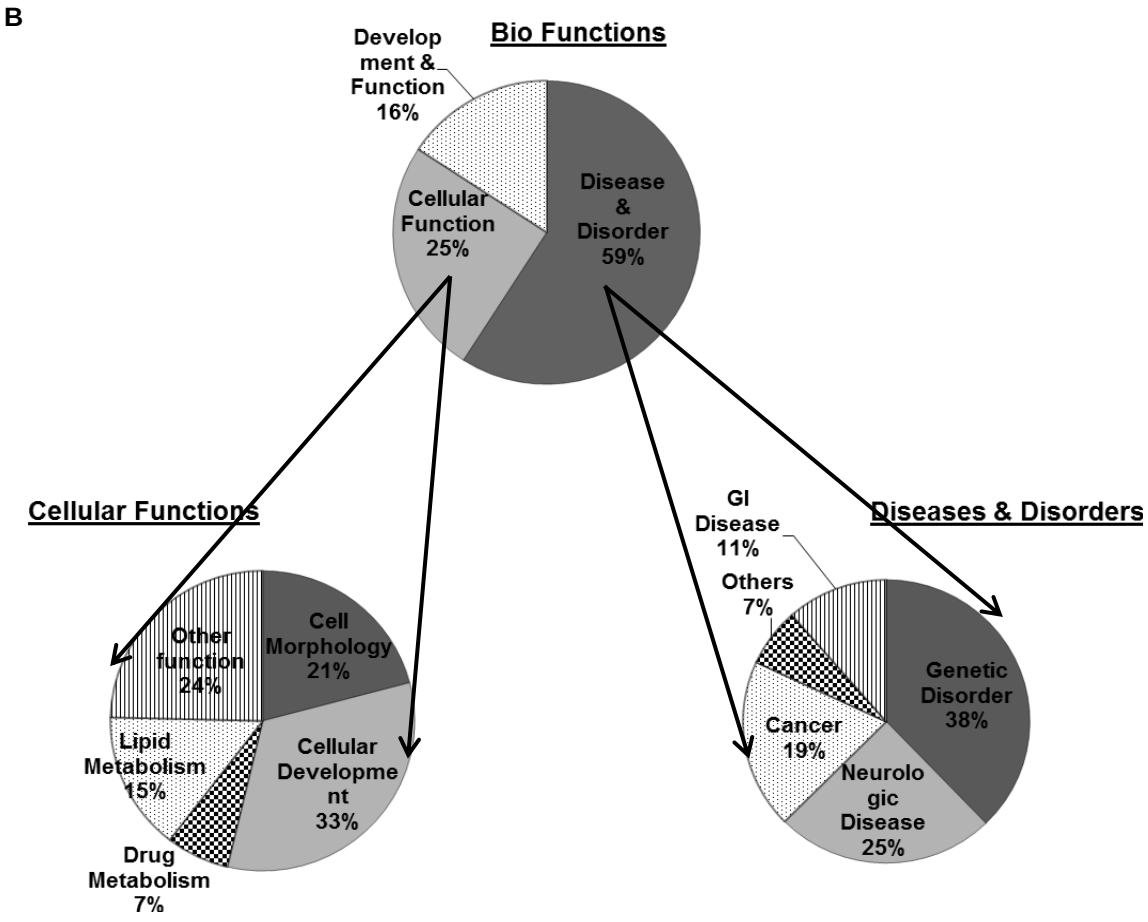


Figure 21. Ingenuity Pathway Analyses showing the top biological functions of the genes identified in genistein- and ICI 182,780- treated tumours.

Up-regulated genes in the genistein-treated group (A) and ICI 182,780 group (B) were ranked and categorized into their biological functions (cellular functions and disease and disorder categories) based on *p*-values.

2.4 Mechanism of genistein-induced metastatic progression via ERβ: non-genomic pathway

The previous sections showed that estrogenic genistein enhanced metastasis, while an anti-estrogen abrogated metastatic spread in the LTL313h prostate cancer xenograft model. In efforts to understand the underlying mechanisms behind the effects of these estrogen agonists/antagonists, a gene expression microarray study was performed using tumours treated with genistein and anti-estrogen (ICI 182,780).

2.4.1 Methods

2.4.1.1 Western blot analysis

To validate the data generated by the microarray analysis, protein expression of the genes identified by the array analysis were measured using the same immunoblotting procedures as described in section 2.2.1. The primary antibodies (phospho-Y397, S732 and total FAK) used in this section were obtained from Upstate.

2.4.2 The focal adhesion kinase pathway

In efforts to delineate which pathways/molecules were involved in genistein-induced metastasis, the microarray data were further analyzed using IPA software. The canonical pathway analysis matched genes identified in the microarray data with known or previously reported pathways and ranked top pathways and networks based on expression levels and *p*-values. Figure 22 shows metastasis-linked pathways and networks that are affected by treatments with genistein and ICI 182,780, respectively. As shown in this diagram, the molecules regulated by the two compounds are not the same; however, both of the treatments targeted the same

pathway, but with opposing effects. For example, genistein up-regulated upstream molecules (i.e. vitronectin, RHAMM and integrin-α) in the FAK pathway, while ICI 182,780 affected downstream molecules (i.e. Rho, MLCK, PTEN) in the same pathway. As depicted in this canonical pathway diagram, most of the molecules in the pathway are involved in metastasis-related processes (i.e. cell survival, adhesion, motility and tissue invasiveness). Through up- or down-regulation of such molecules, genistein and anti-estrogen may modulate the fate of the biology, and in particular the metastasis outcome, of the tumours. In other words, activation of these pathways may lead to cell proliferation, migration and tissue invasion of prostate cancer just as observed in the genistein-treated tumours, whereas inactivation of the pathways may lead to tumour suppression, just as in the ICI 182,780-treated tumours.

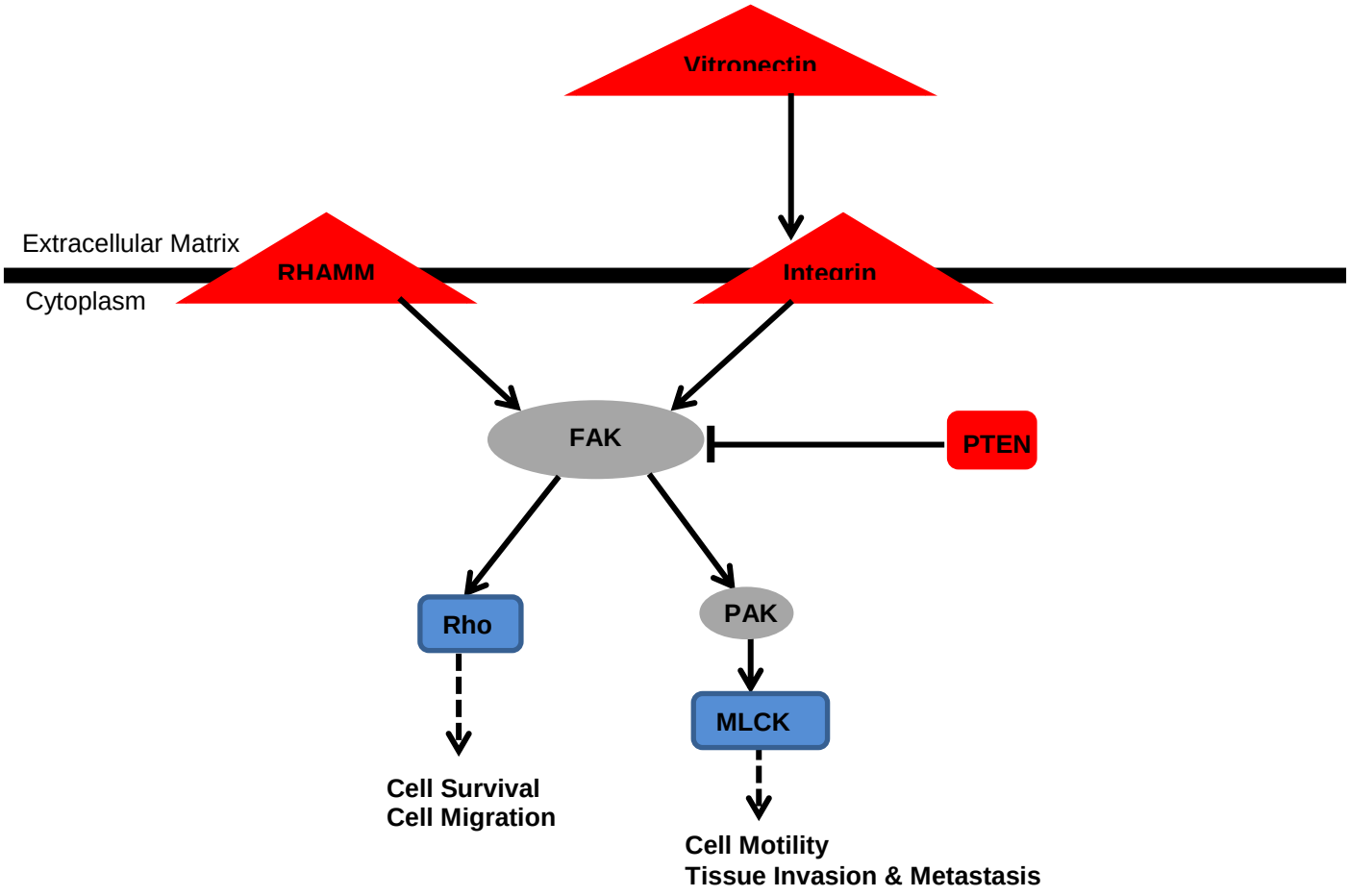


Figure 22. Proposed mechanisms of genistein-induced prostate cancer metastasis via ERß: FAK pathway.

Schematic representation of changes in FAK signaling associated with either genistein or ICI 182,780 treatment as deduced from the Ingenuity Pathway Analysis of Agilent Human gene expression microarray data. Genistein up-regulated FAK upstream molecules, whereas anti-E_2 targeted downstream molecules of the FAK pathway. Triangle: gene expression affected by genistein. Red: up-regulated expression. Blue: down-regulated expression. Grey: no change in expression level detected by the microarray analysis. Square: gene expression affected by ICI.

2.4.3 Validation of the effects of genistein and anti-estrogen (ICI 182,780) on FAK phosphorylation activities

Figure 22 shows that genistein increased expression of upstream molecules involved in integrin/FAK pathway, promoting cell survival and migration, while anti-estrogen targeted downstream of the same pathway, inhibiting metastasis. To validate the effects of genistein and ICI 182,780 on the molecules identified by the IPA analysis, an immunoblotting analysis was performed to examine phosphorylation levels of FAK, which plays the central kinase function in this signaling network. Due to a high amount of hemoglobin in all LTL313b tumour samples, Western blot data were normalized using densitometry as previously described. As shown in Figure 23, genistein-treated LTL313b tumours showed general or overall higher phosphorylation activity at tyrosine 397 (Y397) and at serine 732 (S732) residues, compared to control or ICI 182,780-treated tumours. Although the level of phospho-Y397 was not lower in the ICI 182,780-treated tumours compared to the level in the control, the phospho-S732 in the ICI 182,780-treated tumours was considerably lower than in control or in genistein-treated tumours. Taken together, the data show that genistein indeed stimulates activity of FAK and its signaling pathway, while ICI 182,780 represses such activity although the sites on which these compounds act appear different.

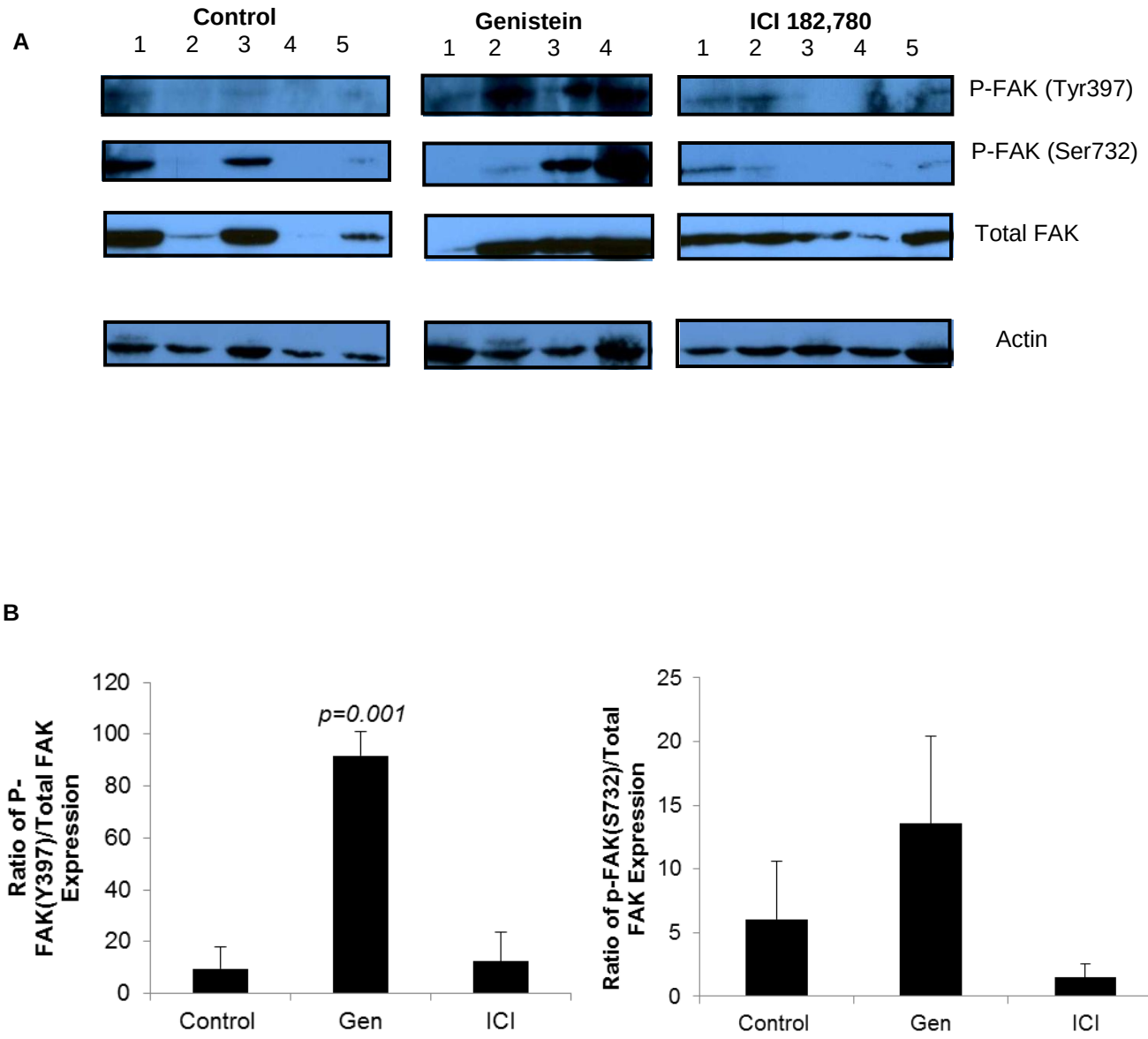


Figure 23. Effects of genistein and ICI 182,780 on FAK phosphorylation.

A. Genistein-treated LTL313b tumours had over-all higher FAK phosphorylation activity at tyrosine 397 and at serine 732, while Anti-E_2 inhibited FAK activation at serine 732. Lanes indicate proteins from individual mice carrying LTL313b tumours.
B. Densitometric quantification of phospho-Y397 and phosphor-S732 FAK. *Columns*: mean ratio of phosphorylated FAK/total FAK protein band intensity ±SD.

2.4.4 Epidermal growth factor receptor pathway

Previous *in vitro* studies have shown that genistein modulates tyrosine signaling pathways by altering kinase activities [290-292]. To determine if genistein alters phosphorylation patterns of other (non-FAK) kinases in the prostate tumours, a Western blot analysis was performed on LTL163a tumour-derived protein lysates using an anti-phosphotyrosine antibody which interacts with a wide range of phosphorylated tyrosine kinases. Immunoblotting results showed that genistein-treated tumours had a higher level of phosphorylation at a 60 kDa protein band relative to the untreated control group. Interestingly, at longer exposures of the x-ray film, more intense band patterns were observed for higher molecular weight proteins in the genistein-treated group compared to the control (figure 24). Of particular interest is a band at 170 kDa. Longer exposure revealed bands in the genistein-treated group at this particular sized protein while no phosphorylation was observed in the control group.

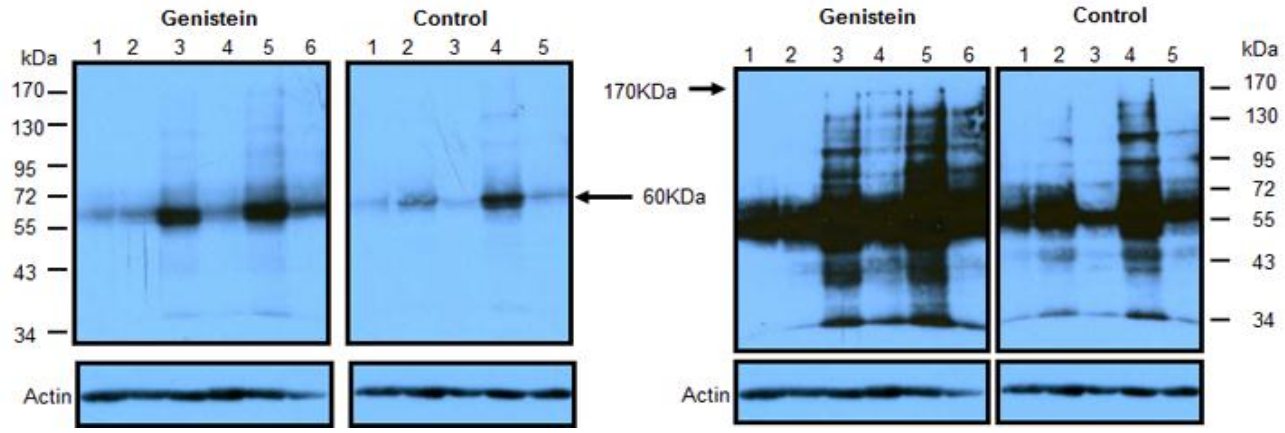


Figure 24. Effects of genistein on tyrosine phosphorylation.

After a three-week treatment of LTL163a xenografts with genistein, tumours were harvested, and protein was extracted from six genistein-treated and five untreated-control mice (one of the control mice died during treatment). Western blot analysis was performed on tumour-derived protein lysates for phosphotyrosine as described in the Methods section. Genistein-treated tumours showed higher phosphorylation levels at 60kDa compared to controls. The same blot was stained with an anti-beta actin antibody to evaluate protein loading. Lanes represent proteins from individual tumours. Right and left panels are from the same blot. Left: 5 second X ray film exposure; right: 30 second exposure.

The potential candidates for the 60 kDa and 170 kDa proteins (in figure 24) are Src and its potential upstream molecule, epidermal growth factor receptor (EGFR). As shown in Figure 25a, genistein increased the total EGFR protein expression slightly when compared to the control. While almost all untreated tumours (except one) showed very little to no phosphorylation of this protein, most genistein-treated tumours showed high band-intensity (figure 25a). Similarly, Src, a target of EGFR, showed slight up-regulation of total protein expression compared to the control group. Again, no phosphorylation of Src was observed in the control group (except for one tumour at a longer exposure), whereas a significantly higher level of Src phosphorylation was observed in the genistein-treated tumour samples (figure 25b). These specific EGFR and Src immunoblot band patterns confirm the general phospho-tyrosine staining patterns observed earlier (figure 24).

The non-biased canonical pathway analysis demonstrated that genistein and anti-estrogen treatments targeted the same signaling pathway involving different molecules, producing opposite effects. Genistein stimulated expression of molecules that reside upstream of the Focal Adhesion Kinase (FAK) pathway, while anti-estrogen down-regulated downstream molecules within the same pathway. The immunoblotting results supported the microarray data demonstrating the ability of genistein to stimulate the FAK phosphorylation activity. This study reveals that genistein indeed stimulates the FAK and EGFR signaling pathways and promotes metastasis in human prostate cancer. In contrast, anti-estrogen treatment inhibits metastasis by impeding the FAK pathway.

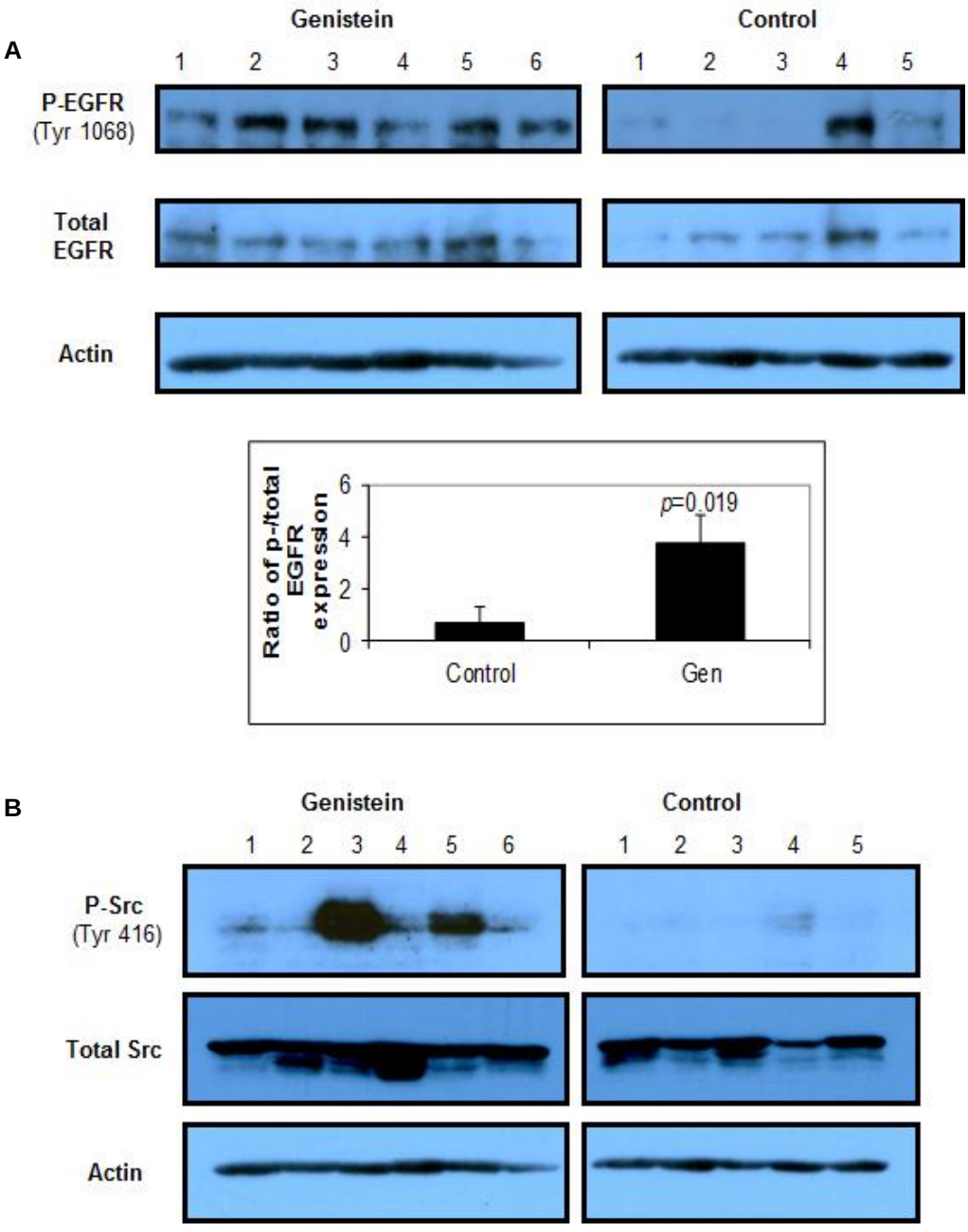


Figure 25. Effects of genistein on EGFR signaling.

A. Genistein increases phosphorylation of EGFR at the tyrosine 1068 residue. Below is the quantification of band intensity for phosphorylated EGFR from protein lysates of 5 untreated control and 6 genistein-treated tumours. *Columns*: mean ratio of phosphorylated EGFR/total EGFR protein band intensity ± SD.

B. Genistein affects phosphorylation of Src at the tyrosine 416 residue. Activated Src expression is significantly higher in genistein-treated tumours than that in the control. Lanes represent proteins from individual tumours.

2.5 Mechanism of genistein-mediated metastatic progression via ERβ: genomic pathway

In contrast to earlier *in vitro* effects reported [260,280-282], genistein exhibited metastasis-promoting effects, which may be mediated by ERβ in the patient-derived prostate cancer xenograft model. Furthermore, an estrogen antagonist, ICI 182,780, effectively inhibited expression of ERβ and metastatic spread in this model (section 2.2). To elucidate which estrogen-linked genes were responsible for these opposing effects by the two compounds, a genome wide expression array analysis of genistein- and ICI 182,780-treated tumours was performed.

2.5.1 Methods

2.5.1.1 siRNA knockdown of ERβ

ERβ and non-target control siRNAs (Accell) were purchased from Thermo/Scientific. The followings are the sequences used for *ERβ* siRNAs.

ESR2-21: 5'-GUGUGAAGCAAGAUCGCUA-3'

ESR2-22: 5'-CCAUCUAGCCUUAAUUCUC -3'

ESR2-23: 5'-GCAACUACUUCAAGGUUUC -3'

ESR2-24: 5'-CCUUUCUCCUUUAGUGGUC -3'

Non-target: 5'-UGGUUUACAUGUUUUCUGA-3'

To assess the efficacy of the siRNAs, PC3 cells were plated in 24-well plates in RPMI-1640, supplemented with 10% FBS and 1% antibiotics. 24 hours after seeding, the siRNA was applied to the cultures by changing the growth media to the Accell Delivery media provided by Accell (Thermo/Scientific). containing 1 μM siRNA. The cultures were incubated at 37 ^{o}C with 5% CO_2 for 72 hours, followed by harvesting and transcript expression analysis by qRT-PCR.

2.5.1.2 Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

RNA was extracted from siRNA-treated cells and LTL 313b treated-tumours in the same manner as described in section 2.3.1. After RNA quality analysis, 1 μg of total RNA was converted to cDNA using a reverse transcriptase by mixing the RNA, the enzyme, reverse transcription (RT) primer mix (Qiagen) and RT buffer. The mixture was then incubated for 15 minutes at 42 ^{o}C and 95 ^{o}C for 3 minutes. The cDNA was diluted 20-fold for PCR amplification. Quantitation of target gene expression was performed using a 7900HT Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). The reaction was carried out in 25 μL volume containing cDNA, 10 μM gene-specific primer pair and Platinum SYBR Green qPCR Master Mix (Invitrogen). At least two replicates of each sample per plate were used for amplification reaction at the cycling parameters of 50 ^{o}C for 2 minutes; 95 ^{o}C for 10 minutes; 95 ^{o}C for 15 seconds (40x); 60 ^{o}C for 1 minute. The averaged data were normalized to a housekeeping gene, *hprt*, which is stably expressed across prostate cancer cell lines and the patient-derived tumour tissue lines (data not shown). Gene expression was quantified using the comparative C_T and standard curve methods and presented as fold-change ± SD. Due to the possibility of mouse cell contamination in the xenografts, all primers were designed human-specific and to span adjacent exons. The following primer sequences were used:

Hprt-F: 5'-GGTCAGGCAGTATAATCCAAAG

Hprt-R: 5'-CGATGTCAATAGGACTCCAGAT

MT2A-F: 5'-GACTCTAGCCGCCTCTTCAG

MT2A-R: 5'- GGAAGTCGCGTTCTTTACATCT

MT1E-F: 5'-AACTGCTCTTGCGCCACT

MT1E-R: 5'- ATCCAGGTTGTGCAGGTTGT

MT1B-F: 5’- TGCTCCTGCACCACAGGT

MT1B-R: 5’- TGATGAGCCTTTGCAGACAC

MT1H-F: 5’- AGTCTCACCTCGGCTTGC

MT1H-R: 5’- ACTTCTCTGACGCCCCTTT

MT1X-F: 5’- GCTGCGTGTTTTCCTCTTGA

MT1X-R: 5’- TCTGACGTCCCTTTGCAGAT

2.5.1.3 RT-PCR

RNA extraction and cDNA conversion were performed as described in the section above. The RT-PCR reaction was carried out using 10 μM gene-specific primer pairs and Platinum Taq DNA Polymerase (Invitrogen) over 35 cycles for *ERβ* and 25 cycles for *GAPDH*.

The following primer sequences were used:

ERβ-F: 5’- CTGTTACTGGTCCAGGTTCAA

ERβ-R: 5’- TCGATTGTACACTGATTTGTAGC

GAPDH-F: 5’CACCAGGGCTGCTTTTAACTC

GAPDH-R: 5’GACAAGCTTCCCGTTCTCAG

2.5.2 Genistein stimulates metallothionein (*MT*) gene expression

The results from Section 2.2. showed that LTL163a and LTL313h tumour tissue lines predominantly express ERβ as distinct from ERα, and that genistein induces estrogen-responsive gene transcription via ERβ. These data suggest that the metastasis-promoting effects of genistein are mediated via ERβ activation. To identify metastasis-linked genes which are regulated by ERβ, a cross-comparison analysis was performed of two gene populations: genistein-up-

regulated genes and ICI 182,780-down-regulated genes (figure 26). If activation of ERβ by genistein promotes cancer metastasis and inactivation of ERβ by ICI 182, 780 leads to inhibition of metastasis, genes that have a critical role in prostate cancer progression may be identified as those commonly shared by the genistein-up-regulated and ICI 182,780-down-regulated gene populations (figure 26). IPA analysis of the gene expression array data revealed six common genes in the two populations, five of which belong to the metallothionein (*MT*) gene family: *MT1B, 1E, 1H, 1X*, and *2A* (Table 5).

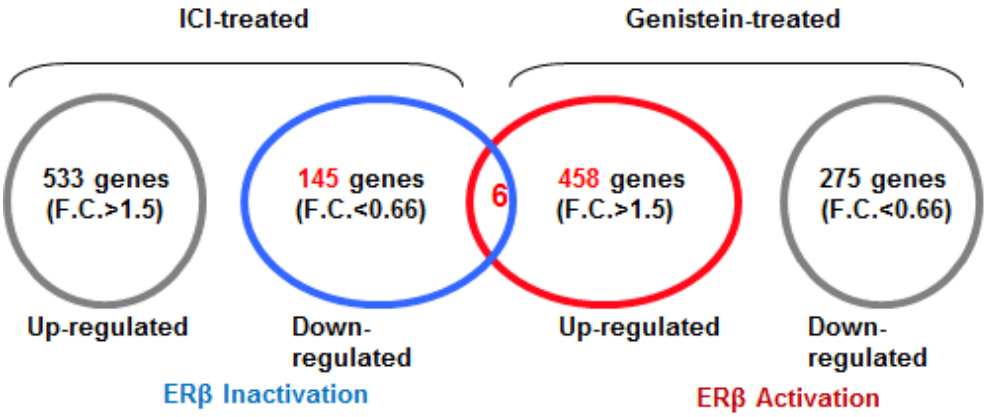


Figure 26. Discovery of metastasis-linked genes regulated by ERβ: cross comparison of the gene populations identified by Ingenuity Pathway Analysis.

Genes which exhibited at least 1.5-fold increase or 0.66-fold decrease were categorized as up-regulated and down-regulated genes, respectively. Six commonly shared genes were identified among genistein-up-regulated and ICI-down-regulated gene sets.

GeneName	Description
metallothionein 2A (FC=1.7)	Homo sapiens **metallothionein 2A** (MT2A), mRNA
metallothionein 1E (FC=1.6)	Homo sapiens **metallothionein 1E** (MT1E), mRNA
metallothionein 1B (FC=1.8)	Homo sapiens **metallothionein 1B** (MT1B), mRNA
metallothionein 1H (FC= 1.7)	Homo sapiens **metallothionein 1H** (MT1H), mRNA
metallothionein 1X (FC=1.8)	Homo sapiens **metallothionein 1X** (MT1X), mRNA
	AGENCOURT_6543460 NIH_MGC_71 Homo sapiens cDNA clone IMAGE:5549618 5', mRNA sequence [BM552308]

Table 5. List of genes shared by genistein-up-regulated and ICI-down-regulated gene populations.

Five of the six commonly shared genes belong to the metallothionein gene family: *MT2A*, *MT1E*, *MT1B*, *MT1H* and *MT1X*. The fold difference between genistein and ICI treated are expressed as FC in brackets.

2.5.3 Validation by qRT-PCR of genistein-induced upregulation of *MT* gene expression

In our *in vivo* experiments with genistein, genistein stimulated PCa metastasis via ERβ activation, whereas ICI 182,780 inhibited metastatic progression by inhibiting expression of ERβ (section 2.2). In search of metastasis-linked genes that are regulated via ERβ, total RNA samples extracted from genistein- and ICI 182,780-treated LTL313b xenografts were subjected to gene expression microarray analysis. A comparison of genistein-up-regulated and ICI-down-regulated gene populations identified 6 commonly shared genes. Five out of the six genes belong to the *MT* gene family. qRT-PCR was carried out to validate altered expression levels of the MT genes. As shown in Figure 27, all five MT genes were up-regulated by genistein relative to the controls, although only the up-regulation of the *MT2A* and *MT1X* genes were statistically significant. Although the expression of the *MT2A, MT1B, MT1E, MT1X* genes in the ICI 182,780-treated xenografts were slightly down, the results were not statistically significant.

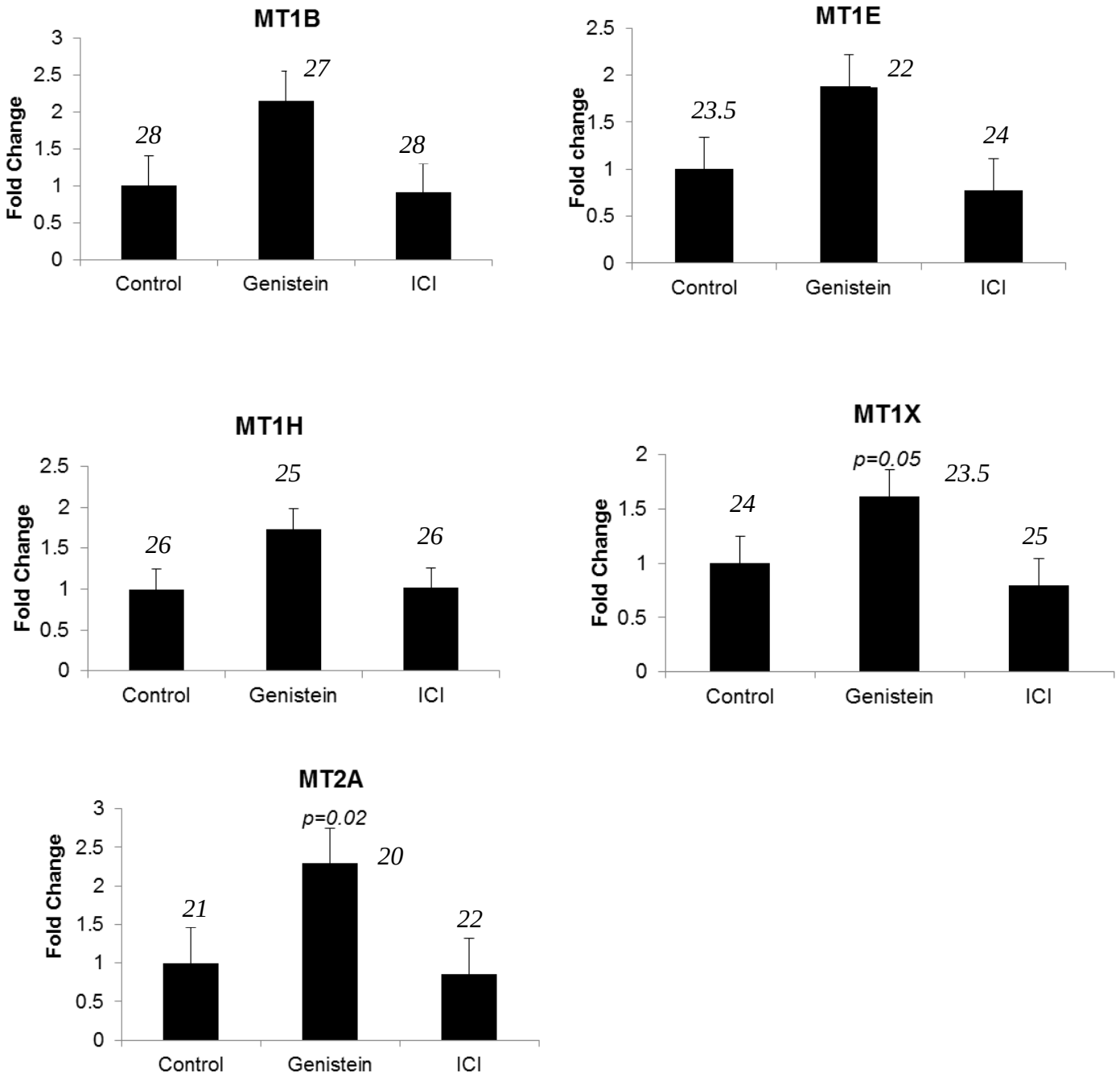


Figure 27.Up- and down-regulation of metallothionein genes (*MT1B, 1E, 1H, 1X* and *2A*) in genistein- and ICI 182,780-treated prostate cancer LTL313h xenografts as determined by qRT-PCR.

Gene expression is presented as fold change relative to control. Values are normalized to *hprt* levels. Genistein increased expression of all *MT* genes tested. There was only slight reduction in *MT* expression by ICI treatment. *Columns*: mean fold change ±SE. *Italic numbers above columns indicate Ct values*

Because genistein-enhanced metastasis of LTL313h xenografts was associated with increased *MT* gene expression, it appeared worthwhile to investigate if *MT* gene expression was higher in this metastatic tumour line than in the non-metastatic LTL313NM line, derived from the same prostate cancer specimen. Figure 28 shows higher expression of the *MT2A, MT1E* and *MT1X* genes in the metastatic tumours compared to the non-metastatic tumours, linking the increased *MT* gene expression to prostate cancer metastasis.

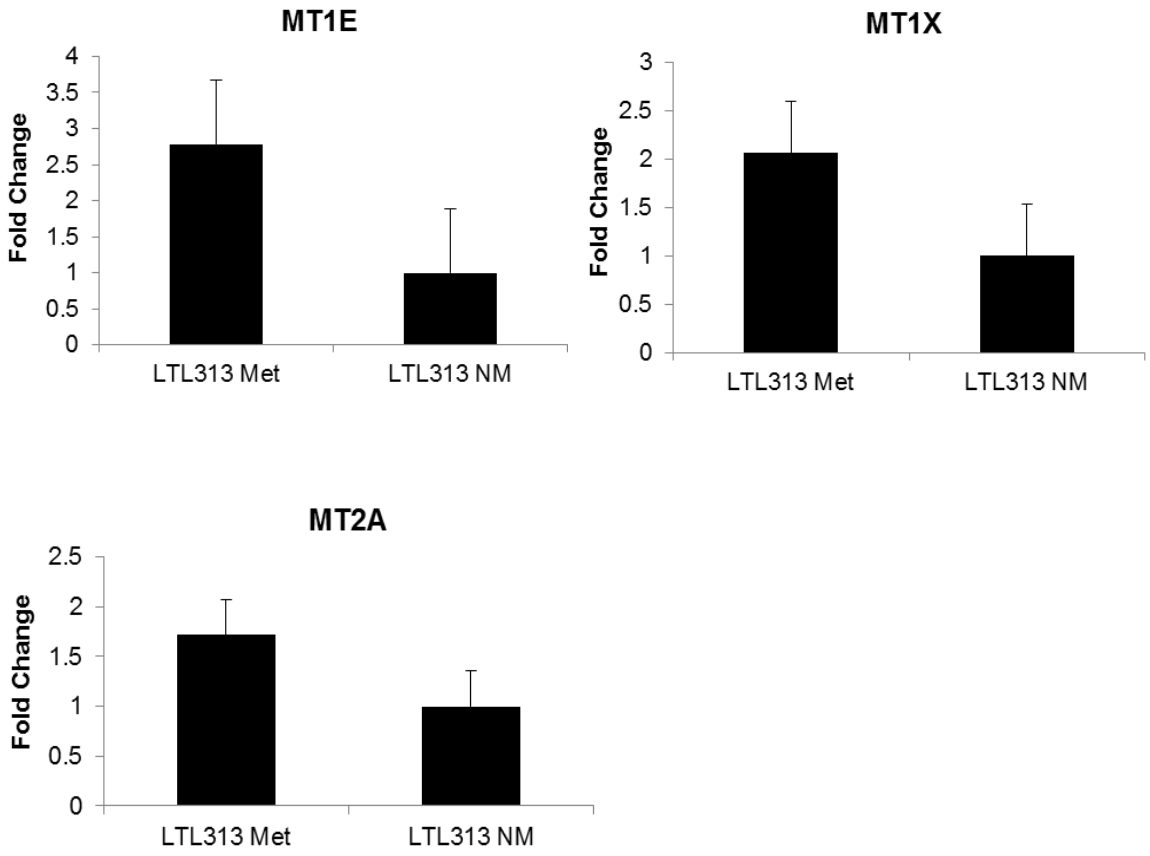


Figure 28. Metastatic (LTL313h Met) tumour line exhibited higher *MT* gene expression compared to non-metastatic (AB313b NM) tumour line.

Gene expression is presented as fold change. Values are normalized to *hprt* levels. *Columns*: mean fold change ±SE.

2.5.4 ERβ knockdown effects on *MT* gene expression

The microarray and qRT-PCR studies showed that expression of the *MT* gene family is modulated by genistein and ICI 182,780. Furthermore, the gene expression data in the metastatic and non-metastatic LTL313h tumour lines suggest that *MT* genes may be linked to metastatic progression of prostate cancer. To determine if *MT* gene expression is regulated via ERβ, siRNA knockdown of ERβ was performed in PC3 cell lines. After 96 hours of incubation with siRNA, the transcript level of ERβ was markedly reduced in contrast to vehicle or non-target siRNA controls (figure 29). Expression analysis by qRT-PCR indicated decreased expression of ERβ and of *MT2A*, *MT1E* and *MT1H* genes in si-ERβ-treated cells compared to vehicle- or non-target siRNA cultures (figure 30). These results suuport ERβ-regulation of *MT* genes.

A cross-comparison of genes up-regulated by genistein (F.C.>1.5) and genes down-regulated by ICI 182,780 (F.C.<0.66), revealed that the two groups had seven genes in common, six of which belonged to the metallothionein (*MT*) gene family. qRT-PCR studies validated the expression levels of the *MT* genes: i.e. their up-regulation by genistein and down-regulation by ICI 182,780. siRNA knockdown of ERβ in PC3 prostate cancer cells showed reduced expression of the *MT* genes compared to non-target siRNA controls, indicating a role for ERβ in the regulation of their transcription.

In summary, genistein stimulated prostate cancer metastasis in our patient-derived tumour models, while ICI 182,780 inhibited metastasis. Since metastatic promotion was observed in two of the tumour lines, this effect is not patient-specific. The western blot analyses using the protein lysates from treated tumours showed that genistein increased phosphorylation levels of tyrosine kinases, which could play major roles in metastatic progression. Also, genistein

upregulated transcription of *MT* genes, which may be responsible for providing tumours aggressive/invasive phenotypes.

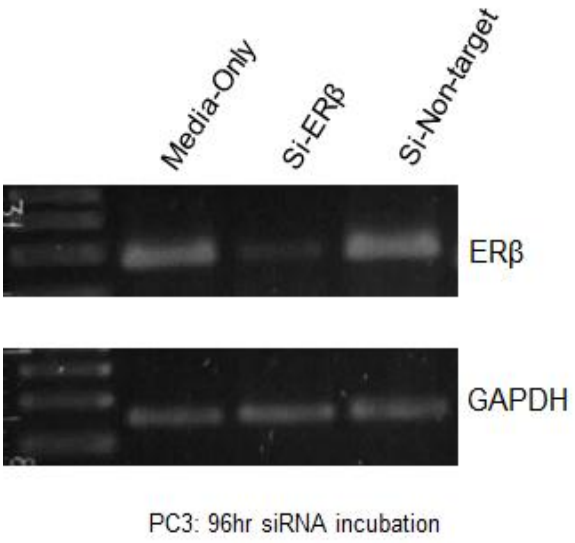


Figure 29. siRNA knockdown of ERß in PC3 cells after 96 hours.

RT-PCR results show reduced transcriptional expression of ERβ in siRNA-treated PC3 cells compared to non-target siRNA and vehicle controls.

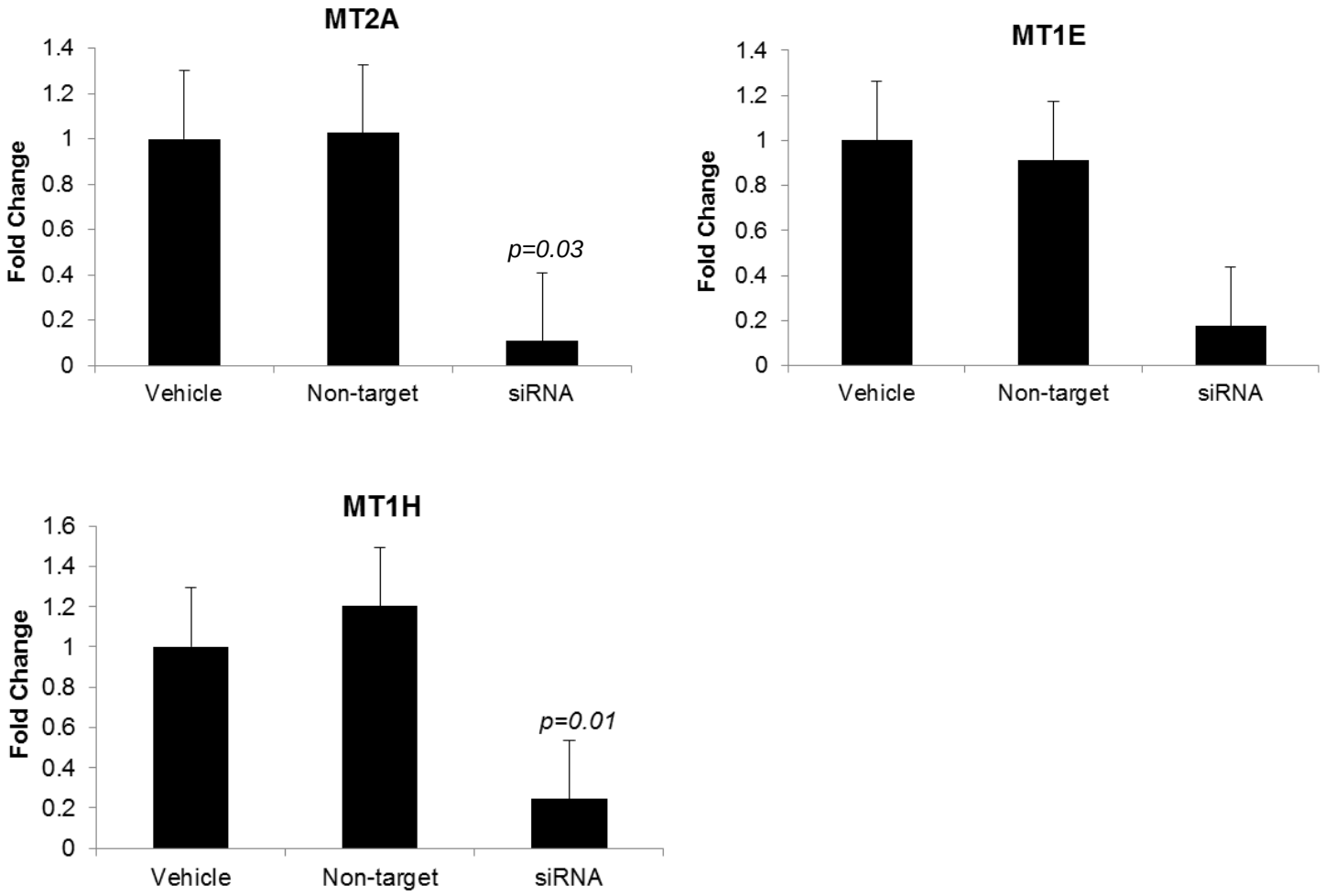


Figure 30. *MT* gene expression following siRNA knockdown of ERß in PC3 cells.

96 hours after incubation with siRNA, the siRNA-treated cells showed reduced expression of *MT* genes (*MT2A, MT1E* and *MT1H*) in contrast to vehicle-or non-target siRNA controls. Gene expression is represented as fold change. Values are normalized to *hprt* levels. *Columns*: mean fold change ±SE.

Chapter 3: CONCLUSION

Prostate cancer is the second leading cause of cancer-related deaths in North America [2]. For over seventy years, hormone therapy has been the gold standard for treatment of advanced PCa and has been proven effective in causing initial tumour regression [149]. Unfortunately, however, the majority of patients will eventually reach castration resistance and succumb to metatstasis [14]. Although new drugs targeting AR or androgen biosynthesis have recently been emerging, there is currently no effective treatment or cure for the metastatic disease [171]. Hence, understanding mechanisms of metastatic progression is important in developing strategies to combat this disease.

Epidemiological data suggest that lower mortality rates of PCa observed in Asia compared to the West are reflected in dietary differences, as an inverse correlation exists between soy consumption and PCa risk [251-253,257]. This has stimulated many investigators to study the primary active component of soy, genistein. *In vitro* evidence indicates that this compound has chemotherapeutic/cytotoxic effects on hormone-dependent cancer cell lines [260,280-282]. However, emerging evidence from recent *in vivo* studies shows contradictory effects on metastasis progression [304-306,324-327]. To resolve the controversy of genistein's effects *in vivo*, a subrenal capsule xenograft technique was utilized to employ a low passage of patient-derived PCa specimen in non-obese diabetic, severe combined immune-deficient (NOD-SCID) mice. The results showed that genistein indeed functioned differently *in vivo* from *in vitro* and promoted metastasis in this clinically relevant xenograft model.

3.1 *In vivo* effects of genistein in human prostate cancer

To investigate the effects of genistein *in vivo,* we have developed clinically relevant xenograft models that have been generated from patient prostatectomy specimens. The resultant patient-derived prostate tumour lines used in this study have retained the original histopathological and genotypic characteristics of the clinical samples [315,316]. Genistein treatment at pharmacological dosages promoted metastasis in the advanced human PCa transplant lines. The use of LTL163a and LTL313h tumour lines, which had been derived from two patients, provided evidence that this stimulatory effect of genistein is not patient-specific.

Although tumour size was not significantly different between control and genistein-treated groups, metastatic incidence was greater in genistein-treated mice vs untreated controls. The metastatic progression observed in our models was characterized by increased cell proliferation and decreased apoptosis for both tumour lines. One of the possibilities for the non-significant difference in tumour size between groups while genistein treatment increased proliferation is that all tumour grafts regardless of treatment had reached the maximum outward growth within the renal capsule, after which point they started to invade inwardly into the kidney, then to LN and to secondary organs.

It is intriguing to note that genistein exhibits biphasic effects in hormone-dependent cancer cell lines, depending on the dosage [306,328,329]. Studies with breast cancer cell lines have shown that low-dose genistein (0.1-25μM) stimulated growth of estrogen-dependent cells, while high-dose genistein (50-100μM) inhibited proliferation [256,328-330]. Wang *et al.* also demonstrated a similar biphasic effect of genistein in a non-cancerous prostate epithelial cell line (RWPE cells) [331]. In our study, however, we did not observe

biphasic effects of genistein with the dose range of 80mg/kg/day and 400mg/kg/day. In order for genistein to exhibit inhibitory effects on advanced PCa *in vivo*, it may require higher concentrations.

Alternatively, genistein's effects may also depend on its food source or stages of cancer. Some epidemiological studies report a positive association between soy and PCa risk [332-336], speculating that fermented soy sources such as natto or miso increase odds ratio [29, 31]. Furthermore, Kurahashi *et al*. suggested that soy's protective role is limited to early, localized PCa and that it may have adverse effects on advanced PCa [32,33]. Their claim was supported by the evidence from Touny and Banerjee's TRAMP study, which indicates a preventive role of genistein if given early (ie, before tumour initiation), but a stimulatory effect if a genistein diet was initiated late in life (ie, after PCa establishment or metastasis) [306].

3.2 Estrogenic effects of genistein and the effects of the anti-estrogen, ICI 182,780

Because genistein has a similar spatial conformation to estradiol, it exhibits a weak estrogenic activity through binding to estrogen receptor (ER) and modulates estrogen-regulated gene transcription in target organs [318,319]. Although genistein's estrogenicity is relatively low (10^{-5} to 10^{-2} of estradiol transcriptional activity on a molecular basis [318,319]), it is known to elicit physiological effects in humans particularly those with high soy consumption. This is because the serum levels of genistein can reach a 100-1,000 times higher level than that of endogenous estrogen in people who have high soy diet [337]. In addition, genistein binds less tightly to serum proteins than estrogens, facilitating easier uptake by cells/tissues [338].

The HPLC-MS analysis in this study revealed that genistein was metabolized to form glucuronide conjugates after consumption, and a small proportion circulated as a free, unbound form in the blood of treated mice. In rodents, it is known that the prostate accumulates a high concentration of aglycone compared to other organs [274,277-279]. Furthermore, the activity of glucuronidase, which converts genistein conjugates to aglycone (the active form) is shown to be higher in tumours than in benign tissues [277]. This high enzymatic activity may increase the proportion of aglycone within tumours. Compared to the conjugates, aglycone has10- to 40-fold higher affinity for ERβ, which is expressed by the epithelial cells of the prostate [275]. It can be speculated that genistein may circulate as a conjugated form; however, upon reaching the target PCa cell/tissue, it gets converted to an active form and binds to ERβ expressed by cancer cells, inflicting its cancer-promoting effects.

Together with the evidence of genistein's preferential binding to ERβ provided by early studies [225,323] and the high and exclusive expression of ERβ in our tumor lines (no ERα expression), it can be hypothesized that genistein's tumour-stimulatory effects observed in this study may be mediated via ERβ activation.

3.2.1 Genistein activates estrogen-responsive gene transcription

To investigate if genistein can bind to ERβ and induce estrogen-responsive gene transcription, primary cultured LTL163a cells were transiently transfected with a reporter construct containing an estrogen response element in a promoter region upstream of a luciferase gene.

The results showed that the genistein-treated cells had a 20-fold increase in ERE-luciferase activity compared to the vehicle control, indicating genistein's ability to bind to and activate ERβ. With the addition of anti-estrogen, ICI 182,780, the genistein-induced luciferase activity was inhibited. This inhibition by ICI 182,780 may be achieved via receptor degradation and/or inactivation of AF-1/2 domains of the receptor (figure 31). Since the tumour lines exclusively express ERβ, it can be concluded that the estrogen-responsive gene transcription is induced by genistein-bound ERβ, and not by ERα. Furthermore, to rule out the possibility that the genistein's effects are mediated by the androgen receptor, LNCaP cells (AR- and ERβ-positive) were transfected with an ARE-reporter construct. As shown in figure 18, genistein did not activate AR or induce androgen- responsive gene transcription. Estradiol treatment, in comparison, increased ARE-luciferase activity slightly, which could be explained by promiscuous binding of mutated AR in LNCaP, which may allow certain non-androgens to bind and activate the receptor [38].

These data clearly demonstrate that genistein does indeed elicit an estrogenic property by binding to ERβ and inducing transcription of estrogen-responsive genes.

3.2.2 Effects of the anti-estrogen, ICI 182,780, on metastasis

In this study, genistein demonstrated tumour-stimulatory effects *in vivo* and estrogenic effects by inducing estrogen-responsive gene transcription *in vitro*. In order to investigate if the enhanced metastatic progression observed in genistein-treated mice was due to genistein's estrogenic property, mice carrying patient-derived PCa xenografts were treated with genistein, an anti-estrogenic compound, ICI 182, 780 and a combination of both.

Figure 19 shows that while genistein promoted lung metastasis in a dose-dependent manner, ICI treatment alone significantly decreased metastatic spread compared to the control. Although the combination treatment (5 mg genistein/day plus 5 mg ICI/week) did not decrease the metastatic incidence, it exhibited a trend towards reduced cell proliferation and enhanced apoptosis compared to the low-dose group (5 mg genistein/day), which was given the same amount of genistein as the combination group. The proliferation index of the combination group was 19.0 % compared to 23.8% of the low-dose group with approaching significance. For apoptosis, the difference was smaller: 1.8% in the combination vs 1.1% in the low-dose group. Even though there was no statistical significance, there is a biological trend to indicate the ICI's tumour-inhibitory effects. One possible explanation for the lack of difference in metastasis between these groups is that the amount of ICI injected per week may have been insufficient to completely block the activity of genistein administered daily. Nevertheless, the group that was given only ICI treatment (5 mg ICI/week) showed a significant reduction in tissue invasiveness compared to all other groups including the control. This signifies an effective inhibitory effect of the anti-estrogen against metastasis in this model.

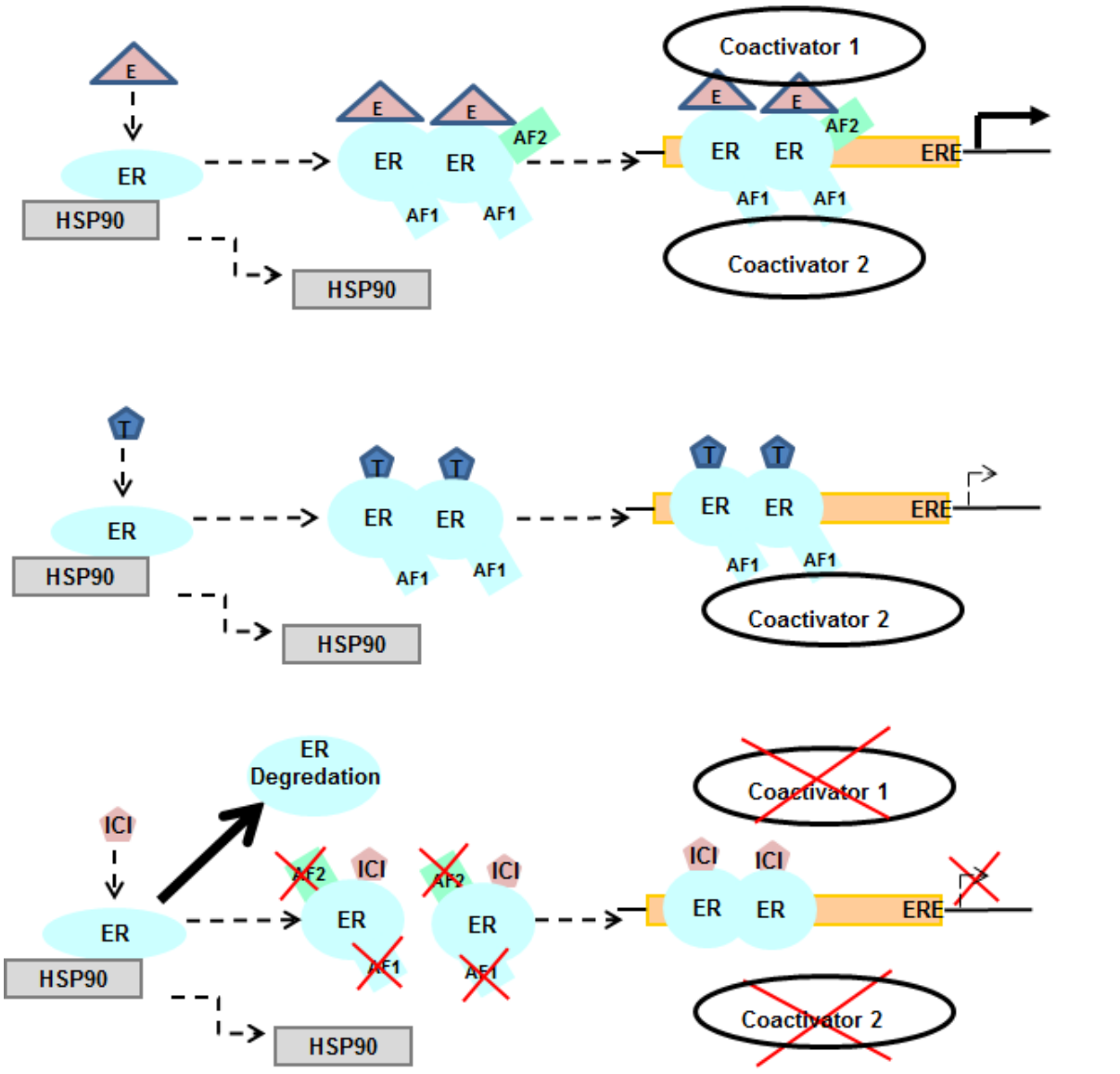


Figure 31. The mechanisms of estrogen-responsive gene transcription and antagonistic effects of SERMs, tamoxifen and ICI 182,780.

E: estradiol. When E binds to the ligand-binding domain of the receptor, it activates AF-2 domain and allows ligand-dependent transcription. T: tamoxifen. When tamoxifen binds to ER, it inactivates AF-2 domain. However, AF-1 domain is still active, which could explain partial agonistic activity in certain tissues. ICI: ICI 182,780 binding to ER induces receptor degradation. (modified from Howell *et al. Cancer*. 2000)

3.3 Gene expression profiles of genistein-treated and anti-estrogen-treated tumours

The results of this study showed that genistein promoted metastatic progression of advanced prostate cancer by stimulating cell proliferation and inhibiting apoptosis; whereas treatment with the anti-estrogen, ICI 182,780, effectively inhibited the spread of invading cancer cells of two of the patient-derived tumours. In order to delineate differential gene expression patterns that underlie the mechanism behind the metastatic promoting effect of genistein and protective effect of ICI 182,780, we performed a whole-genome expression array analysis using an Agilent 4x44K platform.

The array analysis revealed that of all 34,127 human genes screened, genistein stimulated 458 genes and down-regulated 275 genes, while ICI 182,780 up- regulated 533 genes and inhibited 194 genes (figure 20). Using Ingenuity Pathway Analysis, the genes identified by microarray were ranked according to the top functional networks based on p-values, expression levels and the number of molecules appearing in the network. The majority of genes regulated by either treatment (genistein or ICI 182,780) fell into “Cancer” and “Genetic Disorder” categories. Interestingly, the top cellular functions of the genes stimulated by genistein were related to tumour progression : “Cell Cycle”, “Cellular Movement” and “Cell Death” (figure 21). Similarly, those genes regulated by ICI 182,780 fell in the top functional categories that are related to cell movement and morphology (figure 21). These analyses suggest that genistein and the anti-estrogen, ICI 182,780, regulate genes that are central in metastatic progression.

3.4 Mechanism of genistein-mediated metastatic progression via ERβ: non-genomic pathway

ERs are best characterized as transcription factors, whose functions are to bind to a specific region of DNA upon ligand activation and to induce estrogen-responsive gene transcription. However, ERs are also known to have non-genomic functions by modulating signaling pathways [230]. This non-genomic function is important in cancer development and progression as signaling of protein tyrosine kinases (PTKs) regulates critical cellular activities such as proliferation, apoptosis, differentiation and cell survival [293].

Several studies have shown that genistein-bound ER can activate PTKs via non-genomic signaling [331,339]. In a non-tumorigenic prostate epithelial cell line (RWPE-1), which predominantly expresses ERβ, Wang *et al.* showed that genistein at low concentrations (0-12.5μmol/L) increased cell proliferation and the activity of extracellular signal-regulated kinase (ERK)1/2 via ERβ binding. They have also shown that anti-estrogen treatment with ICI 182, 780 inhibited genistein-induced cell proliferation and activities of ERK1/2 [331]. Another study by Migliaccio *et al*. demonstrated that ligand-activated-ERβ/androgen receptor (AR) complex associated with Src, which then activated the Src/Raf-1/Erk-2 pathway, stimulating cell proliferation of LNCaP cells [339].

As demonstrated by the LNCaP and RWPE studies [331,339], genistein-activated ERβ can stimulate various PTKs in PCa cells. Therefore, the increased EGFR/Src signaling observed in genistein treated tumours in this study may have resulted from a non-genomic action of this compound, which led to increased proliferation, reduced apoptosis and tumour progression in our PCa model.

3.4.1 Focal adhesion kinase pathway

The canonical pathway analysis of the microarray data showed that genistein and the anti-estrogen, ICI 182,780, targeted the same PTK pathway, called focal adhesion kinase, but produced opposite effects/outcomes on tumour biology (figures 32 and 33).

As shown in the figure 32, genistein up-regulated the molecules that reside upstream of the FAK pathway, such as integrin and vitronectin, activating the pathway and potentially promoting metastasis in the treated mice. This finding is supported by other studies which reported that the interaction of vitronectin (an ECM protein)-integrin protein complex is important in phosphorylation/activation of FAK, which provides invasive phenotypes in PCa [85,98,99]. In contrast, ICI 182, 780 down-regulated the expression of *Rho* and *MLCK*, FAK downstream molecules, leading to inhibition of cell proliferation and migration (figure 33). Furthermore, ICI 182, 780 treatment up-regulated the expression of *PTEN*, a tumor suppressor gene, whose encoded protein has a phosphatase function. Using glioblastoma and breast cancer cells, Tamura *et al.* have shown that PTEN de-phosphorylated FAK at Y397 and phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which led to inhibition of cell migration and invasion [340]. In our study, the up-regulated PTEN observed in the ICI 182, 780 group may be responsible for inactivation of FAK pathway, or it may indirectly counteract the activity of FAK by inhibiting activation of PI3kinase, thus keeping Akt inactive. This, in return, may have resulted in inhibition of metastasis observed in ICI 182, 780-treated animals.

To validate and compare the activation levels of FAK between treatments, we performed immunoblotting using antibodies specific for FAK -tyrosine and -serine residues, Y397 and S732. At both residues, genistein increased general FAK-phosphorylation levels

compared to control, while the anti-estrogen, ICI 182,780, decreased FAK activity only at ser732 (figure 23). This suggests that genistein and ICI 182, 780 both have an effect on FAK phosphorylation, but that the phosphorylation sites they modulate may be different. Taken together, the microarray and Western blot data show that genistein stimulated the FAK signaling pathway and promoted metastatic progression in human prostate cancer. In contrast, anti-estrogen treatment inhibited metastasis by impeding the FAK pathway.

3.4.1.1 FAK and its link to ERβ

Although precise mechanisms of how genistein and ICI 182, 780 activate or inactivate FAK are unclear, it can be implied that they may do so via non-genomic action of ERβ because both of these compounds target this receptor, which is exclusively expressed in the cancer cells of the tumour lines used.

The evidence to support this claim comes from early studies: in 2007, Ishida *et al*. investigated expression levels of ERs in oral squamous cell carcinoma (SCC) and the effects of an ER agonist and antagonists in cell invasion. They found that ERβ was exclusively expressed in the primary tumors and various SCC cell lines [341]. Treatment of cells with tamoxifen, an ER antagonist, resulted in decreased kinase activity of FAK, which disrupted cytoskeletal actin organization. This actin disorganization led to reduced cell adhesion, inhibiting invasion and causing cell death [341]. These effects were unique to the ER antagonist, and were not observed with estradiol, an ER agonist [341]. The data from the Ishida *et al*. study suggest that inactivation of ERβ in oral squamous carcinoma leads to decreased FAK activity, which reduces cell adhesion and invasive ability of the tumour cells.

Similarly, 17-β estradiol treatment of Ishikawa cells, endometrial adenocarcinoma cells, led to increased FAK phosphorylation at Y397 and 576, which affected cytoskeletal structures and promoted cell motility [342]. Flamini *et al.* showed that phospho-FAK (Y397) was co-localized with actin at pseudopodia in estrogen-treated cells. In contrast, treatment with an estrogen antagonist, ICI 182, 780, inhibited FAK phosphorylation and pseudopodia formation in these cells [342]. Since Ishikawa cells express both ERα and β, previous studies have suggested two separate mechanisms by which each ER activates FAK. As shown in the diagram below (figure 34), it is hypothesized that estrogen activates/phosphorylates FAK via binding of multi-protein complexes such as Src, G-protein and PI3K in ERα-expressing cells [342-345]. In ERβ-expressing cells, no direct interaction between this receptor and PI3K or FAK has been reported, and thus, it is proposed that FAK activation occurs through Src-mediated signaling, which phosphorylates at Y397 [342,346,347].

Together with the evidence from these early studies, it can be postulated that genistein, an ERβ agonist, binds to and activates the β-receptor, which in turn may lead to phosphorylation of FAK and may activate its pathway via non-genomic fashion, increasing cell migration ability (figure 35). In contrast, ICI 182,780, an anti-estrogen, inactivates ERβ, which leads to decreased FAK activity resulting in retarded cell motility.

As depicted in the canonical pathway diagrams, through up-/or down-regulation of FAK pathway-linked molecules, genistein and anti-estrogen may modulate the fate of tumour biology and metastatic outcome. In other words, activation of this FAK pathway will lead to cell survival, migration and invasion just as observed in genistein-treated tumours, whereas inactivation of the pathway will lead to tumour or metastasis suppression, just as in ICI 182,

780-treated tumors. These findings support the clinical relevance of anti-estrogen therapy as a potential treatment of metastatic PCa.

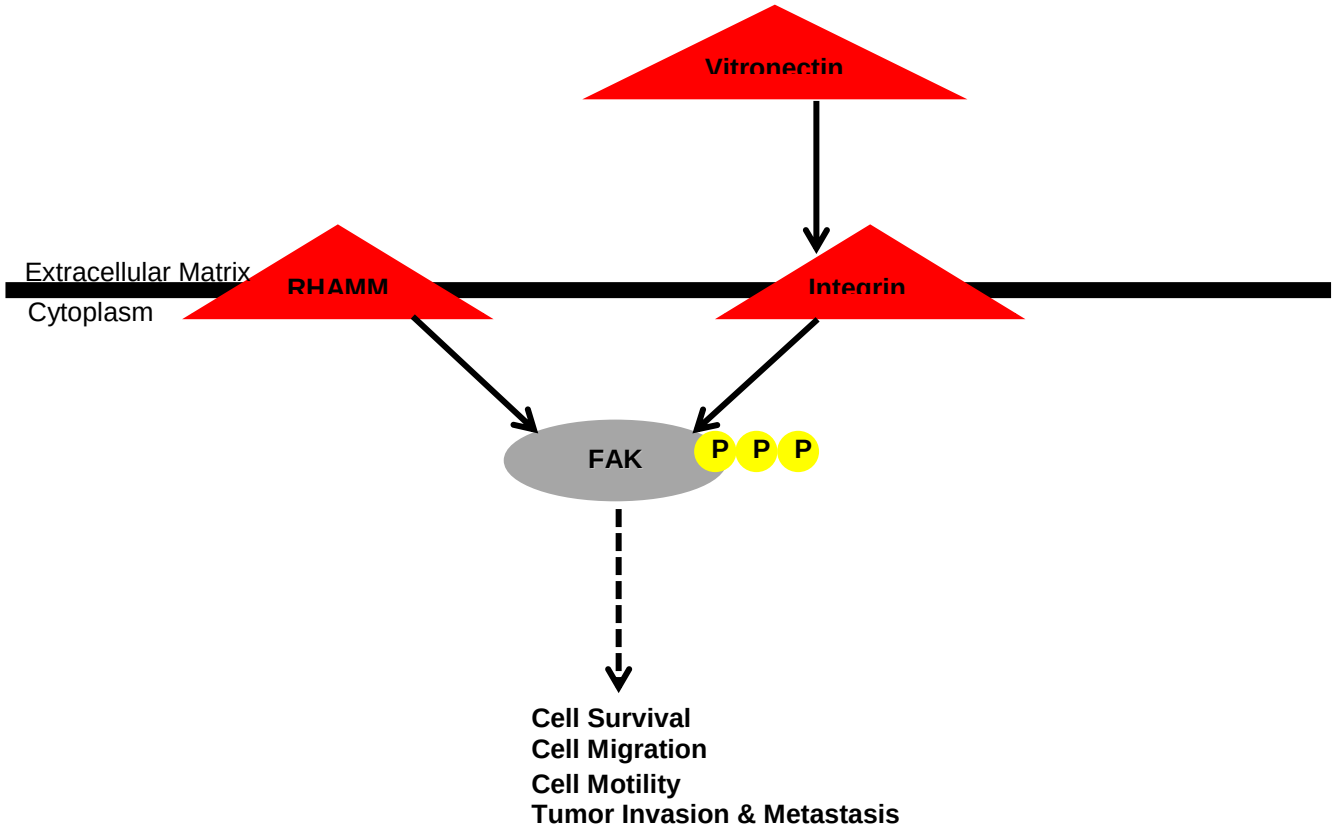


Figure 32. Model depicting the genistein-associated changes of FAK phosphorylation and activity as deduced from Ingenuity Pathway Analysis of Agilent Human gene expression microarray data.

Genistein up-regulated FAK-upstream molecules, leading to PCa progression. Triangle: gene expression affected by genistein. Red: up-regulated expression. Grey: no change in expression level detected by the microarray analysis.

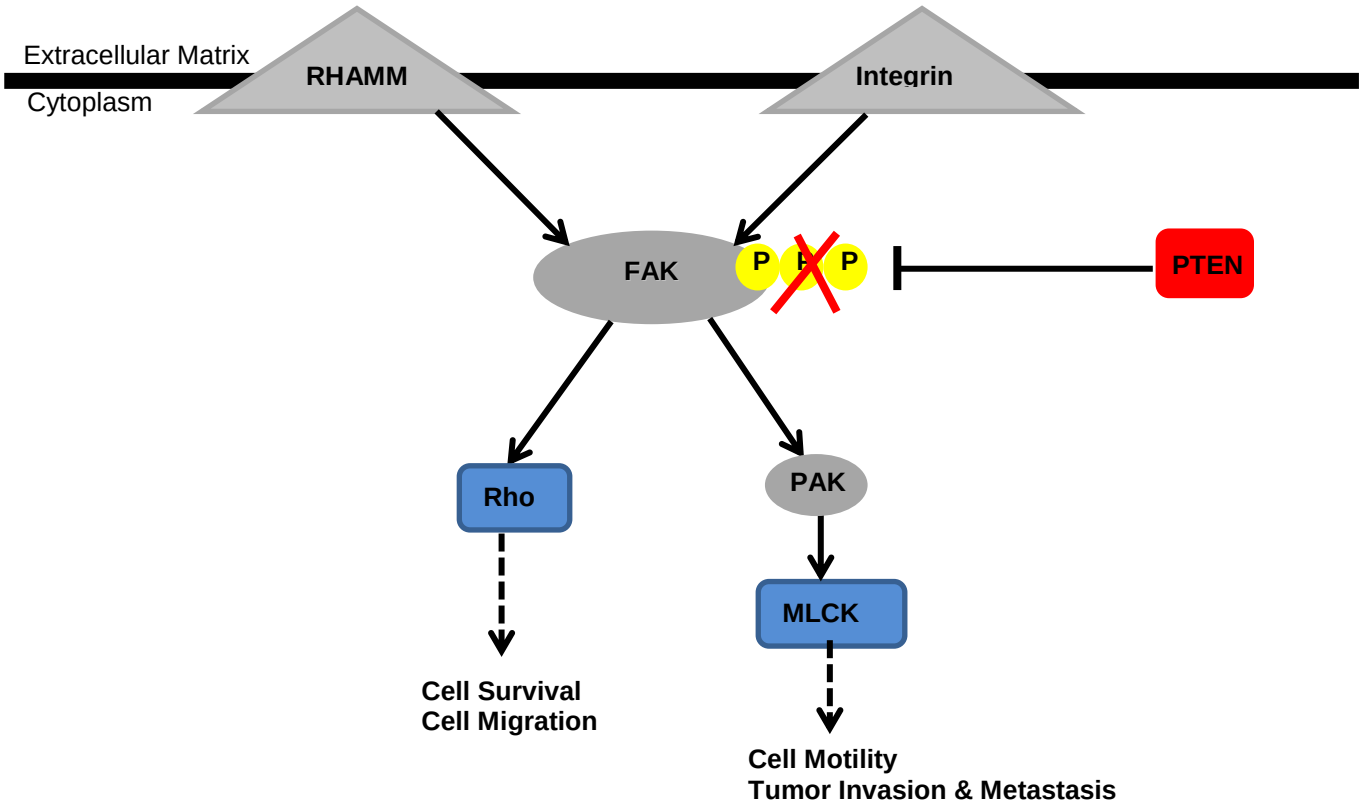


Figure 33. Model depicting the anti-estrogen-associated changes of FAK phosphorylation and activity as deduced from Ingenuity Pathway Analysis of Agilent Human gene expression microarray data.

ICI 182,780 down-regulated expressions of the molecules in the FAK pathway, inhibiting PCa progression. Triangle: gene expression affected by genistein. Square: gene expression affected by ICI 182,780. Red: up-regulated expression. Blue: down-regulated expression. Grey: no change in expression level detected by the microarray analysis.

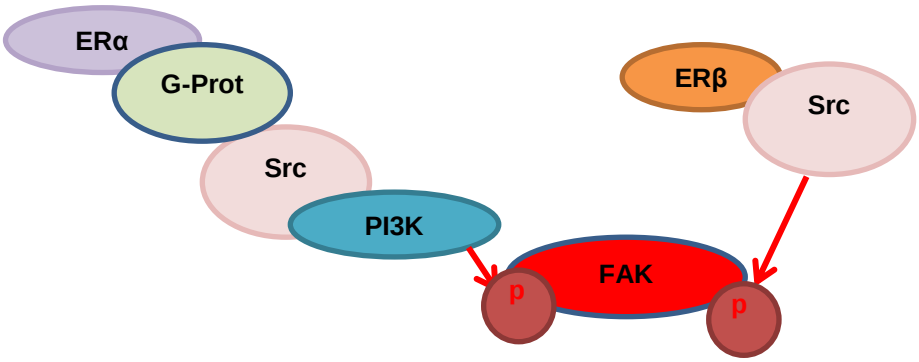


Figure 34. Model depicting indirect activation of the FAK pathway by ERs via a non-genomic mechanism.

It is proposed that ERβ binds to Src, which phosphorylates FAK. In ERα-expressing cells, however, the receptor binds to G-protein, which forms a complex with Src and PI3 kinase, which phosphorylates FAK.

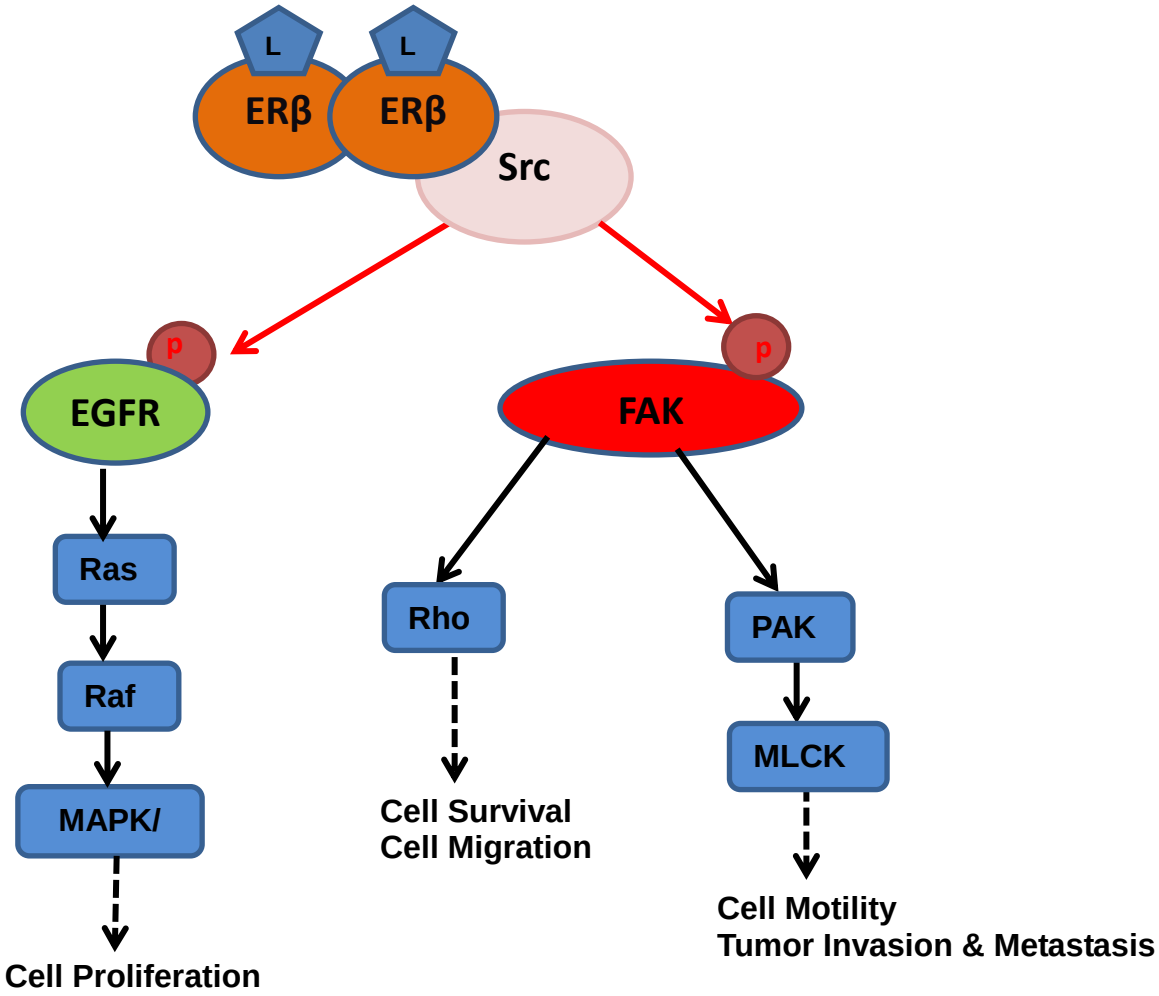


Figure 35. Model depicting non-genomic actions of ERβ.

ERβ-Src complex phosphorylates numerous downstream kinases such as FAK and EGFR.

3.5 Mechanism of genistein-mediated metastatic progression via ERβ: genomic pathway

Using the patient-derived PCa tissue xenograft model, genistein stimulated PCa metastasis via ERβ activation, whereas ICI 182,780 inhibited metastatic progression. The results from section 2.4 of this study suggested that non-genomic action of ERβ activated the FAK pathway, which may have contributed to metastatic promotion in the PCa xenograft model. Beside non-genomic action, ERβ is known to function as a transcription factor by binding to a specific region of DNA called the estrogen-responsive element [226,230]. To delineate which estrogen-linked genes were responsible for the metastasis-stimulatory effects observed in this study, a genome wide expression array was performed using genistein- and ICI 182,780-treated tumours. If activation of ERβ by genistein promoted cancer metastasis and inactivation of ERβ by ICI 182, 780 led to inhibition of metastasis, the genes that were stimulated by genistein but also inhibited by ICI 182, 780 may play important roles in prostate cancer progression. A cross-comparison analysis of the array data revealed six genes that were up-regulated by genistein (F.C.>1.5) and also were down-regulated by ICI 182,780 (F.C.<0.66). Of the six genes identified, five of them belonged to the metallothionein (*MT)* gene family: *MT1B, 1E, 1H, 1X*, and *2A*.

3.5.1 Metallothionein (*MT*) gene family expression

To search for ERβ-linked genes that are important in metastasis, the microarray data were analyzed using an ingenuity pathway analysis tool. A cross-comparison of genistein-up-regulated and ICI-down-regulated gene populations identified 6 commonly shared genes,

five of which belonged to the metallothionein family, whose expression levels were validated by qRT-PCR.

To correlate *MT* expression with metastatic phenotype, *MT* expression levels were compared between metastatic and non-metastatic tumour lines of LTL313h, both of which had been derived from the same PCa patient. Interestingly, the same expression pattern was also observed in this comparison: higher in the metastatic tumour line (LTL313M) than in the non-metastatic LTL313NM line. This provides additional evidence linking the increased *MT* gene expression to prostate cancer metastasis.

There are numerous studies reporting a correlation between *MT* expression and cancer progression and invasion. *In vitro* migration assay and gene expression profiling identified the *MT1E* gene as one of the key genes associated with invasion in bladder cancer [348]. Moreover, clinical evidence showed that elevated expression of MT protein is associated with poor prognosis and recurrence in ductal breast carcinomas and oral squamous carcinomas [349-351]. Immunohistological analysis of clinical PCa specimens revealed an increased MT expression in cancer cells compared to adjacent normal prostatic tissues, which retained only minimal staining in patients after androgen ablation therapy. Such clinical correlation suggests that metallothinein may may play a role in PCa metastatic progression.

3.5.2 ERβ knockdown effects on *MT* gene expression

Gene expression data in the metastatic and non-metastatic LTL313b tumour lines suggest that *MT* genes may be linked to metastatic progression of prostate cancer. Furthermore, the microarray and qRT-PCR analyses showed that expression of the *MT* gene family is modulated by genistein and ICI 182,780, both of which bind to ERβ. These data

may indicate that genistein binds to and activates ERβ, which increases *MT* gene transcription via its genomic action and in turn promotes metastatic progression. To validate a role of ERβ in *MT* gene transcription, other investigators have reported that ERβ forms a complex with another transcription factor, Sp1, which then binds to a GC-rich Sp1-binding sequence in the *MT* promoter region, allowing for its expression [352]. As shown in a study by Hua *et al*., this gene regulational effect was specific to ERβ, and not observed with ERα. It can be speculated that Sp1 may preferentially bind to ERβ, and not ERα, due to the unique protein- binding pocket of the β-receptor. There is a subtle difference between the amino acid sequences within the binding cavity of the β- and α-receptors [323], which may favour Sp1 binding.

This study showed that siRNA knockdown of ERβ reduced *MT* gene expression in PC3 cells. Together with the evidence provided by others, it confirms ERβ regulation of *MT* gene transcription (figure 36).

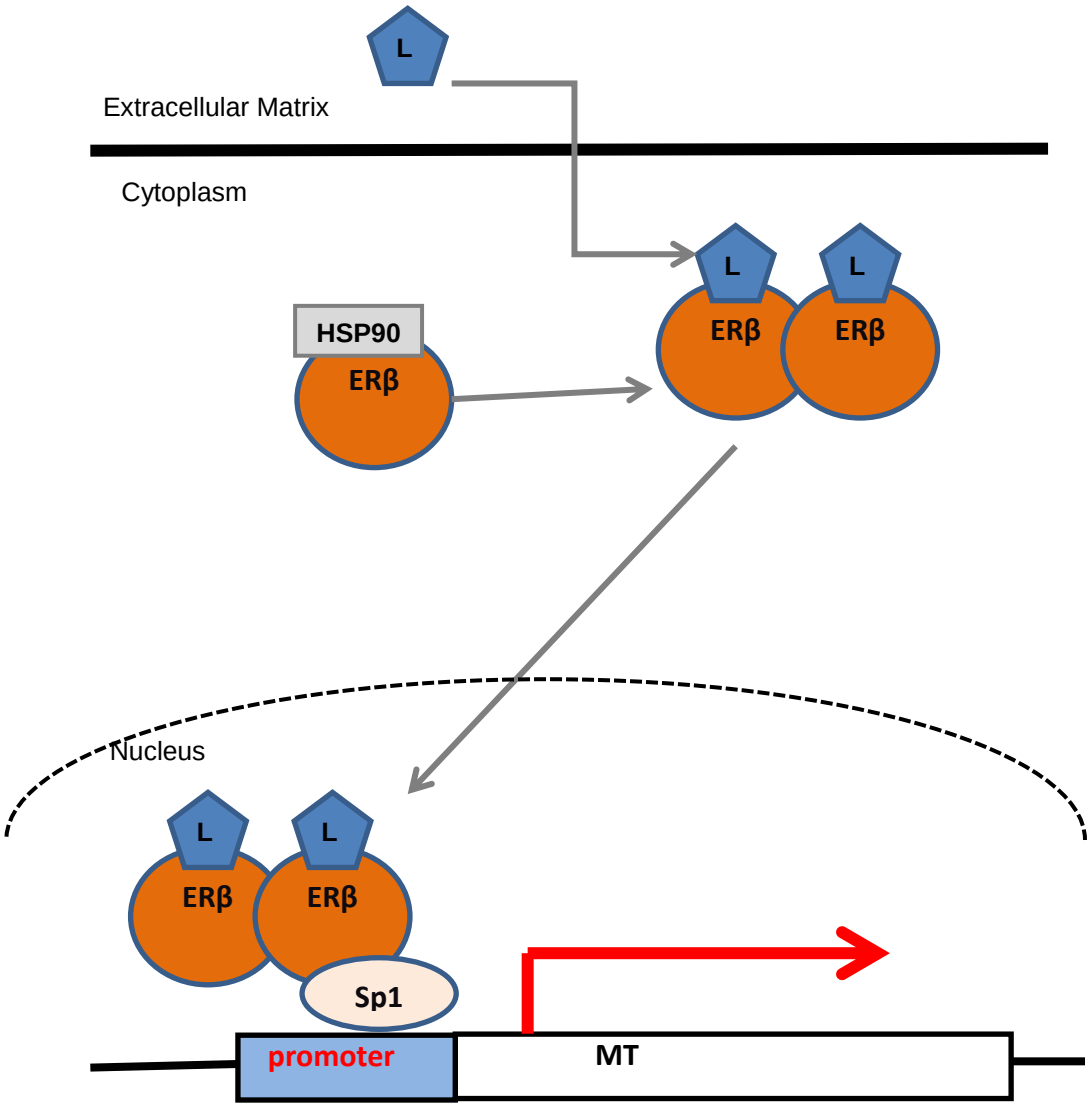


Figure 36. Model depicting genomic actions of ERβ: induction of *MT* gene.

Ligand-activated receptor forms a complex with Sp1, which binds to GC-rich Sp1-binding sequence of *MT* promoter region, inducing transcription. L:ligand. HSP: heat-shock protein.

3.6 Significance and potential application

In this study, genistein promoted metastasis, and anti-estrogen treatment inhibited invasive spread of prostate cancer in the patient-derived PCa tissue tumour xenograft model. The evidence obtained indicates that these compounds affect tumour biology via activation or inactivation of their receptor, ERβ. Through genomic and suggested non-genomic actions of ERβ demonstrated in this study, this receptor may be responsible for activating kinase signaling pathways and for inducing expression of tumour-promoting genes, creating favorable *in vivo* conditions for metastatic progression.

The effects of genistein demonstrated in this study suggest that genistein may have heterogeneous actions which promote cancer growth and progression in certain subtypes of cancers while inhibiting other tumours due to differential ERβ expression among patients. Some PCa cells may have higher expression of ERβ than other cancer cells, favoring the progression of the disease.

Such metastasis-stimulating effects of genistein observed in this *in vivo* study are inconsistent with the anti-cancer effects reported by early *in vitro* studies. However, it is important to note the difference between studies utilizing cultured cells versus *in vivo* models that more closely mimic clinical cancer as demonstrated here. What contributes to this difference may lie in the dynamic tissue interactions that exist in the tumour microenvironment unique to *in vivo* models. For example, growth factors released by the stroma or systemic hormonal influence on cells may affect their biology in ways that cannot be replicated by isolated cancer cells *in vitro*. Data generated from *in vitro* studies have expanded our knowledge of molecular interactions and cellular mechanisms and thus are of unquestionable value; however, its application in the clinical setting is limited. The use of

clinically relevant *in vivo* models as demonstrated in this study, in contrast, serves as a powerful tool for unraveling bona fide effects resulting from intricate interactions between tumour and its microenvironment.

In summary, this project has demonstrated that ERβ is important in metastatic progression of advanced PCa in humans. The significant inhibition of metastasis by anti-estrogen treatment shown here potentiates a promising new selective estrogen receptor modulator treatment for the metastatic disease. Taken together, sub-classification of PCa based on ERβ expression followed by appropriate combination therapy with anti-estrogen and anti-androgen may significantly improve the survival of metastatic PCa patients.

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