INFLUENCE OF SOY ISOFLAVONES ON THE PROLIFERATION AND DIFFERENTIATION OF PROSTATE EPITHELIAL CELLS

DISSERTATION

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By

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ABSTRACT

Epidemiological and experimental evidence suggests that increased consumption of soy can reduce the risk for developing prostate cancer (PCa); the second leading cause of cancer-related deaths in American males. Isoflavones have been identified as one group of biologically active components in soy thought to be responsible, in part, for this anticancer activity. The 3 major isoflavones found in soy are the glycoside derivatives of genistein, daidzein, and glycitein comprising 50%, 40%, and 10% of the total isoflavone profile, respectively. Several lines of evidence suggest that the isoflavones genistein and daidzein may protect against PCa. However, the anticancer of other isoflavones, such as glycitein, have not been extensively investigated.

The objective of the first study was to examine the effects of soy isoflavones on the activation of the extracellular signal-regulated kinase (ERK1/2) pathway in noncancerous prostate epithelial cells (RWPE-1). The ERK1/2 cascade is involved in PCa progression and its activation may be associated with prevention of this disease. Data presented in the first study show that glycitein is the most potent activator of ERK1/2 signaling as compared with genistein, daidzein, and the microbial metabolite of daidzein, equol. Glycitein-induced ERK1/2 activation was sustained for at least 2 hours and decreased cellular proliferation by 40% ($p<0.01$). Glycitein-induced ERK1/2

activation was dependent, in part, by vascular endothelial growth factor receptor (VEGFR) signaling. Sustained ERK1/2 activation is thought to induce cellular differentiation in the prostate epithelium.

The sustained ERK1/2 responsed elicited by glycitein in the first study lead to the hypothesis that glycitein may induce differentiation of the RWPE-1 cell line. Loss of cellular differentiation of the two primary differentiated prostate epithelial cells, luminal and basal cells, contributes to the progression of PCa. Soy isoflavones have been shown to induce cellular differentiation in a number of tissues. However, glycitein-induced differentiation in the prostate has not been examined. Cellular differentiation was characterized by inhibition of cellular proliferation, cell cycle arrest, and cytokeratin expression. Cytokeratins are differentially expressed among epithelial cells with luminal epithelial cells expressing cytokeratins 8/18 while basal epithelial cells express cytokeratins 5/14. Data presented in this study show that glycitein significantly reduced cellular proliferation at concentrations ranging from 0.4 – 50μmol/L. Glycitein significantly reduced the expression of luminal cell markers, cytokeratin 18 and prostate specific antigen (PSA) yet maintained the expression of basal cell markers p63 and cytokeratin 5. These data suggest that glycitein may induce basal cell differentiation in prostate epithelial cells. Prostate epithelial basal cells are disrupted in precancerous

lesions and completely absent in PCa. The ability of soy isoflavones and specifically glycitein to induce basal cell differentiation may represent a novel mechanism of preserving this cell population and reducing PCa risk.

Data from the first two studies of this dissertation show that glycitein is a unique isoflavone that induces a robust ERK1/2 response and induces cellular differentiation of prostate epithelial cells. Structurally, glycitein is the only soy isoflavone with a methoxy group. The third hypothesis was that isoflavones with methoxy groups have greater antiproliferative and ERK1/2 responses in prostate cells as compared with nonmethylated isoflavones. To test this hypothesis, noncancerous, precancerous, and cancerous cells were treated with nonmethylated (genistein, daidzein, equol) and methylated (glycitein, and red clover isoflavones biochanin A and formononetin) isoflavones. The results of this study show that although biochanin A and formononetin are methylated isoflavones, ERK1/2 activation is similar to that of genistein and daidzein. All isoflavones inhibited the proliferation of these cell lines with greater potency in the noncancerous and precancerous cells. However, glycitein-induced ERK1/2 activation was the only isoflavone with associated antiproliferative effects of early stage prostate cells. These results suggest that the position of the methoxy group may be important for the bioactivity of isoflavones. Furthermore, isoflavones may have greater anticancer activity during noncancerous and precancerous stages of PCa.

Dedicated to my father, Robert J. Clubbs, for his support and encouragement throughout my academic career. Although he is no longer with us, his spirit continued to motivate me throughout my journey through the doctoral program.

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FIELDS OF STUDY

Major Field: Ohio State University Nutrition (OSUN)

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CHAPTER 1

INTRODUCTION

Cancer is second only to heart disease as the leading cause of death in the United States (US) [1]. Currently, prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer related deaths in American males [1]. PCa is an age-dependent disease with clinically relevant PCa occurring most frequently in men ≥65 years of age. High grade precancerous lesions of the prostate are most prevalent in men in their 50s. Surprisingly, low grade precancerous lesions have been observed in men as early as 20 and 30 years of age. Clearly, the development and progression from precancerous lesions to clinically relevant PCa can take years or even decades. Therefore, strategies to intervene at different stages of the precancerous process may be appropriate and feasible to prevent the development of PCa.

In the early 1990s, a sharp increased incidence of PCa was observed in the US due in large part to the screening initiative for prostate specific antigen (PSA). PSA is a serum biomarker used by physicians to assess whether or not a biopsy must be performed in order to diagnose or rule out prostate tumors. PSA screening cannot be used to screen for precancerous lesions. In other words, a rise in PSA or a value of 4ng/ml is suggestive

for PCa and other diseases of the prostate but are not suggestive for prostate precancerous lesions. Unfortunately, the only method for screening and diagnosing precancerous lesions is via biopsy.

Not all men that develop precancerous lesions of the prostate will develop clinically relevant PCa. Currently, there are no means of determining which men will and which men will not develop this disease. Treating the prostatic precancerous lesion may be as aggressive as radiation or androgen therapy, or may be as mild as dietary interventions. Dietary intervention is the least invasive treatment for precancerous lesions of the prostate and can be utilized with or without a precancerous lesion diagnosis. Therefore, dietary intervention is an attractive strategy for preventing this disease.

Epidemiological evidence suggests that a diet rich in fruits, vegetables, legumes, and whole grains may reduce the risk of PCa while a diet rich in fat and calories may increase the risk of this disease [2]. Ecologic studies provide the most compelling evidence that diet may influence the risk of PCa. Although East Asians have similar rates of low grade precancerous lesions as compared to the US, the incidence of high grade precancerous lesions and PCa in Americans is tenfold that of East Asian countries [3]. When these people immigrate to the US, their risk of PCa is similar to that of American males [4, 5]. This is most likely due to the abandonment of their traditional diet and the adoption of a more western diet [6]. East Asian diets are rich in soy which is thought to be responsible, in part, for the reduced risk of PCa observed in this population.

A western diet is practically devoid of soy and its absence, along with other dietary modifications, may contribute to the observed increased risk of PCa in this immigrant population.

Dietary soy is the richest source of compounds called isoflavones. It is the isoflavones that are thought to be responsible, in part, for the anticancer effects of soy. Although structurally similar, many studies suggest that isoflavones possess differences in their mechanisms of action for preventing PCa. Recently, studies have found that flavones containing methyl groups appear to be more biologically stable, resistant to phase I and phase II metabolism, and have improved intestinal transport compared to their nonmethylated counterparts [7, 8]. To date however, no studies have compared the effects of methylated and nonmethylated isoflavones, a subfamily of the flavone compounds.

Many studies have utilized *in vitro* and *in vivo* PCa models to measure different anticancer effects of soy isoflavones. These studies have provided valuable information on the potential for treatment of this disease. Although PCa models are appropriate to study the treatment of preexisting PCa, they may not be appropriate to study the prevention of PCa development. Models that mimic a noncancerous or precancerous prostate may better provide details as to how soy isoflavones protect against the development of PCa.

Soy isoflavones have been shown to modulate a number of signaling pathways in cancer development. The extracellular signal-regulated kinase (ERK1/2) cascade is a member of the mitogen-activated protein kinase (MAPK) family and is required for the

normal growth, development and survival of the prostate epithelium [9]. The ERK1/2 pathway is often downregulated during PCa progression and decreased or absent ERK1/2 activity is often an indication of poor prognosis [10 - 13]. Preliminary evidence in this laboratory shows that genistein, the most predominant soy isoflavone, induces ERK1/2 activity at low concentrations and inhibits ERK1/2 activity at higher concentrations in noncancerous prostate epithelial cells (RWPE-1) [14]. It is thought that sustained ERK1/2 activity induces differentiation of the prostate [11]. However the effects of other soy isoflavones on ERK1/2 activity and cellular differentiation have not been examined.

1.1 Hypotheses

1.1.1. Glycitein, a minor soy isoflavone, activates the ERK1/2 signaling cascade via a hormone-independent mechanism in the RWPE-1 cell line.

1.1.2. Glycitein induces terminal differentiation in the RWPE-1 cell line.

1.1.3. Methylated and nonmethylated isoflavones differentially inhibit proliferation and ERK1/2 activity in noncancerous (RWPE-1), precancerous (WPE1-NB14) and cancerous (RWPE-2, PC-3) prostate cell lines.

1.2 Specific Aims and Objectives

1.2.1 Identify the mechanism of glycitein-induced ERK1/2 activation in the noncancerous RWPE-1 cell line.

1.2.1.1 Utilize specific tyrosine kinase inhibitors to identify the mechanism of glycitein-induced ERK1/2 activation.

1.2.1.2 Determine if the effects of glycitein on RWPE-1 cellular proliferation are mediated by glycitein-induced ERK1/2 activation.

1.2.1.3 Characterize the tyrosine kinase receptor mediating glycitein-induced ERK1/2 activation.

1.2.2 Evaluate the effect of glycitein on terminal differentiation in the RWPE-1 cell line.

1.2.2.1 Examine the effects of glycitein on long term cellular proliferation.

1.2.2.2 Examine the effects of glycitein on cell cycle distribution.

1.2.2.3 Determine if glycitein induces cellular differentiation.

1.2.3 Characterize ERK1/2 activity and the antiproliferative effects of methylated and nonmethylated isoflavones in noncancerous (RWPE-1) precancerous (WPE1-NB14) and cancerous (RWPE-2, PC-3) prostate cell lines.

1.2.3.1 Determine antiproliferative effects of individual methylated and nonmethylated isoflavones using cell lines representing different stages of the prostate carcinogenic process.

1.2.3.2 Examine the effects of isoflavones on ERK1/2 activity in cell lines representing different stages of the prostate carcinogenic process.

1.2.3.3 Determine the association between the antiproliferative effects and isoflavone-induced ERK1/2 activity in cell lines representing different stages of the prostate carcinogenic process.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Prostate Anatomy and Physiology

The prostate is a small acorn-shaped exocrine gland that resides at the base of the bladder surrounding the urethra. The only known function of the adult prostate is to secrete proteins in order to maintain fluidity of the seminal fluid. In contrast, the prostate is widely known for harboring many diseases. Interestingly, each disease of the prostate arises in specific regions or zones within this gland. The human prostate contains three distinct morphological zones: peripheral, transitional, and central zones (Fig. 2.1). Approximately 70% of PCa arise from the peripheral zone whereas benign prostatic hyperplasia (BPH), or an enlarged prostate, often arises from the transitional zone [15, 16].

The fibromuscular component of the prostate, the stroma, represents approximately 50% of the prostate weight. The stroma surrounds a specialized extracellular matrix composed of predominantly glycoproteins known as the basement membrane. Structurally, the basement membrane acts as a barrier separating the stroma from the epithelium. Functionally, the basement membrane contributes to the regulation

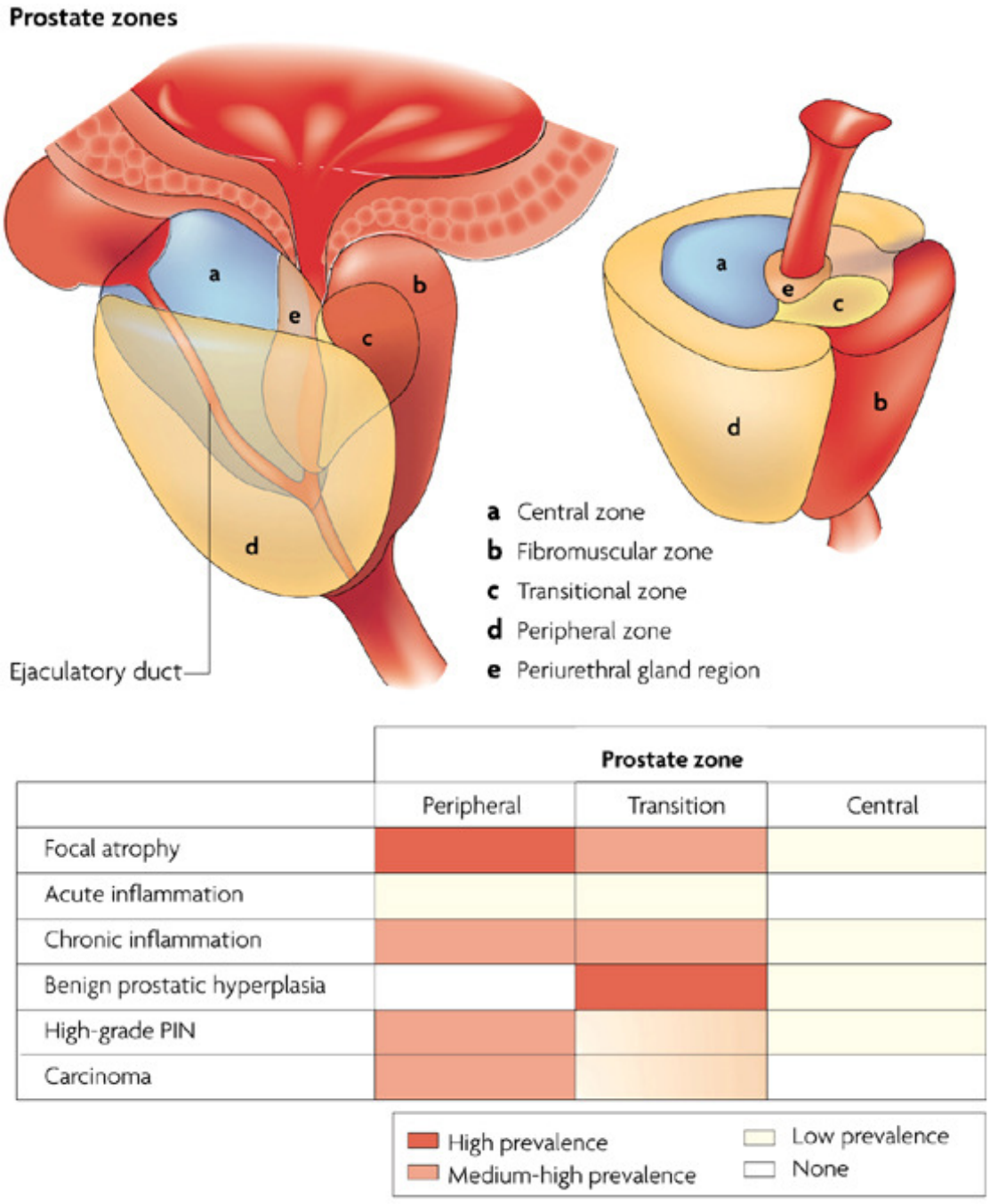


Figure 2.1: Zonal predisposition for prostatic disease. Taken from DeMarzo et al. [16].

of cell behavior and cell signaling [17]. The basement membrane surrounds the prostate epithelium. The epithelium is the site of prostate carcinogenesis and will be the focus of this review.

2.1.1 Prostate Epithelium

The prostate epithelium is composed of two histologically distinct layers, the luminal and basal layers. The luminal cell layer lines the lumen of the prostate duct and is composed of tall columnar cells that secrete proteins, metals, and metabolites into the lumen of the prostate. Just below the luminal cell layer and above the basement membrane lies the basal cell layer. It is thought that the basal cell layer consists of stem, intermediate, and mature basal cells. Another cell type, neuroendocrine (NE) cells, are sparsely scattered between the two epithelial layers. The cells within the prostate epithelium can be distinguished by their unique protein profiles and functional and morphological characteristics.

The terminally differentiated luminal cells are the secretory cells of the prostate epithelium. These cells are positive for androgen receptor (AR) and cytokeratins (CK) 8/18 and upon androgen stimulation, express and secrete PSA and prostatic acid phosphatase (PAP).

The cuboidal basal cells of the prostate separate the luminal cells from the basement membrane. These nonsecretory cells express CK5/14, p63, GSTπ, integrinα_2, bcl-2, and are negative for AR. The function of this cell type is currently unclear. Many studies suggest that basal cells give rise to terminally differentiated luminal cells along an

intermediary pathway [18, 20]. However, evidence also suggests that these cells consist of a mature population of cells independent of luminal differentiation [21, 21]. The function of basal cells are to not only physically separate luminal cells from the basement membrane, but to maintain survival and function of the luminal cells and maintain ductal integrity of the prostate [21].

The origin of NE cells has not been clearly distinquished as arising from the prostate stems cells or arising from the neural crest [22]. Nonetheless, these cells have neuronal and endocrine functions including peptide secretion and regulation of epithelial proliferation and differentiation. NE cells are AR negative, non-proliferating, and PSA negative cells. NE cells express CK8/18 (a marker of luminal cells). Chromogranin A is secreted by this cell population and serves as the gold standard marker for NE detection in the prostate. Other products secreted by these cells include serotonin, bombasin, vasoactive intestinal peptide, neuron-specific enolase, parathyroid hormone-related protein, vascular endothelial growth factor, among others [23].

In the adult prostate, stem cells represent 0.5% of the total epithelial population [21]. Stems cells express the entire repertoire of protein markers found in mature, terminally differentiated prostate epithelial cells. These markers include CK5/14 and 8/18, p63, glutathione-S-transferase pi (GSTπ), among others. Stem cells have the capability to asymmetrically divide in order to maintain the stem cell population and give rise to a daughter cell that enters the differentiation pathway. Unlike the initial development of the prostate in utero, adult stem cells divide only when there is a loss of

the progenitor cells [24]. As the daughter cell migrates along the differentiation pathway, cells bound for luminal differentiation lose the protein markers found in basal cells and cells bound for basal differentiation lose markers of luminal cells.

Intermediate cells are the proliferative component of the adult prostate epithelium. These cells have begun and are committed to the differentiation journey as they are the progenitor of the stem cells. It is thought that intermediate cells maintain this phenotype until called upon to fully differentiate. Several intermediate cell types have been identified by their cytokeratin (CK) profile in vitro including CK5/14/18, CK5/18, CK14/18, CK14/8, CK5/14/8 [23, 25, 26]. However, only the former 2 have been identified *in vivo*.

2.1.2 Hormonal and Epithelial-Stromal Interactions

Both stromal and luminal cells express the AR and respond to androgen stimulation; however, their responses differ. In stromal cells, dihydrotestosterone (DHT) binds to the AR and induces gene transcription for growth factors such as tumor growth factor (TGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF). These growth factors are secreted and regulate prostate epithelial proliferation and differentiation. In prostate epithelial cells, DHT binds to AR and induces gene transcription of PSA, PAP and other secreted proteins. Therefore, the stroma and the epithelial layer intimately interact via hormones and growth factors to reciprocally regulate proliferation and differentiation signals.

Estrogens are also important for the development and maintenance of the prostate. In men, estrogen is primarily produced by conversion of testosterone to estrogen via aromatase in the leydig cells of the testes as well as in extragonadal tissues such as brain, bone, and adipose [27]. Estrogen binds to the estrogen receptor (ER) much like androgens bind to the AR, inducing gene transcription. In the adult male prostate, ERα is expressed in the stromal cells whereas ERβ is expressed in the epithelial cells. Research suggests that activation of ERα by estrogens may actually increase epithelial proliferation and induce metaplastic changes in the prostate epithelium [28, 29]. Furthermore, when androgens and estrogens are given to rats in combination, PCa develops [30 – 35]. However, estrogen activation of ERβ may actually protect against metaplastic changes via induction of epithelial differentiation [36].

2.1.3 Prostate epithelial differentiation

To date, it is unclear at which point during the differentiation process an intermediate cell commits to a specific terminally differentiated cell type (*i.e.* basal, luminal). Furthermore, debate exists as to the differentiation lineage of the prostate. There are two hypotheses of this differentiation lineage: 1) the basal cell population differentiates into a luminal cell population along an intermediate cell pathway [18 – 20]; or 2) basal and luminal cell populations both originate from an intermediate cell and are not dependent on one another for differentiation [21,22]. An illustration of these hypotheses of differentiation is given in Fig. 2.2.

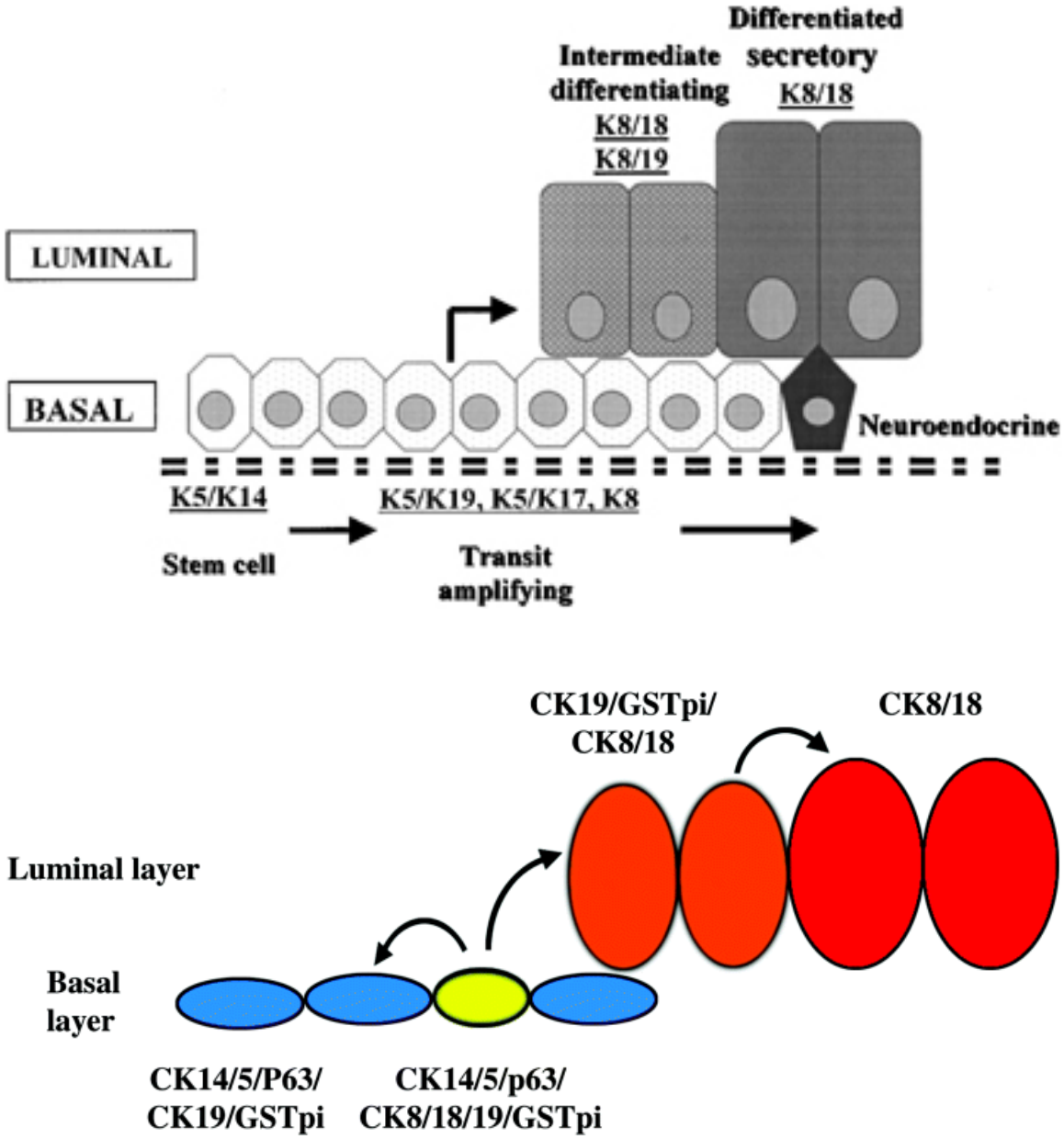


Figure 2.2: Two hypotheses for epithelial differentiation of the prostate. The top schematic was taken from Hudson [19]. The bottom schematic was taken from Wang et al. [22].

The first hypothesis is by far the most widely accepted hypothesis in the prostate research community. One argument for this hypothesis is that a terminally differentiated cell must perform a specialized function(s). Currently, there is no consensus for the function of basal cells. Furthermore, intermediate cells express markers of both basal and luminal cells, weakly suggesting a hierarchical relationship [37]. Finally, the proliferative compartment of the normal adult prostate resides in the basal cell layer [18]. The second hypothesis for the differentiation lineage of the prostate suggests that basal and luminal cells are derived from a common stem/intermediate cell population. During organogenesis, the cells within the premature prostate (the urogenital sinus epithelium) express the entire range of epithelial cell markers and are indicative of a stem cell population [22]. As the prostate matures in utero, stem cells lose the expression of basal cell markers to become luminal cells whereas cells destined for a basal cell phenotype lose the expression of luminal cell markers [22]. In the adult human and murine prostate, the stem cells retaining the expression of both luminal and basal cell markers represent only 0.5% of the epithelium and reside in the basal cell layer [22, 38].

Recently, Lawson et al. [38] used flow cytometric techniques to identify stem cells in the adult murine prostate. His group found that stem cells in the prostate reside in the proximal region of the basal cell layer. In contrast, basal cells found in the distal region of the prostate have little to no regenerative capacity suggesting that basal cells exhibit functionally distinct characteristics based on regional localization in the prostate.

Research from Gerald Cunha's laboratory also suggest a functional role of the basal epithelial cells in the normal developing prostate, further suggesting that basal cells

are a terminally differentiated epithelial cell. Using $p63^{-/-}$ urogenital sinus, his laboratory demonstrated that mice lacking expression of p63 were able to develop luminal and neuroendocrine cells but not basal cells [21]. However, basal cells were required to maintain luminal differentiation and ductal integrity of the mouse prostate. These data suggest that perhaps basal cells mediate signals and/or modulate signals relayed by the stroma to the luminal cells of the prostate and that loss of the basal cells disturbs the intricate intermediary interactions between the luminal and stromal cell compartments.

2.2 Cancer

The prostate epithelium is the site of carcinogenesis. The carcinogenic process is a multistep process of initiation, promotion, and progression and is depicted in Fig. 2.3 [39]. During initiation, the DNA of a normal cell is damaged via endogenous or exogenous carcinogens and goes unrepaired. This initiated cell acquires a growth advantage, allowing for clonal expansion of the genetically damaged cell. The clonal expansion of the initiated cell leads to a preneoplastic lesion and is termed the promotion stage of carcinogenesis. As the cell population expands, additional genetic alterations leads to transformation of the preneoplastic cells to a malignant tumor. This final stage is termed progression.

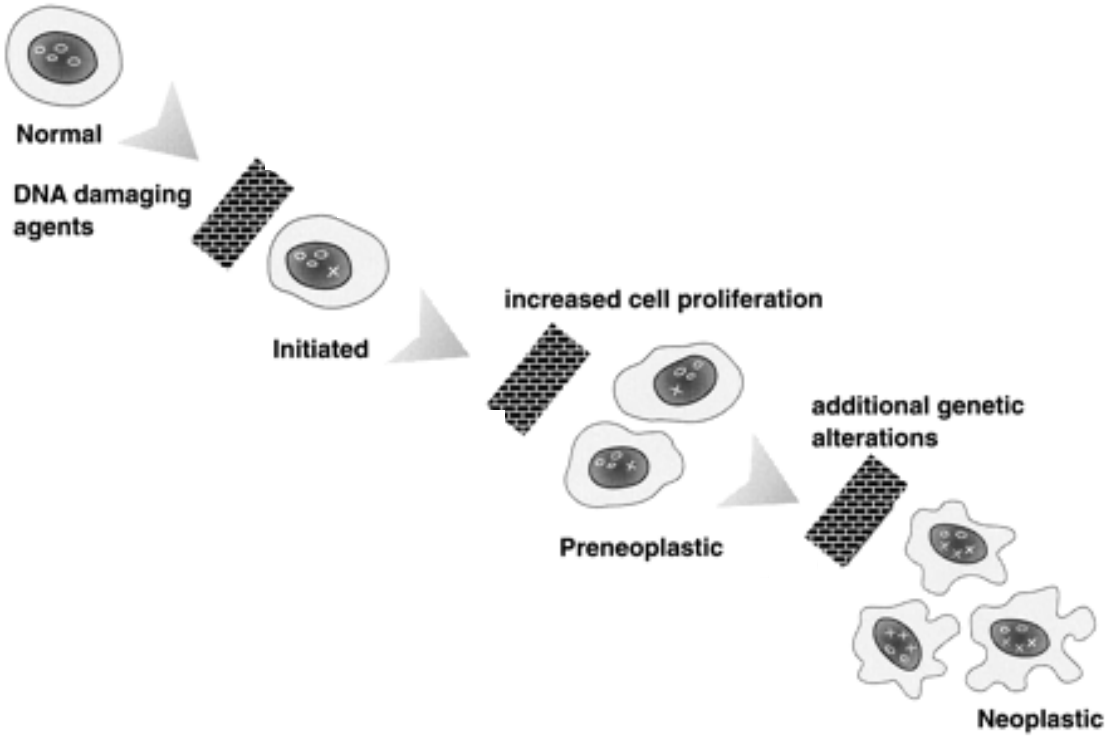


Figure 2.3: The multistage process of carcinogenesis, initiation, promotion, and progression. Taken from Hursting et al. [39].

The series of genetic alterations which lead a normal cell population through the initiation, promotion, and progression stages of cancer have been proposed to be grouped into six categories or hallmarks of cancer [40] and is depicted in Fig. 2.4. These hallmarks can be attributed to all cancers and are described as the following: self-sufficiency in growth signals via oncogenic activation; insensitivity to anti-growth signals via tumor suppressor in activation; evading apoptotic signals; sustaining angiogenesis; limitless replicative potential; and tissue invasion and metastasis.

The initiation, promotion, and progression stages of PCa involve a series of slowly developing preneoplastic events and are described in sections 2.3 and 2.4.1. Genetic alterations acquired during each stage of the prostate carcinogenic process relating various hallmarks of cancer is described in detail in sections 2.4.4 and 2.4.5. The slow progression and acquisition of genetic abnormalities allow for several intervention points that will be discussed throughout this chapter and subsequent chapters.

2.3 Prostate epithelial cell lines representing different cancer stages

The cell line used in the studies described in subsequent chapters is the nontumorigenic prostate epithelial cell line, RWPE-1. The RWPE-1 cell line is a heterogeneous population of predominantly intermediate epithelial cell types that coexist with a subpopulation of prostatic stem cells [41,42]. RWPE-1 cells are characterized by the luminal cell markers PSA and AR, CK 8/18, and the basal cell maker CK 5/14. These cells were obtained from the peripheral zone of the normal prostate of a Caucasian male

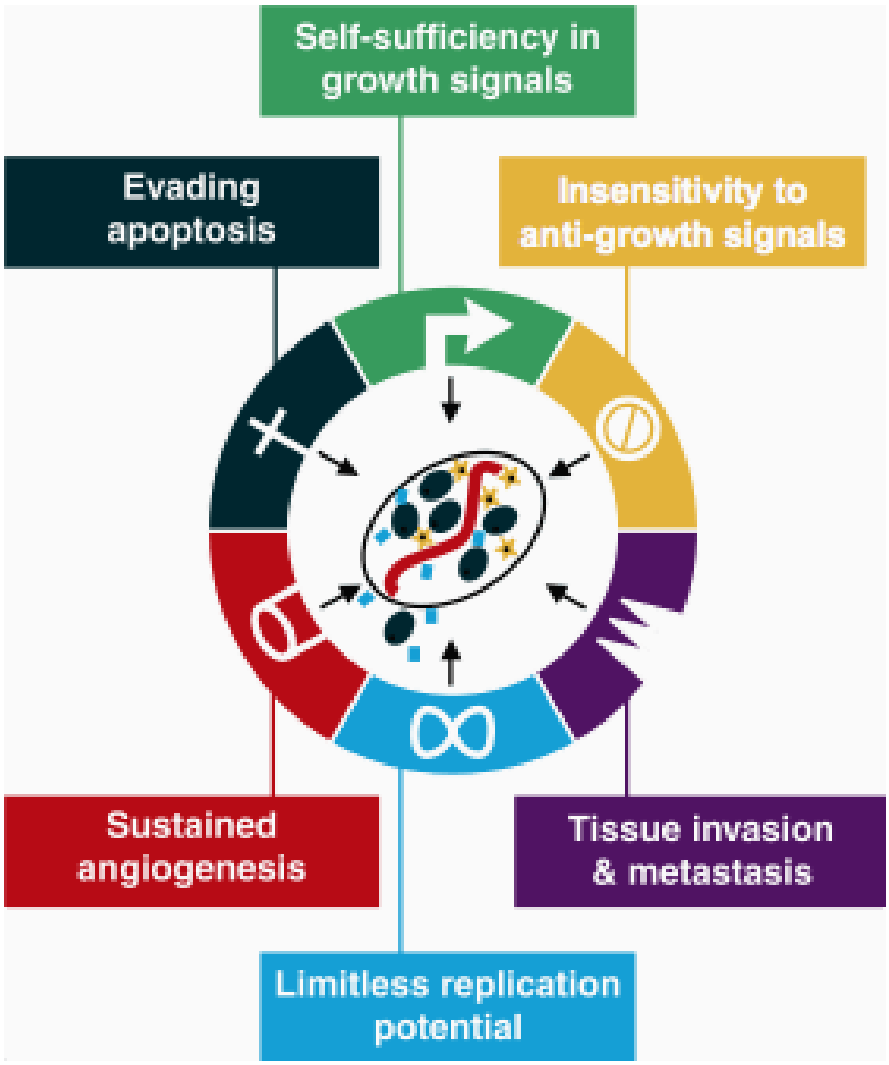


Figure 2.4: Hallmarks of cancer. Taken from Hanahan and Weinberg, [40].

and transformed with the human papilloma virus-18 (HPV-18) for immortalization [41]. RWPE-1 cells do not exhibit anchorage independent growth nor display invasive properties, confirming these cells as nontumorigenic.

The promotional, precancerous stage of the prostate was studied in chapter 5 using the WPE1-NB14 cell line. This cell line was developed by treating the RWPE-1 cell line with 50μg/ml of *N*-methyl-*N*-nitrosourea (MNU) for three cycles and injected into nude mice [43]. The resulting tumor was excised and epithelial cells were collected, injected into nude mice and second generation tumors were excised and epithelial cells collected, giving rise to the WPE1-NB14 cell line. This cell line shows low invasive characteristics *in vitro* and results in a moderated differentiated with no invasive characteristics *in vivo*. The authors suggest that this cell line represents the promotional stages of prostatic intraepithelial neoplasia (PIN) during prostate carcinogenesis.

The RWPE-2 and PC-3 cell lines were used to represent androgen-dependent and androgen-independent cancer, respectively [44]. The RWPE-2 cell line was derived from the RWPE-1 cell line via infection with the Kirsten murine sarcoma virus that contains the activated Ki-ras oncogene [42]. Therefore, this cell line was synthetically driven to represent PCa. However, the PC-3 cell line was taken from a bone metastasized biopsy. This cell line shows loss of the PTEN tumor suppressor protein, allowing for aberrant proliferation [45]. PTEN will be discussed in a subsequent section of this literature review.

2.4 Prostate Cancer

The American Cancer Society estimates that 218,890 men will be diagnosed with and 27,050 men will die from PCa in the US in the year 2007 [1]. In the US, PCa incidence is ten times that of East Asian countries [3]. Interestingly, US immigrants from East Asian countries appear to have an increased risk of PCa compared to their native counterparts, suggesting that environmental factors play a role in the development of this disease[4 – 5]. Age, heredity, and race also influence the risk for PCa.

Age is the single most significant risk factor for PCa. Although low grade prostate precancerous lesions can be observed in men as young as 20 years of age, clinically relevant PCa is not generally presented until the age of 65 or greater. Heredity, another risk factor for PCa, accounts for only 10-15% of PCa incidence. PCa risk is 60% greater in African Americans compared with Caucasians in the US [46], suggesting that race is yet another risk factor for PCa. Caucasians in the US and Europe are at ten times greater risk than East Asians for developing PCa [3]. However when Asians migrate to the US, their risk increases to a rate similar to the US population, suggesting that race and ethnicity play a smaller role than environmental factors in the development of this disease.

Environmental factors, particularly diet, are thought to play a pivotal role with both the induction and prevention of PCa. Diets rich in saturated fat and calories tend to increase the risk of PCa while diets rich in fruits and vegetables tend to decrease the risk for this disease [2]. East Asians tend to eat a diet rich in fruits and vegetables whereas

Americans tend to eat a typical “western” diet rich in meat, saturated fat, and calories. Evidence for the association of diet and prostate cancer will be discussed in subsequent sections of this literature review.

2.4.1 Prostate Tumor Development

PCa follows a protracted series of various grades of precancerous lesions which include proliferative inflammatory atrophy (PIA), low grade prostatic intraepithelial neoplasia (LGPIN), high grade prostatic intraepithelial neoplasia (HGPIN), before finally reaching clinically relevant PCa. The occurrence of precancerous lesions (PIA, LGPIN, HGPIN) is highly prevalent in men by the age of 50 and is estimated that 33% will have one or more precancerous lesions by this point in their lives [47]. However, PCa is significantly less common than that of precancerous lesions. Approximately 1 in 9 men will be diagnosed with clinically relevant PCa by the age of 60 -70. Taken together, early precancerous lesions are three times more common than PCa, suggesting that PCa is not only a consequence of age but a consequence of heredity, race, and environmental factors. The following paragraphs will review the pathology of the PIA, LGPIN, HGPIN and PCa.

Chronic inflammation is implicated in many cancers including, liver, stomach, and urinary bladder [16]. It has been suggested that chronic inflammation may contribute to the development of PCa. The source of inflammation may come from a variety of stimuli including infectious agents, urine reflux, chemical and physical trauma, dietary factors, and estrogens [16]. Focal atrophy is commonly associated with areas of the

prostate where chronic inflammation is observed. These atrophic areas of the prostate epithelium paradoxically have increased proliferative capacity and are therefore called proliferative inflammatory atrophy (PIA). PIA has been detected in both peripheral and transitional zones of the prostate. It is thought that perhaps PIA may be one of the initial events in the development of PCa, although PCa is usually confined to the peripheral zone.

Prostatic intraepithelial hyperplasia (PIN) follows PIA along the carcinogenic pathway. PIN is a condition of abnormal proliferation within the prostate epithelium. PIN has been classified into 2 specific lesions, low grade PIN (LGPIN) and high grade PIN (HGPIN) and is described in Table. 2.1 [48]. LGPIN has been observed in men < 30 years of age, whereas HGPIN, thought to be the immediate precancerous lesion to PCa, is observed in men ≥50 years of age. LGPIN exhibits many characteristics of normal epithelium and therefore the diagnosis is subjective, dependent on the expertise of the pathologist and study conditions [49]. The cytological features of the luminal secretory cells in LGPIN and HGPIN are very similar exhibiting epithelial crowding and stratification (Table 2.1). In addition to epithelial crowding, HGPIN can be characterized as cribiform, tufting, micropapillary, and flat patterns with the tufting pattern seen in almost 97% of all HGPIN cases [50]. Lastly, an intact basal cell layer is observed in LGPIN, where the basal cell layer is commonly disrupted in HGPIN.

LGPIN and HGPIN can only be detected via biopsy. The risk of developing PCa from HGPIN increases with increasing positive core biopsies (>4 of 6 core biopsies) [51, 52]. Traditionally, management of HGPIN includes repeat biopsies, digital rectal exams,

	LGPIN	HGPIN
Architecture	Epithelial cell crowding and stratification with irregular spacing	Similar to LGPIN, more crowding and stratification, 4 patterns: tufting, micropapillary, cribiform, and flat
Cytology		
Nuclei	Enlarged, with marked size variation	Enlarged, some size and shape variation
Chromatin	Normal	Increased density and clumping
Nucleoli	Rarely prominent	Occasionally to frequently large and prominent, similar to invasive carcinoma, sometimes multiple
Basal cell layer	Intact	Disrupted
Basement Membrane	Intact	Intact

Table 2.1: Characteristics of LGPIN and HGPIN. Adapted from Joniau et al. [48]

and PSA analysis. However, men can also choose androgen deprivation therapy or radiation therapy. A less severe approach for HGPIN management is to modify dietary habits. Many studies suggest that a diet low in fat and calories and rich in fruits, vegetables, antioxidants, soy, fish, and nuts, may reduce the risk of HGPIN and PCa [2]. This approach may be more economical and feasible for those patients diagnosed with HGPIN as approximately only one third of men diagnosed with HGPIN will be subsequently diagnosed with PCa.

Several lines of evidence suggest that HGPIN is the most likely precursor for PCa. The frequency of HGPIN increases with increasing age and can precede PCa by more than 10 years [53]. HGPIN is most often confined to the peripheral zone of the prostate as is PCa. Furthermore, HGPIN is observed in approximately 82% of PCa patients at autopsy compared with 43% of patients without PCa at autopsy [54]. Genetic and epigenetic abnormalities that occur both in HGPIN and PCa also support the hypothesis that PCa arises from HGPIN. A summary of genetic alterations during PCa progression is given in Fig. 2.5 [55] and will be discussed in a subsequent section of this literature review.

2.4.2 Role the basal cell layer

Disruption and loss of the basal epithelial cell layer is the most morphologically distinct feature of the development of HGPIN and the transition to PCa. Loss of this cell population has been recognized for approximately twenty years. However, only recently

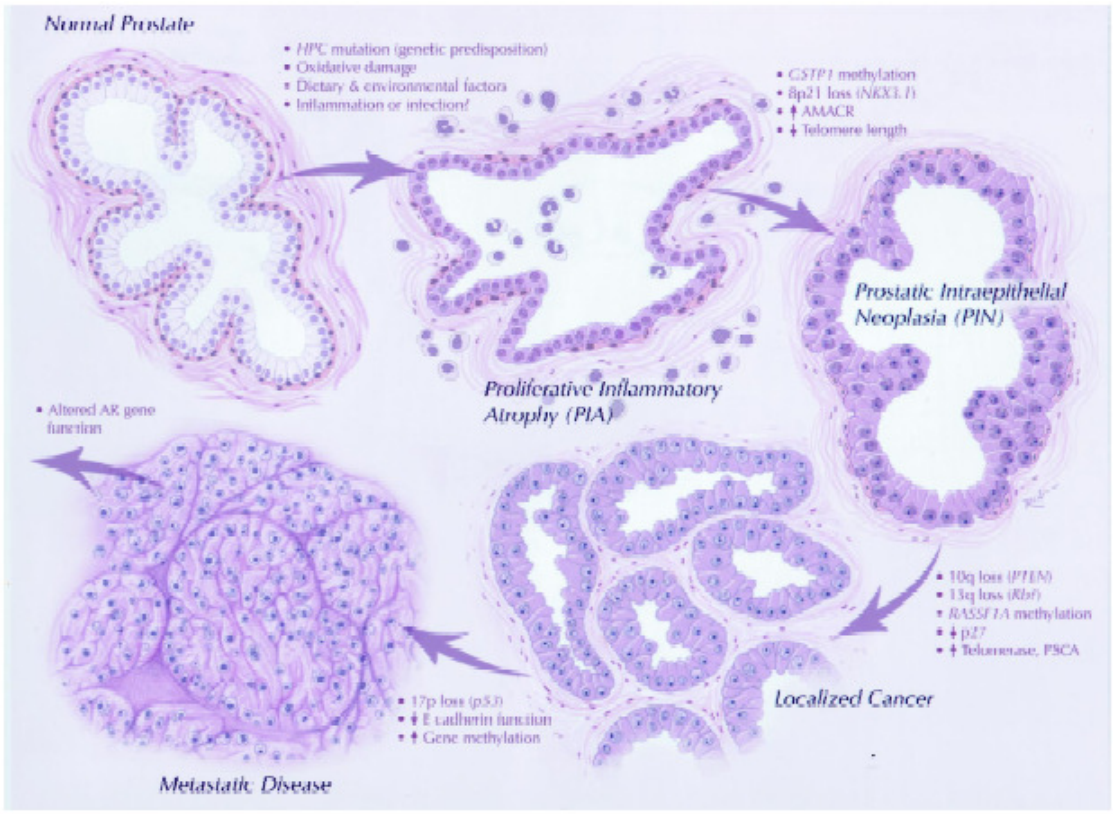


Figure 2.5: Contemporary model of the molecular events associated with prostate carcinogenesis. Taken from Gonzalgo and Isaacs, [55].

the mechanism(s) as to how the basal cells are lost have been investigated. Basal cells harbor many tumor suppressor proteins such as GSTπ, p63, and mapsin [56]. These cells provide a physical barrier between the luminal epithelium and basement membrane and stroma of the prostate. Disruption of the basal cell layer during prostate carcinogenesis allows for a decreased expression of tumor suppressor proteins as well as a direct interaction between luminal and stromal cells.

Recently, an exploratory study examining the prostates of men with pre-invasive and invasive lesions and men without lesions identified early alterations of the basal cell layer and the impact that the loss of this cell populations had on tumorigenesis [56]. These studies hypothesize that basal cells become injured or aged and degenerate, producing focal disruptions within this cell layer. These cells subsequently exhibit increased apoptosis and decreased proliferation. In contrast, cells overlying focal basal cell disruptions showed higher proliferation and increased expression of genes involved with cell proliferation and cell cycle progression. Loss of basal cells allows for loss of tumor suppressor genes, increased interaction between tumor cells and stromal cells, and increased permeability of oxygen and growth factors, thereby aiding growth of tumor cells. Maintenance of the basal cell population may therefore prevent pre-invasive or PIN lesions from developing into clinically relevant PCa [56].

2.4.3 Clinically relevant PCa

PCa originates primarily in the peripheral zone of the prostate and exhibits many morphological characteristics of HGPIN (see table 2.1). However, unlike HGPIN, the

basal epithelium is completely lost and stromal invasion begins to occur [57,58]. Once stromal invasion occurs, tumor cells have the potential to metastasize to other organs such as bone, lymph nodes, and brain via circulation [59].

Between 1969-1974, the Gleason grading system was developed by Donald Gleason to predict the aggressiveness of PCa [60 – 62]. This grading system is based upon the histology of the prostate tumor and assigned a number. Each number or grade corresponds with the degree of differentiation of a specific section of the prostate. Grade 1 corresponds to a well differentiated tumor; grade 2 corresponds to well differentiated sections exhibiting some infiltration; grade 3 corresponds to a moderately differentiated tumor; grade 4 corresponds to a poorly differentiated tumor; and grade 5 corresponds to an undifferentiated tumor. Due to the heterogeneity of prostate tumors, the predominant and second most predominant grades observed in the prostate tumor are added together, resulting in a Gleason score between 2 to 10. Generally speaking, a sum score <6 is considered to be a more indolent tumor with a good prognosis whereas a sum score ≥ 8 is considered to have a poor prognosis.

In addition to the Gleason sum score, physicians will also stage the prostate tumor [63]. Staging is performed using the standard TNM system. TNM staging describes the extent of the primary tumor (T), the absence/presence within the lymph nodes (N), and absence/presence of metastasis (M).

Prostate tumors are initially dependent upon androgen for growth. Often, androgen deprivation therapy is employed to treat androgen-dependent prostate cancer. Upon chemical castration, androgen concentrations are significantly reduced, resulting in

massive apoptosis of the androgen-dependent cells [64]. Unfortunately, this treatment often leads to an androgen-independent PCa most likely due to selective growth of androgen-independent cells that coexisted with androgen-dependent cells prior to androgen ablation [65 – 67].

2.4.4 Molecular Biology of Prostate Cancer

Unlike other preneoplastic lesions and PCa, most PIA lesions do not exhibit somatic genetic alterations. Instead, tumor suppressor genes such as NKX3.1, CDKN1B encoding for p27, and phosphatase and tensin homologue (PTEN) all appear to be down regulated [16]. Glutathione-S-Transferase pi is often upregulated in these lesions which is thought to be a compensatory mechanism to combat the insults that induce inflammation, oxidative stress and genetic damage [68].

The tumor suppressor gene NKX3.1, thought to normally be involved with differentiation, is one of the first tumor suppressor genes lost during the carcinogenic process. Loss of this gene in knockout mice results in defects in prostate ductal morphogenesis and secretory protein function [69]. Therefore, loss of differentiation at the cellular level may contribute to the initial stages of PCa development. Strategies to maintain the differentiation state of the prostate epithelium may inhibit or delay the progression of PCa.

Alpha (α) Methylacyl-CoA racemase is an enzyme that converts R to S-sterioisomers of branched chain fatty acids to allow for β-oxidation and is commonly upregulated in HGPIN and PCa. The main sources for branched chain fatty acids, dairy

and beef products have been associated with an increase risk for PCa [55, 70]. These observations are just one of many suggesting that diet plays a pivotal role in the development of PCa.

Hypermethylation of the GSTP1 gene encoding GSTπ is the most common somatic epigenetic alteration in PCa and is observed in 70% of HGPIN and >90% of PCa samples [55,71]. GSTπ is a phase II enzyme that catalyzes the conjugation of glutathione to electrophilic compounds such as xenobiotics in order to generate a more water soluble compound for excretion.

The tumor suppressor gene PTEN is frequently lost in advanced disease [72,73]. Absent PTEN has been associated with a higher Gleason score and therefore poorer prognosis. PTEN is further discussed in section 2.2.4. In addition, tumor suppressor proteins such as retinoblastoma (Rb), p27, and p53 are lost during PCa progression. The major G_1 cell cycle check point protein, Rb, is either mutated or the expression is lost in 20-50% PCa patients [74 – 76]. The cell cycle inhibitory protein, p27, is commonly downregulated in early stages [77, 78] of PCa whereas p53 is often lost in advanced stages of this disease [79 – 84].

Tumor suppressor genes are commonly downregulated in HGPIN lesions. However, it is not until the development of PCa that oncogenes such as MYC [85] and Bcl-2 [81] are upregulated. The oncogene MYC is a transcription factor that induces transcription of genes involved with cell cycle progression. The amplification of MYC

permits uncontrolled cell cycle progression and subsequent proliferation. The antiapoptotic oncogene Bcl-2 is also often overexpressed during androgen-independent PCa, further perpetuating aberrant proliferation and evading apoptosis [86].

2.4.5 MAPK signaling during prostate carcinogenesis

Signal transduction cascades have emerged as a target for treatment and prevention of PCa, particularly those cascades associated with proliferation, apoptosis, differentiation and senescence. Signal transduction refers to the movement of signals from outside to inside the cell. One family of signal transduction cascades, the MAPK cascade, is important in the regulation of a host of cellular functions including growth, proliferation, apoptosis, inflammation, differentiation and cell cycle progression [reviewed in 9]. The ERK1/2 cascade is one member of the MAPK pathway and is required for the normal growth, development and survival of the prostate epithelium [9]. Activation of ERK1/2 is most commonly associated with cellular proliferation and upregulation of this activity is routinely observed in many malignant tumors with uncontrolled growth. Upregulated ERK1/2 activity in many cancers is a consequence of oncoproteins upstream of ERK1/2 such as receptor tyrosine kinases (RTK) and the oncogene ras. However, the role of this signaling cascade in PCa development and progression is not well characterized. Several studies show decreased ERK1/2 activity with increasing prostatic malignancy [10] while others show increased ERK1/2 activity with increased malignancy [87, 88]. The biological response of ERK1/2 activity is dependent on a multitude of factors such as stimulus, kinetics and duration of the ERK1/2

signal, as well as associated scaffolding proteins associated with and subcellular localization of activated ERK1/2 [89,90]. This may explain, in part, the current controversy of ERK1/2 signaling during prostate carcinogenesis.

It is hypothesized that constitutive activation of the AKT survival pathway may be involved with inhibiting the ERK1/2 pathway during prostate carcinogenesis [12, 13, 91, 92]. AKT is activated upstream via phosphoinositide-3 kinase (PI3K) which converts phosphatidylinositol 4,5 biphosphate (PIP2) to phosphatidylinositol 3,4,5 trisphosphate (PIP3). Activated PIP3 allows for phosphoinositol-dependent kinase (PDK1) to localize to the plasma membrane and phosphorylate AKT. In noncancerous cells, PTEN negatively regulates AKT signaling by dephosphorylating PIP3 to PIP2. However, the expression of PTEN is lost in PCa, subsequently upregulating this survival pathway. The constitutive activation of AKT allows for phosphorylation of Raf-1 [91], an upstream signaling protein in the ERK1/2 pathway. Phosphorylation of Raf-1 inhibits Raf-1 activation, thereby shutting down ERK1/2 activity.

Loss of p53 during the later stages of PCa may also contribute to decreased ERK1/2 activity in PCa [92]. The role of p53 is to induce cell cycle arrest or apoptosis in response to specific stimuli. ERK1/2 activity has been shown to be required for phosphorylation of p53 and subsequent apoptosis in response to certain drugs and chemopreventive agents [93 – 96]. Likewise, p53 induces expression of phosphatases which positively regulate ERK1/2 activity [93, 97 – 99]. During prostate carcinogenesis the p53 gene is frequently altered and inactivated by a variety of mechanisms which may also contribute to the decrease in ERK1/2 activity.

PTEN and p53 regulation of ERK1/2 activity in PCa cells proves to be very complex and not completely understood. A summary of the interactions of these pathways are given in Fig. 2.6 [92]. Nonetheless, it appears that PCa is unique in that activation of ERK1/2 may be beneficial for PCa treatment.

Evidence suggests that ERK1/2 activation may be involved with the differentiation process and that in the prostate, deactivation of ERK1/2 results in a de-differentiation process [11]. This evidence is based on correlations between decreased ERK1/2 activity and poorly differentiated tumors, yet the exact mechanism is currently unknown. The mechanism of ERK1/2 activation and differentiation in other cell types such as neuronal cells has been extensively studied. The duration of ERK1/2 activity appears to be a critical factor that determines cellular response. A transient ERK1/2 signal induces a proliferative response whereas a sustained ERK1/2 signal induces a differentiation response. It is thought that a transient signal does not allow for stabilization of downstream immediate early gene products whereas a sustained signal will allow for downstream stabilization [90]. The stability of downstream targets dictates whether genes encoding for proliferation or differentiation are transcribed.

2.4.6 Screening, Diagnosis, and Treatments

It is recommended by the American Cancer Society that men with a life expectancy greater than 10 years be screened for PCa yearly by the age of 50. The screening process is composed of a digital rectal exam (DRE) and detection of PSA in

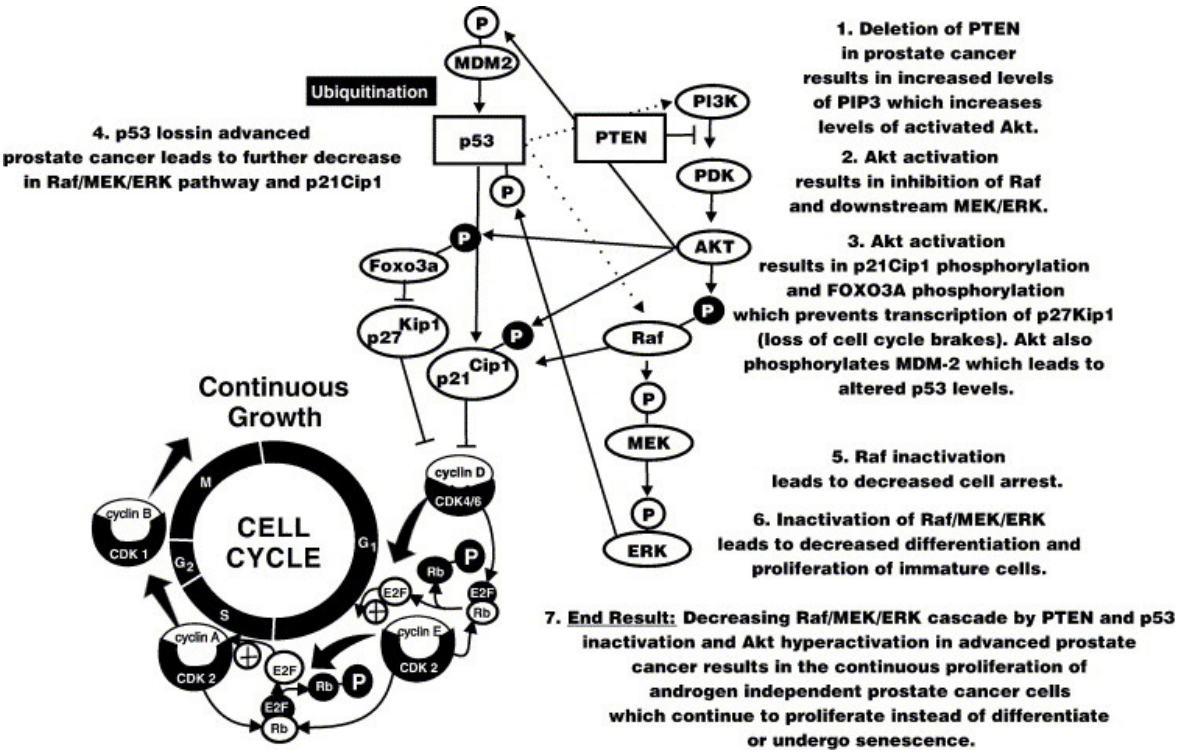


Figure 2.6: Loss of PTEN and p53 expression on ERK1/2 activity during PCa. Taken from McCubrey et al. [92]

serum. Both procedures should be done concurrently as DRE and PSA in combination have a higher predictive value than each test alone [63, 100]. The purpose of the DRE is to check for palpable indurations (hardness), firmness, or asymmetry of the prostate. PSA is a marker in the blood that is present at puberty and continues to rise with age. A PSA value of 4ng/ml is currently the cut-off point for biopsy [63, 101]. When density of PSA is measured, a change in the PSA value of 2ng/ml/year indicates an increased risk for death rate compared to lower PSA velocities [63, 102].

After a biopsy has been performed and a patient is diagnosed with PCa, several options of treatment are discussed. These choices are based on the individual, the family of the patient, prognostic factors (stage and grade), and the patients general physical condition [63]. These choices include: watchful waiting, radical prostatetectomy, interstitial brachytherapy, external beam radiation therapy, and androgen deprivation.

2.4.7 Diet and Prostate Cancer

Several studies suggest that dietary intervention may also prevent and/or treat PCa. Furthermore, excessive energy intake, dietary fat, and calcium have been shown to increase the risk for PCa. Excess energy intake has been intimately linked with the IGF-1/growth hormone (GH) signaling axis [103]. Chronic excessive energy consumption upregulates GH in the pituitary gland which allows for elevated IGF-1 expression in the liver resulting in increased circulating levels of IGF-1. Increased concentrations of IGF-1 are associated with increased risk for PCa [104]. IGF-1 activity induces activation of

proliferation and survival pathways such as MAPK and AKT signaling [105]. Increased consumption of dietary fat has also been implicated with prostate carcinogenesis primarily to due its proxidant effects [106].

Interestingly, the American Institute for Cancer Research has concluded that diets high in calcium may significantly increase the risk for PCa. This conclusion was based on several cohort studies showing a linear relationship between dietary calcium and PCa incidence. The mechanism appears to be related to the reduction of the formation of the bioactive form of vitamin D, 1,25-dihydroxy vitamin D, thereby inhibiting the antiproliferative and differentiation effects observed upon vitamin D treatment [107, 108].

In contrast, The American Institute for Cancer Research has concluded that there is strong epidemiological evidence to support foods containing lycopene, foods containing selenium and selenium may protect against PCa [109]. Lycopene is a carotenoid found primarily in tomato products. Processing the tomato with heat and/or oil increases the bioavailability of lycopene [110, 111]. Recent data suggests that lycopene alone is not as bioactive as the whole tomato product, suggesting that lycopene in combination with other bioactive compounds found in tomatoes may be more beneficial than lycopene provided in supplement form [112]. In contrast, food sources of selenium and selenium alone appear to reduce the risk of PCa. Selenium participates as a cofactor in a number of enzymes responsible for reducing intracellular oxidative stress. Selenium has been shown to induce apoptosis and cell cycle arrest and enhance DNA repair [113].

Other dietary factors that are thought to protect against PCa include vitamin E, vitamin D, green tea, and soy. The protective effect of vitamin E was inadvertently observed during the alpha tocopherol beta carotene (ATBC) study [114]. This study aimed to assess the protective effect of vitamin E and lung cancer. Surprisingly, the investigators found a 30% reduction for the risk of PCa after 4 years and a 41% reduction after 6 years [114]. Since the publication of this study, a host of *in vitro* and *in vivo* studies have been published providing insight into the anticancer mechanism(s) of vitamin E. Vitamin E has been shown to inhibit cell cycle, proliferation and induce apoptosis in prostate cancer cell lines [115].

The chemopreventive mechanisms of vitamin D have recently exploded with great interest and enthusiasm. The observation that geographical incidences in PCa are also related to latitudes further from the equator suggests that the natural production of vitamin D due to sunlight exposure may be protective against this disease [116 – 118]. *In vitro*, vitamin D has been shown to inhibit proliferation and induce apoptosis in PCa cell lines [119, 120]. The potential role of vitamin D on cellular differentiation is also currently being explored.

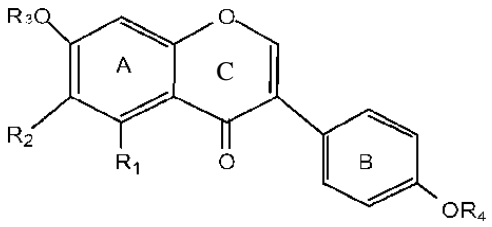
The hypothesis that green tea may reduce the risk of PCa originated from the observation that East Asians consume large quantities of green tea and has a reduced risk of PCa. The most studied antioxidant in green tea has been the catechin epigallocatechin (EGCG) and is attributed in part to the anticancer effects of green tea. EGCG has been shown to induce cell cycle arrest, inhibit IGF-1 synthesis, inhibit proliferation, and induce apoptosis in PCa models [121 – 123].

Unpublished intervention trials suggest that tomato/soy products may also be beneficial with reducing PSA levels in men with advanced PCa [124]. To date, the epidemiologic evidence suggest that soy may protect against PCa is limited [109]. Furthermore, several *in vitro*, *in vivo*, and clinical intervention trials suggest a protective role of soy and compounds found in soy against PCa. The remaining sections of this literature review as well as the following chapters will offer support for the anticancer effects of soy.

2.5 Soy

Soybeans are a species of legumes native to East Asian countries. Soybean cultivars contain approximately 90% cotyledon and 2% hypocotyledon (germ) by weight with the remaining 8% consisting of the hull [125]. Although most legumes are limited in the amino acids methionine and tryptophan, soybeans are a rich source of complete protein and thus do not need complementing [126]. Soybeans provide the most abundant source of isoflavones associated with the protein as compared with other legumes [127]. These attributes make soybeans unique in the legume family. Studies show that the isoflavone content of soy can reach up to 3mg/g [128 – 130]. In addition, soy isoflavones taken from the hypocotyledon can contain upwards of 20mg/g, making the hypocotyledon the most concentrated source of isoflavones [130].

Isoflavones are a subfamily of the flavone compounds that share a basic structure consisting of two benzene rings (A and B) linked through a heterocyclic pyrone C ring (Fig. 2.7) [131]. The benzene B ring position of the isoflavones is in the 3-position, while



R_1	R_2	R_3	R_4	Compounds
H	H	H	H	Daidzein
OH	H	H	H	Genistein
H	OCH_3	H	H	Glycitein
H	H	H	CH_3	Formononetin
OH	H	H	CH_3	Biochanin A

Figure 2.7: Structure of Isoflavones adapted from Chen et al. [131].

the B ring position is in the 2-position of flavones [132]. Soy isoflavones are a family of compounds with structural similarity to estrogen. In the soybean, soy isoflavones can be found in 4 different forms: glycosylated, acetylated, malonylated, and in the aglycone form. The cotyledon contains approximately 88% of the isoflavones whereas the hypocotyledon contains approximately 12% [125]. The predominant isoflavones found in soybeans are in the glycosylated form and include genistin, daidzin, and glycitin, comprising 50, 40, and 10% of the total isoflavone profile. However, in the germ glycitin predominates, representing 47% of the total soy isoflavone profile [125].

2.5.1 Soy isoflavone digestion and absorption

The glycosylated forms of isoflavones cannot be absorbed within the human gut [133]. In order for absorption to occur, the glucose moiety must be hydrolyzed by brush border membrane (BBM) β-glucosidase within the jejunum of the small intestine or within the cytoplasm of the enterocyte [134 – 136]. Recent evidence suggests that transport across the apical membrane is not by passive diffusion but active transport via multidrug resistance related proteins and organic anion transporters [131]. Once the isoflavone has been taken up by the enterocyte, the isoflavones are predominantly glucuronidated by UDP-glucuronyl transferase in order to increase their water solubility for transport within the portal vein [123, 137].

Isoflavones are absorbed and peak in the plasma within 2 hours and peak again within 8-12 hours postprandially [138 – 141] which may be attributed, in part, to enterohepatic circulation [137]. Glucuronidated isoflavones may also be effluxed back

into the intestinal lumen. Glycosylated and glucuronidated isoflavones in the lumen of the small intestine travel to the large intestine where bacterial β-glucosidase and β-glucuronidase hydrolyze glucose and glurcuronic acid, respectively [133]. It is in the large intestine where isoflavones may also be absorbed and/or reabsorbed into circulation, contributing in part to the spike in plasma isoflavone concentrations observed within 8-12 hours after soy consumption [137].

Isoflavone bioavailability is relatively low, with only 1% of ingested isoflavones appearing in plasma. The low bioavailability of isoflavones is attributed, in part, to microbial metabolism of these compounds in large intestine [142, 143]. However, in recent years, researchers have found that isoflavones selectively accumulate in tissues such as the prostate [144, 145]. Individual isoflavones may be ten to thirty fold higher in selective tissues than in plasma [145] which may contribute to relatively low plasma levels of soy isoflavones.

Microbial metabolism of isoflavones results in the production of metabolites which can change the bioactivity of isoflavones. For example, the soy isoflavone daidzein is metabolized to equol by the intestinal microflora of some individuals consuming a diet rich in soy. Equol appears to be more bioactive than daidzein owing to the increased affinity to the ER and increased antioxidant capacity as compared to daidzein [146 – 149]. In contrast, other metabolites such as p-Ethylphenol, a microbial product of genistein, has very little bioactivity. The microbial metabolism of isoflavones in the large intestine therefore plays a major role in both bioavailability and bioactivity of these compounds.

Recent studies suggest that flavones with methoxy groups possess greater bioactivity than nonmethylated flavones. These studies show that methylated flavones are more biologically stable, are resistant to phase I and phase II metabolism, and have improved intestinal transport as compared to their nonmethylated counterparts [7, 8]. Interestingly, glycitein is the only soy isoflavone containing a methoxy group on the sixth carbon of the flavone ring. Glycitein has previously been shown to be more bioavailable than genistein [150] with increased rates of uptake by enterocytes and increased rates of excretion from the basolateral side as measured in the Caco-2 intestinal cell model [131].

2.5.2 Soy and Disease

Soy isoflavones along with soy proteins are thought to possess many health promoting properties such as reducing the risk of heart disease, decreasing the risk of osteoporosis, alleviating menopausal symptoms, increasing cognition, and preventing hormone-dependent cancers. The following paragraphs will briefly highlight the mechanisms by which soy may contribute to these processes.

In 1999 the Food and Drug Administration (FDA) approved the food claim that 25g of soy protein per day may reduce the risk of cardiovascular disease (CVD) [151]. This claim was based on studies suggesting that 25g of soy protein per day decreases total and low density lipoprotein (LDL) cholesterol. It is believed that the cholesterol lowering effect of soy protein is greatest in individuals with elevated levels of serum cholesterol [130, 152 – 155]. The mechanism for lowering cholesterol appears to be mediated by the effects of soy protein on LDL receptor expression and activity [130, 156,

157]. Isoflavones may also decrease the risk of CVD by increasing blood vessel dilation and increasing arterial compliance via its estrogenic activity [158]. In addition to its estrogenic activity, isoflavone antioxidant activity reduces LDL oxidation and increases antioxidant capacity by sparing other antioxidants such as α-tocopherols [156, 159].

During menopause, the protective effects of estrogen on bone health are abruptly reduced. Not only is soy a rich source of calcium, soy isoflavones may also inhibit bone resorption and stimulate bone formation. Isoflavones have been shown to inhibit osteoclast activity (bone resorption cells) by increasing TGFβ production [160] and inhibiting tyrosine kinase activity [130]. Furthermore, isoflavones increase osteoblast activity (bone formation cells) by stimulating IGF-1 production and osteoblast differentiation [161].

Soy isoflavones are thought to reduce the risk of breast cancer when consumed prepubertally and throughout life [162 – 164] which is beneficial for the premenopausal woman. The estrogenic activity induces terminal end bud differentiation leading to inhibited proliferation [162 – 164]. It appears that soy consumption may also reduce circulating levels of estrogen and increase menstrual cycle length which translates into a lower lifetime exposure to estrogens [130, 165 – 169]. Decreased exposure to estrogens throughout life may reduce the risk of hormone-related disease [170].

Soy isoflavones may also stimulate cognitive function, particularly in women [171]. Although the exact mechanisms remain elusive, improved cognitive function has been attributed to both the estrogenic activity of soy isoflavones and the tyrosine kinase inhibitory activity of genistein [172].

2.6 Soy and Prostate Cancer

Differences in clinically relevant PCa incidence and mortality between Asian countries and western countries have been observed as early as 1957 [173]. Evidence that diet influences PCa risk has been observed since 1966 [174]. However, inverse associations between soy consumption and PCa risk were not hypothesized until 1990 [6]. Adlercreutz et al. [175] first hypothesized that the high concentration of isoflavones in soy [176, 177] may lead to increased isoflavone concentrations excreted in the urine of persons consuming a long term diet rich in soy. Based on previous epidemiological studies showing inverse relationships between soy, beans, and lentils and PCa risk [178, 179] Adlercreutz et al. [6] hypothesized that soy may reduce the risk of clinically relevant PCa due to the abundance of isoflavones present in soy [180].

2.6.1 Epidemiological evidence

Two ecological studies have been conducted to test the correlation between soy consumption and PCa risk. Ecological studies examine relationships between factors and disease risk on a population basis. The first study examined the relationship between soy product intake and multiple types of cancers and CVD among a Japanese cohort [181]. This study found no correlation between PCa risk and soy consumption. However, there appeared to be no significant difference between the amount of soy consumed between so called “soy consumers” and “non soy consumers”. The second ecological study examined the association between PCa mortality and environmental factors such as diet, tobacco use, and socioeconomic status [182]. This study found a significant inverse

association between soy consumption and PCa. The discrepancy between these 2 studies can be attributed to a myriad of variables including differences in cohort and study objective.

Case-control studies are another type of epidemiologic study which retrospectively examines relationships between factors such as diet and disease prevalence at an individual level. Nine case control studies have examined the relationship between PCa prevalence and soy consumption. Five of 9 case-control studies showed a decrease in PCa risk with increased soy consumption [183 – 187]. One case-control study found that legume intake was significantly inversely associated with PCa risk but soy was not specifically shown to be more protective than other legumes [188]. In contrast, 3 of the 9 case-control studies showed no significant protective effect. Two of these studies examined the protective effect of specific soy foods such as miso [189] and soymilk [190] but did not examine the health benefits of other types of soy products. The final case control study examined the American population and found that soy did not offer protection against PCa prevalence however the authors recognized that soy intake was reported to be much lower than that of Asian populations [191].

Prospective studies follow individuals forward through time, analyzing specific factors such as diet, and relating these factors to specific disease incidence. Three of 6 prospective trials showed a decrease in PCa incidence upon consumption of tofu [179], soy milk [192], and genistein, daidzein, miso soup, and soy foods [193]. Interestingly Kurahashi et al. [193] found that soy foods may protect against localized PCa but not advanced stage disease. Only 1 study showed no significant association between soy

consumption and PCa incidence among Japanese Americans [194]. This study however specifically examined tofu consumption and no other dietary sources of soy. Two of the 6 studies found inverse correlations between legume consumption and PCa incidence but was not specifically analyzed for soy consumption [178, 195]

2.6.2 *In vitro* and *in vivo* evidence

Many studies have investigated the anticancer properties of individual isoflavones using both *in vitro* and *in vivo* PCa models. Several studies suggest that soy isoflavones decrease the expression of AR [196 – 198] which may explain, in part, for the reduction in the expression of PSA observed upon isoflavone treatment [199]. A reduction in the expression of AR may be due to the affinity that isoflavones have for ERβ as ERβ activation downregulates AR. Furthermore, prostate weight appears to significantly decrease in rats fed an isoflavone rich diet [196, 200, 201]. This observed response may be a consequence, in part, via reduced AR expression. Furthermore, the isoflavone equol appears to bind DHT with high affinity, thereby decreasing the bioactivity of DHT *in vivo* [191]. The latter study describes yet another mechanism by which isoflavones reduce the bioactivity of androgens *in vivo*.

Several *in vitro* studies using PCa cell models support an anticancer effect of soy isoflavones. Akiyama et al. [202] was the first to clearly demonstrate that genistein inhibits tyrosine kinase activity, offering a potential therapeutic target for all cancers given that aberrant tyrosine kinase activity is observed in many cancers. Since this initial observation, genistein has also been shown to inhibit topoisomerase [202], induce

apoptosis [203], and increase antioxidant activity [204] *in vitro*. Several studies have shown that isoflavones, primarily genistein and daidzein, reduce cellular proliferation in a number of prostate cancer cell models such as LNCaP, DU145, and PC-3 [14, 203, 205 – 211]. Interestingly genistein appears to induce a G_2/M cell cycle arrest [203, 208, 210] whereas daidzein and equol induce a G_0/G_1 cell cycle arrest [208]. These studies suggest that individual isoflavones inhibit proliferation via different mechanisms.

Genistein has also been shown to reduce tumor size and number in many *in vivo* studies [212 – 217]. The reduction in tumor size *in vivo* is attributed to in part to a decrease in cellular proliferation, an increase in apoptosis, a decrease in microvessel density, and a decrease in circulating concentrations of IGF-1 [213].

Isoflavones have been shown to modulate the MAPK signaling pathway in PCa cells and animal models. Superphysiologic concentrations of gensitein inhibit ERK1/2 signaling whereas physiologic concentrations of genistein induce ERK1/2 activation [14, 211, 218, 219]. As mentioned previously, the ERK1/2 signaling cascade is downregulated during PCa progression. Upregulation of this signaling cascade may be beneficial for the treatment of PCa.

Animal studies have shown that the timing of exposure to many chemopreventive agents is critical for preventing or reducing tumor number. Exposure to the soy isoflavone genistein during prepubertal and adult life in the female Sprague-Dawley rat treated with dimethylbenz[a]anthracene was most effective for reducing tumor number in

this breast cancer model [162 – 164]. However, in the prostate, genistein is most effective at preventing poorly differentiated tumors but only when exposed throughout life [220].

2.6.3 Clinical Evidence

Studies suggest that the soy isoflavones daidzien and equol selectively accumulate in the prostate, reaching concentrations approximately four to thirteen times greater than in serum [144, 145] whereas the more abundant isoflavone genistein does not significantly accumulate in this tissue. To date, the concentration of glycitein in both serum and prostatitic fluid has not been reported. Nonetheless, the observation that soy isoflavones selectively accumulate in the prostate provides additional support that these compounds may have anticancer activity in this tissue.

Several clinical trials have been conducted assessing the efficacy of soy on specific markers of PCa prevention and treatment. Four clinical studies examined the effects of a soy isoflavone supplement [221, 222] or soy food [223, 224] on circulating levels of PSA in either healthy men or men diagnosed with PCa. A significant decrease, decreasing trend, or stabilization in PSA levels were found in men diagnosed with PCa who were consuming either an isoflavone supplement or soy food [221, 224]. Maskarinec et al. [223] found that circulating PSA levels significantly decreased in men without PCa that were consuming a diet rich in soy. However, Adams et al. [222] found no significant change in PSA levels in men provided with an isoflavone supplement. The differences in outcomes between the latter 2 studies may be attributed to study objective

and study design. Adams et al. [222] utilized an existing cohort of men at risk for colon cancer and therefore the study design was not focused on markers for PCa risk. There was no data on prostate health of the men enrolled in the study. Therefore, there were no inclusion or exclusion criteria specific for prostate health for the subjects in this study [222].

Circulating levels of testosterone and other hormones were also measured in four clinical trials. Three out of the 4 trials showed no change in circulating testosterone levels after soy supplementation [221, 223, 225] whereas one study showed a significant decrease in serum testosterone levels [226]. Discrepancies between the latter study and the former studies may be attributed to differences in amount of isoflavones given per day and the isoflavone profile of the supplement. Van Velhuizen et al. [226] reported supplementing patients with 112 – 224mg isoflavones per day. The lowest dose given exceeded the highest does of isoflavone given in the three trials reporting serum testosterone levels. Furthermore, the isoflavone profile of the supplement was not reflective of an isoflavone profile found in soy foods [226]. The difference in isoflavone profile and large quantity of isoflavones may have contributed to the discrepancies between studies.

Although the majority of studies appear to show no significant decrease in serum testosterone levels, evidence suggest that AR expression significantly decreases upon long term exposure to isoflavones [225]. This suggests that although testosterone levels remain unaffected, the bioactivity of androgens may significantly decrease due to a reduction in receptor availability.

Recently, a study was conducted to determine the effects of a dietary supplement containing vitamin E, selenium, and soy isoflavones on PCa progression in men diagnosed with isolated HGPIN [227]. To date, this is the only study to use this precancerous cohort to test the hypothesis that a dietary supplement may affect PCa incidence. Interestingly, this study found that 24% of the subjects had no detectable HGPIN or PCa after 3 months and 18% after a 6 month repeat biopsy. Although this study is unable to identify which compounds in the supplement may have reversed HGPIN, these data provide support for the hypothesis that soy isoflavones may prevent PCa incidence during early stages of PCa development.

2.7 Summary

The relatively high prevalence and mortality rate of PCa in the US has prompted PCa to be the most studied cancer with regards to prevention and treatment in the year 2007. The prostate carcinogenic process occurs from the second decade of life until diagnosis in the sixth or seventh decade of life. This long latency period of development allows for multiple intervention points. The many studies described herein illustrate that dietary intervention may prevent, delay and/or aid in the treatment of PCa. However, most of these studies utilized a preexisting PCa model. Little is known about the effects of dietary intervention during earlier stages during the prostate carcinogenic process. The following chapters aim to identify the anticancer properties of soy isoflavones in a nontumorigenic prostate epithelial cell line. These studies will offer insight into the mechanisms by which soy isoflavones prevent processes associated with tumorigenesis.

CHAPTER 3

GLYCITEIN ACTIVATES EXTRACELLUAR SIGNAL-REGULATED KINASE (ERK1/2) VIA VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR (VEGFR) SIGNALING IN NONTUMORIGENIC (RWPE-1) PROSTATE EPITHELIAL CELLS

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3.1 Abstract

Increased consumption of soy is associated with a decreased risk for PCa; however the specific cellular mechanisms responsible for this anticancer activity are unknown. Dietary modulation of signaling cascades controlling cellular growth, proliferation and differentiation has emerged as a potential chemopreventative mechanism. The present study examined the effects of four soy isoflavones (genistein, daidzein, glycitein, and equol) on extracellular signal-regulated kinase (ERK1/2) activity in a nontumorigenic prostate epithelial cell line (RWPE-1). All four isoflavones (10µmol/L) significantly increased ERK1/2 activity in RWPE-1 cells as determined by immunoblotting. Isoflavone-induced ERK1/2 activation was rapid and sustained for approximately 2 h post-treatment. Glycitein, the most potent activator of ERK1/2,

decreased RWPE-1 cell proliferation by 40% ($p<0.01$). Glycitein-induced ERK1/2 activation was dependent, in part, on tyrosine kinase activity associated with the vascular endothelial growth factor receptor (VEGFR). The presence of both VEGFR1 and VEGFR2 in the RWPE-1 cell line was confirmed by immunocytochemistry. Treatment of RWPE-1 cells with $VEGF_{165}$ resulted in transient ERK 1/2 activation and increased cellular proliferation. The ability of isoflavones to modulate the ERK 1/2 signaling cascade via VEGFR signaling in the prostate may be responsible, in part, for the anticancer activity of soy.

3.2 Introduction

PCa is the second leading cause of cancer related deaths in the United States [228]. Environmental factors, including diet, can influence the development of this disease. Both epidemiological and experimental evidence suggest that increased consumption of soy and soy-based foods is associated with a decreased risk for PCa [185, 188, 229, 230]. Soy isoflavones are thought to be responsible, in part, for this observed protective effect.

Soy isoflavones (Fig. 3.1) are a family of nonnutritive compounds that can modulate a variety of biological processes associated with carcinogenesis. The three most abundant isoflavones, genistein, daidzein, and glycitein, comprise approximately 50, 40, and 10% of the total soybean isoflavone profile, respectively. The majority of studies examining the anticancer effects of soy have focused on genistein. The anticancer effects of genistein include tyrosine kinase inhibition [202], topoisomerase inhibition

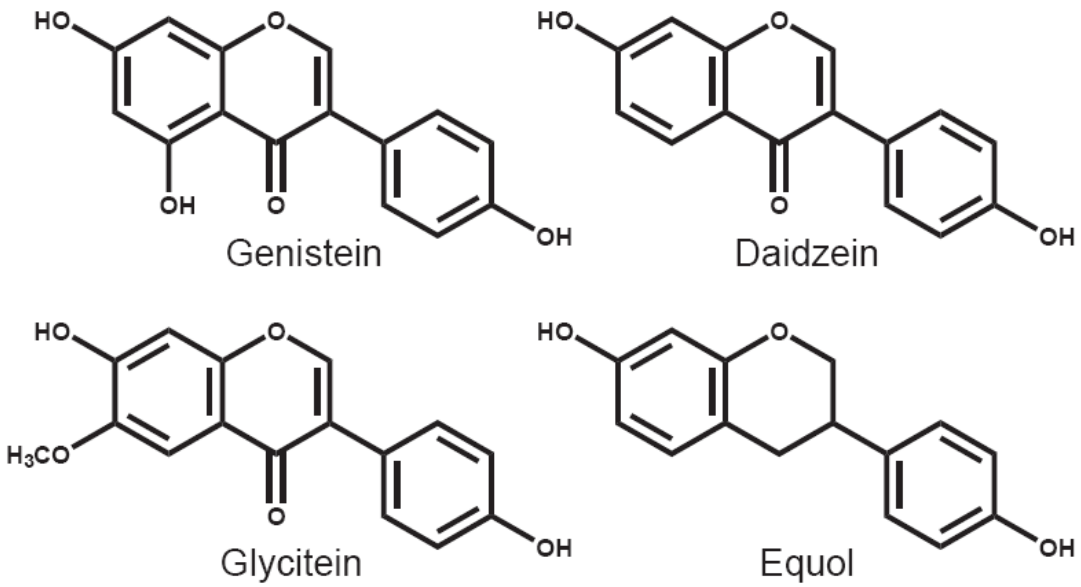

Figure 3.1: Chemical structures of the principle soy isoflavones genistein, daidzein, glycitein, and the daidzein metabolite, equol.

[202], induction of cell cycle arrest [208, 209, 231], apoptosis [203], and increased antioxidant activity [204]. Genistein can also modulate growth factor signaling in transgenic adenocarcinoma mouse prostate model (TRAMP) [218] and reduce the incidence and number of poorly differentiated tumors *in vivo* [164, 212]. The biological effects of genistein as well as daidzein appear to be primarily mediated via sex hormone (i.e. androgen and estrogen) signaling [14, 201, 232 – 235]. However, the specific cellular and molecular mechanisms by which isoflavones exert their anticancer activites are not well characterized.

Dietary and chemotherapeutic modulation of signal transduction cascades controlling cellular proliferation, apoptosis, differentiation, and cell cycle progression has emerged as a potential therapeutic target for treatment and prevention of PCa. The extracellular signal-regulated kinase (ERK1/2) cascade is a member of the mitogen-activated protein kinase (MAPK) family and is required for the normal growth, development and survival of the prostate epithelium [9]. A number of recent studies demonstrate that isoflavones, specifically genistein, modulate ERK1/2 activity in tumorigenic prostate cell lines [14, 198, 210]. The present study examined the effect of four soy isoflavones (genistein, daidzein, equol, and glycitein) on ERK1/2 signaling in nontumorigenic prostate epithelial cells.

3.3 Methods and materials

3.3.1 Materials

Genistein, daidzein, glycitein, equol, and the MEK inhibitor, U0126, were obtained from LC Laboratories (Woburn, MA). Specific chemical inhibitors of tyrosine kinase activity for the following proteins, insulin-like growth factor receptor (IGFR), tumor growth factor β receptor (TGFRβ), steroid receptor coactivator (Src), vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), and platelet derived growth factor receptor (PDGFR), were purchased from EMD Biosciences (LaJolla, CA). The above compounds were prepared as stock solutions in dimethyl sulfoxide (DMSO) and were utilized in cell culture media at appropriate dilutions with a final DMSO concentration of 0.1%. The antiandrogen hydroxyflutamide (HF) (Toronto Research Chemicals, North York, ON) and the antiestrogen ICI 182,780 (Tocris, Ballwin, MO) were dissolved in ethanol and diluted to a final ethanol concentration of 0.1%. Recombinant human $VEGF_{165}$ (R&D systems, Inc. Minneapolis, MN) was dissolved in phosphate buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA). Bicinchoninic acid (BCA) ™ protein assay kit and superSignal ECL™ chemiluminescent substrate kit were obtained from Pierce (Rockford, IL). ECL western blotting detection reagent was obtained from Amersham Biosciences Corporation (Piscataway, NJ). Polyclonal antibodies for phospho- ERK1/2 kinase and total ERK1/2 kinase were obtained from Cell Signaling-Technology (Beverly, MA). Polyclonal antibodies for vascular endothelial growth factor receptor (VEGFR) 1 and 2

were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 3-[4,5- dimethylthiazol-2-yl]-2-,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO) The vectastain® ABC kit was obtained from Vector Labs (Burlingame, CA)

3.3.2 Cell culture

The nontumorigenic human prostate epithelial cell line (RWPE-1) and the metastasized human PCa line (PC-3) were purchased from the American Type Culture Collection (Rockville, MD). RWPE-1 cells were maintained in keratinocyte serum-free medium (KSFM) (GIBCO Laboratories, Grand Island, NY) supplemented with 50mg/L bovine pituitary extract, 5% L-glutamine and 5μg/L epidermal growth factor (EGF). The PC-3 cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The human umbilical vein endothelial cell line (HUVEC) was provided by Dr. Jim Waldman (Department of Pathology, The Ohio State University) and maintained in endothelial cell growth medium consisting of M-199 media, 20% FBS, HEPES buffer (1mol/L), 0.6% bovine brain extract supplemented with heparin, 0.2% penicillin/streptomyosin, and 0.1% fungizone. All cell lines were maintained in a humidified incubator (5% CO_2, 95% O_2) at 37°C.

3.3.3 Immunoblot detection of active and total ERK1/2

RWPE-1 and PC-3 cells were grown in 60mm dishes for 48 h (90% confluent). Growth media was removed and cells were washed with PBS and incubated in

supplement free media for 24 h. Cells were then treated with isoflavones alone or in combination with specific inhibitors (U0126, HF, ICI 182, 780, and chemical tyrosine kinase inhibitors (IGFR, TGFβ, Src, VEGFR, EGFR, and PDGFR)) at appropriate times and concentrations. Following treatments, growth medium was removed and cells were washed with PBS. Crude proteins were isolated and separated on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed using primary antibodies that recognize active (phospho-) and total ERK1/2 at recommended dilutions in TTBS (1 x TBS with 0.1% Tween-20) overnight at 4°C. Following incubation with secondary antibodies, protein signals were visualized on autoradiography film using ECL, and quantified by densitometry using Scion imaging software (Frederick, Maryland). Basal levels of protein expression are given as 100%, and twice this level is reported as 200% of the control.

3.3.4 Immunocytochemical detection of VEGFR1 and VEGFR2

RWPE-1 and HUVEC cells were grown in 6-well plates until 50% confluent. Growth medium was removed and cells were fixed with 4% paraformaldehyde at 4°C and permeabilized with 0.2% Triton-100X. Plates were then blocked with 5.5% normal goat serum, and incubated with primary antibodies overnight at 4°C (1:50 anti-VEGFR1 and anti-VEGFR2). After incubation, samples were incubated with hydrogen peroxide (0.6%) to quench any endogenous peroxidase activity prior to secondary antibody (1:500). Signals were detected with the avidin biotin complex (ABC) and diaminobenzidine (DAB) reagent.

3.3.5 Cellular proliferation

RWPE-1 cells were plated with KSFM with supplements in 48-well plates at an initial density of 1.0 x 10^4 cells per well. Cells were treated with or without glycitein (50μmol/L) or $VEGF_{165}$ (50 μg/L) alone or in combination with U0126 (10μmol/L) and incubated for an additional 72 h. After incubation, cell proliferation was determined by the MTT assay as described previously [14]. Changes in cellular proliferation are expressed as a percent of control (given as 100%).

3.3.6 Statistical Analyses

Statistical significance between groups was determined by one-way analysis of variance (ANOVA) with Tukey's post-hoc comparisons (SigmaStat software; Chicago, IL). Data are presented as percentage of means ± relative standard error (R.S.E.), with α level of $p<0.05$.

3.4 Results

3.4.1 Isoflavones induce ERK1/2 activity in RWPE-1 cells but not PC-3 cells

Isoflavone-induced cytotoxicity was not observed under any experimental conditions (data not shown). Treatment of RWPE-1 cells with daidzein, equol, and glycitein (10 and 50μmol/L, 2 h) resulted in a concentration-dependent activation of ERK1/2 activity (Fig. 3.2A, B). As previously observed [14], treatment of RWPE-1 cells with genistein (2 h) at 10μmol/L activated ERK1/2, while 50μmol/L genistein inhibited

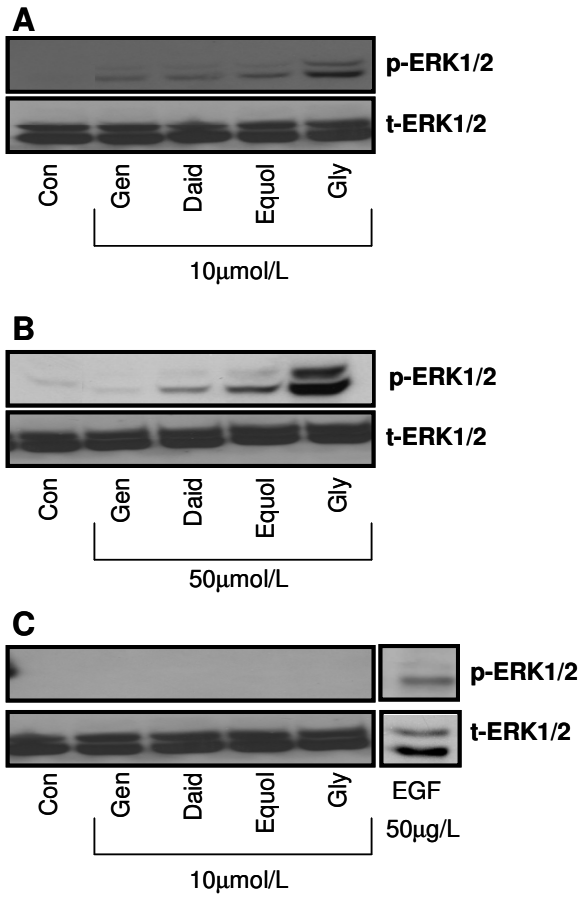

Figure 3.2: Effect of isoflavones on ERK1/2 activity in non-tumorigenic (RWPE-1) and tumorigenic (PC-3) prostate cells. Active ERK1/2 (p-ERK1/2) was measured by immunoblot in RWPE-1 cells following 2 h treatment with either (A) 10μmol/L or (B) 50μmol/L genistein (Gen), daidzein (Daid), equol, glycitein (Gly) or vehicle alone (Con). (C) Active ERK1/2 (p-ERK1/2) was measured by immunoblot in PC-3 cells following 2 h treatment with 10μmol/L genistein (Gen), daidzein (Daid), equol, glycitein (Gly), vehicle alone (Con), or 10 min treatment with 50μg/L epidermal growth factor (EGF). All experiments were performed independently at least 3 times with an n=3/experiment. Total ERK1/2 (t-ERK1/2) was used as the loading control.

ERK1/2. Glycitein was the most active ERK1/2 inducer at both 10 and 50µmol/L (Figure 3.2A, B). Treatment of PC-3 cells with genistein, daidzein, equol, and glycitein (10µmol/L) did not activate ERK1/2 (Fig. 3.2C). No changes in total ERK1/2 (t-ERK1/2) were observed with any treatment. The mechanism by which glycitein activates ERK1/2 was further characterized in the RWPE-1 cell line.

3.4.2 Hormone independent ERK1/2 activation

We have previously shown that genistein induces ERK1/2 activity via an estrogen receptor mediated mechanism in the RWPE-1 cell line [14]. In order to determine if glycitein-induced ERK1/2 activity was also mediated via the estrogen receptor, RWPE-1 cells were treated with glycitein alone (50µmol/L, 2 h) or in combination with the antiestrogen ICI 182, 780 (10µmol/L, 1 h pretreatment) (Fig. 3.3A). Glycitein treatment alone induced ERK1/2 activity, while ICI 182, 780 treatment alone inhibited basal activity. Pretreatment with ICI 182, 780 did not inhibit glycitein-induced ERK1/2 activity.

In order to determine if glycitein-induced ERK1/2 activity was mediated via the androgen receptor, RWPE-1 cells were treated with glycitein alone (50µmol/L, 2 h) or in combination with the antiandrogen hydroxyflutamide (HF) (10µmol/L, 1 h pretreatment) (Fig. 3.3B). Glycitein treatment alone significantly induced ERK1/2 activity ($p<0.01$), while HF pretreatment did not alter basal ERK1/2 activity. Pretreatment with HF did not inhibit glycitein-induced ERK1/2 activation.

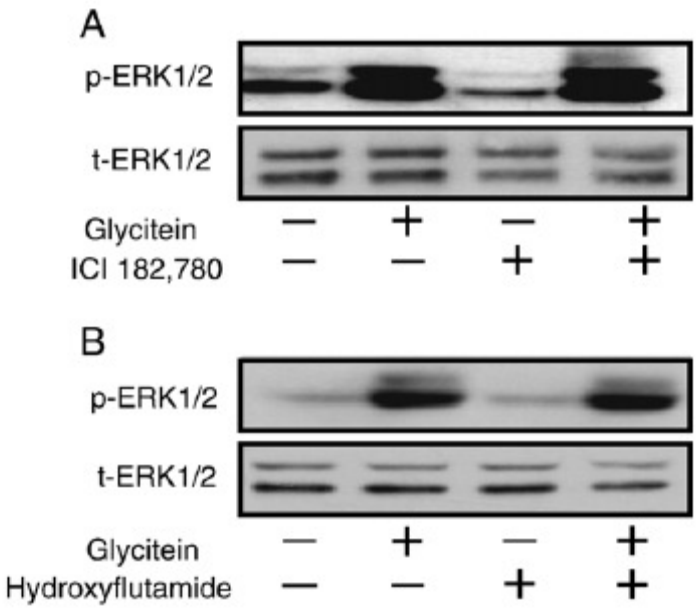

Figure 3.3: Glycitein-induced ERK1/2 activity in RWPE-1 cells is independent of estrogen and androgen receptor activity. Active ERK1/2 (p-ERK1/2) was measured by immunoblot in RWPE-1 cells following treatment with or without (A) ICI 182, 780 (antiestrogen) or (B) hydroxyflutamide (antiandrogen) (10µmol/L, 1 h) prior to glycitein treatment (50µmol/L, 2 h). Data are given as a representative immunoblot. All experiments were performed independently at least 3 times with an n=3/experiment. Total ERK1/2 (t-ERK1/2) was used as the loading control.

3.4.3 Glycitein activates ERK1/2 via a VEFGR-dependent mechanism

To identify the mechanism by which glycitein activates ERK1/2, RWPE-1 cells were pretreated (1h) with several chemical tyrosine kinase inhibitors (IGFR, TGFβ, Src, VEGFR, EGFR, and PDGFR) prior to glycitein (50μmol/L, 2 h) treatment. Of those tested, only the VEGFR tyrosine kinase inhibitor specifically inhibited glycitein-induced ERK1/2 activation ($p<0.01$) (Fig. 3.4A).

Immunocytochemical analysis for VEGFR1 and VEGFR2 is given in Fig. 3.4B. VEGFR1 and VEGFR2 expression was detected in both HUVEC (positive control) and RWPE-1 cells. Samples receiving no VEGFR primary antibody served as the negative control.

Glycitein and $VEGF_{165}$-induced ERK1/2 activity in RWPE-1 cells were both concentration and time dependent (Fig. 3.5A-D). Glycitein-induced ERK1/2 activation was observed at concentrations ranging from 0.01μmol/L to 100μmol/L (Fig. 3.5A). ERK1/2 activity occurred within 10 min and remained active 2 h post treatment. ERK1/2 activity fell below basal levels within 6 h post treatment (Fig. 3.5B). $VEGF_{165}$-induced ERK1/2 activation was observed at concentrations ranging from 10ng/L - 100 μg/L (Figure 3.5C). $VEGF_{165}$-induced ERK1/2 activation peaked within 10 min (Fig. 3.5D) and returned to basal levels within 30 min (data not shown).

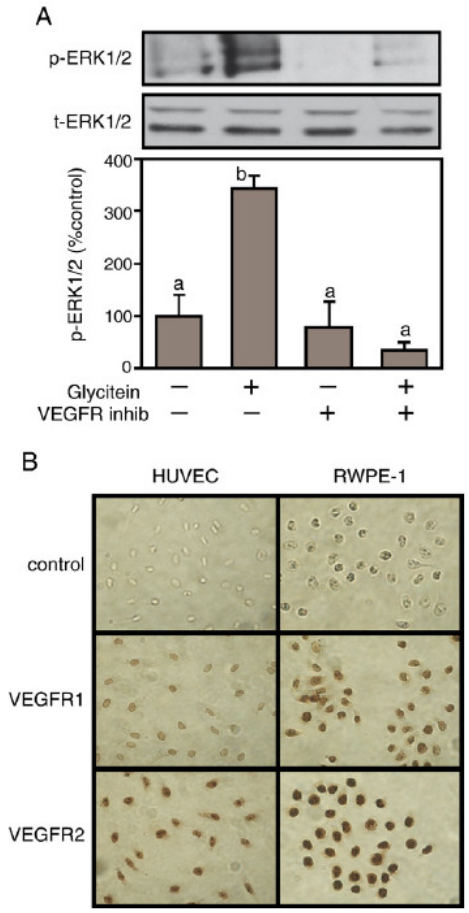

Figure 3.4: Glycitein-induced ERK1/2 activity in RWPE-1 cells is dependent on VEGFR signaling. (A) Active ERK1/2 (p-ERK1/2) was measured by immunoblot in RWPE-1 cells following treatment with or without VEGFR tyrosine kinase inhibitor (0.10µmol/L, 1 h) prior to glycitein (50µmol/L, 2 h). All experiments were performed independently at least 3 times with an n=3/experiment. Data are given by percent of means ± R.S.E.. Mean values not sharing common letter superscript differ significantly ($p<0.05$). Total ERK1/2 (t-ERK1/2) was used as the loading control. (B) Immunocytochemical identification of VEGFR1 and 2 in RWPE-1 cells. Human endothelial vein umbilical cord (HUVEC) cell line served as the positive control for VEGFR1 and 2. Control treatments did not receive primary antibody.

4.4.4 Concentration and time dependent induction of ERK1/2 activity by glycitein and $VEGF_{165}$

Glycitein and $VEGF_{165}$-induced ERK1/2 activity in RWPE-1 cells were both concentration and time dependent (Fig. 5A-D). Glycitein-induced ERK1/2 activation was observed at concentrations ranging from 0.01μmol/L to 100μmol/L (Fig. 5A). ERK1/2 activity occurred within 10 min and remained active 2 h post treatment. ERK1/2 activity fell below basal levels within 6 h post treatment (Fig. 5B). $VEGF_{165}$-induced ERK1/2 activation was observed at concentrations ranging from 10ng/L - 100 μg/L (Figure 5C). $VEGF_{165}$-induced ERK1/2 activation peaked within 10 min (Fig. 5D) and returned to basal levels within 30 min (data not shown).

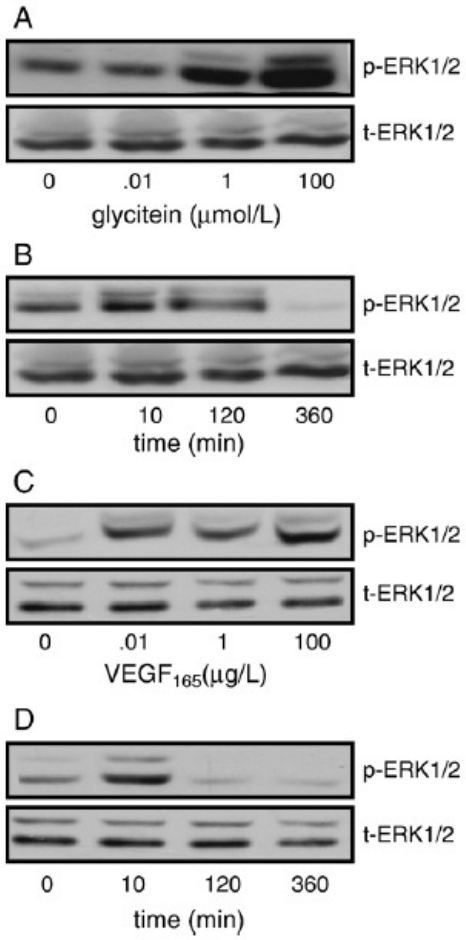

Figure 3.5: Concentration and time-dependent induction of ERK1/2 activity by glycitein and $VEGF_{165}$ in RWPE-1 cells. (A) Active ERK1/2 (p-ERK1/2) was measured by immunoblot in RWPE-1 cells following 2 h treatment with glycitein (0-100μmol/L). (B) Time dependent (0-360 min) activation of ERK1/2 following treatment of cells with 50μmol/L glycitein. (C) Active ERK1/2 (p-ERK1/2) was measured by immunoblot in RWPE-1 cells following 10 min treatment with $VEGF_{165}$ (0-100μg/L). (D) Time dependent (0-360 min) activation of ERK1/2 following treatment of cells with 50μg/L $VEGF_{165}$. All experiments were performed independently at least 3 times with an n=3/experiment. Total ERK1/2 (t-ERK1/2) was used as the loading control.

3.4.5 Effect of $VEGF_{165}$ and glycitein on cellular proliferation

The effects of $VEGF_{165}$ and glycitein induced ERK1/2 activity on RWPE-1 cellular proliferation was determined using the MEK inhibitor, U0126. Treatment of RWPE-1 cells with glycitein (50µmol/L) or U0126 (10µmol/L), reduced cell proliferation by 40% and 45%, respectively ($p<0.01$), 72 h post treatment (Fig. 3.6A, left panel). When treated in combination, glycitein (50µmol/L) and U0126 (10µmol/L) reduced RWPE-1 cell proliferation by 50%, 72 h post treatment ($p<0.01$).

ERK1/2 activation in RWPE-1 cells following treatment with glycitein (50µmol/L, 2 h) and U0126 (10µmol/L, 1 h pretreatment) alone or in combination is given in Fig. 3.6A, right panel. Glycitein treatment alone increased ERK1/2 activity while pretreatment with U0126 inhibited both basal and glycitein-induced ERK1/2 activation.

The effect of $VEGF_{165}$ on RWPE-1 cellular proliferation is given in Fig. 3.6B, left panel. Treatment of RWPE-1 cells with $VEGF_{165}$ (50 µg/L) increased cell proliferation by 23%, while treatment with U0126 (10µmol/L) reduced cell proliferation by 30%, 72 h post treatment ($p<0.01$). Treatment of RWPE-1 cells with $VEGF_{165}$ (50 µg/L) in combination with U0126 (10µmol/L) inhibited cellular proliferation by 25%, compared to controls receiving vehicle alone ($p<0.01$).

ERK1/2 activation in RWPE-1 cells following treatment with $VEGF_{165}$ (50µg/L, 10 min) and U0126 (10µmol/L, 1 h) alone or in combination is given in Fig. 3.6B, right panel. $VEGF_{165}$ treatment alone increased ERK1/2 activity while pretreatment with U0126 inhibited both basal and $VEGF_{165}$-induced ERK1/2 activation.

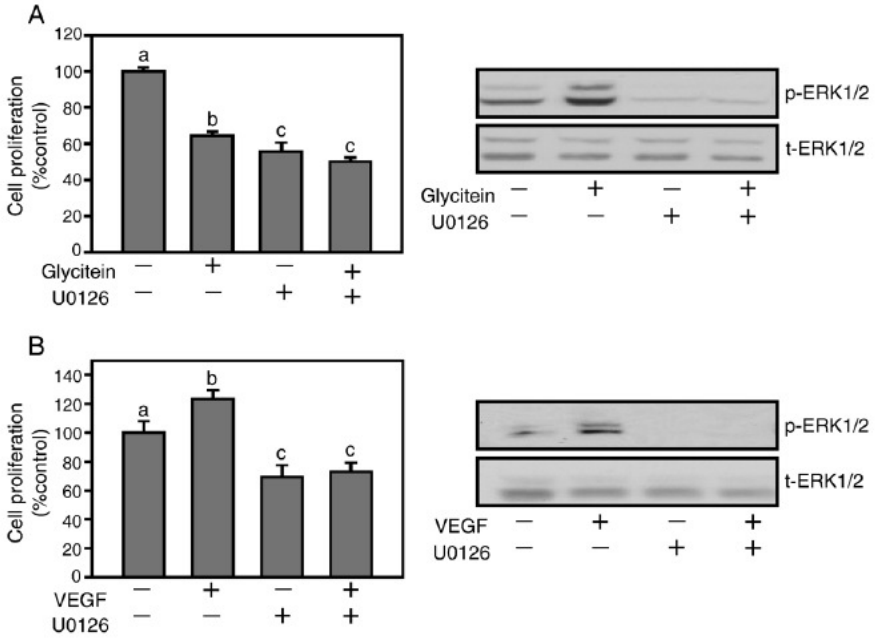

Figure 3.6: Effect of glycitein and $VEGF_{165}$-induced ERK1/2 activation on RWPE-1 proliferation. (A, left) RWPE-1 cells were treated with glycitein (50µmol/L) and U0126 (10µmol/L) alone and in combination for 72 h and proliferation was assessed by MTT assay. (A, right) Active ERK1/2 (p-ERK1/2) was measured by immunoblot in RWPE-1 cells following treatment with or without U0126 (10µmol/L, 1 h) prior to glycitein (50µmol/L, 2 h). (B, left) RWPE-1 cells were treated with $VEGF_{165}$ (50µg/L) and U0126 (10µmol/L) alone and in combination for 72 h and proliferation was assessed by MTT assay. (B, right) Active ERK1/2 (p-ERK1/2) was measured by immunoblot in RWPE-1 cells following treatment with or without VEGFR tyrosine kinase inhibitor (10µmol/L, 1 h) prior to $VEGF_{165}$ (50 µg/L, 10 min). Total ERK1/2 (t-ERK1/2) was used as the loading control. All immunoblot and proliferation experiments were performed independently at least 3 times with an n=3-6/experiment. Data are given by percentage of means ± R.S.E.. Mean values not sharing common letter superscript differ significantly ($p<0.05$).

3.5 Discussion

Total soy isoflavone concentrations in prostatic fluid can reach up to 50μmol/L in persons consuming a soy rich diet [144, 145]. Isoflavones in prostatic fluid are in direct contact with the prostate epithelium, suggesting a potential role for these compounds to modulate epithelial cellular function. The ERK1/2 signaling cascade is necessary, in part, for the survival, growth, and development of the normal prostate epithelium [9]. However, the role of ERK1/2 signaling in prostate carcinogenesis is unclear [9, 10, 13, 87, 88, 236, 237]. Both increased and decreased levels of ERK1/2 activity have been observed with PCa development. We have previously shown that genistein increases ERK1/2 activity and cellular proliferation at physiological concentrations (<12μmol/L) in RWPE-1 cells, while higher concentrations (>12.5μmol/L) decreased ERK1/2 signaling and proliferation of this cell line [14].

Although genistein is the most abundant isoflavone found in soy, it has relatively low concentrations in prostatic fluid (≤1μmol/L) [144, 145]. The isoflavones daidzein and equol have been shown to selectively accumulate in prostatic fluid to a greater extent than genistein, suggesting a potential for greater biological and/or anticancer activity. The concentration of glycitein in prostatic fluid has not been reported. To date, the anticancer activities of isoflavones other than genistein have not been extensively characterized in the prostate. The present study examined the effects of the major and minor soy isoflavones on ERK1/2 signaling and cellular proliferation in nontumorigenic prostate epithelial cells.

Of the isoflavones tested, glycitein-induced ERK1/2 activation in RWPE-1 cells was the most robust. Biological effects of isoflavones are mediated, in part, via modulation of sex hormone (i.e. estrogen) signaling [14, 201, 232 – 235]; however, glycitein-induced ERK1/2 activation was independent of estrogen and androgen receptor signaling. The low estrogenic activity of glycitein is attributed, in part, to the 6-methoxy group interfering with the estrogenic functionality of the 7-hydroxyl group on this isoflavone [238].

Activation of receptor and non-receptor tyrosine kinases is an early initiating event in the activation of multiple cellular signaling pathways, including the ERK1/2 cascade. Genistein is a known inhibitor of tyrosine kinase activity and has been previously shown to inhibit ERK1/2 via this mechanism. However, the effects of other isoflavones on tyrosine kinase and ERK1/2 activation have not been well characterized. We hypothesized that the effects of glycitein on ERK1/2 activity may involve a tyrosine kinase-dependent mechanism. To determine the potential involvement of tyrosine kinases in glycitein-induced ERK1/2 activation, several chemical inhibitors of receptor tyrosine kinase (RTK) and non-RTK activity were utilized. Of those inhibitors tested, only the VEGFR tyrosine kinase inhibitor blocked glycitein-induced ERK1/2 activation. VEGFRs are predominantly expressed in endothelial cell types; however, more recently this family of receptors has been characterized in intestinal epithelial cells, keratinocytes [239, 240] and a variety of cancer cells [241 – 248]. The inability of soy isoflavones to activate ERK1/2 in the PC-3 cell line may be due, in part, to the absence of VEGFRs in this cell line [246, 248, 249].

Activation and signaling via VEGFRs control a host of cellular functions including proliferation, differentiation and vascular permeability. ERK1/2 activation has been shown to mediate many of these VEGFR-associated cellular effects [250]. Data presented in this study demonstrate the presence of both VEGFR isoforms 1 and 2 in the RWPE-1 cell line. In addition, two lines of evidence suggest the functionality of the VEGFR in the RWPE-1 cell line: First, the natural ligand for VEGFR, $VEGF_{165}$, significantly increased RWPE-1 proliferation and second, $VEGF_{165}$-induced cell proliferation was ERK1/2 dependent (Fig. 3.6B). Interestingly, glycitein-induced ERK1/2 was associated with decreased RWPE-1 cell proliferation. We hypothesize that the differential effects of $VEGF_{165}$ and glycitein on cellular proliferation may be explained, in part, by the duration of ERK1/2 activity elicited by each of these compounds. Transient activation of ERK1/2 is associated with increased cellular proliferation, whereas sustained activation of this kinase can lead to increased cellular differentiation [89]. The effects of soy isoflavones on sustained ERK1/2 activation and prostate differentiation have not been examined in vivo. However, the accumulation of isoflavones in prostatitc fluid following long term dietary soy consumption [145] suggest a potential role for these compounds in modulating cell signaling, proliferation and differentiation in the prostate.

The present study demonstrates that glycitein induces sustained ERK1/2 activation at concentrations ranging from 0.01-100μmol/L in RWPE-1 cells. This activation was dependent upon VEGFR signaling, and resulted in decreased cellular

proliferation. These biological effects may contribute, in part, to the anticancer activity of soy in the prostate. The influence of soy isoflavones on prostate epithelial differentiation is currently under investigation in this laboratory.

3.6 Acknowledgements

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CHAPTER 4

BASAL CELL INDUCED DIFFERENTIATION OF NONCANCEROUS PROSTATE EPITHELIAL CELLS (RWPE-1) BY GLYCITEIN

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4.1 Abstract

Increased consumption of soy and soy isoflavones is associated with a reduced risk for prostate cancer (PCa). PCa progression is characterized, in part, by a loss of luminal/basal epithelial differentiation; however, the effects of soy isoflavones on cellular differentiation in the prostate are unknown. The present study examined the effects of the soy isoflavone glycitein on cellular differentiation in prostate epithelial cells (RWPE-1, WPE1-NB14, RWPE-2). Glycitein significantly inhibited RWPE-1 cellular proliferation at concentrations ranging from 0.4 - 50μmol/L. Expression of the luminal epithelial cell marker cytokeratin 18 was not affected by glycitein treatment in the WPE1-NB14 and RWPE-2 cell lines. However, expression of cytokeratin 18 and prostate specific antigen (PSA) were decreased in response to glycitein treatment whereas

the expression of the basal epithelial cell markers p63 and cytokeratin 5 remained unchanged. These data suggest that glycitein may induce basal cell differentiation in the RWPE-1 cell line.

4.2 Introduction

Prostate cancer (PCa) is the second leading cause of cancer related deaths among American males [1]. Although the risk for cancer is multifactorial, substantial portions of cancer incidence rates are related to environmental factors, including diet. Asian populations have lower PCa incidence and mortality as compared to the United States [2 – 5, 134, 250 – 253]. The increased consumption of soy and soy-containing products within these populations may contribute to reduced cancer rates. However, the specific compounds in soy and their mechanisms of action in the prostate are unknown.

Prostate carcinogenesis is characterized as a continuum of impairment of the homeostatic control governing differentiation, proliferation, and apoptosis of the prostate epithelium. The prostate epithelium consists of 2 primary differentiated cell types, luminal and basal and are characterized primarily by their unique cytokeratin profiles. Loss of luminal cell differentiation and a concomitant increase in the proliferation of this cell type is initially observed in low grade prostatic intraepithelial neoplasia (LGPIN) [57, 254, 255]. Progression to high grade PIN (HGPIN) involves disruption and partial loss of the basal cell population. A complete loss of the basal cell population, increased proliferation, decreased apoptosis, and subsequent cancer cell invasion of the basement membrane, stroma, and surrounding tissues is characteristic of PCa. Interestingly,

populations with high soy consumption have a reduced risk for HGPIN and PCa development; however, the incidence of LGPIN is similar to those populations with low soy consumption [256, 257]. This suggests that soy consumption may reduce PCa incidence by maintaining the differentiation state of the prostate epithelium; however, this hypothesis is yet to be tested.

Soy isoflavones have been shown to induce cellular differentiation in many tissues [217, 258, 259]; however, isoflavone-induced differentiation has not yet been examined in the prostate. The objective of this study was to identify the potential of soy isoflavones to induce cellular differentiation of a prostate intermediate cell population.

4.3 Materials and Methods

4.3.1 Materials

Genistein, daidzein and equol were obtained from LC Laboratories (Woburn, MA). Glycitein was purchased from Indofine (Hillsboro, NG). N-(4-hydroxyphenyl) retinamide (4-HPR) was obtained from Tocris (Ballwin, MO). The above compounds were prepared as stock solutions in dimethyl sulfoxide (DMSO) and were utilized in cell culture media at appropriate dilutions with a final DMSO concentration of 0.1%. Bicinchoninic acid (BCA) ™ protein assay kit and superSignal ECL™ chemiluminescent substrate kit were obtained from Pierce (Rockford, IL). ECL western blotting detection reagent was obtained from Amersham Biosciences Corporation (Piscataway, NJ). The monoclonal antibodies for cytokeratin 8/18 and β actin were obtained from Cell

Signaling-Technology (Beverly, MA). The polyclonal antibodies for PSA, vimentin, cytokeratin 5 and p63 were obtained from Abcam Inc (Cambridge, MA). 3-[4,5-dimethylthiazol-2-yl]-2-,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO). DNA flow cytometry analysis kit was purchased from Roche Applied Science (Indianapolis, IN).

4.3.2 Cell culture

The human prostate epithelial cell lines RWPE-1, WPE1-NB14, and RWPE-2 were obtained from the American Type Culture Collection (Rockville, MD) and maintained in keratinocyte serum-free medium (GIBCO Laboratories, Grand Island, NY) supplemented with 50 μg/ml bovine pituitary extract, 5% L-glutamine and 5ng/ml epidermal growth factor (EGF). These cells were maintained in a humidified incubator (5% CO_2, 95% O_2) at 37˚C.

4.3.3 Prostate epithelial proliferation

RWPE-1 cells were plated in 48-well plates at an initial density of 1.0 x 10^4 cells per well with supplements. Cells were treated with or without genistein, daidzein, equol and glycitein alone and in combination at appropriate concentrations for 0-8 days, changing media with treatment every 48 h. After incubation, cell proliferation was determined by the 3-[4,5-dimethylthiazol-2-yl]-2-,5-diphenyltetrazolium bromide (MTT) assay as described previously [14] and quantified spectrophotometrically at 595nm.

4.3.4 Immunoblot analysis

RWPE-1, WPE1-NB14, and RWPE-2 cells were plated in 60mm dishes and treated with or without genistein, daidzein, equol (50μmol/L), glycitein (5 and 50μmol/L) or 4-HPR (1μmol/L) for 8 days, changing media every 48 h. Following treatment, cells were washed with PBS and crude proteins isolated and separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblot was performed using primary antibodies (cytokeratin 8/18, cytokeratin 5/14, p63, PSA, vimentin) at recommended dilutions in TTBS (1 x TBS with 0.1% Tween-20) overnight at 4˚C. Following incubation with secondary antibodies, protein signals were visualized on autoradiography film, and quantified by densitometry using Scion imaging software (Frederick, Maryland).

4.3.5 Morphology

RWPE-1 cells were plated in a 4 well chamber slide and treated with or without glycitein (5 and 50μmol/L) or 4-HPR (1μmol/L) for 8 days. After treatment, cells were washed with PBS and fixed with 1% glutaraldehyde for 30 min at 4˚C. Following fixation, cells were washed with PBS and stained with 0.2% crystal violet for 2 h at room temperature as previously described [260]. Cells were then washed with PBS and examined with Olympus IX50 inverted fluorescent microscope fitted with appropriate filter cubes.

4.3.6 Cell cycle analysis

RWPE-1 cells were plated in 100mm dishes and treated with or without glycitein (5 and 50μmol/L) or 4-HPR (1μmol/L) for 3 days. After treatment, cells were washed with PBS, trypsinized, and fixed in -20˚C ethanol at 4˚C for 30 min. Cells were washed twice with PBS. DNA content was determined using the cellular DNA flow cytometry analysis kit (Roche Applied Science, IN). Briefly, the samples were incubated at 37˚C with RNase A for 30 min and treated with propidium iodide at 4˚C for 2 h. A minimum of 20,000 events per sample was measured for DNA content by propidium iodine staining using the BD FACS Calibur flow cytometer and cell cycle distribution was determined using the software program Modfit®.

4.3.7 Statistical analysis

Statistical significance between groups for proliferation, cell cycle, and immunoblot was determined with either one-way analysis of variance (ANOVA) with Tukey's post-hoc comparisons or 2-sample t-test adjusted for multiple comparisons (SigmaStat software; Chicago, IL). Data are presented as means ± standard error of the mean (S.E.M.) with alpha $p<0.05$ considered significant.

4.4 Results

4.4.1 RWPE-1 cellular proliferation

The antiproliferative effects of genistein, daidzein, equol, and glycitein on RWPE-1 cells is given in Fig. 4.1A - D. RWPE-1 cellular proliferation was significantly reduced upon treatment with 50μmol/L of genistein, daidzein, equol and glycitein by 88.35 ± 8.29%, 29.43 ± 13.73%, 69.21 ± 6.82%, and 84.62 ± 7.88%, respectively, compared to untreated controls ($p<0.001$). A 19.4 ± 1.1% increase in cellular proliferation was observed after treatment with 5μmol/L genistein. Glycitein significantly reduced RWPE-1 proliferation by 21.20 ± 3.01% at concentrations as low as 5μmol/L compared to untreated controls ($p<0.01$).

The cytotoxic effects of genistein and glycitein at 50μmol/L were measured and are given in Fig. 4.2A. After 8 days of treatment, glycitein did not alter the concentration of the initial cell population measured at day 0. Genistein significantly reduced the initial cell population by 25.2 ± 8.30% ($p<0.05$).

The antiproliferative effects of isoflavones in combination and glycitein alone are given in Fig 4.2. B, C. Cells were treated with 0.5μmol/L genistein, 2.8μmol/L daidzein, 2.7μmol/L equol and a range of concentrations of glycitein (0-50μmol/L) for 8 days and cellular proliferation was measured (Fig. 4.2B). Combinations of genistein, daidzein, and equol did not effect the proliferation of the RWPE-1 cell line. However, when glycitein (10 and 50 μmol/L) was added to the isoflavone cocktail, proliferation was reduced 31.7

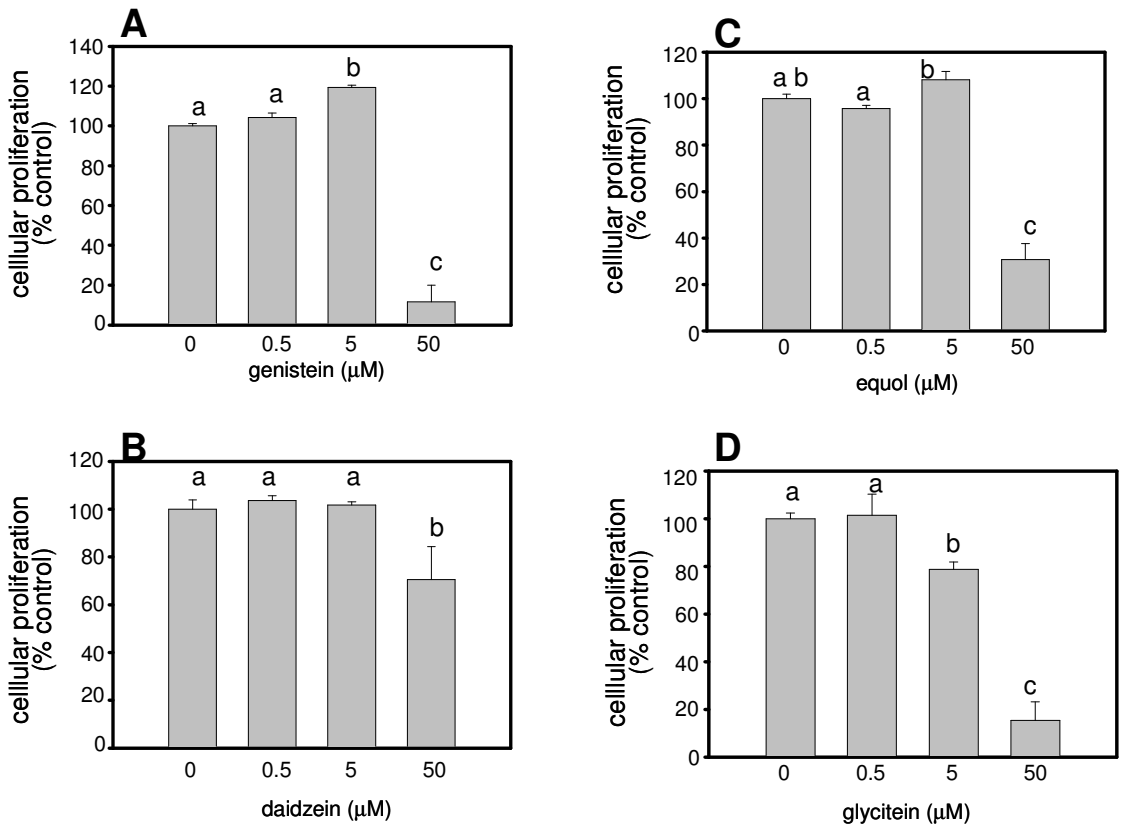

Figure 4.1: Concentration dependent effects of (A) genistien, (B) daidzein, (C) equol, and (D) glycitein on RWPE-1 proliferation. Cell proliferation was measured using the MTT assay. RWPE-1 cells were treated for 8 days with 0-50μmol/L isoflavones, changing media every 48 h. All experiments were performed independently at least 2 times with an n=6/experiment. Mean values not sharing a common letter superscript differ significantly (P<0.05).

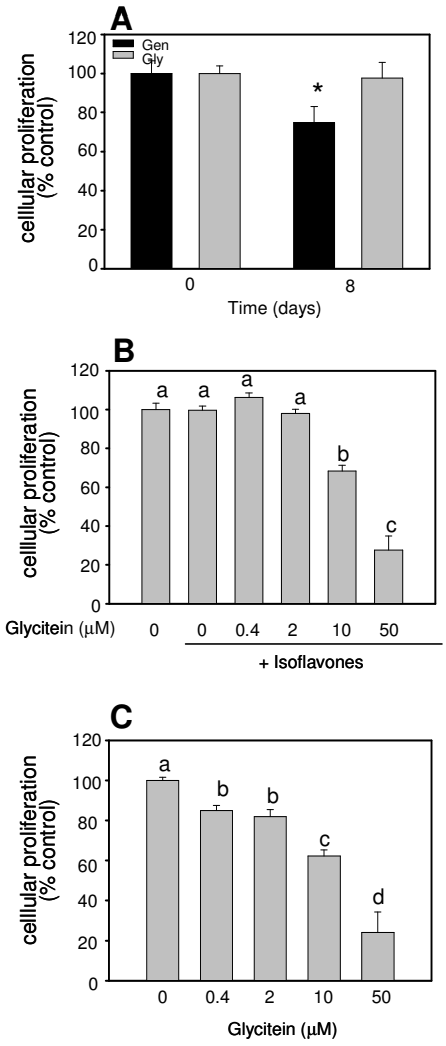

Figure 4.2: Antiproliferative effects of isoflavones alone and in combination (A) RWPE-1 cells were treated for 0 or 8 days with 50μmol/L genistein or glycitein. Cellular proliferation was assessed at day 0 and day 8. Concentration dependent effects of glycitein (0-50μmol/L) (B) in combination with physiologically relevant concentrations of genistein (0.5μmol/L), daidzein (2.8μmol/L), and equol (2.7μmol/L) designated as + isoflavones or (C) alone on RWPE-1 cellular proliferation. Cell proliferation was measured using the MTT assay. RWPE-1 cells were treated for 8 days changing media every 48 h. All experiments were performed independently at least 2 times with an n=6/experiment. Mean values not sharing a common letter superscript differ significantly (P<0.05).

± 3.1% and 72.4 ± 7.2%, respectively ($p<0.05$). Glycitein treatment alone significantly reduced the proliferation of this cell line at all concentrations tested ($p<0.05$) (Fig. 4.2C).

4.4.2 Expression of luminal and basal epithelial cell markers

Effect of isoflavones (50µmol/L) on the expression of cytokeratin 18, a marker of luminal cell differentiation is given in Fig. 4.3A. Genistein, daidzein, and equol induced expression of cytokeratin 18. However, glycitein reduced the expression of cytokeratin 18. Therefore, the effect of glycitein on cellular differentiation was further characterized.

Treatment with 50µmol/L glycitein significantly reduced expression of cytokeratin 18, 8 days post treatment ($p<0.001$) (Fig. 4.3B). 4-HPR (1µmol/L), a known inducer of luminal differentiation, was used as a control and significantly increased expression of cytokeratin 18 ($p<0.005$). Glycitein did not affect the expression of cytokeratin 18 in the WPE1-NB14 and RWPE-2 cell lines (Fig. 4.3C)

In order to further characterize luminal differentiation, RWPE-1 cells were treated with 4-HPR (1µmol/L) or glycitein (50µmol/L) for 8 days, the media was removed, and cells exposed to dihydrotestosterone (DHT) for 24 h (Fig. 4.3D). Upon treatment with DHT, expression of PSA was upregulated in control and 4-HPR treated cells as compared with controls cells not treated with DHT. DHT did not induce the expression of PSA in glycitein treated cells. LNCaP cells were used as the positive control for PSA expression.

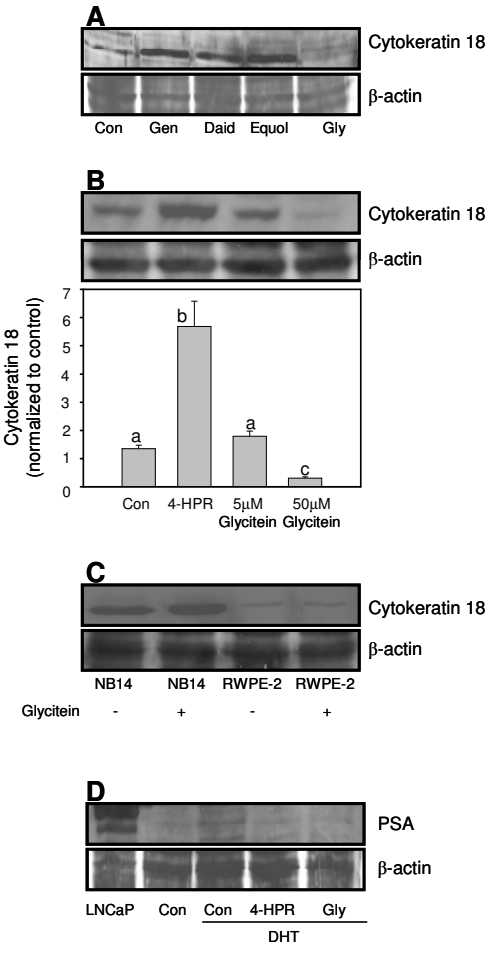

Figure 4.3: Effect of isoflavones on expression of luminal cell differentiation proteins. Expression of CK18 was measured by immunoblot in RWPE-1 cells following 8 day treatment with (A)50μmol/L genistein (Gen), daidzein (Daid), equol, glycitein (Gly) or vehicle alone (Con), and (B) following 8 day treatment with 4-HPR (1μmol/L), glycitein (5 or 50μmol/L) or vehicle alone, changing media every 48 h. Data are quantified and given by percent of means ± S.E.M.. Mean values not sharing common letter superscript differ significantly ($p<0.05$). (C) Expression of CK18 was measured by immunoblot in WPE1-NB14 and RWPE-2 cells following 8 day treatment with glycitein (50μmol/L) or vehicle alone, changing media every 48 h. (D) Expression of PSA was measured by immunoblot in RWPE-1 cells following 8 day treatment with 4-HPR (1μmol/L), glycitein (5 or 50μmol/L) or vehicle alone, changing media every 48 h with subsequent 24 h treatment with DHT. β-actin was used as the loading control for each experiment. All experiments were performed independently at least 3 times with an n=3.

The effect of glycitein on basal cell protein marker expression is given in Fig 4.4. Glycitein (5 and 50µmol/L) maintained expression of cytokeratin 5 (Fig. 4.4A) and p63 (Fig. 4.4B). 4-HPR (1µmol/L) significantly decreased expression of these basal cell makers.

4.4.3 RWPE-1 epithelial cell markers

In order to confirm that glycitein treated cells remained epithelial in origin, expression of vimentin was measured and is given in Fig. 4.5 A. Expression of vimentin remained undetected following 8 day treatment with 4-HPR (1µmol/L) and glycitein (5 and 50µmol/L) compared with untreated controls. Primary dog lens epithelial cell lysate collected at passage 2 was used as the positive control [261]. Furthermore, gross morphology of cells treated with 4-HPR and glycitein resemble untreated controls (Fig. 4.5 B).

4.4.4 RWPE-1 cell cycle distribution

Effects of glycitein on cell cycle distribution are given in Table 4.1. Glycitein did not significantly alter cell cycle distribution at 5µmol/L. However, at 50µmol/L glycitein significantly increased the amount of cells in the G_2-M phase by 5.8 ± 0.7% and decreased S phase by 5.1 ± 0.9% ($p<0.05$). Although these results were statistically

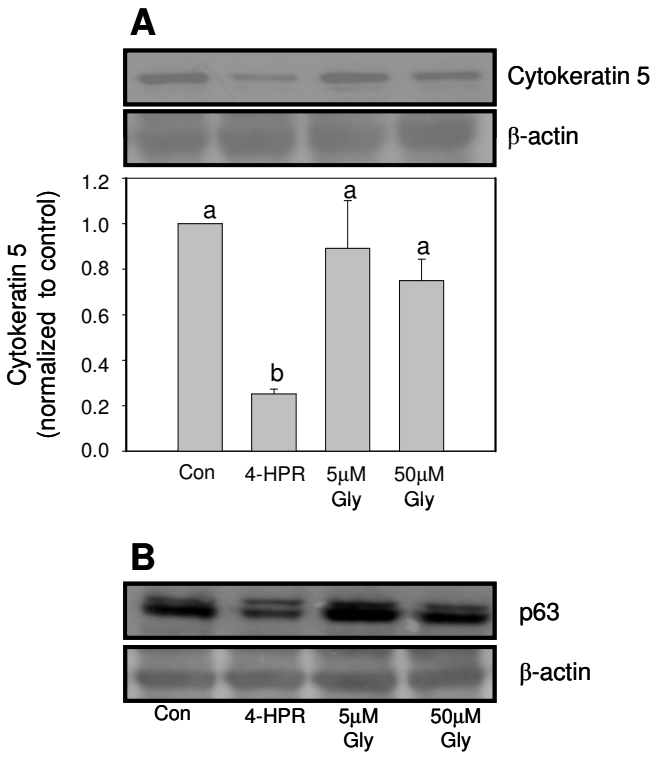

Figure 4.4: Effect of glycitein on expression of basal cell differentiation proteins. (A) Expression of cytokeratin 5 was measured by immunoblot in RWPE-1 cells following 8 day treatment with 4-HPR (1μmol/L), glycitein (5 or 50μmol/L) or vehicle alone, changing media every 48 h. β-actin was used as the loading control. (B) Expression of p63 was measured by immunoblot in RWPE-1 cells following 8 day treatment with 4-HPR (1μmol/L), glycitein (5 or 50μmol/L) or vehicle alone, changing media every 48 h. β-actin was used as the loading control. Data are given as a representative immunoblot. All experiments were performed independently at least 3 times with an n=3. Significant difference from the untreated control is designated by letter superscripts (P<0.05).

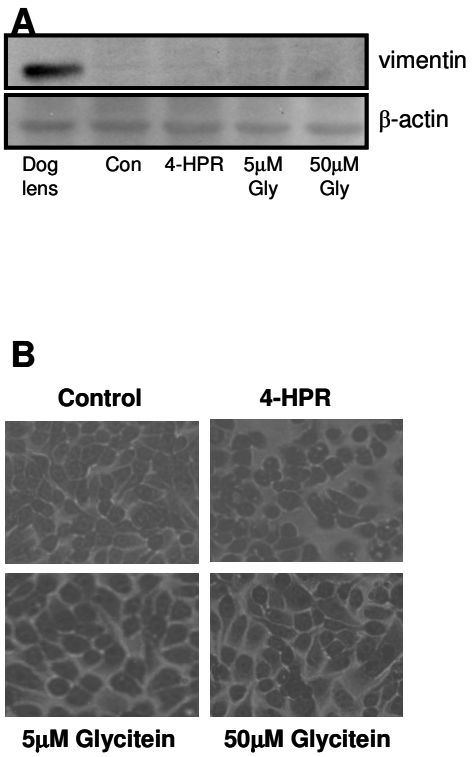

Figure 4.5: Effect of glycitein on epithelial phenotype. (A) Expression of vimentin was measured by immunoblot in RWPE-1 cells following 8 day treatment with 4-HPR (1μmol/L), glycitein (5 or 50μmol/L) or vehicle alone, changing media every 48 h. β-actin was used as the loading control. Data are given as a representative immunoblot. (B) Gross morphology of RWPE-1 cells was measured following 8 day treatment with 4-HPR (1μmol/L), glycitein (5 or 50μmol/L) or vehicle alone, changing media every 48 h. All experiments were performed independently at least 3 times with an n=3.

Cell Cycle Distribution	Cell Cycle Distribution (%)*			
	Control	4-HPR	5µmol/L glycitein	50µmol/L glycitein
G_0-G_1	$63.6^{a} \pm 0.8$	$71.5^{b} \pm 2.7$	$65.9^{a} \pm 1.4$	$62.9^{a} \pm 1.2$
S	$29.2^{a} \pm 2.8$	$12.8^{c} \pm 0.6$	$26.2^{ab} \pm 1.2$	$24.1^{b} \pm 0.9$
G_2-M	$7.2^{a} \pm 1.9$	$15.8^{b} \pm 2.2$	$7.9^{a} \pm 0.2$	$13.0^{b} \pm 0.7$

Table 4.1: Flow cytometric analysis of RWPE-1 cells treated with 4-HPR (1µmol/L), glycitein (5 and 50µmol/L), or untreated control for 3 days.
***All experiments were performed independently at least 3 times with an n=3. Significant difference from the untreated control is designated by letter superscripts ($P<0.05$).**

significant, glycitein did not appreciably alter cell cycle distribution. 4-HPR (1μmol/L), a synthetic retinoid and known inducer of G_0-G_1 cell cycle arrest was used as a positive control.

4.5 Discussion

The objective of the present study was to examine the effects of soy isoflavones, specifically glycitein, on prostate epithelial differentiation. It has been hypothesized that sustained extracellular signal-regulated kinase (ERK1/2) signaling is involved the differentiation process in the prostate epithelium [11]. Previous work in this laboratory demonstrates that glycitein induces a sustained and robust activation of the ERK1/2 signaling cascade in the RWPE-1 cell line [262]. Based on this observation, we hypothesize that soy isoflavones may influence the differentiation of this cell type. We tested this hypothesis using RWPE-1 cells as a model for prostate epithelial cell differentiation. This cell line expresses both luminal and basal cell protein markers and is therefore considered an intermediate prostate epithelial cell type. Research supports the differentiation of this intermediate cell type into either luminal or basal cells during organogenesis [22]. Dysregulated prostate epithelial cell differentiation is commonly observed during prostate carcinogenesis. Therefore, strategies to maintain proper prostate cell differentiation may be beneficial in the treatment and/or prevention of PCa.

The soy isoflavones genistein, daidzein, and the daidzein metabolite equol have been extensively studied for their anticancer properties in several *in vitro* and *in vivo* models [201 – 203, 208, 209, 218, 232 – 234, 263 – 265]. However limited data are

available regarding the anticancer effects of glycitein. Glycitein has been shown to reduce cellular invasion [266, 267] and cell motility [268] in Jurkat T and breast cancer cell lines. In prostate cell lines, glycitein has been shown to induce cell cycle arrest [144], decrease cellular proliferation [144, 262], and modulate signal transduction pathways [262]. The present study examined the antiproliferative effects of genistein, daidzein, equol, and glycitein and found that all isoflavones inhibited proliferation of the nontumorigenic prostate epithelial cell line, RWPE-1, at 50μmol/L. Genistein and glycitein were the most potent inhibitors of cell proliferation at the highest concentration tested. The decrease in cellular proliferation by genistein appears to be cytotoxic while the decrease in cellular proliferation by glycitein appears to be cytostatic. Previous studies in this laboratory support these findings that genistein reduces cell proliferation at >12.5μmol/L, primarily via apoptosis [14].

Glycitein inhibited RWPE-1 proliferation at concentrations tenfold less than the other isoflavones. This observation may be attributed to the methoxy group on the sixth carbon of the flavone ring of glycitein. Recent studies suggest that flavones with methoxy groups are more biologically stable, resistant to metabolism, and have improved intestinal transport as compared to their nonmethylated counterparts [7, 8].

The concentration of genistein, daidzein and equol in prostatic fluid has previously been reported to be an average of 0.5, 2.8, and 2.7μmol/L, respectively [145]. To date, the concentration of glycitein in prostatic fluid has not been reported. Glycitein alone reduced RWPE-1 cell proliferation at all concentrations tested (0 – 50μmol/L).

Interestingly, when glycitein was treated in combination with prostatic fluid concentrations of genistein, daidzein, and equol, the antiproliferative effect of glycitein was reduced. This suggests a possible antagonism between isoflavones. It has been suggested that the antagonistic effects between soy isoflavones may be due to receptor site competition [269]. However, further research is necessary to explore synergistic and antagonistic effects of combinations of physiologically relevant soy isoflavones in the prostate.

The results of the present study show that glycitein altered the expression of specific protein markers consistent with cellular differentiation in the RWPE-1 cell line. Glycitein downregulated the expression of luminal epithelial cell markers and maintained the expression of basal epithelial cell markers. However, glycitein did not alter expression of cellular differentiation markers in the WPE1-NB14 precancerous and RWPE-2 cancerous prostate cell lines, suggesting that the differentiation effects of glycitein may be cancer stage specific. We and others have previously shown that the bioactivity of dietary compounds may be most beneficial during specific stages of PCa and that the timing of exposure is critical for maximizing the anticancer effects [121, 218, 262, 270, 271]. Furthermore, several epidemiological suggest that a diet rich in soy and soy isoflavones may prevent PCa during noncancerous and precancerous stages of the carcinogenic process and that during advanced disease, exposure to soy isoflavones may no longer be beneficial [48 – 50, 68, 183, 193, 272, 273].

To further support our hypothesis that glycitein induces basal cell differentiation in the RWPE-1 cell line, PSA levels were measured following treatment with the

androgen DHT. PSA is a serine protease that is expressed only by luminal and intermediate cells of the prostate. Therefore, PSA is an appropriate marker for distinguishing between luminal and/or intermediate cells from basal cells. The synthetic retinoid 4-HPR induces luminal cellular differentiation in the prostate [271] and therefore was used as a positive control for the expression of PSA. The present study found that DHT did not induce intracellular expression of PSA in glycitein treated cells. However, DHT treatment did induce expression of PSA in vehicle and 4-HPR treated cells. These results further support our observation that glycitein influences the differentiation of an intermediate to a basal cell type.

The process of differentiation involves inhibited proliferation and an exit from the cell cycle as measured by G_0-G_1 cell cycle arrest. These data show that the positive control, 4-HPR, but not glycitein induced cell cycle arrest at the G_0-G_1 phase in the RWPE-1 cell line. Glycitein has shown to induce cell cycle arrest in the G_0-G_1 phase of the cell cycle in primary prostate cell lines [144]. We hypothesize that G_0-G_1 arrest was not observed upon glycitein treatment because immortalization of the RWPE-1 cell line with the human papilloma virus 18 (HPV-18) produces a protein that binds retinoblastoma (pRb), the major G_0-G_1 cell cycle check point protein. Studies suggest that fractions of soy paste induce G_0-G_1 cell cycle arrest in breast cancer cells via activation of pRb [274] and that 4-HPR induced G_0-G_1 cell cycle arrest is independent of pRb activation [275]. This may explain why 4-HPR but not glycitein induced G_0-G_1 cell cycle arrest in our immortalized prostate epithelial cell line; however, the effect of glycitein on pRb activation has yet to be tested.

Disruption and subsequent loss of basal cells within the prostate epithelium is the most prominent morphological feature observed in HGPIN and PCa. Results from the present study suggest that glycitein induces genotypic changes in the RWPE-1 cell line consistent with basal epithelial cell differentiation. We hypothesize that glycitein-induced basal cell differentiation may preserve the basal cell population within the prostate; thus representing a novel mechanism by which dietary soy reduces PCa incidence.

CHAPTER 5

GLYCITEIN-INDUCED ERK1/2 ACTIVATION AND THE ANTIPROLIFERATIVE EFFECTS DURING NONCANCEROUS AND PRECANCEROUS STAGES OF PROSTATE CARCINOGENESIS

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The Ohio State University, Columbus, OH 43210

5.1 Abstract

Increased consumption of soy and soy isoflavones is associated with a reduced risk for prostate cancer (PCa). This observed reduction in the risk for this disease is thought to be due in part to exposure to isoflavones early during the prostate carcinogenic process. Recent evidence suggest that isoflavones with methoxy groups may be more bioactive than there nonmethylated counterparts. The objective of this study was to compare the effects of methylated and nonmethylated isoflavones on the extracellular signal-regulated kinase (ERK1/2) activity and cellular proliferation using noncancerous, precancerous, and cancerous prostate epithelial cell lines. The nonmethylated isoflavone genistein and the methylated isoflavone glycitein significantly reduced the cellular proliferation of all cell lines tested. The antiproliferative effects of the nonmethylated daidzein were only observed in the precancerous prostate epithelial cell line. The

methylated isoflavones biochanin A and formononetin significantly decreased the proliferation of the precancerous and cancerous cell lines. Biochanin A also significantly decreased the proliferation of the noncancerous cell line. Glycitein was the only isoflavone that significantly induced ERK1/2 activity in the noncancerous and precancerous cell lines. Glycitein-induced ERK1/2 activity was associated with the antiproliferative effects of this isoflavone in noncancerous and precancerous prostate epithelial cells. The antiproliferative effects of the other isoflavones were independent of ERK1/2 activation. These data suggest that although isoflavones are structurally similar, their mechanisms of inhibiting proliferation are different. Furthermore, the stage of cancer development may determine the magnitude of the antiproliferative and ERK1/2 response elicited by isoflavones.

5.2 Introduction

Prostate cancer (PCa) is the most commonly diagnosed and the second leading cause of cancer related death in the United States [1]. PCa development follows a series of slowly progressive stages representing normal, precancerous, and finally cancerous stages. Several epidemiologic studies suggest that diet plays a pivotal role in the prevention against this disease [109]. Evidence suggests that timing of dietary intervention during specific stages of the carcinogenic process optimizes the anticancer effects of specific dietary components [121, 218, 262, 270, 261]. Recently epidemiological, *in vivo*, and *in vitro* studies suggest that increased consumption of soy

and/or soy isoflavones early during the prostate carcinogenic process (i.e. normal or precancerous prostate epithelium) may be optimal for preventing PCa [48 – 50, 68, 183, 193, 272, 273].

Isoflavones found in soy are thought to be responsible, in part, for the anticancer effects of soy. The three most abundant isoflavones found in soy are the glycoside derivatives of genistein, daidzein, and glycitein generally comprising 50%, 40%, and 10% of the total isoflavone profile, respectively. Many studies have investigated the anticancer activity of the more predominant soy isoflavones genistein and daidzein. However, studies in this laboratory suggest that glycitein may be more bioactive than genistein and daidzein [262, 276]. Interestingly, glycitein is the only isoflavone found in soy containing a methoxy group. However, isoflavones found in red clover, biochanin A and formononetin, also possess methoxy groups. Recent studies suggest that flavones, the parent family of isoflavones, with methoxy groups possess greater bioactivity than nonmethylated flavones. These studies show that methylated flavones are more biologically stable, are resistant to phase I and phase II metabolism, and have improved intestinal transport as compared to their nonmethylated counterparts [7, 8]. Glycitein has previously been shown to be more bioavailable than genistein [150] with increased rates of uptake by enterocytes and increased rates of excretion from the basolateral side as measured in the Caco-2 intestinal cell model [131]. However, the anticancer activity of glycitien and red clover methylated isoflavones as compared with nonmethylated isoflavones has not been extensively examined in a prostate cell model.

We have previously shown that glycitein is a potent inducer of the extracellular signal-regulated kinase (ERK1/2) cascade and a potent inhibitor of cellular proliferation in the noncancerous prostate epithelial cell line RWPE-1 [262, 276]. Although ERK1/2 signaling is commonly associated with increased proliferation, increased ERK1/2 activity appears to be associated with decreased proliferation in the prostate [10 – 13]. In fact ERK1/2 activity is commonly downregulated or completely lost during PCa progression. The objective of this study was to compare the effects of methylated and nonmethylated isoflavones on proliferation and ERK1/2 activation of prostate epithelial cells representing the normal, precancerous, cancerous, and metastasized stages of the prostate carcinogenic process.

5.3 Materials and Methods

5.3.1 Materials

Genistein, daidzein, glycitein, equol, biochanin A and formononetin were obtained from LC Laboratories (Woburn, MA). The above compounds were prepared as stock solutions in dimethyl sulfoxide (DMSO) and were utilized in cell culture media at appropriate dilutions with a final DMSO concentration of 0.1%. Bicinchoninic acid (BCA) ™ protein assay kit and superSignal ECL™ chemiluminescent substrate kit were obtained from Pierce (Rockford, IL). ECL western blotting detection reagent was obtained from Amersham Biosciences Corporation (Piscataway, NJ). Polyclonal antibodies for phospho- ERK1/2 kinase and total ERK1/2 kinase were obtained from Cell

Signaling-Technology (Beverly, MA). 3-[4,5- dimethylthiazol-2-yl]-2-,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO).

5.3.2 Cell culture

The human prostate epithelial cell lines RWPE-1, WPE1-NB14, and RWPE-2 and the metastasized human prostate cancer line (PC-3) were purchased from the American Type Culture Collection (Rockville, MD). RWPE-1, WPE1-NB14, and RWPE-2 cells were maintained in keratinocyte serum-free medium (KSFM) (GIBCO Laboratories, Grand Island, NY) supplemented with 50mg/L bovine pituitary extract, 5% L-glutamine and 5μg/L epidermal growth factor (EGF). The PC-3 cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). All cell lines were supplemented with 1.0% penicillin/streptomycin and maintained in a humidified incubator (5% CO_2, 95% O_2) at 37°C.

5.3.3 Immunoblot detection of active and total ERK1/2

Cells were grown in 60mm dishes for 48 h (90% confluent). Growth media was removed and cells were washed with PBS and incubated in supplement free media for 24 h. Cells were then treated with isoflavones at appropriate times and concentrations. Following treatments, growth medium was removed and cells were washed with PBS. Crude proteins were isolated and separated on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed using primary

antibodies that recognize active (phospho-) and total ERK1/2 at recommended dilutions in TTBS (1 x TBS with 0.1% Tween-20) overnight at 4°C. Following incubation with secondary antibodies, protein signals were visualized on autoradiography film using ECL, and quantified by densitometry using Scion imaging software (Frederick, Maryland).

5.3.5 Cellular proliferation

Cells were plated with KSFM with supplements in 48-well plates at an initial density of 8.0×10^3 cells per well. Cells were treated with or without isoflavones and incubated for an additional 72 h. After incubation, cell proliferation was determined by the MTT assay as described previously [14]. Changes in cellular proliferation are normalized to the control.

5.3.6 Statistical Analyses

Statistical significance between groups was determined by one-way analysis of variance (ANOVA) with Tukey's post-hoc comparisons (SigmaStat software; Chicago, IL). Data are presented as percentage of means ± standard error of the mean (S.E.M.), with α level of $p<0.05$.

5.4 Results

5.4.1 Prostate epithelial proliferation

The effects of isoflavones on the proliferation of noncancerous, precancerous, and cancerous prostate epithelial cells are given in Fig. 5.1 A – D. The soy isoflavones genistein and glycitein significantly decreased the proliferation of the RWPE-1 cell line by 23% and 24%, respectively ($p < 0.001$) (Fig. 5.1A). Biochanin A, the methylated form of genistein, decreased the proliferation of the RWPE-1 cell line at least 15% more than genistein and glycitein. However, equol significantly increased the proliferation of this noncancerous cell line ($p < 0.001$). Nonmethylated daidzein and its methylated form, formononetin, did not significantly affect the proliferation of the cell line as compared with the control.

All isoflavones significantly altered the proliferation of the noncancerous cell line WPE1-NB14 (Fig 5.1B). Equol was the only isoflavone to significantly increase the proliferation of this cell line ($p < 0.01$). Genistein, glycitein, and biochanin A significantly decreased the proliferation of the precancerous cell line by 25%, 33%, and 33%, respectively ($p<0.01$). Daidzein and formononetin also significantly decreased the proliferation of the WPE1-NB14 cell line as compared with the control ($p < 0.01$).

The antiproliferative effects of isoflavones on the prostate cancer cell line RWPE-2 are given in Fig. 5.1C. Genistein and glycitein significantly decreased the proliferation

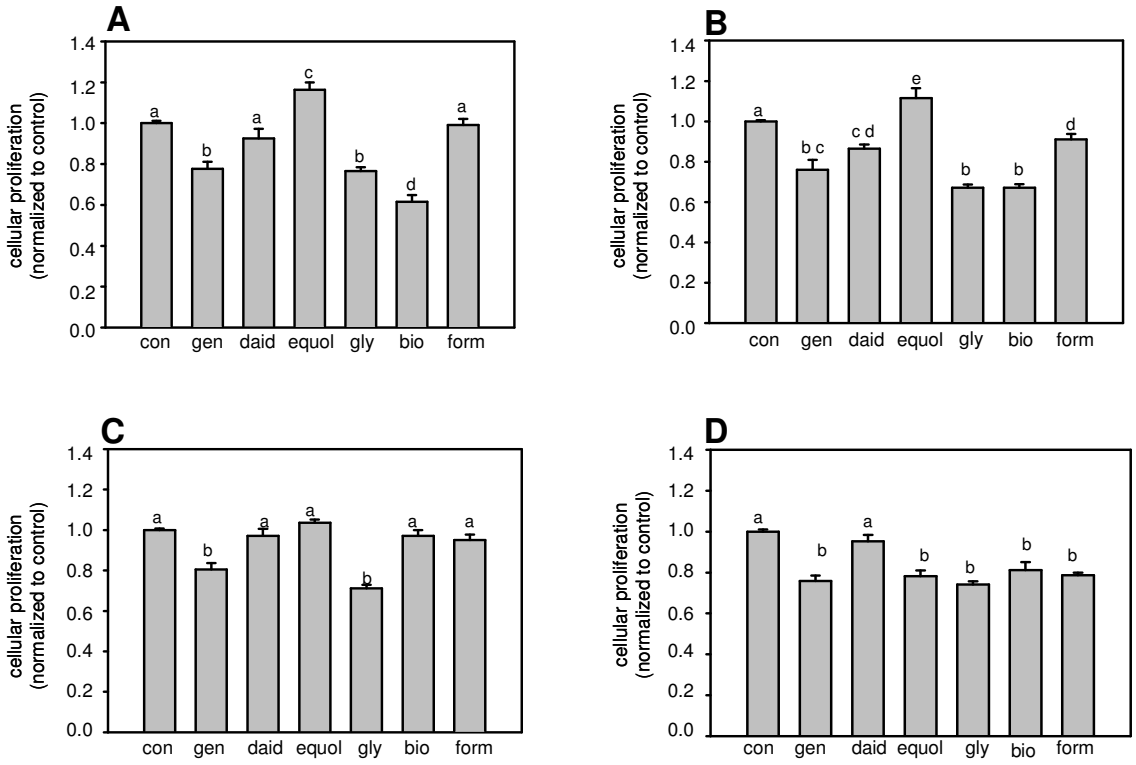

Figure 5.1: The proliferative effects of the isoflavones genistein (gen), daidzein (daid), equol, glycitein (gly), biochanin A (bio), and formononetin (form) on (A) RWPE-1, (B) WPE1-NB14, (C) RWPE-2 and (D) PC-3 cells. Cell proliferation was measured using the MTT assay. Cells were treated for 72 h with 50μmol/L isoflavones. All experiments were performed independently at least 2 times with an n=6/experiment. Mean values not sharing common letter superscript differ significantly ($p<0.05$).

of the RWPE-1 cell line by 20% and 29%, respectively ($p < 0.001$). Daidzein, equol, biochanin A, and formononetin did not significantly affect the proliferation of this cancerous cell line as compared with the control.

The antiproliferative effects of isoflavones on the prostate cancer cell line PC-3 are given in Fig. 5.1D. Unlike the RWPE-2 cell line, genistein, equol, glycitein, biochanin A, and formononetin significantly reduced the proliferation of this cell line ($p < 0.001$). However, daidzein did not significantly alter the proliferation of this cell line.

5.4.2 Effects of isoflavones on ERK1/2 activity

The effects of isoflavones on ERK1/2 activity in noncancerous, precancerous, and cancerous cell lines are given in Fig. 5.2 A – D. Glycitein significantly induced ERK1/2 activity in the RWPE-1 cell line ($p < 0.05$) whereas genistein significantly reduced ERK1/2 activity ($p < 0.05$) (Fig 5.2A). Daidzein and formononetin induced ERK1/2 activity as compared with the control but was not significant.

Glycitein significantly induced ERK1/2 activity in the precancerous cell line WPE1-NB14 as compared with untreated controls ($p < 0.05$) (Fig. 5.2B). Formononetin induced ERK1/2 activity but was not statistically different from that of untreated controls or glycitein treated cells. All other isoflavones did not significantly affect ERK1/2 activity in the precancerous prostate cell line WPE1-NB14.

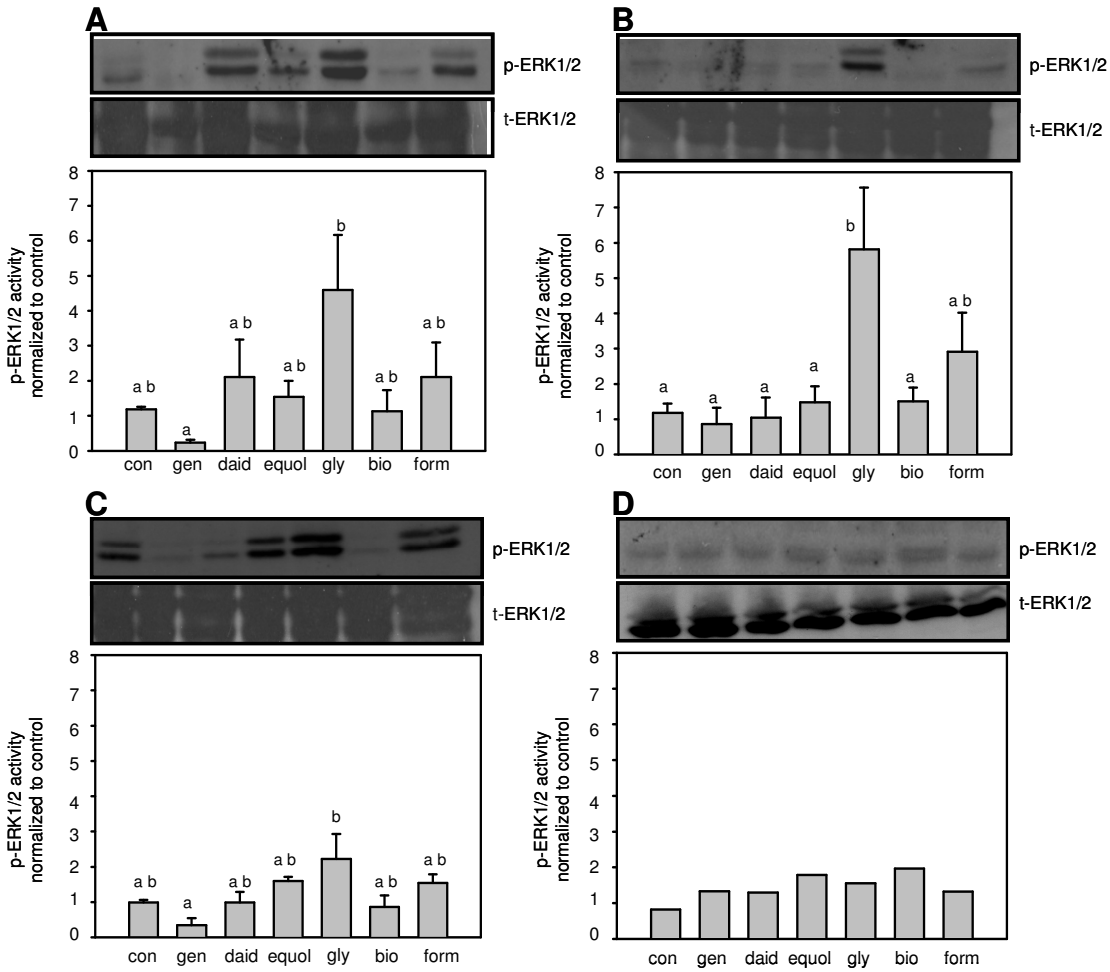

Figure 5.2: Effect of isoflavones on ERK1/2 activity in (A) RWPE-1, (B) WPE1-NB14, (C) RWPE-2 and (D) PC-3 prostate cells. Active ERK1/2 (p-ERK1/2) was measured by immunoblot in RWPE-1 cells following 2 h treatment with either 50µmol/L genistein (Gen), daidzein (Daid), equol, glycitein (Gly), biochanin A (bio), formononetin (form) or vehicle alone (Con). All experiments were performed independently at least 3 times with an n=3/experiment. Mean values not sharing common letter superscript differ significantly ($p<0.05$). Total ERK1/2 (t-ERK1/2) was used as the loading control.

The effects of isoflavones on ERK1/2 activity in the RWPE-2 prostate cancer cell line are given in Fig. 5.2C. Glycitein significantly increased ERK1/2 activity as compared with genistein treated cells. However, the effects of all isoflavones on ERK1/2 activity were not significantly different from untreated controls.

The effects of isoflavones on ERK1/2 activity in the PC-3 prostate cancer cell line are given in Fig. 5.2D. All isoflavones did not significantly alter ERK1/2 activity as compared with untreated controls.

5.4.3 The association between the effects of isoflavones on proliferation and ERK1/2 activity

The association between the effects of isoflavones on ERK1/2 activity and proliferation in noncancerous, precancerous, and cancerous prostate epithelial cells are given in Fig. 5.3 A – D. The data for each panel was taken from the data collected for figures 5.1 and 5.2. Each panel in Fig. 5.3 is divided into four quadrants. The upper left quadrant represents those treatments which induce ERK1/2 activity (> 1.0) and decrease the proliferation (< 1.0) of the cell line. The upper right quadrant represents those treatments which induce ERK1/2 activity (> 1.0) and induce proliferation (>1.0). The lower left quadrant represents those treatments which reduce ERK1/2 activity (<1.0) and reduce proliferation (< 1.0). The lower right quadrant represents those treatments which reduce ERK1/2 activity (<1.0) and induce proliferation (>1.0) of each cell line.

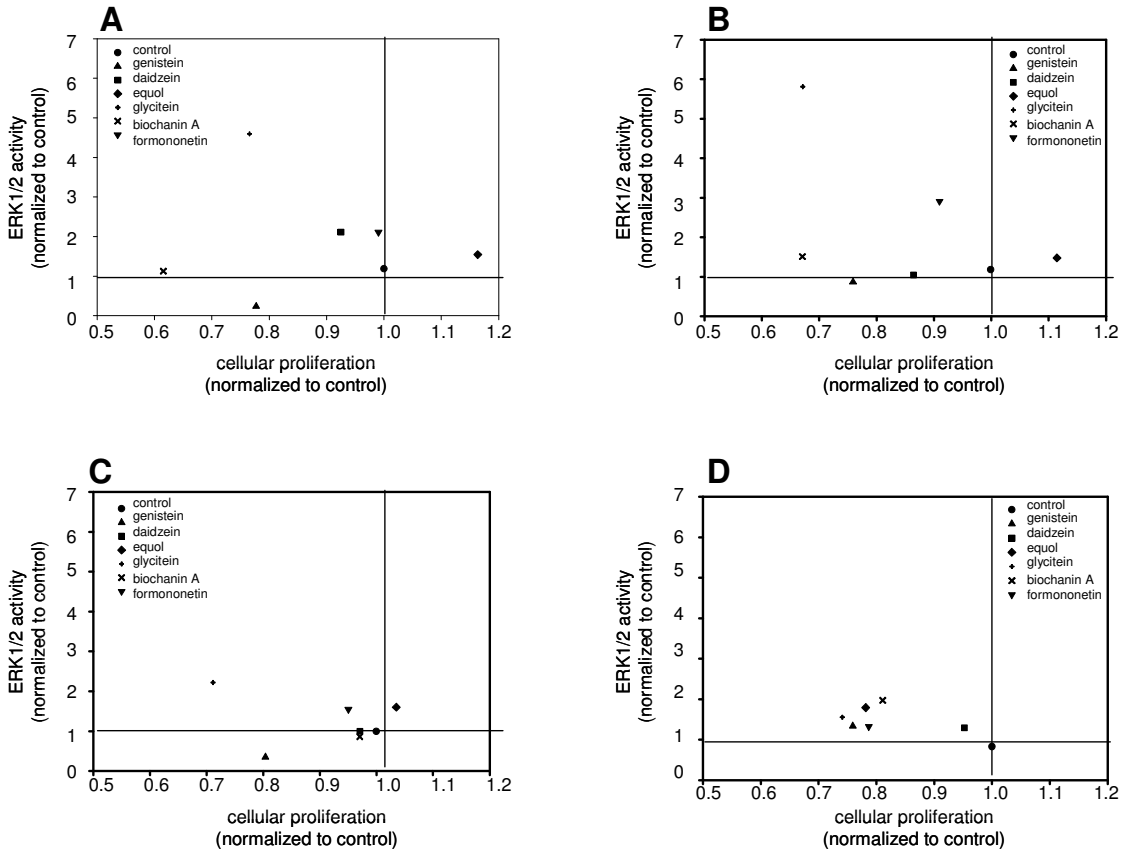

Figure 5.3: Associations between the effects of isoflavones on ERK1/2 activity and cellular proliferation in (A) RWPE-1, (B) WPE1-NB14, (C) RWPE-2, and (D) PC-3 prostate epithelial cells. Cells were treated with 50mmol/L of each isoflavone for either 2 h for ERK1/2 activity or 72 h for proliferation. All experiments were performed independently at least 2 times with an n=6/experiment.

The association between the effects of isoflavones on ERK1/2 activity and cellular proliferation in RWPE-1 cells are given in Fig 5.3A. Glycitein, biochanin A, and genistein all significantly reduced proliferation of the RWPE-1 cell line. The antiproliferative affects of glycitein may be mediated in part by an induction of ERK1/2 activation as the symbol for glycitein is furthest away from both lines for proliferation and ERK1/2 activity as a value of 1.0 results in no significant difference from controls. However, the antiproliferative affects of biochanin A and genistein do not appear to be associated with ERK1/2 activity as both symbols on the graph are not significantly different than 1.0 (Fig. 5.3A and 5.2A). Daidzein and formononetin do not significantly affect proliferation or ERK1/2 activity and therefore no association is observed. Although equol significantly increased proliferation in the RWPE-1 cell line, a significant induction of ERK1/2 activity was not observed and therefore no association can be made.

All isoflavones were shown to significantly affect the proliferation of the precancerous WPE1-NB14 cell line (Fig 5.1B). However, glycitein was the only isoflavone to significantly induce ERK1/2 activity (Fig 5.2B) and as observed in Fig. 5.3B, an association between glycitein-induced ERK1/2 activity and the antiproliferative effects of glycitein can be made. There may also be an association between formononetin-induced ERK1/2 activity and the inhibition of proliferation; however, this observed trend does not appear to be statistically significant.

The association between the effects of isoflavones on ERK1/2 activity and proliferation in RWPE-2 prostate cancer cells are given in Fig. 5.3C. Genistein and glycitein significantly reduced the proliferation of the RWPE-2 cell line (Fig. 5.1C).

Genistein reduced ERK1/2 activity in this cancerous cell line. Therefore, an association between genistein-reduced ERK1/2 activity and the antiproliferative effects of genistein can be made (Fig. 5.3C). In contrast an association between glycitein-induced ERK1/2 activity and the antiproliferative effects of glycitein in the RWPE-2 cell line is observed (Fig. 5.3C). No association between proliferation and ERK1/2 activity can be made for formononetin, biochanin A, daidzein, and equol.

The association between isoflavones and ERK1/2 activity and cellular proliferation of the PC-3 cell line is given in Fig 5.3D. Genistein, glycitein, biochanin A, and formononetin significantly reduced the proliferation of this cell line. However, no association between the antiproliferative affects of these isoflavones and ERK1/2 activity can be made. Daidzein did not affect proliferation or ERK1/2 activity in this cell line.

5.5 Discussion

A considerable amount of literature shows the anticancer activity of the major soy isoflavones genistein and daidzein as well as the microbial metabolite of daidzein, equol, in PCa cell models [201 – 193, 208, 209, 218, 232 – 234, 263 – 265]. Recent studies in this laboratory suggest that the soy isoflavone glycitein may possess greater bioactivity than the nonmethylated isoflavones genistein, daidzein, and equol [262, 276]. The objective of the present study was to investigate the effects that methylated and nonmethylated isoflavones have on the proliferation and ERK1/2 activation of noncancerous, precancerous, cancerous, and metastasized prostate cell lines. The results of the present study suggest that the methylated isoflavones, glycitein and biochanin A,

inhibit the proliferation of noncancerous (RWPE-1), precancerous (WPE1-NB14), and cancerous (PC-3) prostate cell lines. Genistein, a nonmethylated isoflavone, also significantly inhibited the proliferation of all prostate cell lines. The methylated isoflavone formononetin inhibits proliferation during precancerous and cancerous stages of development. In contrast the nonmethylated isoflavone, daidzein significantly inhibited the proliferation of the precancerous cell line alone. Surprisingly after three days of treatment, the nonmethylated metabolite of daidzein, equol, induced the proliferation of noncancerous and precancerous prostate cells; although inhibition of proliferation was observed in the PC-3 prostate cancer cell line.

The RWPE-2 and PC-3 cell lines are both cancerous prostate cell lines yet the effects that isoflavones have on the proliferation of these cell lines is vastly different. One major difference between these cells lines is their basal levels of ERK1/2 activity. The RWPE-2 cell line exhibits upregulated basal levels of ERK1/2 activity whereas PC-3 cells show little to no ERK1/2 activity [28, 277]. The differences in ERK1/2 activation may be due, in part, to the differences observed in response to isoflavone treatment.

Although isoflavone treatment affected proliferation of noncancerous, precancerous, and cancerous cell lines, glycitein was the only isoflavone to significantly affect ERK1/2 activity. Furthermore, glycitein-induced ERK1/2 activity was not observed in either prostate cancer cell line. As mentioned previously, basal ERK1/2 activity is altered in both the RWPE-2 and PC-3 cell lines which may explain why glycitein did not significantly alter ERK1/2 activity. However, glycitein reduced the proliferation of both cancer cell lines, suggesting that the mechanism by which glycitein

inhibits the proliferation of prostate cancer cells cannot be explained entirely by upregulating ERK1/2 activity. However, the mechanism by which glycitein reduces proliferation of prostate cancer cells has yet to be determined.

Previous studies in this laboratory show that glycitein induces ERK1/2 activity and inhibits the proliferation of noncancerous RWPE-1 cells via VEGFR [262]. The sustained ERK1/2 activity elicted by glycitein was shown to induce differentiation of this cell line [276]. In contrast, genistein and daidzein have shown to activate ERK1/2 and induce proliferation via nongenomic ER signaling in the prostate at physiological concentrations [14, 235]. At superphysiological concentrations, genistein inhibits ERK1/2 activity and proliferation via tyrosine kinase inhibition [14]. The mechanism by which formononetin and biochanin A inhibit proliferation has yet to be examined. Interestingly, many studies suggest that formononetin and biochanin A are rapidly demethylated in the liver by the phase I cytochrome p450 isozyme, CYP1A2, resulting in the generation of daidzein, genistein and genistein, respectively [138, 278, 279]. Breast epithelial cells also convert biochanin A to genistein [280], suggesting that extrahepatic tissues may also metabolize methylated isoflavones. These observations would suggest that the antiproliferative effects and ERK1/2 activity elicited by biochanin A would be similar to that of genistein. Similarly, the effects elicited by formononetin would mimic either genistein or daidzein assuming CYP1A2 is active in prostate cells. Our data shows that the antiproliferative and ERK1/2 inhibitory effects of biochanin A as compared with genistein and daidzein versus formononetin are similar in the RWPE-1 cell line. We hypothesize that biochanin A and formononetin are rapidly being converted to genistein

and daidzein in noncancerous prostate cells. Furthermore, there is an insignificant yet subtle difference in ERK1/2 activation between the methylated and nonmethylated isoflavones (biochanin A vs. genistein, formononetin vs. daidzein) in the precancerous cell line. Recently, CYP1A2 mRNA has been detected in the prostate [281]. The mRNA of this enzyme appears to be present in 67% of normal prostate tissue whereas only 43% of malignant tissue. This suggests that the metabolic conversion of the methylated isoflavones may be slowed during the carcinogenic process. However, this hypothesis has yet to be tested.

In contrast, several lines of evidence suggest that the methoxy group on glycitein remains stable and is not removed [138, 282]. It is hypothesized that the position of the methoxy group may determine the stability of the isoflavones. CYP1A2 specifically targets, removes, and hydroxylates the 4' position of the isoflavones structure, the location of the methoxy group on biochanin A and formononetin.

In contrast, the antiproliferative and ERK1/2 inducing affects of glyciten are unique. If glycitein were to be demethylated, the resulting compound would be daidzein. Data presented in this chapter as well as others suggest that glycitein is resistant to metabolism which may be due, in part, to the position of the methoxy group [138, 282]. Glycitein-induced ERK1/2 activation is associated with its antiproliferative affects during noncancerous and precancerous stages of PCa development. Glycitein-induced ERK1/2 activity has been associated with decreased proliferation and increased differentiation of noncancerous prostate epithelial cells [262, 276].

Manufacturers of soy rich products are utilizing the soy germ as the protein and isoflavone component of the food to minimize the "beany" flavor associated with the entire soy bean. Interestingly, although glycitein comprises 10% of the soy bean isoflavone profile, glycitein comprises up to 40% of the isoflavone profile in the soy germ. Therefore, soy food products tend to be enriched with this methylated isoflavone which may provide anticancer benefits above and beyond that of soy protein isolate. However, examining the chemopreventive properties of glycitein and soy germ in controlled *in vivo* and human clinical trials need to be conducted.

CHAPTER 6

EPILOGUE

Several epidemiological studies have indicated that increased consumption of soy and soy isoflavones may reduce the risk for PCa development. Many *in vivo* and *in vitro* studies have examined the anticancer activities of the major isoflavones genistein and daidzein in models with preexisting PCa. Studies which examine the anticancer activity isoflavones using prostate models mimicking earlier stages during the carcinogenic process have not been investigated. The objective of this dissertation was to identify specific isoflavones that possess anticancer activity during early stages of prostate carcinogenesis and characterize a mechanism of action. The first study characterized the mechanism of glycitein-induced ERK1/2 activation in a noncancerous prostate cell line. Based on the results of the first study, the second study examined the effects that glycitein had on the differentiation of this noncancerous prostate cell line. Finally, the third study compared the proliferative and ERK1/2 activation effects of several different isoflavones in prostate cell lines representing noncancerous, precancerous, and cancerous stages of this disease.

Several conclusions can be made from the studies outlined in this dissertation. First, the overall conclusion from these studies suggests that glycitein is a unique bioactive component of soy that may possess greater anticancer activity than the more abundant isoflavones genistein and daidzein. Data from the first study shows that unlike genistein and daidzien signaling through the ER, glycitein induces a strong and sustained ERK1/2 response via VEGFR signaling. This signaling cascade is unique in that VEGFR is not commonly associated with epithelial cells although recent data suggests that this receptor is present on other epithelial cell types. Interestingly, ERK1/2 signaling is commonly lost during the prostate carcinogenic process and restoration of this pathway may aid in the delay of this disease process. Data from the second study showed that glycitein induces basal cell differentiation of prostate epithelial cells. Basal epithelial cells are commonly disrupted in precancerous lesions of the prostate and completely lost in prostate cancer. Glycitein-induced basal cell differentiation may be one mechanism by which soy prevents prostate cancer. Data from the third study showed that the antiproliferative effects of glycitein are associated with glycitein-induced ERK1/2 activity in noncancerous and precancerous prostate cells.

The studies outlined in this dissertation provide important insight and mechanistic plausibility for the anticancer effects of glycitein during early stages of prostate carcinogenesis. These studies are important for two reasons. 1) The anticancer activity of glycitein has not been extensively studied in the prostate or any other cancer model; 2)

The consumption of glycitein rich food products may be on the rise due to the use of the glycitein-rich soy germ. Soy germ seems to minimize the "beany" flavor associated with the entire soy bean in soy food products.

However, many questions still need to be addressed regarding the research outlined in this dissertation. How is glycitein signaling via VEGFR to induce a sustained ERK1/2 activation? Is basal cell differentiation dependent on glycitein-induced ERK1/2 activation in the prostate? Is glycitein able to inhibit or delay the loss of the basal epithelium *in vivo* and in humans? How are glycitein and other methylated isoflavones metabolized in the prostate? Are metabolites more bioactive than the parent compounds? Is the metabolism of isoflavones altered during different stages of PCa development? What is the mechanism of isoflavone metabolism in the prostate?

Answers to these questions may provide better insight and strengthen our knowledge as to how increased consumption of soy may protect against PCa during early stages of the carcinogenic process.

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