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Pomegranate Extract Alters Breast Cancer Stem Cell Properties in Association with Inhibition of Epithelial-to-Mesenchymal Transition

Sameera Nallanthighal[a,b], Kristine M. Elmaliki[a], and Ramune Reliene[a,c]

[a]Cancer Research Center, University at Albany, State University of New York, Rensselaer, New York, USA; [b]Department of Biomedical Sciences, University at Albany, State University of New York, Albany, New York, USA; [c]Department of Environmental Health Sciences, University at Albany, State University of New York, Albany, New York, USA

ABSTRACT

Cancer stem cells (CSCs) have become an important target population in cancer therapy and prevention due to their ability to self-renew, initiate tumors, and resist therapy. We examined whether pomegranate extract (PE) alters characteristics of breast CSCs. Ability to grow as mammospheres is a hallmark of breast CSCs. PE inhibited mammosphere formation in two different cell lines, neoplastic mammary epithelial HMLER and breast cancer Hs578T. In addition, mammosphere-derived cells from PE treatment groups showed reduced mammosphere formation for at least two serial passages. These data indicate that PE inhibits CSC's ability to self-renew. In addition, incubation of mammospheres with PE reversed them into adherent cultures, indicating promotion of CSC differentiation. Epithelial-to-mesenchymal transition (EMT) is a key program in generating CSCs and maintaining their characteristics. Thus, we examined the effect of PE on EMT. PE reduced cell migration, a major feature of the EMT phenotype. In addition, PE downregulated genes involved in EMT, including the EMT-inducing transcription factor Twist family basic helix-loop-helix transcription factor 1 (TWIST1). This suggests that PE suppresses CSC characteristics in part due to inhibition of EMT. The ability of PE to suppress CSCs can be exploited in the prevention of breast cancer.

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Introduction

Cancer stem cells (CSCs) have been identified in various tumors including breast cancers and are thought to be responsible for tumor initiation and progression, therapeutic resistance and disease recurrence (1,2). Breast CSCs are characterized by several functional and cellular markers [reviewed in (3)]. Unlike differentiated epithelial cells, breast CSCs are able to survive and proliferate under nonadherent nondifferentiating conditions as spheroid cultures, termed mammospheres. Mammosphere-forming ability is used as a functional marker of breast CSCs. Mammospheres are enriched in stem and progenitor cells. Stem cells can regenerate mammospheres on serial passage of mammosphere-derived cells, which serves as a marker of stem cell self-renewal, and progenitor cells are capable of differentiation (4). Normal stem/progenitor cells can differentiate along multiple mammary epithelial lineages (4), while most CSCs differentiate into a single cell type—the cells that compose the bulk of the tumor (5). Breast CSCs can display specific cell surface antigens ($CD44^+$, $CD24^-$, $CD49f^+$, $CD29^+$, or $EpCAM^+$) or high expression level and activity of the enzyme aldehyde dehydrogenase (ALDH). Cell sorting based on the expression of cell surface or intracellular markers can be used to isolate breast CSCs. In addition, enrichment in breast CSCs can be achieved through differential trypsinization (6–8). Cells that detach early following trypsin exposure represent CSCs, while the remaining attached cells that are relatively resistant to trypsin represent differentiated cells. Trypsin-sensitive populations display characteristics of CSCs, which include mammosphere-forming ability, higher proportion of $CD44^+/CD24^-/ALDH^+$ cells, and greater tumor-initiating and metastatic potential, compared to trypsin-resistant populations (7). In addition, trypsin-sensitive cells display markers of the epithelial-to-mesenchymal transition (EMT), suggesting a coexistence of CSC and EMT traits (8).

Studies showed that CSCs can arise via activation of EMT (9–13). For example, ectopic expression of EMT inducers such as Twist or Snail transcription factors in a preneoplastic human mammary epithelial (HME) cell

CONTACT Ramune Reliene rreliene@albany.edu Cancer Research Center Rm. 304, University at Albany, State University of New York, 1 Discovery Drive Rensselaer, Rensselaer, New York 12144, USA.

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line, HMLE cells, enhances mammosphere-forming and tumor-initiating ability and appearance of $CD44^+/CD24^-$ cells (9). On the other hand, attenuation of FOXC2 expression inhibits the EMT phenotype and abolishes the CSC properties of mammosphere formation and tumor initiation (13). These studies provided evidence that activation of EMT can generate CSCs. However, not all cells that have undergone an EMT exhibit enhanced stemness (5,14). Other possible mechanisms through which CSCs can arise include genetic and epigenetic alterations in normal tissue stem cells, genetic and epigenetic alterations in cancer cells, and spontaneous dedifferentiation (5). It could therefore be postulated that pharmacological or dietary inhibition of CSC generation *de novo*, suppression of self-renewal of the existing CSC populations, and/or promotion of CSC differentiation may reduce tumor progression at early stages of tumorigenesis and prevent tumor recurrence at advanced stages.

Pomegranate extract (PE) is a mixture of polyphenols, mainly ellagitannins, derived from pomegranates. Ellagitannins include punicalagin isomers, punicalin, gallagic acid, ellagic acid, and ellagic acid glycosides (15,16). Punicalagin is the most abundant polyphenol of pomegranates. The levels of punicalagin in pomegranate juice (PJ) can reach more than 2 g/L juice (17). PE in liquid and powdered forms is commercially available for human consumption and can serve as an alternative to PJ. A study in healthy human volunteers showed that PJ (8 ounces), liquid PE (8 ounces), and powder PE (1,000 mg) contain equivalent levels of polyphenols and their intake provides similar levels of plasma and urinary ellagitannin metabolites (18). Plasma bioavailability, judged based on ellagic acid levels over a 6-h period, and the levels of urolithin-A glucuronide, a urinary metabolite of ellagic acid, were similar in human subjects from the three intervention groups.

PE, PJ, as well as individual pomegranate-specific polyphenols inhibit proliferation and induce apoptosis in vitro in cancer cell lines and suppress inflammation, angiogenesis, and tumor growth in vivo in animal models of cancer [reviewed in (19,20)]. Studies that focused on breast cancer showed that pomegranate polyphenols exhibit antiproliferative and proapoptotic effects in both estrogen-receptor-positive as well as estrogen-receptor-negative human breast cancer cell lines, a stem-like cancer cell line derived from the mouse MMTV-Wnt1 mammary tumors, xenografted tumors, and chemically induced mammary tumors (21–26).

The present studies were conducted to determine whether PE can suppress characteristics of breast CSCs. To assess whether PE modulates CSC self-renewal we determined the effect of PE on mammosphere formation over three serial passages. To assess whether PE promotes CSC differentiation we examined the effect of PE on mammosphere reversal into adherent cultures. In addition, to determine whether PE modulates CSCs via inhibition of EMT we examined the effect of PE on the phenotypic and genetic markers of EMT.

Materials and Methods

Reagents

PE is an aqueous extract of polyphenols (POMx, POM Wonderful, Los Angeles, CA) prepared from pomegranates (*Punica granatum* L.) grown in California (Wonderful variety, Paramount farms, Lost Hills, CA, USA) and consists of 95% glycone ellagitannins (mono- and oligomeric) standardized to about 40% punicalagin and 3.4% ellagic acid (27). PE is commercially available for human consumption in liquid and powdered forms from POM Wonderful, Los Angeles, CA. The preparation of these extracts includes the partial pressing of whole pomegranate fruits to obtain PJ, then PE is prepared from additional pressing and water extraction to produce a liquid extract (POMx liquid), and then further resin purified and dried to produce a powder extract (POMx powder) (18). In this study, we used PE in powdered form. One capsule of PE (1,000 mg of POMx powder) contains equivalent levels of polyphenols as eight ounces (240 ml) of PJ (18), implying that a polyphenol concentration in PJ is about 4.2 mg/ml (1,000 mg: 240 ml = 4.2 mg/ml). Given these estimates, 5–40 μg/ml PE concentrations that were used in our study are equivalent to PJ diluted 840–105 times (0.12–0.95% PJ).

Ellagic acid (#S1327) was obtained from Selleckchem (Houston, TX). Punicalagin (#65995-63-3), trolox (#53188-07-1), and N-acetyl cysteine (#616-91-1) were purchased from Sigma-Aldrich (St. Louis, MI). Stock solutions of PE, punicalagin, N-acetyl cysteine, and trolox were prepared in deionized water and stock solution of ellagic acid was prepared in 1N NaOH.

Cell Culture

HMLER cell line (a gift from Dr. JoEllen Welsh, University at Albany, SUNY, NY) was originally developed in Dr. Weinberg's laboratory (MIT, Cambridge, MA) by immortalization of primary HME cells with the hTERT component of human telomerase followed by oncogenic transformation with the SV40 early region containing large T and small t antigens (HMLE cells) and then by H-*ras*V12 transformation (HMLER cells) (28). HMLER cells are tumorigenic when injected subcutaneously or into the mammary glands of immunocompromised mice (28). HMLER cells were maintained in serum-free Medium 171 (Thermo Fisher Scientific/GIBCO #M-171-

500, Waltham, MA) supplemented with Mammary Epithelial Growth Supplement (MEGS) (Thermo Fisher Scientific/GIBCO #S-O15-5) containing 0.4% bovine pituitary extract, 1 μg/ml recombinant human insulin-like growth factor-I, 0.5 μg/ml hydrocortisone, and 3 ng/ml human epidermal growth factor (29).

Hs578T cell line (a gift from Dr. JoEllen Welsh) is available from American Type Culture Collection (ATCC). Hs578T cell line is derived from a carcinoma of the breast. Hs578T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific/GIBCO #11965-092) supplemented with 0.01 mg/ml bovine insulin (Sigma-Aldrich #I6634, St. Louis, MI) and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific/GIBCO #16140-071). Both cell lines were cultured at 37°C and 5% CO_2 in a humidified incubator and passaged every 3–4 days.

HMLER and Hs578T cell lines were chosen for these studies because they exhibit breast CSC markers including mammosphere-forming ability and a high proportion of $CD44^+/CD24^-$ cells (10,30,31). Other common features include mesenchymal traits, tumorigenicity, estrogen-receptor-negative status, and constitutive H-ras activation (10,28,31–33). A coexistence of mesenchymal and CSC traits in HMLER and Hs578T cells allows studying a connection between EMT and generation of CSCs.

Cell Viability Assay

Cells were seeded at a density of 2,000 cells/well in standard tissue-culture-treated 96-well plates in their respective growth media and allowed to attach overnight followed by PE treatment for 96 h. The number of viable cells was determined by acid phosphatase assay as described elsewhere (23,34). The absorbance was read with Victor^{3}V 1420 Multi-label Counter (PerkinElmer, Waltham, MA) at 450 nm.

Mammosphere Assays

Cells were seeded at a density of 1,000 cells/well (for HMLER cells) and 5,000 cells/well (for Hs578T cells) in serum-free MEGS-supplemented M-171 medium in ultra-low-attachment 24-well plates (Corning Inc., Corning, NY). Primary (P1) mammospheres were allowed to form in the medium with or without PE supplementation. The number of mammospheres that were $\geq$40 μm in diameter was counted at Day 6 under Nikon Eclipse TS100 microscope. To determine the ability of PE to modulate CSC self-renewal, P1 mammospheres from treatment and vehicle control (water) groups were collected and dissociated into single cells by incubation for 5 min at 37°C in StemPro® Accutase® Cell Dissociation Reagent (Thermo Fisher Scientific/GIBCO #A11105-01), followed by mechanical dissociation with 24 gauge $^3/_4$ inch needle (Thermo Fisher Scientific). These mammosphere-forming single cells were re-plated under the same conditions but without PE and were allowed to form secondary (P2) mammospheres. Tertiary (P3) mammospheres were derived from P2 mammospheres. The number of P2 and P3 mammospheres was counted at the end of Day 6 as described above.

To determine whether addition of PE to mammospheres alters their morphology and adherent properties (mimicking differentiation of CSCs), cells were seeded in serum-free medium as above but in standard tissue-culture-treated 24-well plates (Corning Inc., Corning, NY). Cells were allowed to form mammospheres for 4 days and then incubated with PE for an additional 2 days. The number of mammospheres was counted at the end of Day 6. The same protocol was used to study the effect of PE-specific phytochemicals (punicalagin and ellagic acid) and other test agents (FBS and antioxidants—N-acetyl cysteine and trolox).

Migration Assay

HMLER and Hs578T cells were seeded at a density of 500,000 cells/well in 6-well plates in their respective growth media and propagated to 80–90% confluence (1–2 days). Three scratches were made in each well parallel to each other with a new 200-μl pipette tip. Wells were washed with PBS to remove detached cells and replenished with fresh growth medium with or without PE supplementation. The cell migration images were photographed and the gap distance was measured at different time points of incubation (0, 5, 8 and 22 h for HMLER cells and at 0, 5, 10 and 20 h for Hs578T cells). At least four views of each triplicate well were documented. Cell migration was expressed as the percentage of gap closure.

Differential Trypsinization Assay

Hs578T cells were seeded at a density of 10,000 cells/well in standard tissue-culture-treated 96-well plates in PE-supplemented or PE-free DMEM medium in the absence of serum or other external adhesion/differentiation factors. Cells were allowed to attach overnight and then enzymatically dissociated with trypsin (0.05% trypsin, 1 mM EDTA) at different time points (0.5–5 min). The number of remaining attached cells after trypsinization was measured by acid phosphatase cell viability assay as described above.

RNA Extraction and qRT-PCR

For gene expression studies in adherent cultures, cells were seeded at a density of 800,000 cells/well in

10-cm tissue-culture-treated Petri dishes in their respective growth media. Cells were allowed to attach overnight and then incubated with PE (10 μg/ml in HMLER cells and 40 μg/ml in Hs578T cells) for 48 h. For gene expression studies in mammospheres, cells were seeded at a density of 20,000 cells/well in serum-free MEGS-supplemented M-171 medium in standard tissue-culture-treated 24-well plates. Cells were allowed to form mammospheres for 4 days and then incubated with PE (10 μg/ml in HMLER cells and 40 μg/ml in Hs578T cells) for an additional 2 days (48 h). RNA was extracted using Ambion PureLink® RNA Mini Kit (Thermo Fisher Scientific# 12183018A). Purity and concentration of RNA were assessed by NanoDrop 1000 (Thermo Fisher Scientific). Reverse transcription was performed using Multiscribe reverse transcriptase (Thermo Fisher Scientific # 4311235). qRT-PCR was performed using SYBR Green (BioRad# 172–5850, Hercules, CA) in ABI7900 system (Thermo Fisher Scientific/Applied Biosystems). Relative quantification of gene expression was calculated using the 2^{-ddCT} method. GAPDH was used for data normalizations. Primer information is provided below.

ALDH1 – FWD:CTGCTGGCGACAATGGAGT, RWD:GTCAGCCCAACCTGCACAG

AKT1 – FWD: *CCCGGCCCAACACCTT*, RWD: *GCTCCTCAGGAGTCTCCACAT*

CLD1 – FWD:GCGCGATATTTCTTCTTGCAGG, RWD:TTCGTACCTGGCATTGACTGG

CLDN4 – FWD: *GCCCACAACATCATCCAAGA*, RWD: *ATACTTGGCGGAGTAAGGCT*

GAPDH – FWD: *CTCTGCTCCTCCTGTTCGAC*, RWD: *TTAAAAGCAGCCCTGGTGAC*

HMMR – FWD: *ATTCAGTTGTCGAGGAGTGCCAGT*, RWD: *AGTGCAGCATTTAGCCTTGGCTTCC*

PI3KCA – FWD: *CCTGATCTTCCTCGTGCTGCTC*, RWD: *ATGCCAATGGACAGTGTTCCTCTT*

TWIST1 – FWD: *AGTCCGCAGTCTTACGAGGAGT*, RWD: *GGTAGAGGAAGTCGATGTACCTGC*

JNK1 – FWD: *CCAGGACTGCAGGAACGAGT*, RWD: *CCACGTTTTCCTTGTAGCCC*

JNK2 – FWD: *ATGACCCCTTACGTGGTGACA*, RWD: *CATGATGCAACCCACTGACC*

Statistical Analysis

At least three independent experiments were performed for each endpoint and the results are shown as mean ± SEM. Data was analyzed by Student's *t*-test or one-way analysis of variance and Tukey's pot-hoc test for multiple comparisons. *P* values <0.05 were considered statistically significant.

Results

Effect of PE on Mammosphere Formation and Serial Passaging

Unlike differentiated cells, breast CSCs survive and proliferate by forming mammospheres under nonadherent (ultra-low-attachment plates) nondifferentiating (serum-free) conditions and regenerate mammospheres in serial passages, which serves as a marker of CSC self-renewal (3,4). Thus, we examined whether PE alters the ability of CSCs to form mammospheres. We found that PE treatment significantly reduced the number of mammospheres in both HMLER (column P1 in Fig. 1A) and Hs578T (column P1 in Fig. 1B) cells, compared to their respective controls. In addition, we determined whether PE, when added only during passage 1, alters the ability of these cells to form mammospheres in serial passages. We observed that mammosphere-derived cells from PE treatment groups had reduced ability to form secondary and tertiary mammospheres compared to untreated cells (columns P2 and P3 in Fig. 1A and B). These findings demonstrate that PE reduces CSC's ability to self-renew.

While PE had a significant effect on mammospheres, it had little effect on adherent cells. PE concentrations that were used in mammosphere assays (equivalent to 1–4 μM punicalagin) caused only up to 5% growth inhibition in adherent cultures (Suppl. Fig. 1, IC_{50} for growth inhibition was 57 ± 6 μg/ml for HMLER cells and 116 ± 8 μg/ml for Hs578T cells, n = 3–4 experiments), implying that anti-CSC effects of PE are not due to general cytotoxicity.

Effect of PE on Mammosphere Differentiation

Mammospheres are enriched in undifferentiated CSCs, while conditions that induce mammosphere adherence to a substratum promote CSC differentiation (35,36). For example, addition of serum to nonadherent spheroids results in adherence to plastic and differentiated cell characteristics (35,36). We confirmed that incubation of mammospheres with serum (10% FBS) promoted adherence to cell culture plates (Suppl. Fig. 2). Then, we examined whether PE has a similar effect as serum. Cells were propagated as mammospheres and then incubated with PE. Mammospheres reverted into adherent cultures within 48 h after addition of PE, implying that PE promotes CSC differentiation (Fig. 2A). Enumeration of mammosphere counts revealed that the number of mammospheres reduced dramatically after incubation with PE in both cell lines, indicating a significant PE-mediated effect on CSC differentiation (Fig. 2B and C).

To identify a possible bioactive compound of PE that is responsible for this effect, we examined whether

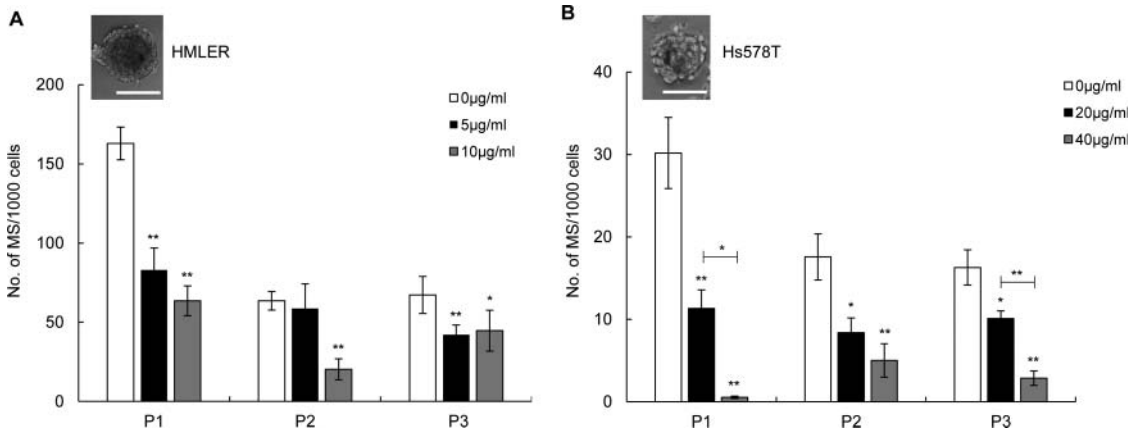

Figure 1. PE inhibits mammosphere formation and serial passaging. A: HMLER cells B: Hs578T cells. Cells were grown under ultra-low attachment serum-free conditions with or without PE for 6 days to form P1 mammospheres. Cells derived from P1 mammospheres were subcultured in P2, and cells derived from P2 mammospheres were subcultured in P3 under the same conditions but in the absence of PE. Mean of number of mammospheres ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to respective controls. Stars above the solid line represent comparison of different concentrations of PE. Insets show mammospheres at 100X magnification. Scale bar: 100 μm. MS, mammospheres.

addition of ellagitannins (punicalagin and ellagic acid) to mammospheres results in their adherence to tissue culture plates. In addition, N-acetyl cysteine and trolox were examined to determine whether this phenomenon could be mimicked by antioxidants. Incubation of mammospheres with punicalagin or ellagic acid induced similar effects as PE, while antioxidants had no effect (Suppl. Fig. 2). These data suggest that ellagitannins are the bioactive compounds of PE which promoted CSC differentiation and that the mechanism is not due to a general antioxidant effect.

Effect of PE on Cell Migration

EMT is a key program in generating breast CSCs (5). Thus, we examined the effect of PE on EMT. We measured cell migration by an in vitro scratch assay, often referred to as a wound healing assay, which is a commonly used functional assay for analysis of the EMT phenotype (37). A scratch was made in nearly confluent adherent cultures and the gap distance was measured at different time points after incubation with PE (Fig. 3A and B). We found that PE significantly reduced cell migration in both cell lines. For example, 50% of the gap was closed in control cells, while only 20% of the gap was closed in PE-treated HMLER cells, at the latest time point measured (Fig. 3C). PE had a similar effect in Hs578T cells (Fig. 3D). These data demonstrate that PE inhibits a major feature of EMT.

Effect of PE on Trypsin-mediated Cellular Detachment

Breast CSCs, which are generated through EMT activation, are sensitive to trypsin-mediated detachment from the cell culture surface, while differentiated cells

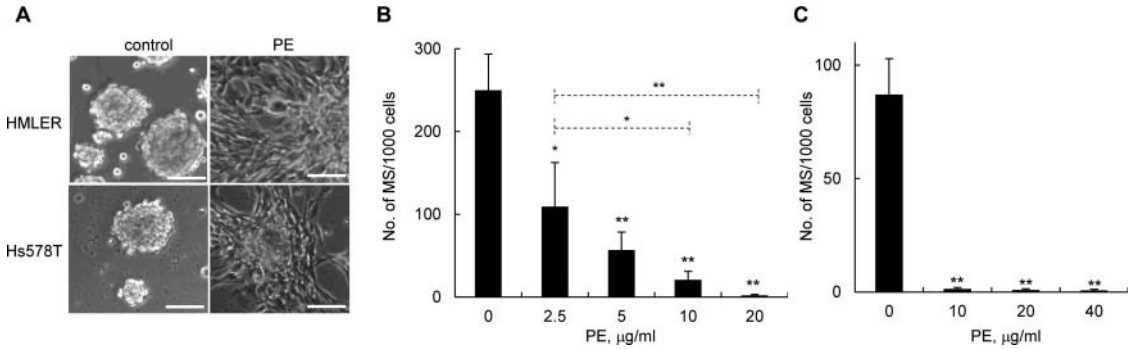

Figure 2. PE promotes mammosphere differentiation. A: Representative images of mammospheres in PE treatment and control groups. Magnification: 100x. Scale bar: 100 μm, B: number of nonadherent mammospheres in HMLER cells, C: number of nonadherent mammospheres in Hs578T cells. Cells were grown for 4 days as mammospheres and then incubated with PE for an additional 2 days. Mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control or different concentrations of PE. Stars above the dotted line represent comparison of different concentrations of PE. MS, mammospheres.

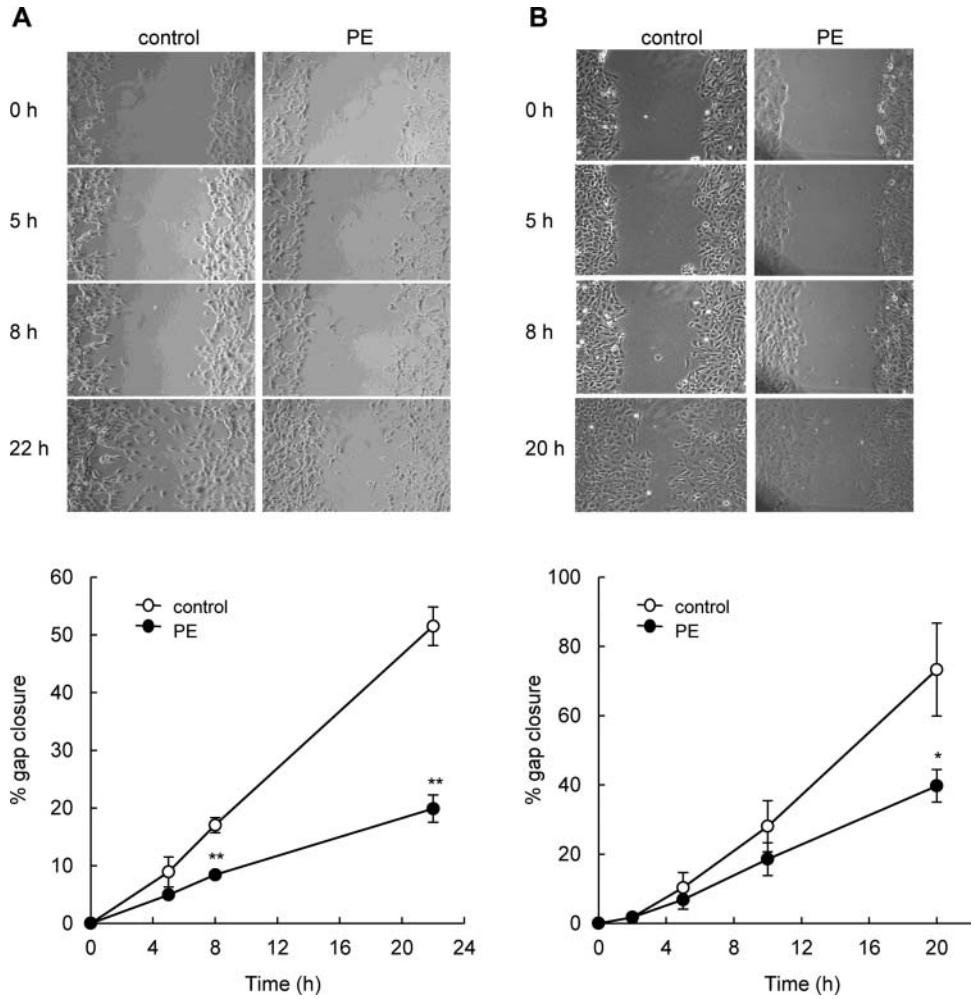

Figure 3. PE inhibits cell migration. A: HMLER cells B: Hs578T cells. Cells were incubated with PE (10 μg/ml in HMLER cells and 40 μg/ml in Hs578T cells) in their respective growth media for indicated time points. Mean ± SEM is shown, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to controls. Upper panels show representative images of the gaps in PE treatment and control groups under 100 × magnification.

are relatively resistant to trypsin (6–8). To assess whether PE increases the proportion of trypsin-resistant cells, Hs578T cells were incubated with PE overnight in DMEM without serum and other adhesion factors and subsequently exposed to trypsin for different durations. We found that PE increased the percentage of trypsin-resistant cells (Fig. 4). The effect was most pronounced at 0.5 min of trypsinization (Fig. 4A). Approximately 50% of untreated cells detached within 0.5 min of trypsin exposure, while only about 20% of PE-treated cells detached within 0.5 min trypsinization (Fig. 4B), suggesting that PE increases the number of differentiated cells and decreases the number of CSCs.

Effect of PE on Gene Expression

To elucidate the molecular mechanisms of the observed effects of PE, we examined whether PE alters expression of EMT and CSC genes, including genes with a dual role in EMT activation and CSC maintenance. To explain the effect of PE on cell migration, gene expression was examined in adherent cultures. To explain the effect of PE on mammosphere differentiation, gene expression was examined in mammospheres.

PE reduced the expression of the EMT-inducing factor TWIST1, a catalytic subunit of PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA), and c-Jun terminal kinases (JNKs), JNK1 and JNK2, in adherent cultures in both cell lines (Fig. 5). Some genes were significantly modulated in one cell line only. For example, PE treatment was associated with reduced expression of AKT serine/threonine kinase 1 (AKT1) in HMLER cells (Fig. 5A). Also, in HMLER cells, PE reduced the expression of hyaluronan-mediated motility receptor (HMMR) gene encoding HMMR involved in cell migration (Fig. 5A). In Hs578T cells, PE upregulated claudin-encoding genes CLDN1 and CLDN4 involved in cell adhesion (Fig. 5A). Thus, PE altered the

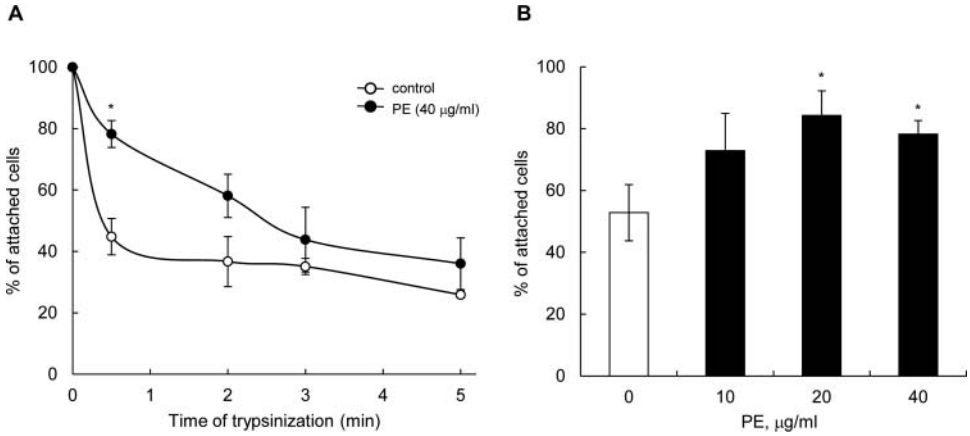

Figure 4. PE increases the percentage of trypsin-resistant cells. A: Percentage of the remaining attached cells after trypsin dissociation for 0.5–5 min, B: effect of PE dose on the percentage of the remaining attached cells after trypsin dissociation for 0.5 min. Hs578T cells were incubated in PE-supplemented or PE-free DMEM medium in the absence of serum overnight and dissociated with trypsin for indicated time points. Percentage of attached cell was calculated by subtracting the percentage of detached cells from the total number of cells. Cell counts were determined by acid phosphatase viability assay. Mean ± SEM is shown, $n = 4$. $^{*}P < 0.05$ compared to control.

expression of upstream regulators and downstream effectors of the EMT program.

Like in adherent cultures, PE downregulated TWIST1 in mammospheres in both cell lines (Fig. 6). Likewise, PE downregulated JNK1, JNK2 and HMMR in HMLER cells (Fig. 6A) and upregulated CLDN4 in Hs578T cells (Fig. 6B). In contrast, PE did not alter expression levels of PI3KCA and/or Akt1 in mammospheres (data not shown). In addition to EMT-associated genes, we examined whether mammospheres treated with PE display reduced expression levels of ALDH1 and CD44 genes that encode CSC intracellular and cell surface markers, respectively. We found that PE reduced the expression of ALDH1 in both cell lines (Fig. 7), while the expression of CD44 did not change (data not show).

Discussion

Several individual phytochemicals such as curcumin, piperine, genistein, and resveratrol have been shown to modulate characteristics of breast CSCs, suggesting that CSCs could be targeted by dietary phytochemicals (38–40). In this study, we examined the effect of a mixture of pomegranate phytochemicals on breast CSCs. We found that PE targets breast CSCs via inhibition of self-renewal and

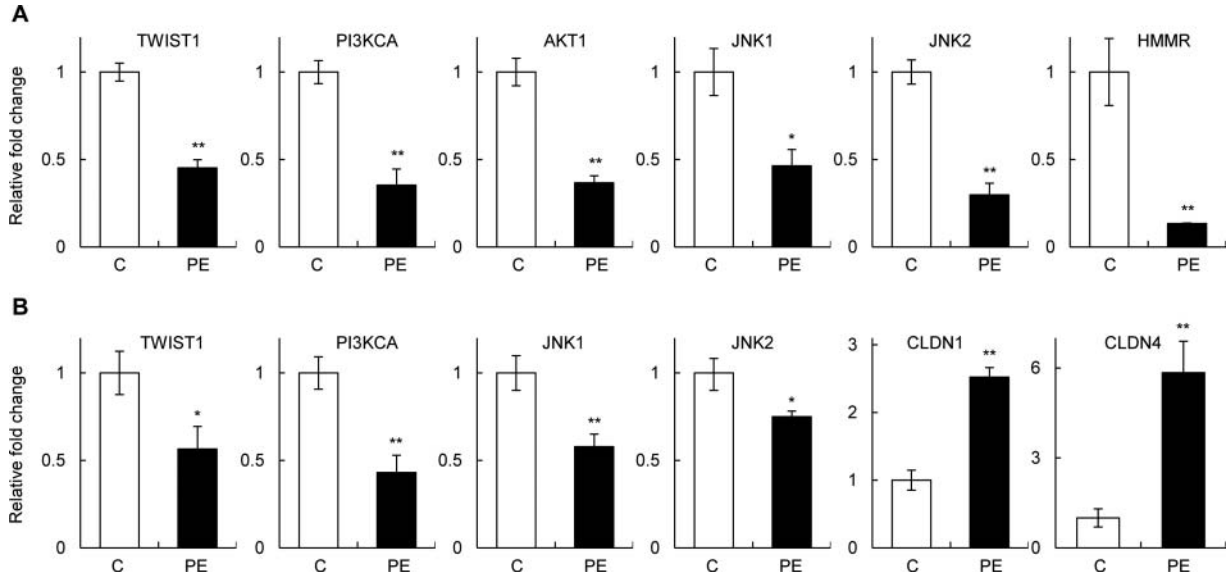

Figure 5. PE modulates EMT genes in adherent cultures. A: HMLER cells B: Hs578T cells. Cells were treated with PE (10 μg/ml in HMLER cells and 40 μg/ml in Hs578T cells) in their respective growth media for 2 days. GAPDH was used for data normalization. Mean ± SEM is shown, $n = 3$–4, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to controls.

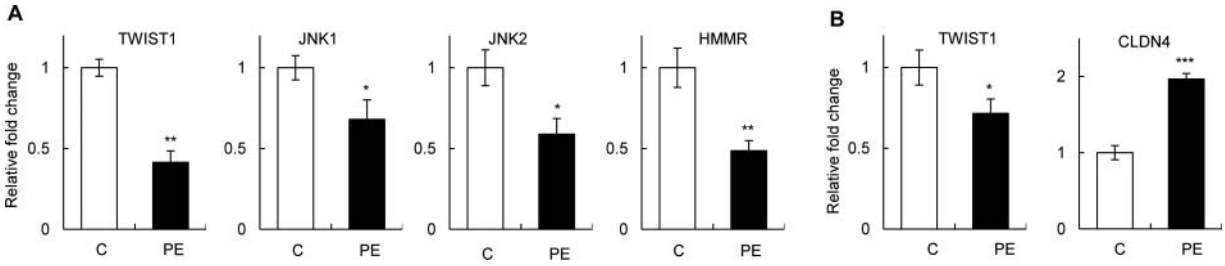

Figure 6. PE modulates EMT genes in mammospheres. A: HMLER cells B: Hs578T cells. Cells were grown for 4 days as mammospheres and then incubated with PE (10 μg/ml in HMLER cells and 40 μg/ml in Hs578T cells) for an additional 2 days. GAPDH was used for data normalization. Mean ± SEM is shown, $n = 3$–4, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls.

promotion of differentiation. These effects were associated with suppression of the EMT phenotype. Given that EMT activation results in the appearance of breast CSCs and, on the contrary, EMT inhibition abrogates CSC characteristics (9–13), the observed anti-CSC effects of PE may be due to EMT suppression.

PE treatment resulted in reduced expression levels of TWIST1 in mammospheres and in adherent cultures in both cell lines studied. Downregulation of TWIST1 was associated with mammosphere differentiation and inhibition of cell migration. These results support work of others that PE, PJ, or a combination of pomegranate-specific phytochemicals such as luteolin, ellagic acid, and punicic acid downregulate TWIST1 (41,42) and reduce cell migration (41–43) in breast cancer and other cancer cell lines. We extend these findings by demonstrating that PE downregulates TWIST1 in mammospheres. TWIST1 has been implicated in the generation of breast CSCs and in the maintenance of their characteristics (12). Thus, these data suggest that anti-CSC effects of PE are due at least in part to transcriptional modulation of TWIST1. Other genes that were downregulated by PE in mammospheres and may explain anti-CSC effects of PE include JNK1 and JNK2, but this effect was observed in one cell line (HMLER cells) only. JNK genes have roles in CSCs, given that pharmacological inhibition or RNA interference-mediated knockdown of JNK1 or JNK2 results in reduced self-renewal and tumor-initiating abilities of CSCs (44,45). In addition, knockdown of JNK1 or JNK2 is associated with reduced expression levels of TWIST1, implying that JNK regulates transcription of TWIST1 (46). Our observation that PE treatment was associated with reduced expression of JNK genes and TWIST1 suggests that PE modulates CSCs by suppressing JNK signaling with TWIST1 being a possible downstream effector. However, because PE modulated JNK genes in mammospheres in HMLER cells but not in Hs578T cells, this potential mechanism of PE cannot be generalized. Some genes including PI3KCA and AKT1 were downregulated by PE in adherent cultures but not in mammospheres. The PI3K/Akt pathway is known to activate EMT (47). Thus, transcriptional modulation of the upstream

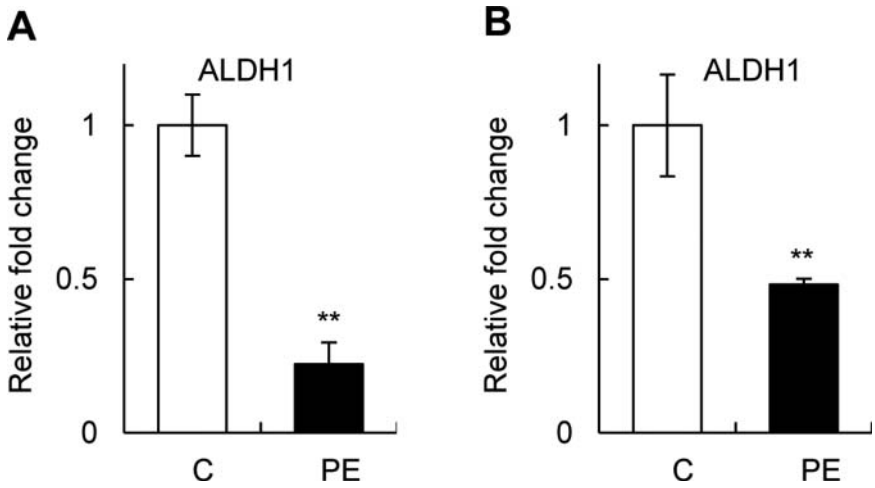

Figure 7. PE modulates CSC genes. A: HMLER cells B: Hs578T cells. Cells were grown for 4 days as mammospheres and then incubated with PE (10 μg/ml in HMLER cells and 40 μg/ml in Hs578T cells) for an additional 2 days. GAPDH was used for data normalization. Mean ± SEM is shown, $n = 3$–4, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls.

components of the PI3K/Akt pathway by PE may play a role in reduced cell migration in our study.

The PE concentrations used in this study had only a minimal effect on differentiated cell viability. Thus, PE targets CSCs at concentrations that are nontoxic to other cell types. To understand whether equivalent concentrations of pomegranate polyphenols can be achieved in humans following PE or PJ intake, we compared ellagitannin concentrations in our in vitro study to concentrations of ellagitannin metabolites in human plasma. In humans, ellagitannins are first hydrolyzed to ellagic and gallagic acids and metabolized to urolithins, and then are absorbed into blood (18,48). A recent study that used a mixture of ellagitannin metabolites, mainly gut microbiota-derived urolithins, showed a reduction in colonosphere formation and ALDH activity at urolithin concentrations (μM range) detected in the human colon following the intake of ellagitannin-containing products, such as pomegranates and walnuts (49). Reduced activity of ALDH due to urolithin exposure in this study is in agreement with reduced ALDH1 expression levels in mammospheres exposed to PE in our study. While individual urolithins in human plasma following PE or PJ intake were detected at lower than μM concentrations (18), the sum of several urolithin metabolites reached μM concentrations (48). Ellagitannin concentrations used in our study are within μM range (1–4 μM punicalagin). This suggests that equivalent levels of plasma ellagitannin metabolites can be achieved following intake of physiological amounts of PE or PJ.

In summary, this study demonstrates that PE modulates breast CSC characteristics in association with inhibition of EMT. This warrants further investigations of anti-CSC effects of pomegranate phytochemicals in different experimental paradigms including animal models for breast cancer and/or clinical trials. To date no clinical trials with PE have been conducted for breast cancer, but clinical trials in prostate cancer patients showed that PE is well tolerated and thus may be appropriate for long-term cancer intervention (50,51).

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