Breast Cancer Res Treat (2012) 136:647–658
DOI 10.1007/s10549-012-2264-5

PRECLINICAL STUDY

Pomegranate juice and specific components inhibit cell and molecular processes critical for metastasis of breast cancer

Ana Rocha · Lei Wang · Manuel Penichet · Manuela Martins-Green

Received: 14 August 2012 / Accepted: 18 September 2012 / Published online: 12 October 2012

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 for breast cancer prevention and treatment. To test this possibility, we have used two 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 inhibiting growth of the breast cancer cells, PJ or a combination of its components luteolin (L) + ellagic acid (E) + 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 SDF1α, 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 SDF1α. 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 indicate that they are potentially a very effective treatment to prevent cancer progression in general.

Keywords Chemotaxis · Adhesion · Migration · Gene expression · Cytokines

A. Rocha · L. Wang · M. Martins-Green (✉)
Department of Cell Biology and Neuroscience, University of California Riverside, BSB Room 2217, 900 University Avenue, Riverside, CA 92521, USA
e-mail: manuela.martins@ucr.edu

M. Penichet
Department of Surgery, Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA 90095, USA

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 [1]. Each year there are more than 10,000 new cases and more than 40,000 women die from this disease [2, 3]. 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 ER 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 [4].

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 [5–10].

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 [8]. The most recent studies involving Pomegranate juice (PJ) or its components focus primarily on its anti-proliferative and apoptotic effects on breast cancer [9, 10]. 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 [9–11]. In their study, Wang et al. [12] 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. Another recent study with luteolin suggests that it induces a caspase-dependent and caspase-independent apoptosis involving AIF nuclear translocation mediated by activation of ERK and p38 in breast cancer cells [13]. It also has been shown [14] that pomegranate ellagitannin-derived compounds are anti-proliferative and inhibit aromatase, an enzyme critically involved in the conversion of androgen to estrogen. 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 [15]. The work we present here goes beyond these findings by showing that PJ and its components have an affect 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 [6, 16]. 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 [17], a chemokine known to be involved in metastasis of hormone refractory prostate cancer cells to the bone [18]. 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 [19]. However, despite these very interesting results this study was very limited and showed only the results of 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 [20]. 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 ER/progesterone receptor negative (ER^-/PR^-) and is associated with an 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 37 °C with 5 % CO_2 in Dulbecco's modified eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, penicillin (100 IU 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 (20 ng/ml), cholera toxin (100 ng/ml), hydrocortisone (0,5 mg/ml), insulin (10 μg/ml), penicillin (100 IU ml/l), and streptomycin (100 mg ml/l).

Growth assay

1×10^5 cells were plated on 6-well plates (B&D Biosciences), allowed to adhere and 24 h 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 h 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 °C.

Adhesion assay

3×10^5 cells were plated on gelatin-coated 6-well plates (B&D Biosciences), allowed to adhere and 24 h later treated with 1 % PJ or L + E + P at 2 or 4 μg/ml each for 24 or 48 h. Cells were then trypsinized and the time required to detach all cells was recorded as an indicator of cell adhesiveness [17, 20].

Migration assay

Confluent cells were scratch wounded using a rubber scraper, washed and treated with 1 % PJ or L + E + P at 2 or 4 μg/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 12, 24, 48, and 72 h after treatment was initiated. Scratch wounded cells without treatment were used as controls [17, 20].

Chemotaxis assay

The upper side of polycarbonate membranes (8 μm pore size) of transwells (BD Biosciences, San Jose, CA) was coated with 50 ng/ml type I collagen (Sigma Chemical Co.). 1×10^5 cells in 100 μl of culture medium were plated on the collagen-coated transwell membranes and were allowed to adhere for 3 h. The wells were introduced into 24-well plates and 1,000 μl of supplemented medium was added to the lower chamber. Cells were treated with 1 % PJ or L + E + P at 2 or 4 μg/ml for 12 h. SDF1α (100 ng/ml) was added to the lower chamber and the cells were allowed to migrate for 4 h 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 eight 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 [17, 20].

Real-time quantitative PCR

Cells were treated with 1 % PJ or the combination of 4 μg/ml each of luteolin, punicic acid, and ellagic acid for 24 h 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 1× 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). 1 μg RNA was reverse-transcribed to cDNA by RETROscript reverse transcription kit (Ambion, Grand Island, NY) at 44 °C for 1 h and 92 °C for 10 min. 2 μl of cDNA from the reverse transcription reaction were added to 23 μl real-time quantitative PCR (RT-qPCR) mixture containing 12.5 μl 2× SYBR Green SuperMix (Bio-Rad, Hercules, CA) and 200 nM 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 s, annealing at 60 °C for 30 s. 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	NAGCTCACTCTTCTCACCAATGCCA	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. 1 μg/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, b). 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 [17, 20]. Both cell lines showed similar results. Although the effects on cell growth were not significantly different at 12 h with any of the treatments, by 24 h differences could be seen. Both 5 % PJ or L + E + P at 8 μg/ml each were inhibitory of growth of the cells by 24 h and by 48 h 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; treatment with L + E + P at 1 or 2 μg/ml each, growth was only slightly diminished (Fig. 1a, b). 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 h of treatment, the cancer cells require up to three times longer to be released by trypsinization and by 48 h this time is further increased (Fig. 1c, d). 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 12 h after treatment (Fig. 2a, b). This pattern of delayed migration continued over time.

SDF1α is known for its ability to attract breast cancer cells to the bone marrow. 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 h prior to initiation of the chemotaxis assay significantly inhibited chemotaxis of the breast cancer cells towards SDF1α (Fig. 2c, d).

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, d), and the effects on adhesion and chemotaxis were minimal and only observed with the high dose of L + E + P (Fig. 3b, c).

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*) [21], claudin 1 (*CLDN1*) [22], and myristoylated alanine-rich protein kinase C substrate (*MARCKS*) [23, 24]; (ii) migration, such as hyaluranan-mediated motility receptor/CD168 (*HMMR*) [25–27], collagen type I

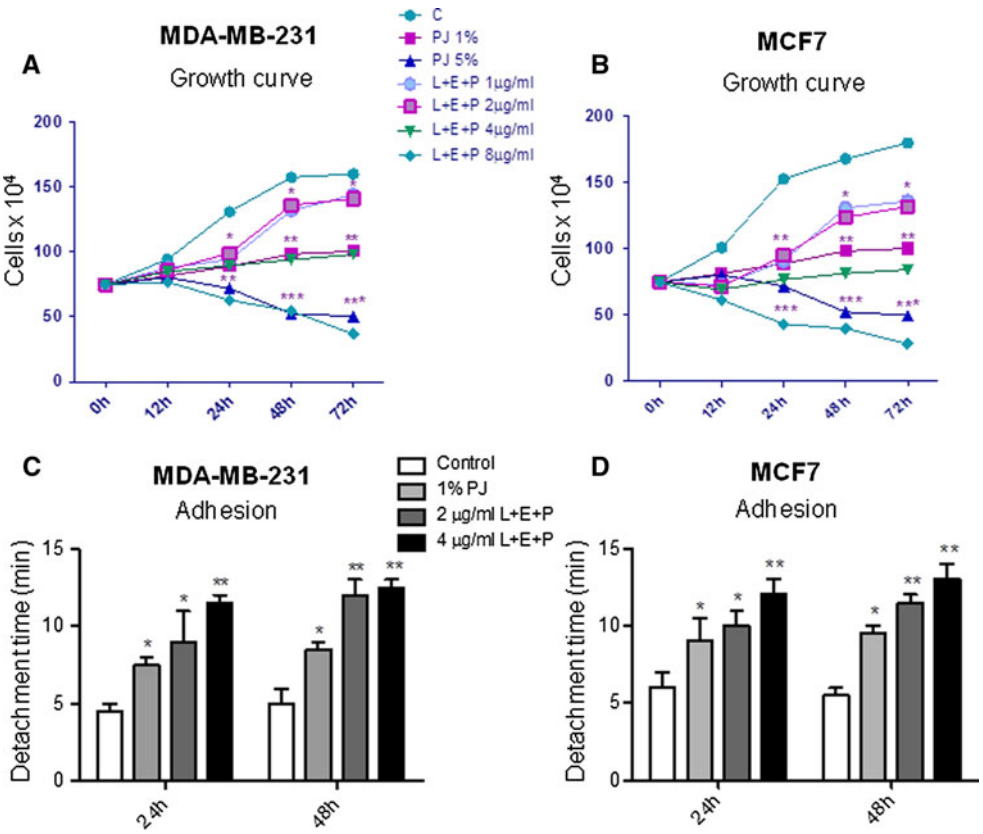

Fig. 1 PJ and the combination of luteolin, ellagic acid, and punicic acid (L + E + P) inhibits growth and stimulates adhesion in breast cancer cells. **a**, **c** MDA-MB-231 (ER^-) and **b**, **d** MCF7 (ER^+) breast cancer cells were treated with 1 or 5 % PJ or L + E + P at 1, 2, 4, or 8 µg/ml each. **a**, **b** For the growth curve, cells were counted at the indicated times after initiation of treatment. Control represents no treatment. 24 h after plating, the media was changed and the appropriate concentration of PJ or its components was added daily thereafter. Repeated three 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 48 h after initiation of treatment. Control represents no treatment. Repeated three times. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$

alpha1 (*COL1A1*) [28], anillin (*ANLN*) [29], and nexilin (*NEXN*) [30]; (iii) cell cycle control, such as N-chimearin (*CHN1*) [31], cyclin E2 (CCNE2) [32], and protein kinase C epsilon (*PRCKE*) [33]; (iv) chemotaxis of the cancer cells to SDF1α through its receptor type 4 (CXCR4) [34]; (v) epithelial-to-mesenchymal transformation, such as twist (TWIST) [35]. 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 4 µg/ml each for 24 h (Table 1). 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 overexpression partially reversed the inhibitory effect of PJ on cell migration (Fig. 4). Scratch wounds were made 24 h after HMMR or TWIST vector transfection and the migrated distances were measured 36 h 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 overexpressing 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, b), 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 [36, 37]. Previously, we have found that in prostate cancer cells PJ and the three components inhibit the repressor for the E-cadherin gene, allowing for elevation of the expression of E-cadherin [20]. 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, b). To determine whether the increase in adhesion stimulated by PJ or L + E + P can be reversed, scratch wounds were made 24 h after E-cadherin siRNA transfection. Distances migrated by the cells were measured 36 h 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

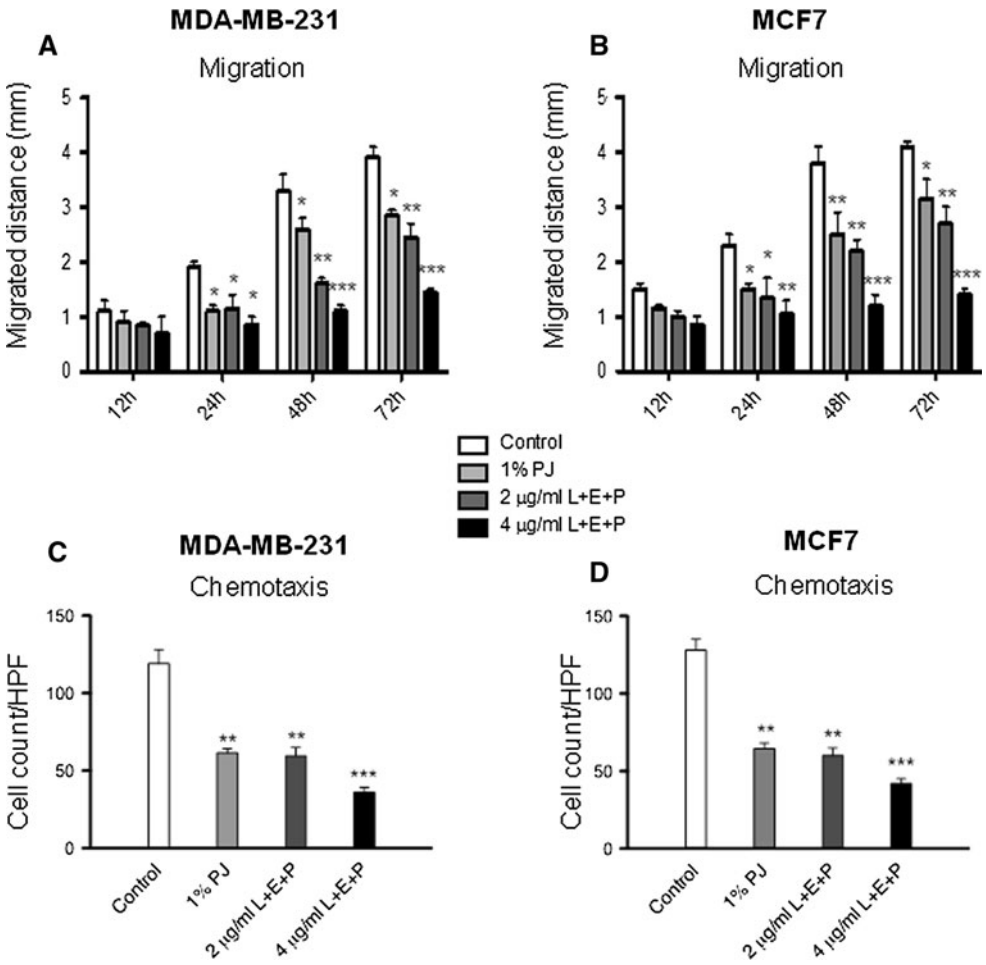

Fig. 2 PJ and the combination of luteolin, ellagic acid, and punicic acid (L + E + P) inhibits breast cancer cell migration and chemotaxis to SDF1α. **a**, **c** MDA-MB-231 and **b**, **d** MCF7 breast cancer cells were treated with 1 % PJ or L + E + P at 2 or 4 μg/ml each for 72 h. **a**, **b** For the migration assay, 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 three 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 h and then treated with 1 % PJ or L + E + P at 2 or 4 μg/ml each for 12 h. At this time, 100 ng/ml of SDF1α was introduced into the lower chamber and the cells found on the bottom of the filter counted 3.5 h later. Control had no treatment. *Bars* represent standard error of the mean. Repeated two times. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$

were treated with PJ or L + E + P (Fig. 5c, d). 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 18 h. 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. 6a–c). However, both cancer cell lines were strongly affected in their expression of IL-8, RANTES, and PDGFB (Fig. 6a–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 epithelial-to-mesenchymal transition; (5) reduces the level of pro-inflammatory cytokines/chemokines while increasing the

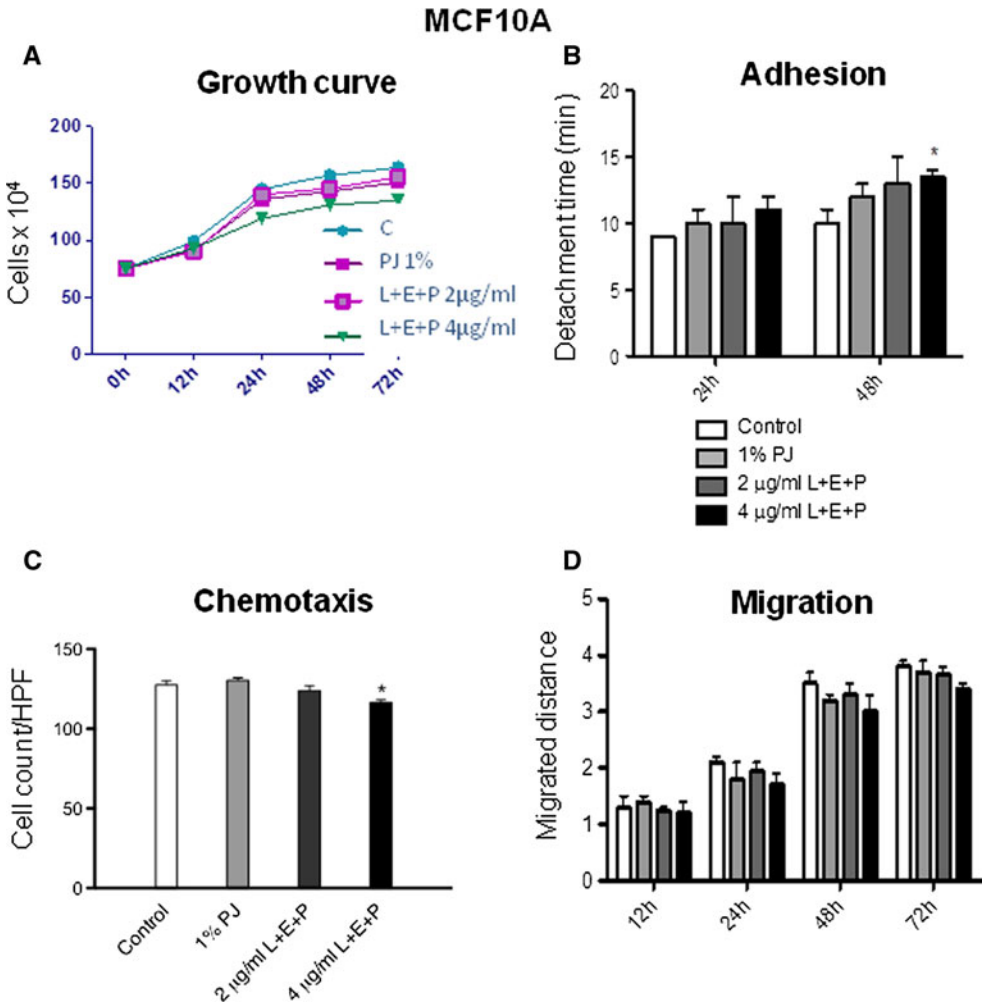

Fig. 3 PJ and the combination of luteolin, ellagic acid, and punicic acid (L + E + P) have no effect on the growth, adhesion, migration, or chemotaxis to SDF1α of non-neoplastic mammary epithelial cells. **a** MCF10A cells were treated with 1 % PJ or L + E + P at 2 and 4 μg/ml each and then counted at the indicated times after initiation of treatment. Control represents no treatment. 24 h after plating, the media was changed and the appropriate concentration of PJ or L + E + P was added. Media, with and without treatment, was changed daily. Repeated three times. **b** MCF10A cells were plated on gelatin-coated dishes and 24 h later media was changed and the cells treated. We tested for adhesion to the substrate at 24 and 48 h 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 three times. **c** MCF10A cells were treated with 1 % PJ or the combination of L + E + P at 2 or 4 μg/ml each for 72 h 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 three times. **d** MCF10A cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 h and then treated with 1 % PJ or L + E + P at 2 or 4 μg/ml each for 12 h. At this time, 100 ng/ml of SDF1α was introduced into the lower chamber and the cells found on the bottom of the filter counted 3.5 h later. Control had no treatment. *Bars* represent standard error of mean. Repeated two times. * $p < 0.05$

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 two 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

Table 1 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	Myristoylated alanine-rich protein kinase C substrate	1.8	2.1	2.2	2.5	Adhesion
CLDNl	Claudin 1	1.5	1.9	1.8	2.1	Adhesion
CHNI	*N*-Chirnearin	−2.1	−2.9	−2.0	−3.1	Cyto skeleton 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
NBXN	Nexilin	−2.7	−3.1	−3.2	−3.4	Migration
COLLAl	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

PJ 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 4 μg/ml L + E + P for 24 h 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 two times

process that involves loss of adhesion and rearrangement of cytoskeletal elements that allow the cells to migrate and invade [38–40]. 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 [21, 23, 24]. The treatment of the breast cancer cells with PJ and L + E + P downregulates 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 [25–27]. 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 [29, 30], and *N*-chimearin, GTPase-activating protein that, when down-regulated, results in loss of filopodia and reduction of migration [31]. 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 EMT [35].

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 [36, 37], 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 [41, 42] are inhibited by PJ or L + E + P treatment. Among the pro-inflammatory cytokines/chemokines

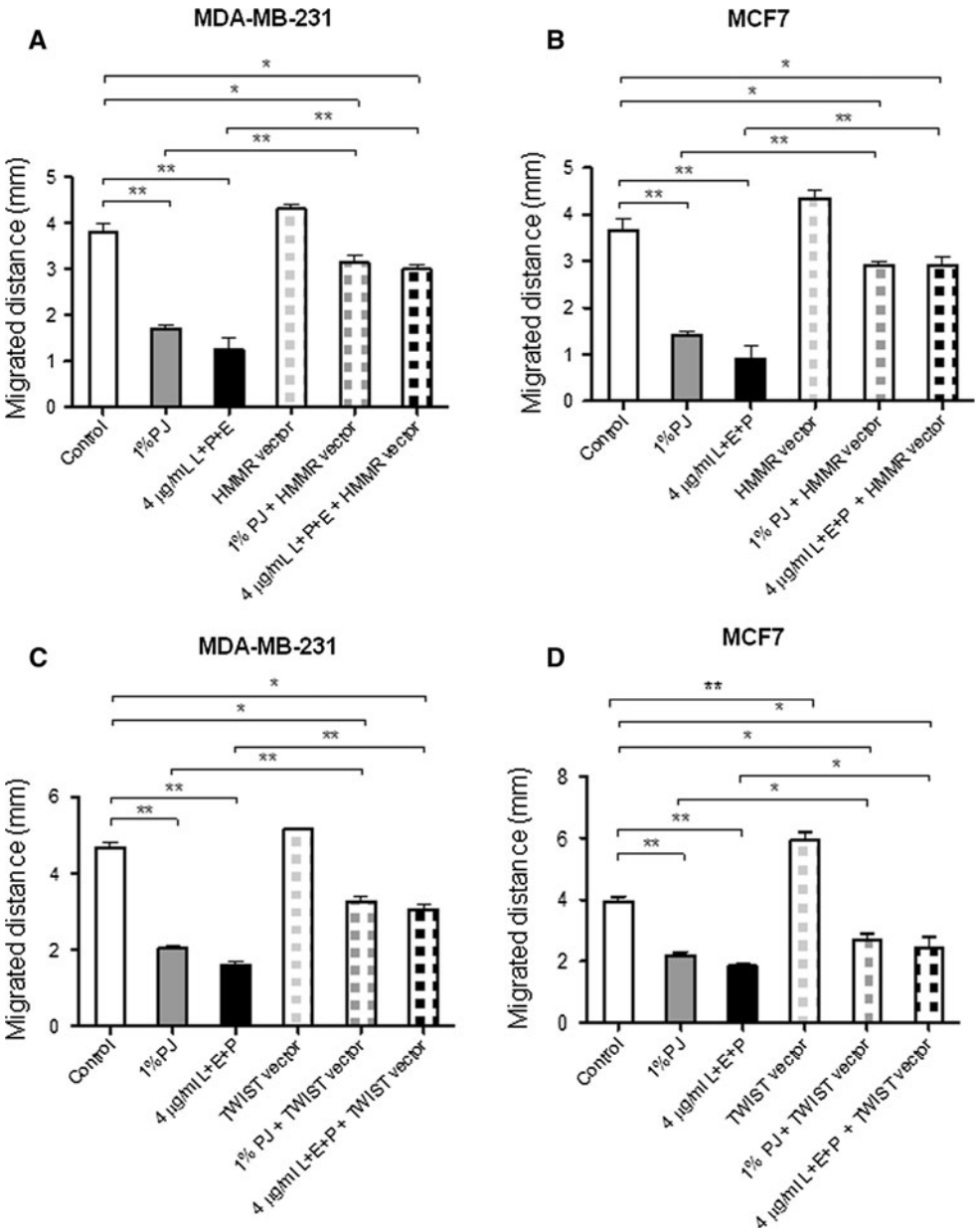

Fig. 4 The effect of PJ and the combination of luteolin, ellagic acid, and punicic acid (L + E + P) on breast cancer cell migration is mediated through HMMR and TWIST. MDA-MB-231 and MCF7 breast cancer cells were transfected with 1 μg/ml of pcDNA3.1 HMMR vector (**a**, **b**) or pcDNA4.1 TWIST vector (**c**, **d**) and 24 h later were scratch wounded and treated with 1 % PJ or L + E + P at 4 μg/ml. The distance migrated by the cells from the wounded edge to the leading edge was measured at 36 h time point. Controls represent no treatment with PJ or its components. *Bars* represent standard error of the mean. Repeated two times. ** $p < 0.01$; * $p < 0.05$ treatment with PJ or its components. *Bars* represent standard error of the mean. Repeated two times. ** $p < 0.01$; * $p < 0.05$

examined with Luminex Multiplex Array assays, the secreted levels of IL-8, RANTES, and 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 [43, 44]. 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 [43, 44]. 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 [44–46]. 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 [47]. In addition, interaction of RANTES with CCR5 on the surface of cancer cells stimulates their invasive capabilities [47]. PDGFBB is a well-known mitogenic and pro-angiogenic factor and has been shown to potentiate cancer growth and progression [48, 49]. 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,

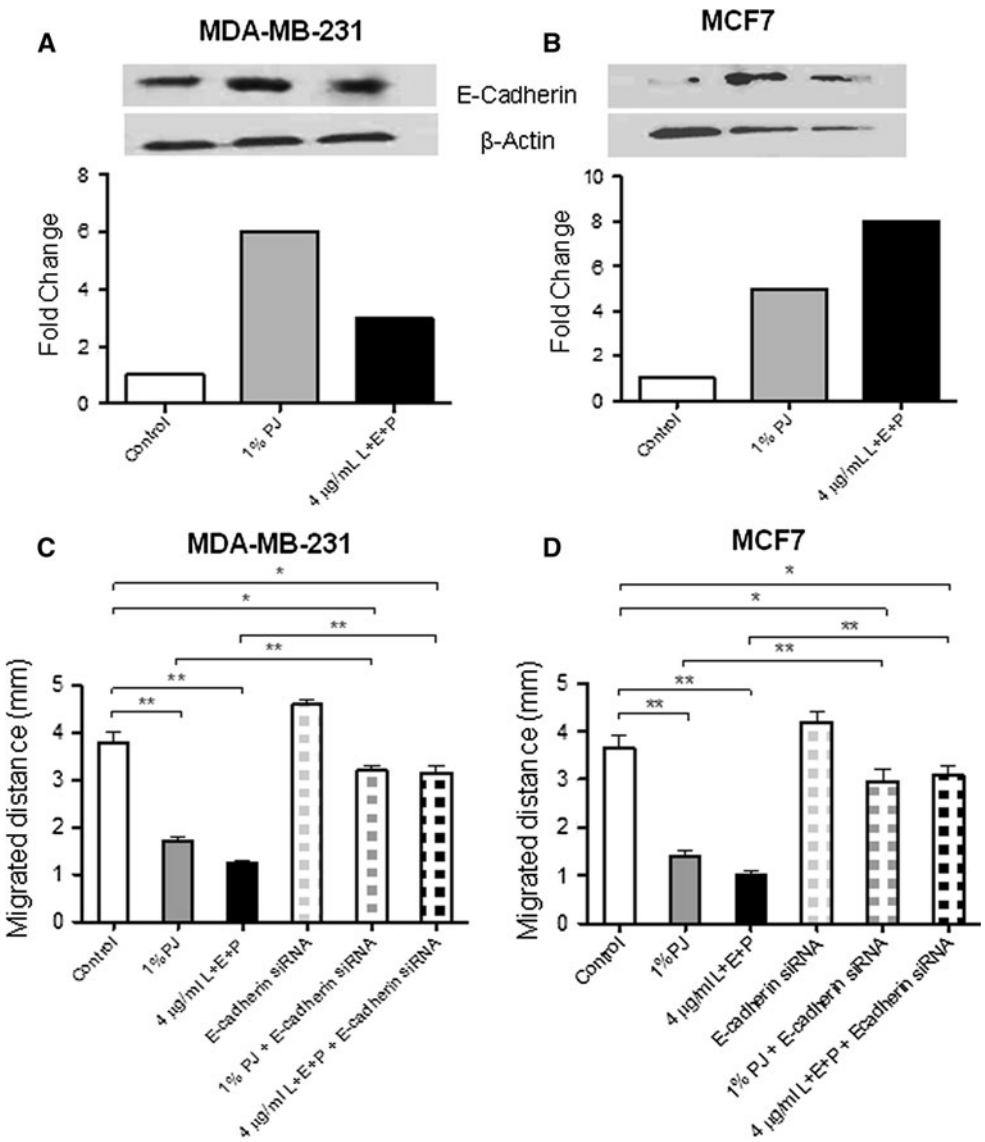

Fig. 5 The effect of PJ and the combination of luteolin, ellagic acid, and punicic acid (L + E + P) on breast cancer cell migration is mediated through E-cadherin. **a** Immunoblot analysis for E-cadherin with protein extracts from MDA-MB-231 and MCF7 cells treated with 1 % PJ or L + E + P at 4 μg/ml. Repeated two times. **b**, **c** MDA-MB-231 and MCF7 breast cancer cells were transfected with 40 nM E-cadherin siRNA. 24 h after transfection, the cells were treated with 1 % PJ or L + E + P at 4 μg/ml. The distance migrated by the cells from the wounded edge to the leading edge was measured at 36 h time point. Control represents no treatment with PJ or its components . *Bars* represent standard error of the mean. Repeated two times. ** $p < 0.01$; * $p < 0.05$

transendothelial migration and mobilization of tumor cells [50, 51]. In addition, tumors of non-neural origin, like prostate, pancreas, and breast carcinoma express high levels of the CX3CR1 receptor [50, 51]. CX3CR1 expression is associated with increased migration and site-specific dissemination. Moreover, fractalkine activates the PI3K/Akt survival pathway in cancer cells [50, 51]. 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.

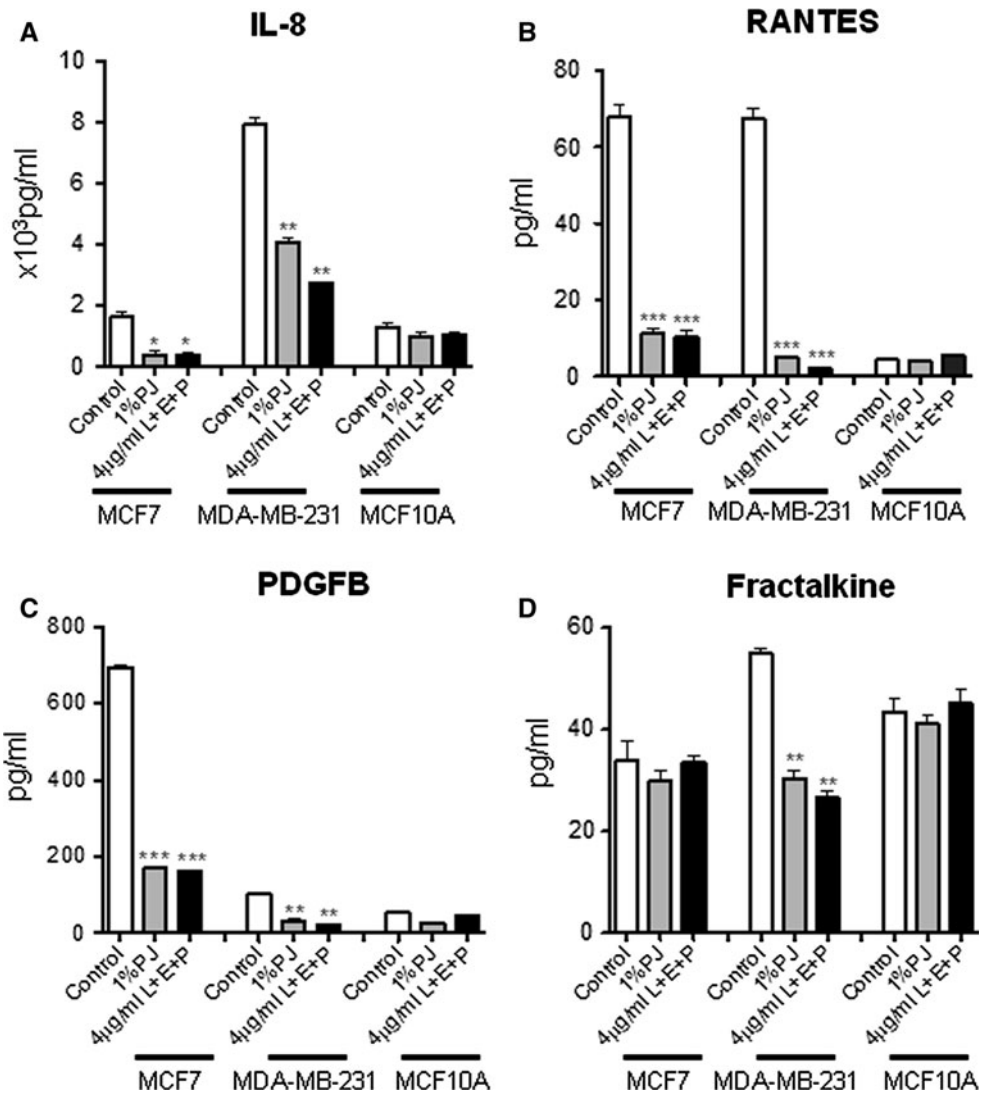

Fig. 6 The effect of PJ and the combination of luteolin, ellagic acid, and punicic acid (L + E + P) on the levels of selected pro-inflammatory cytokines and chemokines. Media collected from MCF7 and MDA-MB-231 and MCF10 cells treated with 1 % PJ or L + E + P at 4 μg/ml for 18 h were analyzed using Luminex Multiplex Array assays. Both MCF7 and MDA-MB-231 significantly decreased IL-8 (**a**), 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. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$

Acknowledgments We thank Dr. E. Turley for the HMMR expression vector and Dr. C. Glackin for the Twist vector. This study was funded by the Portuguese Foundation for Science and Technology.

Conflict of interest None of the authors have conflict of interest related to this study.

References

1. World Health Organization Facts and Figures. http://www.who.int. Accessed July 2012
2. Maughan KL, Lutterbie MA, Ham PS (2010) Treatment of breast cancer. Am Fam Physician 81:1339–1346
3. Siegel R, Naishadham D, Jemal A (2012) Cancer statistics, 2012. Cancer J Clin 62:10–29
4. Cazzaniga M, Bonanni B (2012) Breast cancer chemoprevention: old and new approaches. J Biomed Biotechnol 2012:985620
5. Longtin R (2003) The pomegranate: nature's power fruit? J Natl Cancer Inst 95:346–348
6. Gil MI, Tomas-Barberan FA, Hess-Pierce B, Holcroft DM, Kader AA (2000) Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J Agric Food Chem 48:4581–4589
7. Kim ND, Mehta R, Yu W, Neeman I, Livney T et al (2002) Chemopreventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer. Breast Cancer Res Treat 71:203–217
8. Kim ND, Mehta R, Yu W, Neeman I, Livney T, Amichay A, Poirier D, Nicholls P, Kirby A, Jiang W, Mansel R, Ramachandran C, Rabi T, Kaplan B, Lansky E (2002) Chemopreventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer. Breast Cancer Res Treat 71(3):203–217
9. Dikmen M, Ozturk N, Ozturk Y (2011) The antioxidant potency of *Punica granatum* L. fruit peel reduces cell proliferation and induces apoptosis on breast cancer. J Med Food 12:1638–1646
10. Ozbay T, Nahta R (2011) Delphinidin inhibits HER2 and Erk1/2 signaling and suppresses growth of HER2-overexpressing and triple negative breast cancer cell lines. Breast Cancer 5:143–154
11. Dai Z, Nair V, Khan M, Ciolino HP (2010) Pomegranate extract inhibits the proliferation and viability of MMTV-Wnt-1 mouse mammary cancer stem cells in vitro. Oncol Rep 24(4):1087–1091

12. Wang LM, Xie KP, Huo HN, Shang F, Zou W, Xie MJ (2012) Luteolin inhibits proliferation induced by IGF-1 pathway dependent ERα in human breast cancer MCF-7 cells. Asian Pac J Cancer Prev 13(4):1431–1437
13. Kim MJ, Woo JS, Kwon CH, Kim JH, Kim YK, Kim KH (2012) Luteolin induces apoptotic cell death through AIF nuclear translocation mediated by activation of ERK and p38 in human breast cancer cell lines. Cell Biol Int 36(4):339–344
14. Adams LS, Zhang Y, Seeram NP, Heber D, Chen S (2010) Pomegranate ellagitannin-derived compounds exhibit antiproliferative and antiaromatase activity in breast cancer cells in vitro. Cancer Prev Res 3(1):108–113
15. Grossmann ME, Mizuno NK, Schuster T, Cleary MP (2010) Punicic acid is an omega-5 fatty acid capable of inhibiting breast cancer proliferation. Int J Oncol 36:421–426
16. El Kar C, Ferchichi A, Attia F, Bouajila J (2011) Pomegranate (*Punica granatum*) juices: chemical composition, micronutrient cations, and antioxidant capacity. J Food Sci 76:C795–C800
17. Wang L, Alcon A, Yuan H, Ho J, Li QJ, Martins-Green M (2011) Cellular and molecular mechanisms of pomegranate juice-induced anti-metastatic effects on prostate cancer cells. Integr Biol 3:742–754
18. Duda DG, Kozin SV, Kirkpatrick ND, Xu L, Fukumura D, Jain RK (2011) CXCL12 (SDF1alpha)-CXCR4/CXCR7 pathway inhibition: an emerging sensitizer for anticancer therapies? Clin Cancer Res 17(8):2074–2080
19. Lansky EP, Jiang W, Mo H, Bravo L, Froom P, Yu W, Harris NM, Neeman I, Campbell MJ (2005) Pomegranate (*Punica granatum*) pure chemicals show possible synergistic inhibition of human PC-3 prostate cancer cell invasion across Matrigel (TM). Invest New Drugs 23:11–20
20. Wang L, Ho J, Glackin C, Martins-Green M (2012) Specific pomegranate juice components as potential inhibitors of prostate cancer metastasis. Transl Oncol 5:344–355
21. Roland CL, Harken AH, Sarr MG, Barnett CC Jr (2007) ICAM-1 expression determines malignant potential of cancer. Surgery 141(6):705–707
22. Nakagawa S, Miyoshi N, Ishii H, Mimori K, Tanaka F, Sekimoto M, Doki Y, Mori M (2011) Expression of CLDN1 in colorectal cancer: a novel marker for prognosis. Int J Oncol 39:791–796
23. Aderem A (1992) The MARCKS brothers: a family of protein kinase C substrates. Cell 71:713–716
24. Arbuzova A, Schmitz AAP, Vergeres G (2002) Cross-talk unfolded: MARCKS proteins. Biochem J 362:1–12
25. Sohr S, Engeland K (2008) RHAMM is differentially expressed in the cell cycle and down-regulated by the tumor suppressor p53. Cell Cycle 7:3448–3460
26. Tolg C, Hamilton SR, Morningstar L, Zhang J, Zhang S, Esguerra KV, Telmer PG, Luyt LG, Harrison R, McCarthy JB, Turley EA (2010) RHAMM promotes interphase microtubule instability and mitotic spindle integrity through MEK1/ERK1/2 activity. J Biol Chem 285:26461–26474
27. Hatano H, Shigeishi H, Kudo Y, Higashikawa K, Tobiume K, Takata T, Kamata N (2011) RHAMM/ERK interaction induces proliferative activities of cementifying fibroma cells through a mechanism based on the CD44-EGFR. Lab Invest 91:379–391
28. Helleman J, Jansen MP, Ruigrok-Ritstier K, van Staveren IL, Look MP, Meijer-van Gelder ME, Sieuwerts AM, Klijn JG, Sleijfer S, Foekens JA, Berns EM (2008) Association of an extracellular matrix gene cluster with breast cancer prognosis and endocrine therapy response. Clin Cancer Res 14(17):5555–5564
29. Song L, Rape M (2010) Regulated degradation of spindle assembly factors by the anaphase-promoting complex. Mol Cell 38:369–382
30. Ohtsuka T, Nakanishi H, Ikeda W, Satoh A, Momose Y, Nishioka H, Takai Y (1998) Nexilin: a novel actin filament-binding protein localized at cellmatrix adherens junction. J Cell Biol 143:1227–1238
31. Yang C, Kazanietz MZ (2007) Chimaerins: GAPs that bridge diacylglycerol signaling and the small G-protein Rac. Biochem J 403:1–12
32. Caldon CE, Musgrove EA (2010) Distinct and redundant functions of cyclin E1 and cyclin E2 in development and cancer. Cell Div 17(5):2
33. Gorin MA, Pan Q (2009) Protein kinase C epsilon: an oncogene and emerging tumor biomarker. Mol Cancer 8:9
34. Teicher BA, Fricker SP (2010) CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res 16(11):2927–2931
35. Kang Y, Massague J (2004) Epithelial-mesenchymal transitions: twist in development and metastasis. Cell 118:277–279
36. Yilmaz M, Christofori G (2010) Mechanisms of motility in metastasizing cells. Mol Cancer Res 8:629–642
37. Birchmeier W, Behrens J (1994) Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochim Biophys Acta 1198:11–26
38. Oyama T, Maluf H, Koerner F (1999) Atypical cystic lobules: an early stage in the formation of low-grade ductal carcinoma in situ. Virchows Arch 435:413–421
39. Oyama T, Iijima K, Takei H et al (2000) Atypical cystic lobule of the breast: an early stage of low-grade ductal carcinoma in situ. Breast Cancer 7:326–331
40. Eckhardt BL, Francis PA, Parker BS, Anderson RL (2012) Strategies for the discovery and development of therapies for metastatic breast cancer. Nat Rev Drug Discov 11(6):479–497
41. Dranoff Glenn (2004) Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer 4:11–22
42. Bonecchi R, Locati M, Mantovani A (2011) Chemokines and cancer: a fatal attraction. Cancer Cell 19(4):434–435
43. Inoue K, Slaton JW, Eve BY, Kim SJ, Perrotte P, Balbay MD, Yano S, Bar-Eli M, Radinsky R, Dinney CPN (2000) Interleukin 8 expression regulates tumorigenecity and metastases in androgen-independent prostate cancer. Clin Cancer Res 6:2104–2119
44. Xie K (2001) Interleukin-8 and human cancer biology. Cytokine Growth Factor Rev 2:375–391
45. Araki S, Omori Y, Lyn D, Singh RK, Meinbach DM, Sandman Y, Lokeshwar VB, Lokeshwar BL (2007) Interleukin-8 is a molecular determinant of androgen independence and progression in prostate cancer. Cancer Res 67(14):6854–6862
46. Ahmed OI, Adel AM, Diab DR, Gobran NS (2006) Prognostic value of serum level of inter-leukin-6 and interleukin-8 in metastatic breast cancer patients. Egypt J Immunol 13(2):61–68
47. Soria G, Ben-Baruch A (2008) The inflammatory chemokines CCL2 and CCL5 in breast cancer. Cancer Lett 267:271–285
48. Fredriksson L, Li H, Eriksson U (2004) The PDGF family: four gene products form five dimeric isoforms. Cytokine Growth Factor Rev 15:197–204
49. Song N, Huang Y, Shi H, Yuan S, Ding Y, Song X, Fu Y, Luo Y (2009) Overexpression of platelet-derived growth factor-BB increases tumor pericyte content via stromal-derived factor-1alpha/CXCR4 axis. Cancer Res 69:6057–6064
50. Marchesi F, Locatelli M, Solinas G, Erreni M, Allavena P, Mantovani A (2010) Role of CX3CR1/CX3CL1 axis in primary and secondary involvement of the nervous system by cancer. J Neuroimmunol 224:39–44
51. Jamieson-Gladney WL, Zhang Y, Fong AM, Meucci O, Fatatis A (2011) The chemokine receptor CX3CR1 is directly involved in the arrest of breast cancer cells to the skeleton. Breast Cancer Res 13:R91