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Pomegranate Juice and Pomegranate Derived Natural Products as Alternative Treatment for Cancer Progression and Metastasis

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DEDICATION

This dissertation is dedicated to my wife and my parents. Thank you so much for your endless support and love. I love you.

ABSTRACT OF THE DISSERTATION

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by

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Prostate and breast cancer are the second leading cause of cancer death in American men and women respectively. About 1 man in 6 will be diagnosed with prostate cancer and 1 in 8 women will develop invasive breast cancer during their lifetime. Conventional treatments such as surgery and chemotherapy are available but with severe side effects. Recently, evidences from preclinical and clinical studies demonstrate the potential of pomegranate juice (PJ) as natural remedy to fight cancer. I took an integrative approach by using various cellular assays to study important cellular processes for metastasis, Affimetrix gene arrays to study gene expression, microRNA PCR arrays to study the non-coding RNAs, molecules known to be disregulated in cancer cells, and Luminex multiplex immunoassays to study the level of pro-inflammatory cytokines/chemokines. We found that, in addition to causing cell

death, PJ increases cell adhesion and decreases cell migration of the prostate and breast cancer cells that do not die. PJ also inhibits the ability of the chemokine CXCL12, together with its receptor CXCR4 are critical in cancer metastasis, to chemoattract cancer cells. Since PJ is a very complex mixture of components, it is important to identify specific components that can mimic the effects of the juice. I found that the combination of a polyphenolic compound (luteolin/L), an antioxidant (ellagic acid/E) and a seed oil component (punicic acid/P), individually and in combination, synergistically affect processes critical for metastasis. L+E+P inhibits growth of prostate and breast cancer cells, their migration and their chemotaxis towards CXCL12. These components also increase the expression of cell adhesion genes and decrease expression of genes involved in cell migration and cell cycle control. The *in vivo* studies further support the potential of L+E+P. By using the Severely Combined Immuno-Deficiency (SCID) mouse model in which prostate cancer cells were injected subcutaneously, I show that L+E+P inhibits primary tumor growth, angiogenesis and, more importantly, significantly inhibits metastasis. Furthermore, I show that critical cellular processes for metastasis in human endothelial cells are affected by L+E+P. In conclusion, pomegranate and its components can potentially be used to prevent progression and metastasis of prostate and breast cancer.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Pomegranate fruit from the tree *Punica granatum* has been dubbed as the "nature's power fruit". In modern times, pomegranates are frequently used in the cuisine of Eastern and Middle Eastern countries. In the United States, fresh pomegranate fruit has become increasingly popular over the past decade. The fruit can be divided into three parts: the seeds, the juice, and the peel. Extracts of all parts of the fruit appear to have therapeutic properties. Pomegranate juice has the highest antioxidant capacity as compared to other polyphenol-rich beverages, such as red wine, grape juice, and green tea. The potent antioxidant properties of pomegranate juice have been thought to be responsible for various health benefits. Two major classes of Polyphenols have attracted the interest of researchers: flavonoids, particularly anthocyanidins (delphinidin, cyanidin, and pelargonidin), which are present in the peel or juice, and hydrolyzable tannins, including the ellagitannin, punicalagin, which is unique to pomegranate. Other flavonoids of potential anti-cancer effects include flavonols (kaempferol, quercetin), and flavones (luteolin). Punicic acid, the predominant component in the seed oil, has also been shown to possess anti-cancer property. Recent studies have shown that pomegranate selectively inhibit the growth of breast, prostate, colon, leukemia and lung cancer cells in culture. In preclinical animal studies, oral consumption of pomegranate extract inhibited growth of lung, skin, colon and prostate tumors.

I first review the recent findings on the effects of pomegranate juice and pomegranate extract on prostate cancer. I also discuss the effects of specific

components of the pomegranate fruit how they have been used to study the mechanisms involved in prostate cancer progression and their potential to be used in deterring prostate cancer progression (**Chapter 1**). By taking an integrative approach with various cellular assays to study processes important for metastasis, Affimetrix gene arrays to study gene expression, microRNA arrays to study the non-coding RNAs, and Luminex multiplex immunoassays to study the level of pro-inflammatory cytokines/chemokines, I investigate the cellular and molecular mechanisms of pomegranate juice induced anti-metastatic effect on prostate cancer cells (**Chapter 2**). Since pomegranate juice is a complex mixture of many components, I identify a combination of three components that can mimic the effects of the juice on cancer metastasis by taking a similar integrative approach (**Chapter 3**). I further study the effects of these identified natural products on prostate cancer metastasis by using mouse tumor models (**Chapter 4**). I then review recent findings on the effects of pomegranate and its components on breast cancer (**Chapter 5**) and finally I show that pomegranate juice and its components inhibit cell and molecular processes critical for metastasis of breast cancer (**Chapter 6**).

CHAPTER 1:

Pomegranate and its Components as Alternative Treatment for Prostate Cancer

Abstract

Prostate cancer is the second leading cause of cancer deaths in men in the United States. Although localized prostate cancer can be treated by surgery and/or radiation, when the cancer recurs, early stages can be controlled with hormone ablation therapy to delay the rate of cancer progression but, over time, the cancer overcomes its hormone dependence, becomes highly aggressive and metastasizes. There is a major need for less toxic but yet effective therapies to treat prostate cancer. Pomegranate fruit from the tree *Punica granatum* has been used for centuries for medicinal purposes and is described as "nature's power fruit". The unique biochemical composition of pomegranate fruit, being rich in antioxidant tannins and flavonoids, has recently drawn attention of investigators to study its anti-cancer effects. Recent research has shown that pomegranate juice (PJ) and/or pomegranate extracts (PE) significantly inhibit the growth of prostate cancer cells in culture. In preclinical murine models, PJ and/or PE inhibit growth and angiogenesis of prostate tumors. Results from clinical trials are also promising. PJ and/or PE significantly prolonged the prostate specific antigen (PSA) doubling time in patients with prostate cancer. In this review we discuss data on the effects of PJ and PE on prostate cancer. We also discuss the effects of specific components of the pomegranate fruit how they have been used to study the mechanisms involved in prostate cancer progression and their potential to be used in deterring prostate cancer progression.

Introduction

Prostate cancer (PCa) is the second-leading cause of cancer-related deaths in men in the United States. The American Cancer Society has estimated that a total of 238,590 new cases will be diagnosed and 29,720 men will die of PCa in 2013 (www.cancer.org). Various treatments are available, some more effective than others. To date there is no real cure for the disease beyond surgery and/or radiation when used at early stages of the disease. When recurrence occurs the cancer can be controlled with hormone ablation therapy (Leuprolide/Lupron®), taking advantage of the growth dependence of prostate cancer on testosterone. However, over time, the cancer develops ways to bypass hormone dependence, becoming highly aggressive, castration-resistant prostate cancer (CRPC) that metastasizes to the lung, liver and bone (Chuu et al., 2011; Stavridi et al., 2010). In addition to the hormone ablation, chemotherapy is available today to treat CRPC, but it is not very effective because PCa cells divide slowly and, like with prostatectomy, the treatments are aggressive and have many side effects (Hoffman-Censits and Fu, 2013). As a result, researchers are looking for novel strategies to treat PCa. FDA approved sipuleucel-T (Provenge®) is an autologous cellular immunotherapy to treat metastatic PCa. In the clinical trial on which this approval was granted, the median overall survival rate of patients who received sipuleucel-T improved by only 4.5 months. Treatment is costly but some patients survived much longer than the median (Higano et al., 2010). Novel androgen receptor (AR) antagonists such as enzalutamide (Xtandi®) and androgen biosynthesis

inhibitors such as abiraterone (Zytiga®), have shown great promise as androgen deprivation therapies to prolong overall survival rate among patients with metastatic PCa (Ryan et al., 2013; Sartor and Pal, 2013). Another novel drug, Cabozantinib, is a potent dual inhibitor of the tyrosine kinases MET and VEGFR2, and has been shown to reduce or stabilize metastatic bone lesions in CRPC patients (Smith et al., 2013; Yakes et al., 2011). These treatments against PCa are summarized in Fig 1.1. However, all of these treatments have adverse side effects.

More recently, there has been a renewed push to identify natural remedies to fight prostate cancer. Among the latter is pomegranate juice (PJ) and/or pomegranate extracts (PE). The pomegranate fruit is derived from the tree *Punica granatum*, is edible and is cultivated in Mediterranean countries, Afghanistan, India, China, Japan, Russia, and some parts of the United States (Langley, 2000; Longtin, 2003). Pomegranates have been used in folk medicine for centuries. They possess strong antioxidant, anti-inflammatory, anti-atherogenic effects, and some studies have suggested that they also may have anti-tumorigenic properties (Gil et al., 2000; Khan et al., 2008; Kim et al., 2002; Lansky and Newman, 2007). In fact, the antioxidant activity of pomegranates as been shown to be higher than that of red wine and green tea, two dietary substances that are showing promise in preclinical prostate cancer models and in patients with PCa (Noda et al., 2002). In this review we discuss data on the effects of PJ and PE on PCa cells in cell culture, in animal models and in clinical

trials as well as specific components of the pomegranate fruit and how they have been used to study the mechanisms involved of prostate cancer progression.

PJ and/or PE inhibit PCa cell growth

Considerable amount of evidence is showing that PJ and/or PE are capable of suppressing the growth of human PCa cell lines *in vitro*. About 10 years ago it has been shown that different anatomically discrete fractions of PE-induced cell death of three well-characterized PCa cell lines, LNCaP, PC3, and DU 145 (Albrecht et al., 2004). Each of these cell types has certain advantages as a model. LNCaP has functional androgen receptors and hence it is androgen sensitive and secretes prostate-specific antigen (PSA); PC3 cells are androgen independent and are highly invasive with strong metastatic potential; DU145 cells are also androgen independent and highly proliferative but with moderate metastatic capability. These investigators showed that PE inhibited proliferation, invasion through Matrigel and induced apoptosis of LNCaP, PC3 and DU145 cells (Albrecht et al., 2004). These findings suggest an overall significant anti-proliferative and pro-apoptotic action of PE against human PCa.

Shortly after these studies it was found that PE possesses anti-proliferative and pro-apoptotic effects through modulation of cyclin-dependent kinase (cdk) and the cdk inhibitor machinery in PC3 cells (Malik et al., 2005). They demonstrated that PE inhibited PC3 cell growth by disrupting the cell cycle regulatory molecules in the

G1-phase of the cell cycle. It is well-established that cell cycle progression is regulated by the cyclin and cdk complexes. Cyclins D and E are known to regulate cell cycle progression from G1 to S phase (Sanchez and Dynlacht, 2005). During the progression of the cell cycle, the cdk–cyclin complexes are inhibited via binding to cdk inhibitors such as the p21 and p27 proteins (Harper et al., 1993). These investigators showed that PE significantly down-regulated cyclin D1, D2 and E and cdk2, cdk4 and cdk6 and up-regulated p21 and p27, which may cause a blockage of G1-S phase transition, resulting in a G1-phase arrest and apoptosis (Malik et al., 2005; Malik and Mukhtar, 2006). Furthermore, apoptosis associated proteins such as cleaved PARP and Bax were also found to be up-regulated in PC3 cells by PE whereas apoptosis blocking proteins such as Bcl-2a were down-regulated. Bcl-2 is an upstream effector molecule in the apoptotic pathway and is identified as a potent suppressor of apoptosis. Bcl-2 has been shown to form a heterodimer complex with the pro-apoptotic member Bax, rendering it inactive (Oltersdorf et al., 2005). Therefore, the ratio of Bax to Bcl-2 is a decisive factor and plays an important role in determining whether cells will undergo death or survival. Importantly, in PE treated cells, the ratio of Bax to Bcl-2 was altered in favor of apoptosis (Malik et al., 2005; Malik and Mukhtar, 2006). We showed that 1% or 5% PJ inhibited the growth of PC3, DU145 and LNCaP cells in a dose-dependent manner (Wang et al., 2011). Collectively, these results suggest that PE inhibits the growth of PCa cells through cell

cycle arrest and stimulation of apoptosis. Therefore, PE may have chemopreventive as well as chemotherapeutic effects against PCa in humans.

In vivo studies using mouse xenograft tumor models have met with similar success. In these studies, human PCa cells were transplanted, either subcutaneously or orthotopically, into immunocompromised mice that do not reject human cells. Tumors were developed and the responses to PE and/or PJ treatment were studied. A single subcutaneous administration of PE before PC3 xenograft tumor implantation in nude mice significantly inhibited tumor growth (Albrecht et al., 2004). Similarly, oral consumption of PE inhibited androgen-sensitive CWR22Rv1 xenograft tumor growth in nude mice (Malik et al., 2005) and also inhibited androgen-independent LAPC4 xenograft tumor growth in severe combined immunodeficient (SCID) mice (Rettig et al., 2008). Mouse LAPC4 xenograft tumors are initially androgen-dependent and respond to castration but subsequently become androgen-independent after several weeks (Klein et al., 1997). They found that PE delayed the regrowth of LAPC4 androgen-independent xenograft tumors after castration. In addition, they observed that increase in NF-kB activity during the transition from androgen-dependent to androgen-independent in LAPC4 xenograft tumors, was abrogated by PE.

To evaluate the chemopreventive effects of PE and/or PJ in preclinical animal models, transgenic mouse models that more closely mimic human prostate cancer progression than xenograft models were used. Another advantage of transgenic models is that mice are immunocompetent, hence the tumor microenvironment can be

more accurately simulated. It has recently been found that PE inhibited tumor growth in transgenic adenocarcinoma of mouse prostate (TRAMP) mouse model (Adhami et al., 2012). In the TRAMP model, prostate cancer occurs as a result of the expression of the oncoprotein, SV40 T antigen, which is under the transcriptional control of androgen-regulated prostate-specific probasin promoter (Greenberg et al., 1995). TRAMP mice were fed orally with two doses of PE, equivalent to 250 and 500ml of PJ, and examined for tumor growth (Adhami et al., 2012). These investigators found that PE not only inhibited tumor growth but also enhanced the overall survival of the treated mice. The possible mechanisms by which PE could exert its effects include inhibition of PI3K/Akt/mTOR signaling pathways. It is well established that the downstream effector of PI3K, Akt, is commonly hyperactivated in various human cancers (Hay, 2005). mTOR, one critical downstream effector of Akt, is a kinase that functions as a master switch between catabolic and anabolic metabolism and therefore has emerged as therapeutic anti-cancer target (Faivre et al., 2006). Their findings suggest that PE and/or PJ inhibit PCa development and progression, possibly via targeting the PI3K/AKT/mTOR pathway. The possible mechanisms of the effects of pomegranate on PCa tumor growth are summarized in Fig 1.2. Taken together, the evidence described above establishes the strong potential of PE and/or PJ as chemopreventive agents against PCa.

Effects of PE and/or PJ on the metastatic potential of PCa

Several studies have shown that the invasive capabilities of metastatic cell lines are significantly reduced following PE and/or PJ treatment. The invasiveness was assessed by Matrigel invasion assay that calculates the percentage of PCa cells that are capable of migrating through the Matrigel membrane after PE and/or PJ treatment (Albrecht et al., 2004; Lansky et al., 2005). *The pattern of arachidonic* acid (AA) turnover rate in tumor cells has been shown to be an indicator of invasiveness and metastasis (Honn et al., 1981). The rate of AA turnover in prostate tumor cells is 10-fold higher than its rate in the surrounding normal cells (Chaudry et al., 1991). AA is released from membrane phospholipids by phospholipase A2 (PLA_2). It is then metabolized into prostaglandins and thromboxanes via the cyclooxygenase (COX) system (Hyde and Missailidis, 2009). Products of COX pathways that are secreted by tumor and/or host cells are thought to influence a variety of biological processes such as cell proliferation, cell movement, carcinogenesis, tumor promotion, and tumor metastasis (Khan et al., 2011; Piomelli, 1993; Schneider and Pozzi, 2011). The main product of AA metabolism in PCa cells is PGE_2 (Yang et al., 2012). PGE_2 has been reported to promote tumor progression by stimulating cancer cell survival and invasiveness through the activation of the PI3K/Akt pathway (Sheng et al., 2001). Also, the expression of cytosolic PLA_2 was increased in androgen-independent PCa cells (Ghosh and Myers, 1998; Patel et al., 2008). In addition, inhibitors which selectively inhibit PLA_2 or COX in the prostaglandin synthesis pathway were capable of inhibiting human PCa cell invasion (Attiga et al., 2000). These results suggest that

AA metabolism plays a role in human PCa cell invasion and implicates the involvement of PLA_2 and COX in a specific invasion associated signal cascade. Furthermore, it has been shown that PE significantly reduced the expression level of PLA_2 in PC3 cells (Lansky et al., 2005). Taken together these findings suggest that PE may suppress PCa cell invasion through blocking AA metabolism pathway.

The association of matrix proteolysis and cancer has long been recognized, and destruction of the basement membrane, an ECM structure that underlies epithelia, is a well-established hallmark of malignant invasion (Brinckerhoff and Matrisian, 2002; Kessenbrock et al., 2010). Matrix metalloproteinases (MMPs) are a family of highly homologous Zn2+ endopeptidases that collectively cleave extracellular matrix (ECM) molecules (Page-McCaw et al., 2007). Numerous studies have supported a role for MMPs in tumor invasion and metastasis (Friedl and Wolf, 2008; Overall and Lopez-Otin, 2002; Sternlicht and Werb, 2001). MMPs-2, -7, -9 and -11 have all been shown to contribute to tumor progression in studies using MMP-deficient mice (Nelson et al., 2000). Other studies have shown that MMP-9 was exclusively expressed by malignant prostate tissue and, in particular, by tumors that exhibit aggressive and metastatic phenotype (Hadler-Olsen et al., 2013; Hamdy et al., 1994). It has also been shown that inhibitors of both PLA_2 and COX were able to reduce the production of MMP-2 from PCa cells, indicating that the anti-invasive effect of blocking AA metabolism through the COX pathway is associated with the inhibition of MMPs in tumor progression (Attiga et al., 2000). PE and/or PJ inhibited the

ultraviolet (UV)-induced production of MMP-1, -2,-3,-7 and -9 in reconstituted human skin (Afaq et al., 2009) and inhibited the expression of MMP-1, -3, and -13 in human chondrocytes *in vitro* (Ahmed et al., 2005). Therefore, these studies suggest that the observed anti-invasive effect of PE and/or PJ might involve, at least in part, the reduction of MMP production by PCa cells.

Hyaluronan (HA), also known as hyaluronic acid or hyaluronate, is over-produced by many types of tumor and HA levels are prognostic for malignant progression (Aaltomaa et al., 2002; Auvinen et al., 2000; Lipponen et al., 2001). HA is frequently localized in the stroma of solid tumors, facilitating cell migration, tumor invasion and metastasis (Toole et al., 2005; Turley, 1992). HA interacts with cell surfaces through binding to specific cell-surface receptors, such as hyaluronan-mediated motility receptor (HMMR), to induce the transduction of a range of intracellular signals leading to numerous cellular responses, including those that involve tyrosine kinases, protein kinase C, focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase, nuclear factor-kB and RAS, as well as cytoskeletal components (Turley et al., 2002). Previous studies have revealed that the expression level of HMMR was highly stimulated in CRPC cells (Gust et al., 2009; Lin et al., 2008; Lin et al., 2007). A study using microarray analysis of gene profiles in prostate epithelium obtained from 50 PCa patients showed that major components of the HMMR signaling pathway, such as RhoA-activated kinase (ROK), were consistently overexpressed in metastatic PCa

cells (Lin et al., 2008). Recently, we showed that PJ or a combination of its components decreased cell migration, which is an important cellular process in metastasis of PC3 cells, and the inhibitory effect was mediated via down-regulating the expression of HMMR (Wang et al., 2011; Wang et al., 2012). Therefore, these findings suggest that the anti-metastatic effect of PJ might also occur via targeting HA signaling pathways in PCa cells.

It is known that, with time, cancer cells develop ways to bypass the need for testosterone and then cancer progresses very rapidly. Approximately 80% of patients who have died of advanced CRPC have clinical evidence of bone metastases and 100% have histologic bone involvement (Loberg et al., 2005; Roudier et al., 2003). It has been shown that the CXCL12/CXCR4 axis may play a critical role in the metastasis of prostate cancer to lung, liver and bone (Bubendorf et al., 2000; Furusato et al., 2010; Hirbe et al., 2010; Wang et al., 2006). Other studies showed that activated CXCL12/CXCR4 axis not only greatly increased the invasiveness but also induced the production of MMPs in PCa cells (Taichman et al., 2002). We have recently shown that PJ or a combination of its components inhibits chemotaxis towards CXCL12 in DU145, PC3 and LNCaP cells (Wang et al., 2011; Wang et al., 2012). These findings suggest that targeting the CXCL12/CXCR4 axis in PCa cells by PJ, may lead to inhibition of metastasis.

Recently, we showed that, in addition to causing cell death of androgen-independent PCa cells, PJ also increases cell adhesion and decreases cell

migration which are two important cellular processes involved in metastasis (Wang et al., 2011). PJ up-regulates genes involved in cell adhesion such as E-cadherin, intercellular adhesion molecule 1 (ICAM1) and Myristoylated alanine-rich protein kinase C (MARCKS) and down-regulates genes involved in cell migration such as type I collagen, tenascin C and chimerin 1. In addition, anti-invasive microRNAs such as miR-335, miR-205, miR-200, and miR-126, are up-regulated, whereas pro-invasive microRNA such as miR-21 and miR-373, are down-regulated. Moreover, we found that PJ significantly reduces the level of secreted pro-inflammatory cytokines/chemokines such as IL-6, IL-12p40, IL-1β and RANTES, thereby having the potential for decreasing inflammation and its impact on cancer progression. The possible mechanisms of the anti-metastatic effects of pomegranate are summarized in Fig 1.3. Taken together, these findings strongly suggest the potential of pomegranate as a chemopreventive agent to inhibit metastasis.

Specific components of pomegranate with known effects on PCa

The pomegranate fruit can be divided into three major anatomical components: the juice, the pericarp and the seeds. Anatomically discrete sections of the pomegranate fruit acting synergistically have been found to exert anti-proliferative and anti-invasive effects on PCa cells (Lansky et al., 2005). The juice and pericarp contain a rich complement of two types of polyphenolic components which have attracted interest for recent research: anthocyanins which give the juice its red color

(Hernandez et al., 1999), such as delphinidin, cyanidin and pelargonidin, and hydrolyzable tannins, such as the punicalagin and gallagic acid (Gomez-Caravaca et al., 2013; Lansky and Newman, 2007; Van Elswijk et al., 2004). Other polyphenolic components of possible interest include kaempferol, quercetin and luteolin (Ackland et al., 2005; Qu et al., 2012; Van Elswijk et al., 2004). The seed oil, which is comprised of 65–80% conjugated fatty acids, also contains many compounds of interest with known antioxidant and anti-cancer activities (Schubert et al., 1999). The predominant component among these fatty acids is punicic acid (Gasmi and Sanderson, 2010; Grossmann et al., 2010).

However, because PJ is a very complex mixture of components and is found in many different formulations, it is important to identify specific components that can replace the effects of the juice on growth and metastasis. Ellagitannin, the most abundant polyphenol present in PJ, is hydrolyzed to ellagic acid that is then converted to urolithin A by gut microflora (Heber, 2008; Seeram et al., 2006). Oral administration of ellagitannin-enriched PE not only inhibited LAPC4 xenograft tumor growth in SCID mice (Seeram et al., 2007) but also inhibited tumor-associated angiogenesis (Sartippour et al., 2008). In addition, ellagitannin inhibited the expression of androgen receptor (AR) and androgen synthesizing enzymes, such as 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) and steroid 5α reductase type I (SRD5A1), in PCa cells (Hong et al., 2008). Ellagic acid was shown to possess anti-tumorigenic activities on lung, cervical, colon, breast and prostate cancer cells

(Castonguay et al., 1997; Losso et al., 2004; Narayanan et al., 1999). Recently it was shown that ellagic acid and its metabolite urolithin A synergistically inhibited cell growth and induced apoptosis in DU145 and PC3 cells (Vicinanza et al., 2013). These investigators also found that ellagic acid is more effective than urolithin A.

Several studies have shown anti-proliferative effects of luteolin on human squamous, liver and colon cancer cells (Huang et al., 1999; Lim do et al., 2007; Selvendiran et al., 2006). Using a Matrigel invasion assay, it was shown that luteolin inhibits invasion of PC3 cells via increasing the expression of E-cadherin (Zhou et al., 2009). It is established that decreased expression of E-cadherin, one important cell-cell adhesion molecule (Berx and van Roy, 2009; Perez-Moreno et al., 2003), results in a loss of cell-cell adhesion and increased cell invasion (Thiery, 2002). The investigator found that luteolin induced expression of E-cadherin and knockdown of E-cadherin reversed the effect of luteolin on invasion of PC3 cells. They also found that intraperitoneal administration of luteolin three times a week reduced lung metastasis of PC3 xenograft tumors in nude mice (Zhou et al., 2009). In addition, luteolin has been shown to inhibit PCa tumor growth via targeting angiogenesis. After primary xenograft PC3 tumors have developed in nude mice, they found that intraperitoneal administration of luteolin for 16 days not only inhibited tumor growth but also reduced the number of blood vessels in the tumor (Pratheeshkumar et al., 2012).

Punicic acid, the major component of pomegranate seed oil (70-80%), has been shown to possess anti-cancer effects on PCa. It inhibits cell growth of androgen-dependent LNCaP cells stimulated by dihydrotestosterone (DHT) and inhibited DHT-stimulated androgen receptor nuclear accumulation as well as the expression of androgen receptor-dependent genes (Gasmi and Sanderson, 2010). These investigators also found that punicic acid induced apoptosis via a caspase-dependent pathway in LNCaP cells.

Recently, we showed that luteolin (L), ellagic acid (E) and punicic acid (P), individually and in combination, synergistically affect processes important for metastasis. L+E+P in equal amounts inhibits the growth of hormone-dependent and -independent PCa cells, their migration and their chemotaxis towards CXCL12, a factor that is important in PCa metastasis (Wang et al., 2012). The combination of these components also increases the expression of cell adhesion genes and decreases expression of genes involved in cell cycle control and cell migration. Furthermore, we found that L+E+P increases several well-known anti-invasive miRNAs, such as miR-200c and miR-335, while decreasing several oncogenic miRNAs, such as miR-21 and miR-29b. We have also shown similar anti-metastatic effects of L+E+P on breast cancer cells (Rocha et al., 2012).

Based on these results in cell culture, we investigated whether L+E+P inhibits PCa metastasis *in vivo*. We used a SCID mouse model in which luciferase-expressing human PCa cells were injected ectopically in the region of the prostate. One

advantage of this model is that tumor growth and metastasis can be monitored by bioluminescence imaging. We found that L+E+P significantly inhibited PC-3M-luc primary tumor growth and that none of the tumors treated with L+E+P metastasized. One disadvantage of using this model to study metastasis is the relative low metastasis incidence in untreated mice. However, It has been shown that inhibition of the PTEN/PI3K pathway combined with activation of the Ras/MAPK pathway promotes prostate cancer metastasis (Mulholland et al., 2012). Therefore, we further tested the effects of L+E+P on highly invasive *Pten*$^{-/-}$;*K-ras*^{G12D} PCa cells and found that L+E+P not only inhibited tumor growth but also inhibited lung and liver metastasis. L+E+P also significantly inhibited the CXCL12/CXCR4 axis for metastasis *in vivo*, consistent with our findings *in vitro*. In addition, we found that L+E+P inhibited angiogenesis in PC-3M-luc and *Pten*$^{-/-}$;*K-ras*^{G12D} tumors. To further investigate the mechanisms of the anti-angiogenic effect of L+E+P, we showed that L+E+P decreased human endothelial cell migration and adhesion, disrupted endothelial tube formation and inhibited the production of the angiogenic factors IL-8 or VEGF by the tumors. The possible mechanisms of the anti-metastatic effects of L+E+P are summarized in Fig 1.4. These results show that L+E+P can inhibit PCa progression/metastasis, indicating potential use of this combination of natural products for treatment of PCa in humans.

Clinical trials of PE and/or PJ on PCa

To investigate the effects of PJ consumption on PCa progression in men, a phase II clinical trial for men with rising PSA after surgery or radiotherapy was conducted in 2006 (Pantuck et al., 2006). Patients were treated with 8 ounces of PJ daily until disease progression. During the trial, there were no serious adverse events reported and the treatment was well tolerated. Mean PSA doubling time significantly increased with treatment from a mean of 15 months at baseline to 54 months post-treatment ($P<0.001$). They also found that PJ treatment suppressed cell proliferation and increased apoptosis in prostate cancer cell line LNCaP. In addition, PJ treatment increased serum nitric oxide and reduced the oxidative state and sensitivity to oxidation of serum lipids in patients. No patients developed metastases.

More recently in 2013, another phase II clinical trial of PCa patients with rising PSA received 1g (comparable to about 8oz of PJ) or 3g of PE daily for up to 18 months. PSA doubling time lengthened more than 6 months from 11.9 to 18.5 months ($P<0.001$) with no significant difference between dose groups (Paller et al., 2013). Again, no patient developed metastases. The statistically significant prolongation of PSA doubling time and the lack of metastatic progression in any of the patients in both of these studies, strongly suggests the potential of PJ for treatment of PCa.

Although extremely promising, a major drawback of these two clinical trials was the absence of a proper placebo control group. A recent randomized double blind study showed that men with PCa prior to radical prostatectomy were given PE daily

for up to 4 weeks. No serious side effects were observed and the treatment was well tolerated. The level of 8-hydroxy-2-deoxyguanosine (8-OHdG), an oxidative stress biomarker, was compared between patients in treatment and placebo group. PE lowered the level of 8-OHdG but the difference was not statistically significant, suggesting the need for larger and longer studies in the future (Freedland et al., 2013).

SUMMARY AND CONCLUSION

In summary, the biological activity of pomegranate-derived products, especially the chemotherapeutic and chemopreventive properties, has being investigated in cell, animal and clinical studies. The findings discussed in this review show that pomegranate and its components interfere with multiple biological processes involved in tumor growth, angiogenesis and metastasis of PCa. Therefore, further studies are warranted, including clinical trials using well-characterized and standardized amounts of PJ, PE and specific components as primary or adjuvant therapy in men with PCa. Many of the molecular mechanisms involved in these processes are amenable to drug treatment and to the development of small inhibitory molecules and therefore allow for combination therapy. Therefore, pomegranate and its components can potentially be used to prevent development and progression of PCa as well as other cancers.

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Figure 1.1. Summary of current treatments against prostate cancer: The current treatments include surgery, radiation, chemotherapy, androgen-deprivation therapy (ADT), immunotherapy, and targeted therapy.

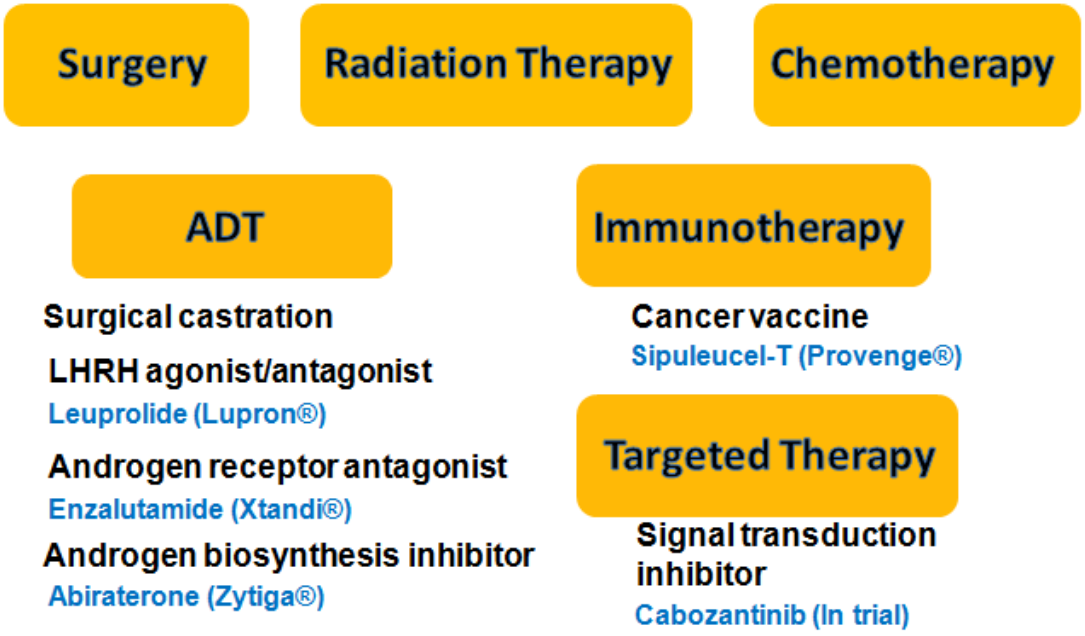
Surgery
Radiation Therapy
Chemotherapy
ADT
Surgical castration
LHRH agonist/antagonist
Leuprolide (Lupron®)
Androgen receptor antagonist
Enzalutamide (Xtandi®)
Androgen biosynthesis inhibitor
Abiraterone (Zytiga®)
Immunotherapy
Cancer vaccine
Sipuleucel-T (Provenge®)
Targeted Therapy
Signal transduction inhibitor
Cabozantinib (In trial)

Figure 1.1

Figure 1.2. Possible mechanisms of the effects of pomegranate on tumor growth: Possible mechanisms include inducing cell cycle arrest and apoptosis, affecting the level of cancer-related miRNAs, decreasing the level of pro-inflammatory cytokines/chemokines, targeting the canonical signaling pathways, and inhibiting angiogenesis.

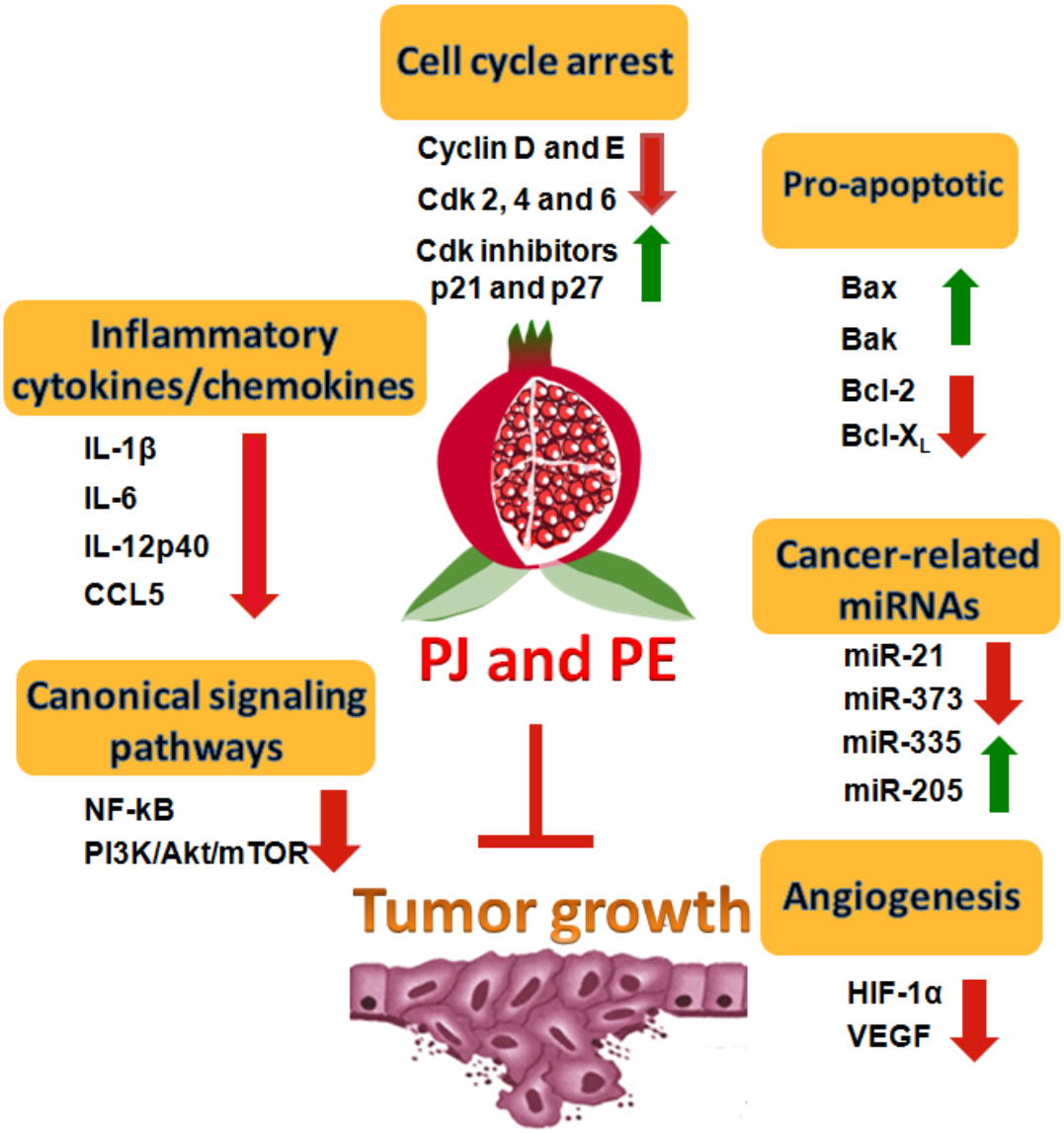
Cell cycle arrest
Cyclin D and E
Cdk 2, 4 and 6
Cdk inhibitors
p21 and p27
Pro-apoptotic
Bax
Bak
Bcl-2
Bcl-XL
Inflammatory cytokines/chemokines
IL-1β
IL-6
IL-12p40
CCL5
PJ and PE
Cancer-related miRNAs
miR-21
miR-373
miR-335
miR-205
Canonical signaling pathways
NF-kB
PI3K/Akt/mTOR
Tumor growth
Angiogenesis
HIF-1α
VEGF

Figure 1.2

Figure 3. Possible mechanisms of the anti-metastatic effects of pomegranate. Possible mechanisms include increasing cell adhesion, decreasing cell migration and CXCL12 chemotaxis, targeting hyaluronan and arachidonic acid pathway, and reducing matrix metalloproteinases (MMPs) production.

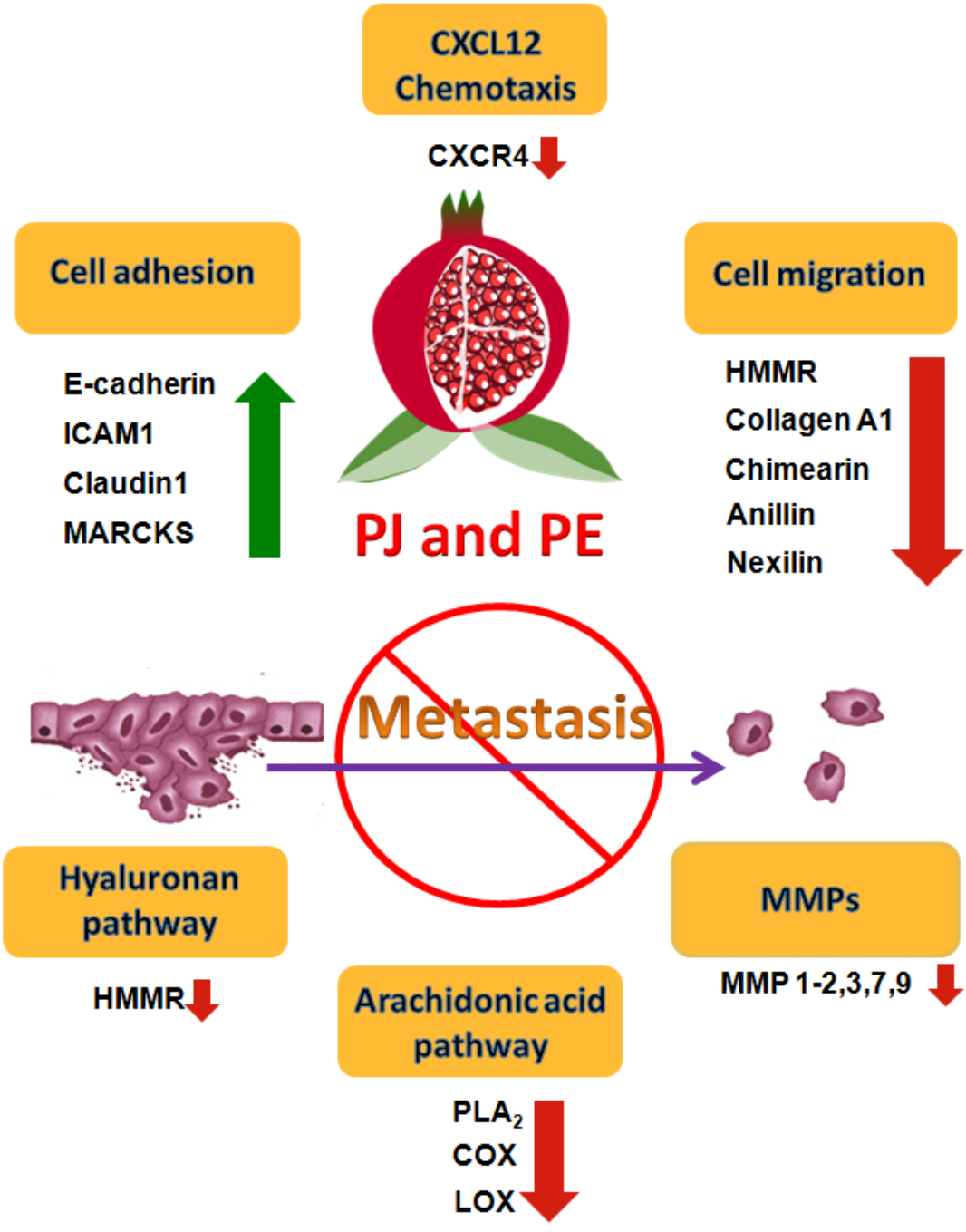
CXCL12
Chemotaxis
CXCR4
Cell adhesion
E-cadherin
ICAM1
Claudin1
MARCKS
PJ and PE
Cell migration
HMMR
Collagen A1
Chimearin
Anillin
Nexilin
Metastasis
Hyaluronan pathway
HMMR
Arachidonic acid pathway
PLA2
COX
LOX
MMPs
MMP 1-2,3,7,9

Figure 1.3

Figure 1.4. Possible mechanisms of the anti-metastatic effects of L+E+P: Possible mechanisms include increasing cell adhesion, decreasing cell migration and CXCL12 chemotaxis, inhibiting proliferation, inhibiting angiogenesis, and inhibiting epithelial-mesenchymal transition (EMT).

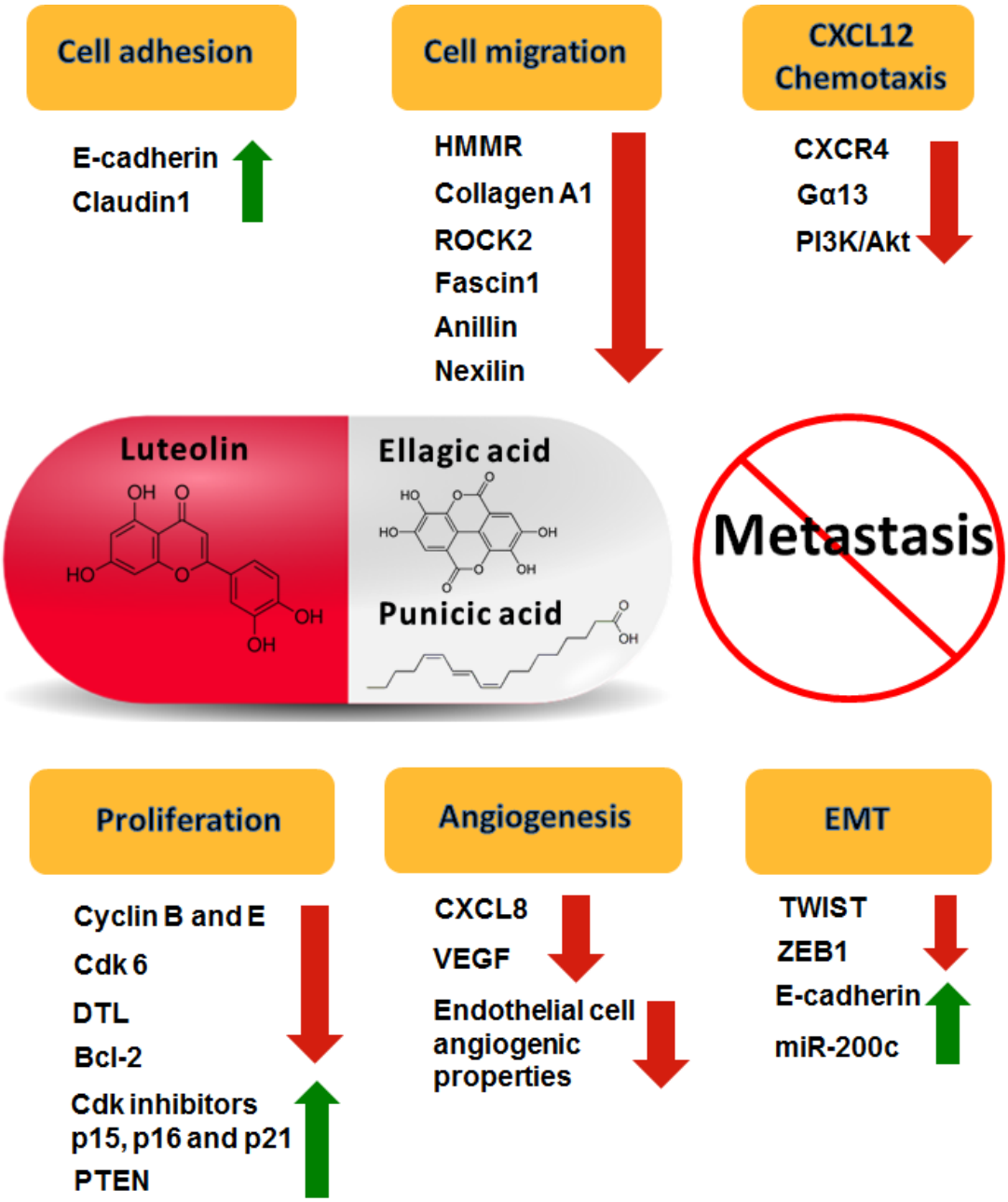
Cell adhesion
E-cadherin
Claudin1
Cell migration
HMMR
Collagen A1
ROCK2
Fascin1
Anillin
Nexilin
CXCL12
Chemotaxis
CXCR4
Gα13
PI3K/Akt
Luteolin
Ellagic acid
Punicic acid
Metastasis
Proliferation
Cyclin B and E
Cdk 6
DTL
Bcl-2
Cdk inhibitors
p15, p16 and p21
PTEN
Angiogenesis
CXCL8
VEGF
Endothelial cell
angiogenic
properties
EMT
TWIST
ZEB1
E-cadherin
miR-200c

Figure 1.4

CHAPTER 2:

Cellular and Molecular Mechanisms of Pomegranate Juice-Induced Anti-Metastatic Effect on Prostate Cancer Cells

Abstract

Prostate cancer is the second leading cause of cancer-related deaths among U.S. males. Pomegranate juice (PJ), a natural product, was shown in a clinical trial to inhibit progression of this disease. However, the underlying mechanisms involved in the anti-progression effects of PJ on prostate cancer remain unclear. Here we show that, in addition to causing cell death of hormone-refractory prostate cancer cells, PJ also increases cell adhesion and decreases cell migration of the cells that do not die. We hypothesized that PJ does so by stimulating the expression and/or activation of molecules that alter the cytoskeleton and the adhesion machinery of prostate cancer cells, resulting in enhanced cell adhesion and reduced cell migration. We took an integrative approach to these studies by using Affimetrix gene arrays to study gene expression, microRNA arrays to study the non-coding RNAs, molecules known to be disregulated in cancer cells, and Luminex Multiplex array assays to study the level of secreted pro-inflammatory cytokines/chemokines. PJ up-regulates genes involved in cell adhesion such as E-cadherin, CD44, and Myristoylated alanine-rich protein kinase C (*MARCKS*) and down-regulates genes involved in cell migration such as type I collagen, tenascin C and chimerin 1. In addition, anti-invasive microRNAs such as miR-335, miR-205, miR-200, and miR-126, were up-regulated, whereas pro-invasive microRNA such as miR-21 and miR-373, were down-regulated. Moreover, PJ significantly reduced the level of secreted pro-inflammatory cytokines/chemokines such as IL-6, IL-12p40, IL-1 and RANTES, thereby having the potential of decreasing inflammation and its impact on cancer progression. PJ also inhibits the ability of the chemokine SDF1 to chemoattract these cancer cells. SDF1 and its receptor CXCR4 are important in metastasis of cancer cells to the bone. Discovery of the mechanisms by which this enhanced adhesion and reduced migration are accomplished can lead to sophisticated and effective prevention of metastasis in prostate and potentially other cancers.

Introduction

Prostate cancer is the second most common cause of cancer-related mortality in U.S. men (13%)(Stavridi et al., 2010). Although localized prostate cancer can be successfully treated by surgery and/or radiation, this is not the case for most locally advanced or widespread disease. Initially, these prostate cancer situations can be controlled with hormone ablation therapy, taking advantage of the growth dependence of prostate cancer on testosterone. However, over time, the cancer develops ways to bypass dependence on circulating hormone (androgen), becoming castration resistant prostate cancer (CRPC) that is highly aggressive and metastatic (Coffey and Pienta, 1987). Chemotherapy has been available for a number of years to treat hormone refractory prostate cancer but such treatment is usually reserved for the more advanced stages of tumor progression when the cancer is already metastasized to the bone. This is so because prostate cancer cells don't grow rapidly until they reach the bone marrow and hence do not respond as well as other cancers to the chemotherapeutic agents. This, coupled with the very serious side effects of chemotherapy, makes it the treatment of choice primarily for advanced cancers (Schrijvers et al., 2010).

As a result, researchers have turned to developing novel strategies to fight prostate cancer. One type of treatment involves developing immunotherapies such as vaccines against PSA (prostate specific antigen), a protein specifically produced by prostate cells, both normal and cancerous. FDA approved therapeutic cancer vaccine

Sipuleucel-T (Provenge®) has been shown to considerably improve the overall survival of some patients, but with a median survival rate of only 4.1 months. Moreover, there was no effect on time of progression in patients who were asymptomatic or with limited metastatic disease and treatment is costly(Higano et al., 2010). Nevertheless, this vaccine is important because it represents a prototype that can be used to develop other cancer vaccines. PROSTVAC-VF, a PSA-based vaccine that uses a small pox-related virus has also been tested in men with metastatic disease and was shown to have an effect on survival(Schenk, 2010). These findings indicate that immunotherapy is promising.

Another very promising therapy is related to the development of novel anti-androgen agents. For example, abiraterone an irreversible inhibitor of CYP17A1 lyase (an enzyme critical for synthesis of androgen in the testes, adrenal glands and prostate tissue) has proved to be effective in improving survival(Shah and Ryan, 2009). Furthermore, Androgen Receptor (AR) antagonist MDV3100 is a very potent inhibitor of AR; it binds to this receptor with a much higher affinity than bicalutamide (Casodex), the currently prescribed androgen receptor inhibitor(Scher et al., 2010).

Recently, there has been a renewed push to identify natural remedies to fight prostate cancer. Among the latter is pomegranate juice (PJ). In a phase II clinical trial performed at the University of California Los Angeles (UCLA), patients with rising PSA were given 8 ounces of pomegranate juice by mouth daily until disease progression. PSA doubling time significantly increased with treatment from a mean of

15 months at baseline to 54 months post-treatment ($P < 0.001$)(Pantuck et al., 2005). Although extremely promising, a major drawback of this study was the absence of a proper placebo control. Nevertheless, statistically significant prolongation of PSA doubling time suggested a potential of pomegranate juice for treatment of human prostate cancer.

As a result of these findings in humans, several studies have shown that PJ has anti-proliferative and pro-apoptotic effects(Albrecht et al., 2004; Rettig et al., 2008; Syed et al., 2008) and that those effects occur through modulation of cyclin kinase inhibitor(Malik et al., 2005)**.** One study using the Matrigel invasion assay showed the capability of PJ to significantly reduce the invasiveness of prostate cancer cells *in vitro*(Lansky et al., 2005). The latter investigators showed that pomegranate juice decreases the levels of phospholipase A2 (PLA_2), which is the enzyme responsible for prostaglandin production in the arachidonic acid (AA) metabolic pathway(Chaudry et al., 1991; Honn et al., 1981; Nie et al., 2001; Piomelli, 1993). Inhibitors of PLA_2 are able to reduce the production of matrix metalloproteinase 2 (MMP-2) by prostate cancer cells(Attiga et al., 2000). Numerous studies support a critical role for MMPs in tumor metastasis(Brinckerhoff and Matrisian, 2002; Hamdy et al., 1994; Kessenbrock et al., 2010). In addition, one component of PJ was shown to stimulate the expression of E-cadherin(Zhou et al., 2009a), a molecule that is important in adhesion of epithelial cells(Thiery, 2002).

Despite these findings, very little is known about the cellular and molecular mechanisms affected by PJ that deter cancer progression, in particular in processes leading to metastasis. We hypothesized that PJ inhibits the migratory and metastatic properties of hormone refractory prostate cancer cells by stimulating the expression and/or activation of molecules that alter the cytoskeleton and the adhesion machinery of the cell, resulting in enhanced cell adhesion and reduced cell migration. To test this possibility we have used prostate cancer cell lines that are hormone refractory and invasive (DU145 and PC3) and applied an integrative approach to discovering the genes, miRNAs and proteins involved in PJ inhibition of prostate cancer progression to uncover pathways towards mechanistic understanding and more sophisticated and effective treatments of the disease.

Materials and Methods

Materials

DU145 and LNCaP prostate cancer epithelial cell lines were purchased from ATCC (Manassas, VA). PC3 prostate epithelial cell line was a gift from A. Walker (UC Riverside). pcDNA 3.1 HMMR vector was a gift from E. Turley (University of Western Ontario). RPMI 1640 media was acquired from Mediatech (Manassas, VA) and fetal bovine serum from Sigma Aldrich (St.Louis, MO). Stromal Derived Factor 1 alpha (SDF1□) was obtained from ProSpec (Boca Raton, FL). Human Genome U133Plus 2.0 Arrays were purchased from Affymetrix (Santa Clara, CA) and the RNeasy RNA Isolation Kit for RNA preparation from Qiagen Inc. (Valencia, CA). The Luminex Multiplex assays were obtained from Millipore (Billerica, MA). E-cadherin Ab was from Cell signaling (Danvers, MA). ICAM1 Ab and Tropomyosin1 Ab were purchased from Developmental Studies Hybridoma Bank (Iowa city, Iowa). Secondary Ab was from Thermo Scientific (Rockford, IL). Detection was done using Supersignal West Dura kit from Thermo Scientific (Rockford, IL). Transfection reagents Lipofectamin^{TM} 2000 were purchased from Invitrogen (Carlsbad, CA). AposcreenTM Annexin V-FITC kit from Southern Biotech (Birmingham, AL). All oligonucleotide primers for qPCR were obtained from IDT (Coralville, IA) and the qPCR iQ SYBR green supermix kit from Bio-Rad (Hercules, CA). CXCR4 specific inhibitor AMD3100 was obtained from Sigma Aldrich (St.Louis, MO). Synthesized RNA duplexes of microRNA mimics and

microRNA inhibitors were purchased from Thermo Scientific (Rockford, IL). E-cadherin siRNA was purchased from Abnova (Taiwan, China). The pomegranate juice was purchased from POMx Wonderful.

Cell culture

DU145 and PC3 are hormone-independent prostate cancer epithelial cell lines. LNCaP is an androgen-responsive prostate cancer epithelial cell line. Cells were cultured at 37°C with 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 I.U/ml) and streptomycin (100 μg/ml) and used at the times indicated in the results.

Adhesion assay

$3x10^5$ DU145, PC3 and/or LNCaP cells were plated on gelatin-coated 6-well plates (B&D Biosciences), allowed to adhere and 24hrs later treated with 1% and/or 5% PJ for 12 hrs and/or 24 hrs. Cells were then trypsinized and the time required to detach all cells was recorded as an indicator of cell adhesiveness. The juice was sterilized by filtration. The filtrate was then centrifuged at 13,000 rpm for 5 min, the supernatant collected to remove any particulate matter and then frozen in small aliquots to reduce the numbers of freeze-thaw cycles that can potentially damage the contents of the juice.

Migration assay

Confluent DU145 and PC3 cells were scratch wounded using a rubber scraper, washed and treated with 1% or 5% PJ. Cell migration was determined by measuring

the distance migrated by the cells from the wounded edge to the leading edge of migration at 12hr, 24h, 48h and 72h after treatments were initiated. Scraped cells without treatment were used as controls.

Chemotaxis assay

The upper side of 8µm pore size polycarbonate membranes of transwells (BD Biosciences, San Jose, CA) were coated with 50 ng/ml type I collagen (Sigma Chemical Co.). DU145, PC3 and/or LNCaP cells ($1x10^5$) in 100 µl culture medium were plated on the upper side of transwell membranes and were allowed to adhere for 3 hours. Then the wells were introduced into 24-well plate and 1000 µl RMPI 1640 with 10% FBS medium was added to lower chamber. Cells were treated with 1% and/or 5% PJ for 12 hours. SDF1α (100ng/ml) was added to the lower chamber and the cells were allowed to migrate for 4 hours at 37°C. The cells on the side of the membrane facing the upper chamber were removed with a cotton swab, and the membranes were then fixed and stained with 2% toluidine blue in 4% paraformaldehyde. Cells were counted in 8 high power fields (HPF)/filter to obtain the average number of cells per field.

Total RNA extraction

DU145 cells were treated with PJ for 12 hrs and total RNA was extracted using the RNeasy RNA isolation Kit according to manufacturer's protocol. Briefly, cells were washed with ice-cold 1X PBS, and lysed on ice with lysis buffer. Cell lysates were

then spun at 12,000rpm for 5 min to remove cell debris, followed by organic extraction to remove proteins. Then lysates were loaded into isolation columns and the final RNA product was dissolved in nuclease-free water. RNA quality was assessed on the Agilent Bioanalyzer 2100 using the Agilent RNA 6000 Nano Assay kit (Agilent Technologies, Waldbronn, Germany) and the concentration determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE USA).

Affymetrix microarray and data analysis

Affymetrix Human Genome U133 Plus 2.0 Arrays, which contain more than 54,000 probe sets representing approximately 38,500 genes and gene sequences, were used. Cells were treated with 5% PJ for 12 hrs and total RNA was extracted and evaluated as described above. Capillary electrophoresis using an Agilent Bioanalyzer 2100 to confirm the RNA quality levels was used before performing the array assays. RNA from cells without treatment was used as control. A single $\log_2$ expression measure for each probe set was calculated from image files (CEL format) using the robust multi-array analysis (RMA) procedure using Agilent GeneSpring GX software. The changes of expression level between untreated and specific PJ components treated sample were compared. Only genes that were over- or under-expressed by >2-fold were considered. Once we identified genes of potential interest, we verified their increase or decrease in expression by RT-qPCR.

Real time quantitative PCR

1μg RNA was reverse-transcribed to cDNA by RETROscript Reverse Transcription Kit (Ambion) at 44°C for 1hr and 92°C for 10min. 2μl of cDNA from the reverse transcription reaction were added to 23μl real-time quantitative PCR mixture containing 12.5μl 2**x** SYBR Green SuperMix (Bio-Rad) and 200nM oligonucleotide primers. PCRs were carried out in a Bio-Rad MyiQ5 Real-Time PCR Detection System (Bio-Rad). The thermal profile was 95°C for 3 min followed by 40 amplification cycles, consisting of denaturation at 95°C for 10 sec, annealing at 60°C for 30 sec. Fluorescence was measured and used for quantitative purposes. At the end of the amplification period, melting curve analysis was done to confirm the specificity of the amplicon. Fold changes of genes after treatment with PJ were calculated by the Pfaffl method to normalize the Ct values to the GADPH internal control. The following primer sequences were designed with IDT PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/) and used for the reactions:

GADPH, TCGACAGTCAGCCGCATCTTCTTT and ACCAAATCCGTTGACTCCGACCTT;

MARCKS, TTGTTGAAGAAGCCAGCATGGGTG and TTACCTTCACGTGGCCATTCTCCT;

ICAM1, ATAACCGCCAGCGGAAGATCAAGA and CGTGGCTTGTGTGTTCGGTTTCAT;

CLDN1, ATGGAAAGGGTGTTGGCATTGGTG and CACTTGGGTGTTTGAGCATTGCCT;

HMMR, ATTCAGTTGTCGAGGAGTGCCAGT and AGTGCAGCATTTAGCCTTGCTTCC;

COL1A1, CAATGCTGCCCTTTCTGCTCCTTT and CACTTGGGTGTTTGAGCATTGCCT;

CHN1, TGAAACTACTGCCACCTGCTCACT and TGGGTCCAAAGACGATTCCAAGGT;

PRCKE, CAACCAAGCAAGCTCTAACCGCAA and TTGTCCTGTAGGAAAGGCCCAGTT;

NEXN, TCAGCCCAAGACCACATAGAGCAA and TCTTTCTTCCCTGGCTCTCTGCAT;

ANLN, AGCTCACTCTTCTCACCAATGCCA and AAGCGGTACCAGGCTGTTCTTGTA.

MicroRNA arrays and data analysis

DU145 cells were treated with 5% pomegranate juice for 12h with the same condition used in the Affymetrix microarray. Total RNA was collected with RNeasy RNA Isolation Kit for microRNA arrays. RNA from cells without treatment was used as control. We then utilized SYBR based real-time PCR to quantify mature microRNA expression (Quantobio Technology). E. Coli polyA polymerase was used to add an adenine tail at the 3' end of RNA molecules that lack a polyA tail. Followed by the oligodT annealing, a universal tag was attached to the 3' end of cDNAs during the cDNA synthesis using retro-transcriptase superscript III (Invitrogen). With this universal tag, qPCR was performed with microRNA-specific forward primer and a reverse universal primer mix.

miRNA mimics, inhibitor, siRNA and vector transfection

DU145 and/or PC3 cells (80-90% confluent) were transfected with Lipofectamine 2000 (Invitrogen) following the manufacture's protocols. 60nM miR-21 miRIDIAN mimics, miR-335 miRIDIAN hairpin inhibitor (Thermo Scientific), 40nM E-cadherin chimera siRNA (Abnova) and 8μg/ml pcDNA3.1 HMMR vectors were transfected. Scratch wound assay was performed as described above, 24 hrs after transfection.

Immunoblotting

DU145 cells were treated with 5 % PJ for 12h, washed with ice cold 1XPBS, and lysed on ice with lysis buffer containing 0.5% Triton X100, 0.5% Nonidet P-40, 10 mM Tris, pH 7.5, 2.5 mM KCl, 150 mM NaCl, 30 mM b-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 0.1% SDS and additional protease inhibitor cocktails (Sigma). Protein concentrations were measured using the DC protein assay kit (Bio-Rad). Equal amounts of protein in the cell extracts were mixed with sample buffer, boiled, and analyzed using 10% acrylamide SDS-PAGE. Immunoblotting was performed with the E-cadherin polyclonal Ab (Cell signaling), ICAM1 polyclonal Ab, Tropomyosin1 polyclonal Ab (Hybridoma Bank) and the HRP-conjugated secondary Ab (Thermo Scientific), followed by incubation with West Dura extended-duration substrate (Thermo Scientific). Blots were then reprobed for histone 2A or GAPDH antibody to show equal loading of proteins.

Statistical Analysis

Data analysis was performed using the one-way ANOVA on raw data using GraphPad Instat software (GraphPad Software Inc.).

Results

Effect of Pomegranate Juice on growth, adhesion, migration and chemotaxis of prostate cancer cells

To set up and standardize the cell culture system to test the effects of PJ on hormone-refractory prostate cancer cell function, we used DU145 and PC3, which are two invasive hormone-independent prostate cancer cell lines. We treated these cells with 1% and 5% filtered pomegranate juice and measured the effect of the juice on cell growth over time (**Fig. 2.1**). Although at 12 hrs there was no measurable effect of the juice on cell growth, by 24 hrs it was clear that the 5% concentration was not allowing the cells to grow and by 48 hrs the cultures contained many floating cells, indicating that this PJ concentration caused the cells to die. In contrast, the cultures treated with 1% PJ continued to grow, although they showed some floating cells. Untreated cells (control) showed no signs of cell death. By 72 hrs, the cultures treated with 5% PJ still contained some cells that were attached to the dish but most of the cells were in suspension (**Fig. 2.1A,B**).

Despite the fact that many cells died with the treatments, especially with 5% treatment, in both sets of treated cultures there were living cells at the end of the experiment. We found that the remaining cells strongly adhered to the gelatin-coated substrate, as indicted by increased time to detach all cells from the culture dish in comparison with untreated cells; by 12 hrs of PJ treatment, the treated cultures required 4-5 times longer to be released by trypsinization and by 24 hrs the time to

release further increased (**Fig. 2.2**). At both time points the cells all still looked healthy and no floating cells were seen in any of the cultures.

The PJ-induced increase in adhesion of the cells that did not die suggested that this juice also might affect cell migration. Indeed, using the scratch wound assay, we found that both 1% and 5% pomegranate juice inhibited the migratory capabilities of these cells. We measured the distance that cells migrated from the wounded edge to the migration front and found it significantly reduced in the treated cells beginning as early as 12hrs after treatment. This pattern of delayed migration continued over time (**Fig. 2.3**).

Because the chemokine Stromal-Derived Growth Factor one alpha (SDF1α) is very important in attracting cancer cells to the bone marrow(Taichman et al., 2002)**,** we tested the possibility that PJ would inhibit chemotaxis towards SDF1α. We first tested whether the cancer cells respond to SDF1α and found that this chemokine chemoattracts these cells (**Fig. 2.4C,D**). This chemoattraction is specific because the specific inhibitor of its receptor, CXCR4, completely obliterates its effect on both cell types. When the cells were pre-treated with PJ for 12h prior to initiation of the chemotaxis assay towards SDF1α, we found that the juice significantly inhibited chemotaxis at both concentrations (**Fig. 2.4A,B**). Therefore, the fluid fraction of PJ has the potential to inhibit metastasis of these cells to bone marrow.

To determine whether these effects of PJ are also found in androgen-sensitive cells, we used LNCaP prostate cancer cells and performed similar studies using the

adhesion and chemotaxis to SDF1α assays. LNCaP cells are less sensitive to PJ-induced increase in adhesion because we see only approximately doubling of the release time for the cells from the substrate as with either DU145 or PC3 cells (**Fig. 2.5A**). In contrast, LNCaP cells are considerably more sensitive to PJ inhibition of SDF1α-induced chemotaxis (**Fig. 2.5B**).

Effect of Pomegranate Juice on the expression of genes involved in functions of the cytoskeleton and in cell adhesion

To understand how PJ inhibits these critical cellular processes involved in metastasis, we used an integrative approach to examine the effects of the juice on gene and miRNA expression and on production of pro-inflammatory cytokines. To study the effect of PJ on cell adhesion and migration related genes, total RNA obtained from DU145 treated with 5% of the aqueous faction of PJ for 12 hrs was analyzed for gene expression using Affymetrix U133 Plus 2.0 microarrays. This time point and this concentration were chosen because under these conditions changes in cell function are already occurring but no cell death is seen, as shown in Figs 2.1&2.2. This was important because we want to determine what genes are modulated by PJ before some of the cells became apoptotic. The data show that PJ stimulates the expression of several genes involved in cell adhesion and inhibits the expression of several genes involved in cytoskeleton function and in cell migration (**Table 2.1**). The genes that are increased significantly all enhance adhesion or are tumor suppressors.

Those that are decreased significantly all are related to migration or to cytoskeletal elements or ECM molecules that facilitate migration.

The reason we picked those genes from Affymetric microarray result is based on the hypothesis that PJ increases cell adhesion while decreases cell migration. Therefore, we focused on those genes with known functions involved in cell adhesion and cell migration. E-cadherin, claudin1 and Intercellular Adhesion Molecule 1 (*ICAM1*) are important tight junction proteins to stabilized cell-cell adhesion; Hyaluranan-mediated motility receptor/CD168 (*HMMR*) is a hyaluronan receptor involved in cell migration. It has been shown that HMMR is overexpressed in various cancer cells and activated HA signaling leads to increased cancer cell invasiveness[22].

To verify the effects of PJ on gene expression shown by Affymetrix mRNA arrays, we examined the mRNA level of Intercellular Adhesion Molecule 1 (*ICAM1*), Myristolytated alanine-rich protein kinase C substrate (*MARCKS*), Claudin 1 (*CLDN1*), Hyaluranan-mediated motility receptor/CD168 (*HMMR*), Collagen Type I alpha1 (*COL1A1*), Protein Kinase C epsilon (*PRCKE*), Anillin (*ANLN*), Nexilin (*NEXN*), N-chimearin (*CHN1*). We used quantitative RT-PCR with total RNA extracted from DU145 cells treated with 5% PJ for 12hrs (**Fig. 2.6A**). The mRNA fold change of these genes was highly consistent with our Affymetrix array results. Because of the complex regulation of E-cadherin expression we thought it to be important to examine the protein levels as well as the gene levels. For the protein levels we used immunoblot analysis with an antibody specific for E-cadherin and

found it to be increased 1.9-fold by 5% PJ after 24h treatment (**Fig. 2.6B**), a result very similar to the array result. In addition, to further confirm the increase in levels of other adhesion/migration proteins we evaluated ICAM1 and tropomyosin. Both proteins were significantly increased by PJ treatment (**Fig. 2.6C,D**).

To determine mechanistically if levels of expression correlate with function, we chose to inhibit E-cadherin (because loss of this adhesion protein is critical for invasion of epithelial tumor cells), and to over-express HMMR (the gene that showed the highest decrease in our gene array), to determine whether these treatments reversed the PJ effects on adhesion and migration, respectively. We find that the inhibitory effect of PJ on cell migration is partially reversed by E-cadherin siRNA and HMMR overexpression (**Fig.2.7**). Scratch wounds on DU145 and PC3 cells were made 24h after E-cadherin siRNA and HMMR vector transfection and the migrated distances were measured 36h later with and/or without 1%PJ treatments. We find that PJ treatment significantly decreases cell migration of untransfected cells but there is no significant difference between cell migration of untreated cells and E-cadherin siRNA (**Fig. 2.7A,B**) and HMMR vector (**Fig. 2.7C**) transfected cells. These results indicate that the effect of PJ on cell migration is mediated through increasing E-cadherin and decreasing HMMR in DU145 cells and through increasing E-cadherin in PC3 cells.

Effect of Pomegranate Juice on the level of microRNAs related to cell adhesion and migration

Micro-RNAs (miRNAs) are naturally-occurring small non-coding RNAs that function as negative regulators of gene expression. They regulate important cellular functions such as cell proliferation, apoptosis, differentiation and development(Calin and Croce, 2006). Mature miRNAs bind to target mRNAs which subsequently results in either direct cleavage of the targeted mRNAs or inhibition of translation. To determine the effects of PJ on adhesion and migration-related miRNAs, we used miRNA arrays to analyze RNA obtained from DU145 cells treated with 5% PJ for 12hrs (**Table 2.2**). We found that miRNAs that regulate the genes we identified as elevated were decreased (miRNA-21 and miRNA-373) whereas those that regulate genes that were decreased show increased expression (miRNA-335 and miRNA-205). An apparent exception to this pattern is the increase in miRNA-200 which regulates E-cadherin, a gene that we identified as having increased expression. This is because miRNA-200 inhibits a repressor for the E-cadherin gene, hence the increase of miRNA-200 is consistent with the pattern of the other miRNAs.

To demonstrate mechanistically if levels of expression of these miRNAs correlate with function, we chose to treat the cells with the mimics for miRNA-21 (because this miRNA is considered to be proinvasive) and to inhibit miRNA-335 (because this miRNA was the most over-expressed). Both of these treatments

reversed the ability of PJ to inhibit cell migration of both DU145 and PC3 cells (**Fig. 2.8 A-D**).

Effect of Pomegranate Juice on the level of selected cytokines and chemokines

Many proinflammatory cytokines and chemokines contribute to cancer progression. To determine the effect of PJ on production of some of these proteins, we analyzed the media collected from DU145 and PC3 cells treated with 1% or 5% PJ for 18 hours, by using Luminex Multiplex Array assays. We tested for the levels of the following pro-inflammatory proteins IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, TNFα, IP-10, and CCL5/RANTES. In both cell lines PJ decreased the levels of IL-6 and IL-12p40 (**Fig. 2.9A,B**) production. In addition, DU145 cells showed decrease in IL-1β (**Fig. 2.9C**) and PC3 cells showed marked decrease in CCL5/RANTES (**Fig. 2.9D**).

Discussion

The studies presented here delineate potential cellular and molecular mechanisms involved in the anti-metastatic effect of PJ on prostate cancer cells. We show that the fluid phase of the juice: (i) stimulates two prostate cancer cells to adhere strongly to the substrate; (ii) inhibits the migratory capabilities of these cells and chemotaxis toward SDF1α; (iii) stimulates expression of genes involved in cell adhesion while reducing expression of genes involved in cytoskeletal functions and in cell migration; (iv) increases the levels of adhesion enhancing miRNAs while reducing the level of pro-invasive miRNAs; (v) reduces the level of the pro-inflammatory cytokines/chemokines IL-6, IL-12p40, IL-1□ and RANTES. The results of this integrative approach are summarized in Figure 12.

During progression of prostate cancer, the epithelial cells of the prostate undergo hyperplasia, followed by prostatic intraepithelial neoplasm, which then develops into invasive adenocarcinoma that finally becomes metastatic leading to spread of the cancer cells primarily to the lymph nodes, bone marrow and lung. Metastatic cells undergo a transition from stationary to migratory. This transition involves loss of adhesion and rearrangement of cytoskeletal elements that allow the cells to move. Our results show that PJ deters that movement by increasing cell adhesion molecules and decreasing molecules that facilitate cell migration. By using an integrative approach to detect gene, miRNA and proinflammatory protein changes induced by the juice, we

have developed a model that may explain how PJ inhibits progression of prostate cancer to metastasis.

The gene microarray results show that expression of genes involved in the cell adhesion machinery are stimulated by PJ treatment whereas genes that stimulate migration are down-regulated. For example, E-cadherin and claudin 1 are important components of tight junction protein complexes that keep epithelial cells together; in this case the epithelial cells of the prostate where the cancer develops. Intercellular adhesion molecule-1 (*ICAM1*) is also important in stabilizing cell-cell interactions. Therefore, PJ might increase cell adhesion through up-regulation of these cell junction proteins preventing the cells from breaking away from the adenocarcinoma. PJ also up-regulates myristoylated alanine-rich protein kinase C substrate (*MARCKS*) which is an actin-binding protein that normally associates with the plasma membrane where vinculin and talin are present in focal adhesions(Aderem, 1992). Phosphorylation of *MARCKS* by protein kinase C inhibits its association with the plasma membrane leading to movement of the protein into the cytosol, resulting in cell spreading onto the substratum(Rosen et al., 1990). *MARCKS* also has been shown to play a role in cell adhesion and cell motility through regulation of the actin cytoskeletal structure(Arbuzova et al., 2002). Our gene microarray results also show that PJ down-regulates the expression of genes related to cell migration. *HMMR* functions as a hyaluronan (HA) receptor and the binding of HA to *HMMR* can stimulate the RhoA-activated protein kinase (ROCK) signal transduction pathway, leading to tumor

cell migration and invasion in various cancers(Lin et al., 2007). In addition, PJ down-regulates actin binding proteins anillin and nexillin which are involved in regulation of the structure of the cytoskeleton(Glotzer, 2005; Ohtsuka et al., 1998b).

MicroRNAs (miRNAs) have been shown to have profound impact on post-transcriptional gene regulation. Aberrant expression of miRNAs occurs in diverse types of human cancer and in different stages of disease progression(Shi et al., 2008; Wu et al., 2007). miRNA-335 has been identified as a metastasis suppressive miRNA in breast cancer by inhibiting type I collagen (*COLA1*) and tenascin C (*TNC*)(Tavazoie et al., 2008b). Type I collagen is an extracellular matrix molecule involved in cytoskeletal control and tenascin C is involved in the regulation of cell migration(Tsunoda et al., 2003). PJ might inhibit the expression of *COL1A1* and *TNC* in prostate cancer cells through up-regulation of the metastasis-suppressive miRNA-335. miRNA-205 has been shown to reduce cell invasion in prostate cancer by inhibiting PKCε (*PRKCE*) and N-chimearin (*CHN1*)(Gandellini et al., 2009). PKCε enhances migration and invasion and promotes autocrine cell-signaling events in prostate cancer cells(Wu et al., 2002). N-chimaerin is a GTPase-activating protein that, if down-regulated, can give rise to the loss of filopodia and reduction of cell migration(Yang and Kazanietz, 2007a). PJ could inhibit the expression of *PRKCE* and *CHN1* in prostate cancer cells through up-regulating miRNA205, which would in turn lead to reduced cell migration and invasion. The miRNA-200 family has been shown to inhibit *ZEB1* and *ZEB2*, which are transcriptional repressors of the E-cadherin

gene(Korpal et al., 2008b). Loss of E-cadherin is one of the molecular hallmarks driving epithelial-mesenchymal transition that is an important process to initiate metastasis(Birchmeier and Behrens, 1994). PJ might stimulate the expression of E-cadherin by inhibiting its transcriptional repression ZEB1 and ZEB through up-regulating miRNA-200 family. This suggestion is consistent with our mRNA microarray result that the expression level of E-cadherin is indeed increased by PJ. miRNA-126 was reported to reduce prostate cancer cell invasiveness by inhibiting prostein(Musiyenko et al., 2008). Prostein is a novel prostate-specific protein but its function is still largely unknown. miRNA-21 was previously identified as a pro-invasive miRNA by inhibiting tropomyosin 1 (*TMP1*), programmed cell death 4 (*PDCD4*) protein and myristoylated alanine-rich protein kinase C substrate (*MARCKS*)(Li et al., 2009; Zhu et al., 2008a). *TPM1* is an actin-binding protein and its overexpression suppresses cell invasion(Perry, 2001). *PDCD4* is a tumor suppressor protein that is capable of interacting with eukaryotic initiation factor 4A (EIF4A) to inhibit protein synthesis(Yang et al., 2003). Our mRNA microarray results show that the mRNA level of *PDCD4* and *MARCKS* are significantly down-regulated, which is consistent with inhibition by miRNA-21. Therefore, PJ might up-regulate *TMP1, PDCD4* and *MARCKS* through down-regulating miRNA-21. miRNA-373 was previously identified as a pro-invasive microRNA by targeting CD44, a transmembrane glycoprotein involved in cell adhesion and cell-stromal interactions(Huang et al., 2008). CD44 also functions as a hyaluoronan receptor in

addition to HMMR(Omara-Opyene et al., 2004). Therefore, the finding that PJ down-regulates miRNA-373 and HMMR suggests that the anti-metastatic effect of PJ may be partially due to inhibiting the hyaluoronan signaling pathway. In summary, PJ significantly up-regulates miRNA-335, miRNA-205 and the miRNA-200 family, all of which have been identified as metastasis suppressor miRNAs and significantly down regulated miRNA-21 and miRNA-373 that have been previously identified as pro-invasive miRNAs.

The molecular mechanisms of the anti-invasive effect of PJ are largely unknown. In addition to the genes and miRNAs we describe here that have the potential to mediate the PJ effects on adhesion and migration, we show that the PJ inhibition of prostate cancer cell migration occurs significantly through increasing E-cadherin and decreasing HMMR levels. We find that cells transfected with E-cadherin siRNA or with a vector that overexpresseses HMMR are no longer able to respond to PJ inhibition of migration. Moreover, we show that miR-21 mimics and a miR-335 inhibitor also partially reverse the inhibitory effect of PJ on cell migration.

It is well known that pro-inflammatory cytokines/chemokines and inflammation promote tumor growth and cancer progression. Among the 14 pro-inflammatory cytokines/chemokines examined with Luminex Multiplex Array assays, the secreted levels of IL-6, IL-12p40, IL-1β and RANTES are significantly reduced by PJ treatment. Elevated levels of IL-6 have been found in the tissues and serum of prostate cancer patients(Steiner et al., 2003) and tumor growth was inhibited by

anti-IL-6-antibody treatment(Culig et al., 2005). Moreover, IL-6 is one of the cytokines that regulates angiogenesis and proliferation in prostate cancer through regulation of VEGF(Steiner et al., 2004). Heterodimeric pro-inflammatory cytokine IL-12 is composed of two subunits. The IL-12p40 subunit can act as a chemoattractant for macrophages and promotes migration of dendritic cells(Cooper and Khader, 2007). It has also been shown that colon cancer metastasis is inhibited by anti-IL-12p40-antibody treatment(Yamamoto et al., 2008). IL-1 is a pleiotropic cytokine that primarily affects inflammatory and immune responses, and also has important influence on diseases and malignancies. It has been reported that secreted IL-1β present in the microenvironment of the tumor cells stimulates inflammation that promotes invasion of fibrosarcoma cells(Apte et al., 2006). RANTES (CCL5) is a potent chemotactic factor for T cells, monocytes and dendritic cells. Expression of RANTES and its receptor, CCR5, has been shown to correlate with prostate cancer progression. In addition, interaction of RANTES with CCR5 on the surface of cancer cells stimulates their invasive capabilities(Vaday et al., 2006). Our findings suggest that the observed anti-metastatic effects of PJ on prostate cancer cells are in part mediated through reducing the production of cancer-related pro-inflammatory cytokines and chemokines.

Conclusion and Future Prospects

To date, there is no cure for prostate cancer when recurrence occurs after surgery and/or radiation. In particular, when it recurs after hormone ablation therapy there are

no other effective treatments for deterrence of cancer progression. Here we have provided an overview of the myriad ways PJ appears to combat prostate cancer and perhaps other cancers, putting into perspective the job ahead. However, we know that there is a light at the end of the tunnel because the UCLA clinical trial has shown that at minimum PJ ameliorates prostate cancer progression and that there is a very high likelihood that it has a significant effect on metastasis in this disease. The data shown in this paper supports and enhances that view. Because PJ interferes with multiple biological processes of cancer cells, stimulation of cell death as shown by ourselves and others, as well as increase in cell adhesion, decrease in cell migration and suppression of pro-inflammatory cytokines and chemokines shown here, PJ holds the promise of preventing or at least slowing down metastasis of prostate cancer. Discovery of the mechanisms by which enhanced adhesion, reduced migration and decrease of pro-inflammatory molecules are accomplished can potentially lead to more sophisticated and effective treatments of the disease when the component(s) of the juice responsible for these effects are identified. Pursuit of many of these aspects will dominate the work in our laboratory for the foreseeable future. We have identified several important components within PJ that might be responsible for the anti-metastatic effect on prostate cancer cells. We have proposed to study the effect of these PJ components on prostate cancer metastasis in vivo by using several well-developed mouse models. However, the in vivo study is beyond the scope of this paper.

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Figure 2.1. Pomegranate Juice inhibits growth of hormone-independent prostate cancer cells. DU145 **(A)** and PC3 **(B)** prostate cancer cells were treated with 1% and 5% PJ and counted for increasing times after initiation of treatment. Controls represent no PJ. Media containing PJ was changed daily. Bars represent Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$. Repeated many times.

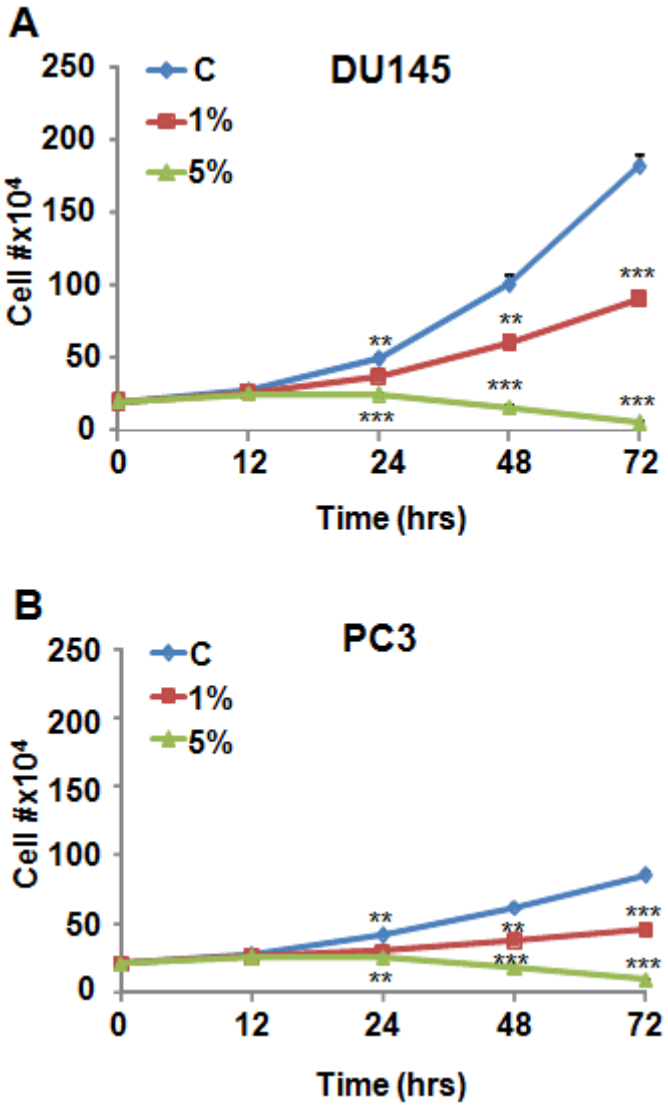
A
DU145
C
1%
5%
Cell #x10⁴
250
200
150
100
50
0
0
12
24
48
72
Time (hrs)
**
**
***
***
***
B
PC3
C
1%
5%
Cell #x10⁴
250
200
150
100
50
0
0
12
24
48
72
Time (hrs)
**
**
***
**
***
***

Figure 2.1

Figure 2.2 Pomegranate Juice stimulates hormone-independent prostate cancer cell adhesion. DU145 **(A)** and PC3 **(B)** cancer cells were plated on gelatin-coated dishes and 24 hrs later media was changed and the cells were treated with 1% or 5% Pomegranate Juice. We tested for adhesion to the substrate at 12 and 24hrs after initiation of treatment by recording the time it took for trypsinization to remove all of the cells from the dish. Control represents no Pomegranate Juice. Within each experiment, the times of trypsinization were the same within 1 minute for each specific treatment. Repeated many times.

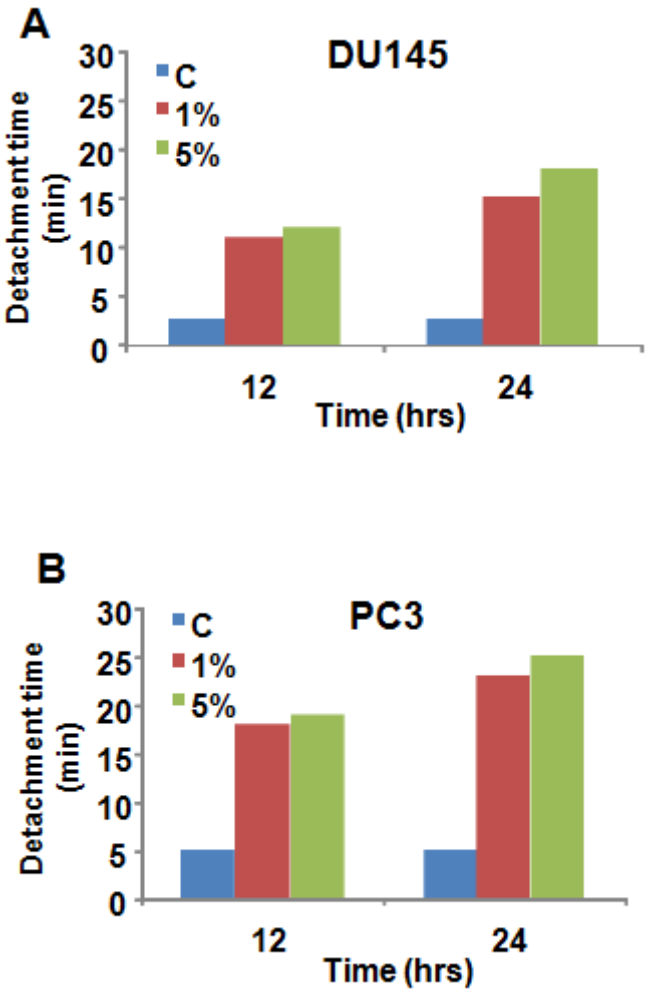
A
DU145
C
1%
5%
Detachment time (min)
30
25
20
15
10
5
0
12
24
Time (hrs)
B
PC3
C
1%
5%
Detachment time (min)
30
25
20
15
10
5
0
12
24
Time (hrs)

Figure 2.2

Figure 2.3. Pomegranate Juice inhibits hormone-independent prostate cancer cell migration. DU145 **(A)** or PC3 **(B)** prostate cancer cells were treated with 1%, and 5% PJ for 72 hrs and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. Controls represent no PJ. Media and PJ were changed daily. Bars represent Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. Repeated at least 3 times.

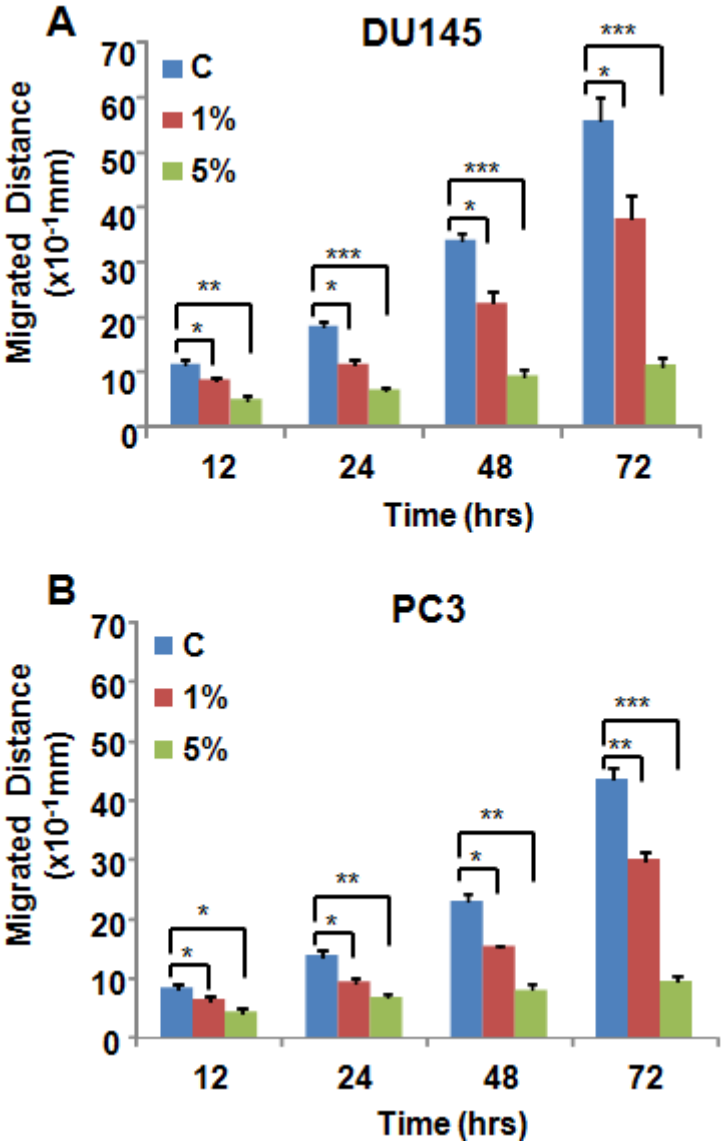
A
DU145
C
1%
5%
Migrated Distance (x10⁻¹mm)
70
60
50
40
30
20
10
0
12
24
48
72
Time (hrs)
B
PC3
C
1%
5%
Migrated Distance (x10⁻¹mm)
70
60
50
40
30
20
10
0
12
24
48
72
Time (hrs)

Figure 2.3

Figure 2.4. Pomegranate Juice inhibits hormone-independent prostate cancer cell chemotaxis to SDF1α. (A,B) DU145 and PC3 cancer cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 hrs and then treated with 1% or 5% PJ for 12hrs. At this time, 100ng/ml of SDF1α were introduced into the lower chamber and the cells found on the bottom of the filter counted 3.5 hrs later. Control had no PJ treatment. **(C,D)** DU145 and PC3 cancer cells were pre-treated with 5μg/ml of a specific CXCR4 inhibitor for 15mins before introducing 100ng/ml of SDF1α into the lower chamber. The number of cells found on the underside of the filter was counted 3.5h later. Negative controls consist of treatment with inhibitor alone. Bars represent Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$. Repeated at least 3 times.

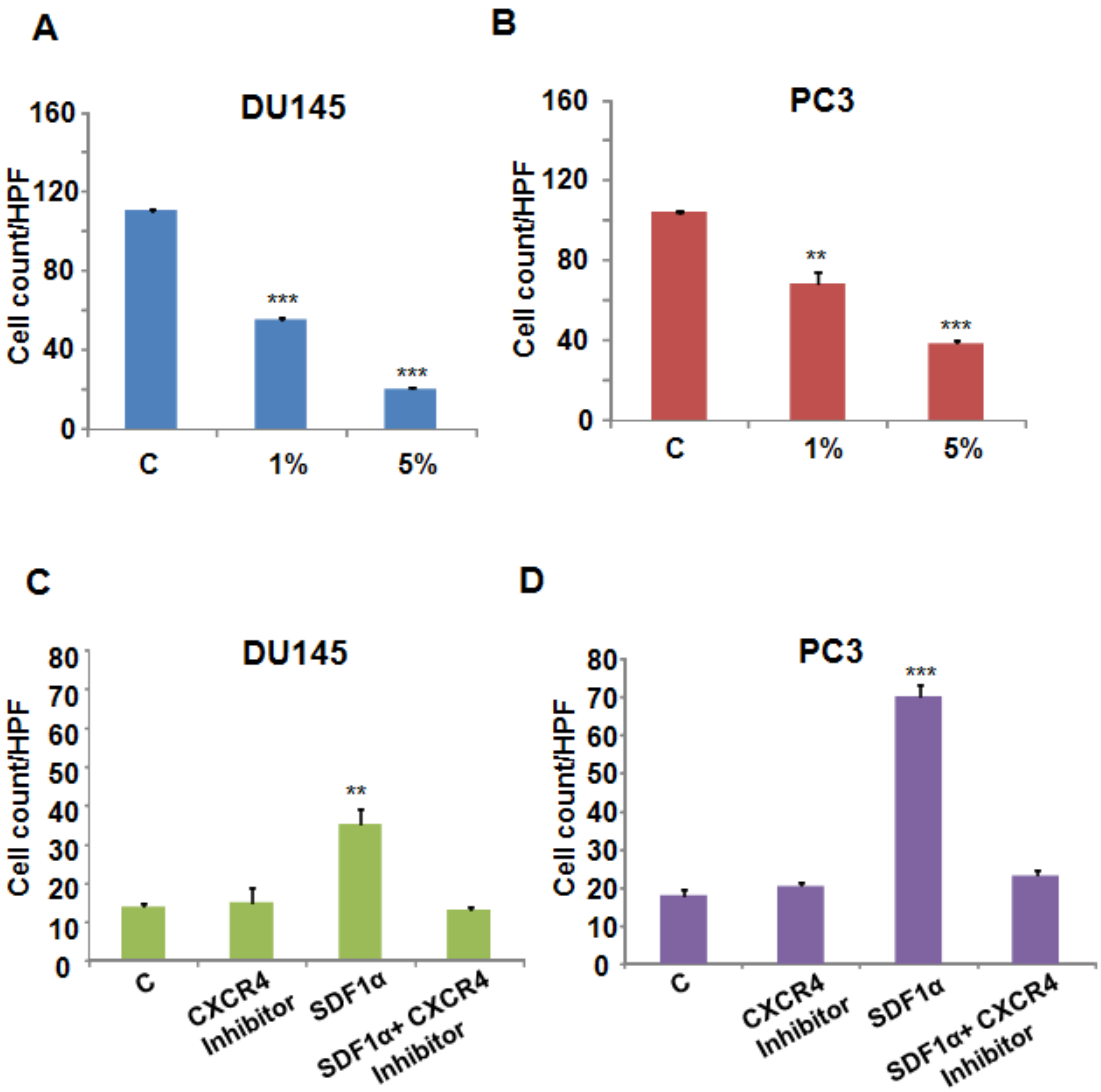
A
DU145
Cell count/HPF
160
120
80
40
0
***
***
C
1%
5%
B
PC3
Cell count/HPF
160
120
80
40
0
**
***
C
1%
5%
C
DU145
Cell count/HPF
80
70
60
50
40
30
20
10
0
**
C
CXCR4 Inhibitor
SDF1α
SDF1α+ CXCR4 Inhibitor
D
PC3
Cell count/HPF
80
70
60
50
40
30
20
10
0
***
C
CXCR4 Inhibitor
SDF1α
SDF1α+ CXCR4 Inhibitor

Figure 2.4

Figure 2.5. Pomegranate Juice stimulates cell adhesion and inhibits chemotaxis to SDF1α of LNCaP prostate cancer cells. (A) LNCaP cancer cells were plated on gelatin-coated dishes and 24 hrs later media was changed and the cells were treated with 1% or 5% Pomegranate Juice. We tested for adhesion to the substrate at 12 and 24hrs after initiation of treatment by recording the time it took for trypsinization to remove all of the cells from the dish. Control represents no PJ treatment. Within each experiment, the times of trypsinization were within 1 minute of each other for each specific treatment. **(B)** LNCaP cancer cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 hrs and then treated with 1% or 5% PJ for 12hrs. At this time, 100ng/ml of SDF1α were introduced into the lower chamber and the cells found on the bottom of the filter counted 3.5 hrs later. Control had no PJ treatment. Bars represent Standard Error of the Mean. ***$p < 0.001$.

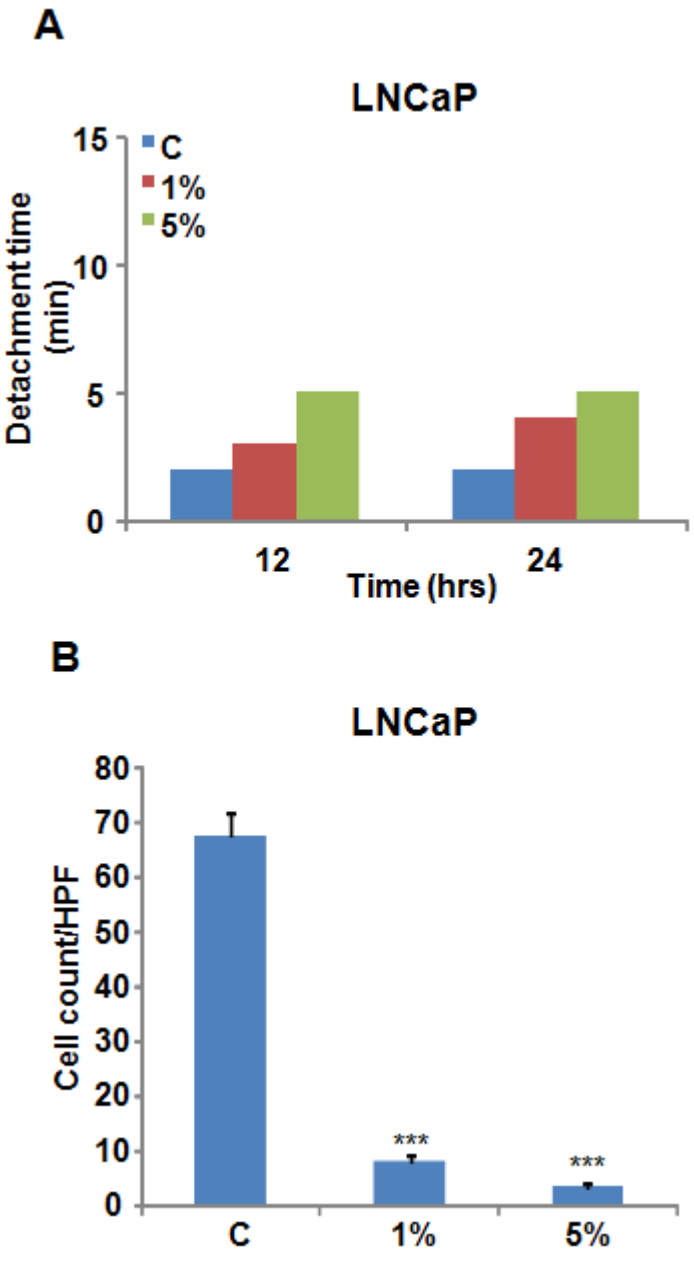
A
LNCaP
15
10
5
0
C
1%
5%
Detachment time (min)
12
24
Time (hrs)
B
LNCaP
80
70
60
50
40
30
20
10
0
Cell count/HPF
***
***
C
1%
5%

Figure 2.5

Figure 2.6. Verification of the effect of Pomegranate Juice on gene expression using RT-qPCR. (A) The mRNA level of *ICAM1*, *MARCKS*, *Claudin1*, *HMMR*, *Collagen IAα*, *PKCε*, *Anillin*, *Nexilin* and *N-chimearin*, were determined by using qPCR with RNA extracted from DU145 cells treated with 5% PJ for 12hrs. **(B-D)** Immunoblot analysis for E-cadherin, ICAM-1 and Tropomyosin1 with protein extracts from DU145 cancer cells treated with 5% pomegranate juice for 24hrs. Performed one time because the results supported the data from the array.

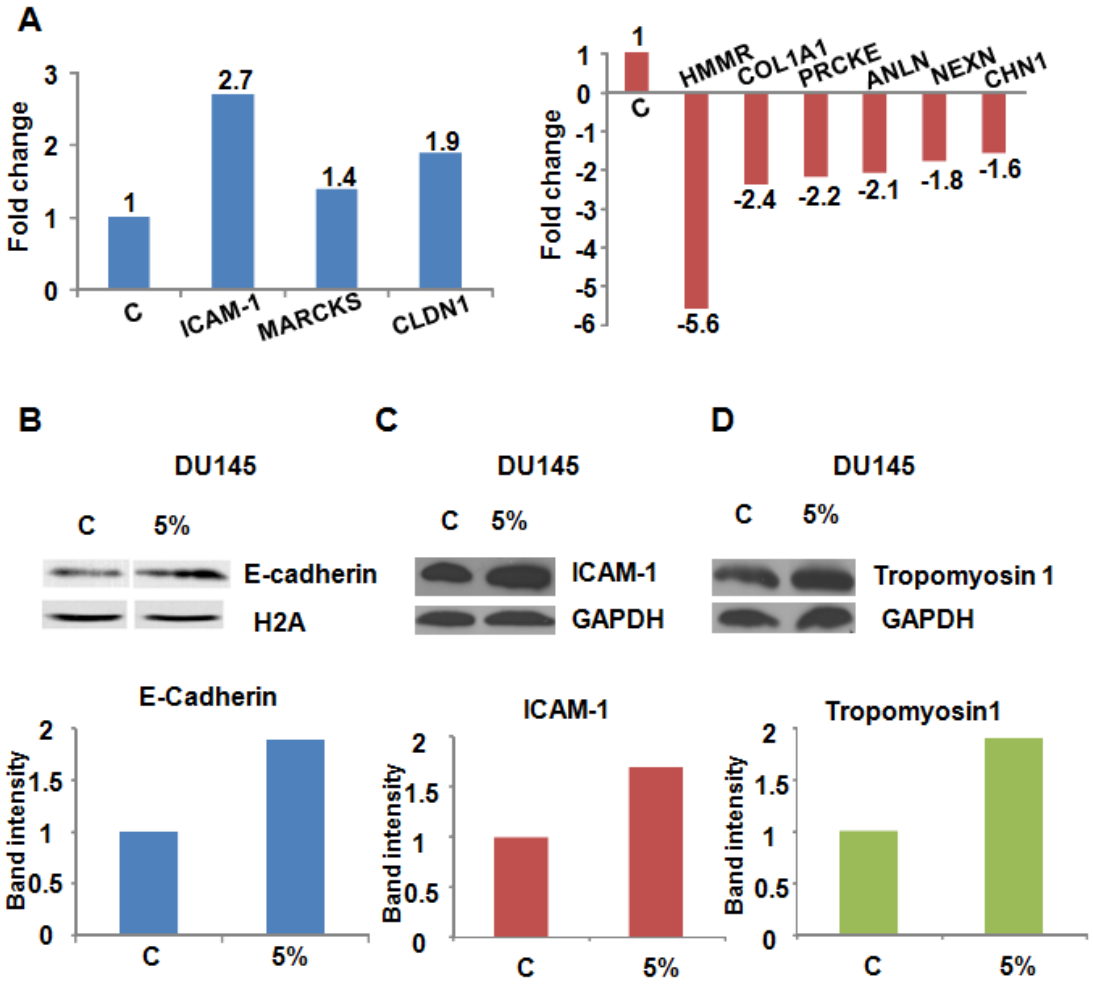
A
Fold change
3
2
1
0
1
2.7
1.4
1.9
C
ICAM-1
MARCKS
CLDN1
Fold change
1
0
-1
-2
-3
-4
-5
-6
1
-5.6
-2.4
-2.2
-2.1
-1.8
-1.6
C
HMMR
COL1A1
PRCKE
ANLN
NEXN
CHN1
B
DU145
C
5%
E-cadherin
H2A
C
DU145
C
5%
ICAM-1
GAPDH
D
DU145
C
5%
Tropomyosin 1
GAPDH
E-Cadherin
Band intensity
2
1.5
1
0.5
0
C
5%
ICAM-1
Band intensity
2
1.5
1
0.5
0
C
5%
Tropomyosin1
Band intensity
2
1.5
1
0.5
0
C
5%

Figure 2.6

Figure. 2.7. Effect of Pomegranate Juice on cell migration is mediated through E-cadherin and HMMR. (A,B) DU145 **(A)** and PC3 **(B)** prostate cancer cells were transfected with 40nM E-cadherin siRNA and 24hrs later a scratch made and treated with 1% PJ for 36 hrs and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. **(C)** DU145 cancer cells were transfected with 8µg/ml of pcDNA3.1 HMMR vector. Controls represent no PJ. Bars represent Standard Error of the Mean. **$p < 0.01$; *$p < 0.05$. Experiment was performed two times.

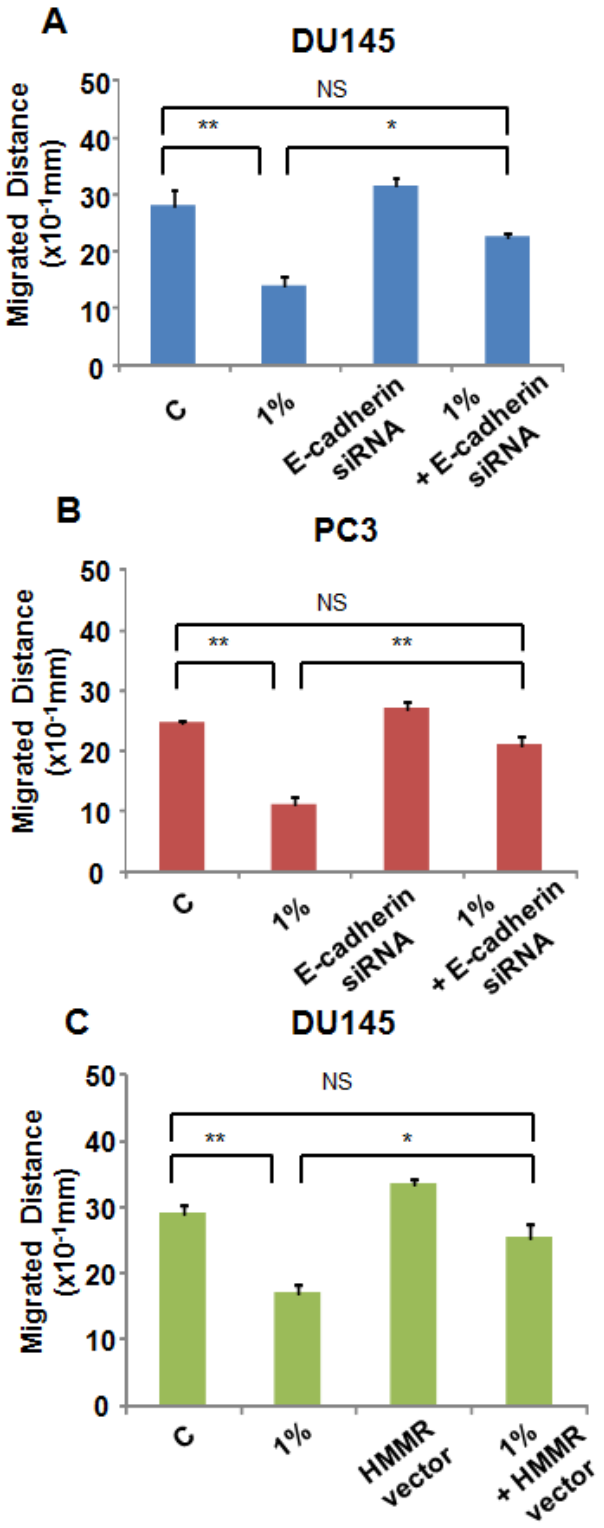
A
DU145
NS
**
*
Migrated Distance (x10-1mm)
50
40
30
20
10
0
C
1%
E-cadherin siRNA
1% + E-cadherin siRNA
B
PC3
NS
**
**
Migrated Distance (x10-1mm)
50
40
30
20
10
0
C
1%
E-cadherin siRNA
1% + E-cadherin siRNA
C
DU145
NS
**
*
Migrated Distance (x10-1mm)
50
40
30
20
10
0
C
1%
HMMR vector
1% + HMMR vector

Figure 2.7

Figure 2.8. Effect of Pomegranate Juice on cell migration is mediated through miRNA-21 and miR-335. (A,B) DU145 **(A)** and PC3 **(B)** cells were transfected with 60nM miR-21 mimics and 24hrs later treated with 1% PJ for 36 hrs and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. **(C,D)** DU145 **(C)** and PC3 **(D)** cells were transfected with 60nM miR-335 inhibitor. Controls represent no PJ. Bars represent Standard Error of the Mean. **$p < 0.01$; *$p < 0.05$. Experiment was performed two times.

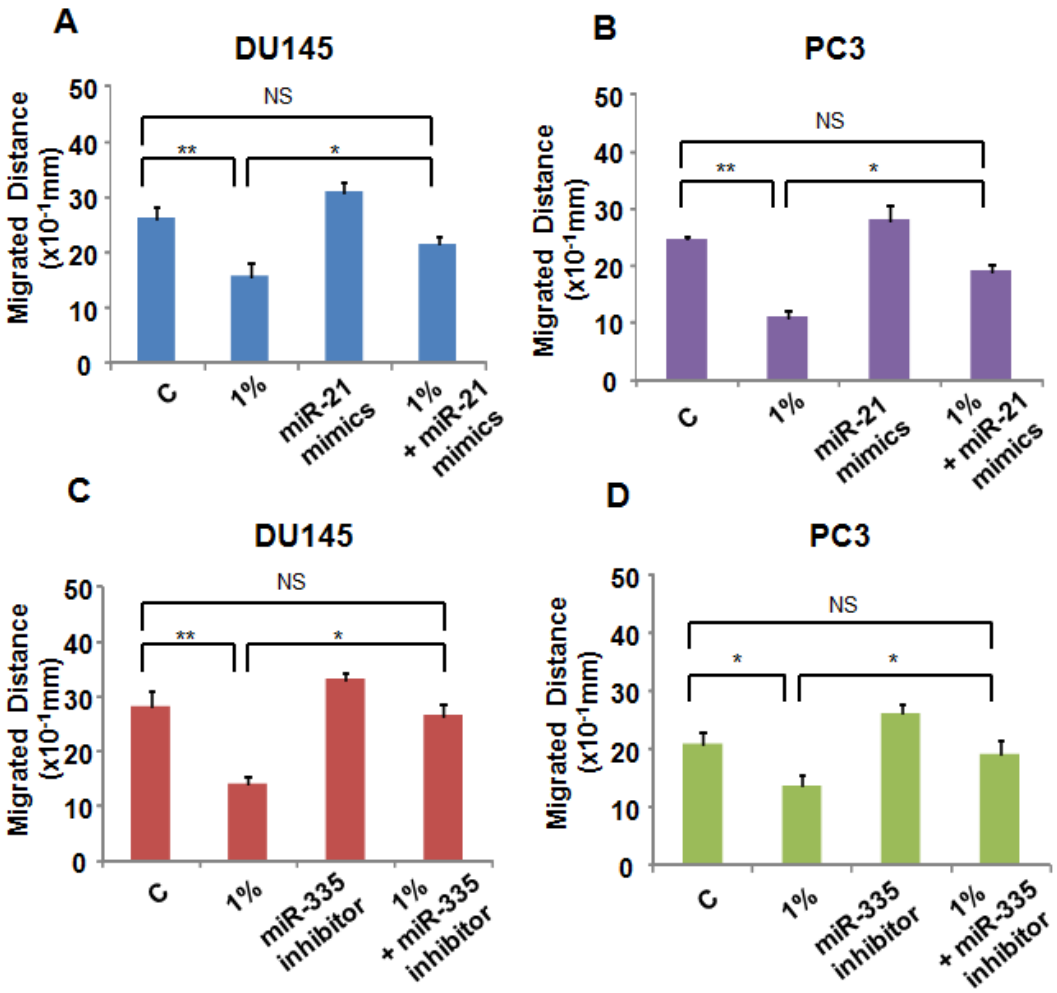
A
DU145
Migrated Distance (x10-1mm)
NS
**
*
C
1%
miR-21 mimics
1% + miR-21 mimics
B
PC3
C
DU145
miR-335 inhibitor
1% + miR-335 inhibitor
D
PC3

Figure 2.8

Figure 2.9. Effects of Pomegranate Juice on the levels of selected pro-inflammatory cytokines and chemokines. Media collected from DU145 and PC3 cancer cells treated with 1% or 5% PJ for 18hrs were analyzed using Luminex Multiplex Array assays. Both DU145 and PC3 significantly decreased IL-6 **(A)** and IL-12p40 **(B)** whereas only DU 145 inhibited production of IL-1β **(C)** and only PC3 inhibited production of RANTES **(D)**. ***$p < 0.001$; *$p < 0.05$. Experiment was performed two times.

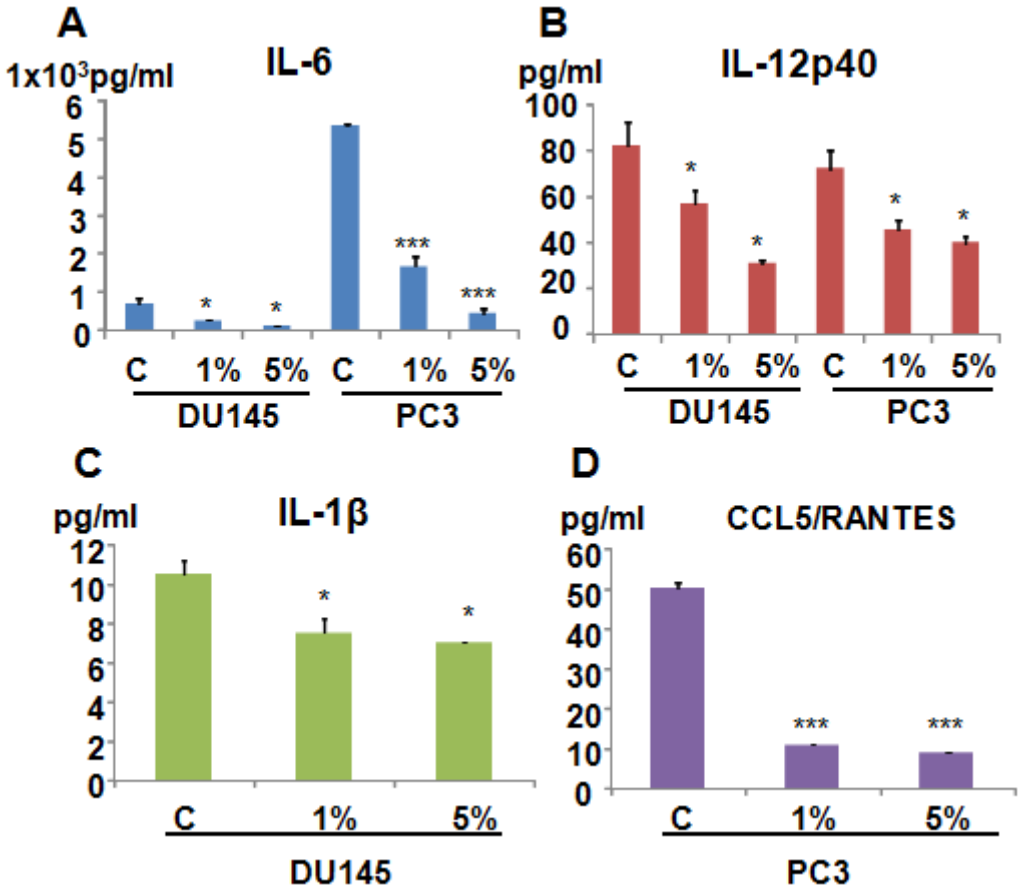
A
1x10³pg/ml
IL-6
6
5
4
3
2
1
0
*
*
***
***
C
1%
5%
C
1%
5%
DU145
PC3
B
pg/ml
IL-12p40
100
80
60
40
20
0
*
*
*
C
1%
5%
C
1%
5%
DU145
PC3
C
pg/ml
IL-1β
12
10
8
6
4
2
0
*
*
C
1%
5%
DU145
D
pg/ml
CCL5/RANTES
60
50
40
30
20
10
0
***
***
C
1%
5%
PC3

Figure 2.9

Figure 2.10. Schematic summary of the effects of Pomegranate Juice on hormone-independent prostate cancer cells. *E-cadherin* is a calcium-dependent cell-cell adhesion glycoprotein and one of the important components in adherens junctions. *Myristoylated alanine-rich protein kinase C substrate (MARCKS)* is localized in the plasma membrane and is an actin filament cross-linking protein. *Intercellular Adhesion Molecule-1 (ICAM-1)* is an endothelial transmembrane protein known for its importance in cell adhesion and in stabilizing cell-cell interactions. *Claudin-1*, a transmembrane protein, is an important component of tight junctions. *Type I collagen* is an important component of ECM with both structural and signaling functions that mediate cell migration and survival. *N-chimearin* is known as a Rho GTPase-activating protein (GAP). Studies showed that N-chimearin cooperated with Rac1 in inducing changes in cytoskeletal morphology. *Protein kinase Cε* (PKCε) is a member of protein kinase C family. Inhibition of PKCε reduces the invasiveness of prostate cancer cells. *Hyaluronan-mediated motility receptor (HMMR)* or *CD168* functions as hyaluronan receptor. *Anillin and nexilin* are actin-binding proteins involved in regulation of cytoskeleton structure. *Tenascin C* is an extracellular molecule known to promote cell migration. *Programmed cell death 4* (Pdcd4) has been identified as a tumor suppressor with the capability to suppress cancer cell invasion through JNK signaling pathway. *miR-335* represses the expression of type I collagen and tenascin C. *miR-205* represses the expression of N-chimearin and PKC-□. *N-chimearin* is a GTPase-activating protein that when down-regulated results in loss of filopodia and reduction of migration. *miR-200 family* represses E-cadherin transcriptional repressor ZEB1 and ZEB2, resulting in up-regulation of E-cadherin. *miR-21* represses expression of Myristoylated alanine-rich protein kinase C (MARCKS), Tropomyosin (TPM1), and Programmed cell death 4 (Pdcd4). MARCKS and TPM1 are actin-filament binding proteins that are involved in the regulation of cell adhesion and cancer cell invasion, respectively. *miR-373* represses transmembrane adhesion glycoprotein CD44 expression, a glycoprotein that mediates cell-cell and cell-stromal interactions, binds HA and several other matrix molecules, and controls cell shape through the cytoskeleton. PJ significantly up-regulates miR-335, miR-205, miR-200 family and miR-126 previously identified as metastasis suppressor microRNAs and significantly down regulated miR-21 and miR-373 previously identified as pro-invasive microRNAs.

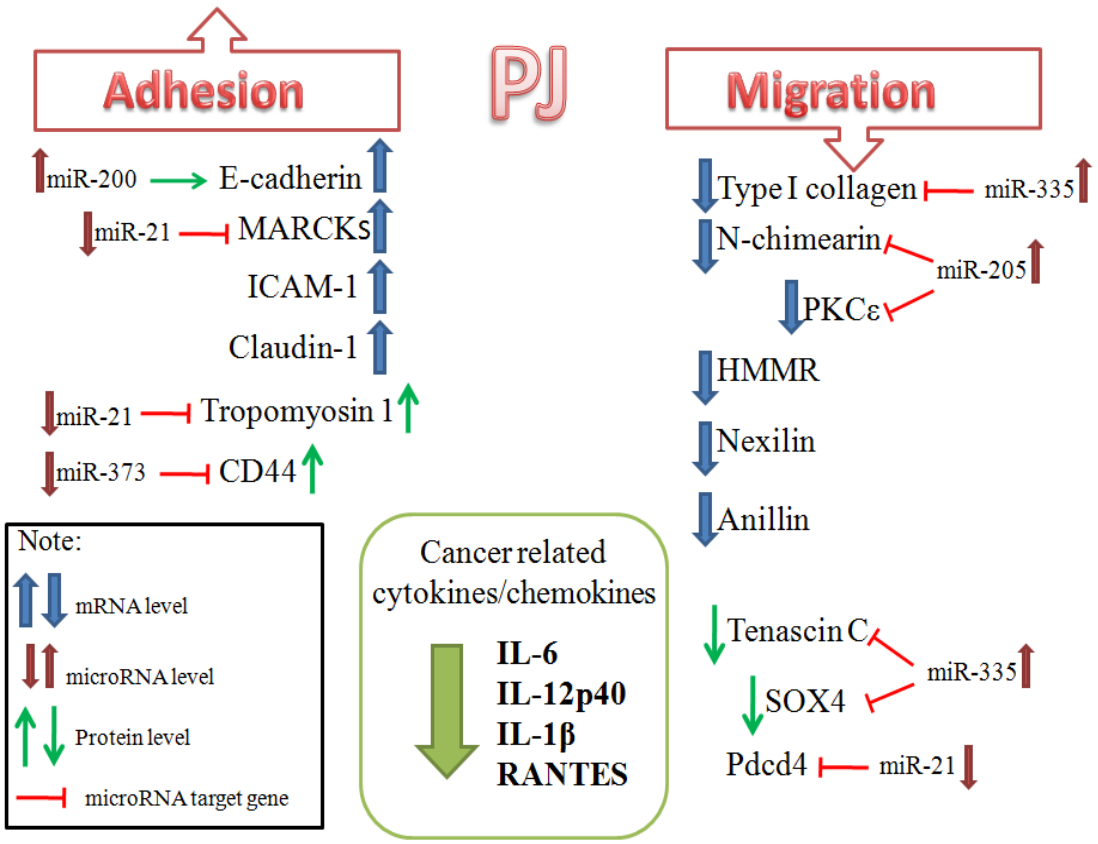
Adhesion
PJ
Migration
miR-200
E-cadherin
miR-21
MARCKs
ICAM-1
Claudin-1
miR-21
Tropomyosin 1
miR-373
CD44
Type I collagen
miR-335
N-chimearin
miR-205
PKCε
HMMR
Nexilin
Anillin
Tenascin C
miR-335
SOX4
Pdcd4
miR-21
Note:
mRNA level
microRNA level
Protein level
microRNA target gene
Cancer related cytokines/chemokines
IL-6
IL-12p40
IL-1β
RANTES

Figure 2.10

Table 2.1. Pomegranate Juice changes the expression profile of genes involved in the cytoskeleton and cell adhesion machinery. RNA was extracted from DU145 cells that had been treated with 5% PJ for 12 hrs and Affymetrix array analysis was performed as described in Materials and Methods. Relative mRNA levels are presented as fold change compared with untreated controls. Only genes with fold increase greater than 2 are depicted. Red line separates genes increased from genes decreased.

List of selected gene expression changes with POMEGRANATE JUICE treatment

Gene Name	Product	Fold-change vs.Control	Function
ICAM-1	Intercellular adhesion molecule 1	**↑3.3**	Cell adhesion
MARCKS	Myristoylated alanine-rich protein kinase C substrate	**↑2.8**	Cell adhesion
PDCD4	Programmed cell death 4	**↑2.4**	Tumor suppressor
CDH1	E-cadherin	**↑2.4**	Cell adhesion
CLDN1	Claudin 1	**↑2.3**	Cell adhesion
HMMR	hyaluronan-mediated motility receptor (CD168)	**↓6.7**	Hyaluronan receptor, cell migration
ANLN	Anillin	**↓2.8**	Actin binding
COL1A1	Collagen type I α1	**↓2.7**	ECM component
PRKCE	Protein kinase C, epsilon	**↓2.4**	Signal transduction
NEXN	Nexilin	**↓2.3**	Actin binding
CHN1	N-chimearin	**↓2.1**	Cell motility

Table 2.1

Table 2.2. Effects of Pomegranate Juice on the levels of metastasis-related miRNAs. RNA from DU145 treated with 5% PJ for 12 hrs was submitted to microRNA array analysis. Relative miRNA levels are shown as fold change compared with untreated control. The predicted targets are: tenascin C (TNC); SRY-related HMG-box (SOX-4); E-cadherin transcriptional repressor zinc finger E-box binding homeobox 1 (ZEB1) and zinc finger E-box binding homeobox 2 (ZEB2), resulting in up-regulation of E-cadherin; Tropomyosin (TPM1); transmembrane adhesion glycoprotein cluster of differentiation 44 (CD44).

List of selected miRNA changes with POMEGRANATE JUICE treatment

miRNA	Fold-change vs. Control	Predicted Target
miR-335	**↑ 411**	COL1A1, TNC, SOX4
miR-205	**↑ 50**	CHN1, PRKCE
miR-200 family	**↑ 3-20**	ZEB1, ZEB2
miR-126	**↑ 11**	SLC45A3 (Prostein)
miR-21	**↓ 12**	MARCKS, PDCD4, TPM1
miR-373	**↓ 10**	CD44

Table 2.2

CHAPTER 3:

Specific Pomegranate Juice Components as Potential Inhibitors of Prostate Cancer Metastasis

ABSTRACT

Pomegranate juice (PJ) is a natural product that inhibits prostate cancer progression; a clinical trial on patients with recurrent prostate cancer resulted in none of the patients progressing to a metastatic stage during the period of the trial. We have previously found that, in addition to causing cell death of hormone-refractory prostate cancer cells, PJ also markedly increases cell adhesion and decreases cell migration of the cells that do not die. However, because PJ is a very complex mixture of components and is found in many different formulations, it is important to identify specific components that can replace the effects of the juice on growth and metastasis. Here we show that the specific PJ components luteolin, ellagic acid and punicic acid together inhibit growth of hormone-dependent and -independent prostate cancer cells, their migration and their chemotaxis towards SDF1α, a factor that is important in prostate cancer metastasis to the bone. These components also increase the expression of cell adhesion genes and decrease expression of genes involved in cell cycle control and cell migration. Furthermore, they increase several well-known tumor-suppression miRNAs, decrease several oncogenic miRNAs and inhibit the CXCR4/SDF1α chemotaxis axis. Our results suggest that these components may be more effective in inhibiting of prostate cancer metastasis than simply drinking the juice. Moreover, because the mechanisms of metastasis are similar for most cancers, these PJ components may also be effective in treatment of metastasis of other cancers.

INTRODUCTION

Prostate cancer is the second leading cause of death by cancer among men in America. It accounts for approximately 30% of all male malignancies; one in six men will be diagnosed with prostate cancer and 1 in 35 will die of the disease (Stavridi et al., 2010). Early stages of localized prostate cancer can be effectively treated with surgery and radiation but a majority of patients develop locally advanced or widespread cancer that requires hormone ablation therapy. Moreover, 80-90% of patients who receive hormone ablation therapy ultimately develop metastatic castration-resistant prostate cancer (CRPC) 12-33 months after initiation of hormone ablation therapy (Chuu et al., 2011; Coffey and Pienta, 1987). Chemotherapy can be used to treat castration-resistant prostate cancer but chemotherapeutic drugs are aggressive and have many side effects (Harzstark and Small, 2010; Petrylak, 1999). Therefore, there is a major need for more effective and less toxic therapies to treat prostate cancer.

Sipuleucel-T (Provenge®), an autologous cellular immunotherapy, was approved by the FDA in 2010 to treat metastatic prostate cancer. The overall survival rate of patients who received Sipuleucel-T was improved but the median survival rate was only improved by 4.5 months. In addition, the effect on time of progression in patients who were asymptomatic or with limited metastatic disease did not reach statistical significance and treatment is costly (Higano et al., 2010). Abiraterone, an inhibitor of androgen biosynthesis, has been shown as very promising anti-androgen therapy to

prolong overall survival rate among patients with metastatic prostate cancer (Shah and Ryan, 2009). Moreover, novel androgen receptor (AR) antagonist MDV3100, which blocks androgen from binding to AR and prevents nuclear translocation, showed promising antitumor effects in recent clinical trials (Scher et al., 2010; Schrijvers et al., 2010). Another novel drug, Cabozantinib, a potent dual inhibitor of the tyrosine kinases MET and VEGFR2, has been shown to reduce or stabilize metastatic bone lesions in CRPC patients (D. C. Smith, 2011; Yakes et al., 2011). However, all of these treatments have adverse side effects. Recently, there has been a renewed push to identify natural remedies to fight prostate cancer. Among the latter is Pomegranate Juice.

Mounting evidence shows that PJ has great potential to inhibit the growth and reduce the invasiveness of prostate cancer cells both *in vitro* and *in vivo* (Albrecht et al., 2004; Rettig et al., 2008; Syed et al., 2008). In a phase II clinical trial patients with rising PSA were given 8 oz of PJ by mouth daily. PSA doubling time significantly increased with treatment from a mean of 15 months at baseline to 54 months post-treatment ($P < 0.001$). This statistically significant prolongation of PSA doubling time and the lack of metastatic progression in any of the patients, strongly suggests a potential of PJ for treatment of prostate cancer (Pantuck et al., 2005). As a result of these findings, several studies have shown that PJ affects many of the cellular processes involved in cell death and also affects signaling pathways that could inhibit cell migration and invasion (Lansky et al., 2005; Malik et al., 2005). We have

shown previously that PJ inhibits the migratory and metastatic properties of hormone refractory prostate cancer cells by stimulating cell adhesion and inhibiting cell migration/chemotaxis (Wang et al., 2011c). However, the soluble phase of PJ contains many components and as a whole it is difficult to determine how to best maximize its use in treating prostate cancer. A way to overcome this challenge is to identify chemical components of PJ that are responsible for the anti-metastatic effect of the whole juice.

The fruit of PJ can be divided into several anatomical compartments: seeds, juice and peel and all these compartments have been reported to possess anti-proliferative and anti-metastatic effect against prostate cancer cells (Adhami et al., 2009; Kim et al., 2002a; Lansky et al., 2005; Syed et al., 2007). The juice is a rich source of polyphenolic compounds including anthocyanins such as delphinidin, cyanidin, and pelargonidin, which give the fruit and juice its red color. It is also rich in punicalin, punicalagin, gallagic, quercetin, ferulic acid, caffeic acid, ellagic acid and luteolin, which largely account for the antioxidant activity of the whole fruit (El Kar et al., 2011b; Gil et al., 2000b; Syed et al., 2008; Zhou et al., 2009b). The seed oil of PJ, which is comprised of 65–80% conjugated fatty acids, also contains many compounds of interest with known anti-cancer activities. The predominant component among these fatty acids is punicic acid (Gasmi and Sanderson, 2010; Grossmann et al., 2010). However, the specific components of PJ that have anti-metastatic effects against prostate cancer are largely unknown. There is a short one and half pages

communication reporting that ellagic acid, caffeic acid, luteolin, and punicic acid, inhibit *in vitro* invasion of human prostate cancer (PC3) cells across matrigel (Lansky and et al., 2005). Their findings are interesting but very limited. No studies were presented on effects on other processes involved in metastasis nor was mechanism of action of these PJ components addressed. Given these findings, we hypothesized that these specific components of PJ are able to replace the effects of the full PJ in inhibiting cellular and molecular processes involved in adhesion, migration and chemotaxis of prostate cancer. Here we show that luteolin (L), ellagic acid (E), punicic (P), but not caffeic acid (C), stimulate molecules cell adhesion, inhibit molecules involved in cell migration and inhibit chemotaxis of the cancer cells via CXCR4/ SDF1α, a chemokine axis that is very important in metastasis of prostate cancer cells to the bone. Our findings strongly suggest that L+E+P can potentially be used to prevent metastasis of prostate cancer.

MATERIALS AND METHODS

Materials

RPMI 1640 media was acquired from Mediatech (Manassas, VA) and fetal bovine serum from Sigma Aldrich (St.Louis, MO). Stromal Derived Factor 1 alpha (SDF1a) was obtained from ProSpec (Boca Raton, FL). The RNeasy RNA Isolation Kit for RNA preparation from Qiagen Inc. (Valencia, CA). Secondary Ab was from Thermo Scientific (Rockford, IL). Detection was done using Supersignal West Dura kit from Thermo Scientific (Rockford, IL). Transfection reagents Lipofectamin^{TM} 2000 were purchased from Invitrogen (Carlsbad, CA). AposcreenTM Annexin V-FITC kit from Southern Biotech (Birmingham, AL). All oligonucleotide primers for qPCR were obtained from IDT (Coralville, IA) and the qPCR iQ SYBR green supermix kit from Bio-Rad (Hercules, CA). RT2 qPCR-Grade miRNA isolation kit and RT2 miRNA PCR Array (MAH-001A) were obtained from SABiosciences (Frederick, MD). The pomegranate juice was purchased from POMx Wonderful.

Cell culture

DU145 and PC3 are hormone-independent prostate cancer epithelial cell lines. LNCaP is an androgen-responsive prostate cancer epithelial cell line. DU145 and LNCaP prostate cancer epithelial cell lines were purchased from ATCC (Manassas, VA). PC3 prostate epithelial cell line was a gift from A. Walker (UC Riverside). Cells were cultured at 37°C with 5% CO_2 in RPMI 1640 supplemented with 10% fetal

bovine serum, penicillin (100 I.U/ml) and streptomycin (100 µg/ml) and used at the times indicated in the results.

Adhesion assay

$3x10^5$ PC3, DU145 or LNCaP cells were plated on gelatin-coated 6-well plates (B&D Biosciences), allowed to adhere and 24hrs later treated with PJ and/or PJ components for 12 hrs and/or 24 hrs. Cells were then trypsinized and the time required to detach all cells was recorded as an indicator of cell adhesiveness.

Migration assay

Confluent PC3, DU145 or LNCaP cells were wounded using a rubber scraper to create a scratch, washed and treated with PJ and/or PJ components at various concentrations. Cell migration was determined by measuring the distance migrated by the cells from the wounded edge to the leading edge of migration at 12hr, 24h, 48h and 72h after treatments were initiated. Scraped cells without treatment were used as controls.

Chemotaxis assay

The upper side of 8µm pore size polycarbonate membranes of transwells (BD Biosciences, San Jose, CA) were coated with 50 ng/ml type I collagen (Sigma Chemical Co.). DU145, PC3 or LNCaP cells ($1x10^5$) in 100 µl culture medium were plated on the upper side of transwell membranes and were allowed to adhere for 3 hours. Then the wells were introduced into 24-well plate and 1000 µl RMPI 1640 with 10% FBS medium was added to lower chamber. Cells were treated with PJ

and/or PJ components for 12 hours. SDF1α (100ng/ml) was added to the lower chamber and the cells were allowed to migrate for 4 hours at 37°C. The cells on the side of the membrane facing the upper chamber were removed with a cotton swab, and the membranes were then fixed and stained with 2% toluidine blue in 4% paraformaldehyde. Cells were counted in 8 high power fields (HPF)/filter to obtain the average number of cells per field.

Total RNA extraction

DU145 and PC3 cells were treated with PJ components for 12 hrs and total RNA was extracted using the RNeasy RNA isolation Kit according to manufacturer's protocol. Briefly, cells were washed with ice-cold 1X PBS, and lysed on ice with lysis buffer. Cell lysates were then spun at 12,000rpm for 5 min to remove cell debris, followed by organic extraction to remove proteins. Then lysates were loaded into isolation columns and the final RNA product was dissolved in nuclease-free water. RNA quality was assessed on the Agilent Bioanalyzer 2100 using the Agilent RNA 6000 Nano Assay kit (Agilent Technologies, Waldbronn, Germany) and the concentration determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE USA).

Affymetrix microarray and data analysis

Affymetrix Human Genome U133 Plus 2.0 Arrays, which contain more than 54,000 probe sets representing approximately 38,500 genes and gene sequences, were used. Cells were treated with PJ components for 12 hrs and total RNA was extracted and

evaluated as described above. Capillary electrophoresis using an Agilent Bioanalyzer 2100 to confirm the RNA quality levels was used before performing the array assays. RNA from cells without treatment was used as control. A single $\log_2$ expression measure for each probe set was calculated from image files (CEL format) using the robust multi-array analysis (RMA) procedure using Agilent GeneSpring GX software. The changes of expression level between untreated and specific PJ components treated sample were compared. Only genes that were over- or under-expressed by >2-fold were considered. Once we identified genes of potential interest, we verified their increase or decrease in expression by RT-qPCR.

Real time quantitative PCR

1μg RNA was reverse-transcribed to cDNA by RETROscript Reverse Transcription Kit (Ambion) at 44°C for 1hr and 92°C for 10min. 2μl of cDNA from the reverse transcription reaction were added to 23μl real-time quantitative PCR mixture containing 12.5μl 2**x** SYBR Green SuperMix (Bio-Rad) and 200nM oligonucleotide primers. PCRs were carried out in a Bio-Rad MyiQ5 Real-Time PCR Detection System (Bio-Rad). The thermal profile was 95°C for 3 min followed by 40 amplification cycles, consisting of denaturation at 95°C for 10 sec, annealing at 60°C for 30 sec. Fluorescence was measured and used for quantitative purposes. At the end of the amplification period, melting curve analysis was done to confirm the specificity of the amplicon. Fold changes of genes after treatment with PJ were calculated by the Pfaffl method to normalize the Ct values to the GAPDH internal control. The

following primer sequences were designed with IDT PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/) and used for the reactions:

GAPDH,TCGACAGTCAGCCGCATCTTCTTT and ACCAAATCCGTTGACTCCGACCTT;

BCL2, TTTCTCATGGCTGTCCTTCAGGGT and AGGTCTGGCTTCATACCACAGGTT;

ROCK2, TTCCAGTGGAGCCAGTTGGAGAAA and TACAAGCCTCACAGTTGGTTGGGA;

EZH2, CAGTTTGTTGGCGGAAGCGTGTAA and AGGATGTGCACAGGCTGTATCCTT;

CDK6, ATTCACTGCCTGGGACACAGTCTT and ACAGGCCACTGTGGTAACTCTCAA;

DTL, ATTTGGATCTGTGCTGCCTTGCTG and AGGTAGCGTTCCACAGCTTTCTGA;

CCNE2, ATGACACCACCGAAGAGCACTGAA and TTGGCTAGGGCAATCAATCACAGC;

FSCN1, CAACGATGGCGCCTACAACATCAA and TGGCCACCTTGTTATAGTCGCAGA;

CDC25B, TCAGGTGCTGTCCATGGGAAAGAT and AACTCAACAGACTGGGCTCTTCCA;

TWIST, ACCATCCTCACACCTCTGCATTCT and TTCCTTTCAGTGGCTGATTGGCAC;

CCNB1, TGTGGATGCAGAAGATGGAGCTGA and TTGGTCTGACTGCTTGCTCTTCCT;

PTEN, GGTTGCCACAAAGTGCCTCGTTTA and AACTGGCAGGTAGAAGGCAACTCT;

CDKN1A,TTAGCAGCGGAACAAGGAGTCAGAand ACACTAAGCACTTCAGTGCCTCCA.

MicroRNA PCR array and data analysis

MicroRNAs were extracted from total RNA using RT2 qPCR-Grade miRNA isolation kit from SABiosciences. For PCR Arrays, miRNAs cDNA (100 ng) were amplified using RT2 miRNA First Strand Kit and RT2 miRNA PCR Array (MAH-001A) from SABiosciences following manufacturer's instructions. The fluorescence threshold

value (Ct) was calculated using SABiosciences web based RT2 profiler PCR array data analysis portal:

http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php

siRNA and vector transfection

PC3 cells (80-90% confluent) were transfected with Lipofectamine 2000 (Invitrogen) following the manufacture's protocols. 40nM E-cadherin chimera siRNA (Abnova) was transfected. 2µg/ml pcDNA3.1 HMMR vector, pcDNA 4.1 TWIST vector or pcDNA3 CCNE2 vector (Zariwala et al., 1998) was transfected. Scratch wound assay, adhesion assay and/or cell growth assay were performed as described above, 24 hrs after transfection.

Immunoblotting

DU145 and PC3 cells were treated with PJ components for 12h, washed with ice cold 1XPBS, and lysed on ice with lysis buffer containing 0.5% Triton X100, 0.5% Nonidet P-40, 10 mM Tris, pH 7.5, 2.5 mM KCl, 150 mM NaCl, 30 mM b-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 0.1% SDS and additional protease inhibitor cocktails (Sigma). Protein concentrations were measured using the DC protein assay kit (Bio-Rad). Equal amounts of protein in the cell extracts were mixed with sample buffer, boiled, and analyzed using 10% acrylamide SDS-PAGE. Immunoblotting was performed with the The HRP-conjugated secondary Ab (Thermo Scientific), followed by incubation with West Dura extended-duration substrate

(Thermo Scientific). Blots were then reprobed for histone 2A or GAPDH antibody to show equal loading of proteins.

Statistical Analysis

Data analysis was performed using the one-way ANOVA on raw data using GraphPad Instat software (GraphPad Software Inc.).

RESULTS

Effect of luteolin, ellagic acid, punicic acid and caffeic acid on prostate cancer cell growth, adhesion, migration and chemotaxis

We treated hormone-independent prostate cancer cells, PC3 and DU145, with the 4 PJ components L, E, P and C **(Fig 3.1A)** at concentrations of both 4µg/ml and 8µg/ml which are relevant to physiological concentration of these components in the juice (Lansky and et al., 2005). We found that this combination significantly inhibited cell growth of both PC3 **(Fig 3.1B)** and DU145 (**Fig 3.2A)**. When compared to PJ treatment, the number of cells floating in the medium after the treatment with the components was much smaller. Trypan blue staining showed that the majority of the cells remained viable after treatment with the components at either 4µg/ml or 8µg/ml **(Fig 3.1C and Fig 3.2B)**. At both 12 and 24hrs, the cells all still looked healthy and no floating cells were seen in any of the cultures. We also found that the cells strongly adhered to gelatin-coated substrate, as indicated by increased time to detach all cells from the culture dish in the presence of treatment with the components; by 12 hrs of 4µg/ml L+E+C+P treatment, the cultures required about double the time to be released by trypsinization **(Fig 3.1D and Fig 3.2C)**. Moreover, the effect was dose dependent with the 8µg/ml of the combination of PJ components being more potent than treatment with either 4µg/ml of the components or 1% PJ. Using the scratch wound assay, we found that the combination of L+E+C+P significantly inhibited the migratory capabilities of these cells. We measured the distance that the cells migrated

from the wounded edge to the migration front and found it to be significantly reduced in the treated cells beginning as early as 12hrs after treatment, continued over 72hrs and the effect was dose-dependent **(Fig 3.1E and Fig 3.2D)**.

Stromal cell-Derived Factor one alpha (SDF1α) is a critical chemokine secreted by bone marrow cells that attracts prostate cancer cells to the bone. Therefore, we tested whether the combination of L+E+C+P also inhibits chemotaxis towards SDF1α. Cells pre-treated with L+E+C+P at 4µg/ml or 8µg/ml for 12h prior to initiation of the chemotaxis assay towards SDF1α, showed significantly inhibited chemotaxis at both concentrations **(Fig 3.1F and Fig 3.2E)**; at 8µg/ml the effects were similar to those of 5%PJ. Therefore, L+E+C+P has the potential to inhibit metastasis of these cells to the bone marrow.

Because the combination of L+E+C+P significantly inhibited processes involved in metastasis, we also tested their effects individually using the scratch migration assay. L, E and P each significantly inhibited cell migration of PC3 and DU145 cells when individually applied at 4µg/ml or 8µg/ml **(Fig 3.3A-C and Fig 3.4A-C)**. However, caffeic acid when applied individually did not show any effects on cell migration of either cell type **(Fig 3.3D and Suppl. Fig 3.4D)**. Based on these findings, we eliminated caffeic acid as a potential anti-cancer component and focused on the effects of the other three components, L, E and P.

To test whether the combination of L+E+P is more potent than the combination of any two components, we treated PC3 and DU145 cells with two-component

combinations (L+E, L+P, and E+P) and three-component combination (L+E+P) at 4μg/ml or 8μg/ml **(Fig 3.5A,B and Fig 3.6A,B)**. Over 72hrs of the scratch wound assay, L+E+P was significantly more potent than any dual combinations at 48hrs and 72hrs. Based on these findings, we performed the remainder of the studies using L+E+P.

L+E+P significantly increased cell adhesion of PC3 **(Fig 3.5C)** and DU145 (**Fig 3.6C)** cells. The detachment time was increased by 4μg/ml or 8μg/ml of L+E+P treatment for 12hrs and 24hrs. When compared to the effect of PJ, L+E+P at 8μg/ml showed similar effect to 5%PJ. In addition, chemotaxis towards SDF1α was significantly inhibited by pre-treatment of PC3 and DU145 cells with L+E+P at 4μg/ml or 8μg/ml **(Fig 3.5D and Fig 3.6D)**. The effect of L+E+P at 8μg/ml mimicked the effect of 5%PJ. We also tested the effects of L+E+P combinations at different ratios on cell migration. However, we found that the ratios of 1:1:1 are the most potent combination.

To determine whether these effects of L+E+P are also found in androgen-sensitive cells, we used LNCaP, a prostate cancer cell line that is responsive to androgen and performed similar studies using growth, migration and SDF1α chemotaxis assays. L+E+P at 4μg/ml significantly suppressed cell growth of LNCaP cells **(Fig 3.7A)**. However, LNCaP cells are considerably more sensitive to these PJ components as we observed that cells were largely killed by L+E+P at 8μg/ml (data not shown). As a result, we used lower concentrations of L+E+P to treat LNCaP cells

in scratch migration assays and chemotaxis assays. L+E+P at 2μg/ml and 4μg/ml significantly inhibited cell migration over 72hrs of treatment **(Fig 3.7B)** and chemotaxis towards SDF1α **(Fig 3.7C)**. These results suggest that L+E+P are even more effective in androgen-sensitive than androgen-independent prostate cancer cells.

Effect of L+E+P on the expression of genes involved in cell growth, adhesion, migration and chemotaxis

To understand how L+E+P inhibit cell growth, cell migration and chemotaxis and increase cell adhesion, we performed Affymetrix microarray analysis to examine their effects on the expression of genes involved in these cell functions. For these studies we used total RNA from PC3 cells treated with L+E+P at 8μg/ml for 12 hrs and analyzed for gene expression using Affymetrix Human U133 Plus 2.0 microarrays. PC3 cells were used because these are the most invasive cells of all of three types of cells we studied. The data show that L+E+P inhibit the expression of several important genes involved in cell growth and cell migration while stimulating the expression of several genes involved in cell adhesion **(Fig 3.8A, blue arrows and Table 3.1)**. The genes we show that are increased all significantly enhance adhesion or are tumor suppressor genes. Those that are decreased are all related to stimulation of migration and cytoskeletal components or ECM molecules that facilitate migration.

To verify the effects of L+E+P on gene expression revealed by the Affymetrix arrays, we used quantitative real-time PCR (RT-PCR) to examine mRNA levels of

specific genes that are upregulated or downregulated in the gene arrays: claudin 1 (*CLDN1*), p21 (*CDKN1A*), Pten *(PTEN*), cyclin E2 (*CCNE2*), anillin (*ANLN*), cyclin B1 (CCNB1), denticleless homologue (*DTL*), hyaluronan-mediated motility receptor (*HMMR*), Twist (*TWIST*), Rock2 (*ROCK2*), cell division cycle homolog B (*CDC25B*), Bcl2 (*BCL2*), nexilin (*NEXN*), cyclin-dependent kinase 6 (*CDK6*), collagen I (COL1A1), fascin1 (*FSCN1*) and enhancer of zeste homolog 2 (*EZH2*). For the RT-PCR, total RNA was extracted from PC3 cells treated with L+E+P at 8µg/ml for 12hrs **(Fig 3.8B)**. The mRNA fold change of these genes was highly consistent with the Affymetrix array results.

Effect of L+E+P on the level of cancer related microRNAs

Micro-RNAs (miRNAs) are naturally-occurring small non-coding RNAs that function as negative regulators of gene expression. They regulate important cellular functions such as cell proliferation, apoptosis, differentiation and development. Mature miRNAs bind to target mRNAs which subsequently results in either direct cleavage of the targeted mRNAs or inhibition of translation. To determine the effects of L+E+P on cancer-related miRNAs and how they correlate with gene expression, we used miRNA PCR arrays to analyze the RNA obtained from PC3 cells treated with L+E+P at 8µg/ml for 12hrs. Among 88 well-known cancer-related miRNAs in the array, we found that those that function as tumor-suppressors are highly increased whereas those that function as oncogenic miRNAs are highly decreased **(Table 3.2)**.

Moreover, we found a consistent correlation between the level of miRNAs and the expression levels of their predicted target genes as shown in the Affymetrix mRNA arrays. These correlations are shown in **Figure 3.8A**.

Testing mechanistically specific genes involved in cell proliferation, adhesion and migration

To determine whether the inhibitory effect of L+E+P on cell proliferation can be reversed, we chose to overexpress cyclin E (CCNE2) which is the most decreased gene shown in the gene array and is known to be critical in cell proliferation. We found that the proliferation inhibitory effect of L+E+P was signifcantly reversed by CCNE2 overexpression **(Fig 3.9A)**.

We also found that L+E+P significantly increases the protein levels of E-cadherin **(Fig 3.9B)**. Loss of this adhesion protein is critical for invasion of epithelial tumor cells. To determine whether the effect of L+E+P on cell adhesion can be reversed, we used siRNA to inhibit E-cadherin and performed adhesion assays 36hrs after PC3 cells were transfected with E-cadherin siRNA. We found that L+E+P treatment significantly increases attachment of the untransfected (control) cells and that the effect is partially reversed by E-cadherin siRNA **(Fig 3.9C)**. These results indicate that the effects of L+E+P on cell adhesion are mediated significantly by increased E-cadherin.

To determine whether the inhibitory effects of L+E+P on cell migration can be reversed, we chose to overexpress HMMR (a gene that is known to promote cell migration and was highly decreased in the gene array) and TWIST (a gene that is known to play a role in epithelial-to-mesenchymal transitions and was also decreased in the gene array). The inhibitory effects of L+E+P on cell migration are partially reversed by overexpression of HMMR or TWIST. Scratch wounds on PC3 cell cultures were made 24h after transfection of HMMR or TWIST and the migrated distances were measured 36h or 48h later. We found that L+E+P treatment significantly decreased cell migration of untransfected cells but the effect was partially reversed by over-expressing HMMR **(Fig 3.9D)** and TWIST **(Fig 3.9E)**. These results indicate that the effects of L+E+P on cell migration are significantly mediated through decreasing HMMR and/or TWIST.

Effect of L+E+P on CXCR4/SDF1α signaling in prostate cancer cells

Approximately 80% of patients who have died of advanced hormone refractory prostate cancer have clinical evidence of bone metastases and 100% have histologic bone involvement(Roudier et al., 2003)**.** Constitutive production of SDF1α by bone marrow stromal cells is a major source of this chemokine. We show here that L+E+P inhibits chemotaxis of hormone independent prostate cancer cells towards SDF1α (**Fig. 3.5D and Fig 3.6D**). Moreover, we found that L+E+P significantly decreased the protein levels of CXCR4, the receptor for SDF1α which is present on the prostate

cancer cells, by 50% in PC3 cells **(Fig 3.10A)** and that they inhibit the downstream signaling pathway of CXCR4/SDF1α. We found that L+E+P inhibits activation of PI3K **(Fig 3.10B)** and abolishes phosphorylation/activation of AKT induced by SDF1α **(Fig 3.10C)**. These findings show that L+E+P inhibits the CXCR4/SDF1α chemotaxis axis both at the receptor level and the downstream signaling pathways, making these components strong contenders for treatment of prostate cancer metastasis.

DISCUSSION

The studies presented here identify L, E and P as potential anti-metastatic components in PJ. We delineate cellular and molecular mechanisms involved in inhibition of processes critical for metastasis of prostate cancer cells and show that L+E+P: (**i**) suppresses the growth of two prostate cancer cell lines that are irresponsive to androgen deprivation and one line that is responsive to androgen deprivation; (**ii**) stimulates prostate cancer cells to adhere strongly to the substrate; (**iii**) inhibits the migratory capabilities of prostate cancer cells and their chemotaxis toward SDF1α; (**iv**) stimulates expression of genes involved in cell adhesion while reducing expression of genes involved in cell migration and cell cycle control; and (**v**) increases the levels of tumor-suppressive miRNAs while reducing the level of oncogenic miRNAs.

During metastasis cancer cells loose adhesion to each other and become migratory. E-cadherin has been shown as a critical adhesion molecule holding epithelial cells together and preventing them from breaking away. We found that the protein levels of E-cadherin are increased whereas the expression levels of Zeb1 (ZEB1), a zinc finger transcription repressor of E-cadherin, is inhibited by L+E+P. Furthermore, when the cells are treated with siRNA for E-cadherin we observe that the effect of the PJ components is reversed strongly suggesting that E-cadherin is a key target of L+E+P. Moreover, the expression of a tight junction protein claudin 1

is increased by treatment with these components. Therefore, L+E+P increase cell adhesion through up-regulation of these cell adhesion proteins.

Several molecules involved in cell migration are downregulated by L+E+P. For example, HMMR binds hyaluronan (HA), a molecule that facilitates cell migration. Binding of HA to HMMR stimulates the RhoA-activated protein kinase (ROCK) signal transduction pathway, leading to tumor cell migration and invasion in various cancers (Lin et al., 2007). We found that the expression of HMMR and ROCK2 were down-regulated by L+E+P. Other proteins such fascin which is an actin-bundling protein, that regulates the actin cytoskeleton and the formation of migration initiating filopodia and actin-binding proteins anillin and nexillin, which are involved in regulation of the structure of the cytoskeleton, (Glotzer, 2005; Ohtsuka et al., 1998a) are down-regulated by L+E+P. Twist (TWIST) is a basic-helix-loop-helix transcription factor that has been implicated in epithelial-to-mesenchymal transition (EMT) characterized by loss of cell adhesion and increased cell motility. (Kang and Massague, 2004b; Thiery et al., 2009).Therefore, our results strongly suggest that L+E+P decrease cell migration through down-regulation of genes involved in cell migration and transcriptional factors involved in EMT.

The three PJ components also inhibit cell division. For example, Cyclins E2 and B1 and cell cycle regulator CDK6 are highly down-regulated by L+E+P treatment. p21, also known as CDK inhibitor 1, binds to and inhibits the activity of cyclin-CDK complex and therefore functions as a key regulator to control cell cycle progression

(Harper et al., 1993). We found that p21 was up-regulated by L+E+P treatment. Denticleless homologue (DTL) is a substrate-specific adaptor of E3 ubiquitin-protein ligase complex required for cell cycle control. DTL mediates the ubiquitination and subsequent degradation of p21 (Higa et al., 2006). Down-regulation of DTL together with up-regulation of p21 by L+E+P can potentially lead to suppression of cell cycle progression. Therefore, L+E+P may inhibit cell growth through down-regulation of genes involved in cell cycle control.

MicroRNAs (miRNAs) have been shown to have profound impact on post-transcriptional gene regulation. Aberrant expression of miRNAs occurs in diverse types of human cancer and in different stages of disease progression. Our miRNA PCR array results show that many well-known tumor-suppression miRNAs are highly up-regulated whereas many oncogenic miRNAs are highly down-regulated by L+E+P. Among the tumor-suppression miRNAs, miR-144 is known to negatively regulate the Notch-1 signaling pathway which has been suggested to be involved in a wide variety of human cancers (Sureban et al., 2011). miR-133b is known to negatively target receptor tyrosine kinase c-Met and actin-bundling protein fascin. Aberrantly activated c-Met (also known as hepatocyte growth factor receptor) has been shown to promote tumor growth, angiogenesis and metastasis in various cancers, and fascin is known as an important regulator of actin cytoskeleton and cell migration (Akcakaya et al., 2011; Kano et al., 2010). miR-1 is known to negatively target cyclin D2, a protein required for cell cycle progression and also CXCR4 and its ligand

SDF1α which are known as important chemotactic proteins in cancer metastasis (Leone et al., 2011; Nohata et al., 2011). miR-212 is known to negatively target anti-apoptotic protein PED and transcriptional factor c-Myc. miR-122 is known to negatively target cyclin B1 (Incoronato et al., 2010) and miR-34c to negatively target transcriptional factor E2F3 and apoptosis key regulator Bcl2 (Cai et al., 2010; Hagman et al., 2010). miR-200c has been shown to inhibit *ZEB1* and *ZEB2*, which are transcriptional repressors of the E-cadherin gene, whose product is critical in cell adhesion (Korpal et al., 2008a). As a result, L+E+P might stimulate the expression of E-cadherin by inhibiting its transcriptional repression ZEB1 and ZEB through up-regulating miR-200c. miR-335 has been identified as a metastasis suppressive miRNA in breast cancer by inhibiting type I collagen and tenascin C. Type I collagen is an extracellular matrix molecule involved in cytoskeletal control and tenascin C is involved in the regulation of cell migration (Tavazoie et al., 2008a). miR-124 is known to negatively regulate ROCK2, a kinase involved in cell migration and CDK6 a key regulator of cell cycle (Zheng et al., 2011). Among the oncogenic miRNAs which are highly down-regulated by L+E+P, miR-21 is one of the first discovered and best-studied oncogenic miRNAs which negatively regulate several tumor suppressor genes including tropomyosin 1 (TMP1) and programmed cell death 4 (PDCD4) protein (Zhu et al., 2008b). miR-9 is known to negatively target E-cadherin (Ma et al., 2010) and miR-29b and miR-181b are oncogenic miRNAs known to negatively target tumor suppressor Pten (Wang et al., 2011a). Interestingly, the mRNA level of Pten is

up-regulated by L+E+P treatment which correlates with the down-regulation of miR-29b and miR-181b. These results show that L+E+P significantly up-regulate many tumor-suppression miRNAs and significantly down-regulate many oncogenic miRNAs.

It is also known that, with time, prostate cancer cells develop ways to bypass the need for testosterone and then the cancer progresses very rapidly. It has been shown that the CXCR4/SDF1α chemotaxis axis may play a critical role in the metastasis of prostate cancer to bone and constitutive production of SDF1α by bone marrow stromal cells is a major source of this chemokine. We have shown that L+E+P is capable of inhibiting prostate cancer migration towards SDF1α as shown in our chemotaxis experiments. In addition, we found that L+E+P significantly reduced the level of CXCR4 in prostate cancer cells and inhibited the phosphorylation/activation of AKT. Therefore, L+E+P may act by suppressing the levels of CXCR4 and inhibiting the downstream signaling pathway of CXCR4/SDF1α.

Conclusion and Future Prospects

To date, there is no cure for prostate cancer when recurrence occurs after surgery and/or radiation. In particular, when it recurs after hormone ablation therapy there are no other effective treatments for deterrence of cancer progression. Here we show that luteolin, ellagic acid and punicic acid, components of pomegranate juice, can potentially be used as anti-metastatic treatments to deter prostate cancer metastasis. L+E+P interfere with multiple biological processes involved in metastasis of cancer

cells such as suppression of cell growth, increase in cell adhesion, inhibition of in cell migration and inhibition of chemotaxis towards proteins that are important in prostate cancer metastasis to the bone.

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Zhou, Q., Yan, B., Hu, X., Li, X.B., Zhang, J., and Fang, J. (2009). Luteolin inhibits invasion of prostate cancer PC3 cells through E-cadherin. Mol Cancer Ther *8*, 1684-1691.

Zhu, S., Wu, H., Wu, F., Nie, D., Sheng, S., and Mo, Y.Y. (2008). MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res *18*, 350-359.

Figure 3.1. L+E+C+P inhibits growth, stimulate adhesion, inhibits migration and inhibits chemotaxis towards of SDF1α of hormone-independent prostate cancer cells. (A) The chemical structure of L, E, P and C. **(B)** Prostate cancer cell PC3 were treated with 4 PJ components L+E+C+P at 4µg/ml and 8µg/ml and counted for increasing times after initiation of treatment. Controls represent no treatment. Media containing L+E+C+P was changed daily. **(C)** PC3 cells were treated with L+E+C+P at 4µg/ml and 8µg/ml and the percentage of dead cells was determined by Trypan blue staining at the indicated time points. **(D)** PC3 cells were plated on gelatin-coated dishes and 24 hrs later media was changed and the cells were treated with L+E+C+P at 4µg/ml and 8µg/ml. We tested for adhesion to the substrate at 12 and 24hrs after initiation of treatment by recording the time it took for trypsinization to remove all of the cells from the dish. Control represents no treatment. The reason for not presenting statistical significance is because the loss of adhesion is very similar from culture to culture and it occurs rapidly when the cells begin to detach. Within each experiment, the times of trypsinization were the same within 1 minute for each specific treatment. **(E)** PC3 cells were treated with L+E+C+P at 4µg/ml and 8µg/ml for 72 hrs and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. Controls represent no treatment. Media containing the components was changed daily. **(F)** PC3 cells were allowed to attach to the top of the filter of the chemotaxis assay chambers for 4 hrs and then treated with L+E+C+P at 4µg/ml and 8µg/ml for 12hrs. At this time, 100ng/ml of SDF1α were introduced into the lower chamber and the cells found on the bottom of the filter counted 3.5 hrs later. Control had no treatment. The number of cells found on the underside of the filter was counted 3.5h later. Bars represent Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

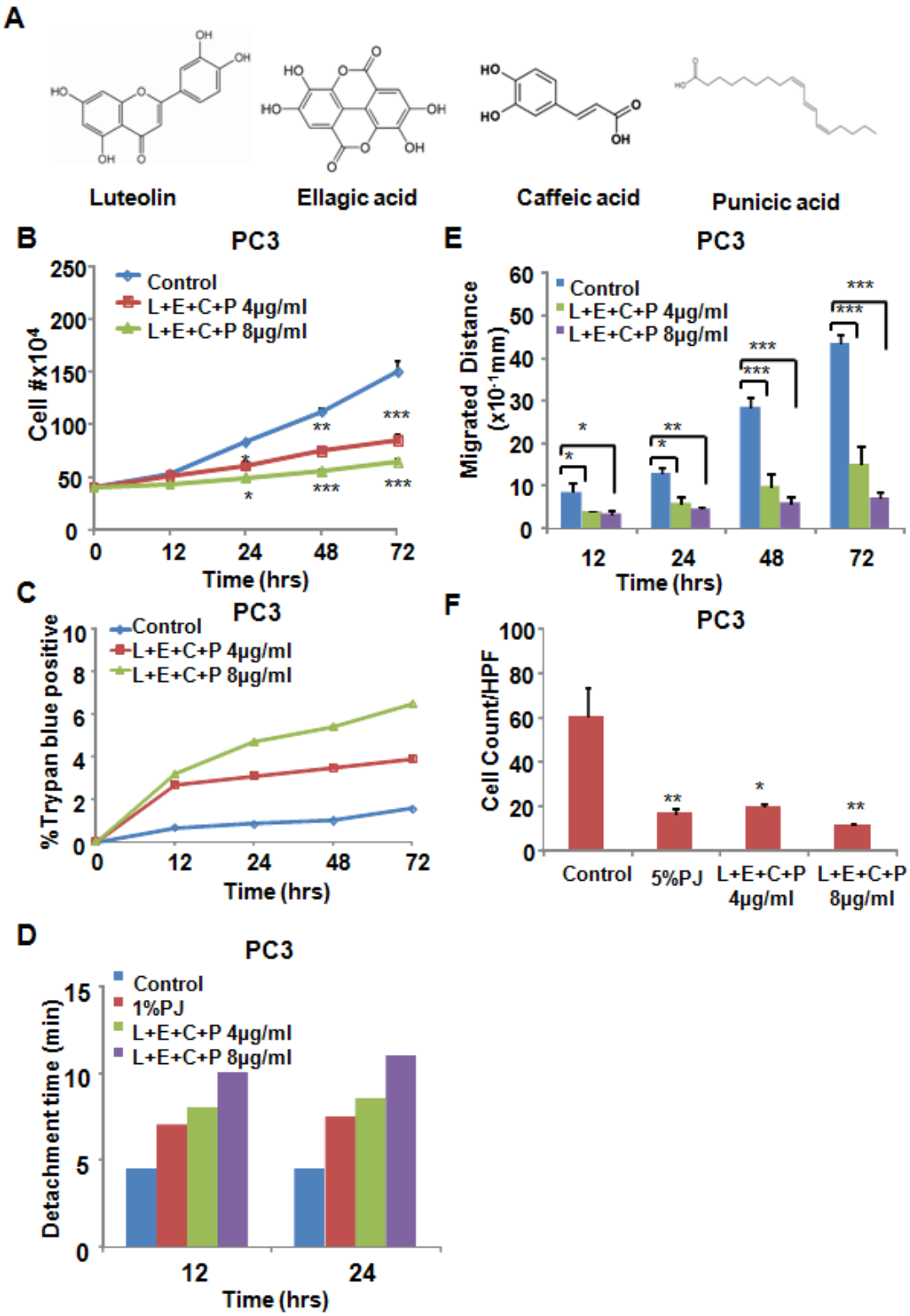
A
Luteolin
Ellagic acid
Caffeic acid
Punicic acid
B
PC3
Control
L+E+C+P 4µg/ml
L+E+C+P 8µg/ml
Cell #x10⁴
Time (hrs)
C
PC3
% Trypan blue positive
Time (hrs)
D
PC3
Control
1%PJ
L+E+C+P 4µg/ml
L+E+C+P 8µg/ml
Detachment time (min)
Time (hrs)
E
PC3
Migrated Distance (x10⁻¹mm)
F
PC3
Cell Count/HPF
Control
5%PJ
L+E+C+P 4µg/ml
L+E+C+P 8µg/ml

Figure 3.1

Figure 3.2. L+E+C+P inhibits growth, stimulate adhesion, inhibits migration and inhibits chemotaxis towards of SDF1α of hormone-independent prostate cancer cells DU145. (A) DU145 prostate cancer cells were treated with 4 PJ components L+E+C+P at 4µg/ml and 8µg/ml and counted for increasing times after initiation of treatment. Controls represent no treatment. Media containing PJ components was changed daily. Bars represent Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$. **(B)** DU145 cancer cells were plated on gelatin-coated dishes and 24 hrs later media was changed and the cells were treated with PJ components L+E+C+P at 4µg/ml and 8µg/ml . We tested for adhesion to the substrate at 12 and 24hrs after initiation of treatment by recording the time it took for trypsinization to remove all of the cells from the dish. Control represents no treatment. Within each experiment, the times of trypsinization were the same within 1 minute for each specific treatment. **(C)** DU145 prostate cancer cells were treated with PJ components L+E+C+P at 4µg/ml and 8µg/ml for 72 hrs and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. Controls represent no treatment. Media containing PJ components were changed daily. **(D)** DU145 cancer cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 hrs and then treated with PJ components L+E+C+P at 4µg/ml and 8µg/ml for 12hrs. At this time, 100ng/ml of SDF1α were introduced into the lower chamber and the cells found on the bottom of the filter counted 3.5 hrs later. Control had no treatment. The number of cells found on the underside of the filter was counted 3.5h later. Bars represent Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

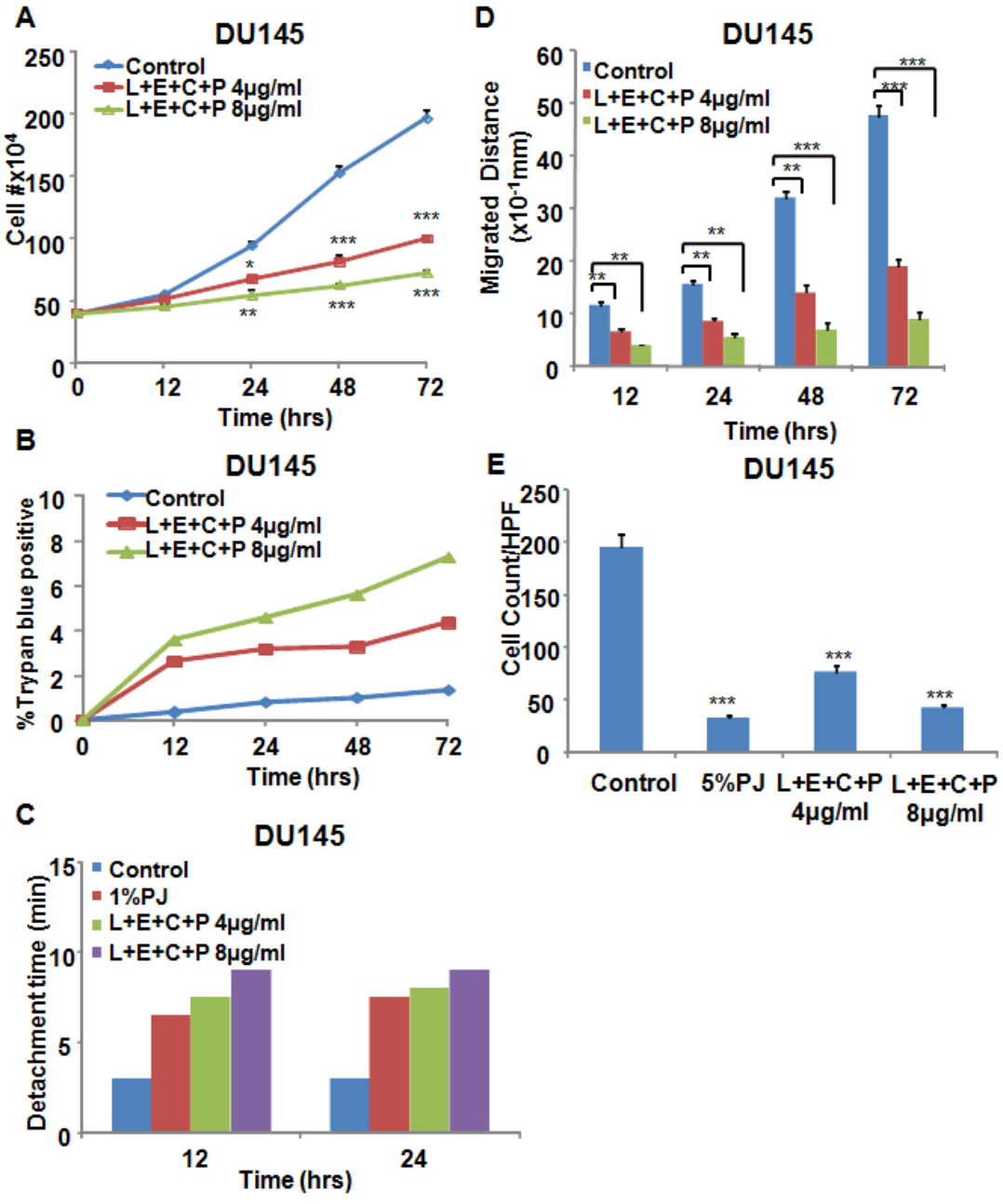
A
DU145
Control
L+E+C+P 4µg/ml
L+E+C+P 8µg/ml
Cell #x10⁴
Time (hrs)
B
DU145
% Trypan blue positive
Time (hrs)
C
DU145
Control
1%PJ
L+E+C+P 4µg/ml
L+E+C+P 8µg/ml
Detachment time (min)
Time (hrs)
D
DU145
Migrated Distance (x10⁻¹mm)
Time (hrs)
E
DU145
Cell Count/HPF
Control
5%PJ
L+E+C+P 4µg/ml
L+E+C+P 8µg/ml

Figure 3.2

Figure 3.3. Luteolin, Ellagic acid, and Punicic acid but not Caffeic acid individually inhibits cell migration of hormone-independent prostate cancer cells PC3. PC3 prostate cancer cells were treated with individually PJ component luteolin (A), ellagic acid (B), punicic acid (C) and caffeic acid (D) at 4µg/ml and 8µg/ml for 72 hrs and migration assay was performed as described in Fig 3.1. Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

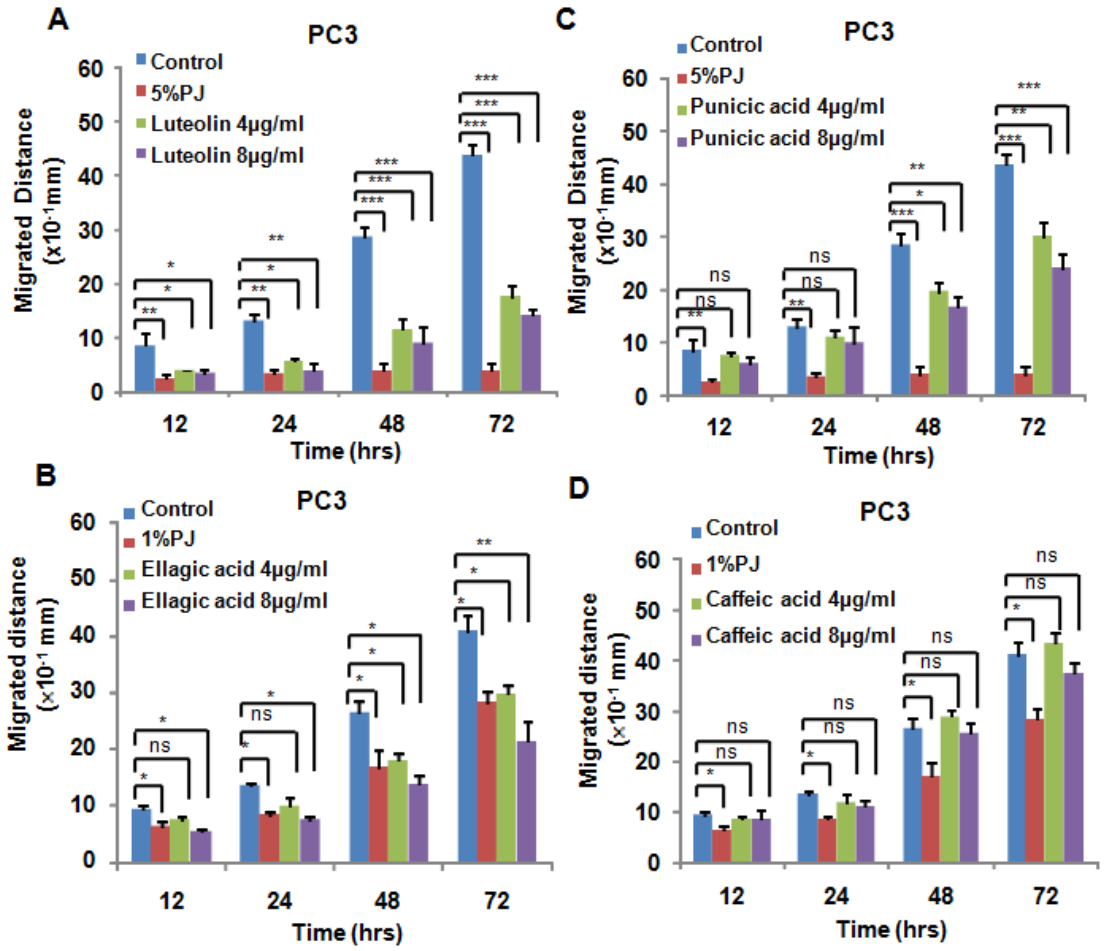
A
PC3
Control
5%PJ
Luteolin 4µg/ml
Luteolin 8µg/ml
Migrated Distance (x10⁻¹mm)
Time (hrs)
B
PC3
Control
1%PJ
Ellagic acid 4µg/ml
Ellagic acid 8µg/ml
Migrated distance (x10⁻¹ mm)
Time (hrs)
C
PC3
Control
5%PJ
Punicic acid 4µg/ml
Punicic acid 8µg/ml
Migrated Distance (x10⁻¹mm)
Time (hrs)
D
PC3
Control
1%PJ
Caffeic acid 4µg/ml
Caffeic acid 8µg/ml
Migrated distance (x10⁻¹ mm)
Time (hrs)

Figure 3.3

Figure 3.4. Luteolin, Ellagic acid, and Punicic acid but not Caffeic acid individually inhibits cell migration of hormone-independent prostate cancer cells DU145. DU145 prostate cancer cells were treated with individually PJ component luteolin (A), ellagic acid (B), punicic acid (C) and caffeic acid (D) at 4µg/ml and 8µg/ml for 72 hrs and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. Controls represent no treatment. Media containing PJ components were changed daily. Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

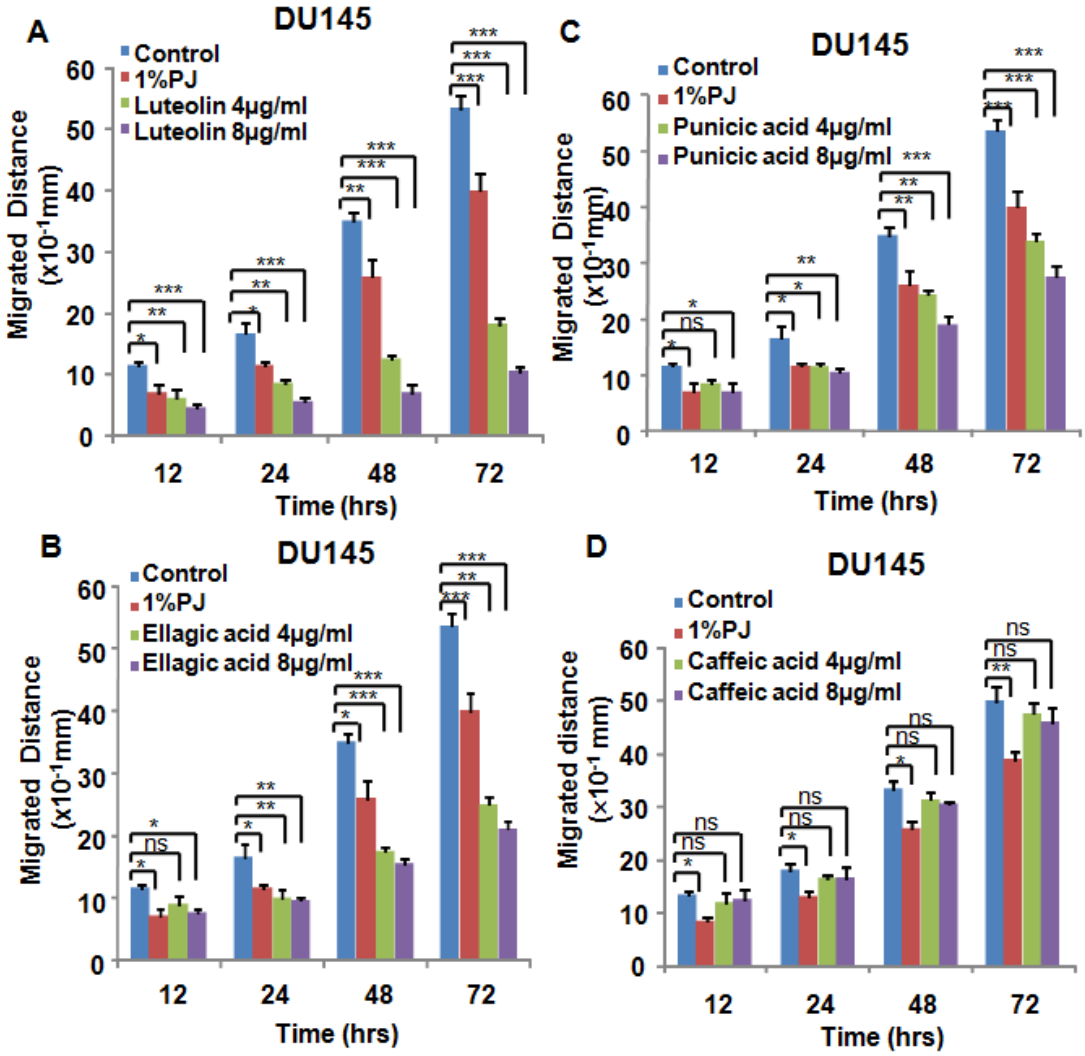
A
DU145
Control
1%PJ
Luteolin 4µg/ml
Luteolin 8µg/ml
Migrated Distance (x10-1mm)
Time (hrs)
C
DU145
Control
1%PJ
Punicic acid 4µg/ml
Punicic acid 8µg/ml
Migrated Distance (x10-1mm)
Time (hrs)
B
DU145
Control
1%PJ
Ellagic acid 4µg/ml
Ellagic acid 8µg/ml
Migrated Distance (x10-1mm)
Time (hrs)
D
DU145
Control
1%PJ
Caffeic acid 4µg/ml
Caffeic acid 8µg/ml
Migrated distance (x10-1 mm)
Time (hrs)

Figure 3.4

Figure 3.5. L+E+P is the most potent combination for inhibition of cell migration and chemotaxis towards SDF1α as well as increasing cell adhesion of hormone-independent prostate cancer cells PC3. PC3 cells were treated with different combination of PJ components L, E and P at 4µg/ml **(A)** and 8µg/ml **(B)** for up to 72 hrs and migration assay was performed as described in Fig 1. **(C)** PC3 cells were plated on gelatin-coated dishes and 24 hrs later media was changed and the cells were treated with PJ components L+E+P at 4µg/ml and 8µg/ml. Adhesion assay was performed as described in Fig 1. **(D)** PC3 cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 hrs and then treated with L+E+P at 4µg/ml and 8µg/ml for 12hrs. Chemotaxis assay was performed as described in Fig 3.1. Bars represent Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

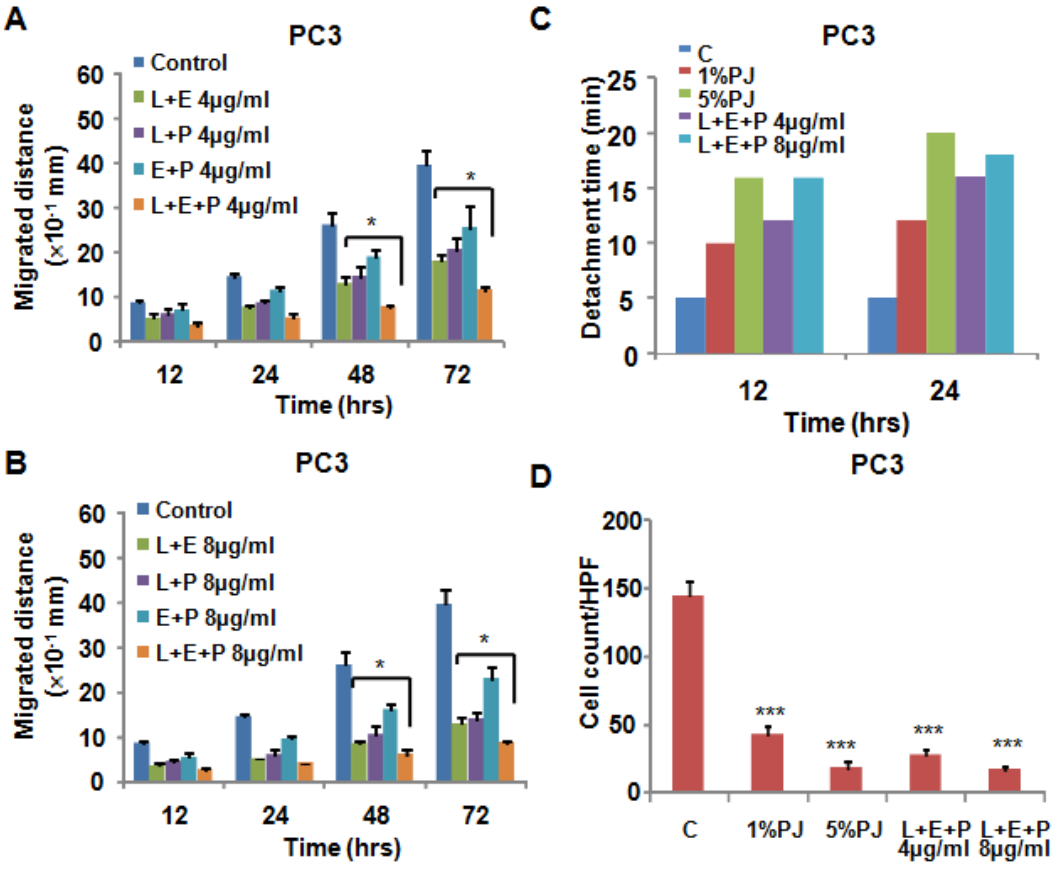
A
PC3
Control
L+E 4µg/ml
L+P 4µg/ml
E+P 4µg/ml
L+E+P 4µg/ml
Migrated distance (×10⁻¹ mm)
Time (hrs)
B
PC3
Control
L+E 8µg/ml
L+P 8µg/ml
E+P 8µg/ml
L+E+P 8µg/ml
Migrated distance (×10⁻¹ mm)
Time (hrs)
C
PC3
C
1%PJ
5%PJ
L+E+P 4µg/ml
L+E+P 8µg/ml
Detachment time (min)
Time (hrs)
D
PC3
Cell count/HPF
C
1%PJ
5%PJ
L+E+P 4µg/ml
L+E+P 8µg/ml

Figure 3.5

Figure 3.6. L+E+P is the most potent combination to inhibit cell migration and its effect on cell adhesion and chemotaxis towards SDF1α of hormone-independent prostate cancer cells DU145. DU145 prostate cancer cells were treated with different combination of PJ components L, E and P at 4μg/ml **(A)** and 8μg/ml **(B)** for 72 hrs and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. Controls represent no treatment. Media containing PJ components were changed daily. Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. **(C)** DU145 cancer cells were plated on gelatin-coated dishes and 24 hrs later media was changed and the cells were treated with PJ components L+E+P at 4μg/ml and 8μg/ml. We tested for adhesion to the substrate at 12 and 24hrs after initiation of treatment by recording the time it took for trypsinization to remove all of the cells from the dish. Control represents no treatment. Within each experiment, the times of trypsinization were the same within 1 minute for each specific treatment. **(D)** DU145 cancer cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 hrs and then treated with PJ components L+E+P at 4μg/ml and 8μg/ml for 12hrs. At this time, 100ng/ml of SDF1α were introduced into the lower chamber and the cells found on the bottom of the filter counted 3.5 hrs later. Control had no treatment. The number of cells found on the underside of the filter was counted 3.5h later. Bars represent Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

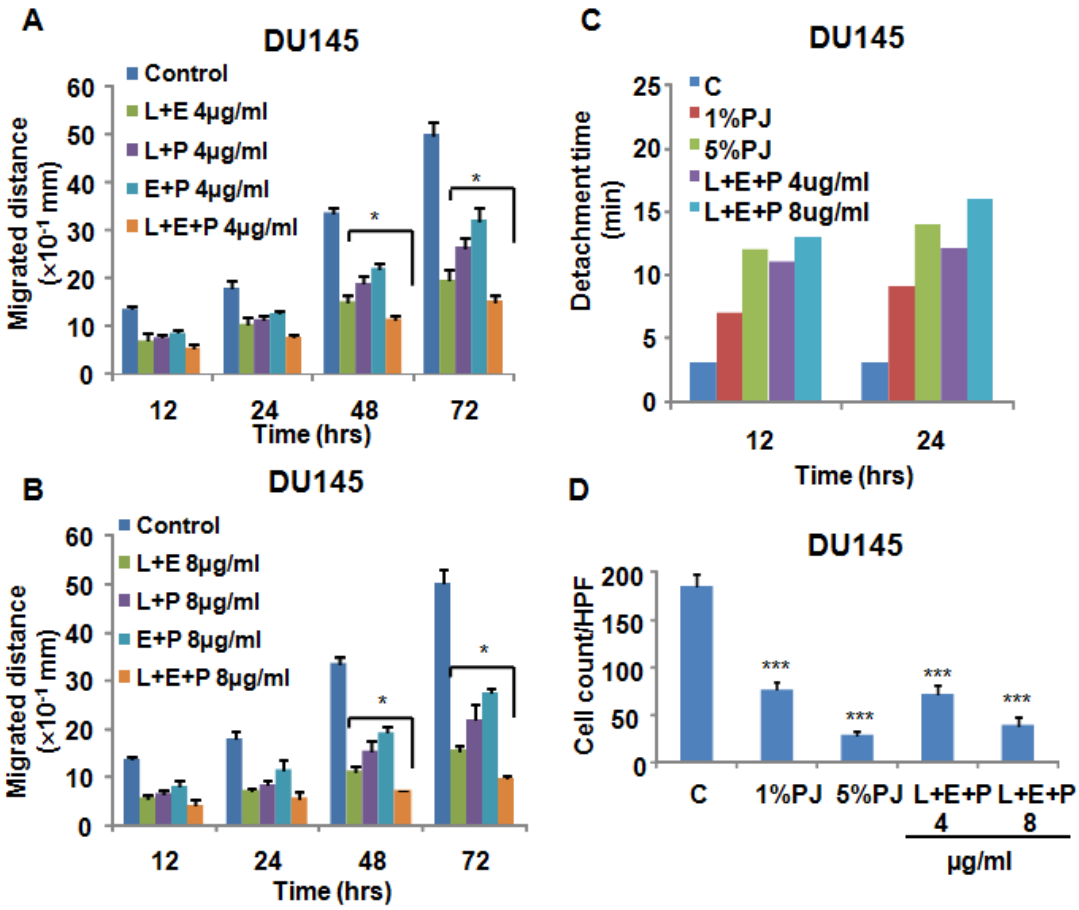
A
DU145
Control
L+E 4μg/ml
L+P 4μg/ml
E+P 4μg/ml
L+E+P 4μg/ml
Migrated distance (×10⁻¹ mm)
Time (hrs)
B
DU145
Control
L+E 8μg/ml
L+P 8μg/ml
E+P 8μg/ml
L+E+P 8μg/ml
Migrated distance (×10⁻¹ mm)
Time (hrs)
C
DU145
C
1%PJ
5%PJ
L+E+P 4ug/ml
L+E+P 8ug/ml
Detachment time (min)
Time (hrs)
D
DU145
Cell count/HPF
C
1%PJ
5%PJ
L+E+P 4
L+E+P 8
μg/ml

Figure 3.6

Figure 3.7. L+E+P suppresses cell growth, stimulates cell adhesion and inhibits chemotaxis to SDF1α of prostate cancer cell LNCaP. (A) Prostate cancer cell LNCaP were treated with L+E+P at 4µg/ml and counted for increasing times after initiation of treatment. **(B)** LNCaP cells were plated on gelatin-coated dishes and 24 hrs later media was changed and the cells were treated with L+E+P at 2µg/ml and 4µg/ml. Adhesion assay was performed as described in Fig 1. **(C)** LNCaP cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 hrs and then treated with L+E+P at 2µg/ml and 4µg/ml for 12hrs. Chemotaxis assay was performed as described in Fig 3.1. Bars represent Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

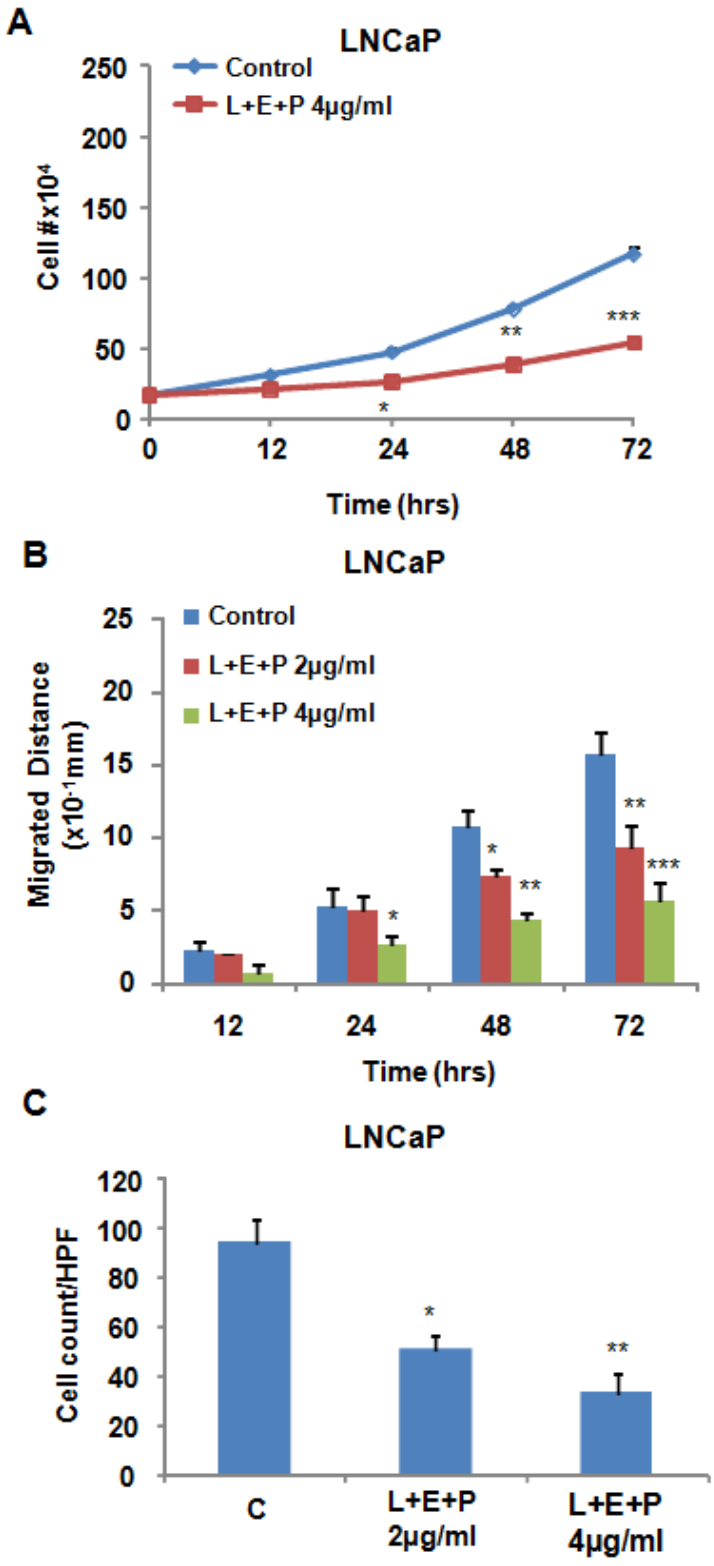
A
LNCaP
Control
L+E+P 4µg/ml
Cell #x10⁴
250
200
150
100
50
0
0
12
24
48
72
Time (hrs)
*
**
***
B
LNCaP
Control
L+E+P 2µg/ml
L+E+P 4µg/ml
Migrated Distance (x10⁻¹mm)
25
20
15
10
5
0
12
24
48
72
Time (hrs)
C
LNCaP
Cell count/HPF
120
100
80
60
40
20
0
C
L+E+P 2µg/ml
L+E+P 4µg/ml

Figure 3.7

Figure 3.8. Effect of L+E+P on genes involved in cell growth, cell adhesion and migration of hormone-independent prostate cancer cells. (A) Schematic summary of the effects of L+E+P on gene and miRNA expression in hormone-independent prostate cancer cells. (**B**) The mRNA level of *CCNE2, ANLN, CCNB1, DTL, HMMR, TWIST, ROCK2, CDC25B, BCL2, NEXN, CDK6, COL1A1, FSCN1, EZH2, PTEN, CDKN1A* and *CLDN1* were determined by using qPCR with RNA extracted from PC3 cells treated with L+E+P at 8µg/ml for 12 hrs.

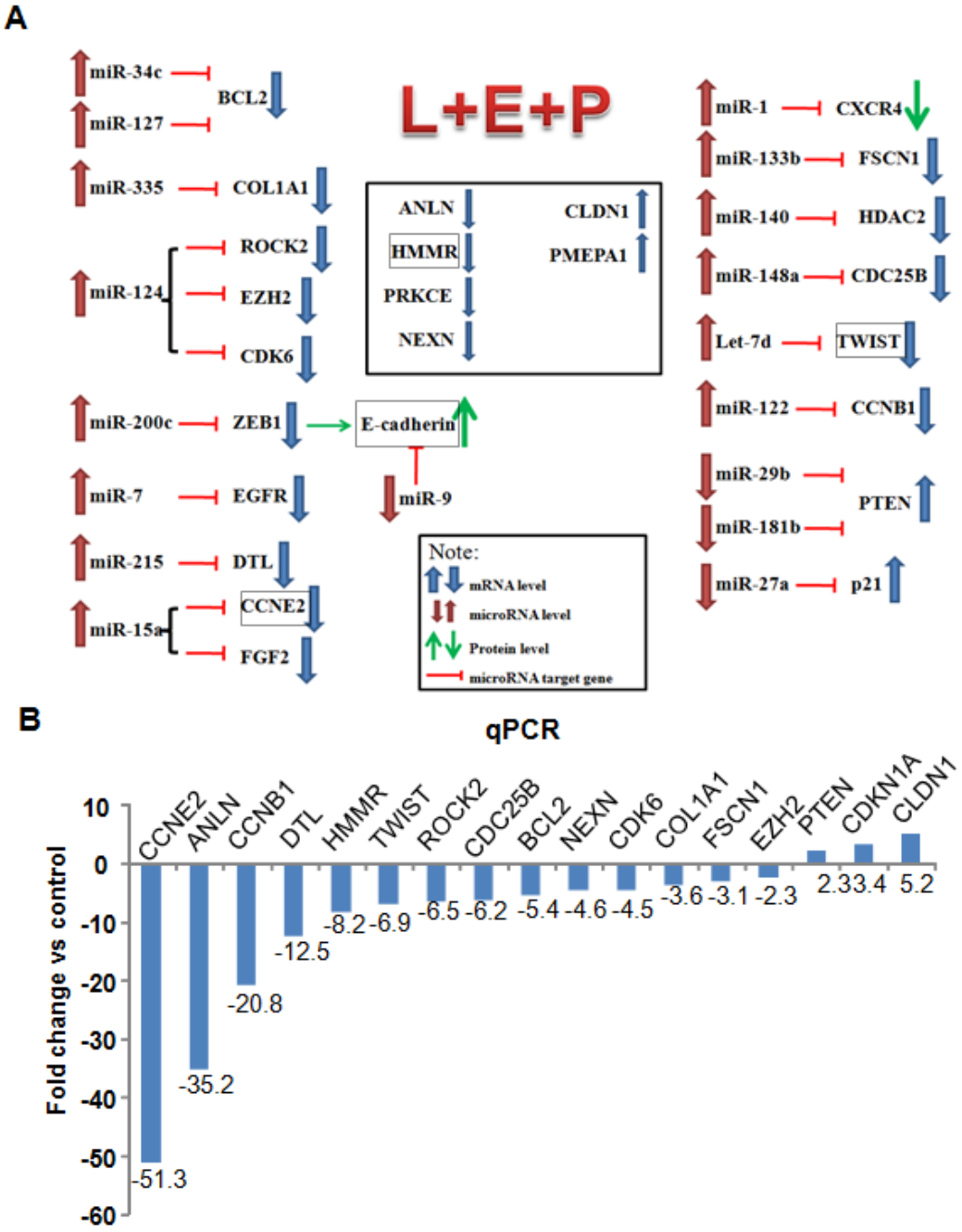
A
L+E+P
miR-34c
miR-127
BCL2
miR-335
COL1A1
miR-124
ROCK2
EZH2
CDK6
miR-200c
ZEB1
E-cadherin
miR-9
miR-7
EGFR
miR-215
DTL
miR-15a
CCNE2
FGF2
ANLN
HMMR
PRKCE
NEXN
CLDN1
PMEPA1
miR-1
CXCR4
miR-133b
FSCN1
miR-140
HDAC2
miR-148a
CDC25B
Let-7d
TWIST
miR-122
CCNB1
miR-29b
miR-181b
PTEN
miR-27a
p21
Note:
mRNA level
microRNA level
Protein level
microRNA target gene
B
qPCR
Fold change vs control
10
0
-10
-20
-30
-40
-50
-60
CCNE2
ANLN
CCNB1
DTL
HMMR
TWIST
ROCK2
CDC25B
BCL2
NEXN
CDK6
COL1A1
FSCN1
EZH2
PTEN
CDKN1A
CLDN1
-51.3
-35.2
-20.8
-12.5
-8.2
-6.9
-6.5
-6.2
-5.4
-4.6
-4.5
-3.6
-3.1
-2.3
2.3
3.4
5.2

Figure 3.8

Figure 3.9. Mechanistically study of the effect of L+E+P on cell growth, cell adhesion and migration of hormone-independent prostate cancer cells. (A) PC3 cells were transfected with pcDNA3 CCNE2 vector (2µg/ml) and treated with L+E+P at 8µg/ml 24hrs after transfection. Cell number was counted at 48hr time point **(B)** Immunoblot analysis for E-cadherin with protein extracts from PC3 cells treated with L+E+P at 8µg/ml for 24hrs. **(C)** PC3 cells were transfected with 40nM E-cadherin siRNA. 24hrs after transfection, cells were treated with L+E+P at 8µg/ml for 12hrs and adhesion assay was performed. PC3 cells were transfected with 2µg/ml of pcDNA3.1 HMMR vector **(D)** or pcDNA4.1 TWIST vector **(E)** and treated with L+E+P at 8µg/ml 24hrs after transfection. Migrated distance was determined at 48hr time point. Bars represent Standard Error of the Mean. $^{**}p < 0.01$; $^{*}p < 0.05$.

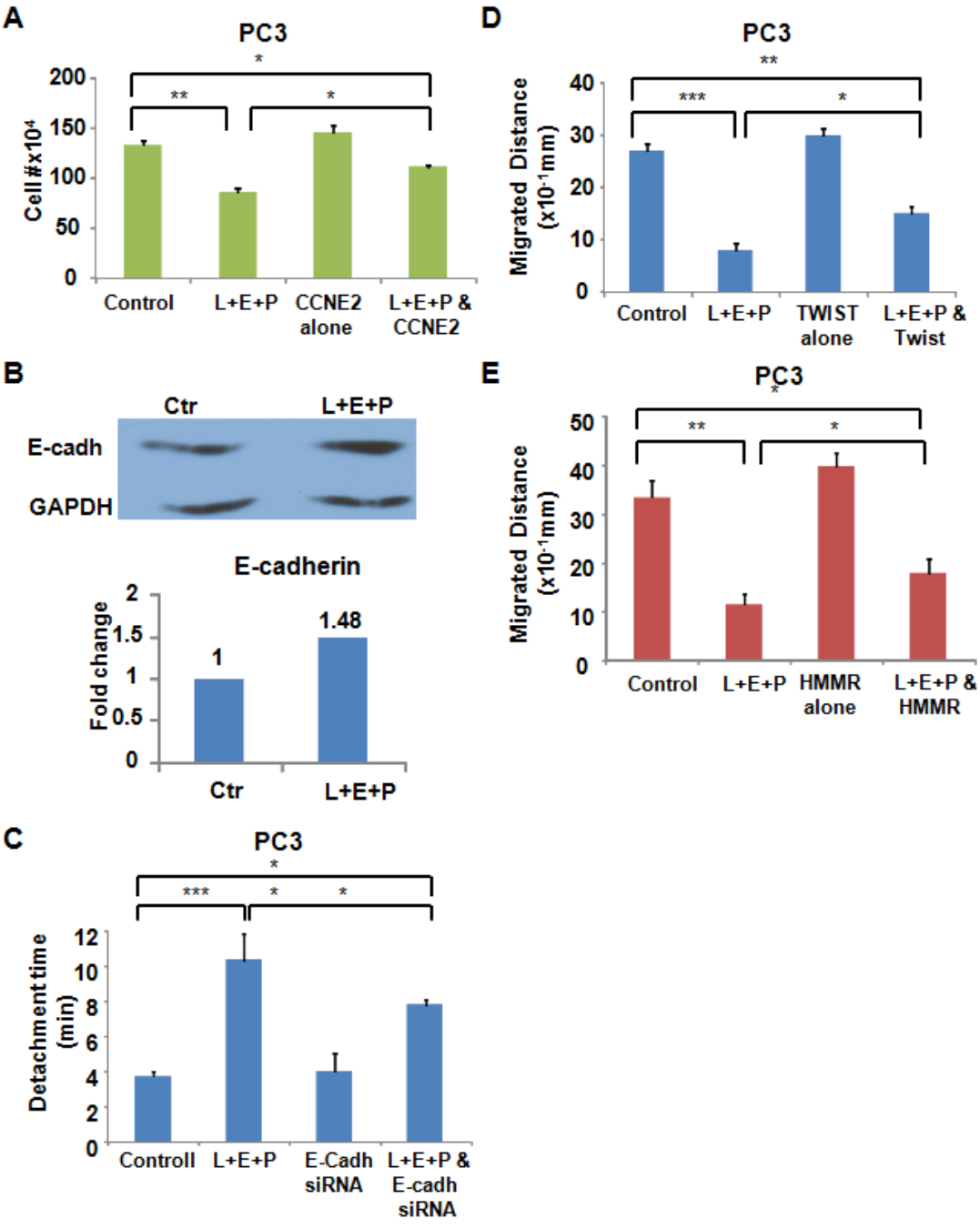
A
PC3
Cell #x10⁴
200
150
100
50
0
Control
L+E+P
CCNE2 alone
L+E+P & CCNE2
B
Ctr
L+E+P
E-cadh
GAPDH
E-cadherin
Fold change
2
1.5
1
0.5
0
1
1.48
Ctr
L+E+P
C
PC3
Detachment time (min)
12
10
8
6
4
2
0
Controll
L+E+P
E-Cadh siRNA
L+E+P & E-cadh siRNA
D
PC3
Migrated Distance (x10⁻¹mm)
40
30
20
10
0
Control
L+E+P
TWIST alone
L+E+P & Twist
E
PC3
Migrated Distance (x10⁻¹mm)
50
40
30
20
10
0
Control
L+E+P
HMMR alone
L+E+P & HMMR

Figure 3.9

Figure 3.10. Effect of L+E+P on the SDF1α chemotaxis in prostate cancer cells (**A)** Immuoblot analysis for CXCR4 in extracts prepared from PC3 cells treated with L+E+P at 8µg/ml for 24 hrs. **(B)** PI3K immunoblotting with protein extract from PC3 cells treated with 200ng/ml SDF1α for 8hrs in the presence of L+E+P at 8µg/ml. (C) phosphor-Akt immunoblotting with protein extract from PC3 cells induced with 200ng/ml SDF1α for 2mins in the presence of L+E+P at 8µg/ml.

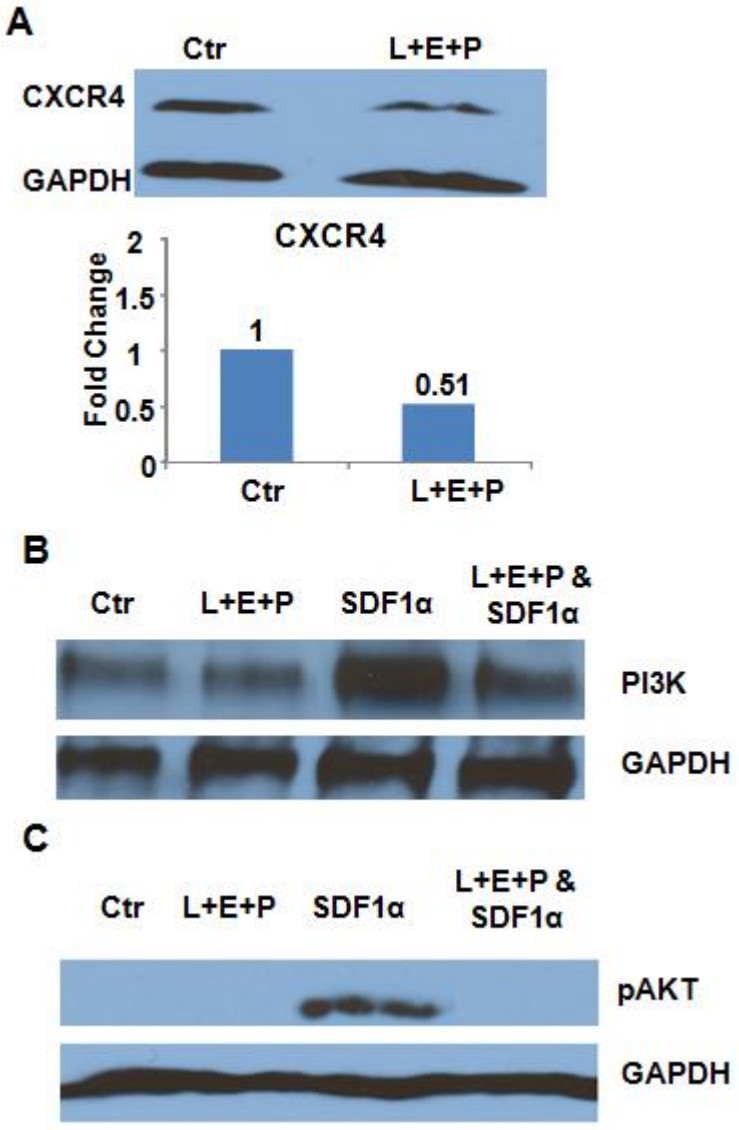
A
Ctr
L+E+P
CXCR4
GAPDH
CXCR4
Fold Change
2
1.5
1
0.5
0
1
0.51
Ctr
L+E+P
B
Ctr
L+E+P
SDF1α
L+E+P &
SDF1α
PI3K
GAPDH
C
Ctr
L+E+P
SDF1α
L+E+P &
SDF1α
pAKT
GAPDH

Figure 3.10

Table 3.1. L+E+P changes the expression profile of genes involved in the cell growth, cytoskeleton and cell adhesion machinery. RNA was extracted from PC3 cells that had been treated with L+E+P at 8μg/ml for 12 hrs and Affymetrix array analysis was performed as described in Materials and Methods. Relative mRNA levels are presented as fold change compared with untreated controls.

Gene analysis of the effects of L+E+P on PC3 cells

Gene Name	Product	Fold change	Function
CDKN2B	Cyclin-dependent kinase inhibitor 2B; p15	↑4.9	Cell cycle control
CLDN1	Claudin1	↑3.6	Adhesion
CDKN2A	Cyclin-dependent kinase inhibitor 2A; p16	↑2.2	Cell cycle control
CDKN1A	Cyclin-dependent kinase inhibitor 1A; p21	↑2	Cell cycle control
PTEN	Pten	↑1.6	Tumor suppressor
CCNE2	Cyclin E2	↓18	Cell cycle control
ANLN	Anillin	↓16	Migration
HMMR	Hyaluronan-mediated motility receptor	↓8	Migration
DTL	Denticleless homolog	↓7	Cell cycle control
CCNB1	Cyclin B1	↓5.1	Cell cycle control
CCNB2	Cyclin B2	↓4.5	Cell cycle control
ROCK2	Rock2	↓4	Migration
ZEB1	Zinc finger E-box binding homeobox 1	↓3	EMT
TWIST	Twist	↓2.6	EMT
CDC25B	Cell division cycle 25 homolog B	↓2.5	Cell cycle control
EZH2	Enhancer of zeste homolog 2	↓2.3	Oncogenic
NEXN	Nexilin	↓2.3	Migration
BCL2	Bcl2	↓2	Anti-apoptotic
COL1A1	Collagen 1	↓2	Migration
CDK6	Cyclin-dependent kinase 6	↓1.8	Cell cycle control
FSCN1	Fascin 1	↓1.7	Migration

Table 3.1

Table 3.2. Effects of Pomegranate Juice on the levels of metastasis-related miRNAs. RNA from PC3 cells treated with L+E+P at 8μg/ml for 12 hrs was submitted to microRNA PCR array analysis. Relative miRNA levels are shown as fold change compared with untreated control.

Effect of L+E+P on cancer-related miRNAs

miRNA	Function	Fold change	Targets
miR144	Tumor-suppressive	↑772941-fold	Notch-1
miR-133b	Tumor-suppressive	↑177812-fold	c-MET, FSCN1
miR-1	Tumor-suppressive	↑66913-fold	Cyclin D2, CXCR4, SDF1α
miR-122	Pro-apoptotic	↑9741-fold	Cyclin B1
miR-34c	Tumor-suppressive	↑6700-fold	E2F3, Bcl2, c-MET
miR-200c	Tumor-suppressive	↑5077-fold	ZEB1, ZEB2, FN1, MSN
miR-127	Tumor-suppressive	↑4067-fold	E2F3,Notch-1, Bcl2
miR-335	Tumor-suppressive	↑3983-fold	COLA1,SOX4,TNC
miR-124	Tumor-suppressive	↑1389-fold	ROCK2,EZH2,CDK6
miR-181a	Tumor-suppressive	↑962-fold	K-ras, Bcl2
miR-7	Tumor-suppressive	↑849-fold	Bcl2,EGFR,IGF1R
miR-215	Tumor-suppressive	↑820-fold	DTL
miR-15a	Tumor-suppressive	↑786-fold	Fgf2,cyclin E2
Let-7d	Tumor-suppressive	↑4-fold	Twist
miR-20a	Oncogenic	↓13587-fold	APP
miR-21	Oncogenic	↓1260-fold	TMP1,Pdcd4, MARCKS
miR-9	Oncogenic	↓173-fold	E-cadherin
miR-29b	Oncogenic	↓48-fold	PTEN
miR-181b	Oncogenic	↓39-fold	PTEN, TIMP3

Figure 3.2

CHAPTER 4:

Luteolin, Ellagic Acid and Punicic Acid are Natural Products that Inhibit Prostate Cancer Metastasis

Abstract

Prostate cancer is the second cause of cancer deaths in men in the United States. When the cancer recurs, early stages can be controlled with hormone ablation therapy to delay the rate of cancer progression but, over time, the cancer overcomes its hormone dependence, becomes highly aggressive and metastasizes. Clinical trials have shown that pomegranate juice (PJ) inhibits prostate cancer progression. We have previously shown that the PJ components luteolin (L), ellagic acid (E) and punicic acid (P) together inhibit growth of hormone-dependent and -independent prostate cancer cells and inhibit their migration and chemotaxis towards CXCL12, a chemokine that is important in prostate cancer metastasis. Based on these findings, we hypothesized that L+E+P inhibit prostate cancer metastasis *in vivo*. To test this possibility, we used a Severely Combined Immuno-Deficiency (SCID) mouse model in which luciferase-expressing human prostate cancer cells were injected subcutaneously. Tumor progression was monitored with Bioluminescence Imaging weekly. We found that L+E+P inhibits PC-3M-luc primary tumor growth, inhibits the CXCL12/CXCR4 axis for metastasis and none of the tumors metastasized. In addition, L+E+P significantly inhibits growth and metastasis of highly invasive $Pten^{-/-}$;$K\text{-}ras^{G12D}$ prostate tumors. Furthermore, L+E+P inhibits angiogenesis *in vivo*, prevents human endothelial cell (EC) tube formation in culture and disrupts pre-formed EC tubes, indicating inhibition of EC adhesion to each other. L+E+P also inhibits the angiogenic factors IL-8 and VEGF as well as their induced signaling pathways in ECs. *In conclusion*, these results show that L+E+P inhibits prostate cancer progression and metastasis.

Introduction

Prostate cancer (PCa) is the most common male malignancy and the second leading cause of cancer-related death among men in America. The American Cancer Society has estimated that a total of 238,590 new cases will be diagnosed and 29,720 men will die of PCa in 2013 (www.cancer.org). To date there is no real cure for the disease beyond surgery and/or radiation. Early stages can be controlled with hormone ablation therapy that suppresses the rate of PCa growth. However, over time, the cancer overcomes its hormone dependence, becomes castration resistant prostate cancer (CRPC) and metastasizes to the lung, liver and bone.

Chemotherapy is available but the chemotherapeutic drugs are aggressive and have many negative side effects. As a result, researchers are looking for novel strategies to treat PCa. FDA approved sipuleucel-T (Provenge®) is an autologous cellular immunotherapy to treat metastatic PCa. The median overall survival rate of patients who received sipuleucel-T was only improved by 4.5 months and treatment is costly but some patients survived much longer (Higano et al., 2010). Novel androgen receptor (AR) antagonists such as enzalutamide (Xtandi®) and androgen biosynthesis inhibitors such as abiraterone (Zytiga®), have shown great promise as androgen deprivation therapy (ADT) to prolong overall survival rate among patients with metastatic PCa (Pal et al., 2013; Ryan et al., 2013; Sartor and Pal, 2013; Scher et al., 2010; Stein et al., 2012). Another novel drug, Cabozantinib, is a potent dual inhibitor

of the tyrosine kinases MET and VEGFR2, that has been shown to reduce or stabilize metastatic bone lesions in CRPC patients (Smith et al., 2013; Yakes et al., 2011). However, all of these treatments have adverse side effects.

Recently, pomegranate juice (PJ) has been identified as a natural agent to fight PCa. Mounting evidence shows that PJ has great potential to inhibit the growth and reduce the invasiveness of PCa cells both *in vitro* and *in vivo* (Albrecht et al., 2004; Malik et al., 2005; Rettig et al., 2008; Syed et al., 2008). In 2006, a 2-year phase II clinical trial of PCA patients with rising PSA were given 8 oz of PJ by mouth daily. PSA doubling time (PSADT) lengthened with treatment from a mean of 15.6 months at baseline to 54.7 months post-treatment ($P < 0.001$) (Pantuck et al., 2006). More recently in 2013, another phase II clinical trial of PCa patients with rising PSA received 1g (comparable to about 8oz of PJ) or 3g of pomegranate extract daily for up to 18 months. PSADT lengthened more than 6 months from 11.9 to 18.5 months ($P<0.001$) with no significant difference between dose groups (Paller et al., 2013). The statistically significant prolongation of PSADT and the lack of metastatic progression in any of the patients, strongly suggests a potential of PJ for treatment of PCa.

We have previously shown that PJ inhibits the migratory and metastatic properties of hormone refractory PCa cells by stimulating cell adhesion and inhibiting cell migration/chemotaxis (Wang et al., 2011). However, PJ contains many components and as a whole it is difficult to determine how to best maximize its use in

treating PCa. A way to overcome this challenge is to identify components of PJ that are responsible for the anti-metastatic effect of the whole juice. The juice contains a rich complement of polyphenolic compounds such as delphinidin, punicalin, punicalagin, quercetin and luteolin (Syed et al., 2008). Pomegranate pericarp is very rich in tannates of gallic acid and ellagic acid, which are strongly antioxidant (Gil et al., 2000). Pomegranate seed oil is rich in steroids and sterols (El Kar et al., 2011) . Remarkably, the oil is comprised of 80% punicic acid, which is a rare C18 octadecatrienoic fatty acid. Many of these compounds have been shown to have anticancer properties (Gasmi and Sanderson, 2010; Hora et al., 2003; Lansky et al., 2005; Zhou et al., 2009). Nevertheless, the specific components of PJ that have anti-metastatic effects against PCa are largely unknown. Recently, we have shown that a combination of a polyphenolic compound (luteolin/L), an antioxidant (ellagic acid/E) and a seed oil component (punicic acid/P), individually and in combination, addictively affect processes critical for metastasis. They stimulate cell adhesion, inhibit cell migration and inhibit chemotaxis of the PCa cells via CXCL12/CXCR4, a chemokine axis that is very important in metastasis of PCa cells (Wang et al., 2012). We have also shown similar anti-metastatic effects of L+E+P on breast cancer cells (Rocha et al., 2012). However, there is no direct evidence yet showing that L+E+P inhibits PCa metastasis *in vivo*. Therefore, in the present study, we tested the effect of L+E+P on metastasis of PCa in a mouse tumor model. We found that L+E+P administration inhibits growth and metastasis of luciferase expressing human PCa

(PC-3M-luc) xenograft tumors in severe combined immunodeficiency (SCID) mice. L+E+P treatment also inhibits growth and metastasis of allograft tumors of the highly invasive mouse PCa cells with PTEN deletion and K-Ras activation. In addition, L+E+P treatment inhibits tumor angiogenesis and angiogenesis-related molecular properties of endothelial cells. Our findings strongly suggest that L+E+P can potentially be used to deter metastasis of PCa.

Materials and Methods

Materials

Severe combined immunodeficiency (SCID) mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1wjl}/szJ, Cat No. #005557) were purchased from Jackson Laboratories (Bar Harbor, ME). Luteolin and ellagic acid were purchased from Sigma Aldrich (St. Louis, MO). Punicic acid was purchased from Larodan Fine Chemicals (Malmo, Sweden). D-luciferin was purchased from Biosynth International Inc. (Itasca, IL). RPMI 1640 media was acquired from Mediatech (Manassas, VA) and fetal bovine serum from Sigma Aldrich (St.Louis, MO). CXCL12 was obtained from ProSpec (Boca Raton, FL). Secondary Abs were purchased from Thermo Scientific (Rockford, IL). Detection was done using Supersignal West Dura kit from Thermo Scientific (Rockford, IL.). Transfection reagents Lipofectamin^{TM} 2000 were purchased from Invitrogen (Carlsbad, CA). Primary antibodies were obtained as follows: anti-AKT, anti-phospho-AKT, anti-ERK, anti-phospho-ERK, anti-PI3K, anti-GAPDH from Cell Signaling Technology (Danvers, MA); anti-CXCR4, anti-VEGFR2, anti-phospho-VEGFR2, anti-VE-Cadherin, anti-Ki67 from Abcam (Cambridge, MA); anti-CD31 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Gα13 from Neweast Biosciences (King of Prussia, PA); anti-αSMA (alpha-smooth muscle actin) from Sigma Aldrich (St.Louis, MO).

Cell culture

The luciferase-expressing human PCa cell line PC-3M-luc2 was purchased from Caliper Life Sciences (Waltham, MA) and was cultured in EMEM supplemented with 10% fetal bovine serum (FBS). The human endothelial cell line HMVEC-1 was obtained as a gift from Centers for Disease Control and Prevention (Atlanta, GA) and was cultured in DMEM supplemented with 10% FBS. The human PCa cell line PC3, was obtained as a gift from A. Walker (UC Riverside) and was cultured in RPMI 1640 supplemented with 10% fetal bovine serum. The *Pten*$^{-/-}$;*K-ras*^{G12D} cell line was isolated from 10-week *C*$^{+}$;*Pten*$^{L/L}$;*K-ras*$^{L/W}$ mutant mouse prostate (Mulholland et al., 2012). U373-MAGI-CXCR4$_{CEM}$ cells were obtained from NIH AIDS Research and Reference Reagent Program contributed by Dr. A. Geblle. Cells were cultured at 37°C with 5% CO_2 in penicillin (100 I.U/ml) and streptomycin (100 μg/ml).

Prostate cancer patient samples

Human prostate tumor samples were obtained through an IRB-approved protocol. Matched normal and tumor tissues from a single patient were obtained at the time of prostatectomy. The fragments used were shown by frozen section to contain or not contain tumor. Two patient-derived xenograft tumors were also utilized. Tissue was removed from subcutaneous xenografts in SCID mice, at 6th passage level.

Prostate cancer cell injection and monitoring of tumor size

Experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Riverside. Male SCID mice (5-6 weeks old)

were anesthetized and were injected ventrally under the skin with 2×10^6 PC-3M-luc2 cells or 1×10^6 *Pten*$^{-/-}$;*K-ras*^{G12D} cells suspended in 100µl Dulbecco's Phosphate Buffered Saline (DPBS). For the weekly imaging, mice were anesthetized and were given the substrate D-luciferin by intraperitoneal injection at 150mg/kg in DPBS 5-10 min before imaging. Bioluminescent imaging was performed with a bioluminescent imaging system (ONYX, Stanford Photonics) comprised of a highly sensitive cooled CCD camera mounted in a light-tight specimen box. Images and measurements of BLI were acquired and analyzed using WinView software. The tumor diameters were measured and the volumes were calculated by the formula: Volume=$(\text{width})^2\times\text{length}/2$ (Euhus et al., 1986).

Adhesion assay

Three hundred thousand HMVEC or *Pten*$^{-/-}$;*K-ras*^{G12D} cells were plated on gelatin-coated 6-well plates (BD Biosciences, San Jose, CA), cultured for 24hrs and then treated with PJ components for 12 hrs or 24 hrs. Cells were then trypsinized and the time required to detach all cells was recorded as an indicator of cell adhesiveness as previously described (Rocha et al., 2012; Wang et al., 2011; Wang et al., 2012).

Migration assay

Confluent HMVEC or *Pten*$^{-/-}$;*K-ras*^{G12D} cell cultures were wounded using a rubber scraper to create a scratch, washed and treated with PJ components at various concentrations. Cell migration was determined by measuring the distance migrated by

the cells from the wounded edge to the leading edge of migration at 24h and 48h after treatment was initiated. Scraped cell cultures without treatment were used as controls.

Chemotaxis assay

The upper side of 8µm pore size polycarbonate membranes of transwells (BD Biosciences, San Jose, CA) were coated with 50 ng/ml type I collagen (Sigma Chemical Co.). HMVEC or *Pten*$^{-/-}$;*K-ras*^{G12D} cells (1x10^5) in 100 µl culture medium were plated on the upper side of transwell membranes and were allowed to adhere for 3 hours. Then the wells were introduced into a 24-well plate and 1000 µl of medium with 10% FBS medium was added to lower chamber. Cells were treated with PJ components for 12 hours. CXCL12 (100ng/ml) or PC3 cells conditioned medium was added to the lower chamber and the cells were allowed to migrate for 4 hours at 37°C. The cells on the side of the membrane facing the upper chamber were removed with a cotton swab, and the membranes were then fixed and stained with 2% toluidine blue in 4% paraformaldehyde. Cells on the underside of the membrane were counted in 8 high power (40X) fields (HPF)/filter to obtain the average number of cells per field.

Tube formation assay

50µl of Matrigel (BD Biosciences, San Jose, CA) was applied to the center of 35-mm cell culture dishes to evenly coat a 1.5cm diameter area. The coated plates were incubated for 30 min at 37°C. 3×10^4 HMVECs suspended in 200µl DMEM were plated onto the Matrigel coated area. To study the effect of PJ components on tube formation, HMVECs were treated after the cells adhered for 1 hour and then observed

for tube formation. To determine whether the PJ components disrupt pre-formed tubes, HMVECs were treated after the cells had formed tubes and observed for tube disruption. The tubes were observed with an inverted phase contrast microscope intermittently for 12hrs. The number of formed tubes was recorded.

In vivo angiogenesis

C57BL mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All experimental protocols were approved by the UCR IACUC. The mice were treated with L+E+P (64µg of each) by intraperitoneal injection (IP) daily for 2 weeks. The hair was then removed from the back of the mice using Nair hair remover (Madera, CA) and they were injected with IL-8 (100ng/20µl saline) or VEGF (200ng/20µl saline) using an insulin syringe. IL-8 and VEGF were each injected in two sites symmetrically located on the back of each mouse every 24 hrs for 4 days. Injection sites were labeled using a permanent marker to ensure that repeated injections for the same site all took place in the same spot; on each animal, the pair of injection sites for IL-8 and the pair for VEGF were on opposite sides of the backbone and at least 2 cm apart to avoid possible cross-over effects. Skin samples from the injected areas were collected and observed at day 5.

Immunolabeling

Frozen tissue sections were immunolabeled with Ki67 or αSMA antibodies, mounted with Vectashield, and viewed using Nikon Microphot-FXA fluorescence microscope with a Nikon DS-Fi1 digital camera and Nikon NIS-Elements software.

Immunoblotting

HMVEC cells were treated with PJ components for 12h, washed with ice cold PBS, and lysed on ice with lysis buffer containing 0.5% Triton X100, 0.5% Nonidet P-40, 10 mM Tris, pH 7.5, 2.5 mM KCl, 150 mM NaCl, 30 mM b-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 0.1% SDS and additional protease inhibitor cocktails (Cell Signaling, Danvers, MA). Protein concentrations were measured using the DC protein assay kit. Equal amounts of protein in the cell extracts were mixed with sample buffer, boiled, and analyzed using 10% acrylamide SDS-PAGE. Immunoblotting was performed with the HRP-conjugated secondary Ab, followed by incubation with West Dura extended-duration substrate. Blots were then reprobed for GAPDH or histone 2A antibody to show equal loading of proteins.

Statistical Analysis

Data analysis was performed using the Student's *t*-test or one-way ANOVA on raw data with GraphPad Instat software (GraphPad Software, La Jolla, CA).

Results

L+E+P inhibits tumor growth, metastasis and angiogenesis of prostate xenograft and allograft tumors

To study the effects of L+E+P on the metastasis of PCa *in vivo*, male SCID mice were injected ventrally under the skin with luciferase-expressing human PCa cells (PC-3M-luc). One day after the injection of the tumor cells, mice were randomly divided into two groups. One group of mice received L+E+P treatment once a day, five days/week for 8 weeks and the other received only vehicle (DPBS) for the same period of time, via intraperitoneal (IP) injection. Tumor growth and progression to metastasis of both treated and non-treated groups, were monitored weekly by bioluminescence imaging (BLI). Without treatment the tumors grew to the maximum size allowed by the IACUC in 8 weeks (**Fig. 4.1A)**.

We had previously determined *in vitro* that the most effective combination of L+E+P was when the components were used at equal doses in the mixture. Therefore, we tested increasing doses of the mixture in a ratio of 1:1:1 starting with 16µg per component which was the most effective dose in *in vitro* assays (Wang et al., 2012) . We tested L+E+P at 16, 32, 64 and 128µg of each component and found that 16 µg of each was ineffective in deterring tumor growth, 32µg had a very small effect and 128µg killed the mice. We therefore used a dose of 64µg of each component for all experiments. During the 8 weeks of treatment, no apparent toxicity was observed with 64 µg/component/day in any of the L+E+P treated mice, whereas this

concentration significantly inhibited tumor growth as indicated by the decreased level of BLI (**Fig. 4.1B)**. We also found that L+E+P was effective in deterring tumor growth when treatment was started at week 5 of tumor development (**Fig. 4.1C)**. At week 8, all the mice were euthanized and the volume of the tumors was measured after excision. L+E+P treatment significantly decreased tumor volume when compared to mice treated with the vehicle (**Fig. 4.1D**) and it completely inhibited metastatic incidence in PC-3M-luc xenograft tumors as shown by BLI when the signal from the primary tumor was blocked (**Fig. 4.1E**). Among the control vehicle-treated group, tumors in 5 out of 7 mice metastasized by week 8. However, none of the tumors in 7 mice treated with L+E+P metastasized by week 8 (**Fig 4.1F**).

Because we saw a decrease in tumor size, we used a marker for Ki67 to determine whether L+E+P affected tumor cell proliferation and observed significant inhibition of tumor cell proliferation **(Fig. 4.2A, B).** To determine whether L+E+P treatment affects angiogenesis in the tumor, sections from the control and treated groups were stained with Masson's trichrome to highlight the collagen surrounding the blood vessels. We found that the number of blood vessels in treated mice was significantly lower than in the vehicle-treated mice **(Fig. 4.2C)**. The effects of L+E+P on tumor biology are summarized in **Fig. 4.2D**.

Because not all xenograft tumors from PC-3M-luc cells in SCID mice metastasized, we injected the highly invasive mouse cancer cell line with PTEN deletion and K-RAS activation ($Pten^{-/-}$;$K\text{-}ras^{G12D}$) to evaluate the effects of L+E+P on

metastasis of this aggressive PCa cell line (Mulholland et al., 2012). To initiate these studies we tested the effect of L+E+P on cultured cells and found that L+E+P increases cell adhesion, decreases cell migration and decreases chemotaxis towards CXCL12 much like it did on PC3 cells (**Fig. 4.3A-C**). For the experiments *in vivo*, SCID mice were injected with *Pten*$^{-/-}$;*K-ras*^{G12D} cells subcutaneously in the region of the prostate, and then divided into two groups. One group of mice received L+E+P treatment (64µg/component/day five days a week) and the other group received only the vehicle. Because of the tumor aggressiveness, all mice were treated for only 4 weeks and euthanized at the end of the 4th week. We found that L+E+P significantly decreases the size of the tumor compared to the vehicle-treated group (p=0.0006) (**Fig. 4.4A**). By the end of week 4, all tumors in the vehicle-treated group had metastasized and the major sites of metastasis were the lung and liver. The number of metastatic lesions was counted macroscopically; L+E+P treatment significantly inhibited metastasis to both the lung (**Fig. 4.4B,C**) and the liver (**Fig. 4.4B,D**). We summarize and compare lung and liver metastases between control and L+E+P group (**Fig 4.4E)**. In addition, we found that L+E+P reduced the number of blood vessels in the *Pten*$^{-/-}$;*K-ras*^{G12D} tumors (**Fig. 4.5A**). L+E+P also inhibited tumor cell proliferation as shown by reduced Ki67 positive cells (**Fig. 4.5B**). These findings show that L+E+P effectively inhibits metastasis in both the PC-3M-luc tumors and the highly invasive *Pten*$^{-/-}$;*K-ras*^{G12D} tumors.

L+E+P inhibits the CXCL12/CXCR4 chemotactic axis

It has been well established that the CXCL12/CXCR4 signaling axis plays a critical role in cancer metastasis. We have previously shown that L+E+P reduces CXCR4 levels and inhibits the down-stream signaling pathways of CXCL12 in human endothelial cells *in vitro* (Wang et al., 2012). To investigate the effect of L+E+P on CXCL12/CXCR4 axis *in vivo*, we examined the protein levels of CXCR4 in the L+E+P treated PC-3M-luc tumors and found that treatment with these components significantly decreased the protein levels of CXCR4. Furthermore, we also found that $G\alpha_{13}$, PI3K and AKT, proteins involved in the signaling downstream of CXCR4, were also decreased (**Fig 4.6A,B**). These findings show that L+E+P inhibits the CXCL12/CXCR4 chemotactic axis in the tumor and are consistent with the observed L+E+P inhibition of metastasis.

L+E+P alters the angiogenic properties of HMVECs

To study the molecular mechanisms of inhibition of angiogenesis by L+E+P as observed in the tumors, we performed a variety of cellular and molecular assays *in vitro* using human microvascular endothelial cells (HMVEC). These cells were treated with L+E+P at 2 or 4μg/ml (higher concentrations were detrimental to these cells) and performed adhesion assays, much like we did for the tumor cells, using a gelatin-coated substrate and the trypsinization time required to completely detach all cells from the culture dish as an indicator of cell adhesion strength (Rocha et al., 2012;

Wang et al., 2011; Wang et al., 2012). Treatment with L+E+P for 12h or 24h decreased the ability of HMVECs to adhere to the substrate (**Fig. 4.7A**). Moreover, using the scratch wound assay, we found that L+E+P treatment significantly decreased cell migration of HMVECs in a dose-dependent manner (**Fig. 4.7B**).

It is well established that tumor cells can attract endothelial cells through chemotaxis. To investigate the effects of L+E+P on chemotaxis of endothelial cells towards the tumor cells, we performed chemotaxis assays with PC3 conditioned medium and found that L+E+P treatment greatly inhibited the PC3 conditioned medium-induced chemotaxis (**Fig. 4.7C,D**).

To further investigate the molecular mechanisms of L+E+P on HMVECs, we determined the effect of these components on levels of VE-cadherin and CD31, which are two important proteins in endothelial cell adhesion to each other and in tube formation in blood vessels. Treatment for 24 or 48h with L+E+P decreased the levels of both proteins and did so in a time-dependent manner (**Fig. 4.7E,F**). Given these results, we also tested the possibility that L+E+P could have an effect on endothelial cell tube formation. HMVECs were treated with L+E+P after the tubes were allowed to form and found that these components significantly disrupted the formed tubes (**Fig. 4.7G,H**). Alternatively, L+E+P treatment after the cells have adhered to the substrate prevented tube formation **(Fig. 4.8A,B).** Both effects occurred in a dose-dependent manner. These results suggest that L+E+P inhibits tumor-induced

angiogenesis through changing endothelial cell processes that are involved in cell migration, chemotaxis and tube formation.

It is well established that IL-8 and VEGF are potent angiogenesis inducers and that tumor cells in general produce high levels of both. We examined the levels of IL-8 and VEGF in human normal prostate tissues and prostate tumors. Levels of both growth factors are very low in the normal prostate tissue but are consistently elevated in the human prostate tumors (**Fig. 4.9A, B**). To determine the effect of L+E+P on IL-8 and VEGF production, we treated PC3 cells with L+E+P and found that these PJ components significantly reduce the IL-8 and VEGF production (**Fig. 4.9C, D**).

We have previously shown that IL-8-induced angiogenesis occurs via transactivation/ phosphorylation of VEGFR2 (Petreaca et al., 2007). To determine whether L+E+P inhibits IL-8-induced angiogenesis via this signaling mechanism, we examined whether IL-8 induces the phosphorylation/activation of VEGFR2 and subsequent activation of AKT. Treatment with L+E+P strongly inhibits IL-8-induced phosphorylation of VEGFR2 and phosphorylation of AKT at 15-60 min (**Fig. 4.9E**). VEGF is known to promote endothelial cell proliferation through activation of the PI3K/AKT and MEK/ERK signaling pathways (Marshall, 1995). L+E+P inhibits VEGF-induced phosphorylation of ERK and AKT (**Fig. 4.9F**), suggesting that L+E+P inhibits angiogenesis by inhibiting the response of endothelial cells to the pro-angiogenic factors IL-8 and VEGF.

To study whether L+E+P inhibit IL-8 or VEGF induced angiogenesis *in vivo*, C57BL mice were injected for 2 weeks with 64µg each of L+E+P once/day. IL-8 or VEGF was injected under the skin once/day for 4 consecutive days to examine whether the angiogenic effects were inhibited by systemically pre-treating the animals with L+E+P. The skin samples were collected, imaged and the blood vessels were manually highlighted. We found that angiogenesis induced by IL-8 or VEGF was inhibited by L+E+P as shown by reduced number of blood vessels in L+E+P treated skin samples (**Fig. 4.9G** and **Fig. 4.10A-B)**.

Discussion

Prostate cancer that has become metastasized has a poor prognosis and remains a significant therapeutic challenge. Here we have tested the hypothesis that L+E+P has anti-metastatic effects *in vivo* and have found that a 1:1:1 combination of these PJ components (i) Inhibits growth of primary tumors; (ii) inhibits cellular and molecular processes critical for metastasis; (iii) inhibits tumor angiogenesis and changes angiogenesis-related properties of human endothelial cells; (iv) inhibits the CXCL12/CXCR4 chemotactic signaling axis which is known to be important in cancer metastasis.

Using SCID mice and a luciferase-expressing human prostate cancer cell line to obtain xenograft tumors, we investigated the potential for tumor metastasis. The advantage of this model is that tumor growth and metastasis can be monitored in real time. Treatment with L+E+P significantly suppressed tumor growth but more importantly, it completely eliminated metastasis of these tumors. However, the fact that in the control tumor progression to the metastatic stage did not occur 100% of the time limits the power of this result. This led us to test the effects of these components on a more aggressive tumor.

It has been shown that inhibition of the PTEN/PI3K pathway combined with activation of the Ras/MAPK pathway promotes prostate cancer metastasis (Mulholland et al., 2012). As a result, we also investigated a highly invasive mouse prostate cancer cell line which has PTEN deletion and K-ras activation

(*Pten*$^{-/-}$;*K-ras*^{G12D}) to generate allograft tumors. In this model, all control mice metastasized by the end of week 4 and we observed abundant metastases in lung and liver. In the corresponding mice treated with L+E+P by IP injection, tumor growth was again very significantly suppressed but in this case metastasis to lung and liver also occurred. Nevertheless, the overall number of metastases was greatly reduced.

Detectable ellagic acid and metabolites can be identified briefly and in low quantity in plasma and urine within an hour of both oral and IP administration. When given orally most ellagic acid appears to be converted by intestinal microorganisms to urolithins and their glucuronides (Gonzalez-Sarrias et al., 2010; Seeram et al., 2007; Vicinanza et al., 2013). These urolithin glucuronides are then absorbed and concentrated in prostate tissue, where they may exert anti-proliferative and anti-oxidant effects. Thus ellagic acid itself does not appear to be a major, biologically-effective component of orally-administered PJ. When injected IP, ellagic acid itself ends up in the plasma and in the prostate of mice (Seeram et al., 2007). Much less data exist to describe the bioavailability and activity of luteolin or punicic acid after oral ingestion by humans. Our data suggest that a combination of PJ constituents may have anti-tumor activity when administered parenterally at a non-toxic dose. While not as convenient as oral administration, parenteral dosing of PJ components may have novel biologic effects by bypassing intestinal metabolism. Comparative studies of the biologic activities of L, E, and P by both routes will be necessary to define the optimum methods to use these compounds in clinical cancer care.

There is a limit to how large a tumor can grow without angiogenesis (Carmeliet and Jain, 2000). Indeed, angiogenesis plays a crucial role in the survival, proliferation and metastatic potential of PCa tumors through providing nutrients and oxygen (Weis and Cheresh, 2011). Therefore, angiogenesis is an attractive treatment target in many types of solid tumors, including PCa tumors. To date, the most successful anti-angiogenic agent is bevacizumab, a monoclonal antibody against VEGF (Aragon-Ching and Dahut, 2008). Natural agents such as pomegranate extract have been shown to inhibit angiogenesis in prostate cancer (Sartippour et al., 2008) but the active components responsible for the anti-angiogenic effects are largely unknown. We have shown here that L+E+P inhibits angiogenesis by reducing the number of blood vessels in the tumors. New blood vessels are formed in response to interaction between tumor cells and endothelial cells, growth factors, and extracellular matrix components (Jahroudi and Greenberger, 1995). In cultured endothelial cells, we show that L+E+P significantly, and in a dose-dependent manner, decreases endothelial cell adhesion, migration and tube formation, all of which are important processes in angiogenesis. In addition, VE-cadherin and CD31, two of the important endothelial-specific adhesion proteins that maintain the integrity of blood vessels at adherens junctions (Vestweber et al., 2009) are completely or significantly inhibited, respectively, by L+E+P treatment. These findings suggest that the anti-angiogenic effects of L+E+P are at least partially due to directly changing the cellular and molecular properties of endothelial cells.

It is well established that tumor-secreted factors can chemoattract local stromal cells, such as fibroblasts and macrophages, and distant cells such as endothelial cells (Wels et al., 2008). We show that L+E+P inhibits chemotaxis of endothelial cells towards PC3 conditioned medium, suggesting that these components inhibit angiogenesis also through perturbing the communication between tumor and endothelial cells. Many studies have demonstrated that tumor cells secrete growth factors such as IL-8 and VEGF to stimulate the migration and proliferation of endothelial cells (Ahmad et al., 2002). IL-8 and VEGF are known as potent promoters of angiogenesis and the level of IL-8 and VEGF are in general elevated in various tumors (Waugh and Wilson, 2008). IL-8 has been shown to stimulate cell migration via the PI3K/AKT signaling pathway (Lai et al., 2011) and VEGF is known to promote endothelial cell proliferation through activation of the PI3K/AKT and MEK/ERK signaling pathways (Marshall, 1995). We show that L+E+P treatment significantly reduces the production of IL-8 and VEGF in PC3 cells and inhibits the endothelial cell response to IL-8 and VEGF.

It has been well established that prostate cancer cells develop ways to bypass the need for testosterone and then the cancer progresses very rapidly. The CXCL12 receptor CXCR4 is widely expressed in various tumor cells and is responsible for metastasis to the most common destinations such as lung and liver (Taichman et al., 2002; Zlotnik et al., 2011). As a result, the CXCL12/CXCR4 signaling axis has become an attractive therapeutic target for metastasis. Neutralizing CXCR4 function

with an antagonist or an antibody has been shown to inhibit prostate and breast cancer metastasis (Huang et al., 2009; Taichman et al., 2002). We show that L+E+P reduces the level of CXCR4 in prostate tumors and inhibits the CXCR4 downstream signaling Gα13 which is the G protein α subunit involved in CXCL12 induced chemotaxis (Tan et al., 2006). These results strongly suggest that L+E+P inhibits prostate cancer metastasis also through targeting CXCL12/CXCR4 chemotaxis axis.

In conclusion, L+E+P can be used in combination to prevent prostate cancer growth and metastasis and because these are natural products they could be used in humans in the very near future. Furthermore, it may be possible to develop them into novel drugs that can be made more effective than the natural products in preventing cancer progression.

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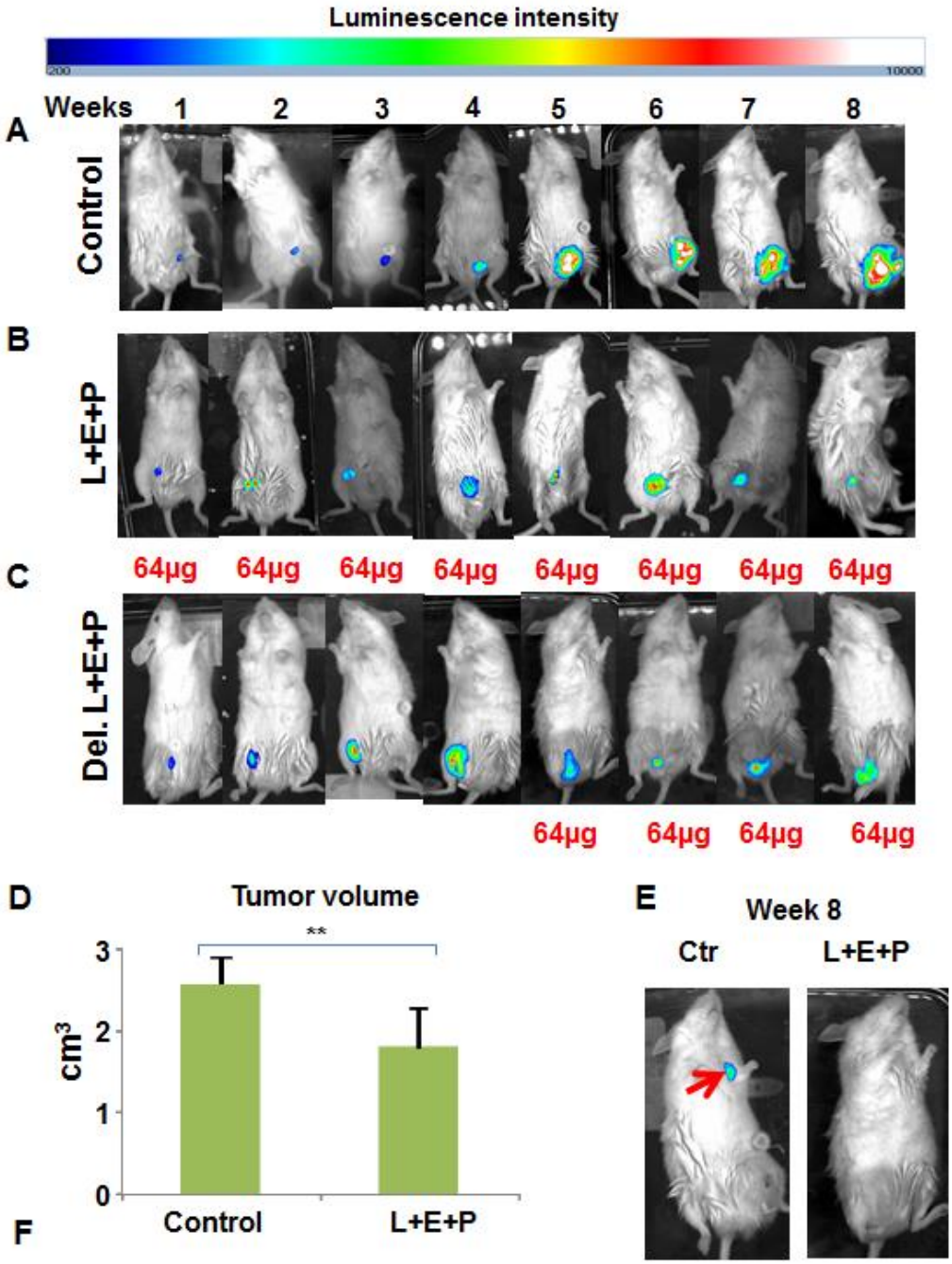
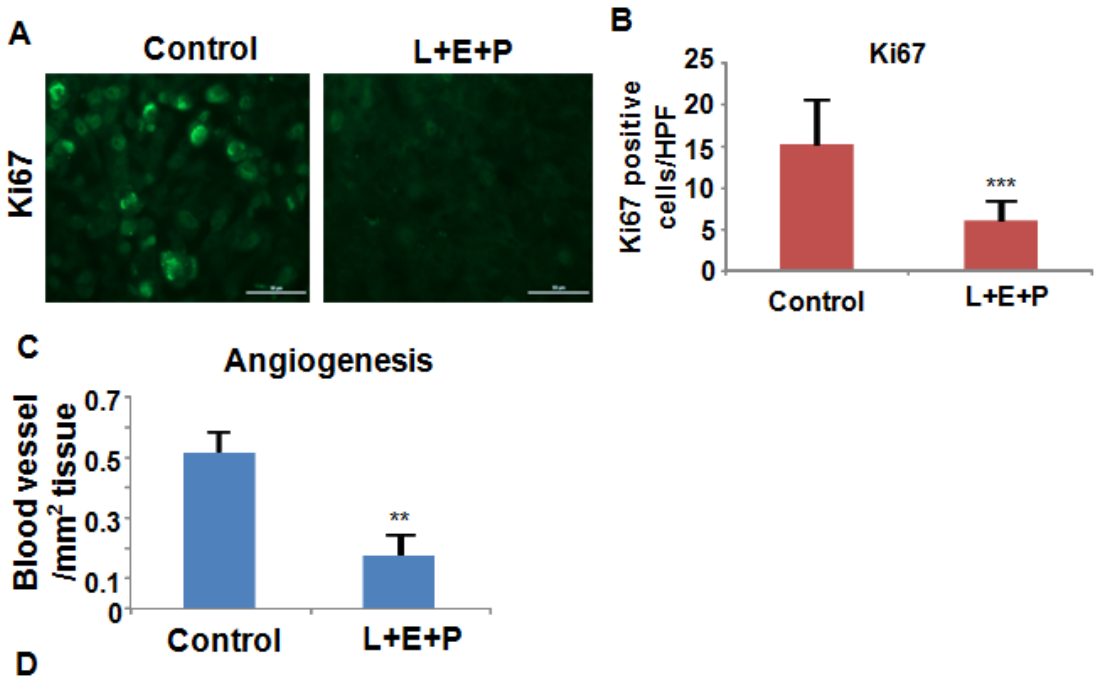
Figure 4.1. L+E+P inhibits growth and metastasis of PC-3M-luc xenograft tumors in SCID mice. 2×10^6 PC-3M-luc cells were injected into SCID mice subcutaneously near the prostate region. The treated mice received 64µg each of L+E+P IP once a day, 5 days/week for 8 weeks and the control mice received only vehicle (DPBS). Mice with delayed L+E+P treatment (Del. L+E+P) received 64µg L+E+P treatment starting at week 5. (Control group n=7, L+E+P group n=7, Delayed L+E+P group n=5). **(A-C)** Tumor growth and progression in Control, L+E+P and Delayed L+E+P groups of mice were monitored by BLI weekly. **(D)** Mice were euthanized at the end of the 8^{th} week and tumor volume was determined using the formula: Volume=$(\text{width})^2\times\text{length}/2$ (p=0.0089). **(E)** Tumors from mice in the control group metastasized by week 8 as shown by the BLI when the luminescence signals from primary tumors were blocked. Arrow indicates a metastasis. **(F)** Comparison of number of mice showing metastases between control and L+E+P group was quantified using the Fisher's Exact Test (p=0.021). Bars represent Standard Error of the Mean. **p < 0.01.



Groups	Mice with metastases	p-value
Control	5 out of 7	
L+E+P	0 out of 7	0.021

Figure 4.1

Fig 4.2. L+E+P inhibits angiogenesis of PC-3M-luc xenograft tumors in SCID mice. Tumors collected from the control and L+E+P treated group were fixed and sectioned. **(A)** Ki67 immunolabeling of tumors from control and L+E+P group. **(B)** The numbers of Ki67 positively stained cells were averaged in 10 high-power (40X) fields (HPF). **(C)** Quantification of blood vessels in each section. The total numbers of blood vessel counted/mm^2 of tumor tissue. **(D)** Summary of the observed differences between tumors from control and L+E+P treated group. Bars represent Standard Error of the Mean. **$p < 0.01$, ***$p< 0.001$.



Tumors in Control mice	Tumors in Treated mice
Bigger	Smaller
Very rigid	Relatively soft
Blood vessels	Fewer blood vessels
Some dead tissues	More dead tissues
Metastasized	No metastasis
High bioluminescence signal	Low bioluminescence signal

Figure 4.2

Fig 4.3. L+E+P stimulates adhesion, and inhibits migration and chemotaxis of *Pten*$^{-/-}$;*K-ras*^{G12D} prostate cancer cells towards of CXCL12. (A) *Pten*$^{-/-}$;*K-ras*^{G12D} cells were plated on gelatin-coated dishes and 24 hrs later media was changed and the cells were treated with L+E+P each at 4µg/ml and 8µg/ml. We tested for adhesion to the substrate at 12hrs after initiation of treatment by recording the time it took for trypsinization to remove all of the cells from the dish. Control represents no treatment. **(B)** *Pten*$^{-/-}$;*K-ras*^{G12D} cells were treated with L+E+P each at 2µg/ml, 4µg/ml or 8µg/ml for 72 hrs and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. Controls represent no treatment. Media containing PJ components were changed daily. **(C)** *Pten*$^{-/-}$;*K-ras*^{G12D} cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 hrs and then treated with L+E+P each at 8µg/ml for 12hrs. At this time, 100ng/ml of CXCL12 were introduced into the lower chamber and the cells found on the bottom of the filter counted 4 hrs later. Control had no treatment. Bars represent Standard Error of the Mean. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

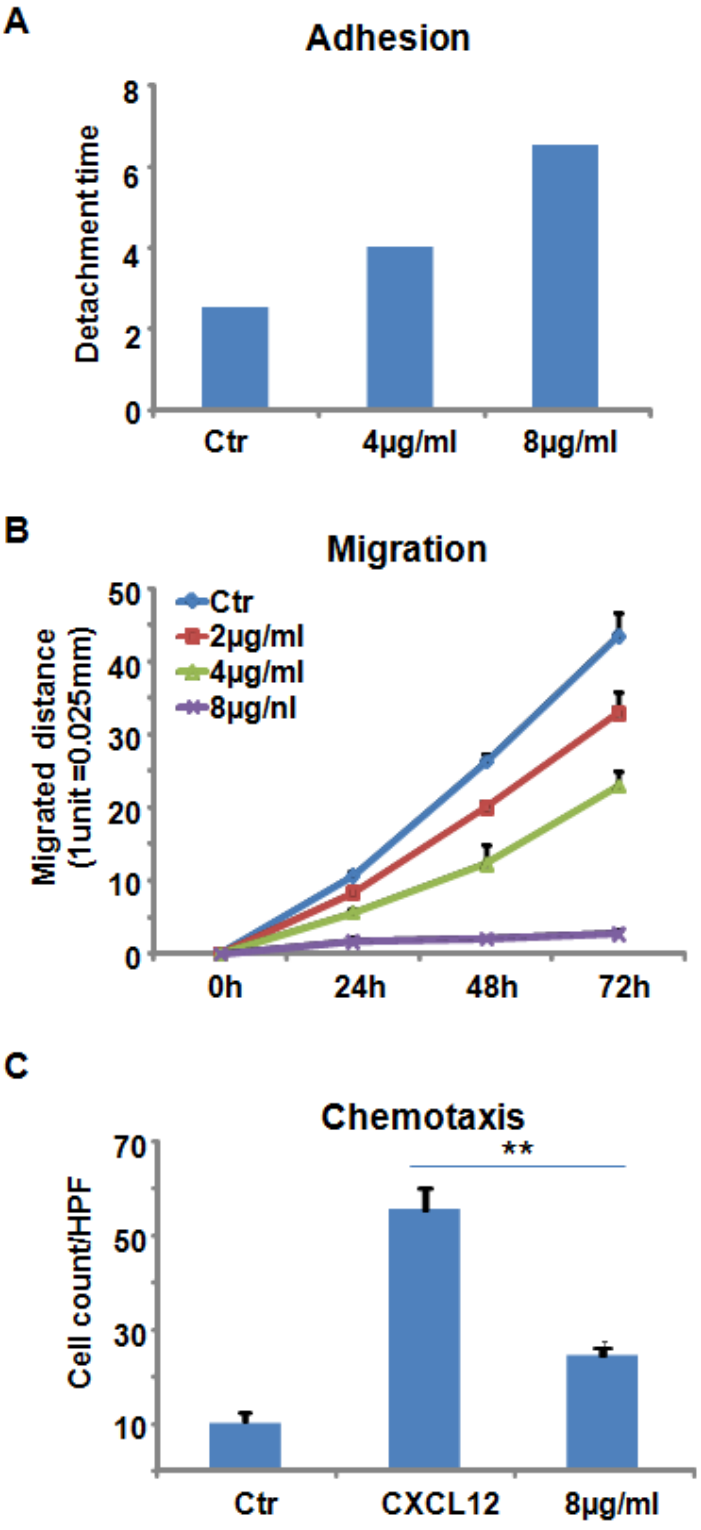
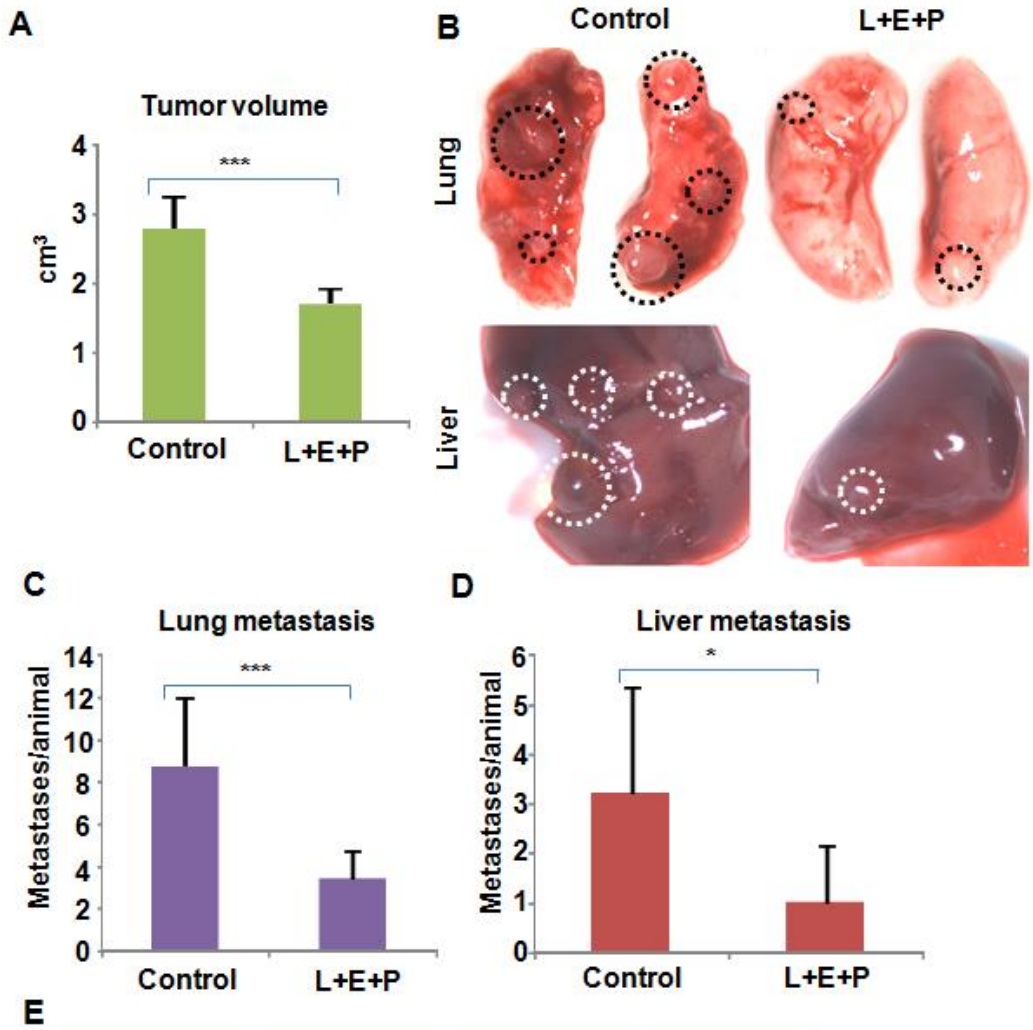

A
Adhesion
Detachment time
8
6
4
2
0
Ctr
4μg/ml
8μg/ml
B
Migration
Migrated distance
(1unit=0.025mm)
50
40
30
20
10
0
Ctr
2μg/ml
4μg/ml
8μg/nl
0h
24h
48h
72h
C
Chemotaxis
**
Cell count/HPF
70
50
30
10
Ctr
CXCL12
8μg/ml

Figure 4.3

Fig 4.4. L+E+P inhibits metastasis of *Pten*$^{-/-}$;*K-ras*^{G12D} mouse cancer cell allograft tumors. 1×10^6 *Pten*$^{-/-}$;*K-ras*^{G12D} mouse cancer cells were injected into SCID mice subcutaneously near the prostate region. L+E+P group of mice received 64µg each L+E+P once a day, five days/week for 4 weeks and the control group received only the vehicle (DPBS) (Control group n=6, L+E+P group n=7). **(A)** Mice were euthanized at the end of the 4th week and tumor volume was determined using the formula: Volume=(width)$^2\times$length/2 (p=0.0006). **(B)** Lung and liver metastases in mice from control and L+E+P group. Circles indicate the metastatic lesions. **(C-D)** The numbers of metastatic lesions in lung **(C)** (p=0.002) and liver **(D)** (p=0.04) were counted macroscopically in each animal. **(E)** Summary of the number of metastases in lung and liver. Bars represent Standard Error of the Mean. *p < 0.05, ***p< 0.001.



E

Groups	Mice with lung metastases	Metastases per animal (mean±SD)	p-value
Control	6 out of 6	8.7±3.2	
L+E+P	6 out of 7	3.4±1.3	0.002

Groups	Mice with liver metastases	Metastases per animal (mean±SD)	p-value
Control	5 out of 6	3.2±2.1	
L+E+P	4 out of 7	1±1.2	0.04

Figure 4.4

Fig 4.5. L+E+P inhibits angiogenesis of *Pten*$^{-/-}$;*K-ras*^{G12D} allograft tumors in SCID mice. 1×10^6 *Pten*$^{-/-}$;*K-ras*^{G12D} mouse cancer cells were injected into SCID mice subcutaneously in the region of the prostate. One group of mice received 64µg each L+E+P treatment once a day, five days/week for 4 weeks and the other group received only the vehicle (DPBS) **(A)** Quantification of blood vessels in each section. The total numbers of blood vessel counted/mm^2 of tumor tissue. **(B)** The numbers of Ki67 positively stained cell were counted and averaged in 10 high-power (40X) fields (HPF). Bars represent Standard Error of the Mean. *$p < 0.05$, **$p < 0.01$.

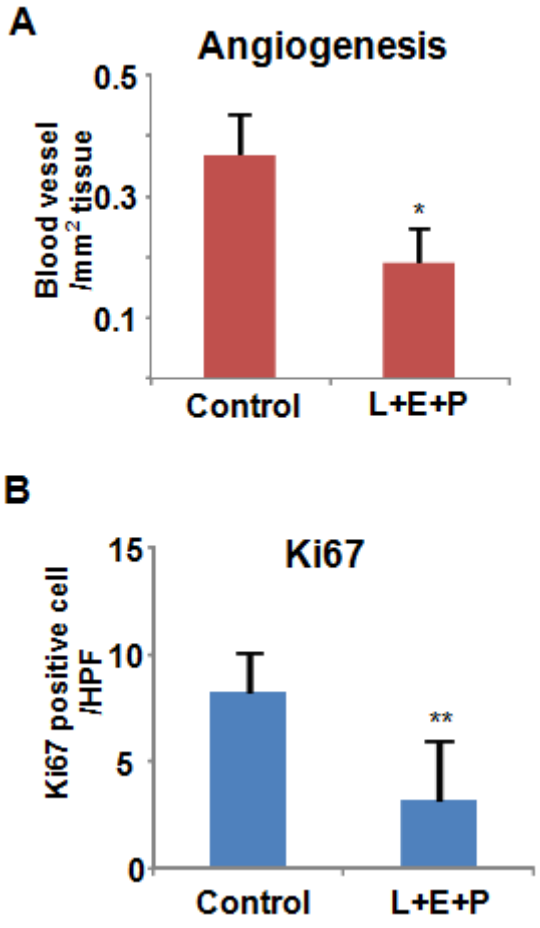

A
Angiogenesis
Blood vessel /mm2 tissue
0.5
0.3
0.1
*
Control
L+E+P
B
Ki67
Ki67 positive cell /HPF
15
10
5
0
**
Control
L+E+P

Figure 4.5

Fig 4.6. L+E+P inhibits CXCL12/CXCR4 axis *in vivo*. PC3M-luc tumors from control and L+E+P groups of mice were collected and total protein was extracted and analyzed by immunoblotting. **(A)** Immunoblotting for CXCR4, Gα13, PI3K and p-AKT (S473). **(B)** Densitometry of the bands. Bars represent Standard Error of the Mean. ***p< 0.001.

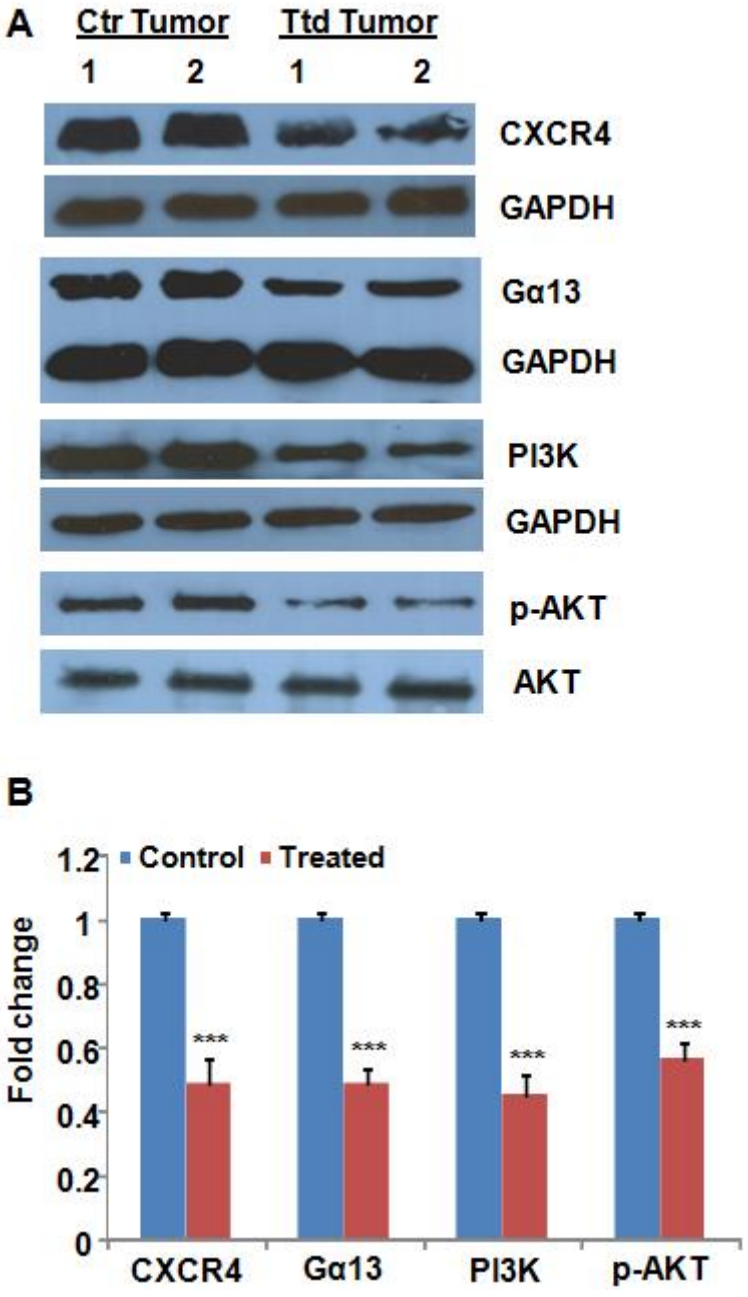

A
Ctr Tumor
Ttd Tumor
1
2
1
2
CXCR4
GAPDH
Gα13
GAPDH
PI3K
GAPDH
p-AKT
AKT
B
Control
Treated
Fold change
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
***
CXCR4
Gα13
PI3K
p-AKT

Figure 4.6

Fig 4.7. L+E+P changes angiogenesis-related properties of HMVEC cells. (A) HMVEC cells were plated onto gelatin-coated plates and were treated with L+E+P at 2µg/ml or 4µg/ml for 12h or 24h. Time required to completely trypsinize all cells from the plate was recorded. **(B)** Confluent HMVEC-1 cultures were scratch wounded and were treated with L+E+P at 2µg/ml or 4µg/ml for 24h or 48h. The distance migrated from the wounded edge was recorded at indicated time points. **(C)** HMVECs were seeded onto the collagen-coated upper side of 8µm pore size membranes of inserts inside transwell units and were treated with L+E+P at 4µg/ml for 12h. Conditioned medium collected from PC3 culture was introduced into the lower chamber to induce chemotaxis for 4h. L+E+P inhibited chemotaxis of the ECs towards the conditioned media of the cancer cells. **(D)** The numbers of cells that migrated through the pores were counted and averaged in 10 high-power (40X) fields (HPF). **(E)** HMVECs were treated with L+E+P at 4µg/ml for 24h or 48h and protein extracts were analyzed by immunoblotting for VE-cadherin and CD31. **(F)** Densitometry of the bands in **E**. **(G)** HMVEC-1 cells were plated onto Matrixgel-coated plates and allowed to form tubes for 4h. Then, the tubes were treated with L+E+P at 2µg/ml or 4µg/ml for 4h. L+E+P treatment resulted in dismantling of the endothelial tubes. **(H)** Tube number per 4X power field. Bars represent Standard Error of the Mean. *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

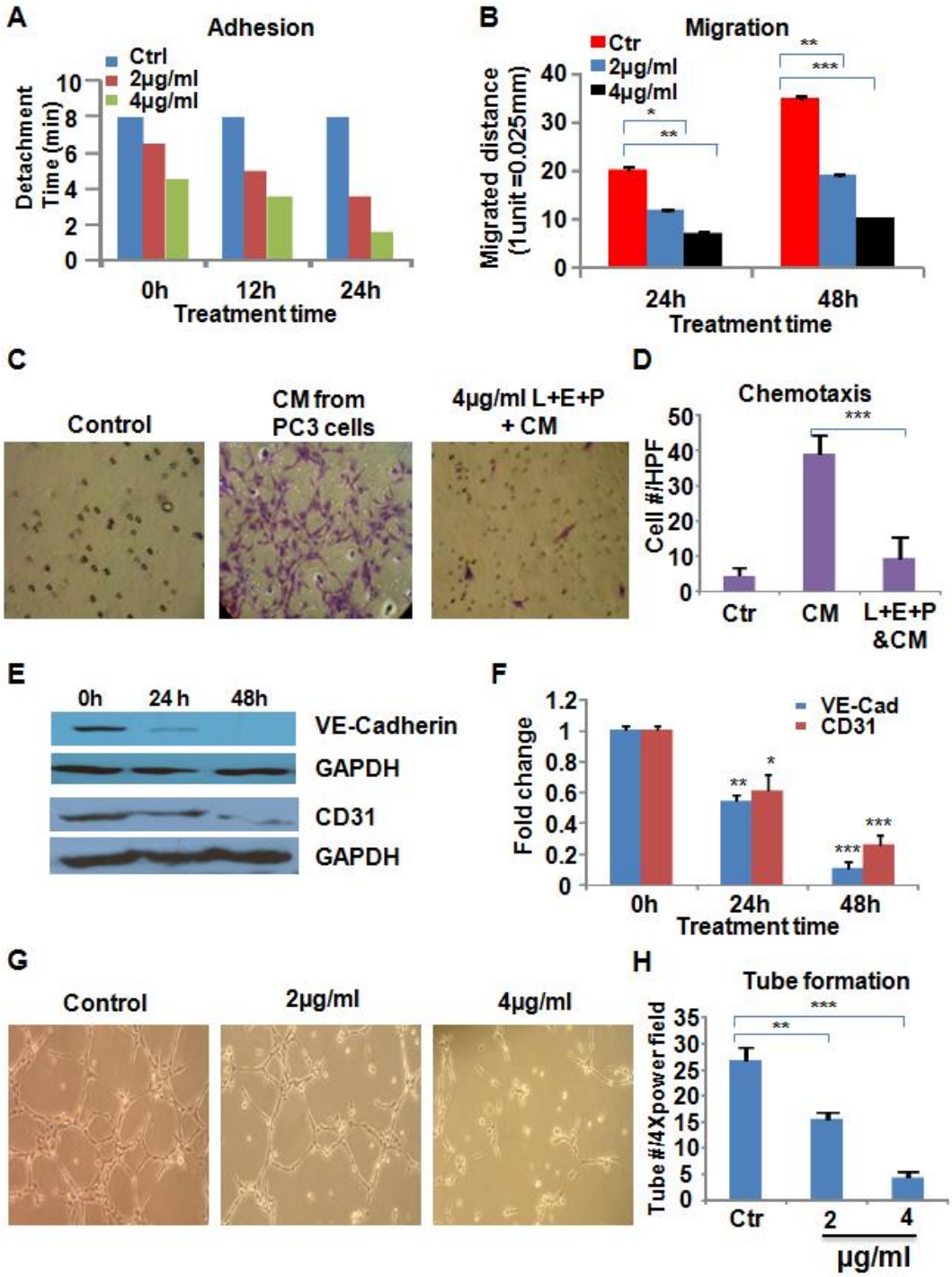

A
Adhesion
Ctrl
2μg/ml
4μg/ml
Detachment Time (min)
0h
12h
24h
Treatment time
B
Migration
Ctr
2μg/ml
4μg/ml
Migrated distance (1unit =0.025mm)
24h
48h
Treatment time
C
Control
CM from PC3 cells
4μg/ml L+E+P + CM
D
Chemotaxis
Cell #/HPF
Ctr
CM
L+E+P &CM
E
0h
24 h
48h
VE-Cadherin
GAPDH
CD31
GAPDH
F
VE-Cad
CD31
Fold change
0h
24h
48h
Treatment time
G
Control
2μg/ml
4μg/ml
H
Tube formation
Tube #/4Xpower field
Ctr
2
4
μg/ml

Figure 4.7

Fig 4.8. L+E+P prevents tube formation in HMVEC cells. (A-B) HMVEC cells were plated onto Matrixgel-coated plates and allowed to adhere for 1h. The cells were then treated with L+E+P at 2µg/ml or 4µg/ml for 4h and tube number counted. Bars represent Standard Error of the Mean.; **p < 0.01, ***p < 0.001.

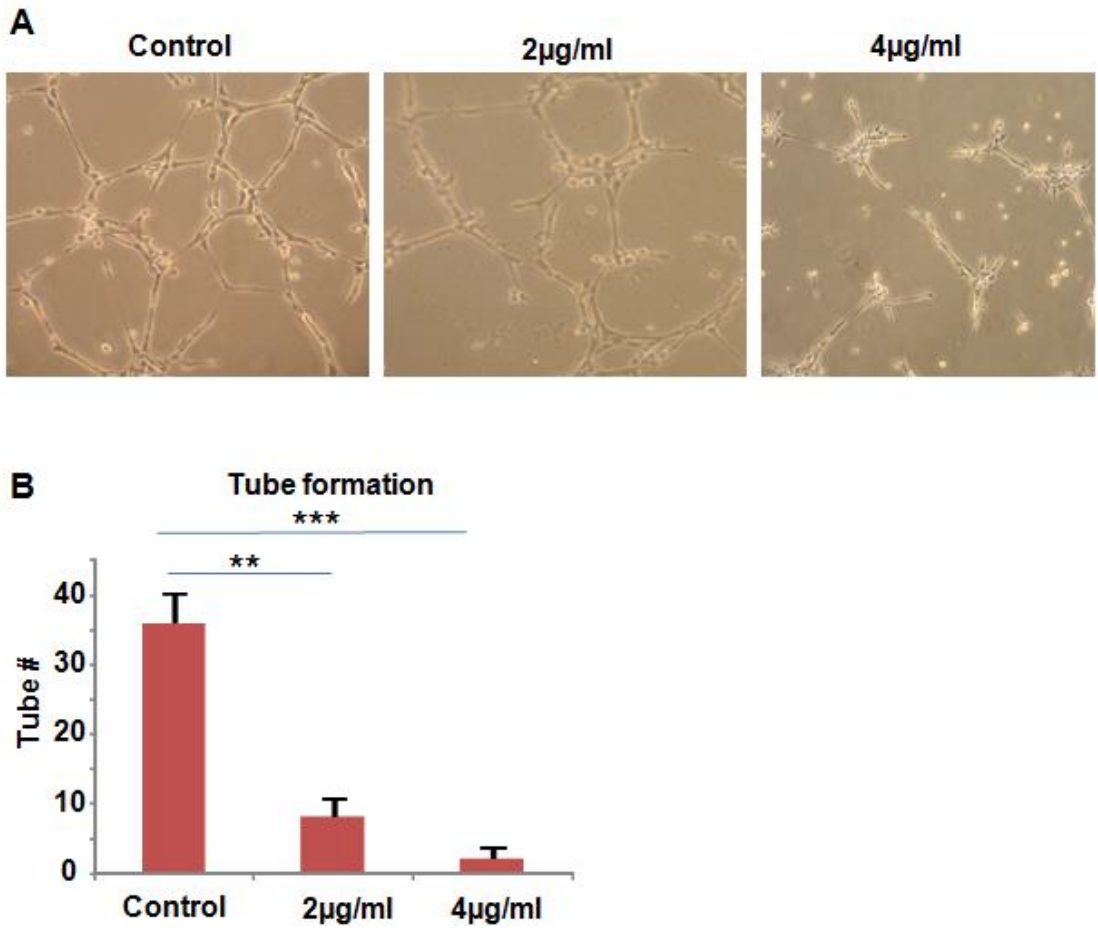

A
Control
2µg/ml
4µg/ml
B
Tube formation
***
**
Tube #
40
30
20
10
0
Control
2µg/ml
4µg/ml

Figure 4.8

Fig 4.9. L+E+P inhibits production of pro-angiogenic factors in tumors and inhibits signaling in HMVEC cells. (A-B) Levels of IL-8 and VEGF in normal human prostate tissue and in three different human prostate tumors. **(C-D)** IL-8 and, especially, VEGF production from PC3 cells is greatly reduced when treated with 8µg/ml L+E+P in comparison to untreated tumors. **(E)** HMVEC cells were pre-treated with 4µg/ml L+E+P for 12 h and then treated with IL-8 at 100ng/ml for 60 min in the presence or absence of L+E+P. Protein extracts were analyzed by immunoblotting for p-AKT (S473) and VEGFR2 phosphorylation (Y1054). **(F)** HMVEC cells were pre-treated with 4µg/ml L+E+P for 12 h and then treated with VEGF at 200ng/ml for 60 min in the presence or absence of L+E+P. Protein extracts were analyzed by immunoblotting for p-AKT (S473) and p-ERK (T202/Y204). **(G)** C57BL mice were treated with 64µg of each L+E+P for 2 weeks and then injected under the skin on the back (after hair removal) with IL-8 (100ng/20µl saline) or VEGF (200ng/20µl saline) at symmetric sites (see text) every 24 hrs for 4 days. Skin samples from the injected areas were collected at day 5 and photographed. The blood vessels were manually highlighted. Bars represent Standard Error of the Mean. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

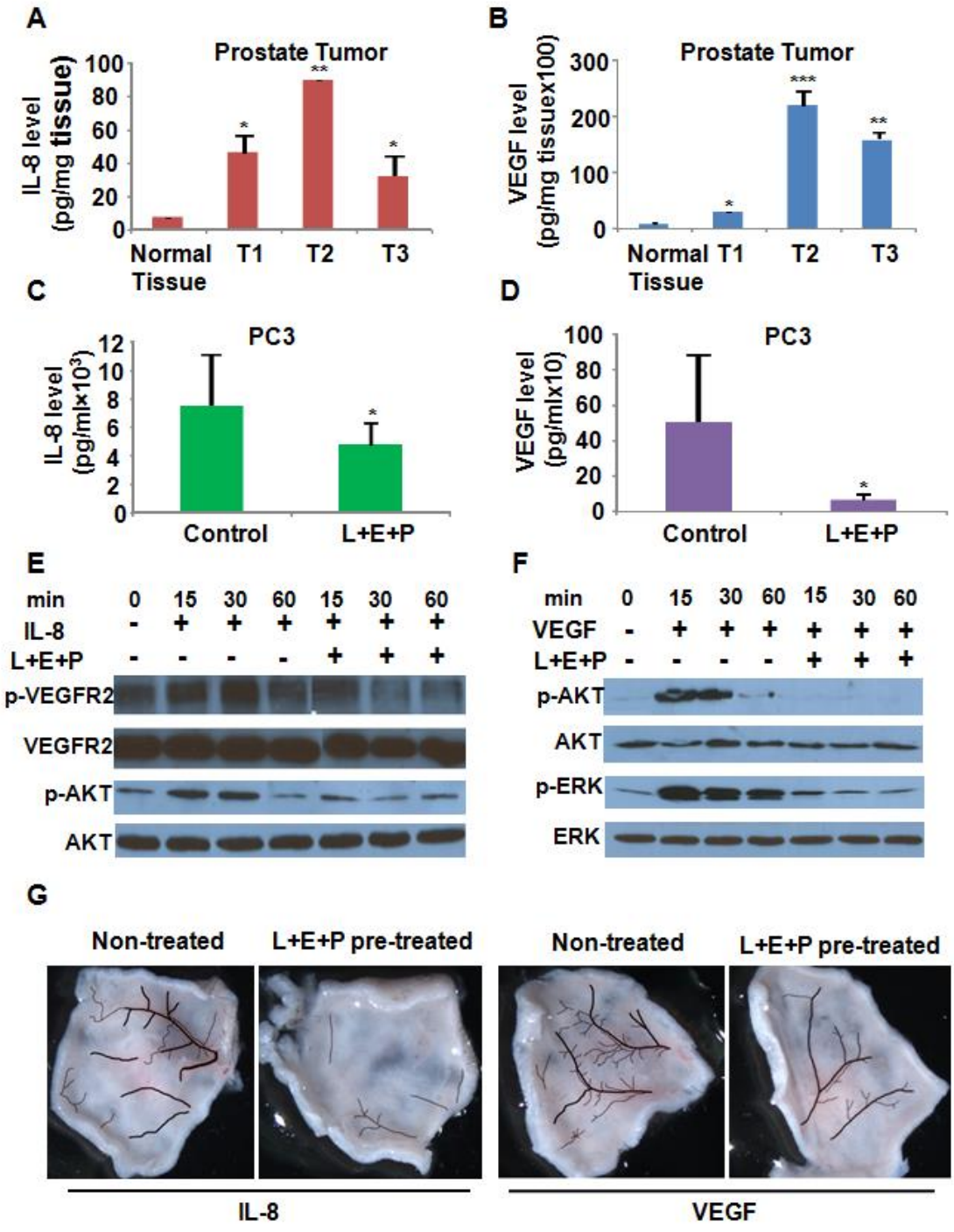

A
Prostate Tumor
IL-8 level (pg/mg tissue)
100
80
60
40
20
0
Normal Tissue
T1
T2
T3
B
Prostate Tumor
VEGF level (pg/mg tissuex100)
300
200
100
0
Normal Tissue
T1
T2
T3
C
PC3
IL-8 level (pg/ml×10³)
12
10
8
6
4
2
0
Control
L+E+P
D
PC3
VEGF level (pg/mlx10)
100
80
60
40
20
0
Control
L+E+P
E
min 0 15 30 60 15 30 60
IL-8 - + + + + + +
L+E+P - - - - + + +
p-VEGFR2
VEGFR2
p-AKT
AKT
F
min 0 15 30 60 15 30 60
VEGF - + + + + + +
L+E+P - - - - + + +
p-AKT
AKT
p-ERK
ERK
G
Non-treated
L+E+P pre-treated
Non-treated
L+E+P pre-treated
IL-8
VEGF

Figure 4.9

Fig 4.10. L+E+P inhibits IL-8 or VEGF induced angiogenesis. C57BL mice were treated with 64μg each of L+E+P for 2 weeks and injected with IL-8 (100ng/20μl saline) or VEGF (200ng/20μl saline) at symmetric sites (see text) on the back every 24 hrs for 4 days, and skin samples from the injected areas were collected at day 5 and photographed **(A)** under direct light and **(B)** vessels were highlighted using Photoshop.

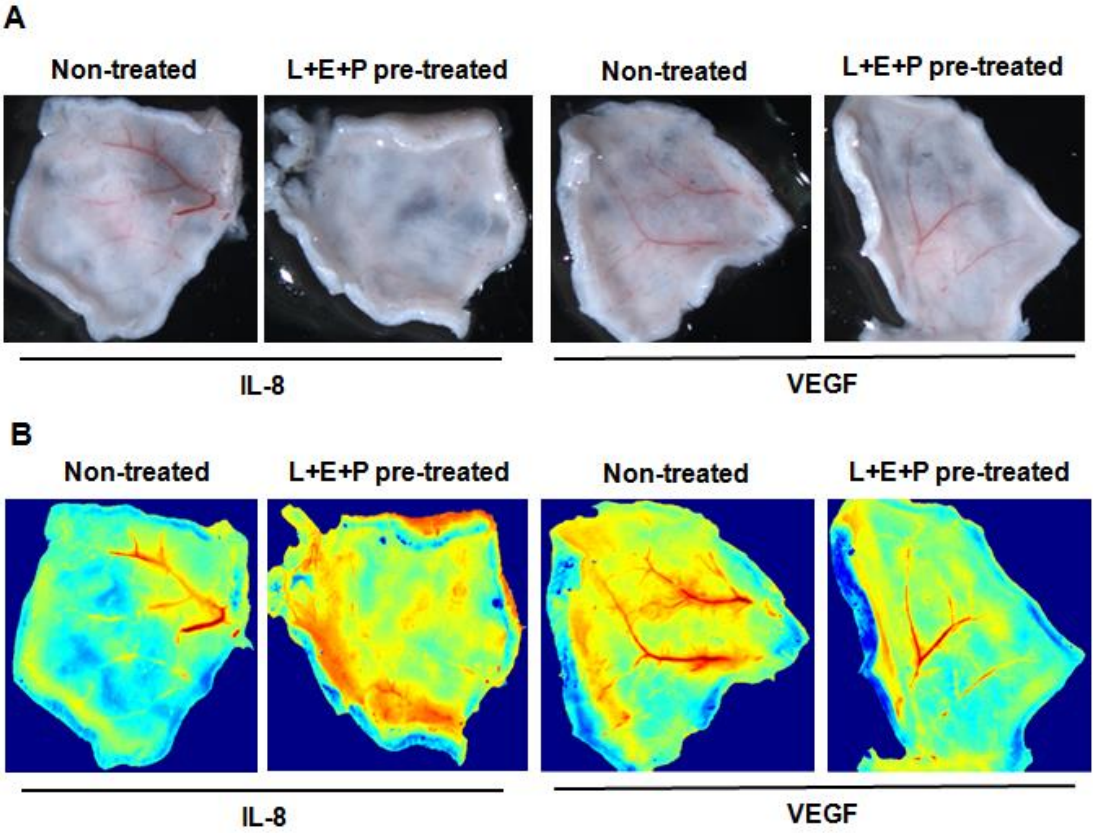

A
Non-treated
L+E+P pre-treated
Non-treated
L+E+P pre-treated
IL-8
VEGF
B
Non-treated
L+E+P pre-treated
Non-treated
L+E+P pre-treated
IL-8
VEGF

Figure 4.10

CHAPTER 5:

The Potential of Pomegranate and its Components for Prevention and Treatment of Breast Cancer

ABSTRACT

Breast cancer is the most common malignancy women in the United States, and the second leading cause of cancer-related death in women. There is a major need for more effective and less toxic therapies to treat breast cancer. Pomegranate fruit from the tree *Punica granatum* has been used for centuries for medicinal purposes and is described as the "nature's power fruit". Recent research has demonstrated that pomegranate extracts possess polyphenolic and other compounds with anti-proliferative, pro-apoptotic and anti-angiogenic effects on breast, prostate, lung, and other cancers. This review focuses on recent investigations into the effects of pomegranate and its components on breast cancer.

SIGNIFICANCE AND SCOPE

Breast cancer is the most common cancer and the second leading cause of cancer death and morbidity among women in the western world . About 1 in 8 (12%) women in the US will develop invasive breast cancer during their lifetime. It is estimated that there will be about 232,340 new cases of invasive breast cancer diagnosed in women and about 39,620 women will die from breast cancer in 2013 .

In addition to surgery and chemotherapy, hormone therapy has been used to treat breast cancer because hormone-dependent breast cancer cells require estrogen to grow. Tamoxifen, an antagonist of estrogen receptor (ER), is used to treat early and advanced ER positive breast cancer (Jordan, 2006). Aromatase inhibitors block estrogen production through inhibiting the enzyme aromatase, which converts androgen into estrogen (Miller et al., 2008). Human epidermal growth factor receptors HER1, HER2, HER3 and HER4, also known as ErbB1, ErbB2, ErbB3 and ErbB4, are transmembrane tyrosine kinase receptors that regulate cell proliferation and survival (Yarden, 2001). Receptor hetrodimerization or homodimerization induced by extracellular ligand binding activates the intracellular tyrosine kinase domain. In contrast to the other three HER receptors, HER2 can dimerize in the absence of ligand binding because the conformation of its extracellular domain resembles the ligand-activated state (Brennan et al., 2000). HER2 activation leads to downstream signaling pathways promoting cell survival and inhibiting cell death (Yarden and Sliwkowski, 2001) and is overexpressed in 20-30% of invasive breast cancer (Slamon

et al., 1987). As a result, specifically targeting HER2 is another important strategy to treat breast cancer.

Trastuzumab (Herceptin®) is a humanized monoclonal antibody that binds to the extracelullar domain of HER2 and inhibits the proliferation and survival of HER2-dependent tumors (Hudis, 2007). The mechanism of action of Trastuzumab includes preventing HER2 dimerization, blocking the cleavage of HER2 and activating cell-mediated cytotoxicity (Scheuer et al., 2009; Seidman et al., 2001). Because dimerization is required for HER2 receptor to initiate intracellular signaling pathways that regulate cancer cell proliferation, inhibition of receptor dimerization is a potential strategy for inhibition of HER2 dependent cancer progression.

Pertuzumab (Perjeta®) is a newer class of drugs (monoclonal antibody) that inhibits HER2 dimerization (Adams et al., 2006). Because Pertuzumab binds to a different epitope of HER2 extracellular domain than trastuzumab, combination therapy with Pertuzumab and Trastuzumab may enhance the efficiency in blocking HER2 signaling. HER1 (or EGFR) signaling pathway is well-known to be involved in cell proliferation and survival (Olayioye et al., 2000). Dual inhibition of HER1 and HER2 has been found to exert greater biological effects in the inhibition of cancer cell proliferation and survival than inhibition of either receptor alone. Lapatinib (Tykerb®) is a dual tyrosine kinase receptor inhibitor that inhibits both HER1 and HER2 (Burris, 2004).

Despite significant improvement in early diagnosis, aggressive surgical treatment and application of additional nonsurgical modalities, many patients experience disease recurrence as a consequence of drug resistance (Cazzaniga and Bonanni, 2012). As a result, it becomes of primary importance to search for new therapeutic agents that may lead to better disease-free and overall survival. Over the past decade, the use of new therapeutic approaches based on plant-derived natural products for the prevention and treatment of breast cancer has increased in the US. One such approach has been by using pomegranate juice or its components to potentially prevent or treat breast cancer as described below. In this short article we will discuss current literature on the use of the juice itself or some of its components and their effects on breast cancer biology and finish with speculation on the potential for translational research and for the clinic.

DISCUSSION OF CURRENT LITERATURE ON POMEGRANATE AND ITS COMPONENTS

Pomegranates are the fruit of the tree *Punica granatum* which is cultivated in Mediterranean countries, Afghanistan, India, China, Japan, Russia, and some parts of the US. It has been shown that the antioxidant activity of the pomegranate fruit is higher than that of red wine and green tea, two beverages which are showing promise in preclinical cancer models and in cancer patients (Khan et al., 2008).

The pomegranate fruit can be divided into three major anatomical components: the juice, the pericarp and the seeds. The juice and pericarp contain a rich complement of two types of polyphenolic components which have attracted interest of recent research: anthocyanins which give the juice its red color (Hernandez et al., 1999), such as delphinidin, cyanidin and pelargonidin, and hydrolyzable tannins, such as the punicalagin and gallagic acid (Gomez-Caravaca et al., 2013; Lansky and Newman, 2007; Van Elswijk et al., 2004). Other polyphenolic components of possible interest include kaempferol, quercetin and luteolin (Ackland et al., 2005; Qu et al., 2012; Van Elswijk et al., 2004). The seed oil, which is comprised of 65–80% conjugated fatty acids, also contains many compounds of interest with known anti-cancer activities. The predominant component among these fatty acids is punicic acid (Gasmi and Sanderson, 2010; Grossmann et al., 2010). Several of these components of pomegranate appear to exhibit anti-cancer effects on human breast cancer cells that potentially can be used as alternatives to treat breast cancer.

Effects of pomegranate juice and various extracts on breast cancer: In 2002, it was shown that pomegranate has chemopreventive and therapeutic potential for breast cancer (Kim et al., 2002b). In this study, pomegranates were processed into three fractions: fermented juice, pericarp extract and seed oil. Polyphenols from fermented juice and pericarp inhibited aromatase activity by 51% and 24% respectively as compared to 65% inhibition by a known aromatase inhibitor, aminoglutethimide [20]. They also showed that fermented juice, pericarp polyphenols and whole seed oil inhibited the activity of 17β-hydroxysteroid dehydrogenase, the enzyme that catalyzes the conversion of estrone to 17β-hydroxysteroid (17β-Estradiol), which is the most biologically active form of estrogen (Labrie et al., 1997). Interestingly, the inhibitory effect of seed oil was much more potent than the juice and pericarp [20]. It was also shown that fermented juice, pericarp polyphenol and seed oil inhibited cell proliferation of ER positive (MCF-7) and ER negative (MB-MDA-231) breast cancer cell lines but showed no effect on normal breast epithelium (MCF-10A). Proliferation of the ER positive MCF-7 cells was more sensitive to the pomegranate treatment than MB-MDA-231 cells. The seed oil also significantly inhibited the invasion of MCF-7 cells across a Matrigel membrane. These findings suggest that pomegranate juice, pericarp and seed oil possess anti-estrogenic effects by inhibiting estrogen-induced functions related to proliferation and invasion.

In 2004, studies were performed to determine the chemopreventive efficacy of a purified peak of pomegranate fermented juice containing polyphenols (Mehta and Lansky, 2004). In this study, mouse mammary glands were removed from female mice, exposed to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) and allowed to develop cancerous lesions (Mehta, 2000). The mammary glands were then treated with the high-performance liquid chromatographic (HPLC) peak separated from pomegranate fermented juice polyphenols. This fraction suppressed 75-90% of mammary lesion formation whereas the juice polyphenols only showed 40% inhibition, suggesting that the purified peak component has greater chemopreventive potential than the juice itself (Mehta and Lansky, 2004). Interestingly, pomegranate seed oil was remarkably more effective in suppressing lesion formation (~ 90%) showing even greater chemopreventive potential than the polyphenolic chromatographic peak (Mehta and Lansky, 2004).

It is well established that elevated levels of nuclear factor-kB (NF-kB) are frequently found in various cancers including breast cancer (Nakshatri and Goulet, 2002). NF-kB is a key player in regulation of genes important for angiogenesis, invasion and metastasis of breast cancer (Biswas et al., 2004; Hayden and Ghosh, 2004; Karin, 2006). The NF-kB signaling pathway is constitutively active in aggressive breast cancer cell lines such as SUM149 and MDA-MB-231 (Streicher et al., 2007). In 2009, the effects of pomegranate fruit extracts, consisting of both fermented juice and seed oil, on the NF-kB pathway in breast cancer, was examined

(Khan et al., 2009). These investigators found that the pomegranate fruit extracts suppressed the constitutive NF-kB activity in both SUM149 and MDA-MB-231 cell line and did so in a dose-dependent manner. The invasiveness and motility of these two aggressive breast cancer cell lines were significantly inhibited by the pomegranate fruit extracts. These investigators found that ellagitannins and phenolic acids were the major components in the aqueous phase, and conjugated octadecatrienoic acids, such as punicic acid, were abundant in the lipid phase derived from seeds [36].

More recently, several other studies have shown the anti-cancer effects of pomegranate on breast cancer. In 2012, it was shown that the anticancer effects of pomegranate extracts are partially due to targeting the microRNAs miR-155 and miR27a (Banerjee et al., 2012). In this study, the authors showed that pomegranate extract significantly decreased specificity protein (Sp) transcription factors (Sp1, Sp3, Sp4) as well as miR-27a and miR-155 in breast cancer cells. Sp1, Sp3, Sp4 are widely overexpressed in various cancers. These transcription factors regulate genes involved in proliferation, survival, and angiogenesis (Wang et al., 2003). Sp1 is also known to regulate NF-kB through a GC-rich binding site in the promoter region of the NF-kB p65 subunit (Hirano et al., 1998). As mentioned above pomegranate extracts down-regulate NF-kB activity, thus, the down regulation of NF-kB by these extracts could be mediated through down regulating of the Sp transcription factors.

In breast cancer cells, the high expression of Sp transcription factors is due to suppression by miR-27a of the Sp-repressor ZBTB10. It has been shown that several anticancer agents down regulate miR-27a while they up regulate ZBTB10 (Mertens-Talcott et al., 2007; Mertens-Talcott et al., 2012). Pomegranate extracts also increased the expression of the transcriptional repressor ZBTB10. In addition, pomegranate extracts increased the expression of inositol 5'-phosphatase SHIP-1, a target gene of miR-155. This lipid phosphatase is a critical negative regulator of phosphatidylinositol-3,4,5-trisphosphate (PI3K)/AKT signaling pathway (Kerr, 2011), which promotes cell proliferation and survival and is commonly activated in various tumors (Vivanco and Sawyers, 2002). These findings suggest that the anti-cancer effects of pomegranate extract on breast cancer cells are partially due to decreasing the levels of miR-27a and miR-155.

Effects of specific pomegranate components on breast cancer: The majority of the work performed until recently was performed with extracts of the various compartment of the pomegranate fruit or with chromatographically purified fractions of the juice. However, more recently studies have been performed using single components or combination of components. We found that three pomegranate components, luteolin (L), ellagic acid (E) and punicic acid (P), possess promising anti-cancer effects on prostate cancer cells(Wang et al., 2012a) and on breast cancer cells(Rocha et al., 2012). In breast cancer, using estrogen insensitive MDA-MB-231

cells (ER-), estrogen sensitive MCF7 cells (ER+), and the non-neoplastic cell line MCF10A, we showed that, in addition to inhibiting growth of the breast cancer cells, L+E+P in equal amounts increased cancer cell adhesion and decreased cancer cell migration but did not affect normal cells (Rocha et al., 2012). L+E+P also increased the expression of genes involved in cell adhesion, such as intercellular adhesion molecule 1 (ICAM1) and claudin 1(CLDN1), and decreased the expression of genes involved in cell migration, such as hyaluronan-mediated motility receptor (HMMR). These components also inhibited chemotaxis of the cancer cells to CXCL12, a chemokine that plays a critical role in breast cancer metastasis (Liang et al., 2005; Mueller et al., 2001). It is well known that loss of adhesion, increase in cell migration and increase in CXCL12 chemotaxis are key processes that lead to metastasis in breast cancer. We also found that L+E+P increased the expression of E-cadherin and decreased the expression of TWIST, two important genes involved in epithelial-to-mesenchymal transitions. Moreover, we found that E-cadherin knockdown by siRNA or overexpression of TWIST partially reversed the inhibitory effect of L+E+P on cancer cell migration. In addition, by using cytokine/chemokine multiplex arrays, we found that secreted levels of pro-inflammatory cytokines/chemokines, such as IL-8 and RANTES, were significantly reduced by L+E+P, thereby having the potential to decrease inflammation and its impact on cancer progression.

The promising anti-cancer effects of luteolin, ellagic acid and punicic acid on breast cancer cells have also been supported by other studies. It has been shown that luteolin suppresses insulin-like growth factor 1 (IGF-1)-stimulated MCF-7 cell proliferation in a dose-dependent manner (Wang et al., 2012b). They showed that luteolin markedly decreased IGF-1-dependent IGF-1R and AKT phosphorylation. It was also shown that ER is directly involved in the inhibitory effects of luteolin on IGF-1- induced cell proliferation. Luteolin significantly decreased ER expression. Knocking down the ER in MCF-7 cells by an ER-specific siRNA decreased the IGF-1-induced cell proliferation inhibitory effects of luteolin. Therefore, ER is a possible molecular target of luteolin. These findings indicate that the inhibitory effect of luteolin on the growth of MCF-7 cells occurs via inhibition of the IGF-1-mediated PI3K-Akt pathway.

Ellagic acid has been shown to possess an anti-angiogenesis effect on breast cancer (Wang et al., 2012c). It was shown that ellagic acid significantly inhibited vascular endothelial growth factor (VEGF)-induced cell proliferation, migration and tube formation in human endothelial cells. Furthermore, ellagic acid inhibited VEGFR-2 tyrosine kinase activity and its downstream signaling pathways including MAPK and PI3K/AKT in endothelial cells. These investigators also showed that ellagic acid inhibited MDA-MB-231 xenograft tumor growth and reduced the level of phospho-VEGFR2. In-silico analysis using molecular docking simulation indicated that ellagic acid could interact with the ATP-binding region of the VEGFR-2 (Wang et

al., 2012c). These findings suggest that the anti-angiogenesis effect of ellagic acid could be mediated through targeting VEGFR-2 signaling pathway in breast cancer.

Punicic acid, the most abundant component in seed oil, has been reported to have anti-cancer effects on breast cancer cells. It has been shown that punicic acid inhibits the proliferation of both an estrogen insensitive breast cancer cell line (MDA-MB-231) and an estrogen sensitive cell line developed from the MDA-MB-231 cells (MDA-ERα7) (Grossmann et al., 2010). Furthermore, punicic acid induced apoptosis in the MDA-MB-231 and MDA-ERα7 cells and disrupted cellular mitochondrial membrane potential. They also showed that lipid oxidation was required for the effects of punicic acid on cell proliferation and apoptosis and that this effect was partially blocked in both the MDA-MB-231 and MDA-ERα7 cells by PKC inhibitor. Their findings suggest that the anti-breast cancer effects of punicic acid are partially dependent on lipid oxidation and the PKC pathway.

SUMMARY AND CONCLUSION

The findings discussed above show that pomegranate juice, some of its fractions, and L, E and P singly or in combination, interfere with multiple biological processes involved in tumor growth, angiogenesis and metastasis of breast cancer cells (**Figure 5.1**). They suppress cell growth, increase cell adhesion, inhibit cell migration and inhibit chemotaxis of tumor cells towards proteins that are important in breast cancer metastasis. Many of the molecular mechanisms involved in these processes are amenable to drug treatment and to the development of small inhibitory molecules. The findings describe here on the effects of pomegranate juice, when coupled with similar results on prostate cancer, from our laboratory and elsewhere, strongly suggest that pomegranate juice and its components can potentially be used to prevent progression of breast and prostate cancer and well as other cancer(Rocha et al., 2012; Wang et al., 2011; Wang et al., 2012a).

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Figure 5.1. Summary of the anti-cancer effects of pomegranate and its components on breast cancer. Pomegranate and its components inhibit tumor growth by decreasing cell proliferation through decreasing cyclin E, PKCε, NF-kB, miR-27a and miR-155; inhibiting angiogenesis through decreasing CXCL8, platelet derived growth factor (PDGF) and VEGFR2; inhibiting the production of pro-inflammatory cytokines such as CCL5 and CX3CL1; inhibiting estrogen synthesis through decreasing aromatase and 17β-hydroxysteroid dehydrogenase. Pomegranate and its components inhibit metastasis by stimulating cell adhesion through increasing intercellular adhesion molecule 1(ICAM1), claudin 1 and myristoylated alanine-rich protein kinase C substrate (MARCKS); inhibiting CXCL12 chemotaxis through decreasing CXCL12 receptor CXCR4; inhibiting cell migration through decreasing hyaluronan mediated motility receptor (HMMR), N-chimearin, nexilin, anillin and collagen I; inhibiting epithelial-mesenchymal transition (EMT) through increasing E-cadherin and decreasing Twist.

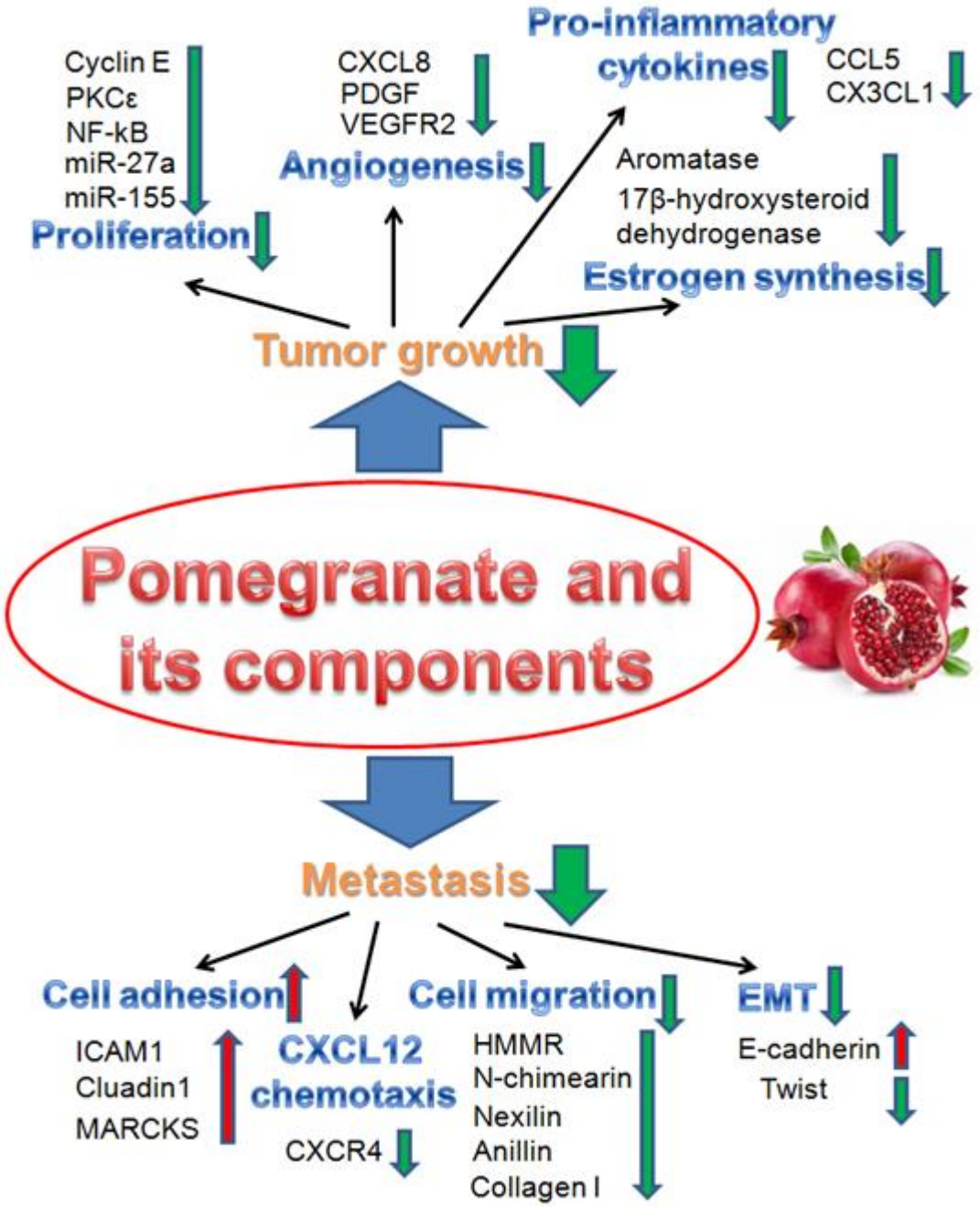
Pro-inflammatory cytokines
Cyclin E
PKCε
NF-kB
miR-27a
miR-155
Proliferation
CXCL8
PDGF
VEGFR2
Angiogenesis
CCL5
CX3CL1
Aromatase
17β-hydroxysteroid dehydrogenase
Estrogen synthesis
Tumor growth
Pomegranate and its components
Metastasis
Cell adhesion
ICAM1
Cluadin1
MARCKS
CXCL12 chemotaxis
CXCR4
Cell migration
HMMR
N-chimearin
Nexilin
Anillin
Collagen I
EMT
E-cadherin
Twist

Figure 5.1

CHAPTER 6:

Pomegranate Juice and Specific Components Inhibit Cell and Molecular Processes Critical for Metastasis of Breast Cancer

ABSTRACT

Breast cancer is the most common cancer and the second leading cause of cancer death and morbidity among women in the western world. Pomegranate juice (PJ) and three of its specific components have been shown to inhibit processes involved in prostate cancer metastasis. If this also proves to be true for breast cancer, these natural treatments will be promising agents against breast cancer that can serve as potentially effective and nontoxic alternatives or adjuncts to the use of conventional selective estrogen receptor modulators (SERMs) for breast cancer prevention and treatment. To test this possibility, we have used 2 breast cancer cell lines, MDA-MB-231 cells (ER-) and MCF7 (ER+), and the non-neoplastic cell line MCF10A. We show that, in addition to ihibiting growth of the breast cancer cells, PJ or a combination of its components Luteolin (L) plus Ellagic Acid (E) plus Punicic Acid (P) increase cancer cell adhesion and decrease cancer cell migration but do not affect normal cells. These treatments also inhibit chemotaxis of the cancer cells to SDF1□, a chemokine that attracts breast cancer cells to the bone. We hypothesized that PJ and L+E+P stimulate expression of genes that increase adhesion and inhibit genes that stimulate cell migration and inhibit chemotaxis to SDF1□. Using qPCR, we confirmed these proposed effects on gene expression and in addition we found that a gene important in epithelial-to-meshenchymal transitions is decreased. We also found that pro-inflammatory cytokines/chemokines are significantly reduced by these treatments, thereby having the potential to decrease inflammation and its impact on cancer progression. Discovery that PJ and L+E+P are inhibitory of metastatic processes in breast cancer cells in addition to prostate cancer cells indicates that they are potentially a very effective treatment to prevent cancer progression in general.

INTRODUCTION

Breast cancer continues to be the most common cancer and the second leading cause of cancer death and morbidity among women in the western world (WHO, 2013). Each year there are more than 10,000 new cases and more than 40,000 women die from this disease (Maughan et al., 2010; Siegel et al., 2012). As a result, it becomes of primary importance to search for new therapeutic agents that may lead to better disease-free and overall survival. Chemopreventive agents, tamoxifen and raloxifene, have been shown to reduce the risk of estrogen receptor (ER) positive breast cancers by 50% in high-risk women but, unfortunately, their use is associated with major side effects. In addition, they do not prevent estrogen receptor negative breast cancers. Despite significant improvement in early diagnosis, aggressive surgical treatment and application of additional nonsurgical modalities, many patients experience disease recurrence as a consequence of drug resistance (Cazzaniga and Bonanni, 2012).

Over the past decade, the use of new therapeutic approaches based on plant-derived natural products for the prevention and treatment of cancer has increased in the United States. Pomegranate (*Punica granatum*) is a rich source of many phenolic compounds including flavonoids like anthocyanins, hydrolyzable tannins, flavonols and flavones. These pomegranate compounds appear to exhibit anti-inflammatory and therapeutic effects on human breast cancer cells that could serve as effective, yet nontoxic, alternatives or adjuncts to the use of conventional

treatment for breast cancer (Dikmen et al., 2011; Gil et al., 2000a; Kim et al., 2002b; Longtin, 2003; Ozbay and Nahta, 2011).

Some of the initial studies on pomegranate extracts showed that these extracts had significant anti-proliferative and pro-apoptotic effects against MCF-7 (ER^+) and MD-MBA-231 or MD-MBA-435 (ER^-) human breast cancer cell lines (Dikmen et al., 2011). The most recent studies involving PJ or its components focus primarily on its antiproliferative and apoptotic effects on breast cancer (Ozbay and Nahta, 2011). These studies show that pomegranate extracts or some of its specific polyphenolic components have growth inhibitory effects on breast cancer cells and can even prevent the proliferation of cancer stem cells (Dai et al., 2010). In their study, Wang et al indicate that the inhibitory effect of luteolin on the growth of MCF-7 cells occurs via inhibition of the IGF-1 mediated PI3K-Akt pathway dependent on ERα expression (Wang et al., 2012b). Another recent study with luteolin suggests that it induces a caspase-dependent and -independent apoptosis involving AIF nuclear translocation mediated by activation of ERK and p38 in breast cancer cells (Kim et al., 2012). It also has been shown that pomegranate Ellagitannin-derived compounds are anti-proliferative and inhibit aromatase, an enzyme critically involved in the conversion of androgen to estrogen(Adams et al., 2010). These authors suggest that these compounds may have the potential to affect estrogen-responsive breast cancers. Studies with punicic acid have shown that this component of the juice inhibits breast cancer cell proliferation through its lipid peroxidation properties and also by affecting

the PKC pathway (Grossmann et al., 2010). The work we present here goes beyond these findings by showing that PJ and its components have an effect on several critical processes involved in breast cancer metastasis.

PJ is rich in antioxidants in particular in punicalin, acid, caffeic acid, ellagic acid and luteolin (El Kar et al., 2011a). Our group has previously shown that the aqueous portion of PJ stimulates adhesion, inhibits growth and migration of DU 145 and PC3 cells and inhibits chemotaxis of these prostate cancer cells towards SDF1α (Wang et al., 2011b), a chemokine known to be involved in metastasis of hormone refractory prostate cancer cells to the bone (Duda et al., 2011). All of these processes are critical for cancer metastasis. However, the soluble phase of PJ contains many components that vary with variety, cultivation, extraction, etc. and therefore it is difficult to determine how best to maximize its use in treating prostate cancer. A short communication reported that Luteolin (L) ellagic acid (E), caffeic acid (C), and punicic acid (P), inhibit *in vitro* invasion of human prostate cancer (PC3) cells across matrigel (Lansky and et al., 2005). However, despite these very interesting results this study was very limited and showed only the results of a a few chemotaxis chamber invasion assays. Using these components we showed that L+E+P together mimic the anti-metastatic effect of the whole juice, whereas C was ineffective (Wang et al., 2012a). Because loss of adhesion, increase in migration and increase in chemotaxis are hallmarks of cancer metastasis, we hypothesized that the same three components were likely to affect these processes in breast cancer cells. If so, these natural

treatments will be promising agents not only against breast cancer but also potentially an effective treatment to prevent cancer progression in general.

Using an ER^+ cell line (MCF-7), an ER^- cell line (MD-MBA-231), and a normal breast cell line (MCF10A), we show here that PJ inhibits growth, increases adhesion, decreases migration and inhibits chemotaxis to SDF1α of the cancer cells but not of the normal cells. We show further that L+E+P is equally effective. We conclude that these three components in combination strongly affect processes that are critical for growth and metastasis of breast cancer and potentially can be generalized to affect metastasis of other cancers. Given these findings, we also investigated what could be the possible mechanism of action of the PJ and its components and found that they stimulate molecules involved in cell adhesion and inhibit molecules involved in cell migration. Furthermore, we also found that both the juice and the three components inhibit the production of pro-inflammatory cytokines/chemokines in the cancer cells but not in the non-neoplastic cells. Our findings strongly suggest that L+E+P can potentially be used to prevent growth and metastasis of breast cancer and could be used in chemoprevention and/or as a co-adjuvant to traditional therapy.

MATERIALS AND METHODS

Cell Lines: MCF7, MDA-MB-231 and MCF10A human cell lines were purchased from ATCC (Manassas, VA) and cultured according to the procedures provided by the company. MCF7 is an estrogen/progesterone receptor positive (ER^+/PR^+) cell line. The MDA-231 cell line is estrogen receptor/progesterone receptor negative (ER^-/PR^-) and is associated with aggressive cancer phenotypes with increased invasion, and motility. This cell line demonstrates marked tumorigenicity with increased metastatic potential *in vivo*. The MCF10A line consists of spontaneously immortalized mammary epithelial cells (derived from a patient with benign fibrocystic breast disease) and was used as non-neoplastic control. MCF7 and MDA-MB-231 cells were cultured at 37ºC with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum, penicillin (100 I.U ml/L) and streptomycin (100 mg ml/L). Cells of the MCF10A line were grown in DMEM/F12 supplemented with 5% horse serum, extracellular growth factor (20ng/mL), choleratoxin (100ng/ml), hydrocortisone (0,5mg/ml), insulin (10 µg/mL), penicillin (100 I.U ml/L) and streptomycin (100 mg ml/L).

Growth assay: $1x10^5$ cells were plated on 6-well plates (B&D Biosciences), allowed to adhere and 24hrs later treated with 1% and 5% PJ or with L+E+P at 1, 2, 4 or 8 µg/ml each. The number of proliferative cells on the plate was accessed by trypsinization after 12, 24, 48 and 72 hrs using trypan blue staining. The juice was sterilized by filtration. The filtrate was then centrifuged, the supernatant collected to

remove any particulate matter and then frozen in small aliquots to reduce the numbers of freeze-thaw cycles that can potentially damage the contents of the juice and stored at -20^{o}C.

Adhesion assay: 3x10^{5} cells were plated on gelatin-coated 6-well plates (B&D Biosciences), allowed to adhere and 24hrs later treated with 1% PJ or L+E+P at 2 or 4µg/mL each for 24 hrs or 48 hrs. Cells were then trypsinized and the time required to detach all cells was recorded as an indicator of cell adhesiveness (Wang et al., 2011c; Wang et al., 2012a).

Migration assay: Confluent cells were scratch wounded using a rubber scraper, washed and treated with 1% PJ or L+E+P at 2 or 4µg/ml each. Cell migration was determined by measuring the distance migrated by the cells from the wounded edge to the leading edge of migration at 12hrs, 24h, 48h and 72h after treatment was initiated. Scratch wounded cells without treatment were used as controls (Wang et al., 2011c; Wang et al., 2012a).

Chemotaxis assay: The upper side of polycarbonate membranes (8µm pore size) of transwells (BD Biosciences, San Jose, CA) was coated with 50ng/ml type I collagen (Sigma Chemical Co.). 1x10^{5} cells in 100µl of culture medium were plated on the collagen-coated transwell membranes and were allowed to adhere for 3 hrs. The wells were introduced into 24-well plates and 1000 µl of supplemented medium was added to the lower chamber. Cells were treated with 1% PJ or L+E+P at 2 or 4µg/ml for 12 hrs. SDF1α (100ng/ml) was added to the lower chamber and the cells were allowed to

migrate for 4 hrs at 37°C. The cells on the side of the membrane facing the upper chamber were removed with a cotton swab, and the membranes were then fixed and stained with 2% toluidine blue in 4% paraformaldehyde. Cells were counted in 8 high-power fields (HPF)/filter to obtain the average number of cells per field that migrate from the top of the membrane to the underside (Wang et al., 2011c; Wang et al., 2012a).

Real time quantitative PCR: Cells were treated with 1% PJ or the combination of 4μg/ml each of Luteolin, Punicic Acid and Ellagic Acid for 24 hrs and total RNA was extracted using the RNeasy RNA isolation Kit according to manufacturer's protocol (QIAGEN, Sciences, Maryland, USA). Briefly, cells were washed with ice-cold 1X PBS, and lysed on ice with lysis buffer. Cell lysates were then centrifuged to remove cell debris, followed by organic extraction to remove proteins. Then lysates were loaded into isolation columns and the final RNA product was dissolved in nuclease-free water. RNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE USA). 1μg RNA was reverse-transcribed to cDNA by RETROscript Reverse Transcription Kit (Ambion, Grand Island, NY) at 44°C for 1hr and 92°C for 10min. 2μl of cDNA from the reverse transcription reaction were added to 23μl real-time quantitative PCR mixture containing 12.5μl 2X SYBR Green SuperMix (Bio-Rad, Hercules, CA) and 200nM oligonucleotide primers. PCR was carried out in a Bio-Rad MyiQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The thermal profile was 95°C for 3

min followed by 40 amplification cycles, consisting of denaturation at 95°C for 10 sec, annealing at 60°C for 30 sec. Fluorescence was measured and used for quantitation purposes. At the end of the amplification period, melting curve analysis was done to confirm the specificity of the amplicon. Fold-changes of genes after treatment with PJ were calculated by the Pfaffl method to normalize the Ct values to the GADPH internal control. The following primer sequences were designed with IDT PrimerQuest and used for the reactions:

GADPH, TCGACAGTCAGCCGCATCTTCTTT and
ACCAAATCCGTTGACTCCGACCTT;

MARCKS, TTGTTGAAGAAGCCAGCATGGGTG and
TTACCTTCACGTGGCCATTCTCCT;

ICAM1, ATAACCGCCAGCGGAAGATCAAGA and
CGTGGCTTGTGTGTTCGGTTTCAT;

CLDN1, ATGGAAAGGGTGTTGGCATTGGTG and
CACTTGGGTGTTTGAGCATTGCCT;

HMMR, ATTCAGTTGTCGAGGAGTGCCAGT and
AGTGCAGCATTTAGCCTTGCTTCC;

COL1A1, CAATGCTGCCCTTTCTGCTCCTTT and
CACTTGGGTGTTTGAGCATTGCCT;

CHN1, TGAAACTACTGCCACCTGCTCACT and
TGGGTCCAAAGACGATTCCAAGGT;

PRCKE, CAACCAAGCAAGCTCTAACCGCAA and TTGTCCTGTAGGAAAGGCCCAGTT;

NEXN, TCAGCCCAAGACCACATAGAGCAA and TCTTTCTTCCCTGGCTCTCTGCAT;

ANLN, AGCTCACTCTTCTCACCAATGCCA and AAGCGGTACCAGGCTGTTCTTGTA.

Treatment with siRNA, inhibitors or with expression vectors containing the gene of interest: Cells (80–90% confluent) were transfected with Lipofectamine 2000 (Invitrogen, Grand Island, NY) following the manufacture's protocols. 40 nM E-cadherin chimera siRNA (Abnova, Jhongli, Taiwan) was transfected. 1μg/ml pcDNA3.1 HMMR vector and pcDNA4.1 TWIST vector were transfected. Scratch wound assay was performed as described above, 24 h after transfection.

Statistical Analysis: Data are shown as mean ± standard deviation (SD). Data analysis was performed using the unpaired Student's *X*-test on raw data using GraphPad Instat software (GraphPad Software Inc.). Statistical analysis between more than two groups was performed by One-way ANOVA.

RESULTS

Effect of PJ and L+E+P on growth, adhesion, migration and chemotaxis of ER^+/PR^+, ER^-/PR^- and non-neoplastic breast cancer cells

MDA-MB-231 (ER^-/PR^-) and MCF7 (ER^+/PR^+) breast cancer cells were treated with 1% and 5% filtered PJ or L+E+P at 1, 2, 4 or 8 µg/ml each and the effects on cell growth measured over time (Fig. 1A and 1B). The choice of these doses was based on previous studies and are designed to test for the best dose that still keeps the cells healthy so that processes involved in metastasis can be tested (Wang et al., 2011c; Wang et al., 2012a). Both cell lines showed similar results. Although the effects on cell growth were not significantly different at 12 hrs with any of the treatments, by 24 hrs differences could be seen. Both 5% PJ or L+E+P at 8 µg/ml each were inhibitory of growth of the cells by 24hrs and by 48 hrs the cultures contained many floating cells indicating that cell death was occurring. In contrast, treatment with 1% PJ or L+E+P at 4 µg/ml each completely stopped cell growth but did not cause cell death; treastment with L+E+P at 1 or 2 µg/ml each, growth was only slightly diminished (Fig. 1A and 1B). Therefore, we performed the remainder of the experiments at doses no higher than 1% PJ or L+E+P at 4 µg/ml each and measured time of cell release by trypsinization. The results show that after 24 hrs of treatment, the cancer cells require up to three times longer to be released by trypsinization and by 48hrs this time is further increased (Fig. 1C and 1D). In contrast, adhesion of the non-neoplastic

cells to the culture dish was not affected. The cells remained healthy at all times with no floating cells present in any of the cultures.

The increase in adhesion of the cells by PJ or L+E+P suggested that both potentially affect cell migration. To measure migration we used the scratch wound assay, measured the distance that the cells migrated from the wounded edge to the migration front and found that treatment with 1% PJ or L+P+E at 2 or 4 µg/ml each inhibited the migratory capabilities of both types of breast cancer cells as early as 12hrs after treatment (Fig. 2A and 2B). This pattern of delayed migration continued over time.

SDF1α is known for its ability to attract breast cancer cells to the bone marrow(Taichman et al., 2002)(Taichman et al., 2002). With this in mind, we tested the possibility that PJ or L+E+P would inhibit chemotaxis of the breast cancer cells towards SDF1α. Indeed, treatment with 1% PJ or L+E+P at 2 or 4 µg/ml each for 12 hrs prior to initiation of the chemotaxis assay significantly inhibited chemotaxis of the breast cancer cells towards SDF1α (Fig. 2C and 2D).

To determine whether these effects are specific for the cancer cells we treated MCF10A breast epithelial cells with 1% PJ or L+E+P at 2 or 4 µg/ml each and measured their effect on cell growth, cell adhesion, cell migration and chemotaxis to SDF1α over time (Fig. 3). Treatment of these non-neoplastic cells with the juice or its components showed no significant effects on growth or migration (Fig. 3A and 3D)

and the effects on adhesion and chemotaxis were minimal and only observed with the high dose of L+E+P (Fig 3B and 3C).

Effect of PJ or L+E+P on the expression of genes involved in processes critical for metastasis

To better understand the effects of PJ and L+E+P on functions involved in the metastatic process, we examined expression of specific genes involved in the following: (i) adhesion, such as intercellular adhesion molecule 1 (*ICAM1*) (Roland et al., 2007), claudin 1 (*CLDN1*) (Nakagawa et al., 2011) and myristolytated alanine-rich protein kinase C substrate (*MARCKS*) (Arbuzova et al., 2002); (ii) migration, such as hyaluranan-mediated motility receptor/CD168 (*HMMR*) (Hatano et al., 2011; Lin and Ying, 1999; Tolg et al., 2010), collagen type I alpha1 (*COL1A1*) (Helleman et al., 2008), anillin (*ANLN*) (Song and Rape, 2010) and nexilin (*NEXN*) (Ohtsuka et al., 1998a); (iii) cell cycle control, such as N-chimearin (*CHN1*) (Yang and Kazanietz, 2007b), cyclin E2 (*CCNE2*) (Caldon and Musgrove, 2010) and protein kinase C epsilon (*PRCKE*) (Gorin and Pan, 2009); (iv) chemotaxis of the cancer cells to SDF1α through its receptor type 4 (*CXCR4*) (Teicher and Fricker, 2010); (v) epithelial-to-mesenchymal transformation, such as twist (*TWIST*) (Kang and Massague, 2004a). We used quantitative RT-PCR with total RNA extracted from MDA-MB-231, MCF7 and MCF10A cells treated with 1% PJ or L+E+P at 4μg/ml each for 24hrs (Table I). We found that this treatment increases expression of the adhesion genes, decreases the cell cycle and the migration-inducing genes and

strongly decreases the epithelial-to-mesenchymal transition (EMT) gene we tested (*TWIST*).

To mechanistically test whether HMMR (migration-inducing) and TWIST (EMT) genes are critical in the inhibitory effects of PJ or L+E+P, we overexpressed each independently in the cancer cells and showed that over-expression partially reversed the inhibitory effect of PJ on cell migration (Fig. 4). Scratch wounds were made 24h after HMMR or TWIST vector transfection and the migrated distances were measured 36h later with or without 1% PJ or L+E+P at 2 or 4 μg/ml each. Neither HMMR- nor TWIST-transfected MDA-MB-231 migrate significantly different from the untreated cancer cells. However, MCF7 cells are able to migrate further than the control when transfected with TWIST (Fig 4D). In all cases the inhibitory effects of PJ and L+E+P on cell migration are partially reversed by the treatment of cells over-expressing of HMMR or TWIST (Fig.4). These results indicate that the effects of PJ or its components on cell migration are mediated, at least in part, through a decrease in HMMR and TWIST in breast cancer cells.

To further extend these studies, we evaluated the protein levels of E-cadherin (Fig. 5A and 5B), a cell adhesion molecule that is important in keeping mammary epithelial cells together, thereby preventing their migration; loss of E-cadherin is critical for invasion of epithelial tumor cells (Birchmeier and Behrens, 1994; Yilmaz and Christofori, 2010). Previously, we have found that in prostate cancer cells PJ and the three componentes inhibit the repressor for the E-cadherin gene, allowing for

elevation of the expression of E-cadherin (Wang et al., 2012a). We found that PJ and L+E+P significantly increase the protein levels of E-cadherin in both types of breast cancer cells (Fig 5A and 5B). To determine whether the increase in adhesion stimulated by PJ or L+E+P can be reversed, scratch wounds were made 24h after E-cadherin siRNA transfection. Distances migrated by the cells were measured 36h later with or without 1% PJ or L+E+P treatments. We find that the distance migrated by the transfected cells was not significantly different from control but migration was partially reversed by E-cadherin siRNA when the transfected cells were treated with PJ or L+E+P (Fig. 5C and 5D). These results indicate that the effects of PJ or L+E+P on cell migration are mediated significantly by an increase in E-cadherin.

Effect of PJ or L+E+P on the level of pro-inflammatory cytokines and chemokines.

Many pro-inflammatory cytokines and chemokines contribute to cancer progression. To determine the effect of PJ or L+E+P on production of some of these proteins, we used Luminex Multiplex Array assays to analyze the media collected from cells treated with 1% PJ or 4 μg/ml each of L+P+E for 18h. We tested for the levels of the following pro-inflammatory proteins: IFN-α, IFN-γ, IL-1α, IL-1β, IL1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-12p40, IL-13, IL-15, IL-17, IP-10, RANTES, TNF-α, TNF-β, EGF, Eotaxin, FGF3, Flt3, Fractalkine, G-CSF, G-MCSF, GRO, MCP1, MCP3, MDC, MIP1α, MIP1β, PDGFA,

PDGFB, sCD40L, sIL2RA, TGFα and VEGF. Production of these cytokines in MCF-10A cells was not affected by treatment with either the juice or the components (Fig.6 A-C). However, both cancer cell lines were strongly affected in their expression of IL-8, RANTES and PDGFB (Fig. 6 A-C). The more aggressive cells, MDA-MB-231 that are ER^-, also showed marked decrease in the chemokine Fractalkine when the cells were treated with PJ or L+E+P (Fig. 6D).

DISCUSSION

In this study, we used two breast cancer cell lines that have been used previously in many other breast cancer studies. We show the biological effects, both cellular and molecular, of PJ and some of its specific components in these breast cancer cell lines. We find that PJ or L+E+P: (**1**) increases adhesion of the breast cancer cells; (**2**) inhibits the migratory capability of these cells; (**3**) inhibits their chemotaxis toward SDF1α; (**4**) stimulates the expression of genes involved in cell adhesion and inhibits expression of genes involved in cell migration and in epithelia-to-mesenchymal transition; (**5**) reduces the level of pro-inflammatory cytokines/chemokines while increasing the levels of anti-inflammatory cytokines. None of these effects was observed in the non-neoplastic MCF10A breast epithelial cell line. All together these results provide compelling evidence that PJ and its components L+E+P specifically target breast cancer cells without toxicity to non-cancerous breast epithelial cells. Furthermore, despite the fact that MDA-MB-231 (ER-) are highly aggressive invasive cells and the MCF7 (ER+) cells are not as aggressively invasive, the response of the 2 cell types to PJ or L+E+P treatment is very similar, strongly suggesting that these effects of the juice are not ER-dependent.

The current model of human breast cancer progression proposes a linear multi-step process which initiates as flat epithelial atypia (FEA), progresses to atypical ductal hyperplasia (ADH), evolves into ductal carcinoma *in situ* (DCIS) and culminates in the potentially lethal stage of invasive ductal carcinoma. In this cancer

progression, stationary cancer cells transform into migratory cells in a process that involves loss of adhesion and rearrangement of cytoskeletal elements that allow the cells to migrate and invade (Eckhardt et al., 2012; Oyama et al., 2000; Oyama et al., 1999). Our results show that PJ or L+E+P can inhibit cell movement by increasing cell adhesion molecules and decreasing molecules that facilitate cell migration. Although the ratios of L+E+P in the juice are 1:50-200:4 we find that the use of equal proportion of the components is more effective to inhibit the cellular and molecular processes presented here.

Increase in the expression of genes involved in cell adhesion and down-regulation of genes that stimulate migration is shown by qPCR. In breast cancer cells, claudin 1, the major component of the tight junctions in epithelial cells, prevents the cells from being able to separate from each other, invade other tissues and metastasize. PJ and L+E+P stimulate Intercellular Adhesion Molecule-1 (ICAM-1), important in stabilization of cell–cell adhesion, and myristoylated alanine-rich protein kinase C substrate (MARCKS), an actin-binding protein that normally associates with the plasma membrane where vinculin and talin are present in focal adhesions (Aderem, 1992; Arbuzova et al., 2002; Roland et al., 2007). The treatment of the breast cancer cells with PJ and L+E+P down-regulates the expression of HMMR. This molecule functions as a hyaluronan (HA) receptor and the binding of HA to HMMR can stimulate the RhoA-activated protein kinase (ROCK) signal transduction pathway, leading to tumor cell migration and invasion in various cancers (Hatano et al., 2011;

Sohr and Engeland, 2008; Tolg et al., 2010). In addition, PJ or L+E+P downregulates anillin and nexillin, actin-binding proteins that are known to be involved in regulation of the structure of the cytoskeleton (Ohtsuka et al., 1998a; Song and Rape, 2010), and N-chimearin, GTPase-activating protein that, when down-regulated, results in loss of filopodia and reduction of migration (Yang and Kazanietz, 2007b). Also, treatment with PJ or L+E+P downregulates TWIST, a basic-helix-loop-helix transcription factor that has been implicated in the loss of cell adhesion and increased cell motility that are characteristics of epithelial-to-mesenchymal transition (EMT) (Kang and Massague, 2004a).

Apart from the genes described here that might mediate the effects of PJ or L+E+P on cell adhesion, migration and chemotaxis, we also find that these treatments act on breast cancer cell migration through significantly increasing E-cadherin, a molecule that is important in cell adhesion, ensuring that cells in epithelia are adherent to each other (Birchmeier and Behrens, 1994; Yilmaz and Christofori, 2010), and decreasing HMMR and TWIST levels. We also find that cell transfection with E-cadherin siRNA or with a vector that overexpresses HMMR or TWIST partially reverses the inhibitory effects of PJ or L+E+P on cancel cell migration.

The cytokine array results show that pro-inflammatory cytokines/chemokines known to promote tumor growth and cancer progression (Bonecchi et al., 2011; Dranoff, 2004) are inhibited by PJ or L+E+P treatment. Among the pro-inflammatory cytokines/chemokines examined with Luminex Multiplex Array assays, the secreted

levels of IL-8, RANTES, PDGFB, are significantly reduced by PJ or L+E+P treatment in both ER^+ and ER^- cells. IL-8 is a potent leukocyte chemoattractant and has also been shown to contribute to human cancer progression through its potential functions as a mitogenic and angiogenic factor. Over-expression of IL-8 is associated with increasing tumor stage and disease progression and recurrence in human melanoma, breast, gastric, ovarian, and prostate cancer (Inoue et al., 2000; Xie, 2001). Furthermore, there is a direct correlation between high levels of IL-8 and tumor angiogenesis, progression, and metastasis in nude xenograft models of human cancer cells (Ahmed et al., 2006; Araki et al., 2007). RANTES (CCL5) is a potent chemotactic factor for T cells, monocytes and dendritic cells. Expression of RANTES and its receptor, CCR5, have been shown to correlate with cancer progression. In addition, interaction of RANTES with CCR5 on the surface of cancer cells stimulates their invasive capabilities (Soria and Ben-Baruch, 2008). PDGFBB is a well-known mitogenic and pro-angiogenic factor and has been shown to potentiate cancer growth and progression (Fredriksson et al., 2004; Song et al., 2009). Also, the more aggressive cell line, MDA-MB-231, when treated with PJ or L+E+P, showed marked decrease in Fractalkine. Fractalkine can exist either in a soluble form, like all the other chemokines, or as a cell membrane molecule. Recent evidence has implicated this chemokine and its cognate receptor CX3CR1 in cancer. Tumors of neural origin (glioma, neuroblastoma) express CX3CR1 which is involved in the adhesion, transendothelial migration and mobilization of tumor cells. In addition, tumors of

non-neural origin, like prostate, pancreas and breast carcinoma express high levels of the CX3CR1 receptor. CX3CR1 expression is associated with increased migration and site-specific dissemination. Moreover, fractalkine activates the PI3K/Akt survival pathway in cancer cells (Jamieson-Gladney et al., 2011; Marchesi et al., 2010). These findings seem to indicate that the observed anti-metastatic effects of PJ or L+E+P on breast cancer cells are in part mediated through reducing the production of cancer-related pro-inflammatory cytokines and chemokines.

Interestingly, the effects of PJ or L+E+P on breast cancer cell lines are in sharp contrast to the observation on the non-neoplastic control cells, MCF10A, where the PJ or its components seem to have no significant effect. *This raises the exciting possibility of a window of therapeutic opportunity for preferentially eliminating breast cancer cells with minimal damage to the surrounding normal mammary tissue.*

All together, our findings show, for the first time, that PJ in general and L+E+P in particular interfere with multiple biological processes involved in metastasis of breast cancer cells such as suppression of cell growth, increase in cell adhesion, inhibition of cell migration and inhibition of chemotaxis towards proteins that are important in breast cancer metastasis. Our findings presented here, when coupled with the similar results on prostate cancer in our laboratory and elsewhere, strongly suggest these results will be applicable to other cancers.

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Figure 6.1. Pomegranate juice and the combination of Luteolin, Ellagic Acid and Punicic Acid (L+E+P) inhibits growth and stimulates adhesion in breast cancer cells. (**A, C**) MDA-MB-231 (ER^-) and (**B, D**) MCF7 (ER^+) breast cancer cells were treated with 1% or 5% PJ or L+E+P at 1, 2, 4 or 8 µg/ml each. (**A, B**) For the growth curve, cells were counted at the indicated times after initiation of treatment. Control represents no treatment. 24hrs after plating, the media was changed and the appropriate concentration of PJ or its components was added daily thereafter. Repeated 3 times. (**C, D**) To test the adhesion to the substrate we recorded the time it took to remove all of the cells from the dish by trypsinization at 24 and 48hrs after initiation of treatment. Control represents no treatment. Repeated 3 times. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

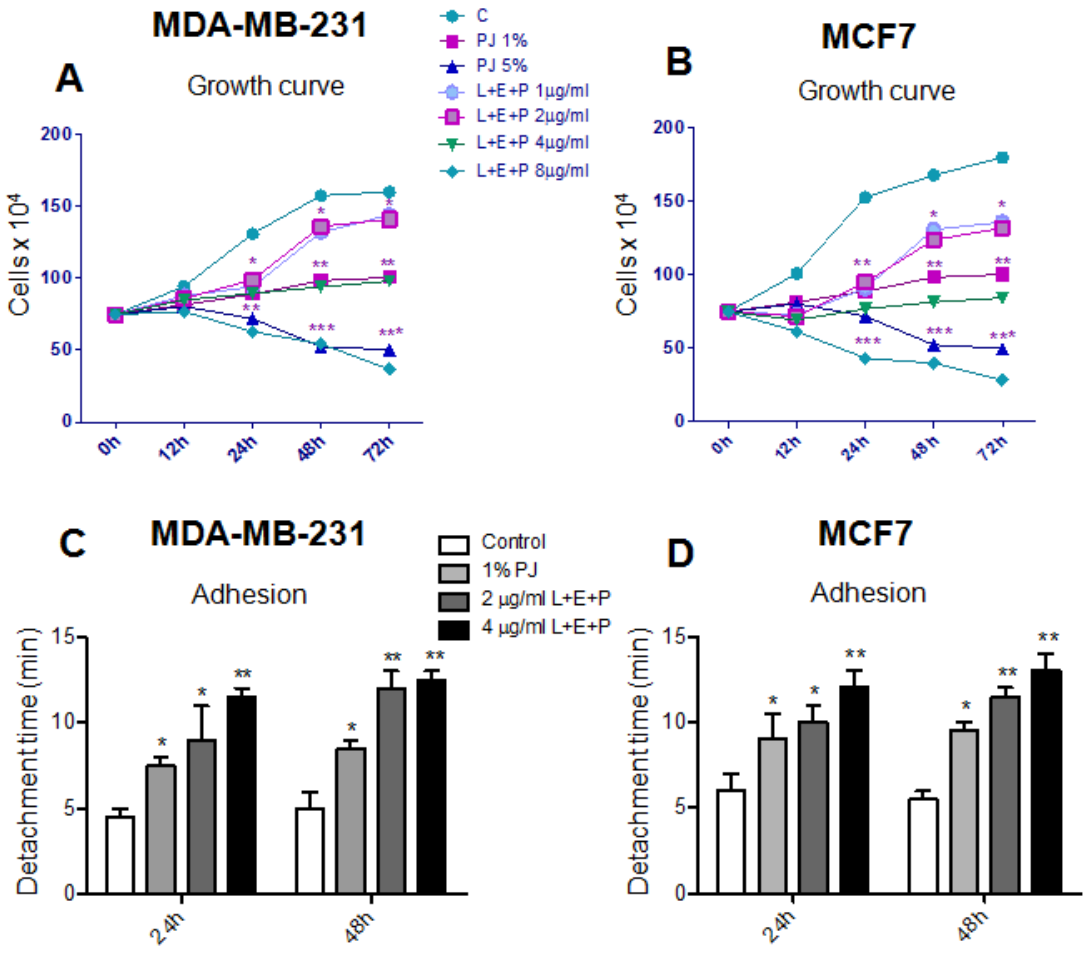
MDA-MB-231
A
Growth curve
C
PJ 1%
PJ 5%
L+E+P 1µg/ml
L+E+P 2µg/ml
L+E+P 4µg/ml
L+E+P 8µg/ml
Cells x 10⁴
0h
12h
24h
48h
72h
MCF7
B
Growth curve
C
MDA-MB-231
Adhesion
Control
1% PJ
2 µg/ml L+E+P
4 µg/ml L+E+P
Detachment time (min)
24h
48h
D
MCF7
Adhesion

Figure 6.1

Figure 6.2. Pomegranate juice and the combination of Luteolin, Ellagic Acid and Punicic Acid (L+E+P) inhibits breast cancer cell migration and chemotaxis to SDF1α. (**A, C**) MDA-MB-231 and (**B, D**) MCF7 breast cancer cells were treated with 1% PJ or L+E+P at 2 or 4μg/ml each for 72 hrs. For the migration assay (**A, B**) the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. Control represents no treatment. Media with and without treatment was changed daily. Repeated 3 times. (**C, D**) For the chemotaxis assays MDA-MB-231 and MCF7 cancer cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 hrs and then treated with 1% PJ or L+E+P at 2 or 4μg/ml each for 12hrs. At this time, 100ng/ml of SDF1α was introduced into the lower chamber and the cells found on the bottom of the filter counted 3.5 hrs later. Control had no treatment. Bars represent standard error of the mean. Repeated 2 times. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

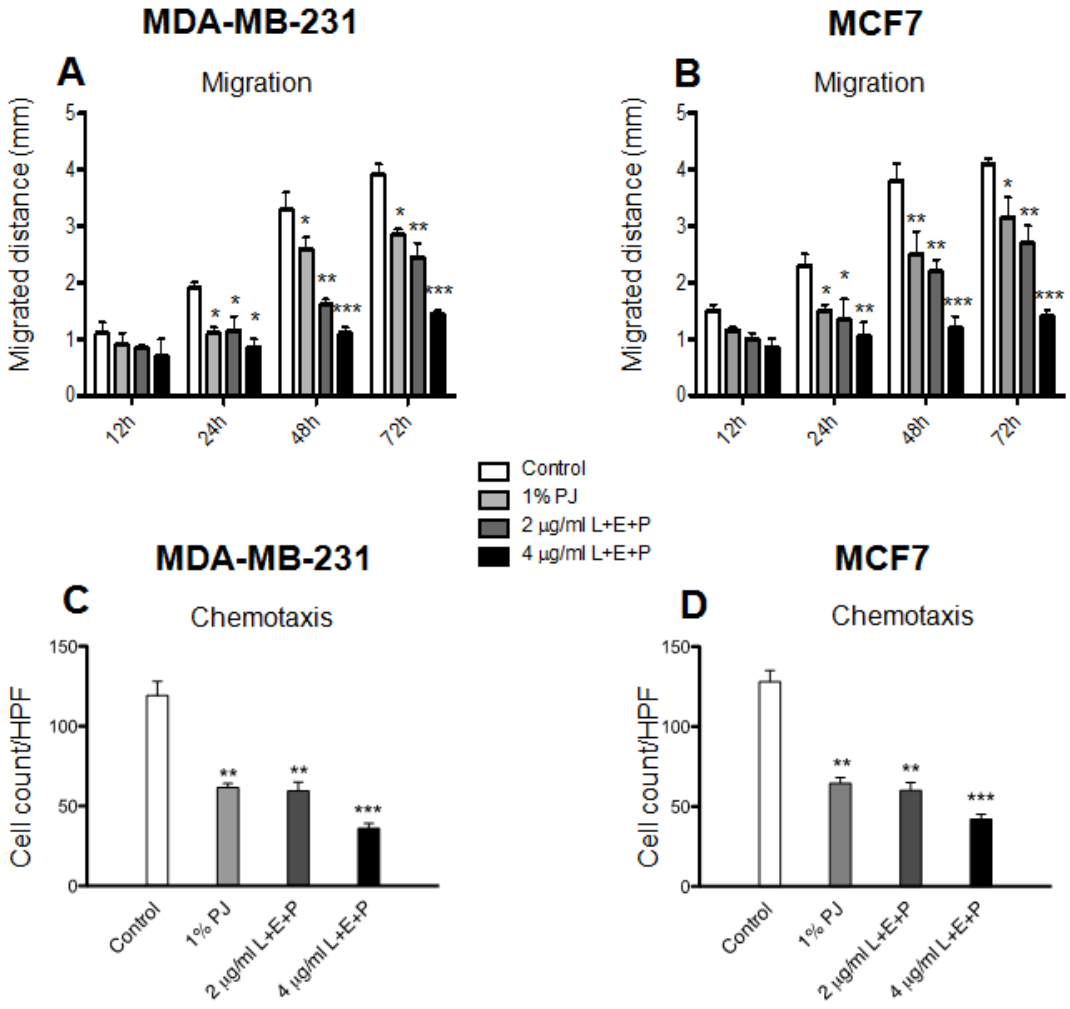
MDA-MB-231
A
Migration
Migrated distance (mm)
12h
24h
48h
72h
MCF7
B
Migration
Migrated distance (mm)
Control
1% PJ
2 µg/ml L+E+P
4 µg/ml L+E+P
MDA-MB-231
C
Chemotaxis
Cell count/HPF
Control
1% PJ
2 µg/ml L+E+P
4 µg/ml L+E+P
MCF7
D
Chemotaxis
Cell count/HPF

Figure 6.2

Figure 6.3. Pomegranate juice and the combination of Luteolin, Ellagic Acid and Punicic Acid (L+E+P) has no effect on the growth, adhesion, migration or chemotaxis to SDF1α of non-neoplastic mammary epithelial cells. (**A**) MCF10A cells were treated with 1% PJ or L+E+P at 2 and 4 µg/ml each and then counted at the indicated times after initiation of treatment. Control represents no treatment. 24hrs after plating the media was changed and the appropriate concentration of PJ or L+E+P were added. Media, with and without treatment, was changed daily. Repeated 3 times. (**B**) MCF10A cells were plated on gelatin-coated dishes and 24 hrs later media was changed and the cells treated. We tested for adhesion to the substrate at 24 and 48hrs after initiation of treatment by recording the time it took for trypsinization to remove all of the cells from the dish. Control represents no treatment. Repeated 3 times. (**C**) MCF10A cells were treated with 1% PJ or the combination of L+E+P at 2 or 4µg/ml each for 72 hrs and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. Control represents no treatment. Media with and without treatment was changed daily. Repeated 3 times. (**D**) MCF10A cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 hrs and then treated with 1% PJ or L+E+P at 2 or 4µg/ml each for 12hrs. At this time, 100ng/ml of SDF1α was introduced into the lower chamber and the cells found on the bottom of the filter counted 3.5 hrs later. Control had no treatment. Bars represent standard error of mean. Repeated 2 times. *$p < 0.05$.

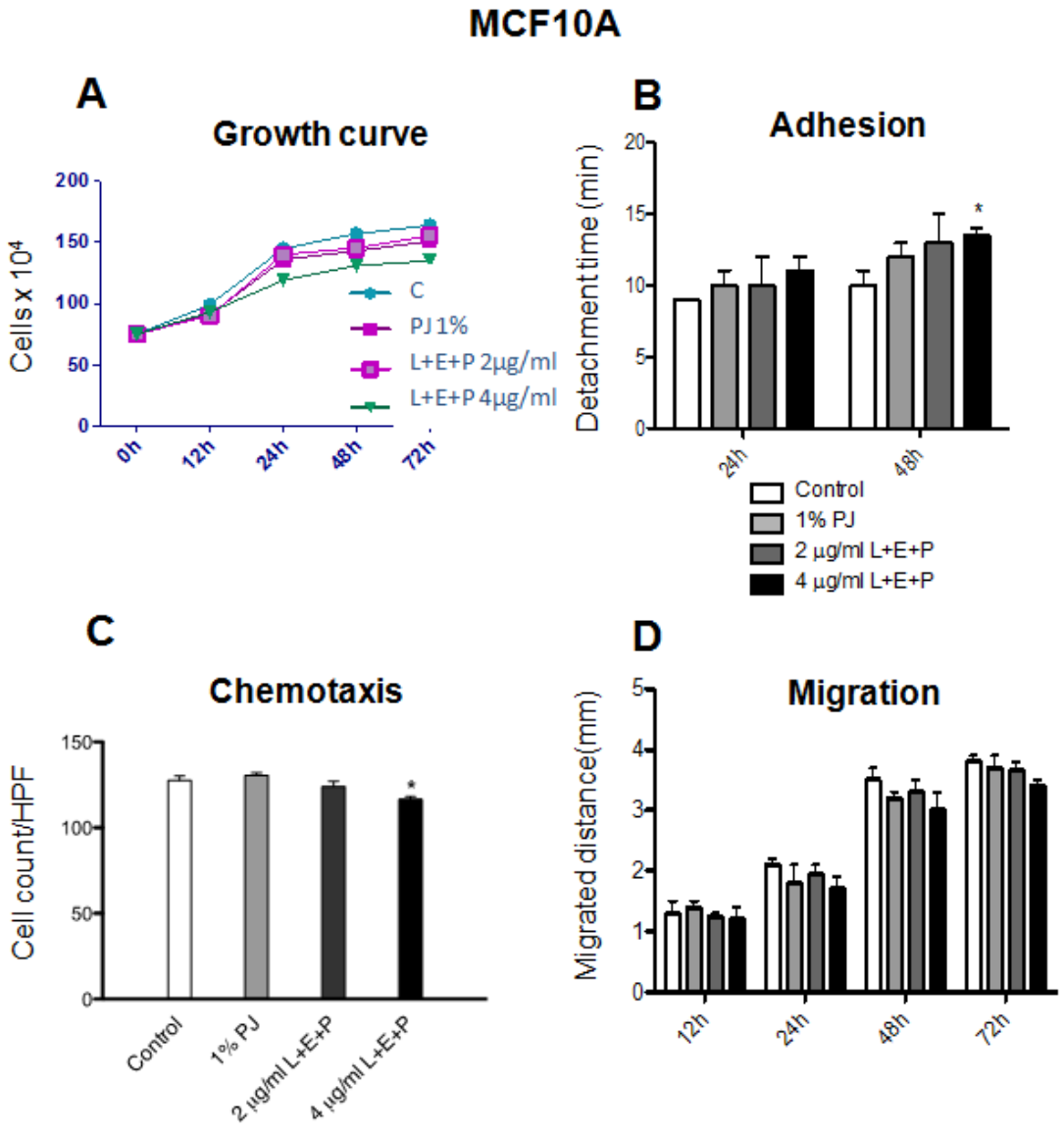
MCF10A
A
Growth curve
Cells x 10⁴
200
150
100
50
0
0h
12h
24h
48h
72h
C
PJ 1%
L+E+P 2µg/ml
L+E+P 4µg/ml
B
Adhesion
Detachment time (min)
20
15
10
5
0
24h
48h
*
Control
1% PJ
2 µg/ml L+E+P
4 µg/ml L+E+P
C
Chemotaxis
Cell count/HPF
150
100
50
0
*
Control
1% PJ
2 µg/ml L+E+P
4 µg/ml L+E+P
D
Migration
Migrated distance(mm)
5
4
3
2
1
0
12h
24h
48h
72h

Figure 6.3

Figure 6.4. The effect of Pomegranate juice and the combination of Luteolin, Ellagic Acid and Punicic Acid (L+E+P) on breast cancer cell migration is mediated through HMMR and TWIST. MDA-MB-231 and MCF7 breast cancer cells were transfected with 1µg/ml of pcDNA3.1 HMMR vector (**A, B**) or pcDNA4.1 TWIST vector (**C, D**) and 24hrs later were scratch wounded and treated with 1% PJ or L+E+P at 4µg/ml. The distance migrated by the cells from the wounded edge to the leading edge was measured at 36 hrs time point. Controls represent no treatment with PJ or its components. Bars represent standard error of the mean. Repeated 2 times. **$p < 0.01$; *$p < 0.05$.

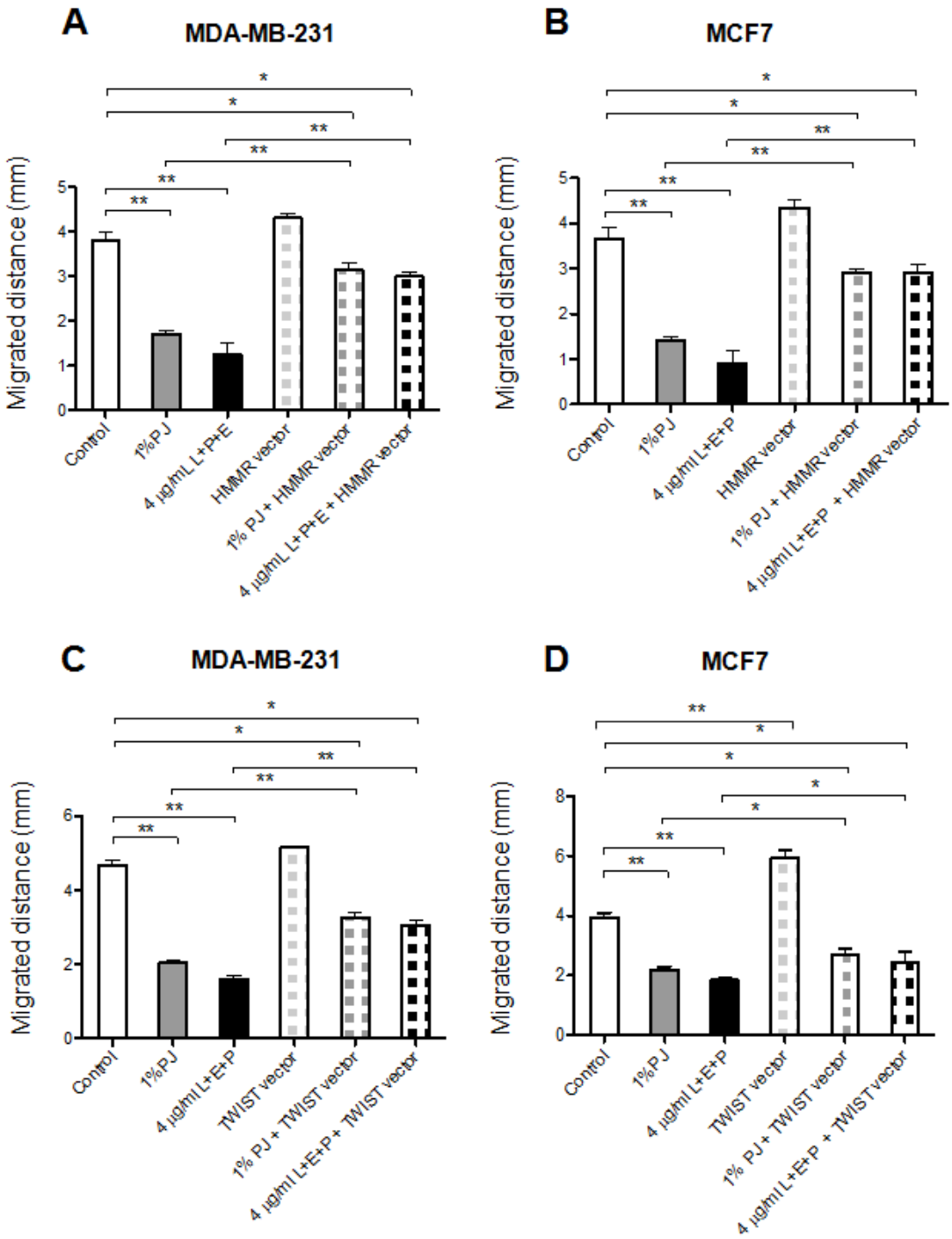
A
MDA-MB-231
Migrated distance (mm)
Control
1%PJ
4 µg/ml L+P+E
HMMR vector
1% PJ + HMMR vector
4 µg/ml L+P+E + HMMR vector
B
MCF7
Migrated distance (mm)
Control
1%PJ
4 µg/ml L+E+P
HMMR vector
1% PJ + HMMR vector
4 µg/ml L+E+P + HMMR vector
C
MDA-MB-231
Migrated distance (mm)
Control
1%PJ
4 µg/ml L+E+P
TWIST vector
1% PJ + TWIST vector
4 µg/ml L+E+P + TWIST vector
D
MCF7
Migrated distance (mm)
Control
1%PJ
4 µg/ml L+E+P
TWIST vector
1% PJ + TWIST vector
4 µg/ml L+E+P + TWIST vector

Figure 6.4

Figure 6.5. The effect of Pomegranate juice and the combination of Luteolin, Ellagic Acid and Punicic Acid (L+E+P) on breast cancer cell migration is mediated through E-Cadherin. (**A**) Immunoblot analysis for E-cadherin with protein extracts from MDA-MB-231 and MCF7 cells treated with 1% PJ or L+E+P at 4μg/ml. Repeated 2 times. **(B, C)** MDA-MB-231 and MCF7 breast cancer cells were transfected with 40nM E-cadherin siRNA. 24hrs after transfection, cells were treated with 1%PJ or L+E+P at 4μg/ml. The distance migrated by the cells from the wounded edge to the leading edge was measured at 36 hrs time point. Control represents no treatment with PJ or its components. Bars represent standard error of the mean. Repeated 2 times. $^{**}p < 0.01$; $^{*}p < 0.05$.

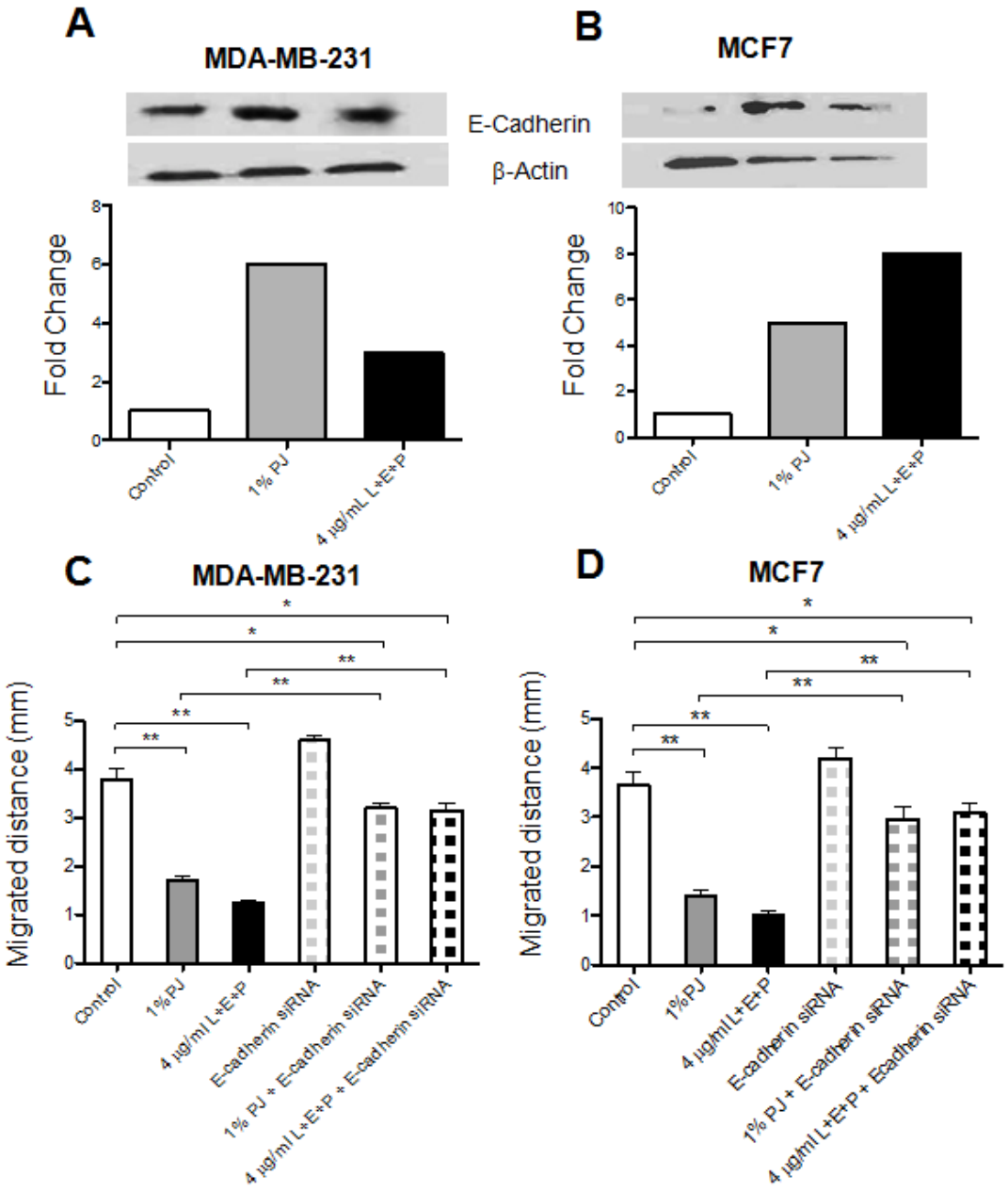
A
MDA-MB-231
E-Cadherin
β-Actin
Fold Change
Control
1% PJ
4 µg/mL L+E+P
B
MCF7
Fold Change
Control
1% PJ
4 µg/mL L+E+P
C
MDA-MB-231
Migrated distance (mm)
Control
1% PJ
4 µg/ml L+E+P
E-cadherin siRNA
1% PJ + E-cadherin siRNA
4 µg/ml L+E+P + E-cadherin siRNA
D
MCF7
Migrated distance (mm)
Control
1% PJ
4 µg/ml L+E+P
E-cadherin siRNA
1% PJ + E-cadherin siRNA
4 µg/ml L+E+P + Ecadherin siRNA

Figure 6.5

Figure 6.6 The effect of Pomegranate juice and the combination of Luteolin, Ellagic Acid and Punicic Acid (L+E+P) on the levels of selected proinflammatory cytokines and chemokines. Media collected from MCF7 and MDA-MB-231 and MCF10 cells treated with 1% PJ or L+E+P at 4µg/ml for 18h were analyzed using Luminex Multiplex Array assays. Both MCF7 and MDA-MB-231 significantly decreased IL-8 (A) and Rantes (B) and PDGFB (C) whereas only MDA-MB-231 inhibited production of Fractalkine (D). Production of the analyzed cytokines or chemokines by the non-neoplastic MCF10A cells was not affected by either treatment. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

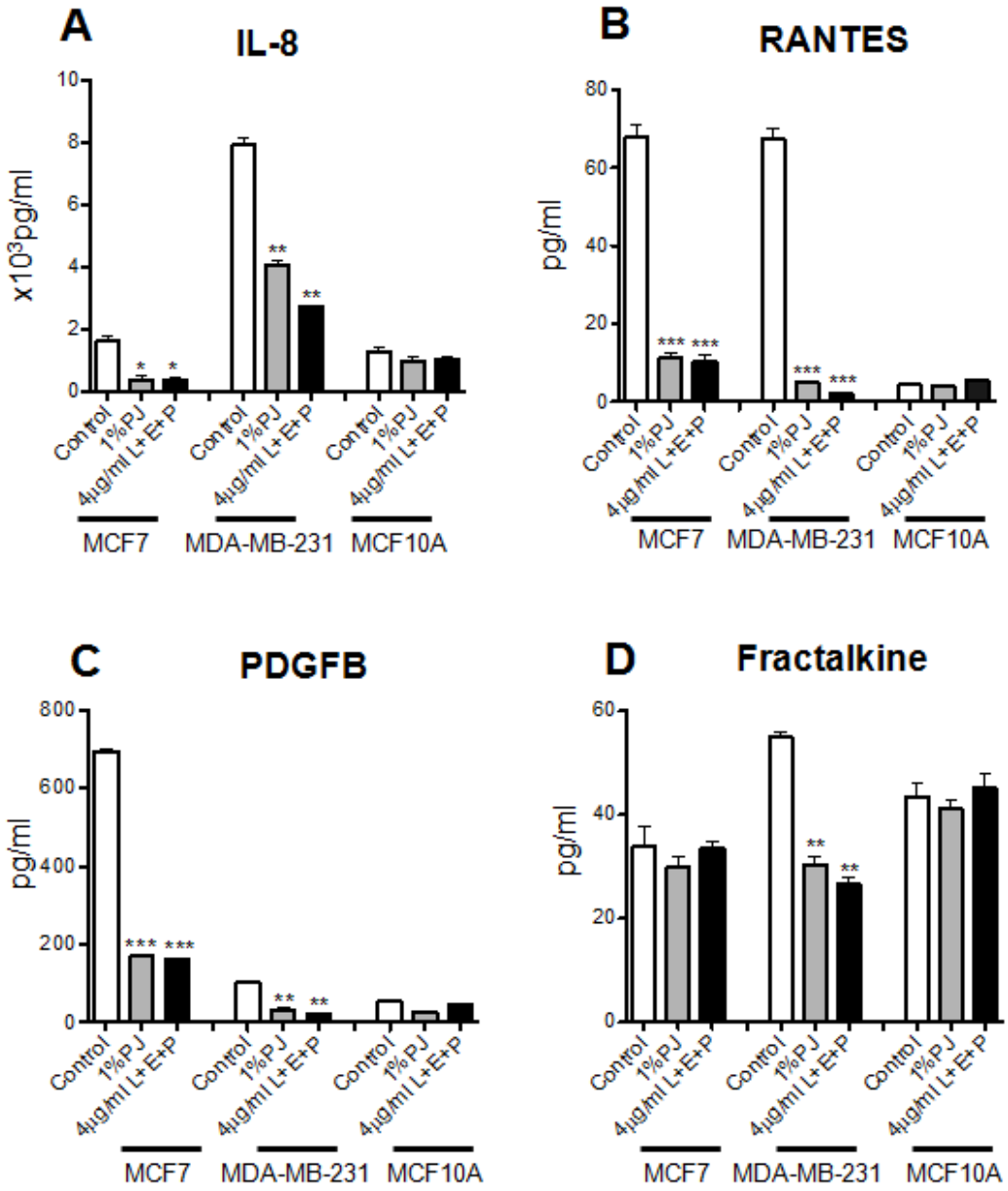
A
IL-8
x10³pg/ml
10
8
6
4
2
0
*
*
**
**
Control
1%PJ
4µg/ml L+E+P
MCF7
MDA-MB-231
MCF10A
B
RANTES
pg/ml
80
60
40
20
0
***
***
***
***
C
PDGFB
pg/ml
800
600
400
200
0
***
***
**
**
D
Fractalkine
pg/ml
60
40
20
0
**
**

Figure 6.6

Table 6.1. Pomegranate juice and the combination of Luteolin, Ellagic Acid and Punicic Acid (L+E+P) alter the gene expression profile of genes involved in cell growth, adhesion and migration. RNA was extracted from MDA-MB-231 and MCF7 cells that had been treated with 1%PJ or 4μg/ml L+E+P for 24 hrs and RT-qPCR was performed as described in Materials and Methods. Relative levels are presented as fold change compared with untreated controls. qPCR was repeated twice for each gene. Repeated 2 times.

Effects of PJ and L+E+P on gene expression of breast cancer cells

Gene Name	Product	Fold Change - MDA Cells with 1%PJ	Fold Change - MDA Cells with L+E+P	Fold Change - MCF7 cells with 1%PJ	Fold Change - MCF7 cells with L+E+P	Function
ICAM	Intercellular adhesion molecule 1	2.8	3.1	3.1	3.3	Adhesion
MARCKS	Myristolytated alanine-rich protein kinase C substrate	1.8	2.1	2.2	2.5	Adhesion
CLDN1	Claudin 1	1.5	1.9	1.8	2.1	Adhesion
CHN1	N-chimearin	-2.1	-2.9	-2.0	-3.1	Cytoskeleton morphology
CCNE2	Cyclin E2	-38	-39.8	-26.4	-26.1	Cell cycle control
PRCKE	Protein kinase C epsilon	-2.3	-2.9	-1.8	-2.5	Cell cycle control
ANLN	Anillin	-2.2	-2.7	-1.9	-2.8	Migration
HMMR	Hyaluronan-mediated motility receptor	-3.2	-3.8	-2.9	-3.3	Migration
NEXN	Nexilin	-2.7	-3.1	-3.2	-3.4	Migration
COL1A1	Collagen 1	-2.2	-2.7	-2.3	-3.1	Migration
TWIST	Twist	-21.4	-22.4	-14.9	-14.5	EMT
CXCR4	Chemokine receptor type 4	-2.6	-3.4	-2.2	-3.3	Metastasis

Table 6.1

CONCLUSION

To date, there is no cure for prostate cancer when recurrence occurs after surgery and/or radiation. In particular, when it recurs after hormone ablation therapy there are no other effective treatments for deterrence of cancer progression. I have provided an overview of the myriad ways pomegranate juice appears to combat prostate cancer and perhaps other cancers, putting into perspective the job ahead. Because pomegranate juice interferes with multiple biological processes of cancer cells, stimulation of cell death as shown by ourselves and others, as well as increase in cell adhesion, decrease in cell migration and suppression of pro-inflammatory cytokines and chemokines shown here, pomegranate juice holds the promise of preventing or at least slowing down metastasis of prostate cancer.

I then showed that luteolin, ellagic acid and punicic acid, components of pomegranate juice, can potentially be used as anti-metastatic treatments to deter prostate cancer metastasis. L+E+P interfere with multiple biological processes involved in metastasis of cancer cells such as suppression of cell growth, increase in cell adhesion, inhibition of in cell migration and inhibition of chemotaxis towards proteins that are important in prostate cancer metastasis.

More importantly, I tested the effect of L+E+P on prostate cancer metastasis in mouse tumor models. L+E+P can be used in combination to prevent prostate cancer growth and metastasis and because these are natural products they could be used in humans in the very near future. Furthermore, it may be possible to develop them

into novel drugs that can be made more effective than the natural products in preventing cancer progression.

The findings on breast cancer cells are also promising. I showed that pomegranate juice in general and L+E+P in particular interfere with multiple biological processes involved in metastasis of breast cancer cells such as suppression of cell growth, increase in cell adhesion, inhibition of cell migration and inhibition of chemotaxis towards proteins that are important in breast cancer metastasis. THE findings coupled with the similar results on prostate cancer in our laboratory and elsewhere, strongly suggest these results will be applicable to other cancers.

Pomegranate is an ancient fruit with an illustrious medical history. The biological activity of pomegranate juice and pomegranate derived natural products, especially the chemotherapeutic and chemopreventive properties, has being investigated in cell, animal and clinical studies. Pomegranate juice has shown an initial promise in recent clinical trials against prostate cancer but clinical studies against breast cancer are still sorely lacking. At this stage it is important that the *in vitro* findings in breast cancer cell lines be corroborated through clinical trials. Therefore, further studies are warranted, including clinical trials using well-characterized and standardized amounts of pomegranate juice and specific components as primary or adjuvant therapy. It is anticipated that in-depth research into the anticancer activities of pomegranate derived natural products would enable one day to develop a cocktail of such molecules for

effective prevention cancers. Therefore, pomegranate and its components can be used to prevent development and progression of prostate and breast cancer.