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RESEARCH PAPER

The soybean peptide lunasin promotes apoptosis of mammary epithelial cells via induction of tumor suppressor PTEN: similarities and distinct actions from soy isoflavone genistein

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Abstract Breast cancer is the leading cause of cancer deaths in women. Diet and lifestyle are major contributing factors to increased breast cancer risk. While mechanisms underlying dietary protection of mammary tumor formation are increasingly elucidated, there remains a dearth of knowledge on the nature and precise actions of specific bioactive components present in foods with purported health effects. The 43-amino acid peptide lunasin (LUN) is found in soybeans, is bioavailable similar to the isoflavone genistein (GEN), and thus may mediate the beneficial effects of soy food consumption. Here, we evaluated whether LUN displays common and distinct actions from those of GEN in non-malignant (mouse HC11) and malignant (human MCF-7) mammary epithelial cells. In MCF-7 cells, LUN up-regulated tumor suppressor phosphatase and tensin homolog deleted in chromosome ten (PTEN) promoter activity, increased PTEN transcript and protein levels and enhanced nuclear PTEN localization, similar to that shown for GEN in mammary epithelial cells. LUN-induced cellular apoptosis, akin to GEN, was mediated by PTEN, but unlike that for GEN, was p53-independent. LUN promoted E-cadherin and β-catenin non-nuclear localization similar to GEN, but unlike GEN, did not influence the proliferative effects of oncogene Wnt1 on HC11 cells. Further, LUN did not recapitulate GEN inhibitory effects on expansion of the cancer stem-like/progenitor population in MCF-7 cells. Results suggest the concerted actions of GEN and LUN on cellular apoptosis for potential mammary tumor preventive effects and highlight whole food consumption rather than intake of specific dietary supplements with limited biological effects for greater health benefits.

Keywords Lunasin · Genistein · Soy · Apoptosis · PTEN · Mammary epithelial · Breast cancer

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Introduction

Diet and lifestyle are major contributing factors to increased breast cancer risk in the US, currently estimated at 1 in 8 women over the course of her lifetime (Siegel et al. 2012). Epidemiological studies have shown that only 5–10 % of breast cancers can be linked to heritable gene

mutations (Lichtenstein et al. 2000); approximately 85 % of breast cancers occur in women with no family history of the disease (Colditz et al. 2012), and obese, postmenopausal women are more predisposed to developing breast cancer when compared to lean women (Rose and Vona-Davis 2010; Ligibel 2011). On the basis of data obtained from these analyses, many experimental and clinical investigations have been conducted to mechanistically evaluate the linkages of dietary effects, dietary preferences, and amounts of food intake to breast cancer risk (Key et al. 2011; Shikany et al. 2011; Irwin et al. 2011; Boggs et al. 2010). Such studies are particularly relevant to breast cancer prevention and therapy since the understanding of diet-associated mechanisms that limit the initiation and propagation of cells with tumorigenic potential is requisite to improving breast health in unaffected women and outcome in breast cancer patients.

Over the past several years, we and others have achieved important insights into mechanisms underlying mammary tumor protection by dietary factors in rodent models and established cell lines. Bioactive components present in soy foods, specifically the soy isoflavone genistein (GEN) have been a focus of our studies given the many health benefits attributed to dietary soy intake (Dong and Qin 2011; Jin and MacDonald 2002; Chiesa et al. 2008; Caan et al. 2011; Shu et al. 2009). These beneficial effects are considered to be particularly manifest in the lower risk of breast cancer reported for Asian women who consume soy-based foods as part of their regular diets beginning in early life (Korde et al. 2009; Dong and Qin 2011). We (Simmen et al. 2005; Dave et al. 2005; Su and Simmen 2009; Rahal and Simmen 2010, 2011; Montales et al. 2012) and others (Ullah et al. 2011; Anastasius et al. 2009; Kazi et al. 2003; Vinall et al. 2007) have shown that specific bioactive components of soy foods function by regulating cellular networks and pathways that promote apoptosis, inhibit proliferation, induce differentiation, and suppress inflammation, to collectively hinder tumor development and growth.

Soy foods contain bioactive components other than GEN that have been linked to cancer protection (Boué et al. 2009; Wang et al. 2008; Zhou et al. 2002). Lunasin (LUN) is a 43-amino acid peptide component of post-translationally processed 2S albumin, initially identified in soybeans (Galvez and de Lumen 1999) and also present in barley, wheat and other seeds (Jeong et al. 2007, 2010; Silva-Sanchez et al. 2008). The anti-carcinogenic properties of LUN have been demonstrated in rodent models of skin (Galvez et al. 2001) and breast (Hsieh et al. 2010a, b) cancers and in colon and breast cancer cell lines (Dia and Mejia 2010; Hsieh et al. 2010c; Dia and Gonzalez de Mejia 2011). Thus far, it is not known how LUN measures up to GEN as a dietary factor with anti-cancer preventative activities. The present study examines LUN effects on mammary epithelial cells, in the context of the functional pathways previously described to be GEN-regulated. We report here that LUN exhibits some but not all of the biological activities attributed to GEN in malignant (MCF-7) and non-malignant (HC11) mammary epithelial cells, which have important implications in mammary tumor protection.

Materials and methods

Animals

Procedures for animal care followed the guidelines approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee. Rats (Sprague–Dawley; Charles River Laboratories, Wilmington, MA, USA) were housed in polycarbonate cages under conditions of 24 °C, 40 % humidity, and a 12-h light–dark cycle with ad libitum access to food and water. Dams during pregnancy and lactation were assigned to one of two semi-purified, isocaloric diets made according to the AIN-93G formulation (Reeves et al. 1993), with corn oil substituting for soybean oil and containing as sole protein source either casein (CAS; New Zealand Milk Products, Santa Rosa, CA, USA) or soy protein isolate (SPI; Solae Company, St Louis, MO). SPI contains 394 ± 16 mg total isoflavones/kg diet, including genistein (216 ± 2 mg/kg) and daidzein (160 ± 6 mg/kg), both expressed as aglycone equivalents (Simmen et al. 2005) and lunasin (38 ± 4 mg/g extracted protein) (De Mejia et al. 2004). Pups from dams of the same diet groups were pooled at delivery and randomly assigned to dams for suckling (10 pups/dam; 5 males and 5 females). Female pups were weaned at postnatal day (PND) 21 to the same diets as their dams and continued on these diets until PND 50, when sera were collected. Male pups were used in unrelated studies.

Cell culture and treatments

Human MCF-7 mammary carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and propagated at 37 °C in Minimal Essential Medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10 % fetal bovine serum (FBS; GIBCO/Invitrogen, Carlsbad, CA, USA) and antibiotic–antimycotic mixture (ABAM; GIBCO/Invitrogen) (Dave et al. 2005). Prior to treatments, cells were plated at a density of 1×10^5 per well in six-well plates, and serum-starved for 18 h in MEM containing 0.5 % charcoal-stripped FBS. Equal volumes of sera were pooled from 6 to 8 PND 50 female rats of the same diet group; pooled sera were used at 1 % final concentration. In studies comparing the effects of genistein (GEN; Sigma-Aldrich, St. Louis, MO, USA) and

Lunasin (LUN; American Peptide Co., Sunnyside, CA, USA), cells were treated with GEN (20 nM, 2 μM) dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich); LUN (50 nM, 2 μM) dissolved in PBS-0.1 % DMSO; or vehicle alone (PBS-0.1 % DMSO). The mouse HC11 mammary epithelial cell line was kindly provided by Dr. Jeffrey Rosen (Baylor College of Medicine, Houston, TX, USA). HC11 cells were propagated and treated with recombinant Wnt1 protein (10 ng/ml) (Invitrogen) as previously described (Su and Simmen 2009) in the presence and absence of LUN (1 μM). Treated cells were collected for analyses at the time points indicated under each study.

TUNEL assay and immunofluorescence

MCF-7 cells were seeded at a density of 1.2×10^5 per well on immunofluorescence chamber slides (Fisher Scientific, Waltham, MA, USA) for 24 h at 37 °C. The cells were washed with PBS and serum-starved in 0.5 % charcoal-stripped FBS/MEM for overnight. After the respective treatments for 24 h at 37 °C, cells were rinsed with PBS and sequentially treated with 4 % paraformaldehyde (30 min, 24 °C), 0.1 % Triton X-100/PBS (15 min, room temperature), and Proteinase K (Sigma-Aldrich; 5 min, 37 °C), with PBS washes in-between treatments. Cells were incubated with fluorescein-labeled TdT reagent (Oncogene Science Diagnostics, Cambridge, MA, USA) in a humidified chamber at 37 °C for 1 h and with rabbit monoclonal anti-human PTEN (1:200; Cell Signaling Technology, Danvers, MA, USA) at 4 °C for overnight. Thereafter, cells were sequentially treated for 30 min each at room temperature with biotinylated secondary anti-rabbit antibody (Vectastain elite ABC kit; Vector Laboratories, Burlingame, CA, USA) and with rhodamine (tetramethylrhodamine isothiocyanate)-conjugated streptavidin (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted using Vectashield mounting medium (Vector Laboratories). Fluorescence images were acquired using a Zeiss AXiOVERT 200 M inverted microscope (Carl Zeiss AG, Oberkochen, Germany) at 400× magnification. Labeled nuclei were counted in five separate fields (200×), each containing 200–300 cells. Data are presented as the percentage of labeled nuclei from the total number of cells counted. Densitometric measurements of labeled cells were obtained using AxioVision 4 software (Carl Zeiss).

RNA interference

MCF-7 cells were seeded in six-well plates in charcoal-stripped 0.5 % FBS/MEM and grown to 50–70 % confluence. Commercially available human PTEN siRNAs or non-targeting (scrambled) siRNAs (Ambion, Inc., Austin, TX, USA) were transfected at 50 nM final concentration using Lipofectamine (Invitrogen) in Opti-MEM-1 Reduced Serum Medium (Invitrogen), following the manufacturer's recommendations. After incubation for 6 h at 37 °C, cells were treated with vehicle (PBS) or 2 μM LUN in PBS. Cells were incubated for an additional 24 h at 37 °C and processed for TUNEL assay as described above.

RNA isolation, reverse transcription and qRT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen). Integrity of total RNA was monitored on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). One microgram of total RNA was reverse transcribed using *Iscript* reagent (Bio-Rad, Hercules, CA, USA). Human primer sequences were [gene, forward and reverse primer; amplicon size (bp)]: *PTEN* (5′-GCT ATG GGA TTT CCT GCA GAA-3′ and 5′-GGC GGT GTC ATA ATG TCT TTCA-3′; 138 bp), *p53* (5′-GGC GCA CAG AGG AAG AGAAT-3′ and 5′-GGA GAG GAG CTG GTG TTG TTG-3′; 103 bp), *C-MYC* (5′-ACA GCT ACG GAA CTC TTG TGC GTA-3′ and 5′-GCC CAA AGT CCA ATT TGA GGC AGT-3′; 186 bp), *CCND1* (5′-CTG GCC ATG AAC TAC CTG-3′ and 5′-GTC ACA CTT GAT CAC TCT GG-3′; 482 bp) and *18S* (5′-TCT TAG CTG AGT GTC CCG CG-3′ and 5′-ATC ATG GCC TCA GTT CCGA-3′; 150 bp). Mouse primer sequences for *C-MYC* and *18S* were as previously described (Su and Simmen 2009). The primers were designed to span introns, using Primer Express (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was performed with the SYBR Green detection system (Applied Biosystems) using an ABI Prism 7000 cycler under previously described conditions (Su and Simmen 2009; Rahal and Simmen 2010). Relative gene expression was normalized to *18S* mRNA.

Immunoprecipitation and immunoblotting

Total cell lysates (RIPA buffer; Sigma-Aldrich), cytoplasmic extracts, and nuclear fractions (NE-PER; Pierce Biotechnology, Rockford, IL, USA) were prepared following the manufacturer's instructions. Protein concentrations were quantified by bicinchoninic acid assay (Bio-Rad) using BSA as standard. Immunoprecipitation followed by Western blotting or Western blotting without immunoprecipitation were performed as previously described (Su and Simmen 2009; Rahal and Simmen 2010). Primary antibodies used were as follows: PTEN (Cell Signaling Technology, Danvers, CA, USA); p53 (Cell Signaling Technology); β-catenin (Cell Signaling Technology); E-cadherin (BD Transduction Laboratory,

San Jose, CA, USA); Lamin A (Sigma-Aldrich); Lamin B1 (Abcam, Cambridge, MA, USA); and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive proteins were detected using horseradish peroxidase-linked anti-rabbit secondary antibody (Bio-Rad Laboratories) and enhanced chemiluminescence using Amersham ECL Plus (GE Healthcare Life Sciences, Piscataway, NJ, USA). Densitometric values of immunoreactive bands were quantified using GE ImageScanner III detection system (GE Healthcare Life Sciences) and Quantity One Software (Bio-Rad).

Transient transfection and luciferase assay

The PTEN-luciferase (PTEN-Luc) reporter construct, human PTEN in pGL3basic vector, was a generous gift from Dr. Eileen D. Adamson (The Burnham Institute, La Jolla, CA, USA) (Virolle et al. 2001). MCF-7 cells (1×10^4 per well) were plated into 24-well plates and serum-starved overnight in 0.5 % FBS/MEM. Cells were transfected with PTEN-Luc reporter plasmid or empty vector plasmid (pGL3basic; Promega, Madison, WI, USA) (0.25 μg/well) using Lipofectamine 2000 reagent (Invitrogen) in OPTI-MEM 1 reduced serum-containing medium (Invitrogen). The Renilla Luciferase reporter construct (pRL-SV40; Promega) was co-transfected in all cases as internal control for transfection efficiency. After 6 h, cells were washed with PBS, and the medium was replaced with 0.5 % FBS/MEM. Twenty-four hours later, cells were incubated in fresh medium containing 2 μM LUN or vehicle (PBS) for another 24 h. Luciferase activity in lysates (Lysis Buffer; Promega) was measured using the Dual Luciferase Reagent kit (Promega) and a MLX Microplate Luminometer (Dynex Technologies, Inc., Chantilly, VA, USA). Results were calculated as the ratio of PTEN-Luc to Renilla luciferase activity. Values for relative luciferase activity were obtained from two independent transfection experiments, with each experiment carried out in quadruplicate.

Mammosphere formation assay

The formation of mammospheres by MCF-7 cells followed previously described protocols (Montales et al. 2012). Plating medium for mammosphere formation consisted of phenol red-free, serum-free Minimal Essential Media (MEM; GIBCO) supplemented with B27 (1X; Invitrogen), 20 ng/ml human basic fibroblast growth factor (Invitrogen), 20 ng/ml human epidermal growth factor (Invitrogen), 10 μg/ml heparin (Sigma-Aldrich), 1 % antibiotic–antimycotic solution (Invitrogen), and 100 μg/ml gentamicin (Sigma-Aldrich). The cells were treated once at initial plating (Passage 1, P1) with vehicle, 2 μM LUN or LUN (2 μM) + GEN (40 nM). Mammospheres with diameters of ≥100 μm were manually counted after 5 days using a Zeiss Axiovision microscope (Carl Zeiss). To assess the relative sphere numbers over the second passage (P2), mammospheres from the initial plating (P1) were collected at day 5, dissociated with 0.05 % trypsin (Invitrogen), filtered using a 40 μM sieve, and replated in ultra-low-attachment plates at a density of 5,000 cells per well. Mammosphere formation in P2 was evaluated at day 7 post-plating. Treatment effects were determined from two independent experiments, with quadruplicates performed for each experimental group.

Statistical analysis

At least two independent experiments were conducted for each study, with each experiment performed in triplicate or quadruplicate. Data are presented as mean ± SEM, relative to the control group. Statistical analysis was performed using SigmaStat Software 3.5 (SPSS, Chicago, IL, USA), and significance between treatment groups ($P \leq 0.05$) was determined by using two-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis or Student's *t* test.

Results

Sera from SPI-fed young adult rats increased PTEN expression and enhanced apoptosis of MCF-7 cells

We previously showed that lifetime exposure to dietary SPI increased the apoptotic status of epithelial cells in distinct mammary compartments (Terminal end buds, Lobules, Ductal epithelium) of young adult (PND 50) female rats coincident with these cells' increased expression of tumor suppressor PTEN (Dave et al. 2005). To evaluate the effects of dietary exposure to SPI relative to CAS on breast cancer cells ex vivo, sera pools from female rats of the two dietary groups ($n = 6$–8 per group) were added at 1 % final concentration to MCF-7 cells. Treated cells were evaluated after 24 h for apoptotic status (TUNEL, T) and PTEN expression (immunoreactive PTEN, P) by immunofluorescence (Fig. 1a). Nuclear PTEN + TUNEL co-localization (D + T + P) was further analyzed by dual immunofluorescence. Cells treated with CAS-sera and SPI-sera both showed nuclear-localized TUNEL and PTEN immunostaining (Fig. 1a), which spatially overlapped. Cells incubated with SPI-sera had a higher percentage of TUNEL- (by threefold) and PTEN (by twofold)-positive cells than corresponding CAS-sera-treated cells (Fig. 1b, c). Densitometric analyses of PTEN-positive cells indicated higher amounts (by 1.5-fold) of nuclear PTEN with SPI-sera than with CAS-sera treatment (Fig. 1d).

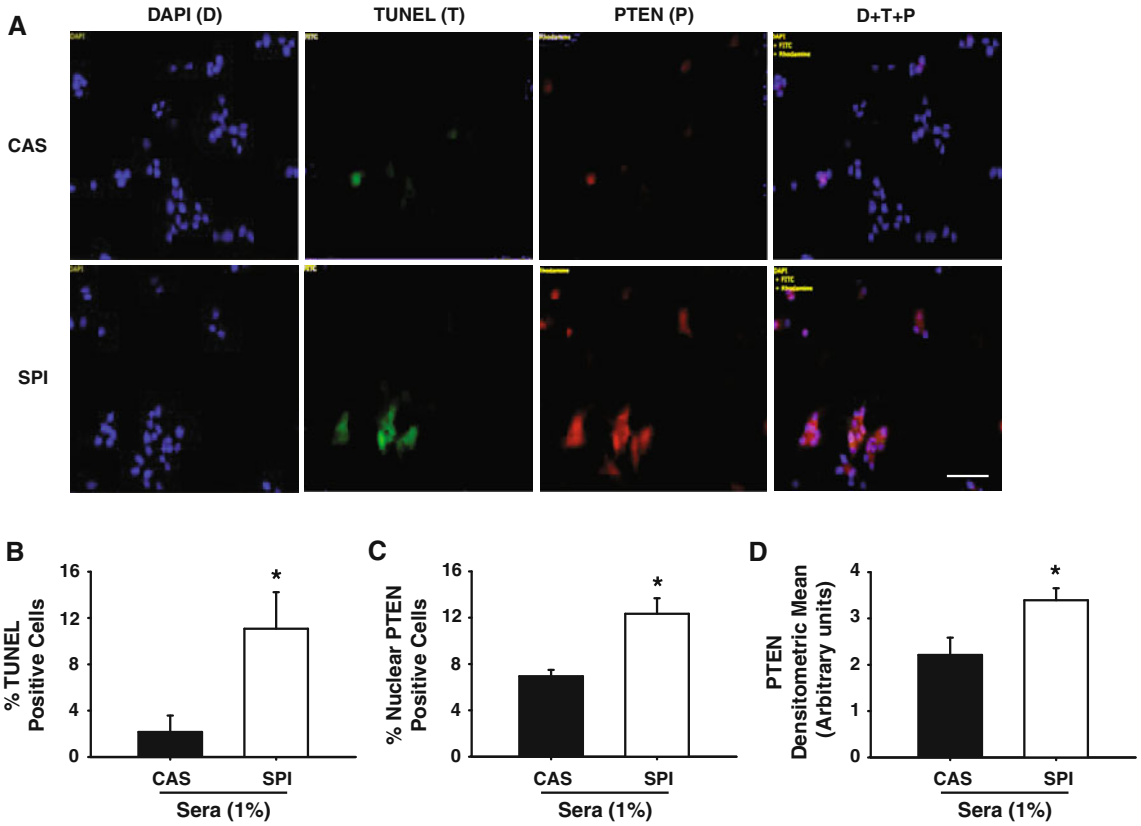
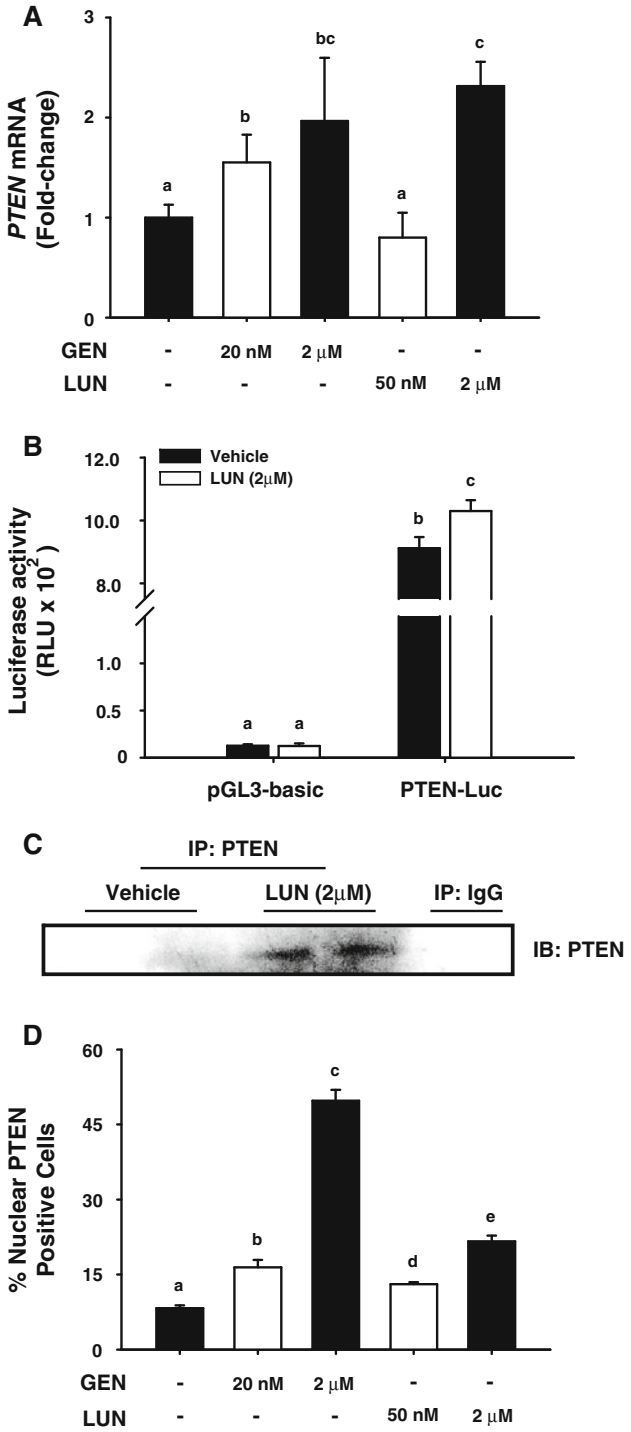


Fig. 1 SPI sera promote apoptosis and nuclear PTEN localization in MCF-7 cells. **a** Dual immunofluorescent staining of MCF-7 cells treated with sera from CAS or SPI-fed adult rats and visualized using fluorescein-labeled TdT reagent for apoptotic cells (*green*) and rhodamine (*red*) for PTEN-positive cells prior to counterstaining with 4′,6-diamidino-2-phenylindole (DAPI; *blue*). Co-localization of TUNEL (apoptotic cells; T) and PTEN-stained cells (P) coincide with DAPI (D + T + P), the latter shown as *purple* color. Representative images for each group are depicted at 400× magnification from three independent experiments; *Bar* 100 μm. (**b**–**d**). The percentages of TUNEL and nuclear PTEN-positive cells were calculated by counting at least 500 cells from five random areas per slide with at least three slides per treatment group. The densitometric means for nuclear PTEN-positive cells were quantified using Axiovision software. Means (±SEM) with *asterisk* are significantly different ($P < 0.05$, Student's *t* test) from the control CAS group

Lunasin increased PTEN expression and nuclear localization

We previously showed that one mechanism by which the major soy isoflavone genistein (GEN) elicits its antitumor properties is through PTEN pathway activation (Dave et al. 2005; Rahal and Simmen 2010). To evaluate the possibility that the soy peptide Lunasin (LUN) exerts mammary tumor-inhibitory activities by influencing PTEN expression similar to GEN, MCF-7 cells treated with LUN were evaluated for effects on PTEN transcript and promoter activity, PTEN protein levels, and PTEN nuclear localization, relative to cells treated with vehicle and in some cases, GEN. For these studies, synthetic LUN was used at concentrations (50 nM or 2 μM) higher than its range of detection in sera of humans and rodents exposed to dietary soy (Jeong et al. 2007; Dia et al. 2009), since LUN is readily degraded in the absence of Bowman-Birk protease inhibitor (Hsieh et al. 2010c), and this inhibitor was not included in the culture medium. Synthetic LUN and LUN purified from soybeans were previously demonstrated to exhibit similar bioactivities in L2110 leukemia cells (De Mejia et al. 2010). GEN, when added, was used at 20 nM and 2 μM, approximating serum levels of occasional and regular soy consumers, respectively (Andrade et al. 2010; Iwasaki et al. 2008). Treatment with LUN at 2 μM increased PTEN transcript levels and PTEN promoter activity (Fig. 2a, b). The induction of PTEN gene expression with 2 μM LUN was confirmed at the level of PTEN protein (Fig. 2c) and was accompanied by increased nuclear PTEN localization (Fig. 2d). Relative to the higher dose, the lower dose of LUN (50 nM) was ineffective in increasing PTEN transcript levels (Fig. 2a) but was modestly effective in inducing PTEN nuclear localization (Fig. 2d). GEN at the two doses examined, significantly increased PTEN transcript levels (Fig. 2a) and nuclear localization (Fig. 2d). While GEN induction of PTEN protein levels was not quantified by Western blotting in the



◀ **Fig. 2** Lunasin induces PTEN expression and nuclear localization. **a** Transcript levels of *PTEN* in MCF-7 cells treated with GEN (20 nM and 2 μM) or Lunasin (50 nM and 2 μM) were quantified by qRT-PCR, normalized to that of 18S, and renormalized to those of the vehicle-treated group. Each *bar* represents mean ± SEM, expressed as fold-change. Results are representative of three independent experiments, with each experiment performed in triplicates. *Different superscripts* are different at $P < 0.05$ by one-way ANOVA. **b** MCF-7 cells were co-transfected with PTEN promoter–reporter construct (PTEN-Luc) or empty vector (pGL3-basic) and pRL-SV40 and then treated with vehicle or Lunasin (LUN) for 24 h. Luciferase activity is expressed as relative luminescence units (RLU) normalized to pRL-SV40. Means (±SEM) with *different superscripts* are significantly different ($P < 0.05$ by two-way ANOVA). **c** Total cell lysates from vehicle or LUN (2 μM)-treated cells were immunoprecipitated (IP) with PTEN anti-mouse antibody or control IgG followed by western blot analysis (IB) using PTEN anti-rabbit antibody. A representative blot is shown, with each lane representing individual samples. **d** The percent of nuclear PTEN-positive staining MCF-7 cells after treatments with GEN or LUN for 24 h was determined by counting five random areas per slide, with at least three slides per treatment group. Means (±SEM) with *different superscripts* are significantly different ($P < 0.05$, one-way ANOVA)

present study, we previously showed that GEN-induced increase in PTEN mRNA levels occurs coincident with the corresponding protein in mammary epithelial cells (Rahal and Simmen 2010). Moreover, densitometric analyses of nuclear PTEN-staining cells (Fig. 2d) indicated that while GEN increased PTEN protein levels by twofold (20 nM) and fivefold (2 μM), respectively, relative to control cells, LUN induction of PTEN protein levels relative to control cells was 1.5-fold and threefold, respectively, for 50 nM and 2 μM (data not shown).

Lunasin induced apoptosis via PTEN

We previously showed that GEN induction of apoptosis in MCF-7 cells was mediated by PTEN (Dave et al. 2005). To evaluate whether LUN can induce apoptosis, and if so, whether this activity is mediated by PTEN, LUN-treated MCF-7 cells (2 μM) were examined for apoptotic status by TUNEL, with and without PTEN silencing achieved, respectively, by transfection with PTEN-specific siRNAs or non-targeting siRNAs. LUN increased the percentage of nuclear PTEN-expressing cells (Figs. 2d, 3a) and apoptotic (TUNEL-positive) cells (Fig. 3a, b). The induction of apoptosis by LUN was highly dose-dependent (50 nM < 2 μM; $P < 0.001$) (Fig. 3b) and was more dramatic (13-fold for 50 nM; 21-fold for 2 μM) than the 2–3-fold induction in nuclear-localized PTEN-positive cells (Fig. 2d) using the same LUN doses. LUN-induced apoptosis was significantly, but not exclusively inhibited by PTEN knock-down (by >90 % at the transcript level, data not shown); by contrast, knockdown with non-targeting siRNAs had no effect on the apoptotic status of LUN-treated cells (Fig. 3c).

In a previous study (Rahal and Simmen 2010), we showed that PTEN-mediated induction of apoptosis in the non-malignant human mammary epithelial cell line MCF-10A by GEN involved the contribution of the tumor suppressor p53. Specifically, GEN induction of PTEN was accompanied by increased p53 nuclear localization, and decreased expression of pro-proliferative gene markers. To evaluate whether LUN mediates a similar mechanism,

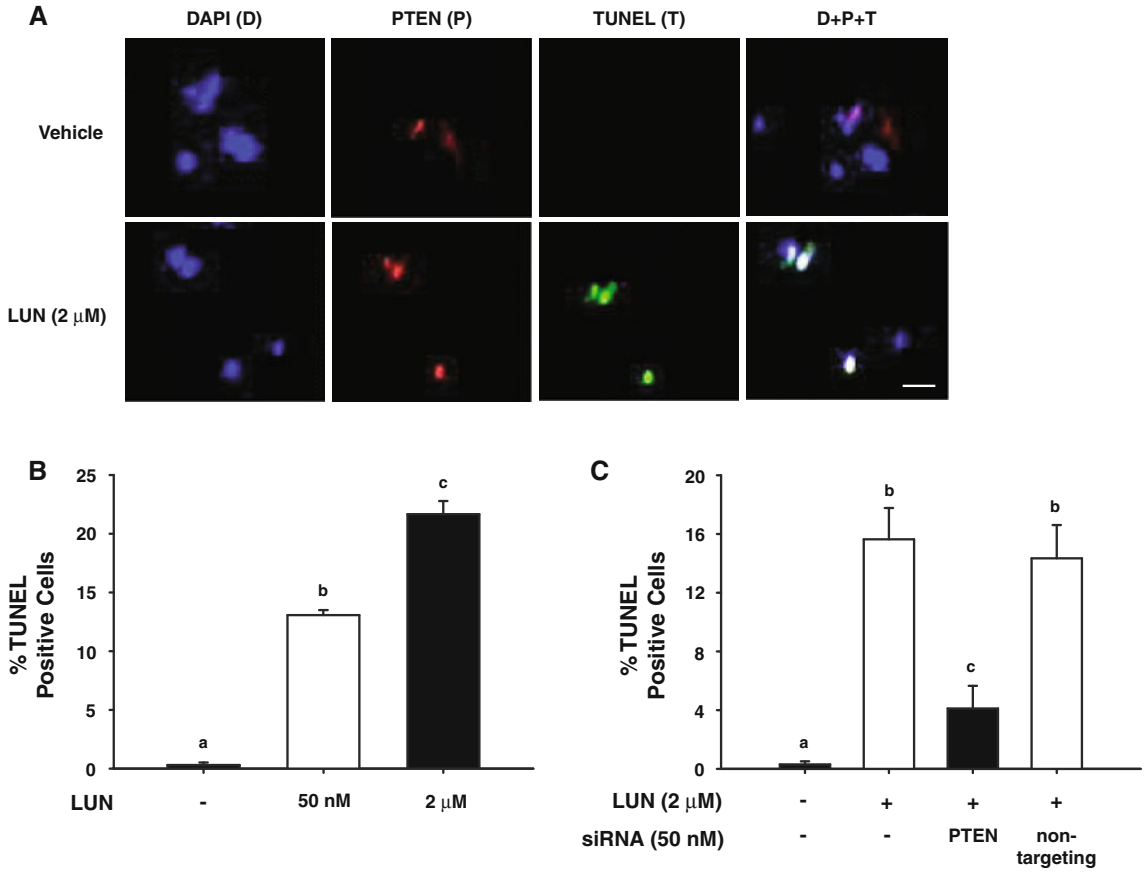


Fig. 3 Lunasin promotion of MCF-7 cell apoptosis is mediated by PTEN. **a** Representative images of dual immunofluorescent-stained MCF-7 cells treated with vehicle or Lunasin (LUN; 2 μM). Stained cells indicate: PTEN-positive (P, *Red*), Apoptotic (T, *green*), and nuclear compartment (D; *blue*). Nuclear co-localization of PTEN- (P) and TUNEL (T)-positive cells is shown as D + P + T; *Bar*, 50 μm. **b** Dose-dependent induction of apoptosis of MCF-7 cells by LUN is shown as percent TUNEL-positive cells. **c** MCF-7 cell apoptotic status after treatment for 24 h with LUN (50 nM and 2 μM) in the presence of PTEN siRNAs or non-targeting (control) siRNAs. The percent of TUNEL-positive cells was determined by counting five random areas per slide and at least three slides per treatment group. Means (±SEM) with *different superscripts* are significantly different ($P < 0.05$, one-way ANOVA)

nuclear extracts from control and LUN-treated MCF-7 cells were evaluated for p53 protein by Western blots. When normalized to those of Lamin A nuclear protein, p53 levels did not differ in control and LUN-treated cells (Fig. 4a). Similarly, LUN treatment had no significant effects on the transcript levels of *p53* and pro-proliferative marker *C-MYC* and cyclin D1 (*CCND1*) genes (Fig. 4b). Further, LUN did not alter the proliferation status of MCF-7 cells as measured by the MTT assay (data not shown).

Lunasin promoted E-cadherin expression and failed to inhibit mammosphere formation

To further define common and distinct pathways between LUN and GEN in mammary epithelial cells, the effects of LUN on two other pathways previously described by our group to be influenced by GEN were evaluated. The first pathway involved GEN up-regulation of E-cadherin expression to attenuate β-catenin signaling in non-malignant mammary epithelial cells (Su and Simmen 2009), and the second pathway relates to our recent report of GEN inhibition of mammosphere formation by MCF-7 cells (Montales et al. 2012). For the first pathway, mouse HC11 mammary epithelial cells were treated with vehicle or LUN (1 μM) for 6 h, and non-nuclear proteins (membrane/cytosolic fractions) were evaluated for levels of E-cadherin and β-catenin by Westerns. Cells treated with LUN showed numerically higher ($P = 0.065$) E-cadherin and significantly higher ($P < 0.05$) β-catenin levels in cytosolic/membrane (non-nuclear) fraction than vehicle-only-treated cells (Fig. 5a). The increase in β-catenin protein was specific to this cellular fraction, since corresponding nuclear proteins from control and LUN-treated cells did not differ in β-catenin levels (Fig. 5b). LUN had no effect on *C-MYC* expression in HC11 cells (Fig. 5c), recapitulating its absence of effect on *C-MYC* expression in MCF-7 cells (Fig. 4b) and did not suppress Wnt-1-induced expression of *C-MYC* (Fig. 5c).

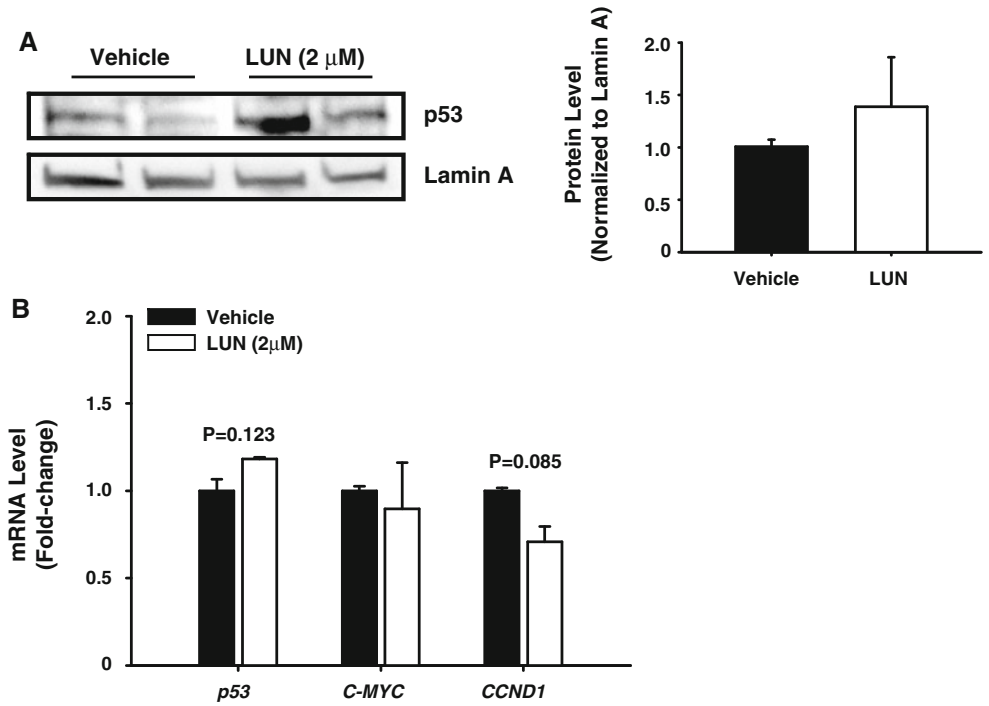


Fig. 4 Lunasin effects on MCF-7 cells are not p53-mediated. **a** Nuclear extracts isolated from MCF-7 cells treated with vehicle or 2 μM Lunasin (LUN) for 24 h were analyzed by western blotting for p53 protein. Lamin A was used as loading control. A representative western blot is shown, with each lane representing samples from an independent experiment. *Bar graph* represents densitometric scan of p53 bands normalized to Lamin A. Means values (±SEM) are not different ($P > 0.05$, Student's *t* test). **b** Gene expression levels of *p53*, *C-MYC,* and *CCND1* in MCF-7 cells treated with vehicle or 2 μM LUN for 24 h were analyzed by qRT-PCR. Transcript levels were normalized with *18S* and renormalized to vehicle group. Means values (±SEM) are not different ($P > 0.05$, Student's *t* test)

To assess the second pathway, MCF-7 cells were treated at plating with vehicle alone, LUN (2 μM) and LUN (2 μM) + GEN (40 nM) and evaluated for numbers of mammospheres formed at passage 1 (P1) and passage 2 (P2). LUN did not influence the number of mammosphere-forming units in P1 (not shown) and P2 (Fig. 5d). We have reported recently that GEN at 40 nM significantly reduced mammosphere formation of MCF-7 cells (Montales et al. 2012). The decrease in mammosphere-forming units by GEN (data not shown) was not affected by LUN (Fig. 5d).

Discussion

In this study, we sought to determine whether the soybean peptide LUN exerts its breast cancer protective effects via mechanisms similar to those described for the major soy isoflavone GEN. Our results show that LUN exhibits some but not all of the biological activities attributed to GEN in malignant (human MCF-7) and non-malignant (mouse HC11) mammary epithelial cells. These cell lines, while derived from different species, are both estrogen receptor positive, in contrast to the human MCF10A, which is estrogen receptor-negative (Rahal and Simmen 2011). A shared pathway influenced by LUN and GEN is PTEN-mediated apoptosis. LUN, similar to GEN, up-regulated PTEN promoter activity, increased PTEN transcript and protein levels, and enhanced nuclear localization of PTEN, leading to induction of the apoptotic status of MCF-7 breast carcinoma cells. The higher apoptotic index induced by LUN was mechanistically linked to LUN-mediated up-regulation of PTEN expression since PTEN siRNA targeting resulted in the dramatic diminution of cellular apoptosis in LUN-treated cells. Another pathway influenced by both LUN and GEN involves the induction of E-cadherin and β-catenin protein levels in non-nuclear (cytosolic/membrane) fractions of mammary epithelial cells. E-cadherin forms a complex with β-catenin outside the nucleus and, in so doing, restricts the migration of β-catenin to the nuclear compartment where it binds the LEF/TCF-1 proteins to induce cellular proliferation via C-MYC activation (Orsulic et al. 1999). While we found vehicle- and LUN-treated cells to exhibit comparable levels of nuclear β-catenin and of pro-proliferative marker genes *C-MYC* and *Ccnd1,* LUN was ineffective in inhibiting Wnt1-induced cellular proliferation (*C-MYC* as surrogate marker) in non-malignant HC11 mammary epithelial cells. This differed from previously demonstrated GEN inhibition of Wnt1-induced *C-MYC* expression (Su and Simmen 2009) but is consistent with the lack of effect

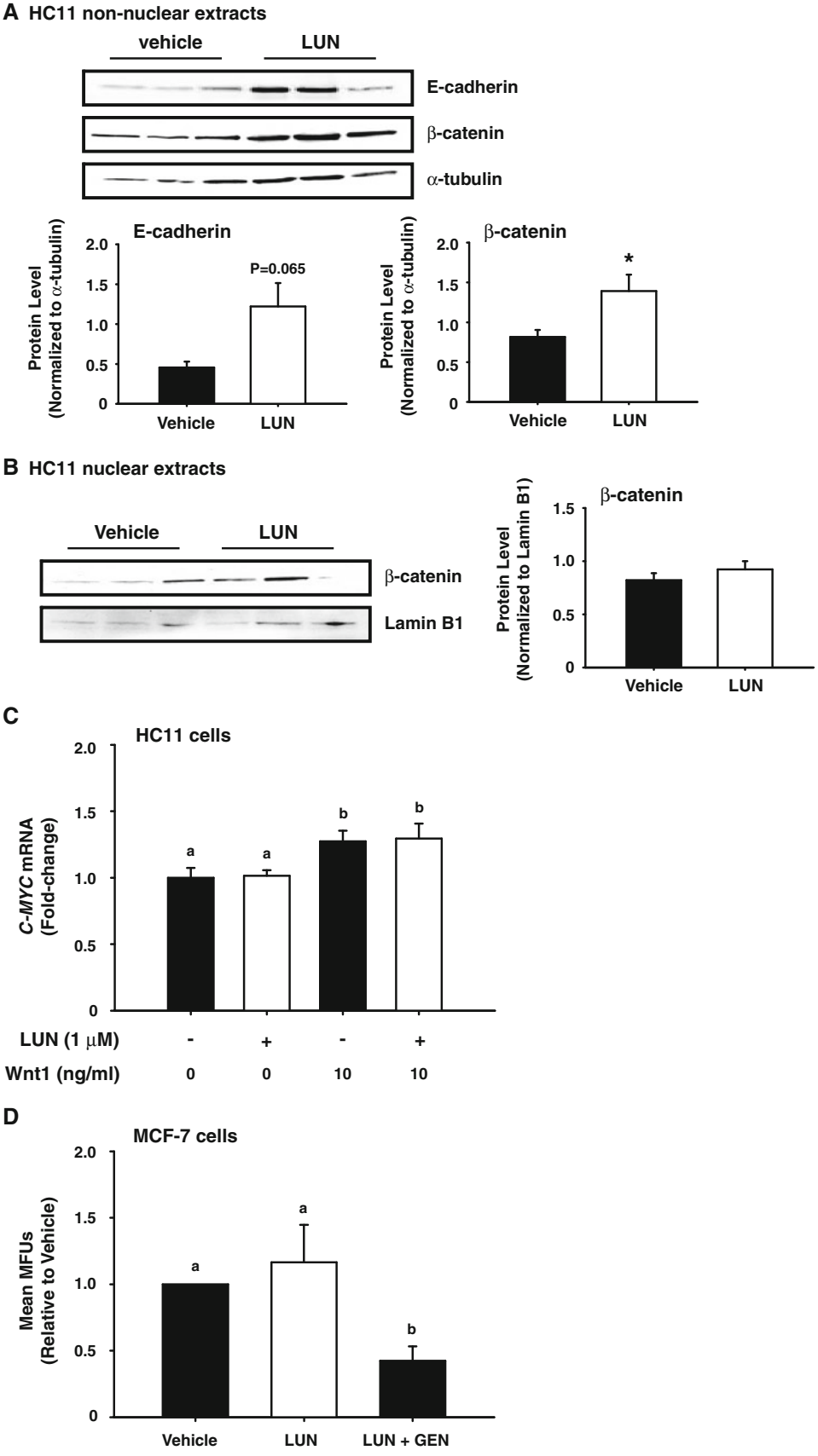


Fig. 5 Lunasin increases E-cadherin expression in mouse HC11 mammary epithelial cells and does not affect mammosphere formation of MCF-7 cells. Non-nuclear (**a**) and nuclear (**b**) extracts were isolated from HC11 cells treated with vehicle or 1 μM Lunasin (LUN) for 6 h and analyzed for E-cadherin and *β*-catenin proteins by western blots. Loading controls used were α-tubulin (**a**) and Lamin B1 (**b**). Representative western blots are shown, with each lane representing samples from an independent experiment. Densitometric scans are normalized to α-tubulin or Lamin B1 (mean ± SEM). *Significant difference at $P < 0.05$, using Student's *t* test. **c** Transcript levels for *C-MYC* in HC11 cells treated with vehicle or LUN (1 μM) with and without added recombinant Wnt1 (10 ng/ml) were quantified by qRT-PCR, Values were normalized to those of corresponding *18S* and renormalized to vehicle-treated group. Means (±SEM) that were expressed as fold-change are from three independent experiments, with each experiment performed in triplicate. *Different superscripts* indicate significant differences ($P < 0.05$, one-way ANOVA). **d** MCF-7 cells were treated with vehicle, 2 μM LUN or 2 μM LUN + 40 nM genistein (LUN + GEN) in low-attachment plates. Primary (P1) mammospheres were collected at day 5 and passaged for secondary (P2) mammospheres without additional treatments. Mammosphere formation at P2 was evaluated at day 7 post-plating. Mean MFUs (±SEM), relative to vehicle-only-treated cells, are from P2 mammospheres of two independent experiments, with each experiment performed in quadruplicate. Means with *different superscripts* are significantly different ($P < 0.05$, one-way ANOVA)

of LUN on MCF-7 cell proliferation as measured by MTT assay (data not shown). Also in contrast to GEN, LUN failed to attenuate the expansion of stem cell-like/progenitor cells with tumor initiating potential in MCF-7 cells (Montales et al. 2012; Fillmore and Kuperwasser 2008). Our collective data, thus, suggest that LUN preferentially promotes apoptotic over inhibiting proliferative pathways in mammary epithelial cells, although this anti-apoptotic activity of LUN is not exclusively PTEN-mediated and may involve other yet-to-be defined mechanisms. These findings are consistent with previous reports showing that LUN stimulated apoptosis in human colon and breast cancer cells (Dia and Mejia 2010; Hsieh et al. 2010a) but had no effect on the growth of numerous immortalized and established cancer lines (Lam et al. 2003).

Our findings that sera from SPI-fed rats significantly influenced PTEN expression, PTEN nuclear localization, and cellular apoptosis, relative to sera from control diet (CAS)-fed mice are consistent with studies reporting the presence of bioactive components such as the isoflavones genistein and daidzein, and LUN in human and rodent sera after dietary soy protein intake (Jeong et al. 2007; Jeong et al. 2010; Dia et al. 2009). Based on the reported LUN concentrations of 14 nM in plasma of men after soy consumption (Dia et al. 2009) and the potential for degradation of LUN when dissociated from its complex with the Bowman-Birk protease inhibitor (Hsieh et al. 2010a), the latter a component of soy proteins, we used LUN at higher concentration (50 nM and 1–2 μM) for evaluating its effects on mammary epithelial cells, since these cells were not co-treated with the protease inhibitor. While the higher LUN dose elicited more robust responses than the lower dose and hence was used for all the assays described here, the 50 nM dose was equally effective in inducing nuclear PTEN localization and apoptosis. Given commercial soy products to contain sufficient amounts of LUN ($\sim$17 mg of LUN per g of soy protein concentrate) (De Mejia et al. 2004) and thus, consumption of 25 g of soybean protein per day will result in a total intake of 0.93 g of LUN (De Mejia et al. 2010), our results suggest that the mammary tumor preventive effects ascribed to soy protein consumption in many epidemiological studies may be attributed in part, to LUN induction of PTEN-mediated apoptosis of neoplastic mammary epithelial cells.

Our novel finding that LUN can up-regulate PTEN promoter activity leading to increased PTEN mRNA and protein levels raises the question of how LUN may mechanistically activate PTEN transcription. While the mechanisms by which LUN crosses the plasma membrane to enter cells and gets transported to the nucleus remain unclear (Galvez and de Lumen 1999), nuclear-localized LUN has been shown to bind to deacetylated histones and to inhibit core histones H3 and H4 acetylation (Galvez et al. 2001), the latter suggesting LUN to function as a histone deacetylase. Numerous studies have shown that promotion of histone acetyl transferase activity, inhibition of histone deacetylase-1 activity, and PTEN acetylation are associated with increased PTEN expression and function (Okomura et al. 2006; Ikenoue et al. 2008; Xiong et al. 2009). While counter-intuitive to LUN effects on PTEN-mediated apoptosis, these findings imply that while LUN may bind to chromatin, its effects on PTEN transcription may not require its direct binding to PTEN gene/promoter regulatory sequences. We have previously shown that GEN up-regulates PTEN transcription by an auto-regulatory loop involving p53 and PTEN; in this mechanism, GEN promotes PTEN-p53 physical association and the recruitment of the PTEN-p53 complex to the p53 binding sites in the PTEN promoter, leading to PTEN transcriptional activation (Rahal and Simmen 2010). LUN effects are not likely to occur through this mechanism since LUN, distinct from GEN, did not promote p53 localization to the nucleus. Further studies to identify the molecular targets of LUN may shed more light on this subject.

Our findings that GEN and LUN exert common and distinct biological effects may have important implications for mammary tumorigenesis and breast cancer prevention. First, the data highlight the necessity for a balance in nutritional uptake to maintain good health and support the notion that consumption of healthy foods with its diverse bioactive components is more advantageous than intake of high amounts of specific dietary supplements with limited biological effects. Second, since bioactive components function through distinct intermediary molecules that ultimately converge at common signaling pathways, the complete understanding of how these molecules facilitate signaling may lead to the development of drugs that target redundant pathways for increased efficacy. In this regard, while it is known that GEN binds to estrogen receptor-β in mammary epithelial cells to promote ER-β signaling and enhance PTEN expression (Rahal and Simmen 2011), molecules mediating entry of LUN into nucleus and its binding to chromatin remain unknown. Finally, because cancer cells evolve to survive and, in the process, utilize numerous survival pathways, it is imperative to evaluate the in vivo combinatory effects of identified bioactive components with distinct biological pathways for their utility in breast cancer prevention and therapy.

In summary, our results demonstrate the ability of soybean peptide LUN to induce PTEN-mediated apoptosis of MCF-7 breast cancer cells. This anti-mammary tumor preventive mechanism of LUN recapitulates a major pathway by which the soy isoflavone GEN may also elicit anti-tumor protection. Our findings are highly relevant to understanding the health effects of soy proteins and suggest the potential benefits of the concerted actions of GEN and LUN for

developing therapeutic strategies against breast cancer. Additional studies using other available breast cancer cell lines of distinct steroid receptor profiles and invasive properties and existing mouse models of human breast cancer should be conducted to confirm these possibilities.

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