Repression of mammary adipogenesis by genistein limits mammosphere formation of human MCF-7 cells

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Abstract

Mammary adipose tissue may contribute to breast cancer development and progression by altering neighboring epithelial cell behavior and phenotype through paracrine signaling. Dietary exposure to soy foods is associated with lower mammary tumor risk and reduced body weight and adiposity in humans and in rodent breast cancer models. Despite the suggested linkage between obesity and breast cancer, the local influence of bioactive dietary components on mammary adiposity for antitumor effects remains unknown. Herein, we report that post-weaning dietary exposure to soy protein isolate and its bioactive isoflavone genistein (GEN) lowered mammary adiposity and increased mammary tumor suppressor PTEN and E-cadherin expression in female mice, relative to control casein diet. To ascertain GEN's role in mammary adipose deposition that may affect underlying epithelial cell phenotype, we evaluated GEN's effects on SV40-immortalized mouse mammary stromal fibroblast-like (MSF) cells during differentiation into adipocytes. MSF cells cultured in a differentiation medium with 40 nM GEN showed reductions in mature adipocyte numbers, triglyceride accumulation, and *Ppar*γ (*Pparg*) and fatty acid synthase transcript levels. GEN inhibition of adipose differentiation was accompanied by increased estrogen receptor β (*Er*β (*Esr2*)) gene expression and was modestly recapitulated by ERβ-selective agonist 2,3-bis-(4-hydroxyphenyl)-propionitrile (DPN). Reduction of *Er*β expression by siRNA targeting increased *Ppar*γ transcript levels and stromal fibroblast differentiation into mature adipocytes; the latter was reversed by GEN but not by DPN. Conditioned medium from GEN-treated adipocytes diminished anchorage-independent mammosphere formation of human MCF-7 breast cancer cells. Our results suggest a mechanistic pathway to support direct regulation of mammary adiposity by GEN for breast cancer prevention.

Key Words

- genistein
- mammary adipocytes
- estrogen receptor-β
- stem/progenitor cells
- breast cancer

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Introduction

Obesity is a major health problem in the United States and globally (Fiegel *et al.* 2010, Swinburn *et al.* 2011). The rising trend in its incidence and prevalence (Parikh *et al.* 2007) warrants serious attention, given its linkage to increased risks for many chronic diseases (Calle & Kaaks 2004, Flynn *et al.* 2006) including breast cancer, the second leading cause of cancer deaths among women (Ligibel 2011). Obesity is an independent risk factor for the development of hormone receptor-positive breast cancer in postmenopausal women (Key *et al.* 2003) and of basal-like breast cancer in premenopausal African–American women (Millikan *et al.* 2007, Rose *et al.* 2007). The connection between obesity and breast cancer has been attributed in part to adipose tissue dysfunction characterized by ectopic fat deposition in abdomen and liver, leading to increased systemic inflammation, oxidative stress, and insulin resistance (Bastard *et al.* 2000, Van Kruijsdijk *et al.* 2009, Olefsky & Glass 2010). In this regard, the pro-inflammatory cytokine interleukin-6, whose levels increase with body mass (Hoene & Weigert 2008), is considered to constitute a viable marker for poor prognosis in breast cancer patients (Hong *et al.* 2007).

Similar to other adipose tissue depots, mammary adipose tissue actively secretes factors including chemokines and cytokines that likely exert paracrine effects on neighboring epithelial cells (Park *et al.* 2011). However, it remains unclear to what extent BMI influences mammary adipose biology and how this relates to breast cancer risk. Recently, a positive association was found between BMI and breast adipocyte size (Morris *et al.* 2011). Moreover, breast tissues of obese women (Morris *et al.* 2011) as well as mammary glands of genetically modified and diet-induced obese mice (Subbaramaiah *et al.* 2011) were shown to display subclinical inflammation characterized by increased P450 aromatase activity and enhanced NF-kB activity. As inflammation is a hallmark of cancer, the correlation of obesity with 'local' mammary adipocyte dysfunction may provide a rational basis for the increased risk and poor prognosis of breast cancer associated with obesity (Anderson & Neuhouser 2012).

Epidemiological, clinical, and preclinical (animal) studies have provided ample evidence indicating that breast cancer development can be influenced by diet and lifestyle (Brennan *et al.* 2010, Patterson *et al.* 2010). High consumption of soy-rich foods containing genistein (GEN) is considered to partly underlie the lower breast cancer incidence in Asian women than in their western counterparts (Wu *et al.* 2008, Hooper *et al.* 2010). The antitumor effects of soy foods and bioactive GEN have been demonstrated in many rodent models of breast cancer (Jin & MacDonald 2002, Simmen *et al.* 2005, Su *et al.* 2007*b*, Verheus *et al.* 2007, Wu *et al.* 2008) to occur through multiple mechanisms. These include tumor suppressor induction and oncogene downregulation (Dave *et al.* 2005, Su *et al.* 2007*a*, Su & Simmen 2009), strong antioxidant activity through redox signaling (Djuric *et al.* 2001), anti-inflammatory actions through inhibition of pro-inflammatory cytokine expression (Chan *et al.* 2009, Hsu *et al.* 2010), increased insulin sensitivity (Fu *et al.* 2012), and anti-adipogenic capacity (Su *et al.* 2009). In the context of obesity, GEN has been shown to decrease body and fat pad weights in rodent models and in humans (Kim *et al.* 2006, Orgaard & Jensen 2008, Park *et al.* 2008) and inhibit adipogenesis (Kandulska *et al.* 1999, Park *et al.* 2008, Zhang *et al.* 2009) and lipid metabolism characterized by decreased expression of key lipogenic enzyme genes such as fatty acid synthase and malic enzyme 1 (Su *et al.* 2009, Takahashi *et al.* 2009). Nevertheless, given that anatomically distinct fat depots exhibit dissimilar biological effects, expression patterns, and abundance of markers of adiposity (McTernan *et al.* 2002, Shi *et al.* 2006, Fain *et al.* 2008), and as the majority of studies evaluating the effects of GEN were previously conducted on non-mammary adipose depots (Park *et al.* 2008, Su *et al.* 2009), the exact function of GEN in mammary adipocytes as a direct conduit to influence normal and pathological mammary epithelial cell behavior is yet to be understood.

Our goals in this study were to determine whether GEN directly inhibits mammary adipogenesis and to evaluate the biological consequence of GEN-mediated adipogenic changes on the expansion of mammary cancer stem-like/progenitor cells. Dysregulation of the biology and function of these mammary epithelial subpopulations is widely considered to initiate breast cancer (Cho *et al.* 2008, Visvader 2009). We here show that limited dietary exposure of mice beginning at post-weaning to soy protein isolate (SPI) and to GEN-fortified Casein (CAS) decreased mammary adipocyte cell size. Using a mouse mammary stromal fibroblastic cell line (MSF) that can differentiate into mature adipocytes under suitable culture conditions (Nakatani *et al.* 2010), we further show that GEN inhibition of mammary adipogenesis is associated with induction of *Erβ* (*Esr2*) expression and inhibition of *Pparγ* (*Pparg*) and *Fasn* expression. Importantly, conditioned

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media from GEN-treated MSF cells showing reduced adipocyte differentiation blocked mammosphere formation, an accepted surrogate of tumorigenesis (Al-Hajj *et al.* 2003, Cho *et al.* 2008), in estrogen receptor (ER+) human breast cancer MCF-7 cells. Our results suggest that GEN inhibition of mammary adipocyte differentiation may alter cancer stem-like expansion, thus linking GEN's 'local' anti-adipogenic effects with potential antitumor actions for breast cancer prevention.

Materials and methods

Animals and diets

Animal experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee, University of Arkansas for Medical Sciences. Mice (B6SJL/J; Jackson Laboratories, Bar Harbor, ME, USA) were housed in polycarbonate cages under conditions of 24 °C, 40% humidity, and a 12 h light:12 h darkness cycle. Dams were fed a semi-purified isocaloric, American Institute of Nutrition (AIN)-93G-based diet containing casein (New Zealand Milk Products, Santa Rosa, CA, USA) (designated CAS diet) as the sole protein source during pregnancy and lactation. At weaning (postnatal day (PND) 21), female pups were exposed to either CAS diet, AIN-93G-based diet containing SPI (Harlan Laboratories, Madison, WI, USA) as the sole protein source, or CAS diet with added GEN (Simmen *et al.* 2005). Mice were allowed to consume food and water *ad libitum*. Mice were killed at PND 35, and for each mouse, the right mammary inguinal gland (#4) was fixed for histological analysis, while the left mammary inguinal gland was harvested for protein analysis by western blotting.

Histological analysis

To determine adipocyte cell size of mouse mammary glands, sections of the right inguinal (#4) mammary gland from PND 35 mice were stained with hematoxylin and eosin. Adipocyte areas were measured in two to three random fields per slide (~200–300 cells per field) from four individual mice per diet group, using Axiovision software (Carl Zeiss AG, Oberkochen, Germany), as described previously (Su *et al.* 2009).

Cell culture and induction of adipogenic differentiation

The mouse MSF cell line has been described previously (Nakatani *et al.* 2010). Cells were propagated in DMEM (Invitrogen) supplemented with 10% FCS (Gibco) and 1% antibiotic–antimycotic solution (Gibco) in 10 cm dishes in an atmosphere of 5% CO_2:95% air at 32 °C. Two days after confluence, cells were plated at a density of 5×10^4 cells/well (in six-well plates), transferred to a 39 °C incubator, and induced to differentiate into adipocytes using the propagation culture medium (above) supplemented with a cocktail containing 1 μg/ml hydrocortisone (Sigma Chemical Co.), 5 μg/ml insulin (Sigma), and 2.5 μg/ml troglitazone (Cayman Chemical, Ann Arbor, MI, USA). GEN (Sigma) dissolved in dimethylsulfoxide (DMSO; Sigma) was added at 40 nM or 2 μM final concentrations, while the ERβ-selective agonist 2,3-bis-(4-hydroxyphenyl)-propionitrile (DPN; Sigma) in DMSO was used at a 50 nM final concentration. The medium was changed every 3 days, and on day 14, cells were collected and processed for various morphological and biochemical determinations (described below).

Oil Red O staining

Differentiated MSF cells grown in six-well plates were fixed with formalin and stained with 0.5% Oil Red O (Sigma). Stained adipocytes were identified in four microscopic fields/well ($n=4$ wells/treatment group) using Axiovision software.

RNA preparation and quantitative real-time PCR (QPCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen) and reverse transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad Laboratories). Gene-specific primers (Supplementary Table 1, see section on supplementary data given at the end of this article) were designed using Primer Express (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). QPCR was performed with SYBR Green Supermix (Bio-Rad Laboratories) and ABI Prism 7000 Detection System (Applied Biosystems), as described previously (Rahal & Simmen 2011). The expression of each target mRNA was calibrated to a standard curve using pooled cDNA stocks and normalized to that of TATA-box binding protein (*Tbp*).

Transient transfection

MSF cells were propagated in culture medium in six-well plates as described above. Cells were serum-starved for 24 h overnight in phenol red-free medium supplemented with charcoal-stripped FCS (0.5% charcoal-stripped FCS;

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Gibco). For siRNA treatment, a pool of double-stranded siRNAs targeting mouse *Erβ* (Dharmacon, Lafayette, CO, USA) at 50 nM final concentration was used. Non-targeting siRNA pool (scrambled (sc) siRNAs; siCONTROL, Dharmacon) was used at the same concentration. The siRNAs were introduced by Lipofectamine 2000 (Invitrogen) in Gibco OPTI-MEM I-reduced serum medium (Invitrogen). Forty-eight hours post-transfection, MSF cells were treated with differentiation medium with or without added GEN (40 nM) or DPN (50 nM) and harvested 14 days later.

Triglyceride quantification assay

Triglyceride content of pre-adipose or adipose cells was quantified in cell extracts using a Biovision triglyceride assay kit (Biovision, Inc., Milpitas, CA, USA), following the manufacturer's instructions. Absorbance was read at 570 nm.

Western blot analysis

Whole cell extracts from mammary tissues and differentiated MSF cells were prepared and subjected to immunoblotting as described previously (Su *et al*. 2009). Primary antibodies used were anti-PTEN (1:1000; Cell Signaling, Danvers, MA, USA), anti-E-cadherin (1:1000; BD Transduction Laboratory, San Jose, CA, USA), anti-ERβ (1:1000; Santa Cruz Biotechnology), anti-α-tubulin (1:2000; Santa Cruz Biotechnology), and anti-β-actin (1:2000; Sigma–Aldrich). Blots were stripped with Restore Western blot stripping buffer (Pierce Biotechnology, Rockford, IL, USA) before reprobing with a new antibody. Immunoreactive proteins were visualized with Amersham ECL Plus kit (GE Health Care Life Sciences, Piscataway, NJ, USA). Digital images were captured using the GE Image Scanner III detection system and quantified using Quantity One software (Bio-Rad Laboratories).

Adipose-conditioned medium

Conditioned medium (CM) was prepared from differentiated MSF cells following published protocols (Su *et al*. 2009). Briefly, cells were treated with GEN (40 nM) or DMSO (vehicle) starting at day 12 of the differentiation protocol. Following treatments for 48 h, cells were then washed with PBS to remove adherent traces of GEN. Washed cells were further incubated in low-serum (0.5% FCS)-containing DMEM overnight. CM was collected, passed through a 40 μm filter (Fisher-Scientific, Waltham, MA, USA), and stored at −80 °C before use in cell viability and mammosphere assays.

Cell viability assay

The human breast cancer cell line MCF-7 was purchased from American Type Tissue Collection (ATCC, Manassas, VA, USA) and propagated in DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic solution at 37 °C. Cells were seeded in 96-well plates (5×10^3cells/well) at the same temperature and cultured for 5 days with adipose-CM (1:1 vol:vol with culture medium; prepared above) from MSF treated with and without GEN (40 nM). Cell viability was evaluated using 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay (MTT; ATCC) following the manufacturer's protocol. Absorbance (570 nm) reflects the amount of formazan formed and was considered a direct measure of metabolically active cells in culture.

Mammosphere formation assay

Formation of mammospheres by MCF-7 cells followed previously described protocols (Montales *et al*. 2012). Adipose-CM from MSFs treated or not with GEN (40 nM) was mixed with mammosphere-plating medium at 1:1 vol:vol ratio. Mammosphere-plating medium consisted of phenol red-free, serum-free minimal essential medium (MEM), supplemented with B27 (1×; Invitrogen), 20 ng/ml human basic fibroblast growth factor (Invitrogen), 20 ng/ml human epidermal growth factor (Invitrogen), 10 μg/ml heparin (Sigma), 1% antibiotic–antimycotic solution (Invitrogen), and 100 μg/ml gentamicin (Sigma). MCF-7 cells were cultured in the mixed medium in ultra-low attachment plates (Corning, Inc., Corning, NY, USA). Medium was refreshed after 3 days and the appearance of primary spheres (P1) was evaluated at day 5. To assess sphere numbers over the second passage (P2), P1 mammospheres were collected at day 5, dissociated with 0.05% trypsin (Invitrogen) into single cell suspensions, filtered using a 40 μm sieve, and replated in ultra-low attachment plates, with no additional treatments. P1 and P2 (collected at day 7) mammospheres were manually counted and sphere diameter was measured using Axiovision software.

To evaluate whether the inflammatory cytokine interleukin-6 secreted by mature adipocytes could reverse the direct effect of GEN on mammosphere-forming ability, MCF-7 cells were cultured in mammosphere-plating

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medium alone or with added IL6 (50 ng/ml; R&D Systems, Minneapolis, MN, USA) in the presence or absence of GEN (40 nM) for 5 days. P1 mammospheres were collected at day 5 and replated for a second passage (P2) with no additional treatments. P2 mammospheres were manually counted at 7 days post-plating.

Statistical analysis

All *in vitro* experiments were conducted at least two times, each in quadruplicate. Results are expressed as the means ±S.E.M. Data were analyzed using Student's *t*-test or one-way ANOVA. Statistical significance of differences between groups was evaluated using SigmaStat version 3.5 for Windows. $P \leq 0.05$ values were considered to be significant.

Results

Dietary SPI and GEN exposure reduce mammary adipocyte cell size and increase mammary expression of tumor suppressor genes

Dietary exposure of female mice to SPI or GEN from PND 21 (weaning) to PND 35 did not affect body weights, relative to those of CAS diet-fed mice (data not shown). However, mice in the SPI and GEN diet groups showed smaller mammary fat cell sizes compared with those of mice in the CAS group. Representative pictures of H&E-stained sections of the mammary fat pads for each dietary group are shown in Fig. 1A. The majority (~90–95%) of mammary adipocytes in the SPI and GEN groups were in the size range of <500–1500 μm^2 while only 60% of the

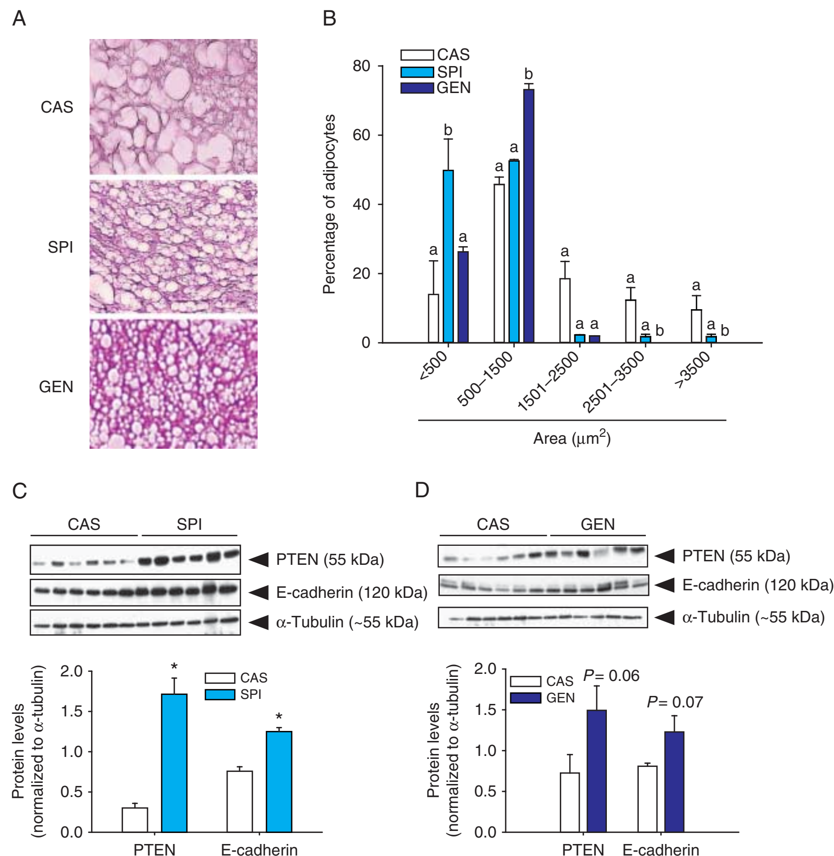

Figure 1
Dietary effects of CAS, SPI, and GEN on mouse mammary adipocyte size and tumor suppressor expression. Mammary tissues were collected from female mice at postnatal day (PND) 35 after exposure to CAS, SPI, or CAS+GEN (designated GEN) diets beginning at PND 21 (weaning). (A) Representative hematoxylin–eosin-stained sections of mammary fat pads of mice from the three diet groups. Magnification 40×. (B) Dietary exposure to SPI and GEN reduced fat cell sizes compared with CAS diet. Values are means±S.E.M., $n=4$ mice/dietary group. Superscripts with different letters differed at $P \leq 0.05$. (C and D) Western blot analysis of PTEN and E-cadherin protein levels in mammary tissue of mice from CAS and SPI (C) and CAS and GEN (D) diet groups. Each lane represents an individual mouse and contains 50 μg of whole tissue extract protein. α-Tubulin was used as normalizing control for protein loading. Immunoreactive bands were quantified by densitometry. Normalized values relative to α-tubulin are presented as histograms. $^*P \leq 0.05$ (relative to CAS).

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adipocytes in the CAS group fell in this range. By contrast, the CAS group had 40% of the adipocytes in the size range of >1500–3500 μm^2 compared with <5% in the SPI and GEN groups. The decreased adipocyte size with dietary SPI exposure was accompanied by increased expression of tumor suppressors PTEN and E-cadherin in mammary tissue (Fig. 1C). Similar increases in the expression levels of PTEN and E-cadherin proteins were observed in the GEN group, although these were not as robust as those found in the SPI group (Fig. 1D).

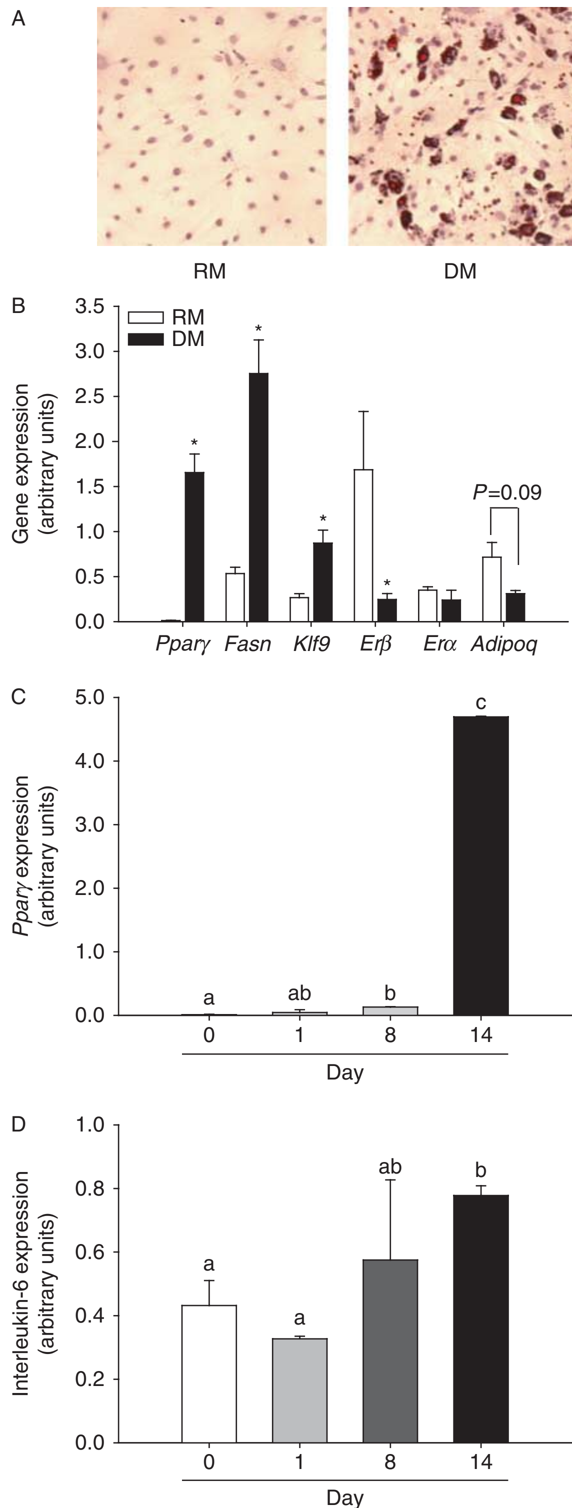

Figure 2
Hormone-induced differentiation of mouse stromal fibroblast-like (MSF) cells to adipocytes. (A) Representative pictures of undifferentiated (left panel) and differentiated (right panel) MSF cells after 14 days of culture in regular (RM) and differentiation (DM) medium respectively. Cells were stained with Oil Red O. Magnification 100×. (B) Transcript levels of *Pparγ*, *Fasn*, *Klf9*, *Erβ*, *Erα*, and *Adipoq* genes were quantified in undifferentiated MSFs (RM) and mature adipocytes (DM) by QPCR. Transcripts were normalized to that of TATA-box binding protein (*Tbp*). Results (means ± S.E.M.) shown are representative of three independent experiments, each done in quadruplicate. *Different from control, $P \leq 0.05$ using *t*-test (B). (C) Time course of *Pparγ* gene expression during differentiation of MSF cells. (D) Time course of *Il6* gene expression during differentiation of MSF cells. For (C and D), transcript levels were quantified by QPCR and normalized to that of *Tbp*. Results (means ± S.E.M.) shown are representative of two independent experiments, each done in quadruplicate. Superscripts with different letters differed at $P \leq 0.05$ by one-way ANOVA.

MSF cells differentiate to mature adipocytes after hormonal induction

In this study, we utilized a recently characterized MSF cell line established from primary cultures of mouse mammary fat pads by infection with the temperature-sensitive SV40LT vector pMESVTS (Nakatani *et al*. 2010). Unlike in the initial report wherein MSF cells were cultured in extracellular matrix, hormonal induction of MSF cells into adipocytes in the present study was carried out in culture dishes at 39 °C. The shift to higher incubation temperature results in the inactivation of the temperature-sensitive simian virus-40 large T antigen (tsSV40LT), allowing the cells to differentiate with the hormonal treatments. To demonstrate that MSF cells are converted into mature adipocytes under these conditions, cells grown to confluence at 32 °C were treated with the hormonal cocktail of glucocorticoids (troglitazone and hydrocortisone) and insulin and incubated at 39 °C. As control, a parallel set of cells grown at 39 °C in medium without the added hormones (regular medium, RM) was used. When cultured in the presence of the differentiation cocktail (designated DM) for 14 days, MSF cells underwent significant phenotypic changes with accompanying lipid accumulation (measured by Oil Red O staining), indicative of conversion to mature adipocytes, compared with those grown in RM (Fig. 2A). Lipid accumulation was associated

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with the dramatic induction in gene expression of molecular markers of adipocyte differentiation namely *Pparγ*, fatty acid synthase (*Fasn*), and the pro-adipogenic Pparγ transcription factor Krüppel-like factor 9 (*Klf9*; Rosen & Spiegelman 2000, Pei *et al.* 2011; Fig. 2B). Transcript levels for *Erβ* (*Esr2*), known to inhibit PPARγ activity (Foryst-Ludwig *et al.* 2008), were downregulated upon MSF differentiation to mature adipocytes. *Erα* (*Esr1*) gene expression did not differ in undifferentiated and differentiated MSF while adiponectin expression (*Adipoq*) tended to decrease ($P=0.09$) with differentiation (Fig. 2B).

To determine whether incubation for 14 days constitutes an optimal period for MSF differentiation, *Pparγ* expression at different time points (days 1, 8, and 14) during differentiation was evaluated by QPCR. *Pparγ* transcripts were dramatically induced between days 8 and 14 of culture (Fig. 2C), confirming maximal MSF conversion into mature adipocytes at day 14 (Fig. 2A). The transcript levels for the mammary adipogenic factor *Il6* (Walter *et al.* 2009) similarly displayed modest but significant increases during this period (Fig. 2D).

Soy isoflavone GEN dose dependently inhibits MSF differentiation to mature adipocytes

Given that dietary exposure to SPI and GEN is protective against mammary tumorigenesis in rodent models of breast cancer (Fritz *et al.* 1998, Jin & MacDonald 2002, Simmen *et al.* 2005, Su *et al.* 2007*b*) and that dietary exposure to GEN resulted in decreased mammary adipocyte size (Fig. 1A and B), we evaluated whether mammary adipocytes constitute relevant direct targets of GEN for mammary tumor protection. MSF cells were grown to confluence and then differentiated with the addition of hormone cocktail in the presence or absence of physiologically relevant (40 nM) and supra-physiological (2 μM) levels of GEN (Rahal & Simmen 2010, Montales *et al.* 2012). As shown in Fig. 3A and B, the phenotypic formation of mature adipocytes (evaluated by Oil Red O staining) was significantly inhibited by 40 nM but not by 2 μM GEN. Expression of adipogenic and lipogenic genes was measured in control and GEN-treated (40 nM, 2 μM) cells after day 14 of culture (Fig. 3C). *Pparγ* and *Fasn* gene expression in differentiated MSF cells were decreased by 40 nM GEN while GEN at 2 μM had no measurable effect on these genes' expression. *Erβ* transcript levels were upregulated with 40 nM but not with 2 μM GEN treatment, while those for *Erα* were unaffected by both GEN concentrations. Western blotting showed that GEN-treated mature adipocytes (40 nM GEN) had lower ERβ protein levels than non-treated mature adipocytes in whole cell extracts (Fig. 3D and E), in contrast to the increased levels of corresponding transcript (Fig. 3C).

GEN inhibition of adipocyte differentiation involves ERβ signaling

ERβ is known to exert anti-proliferative (Vivar *et al.* 2010) and anti-adipogenic (Foryst-Ludwig *et al.* 2008) effects. To further investigate whether GEN inhibition of adipogenesis is mediated solely by ERβ signaling, we performed two studies. First, we determined whether the ERβ-selective ligand DPN mimics the effects of GEN on adipocyte differentiation. MSF cells were cultured from confluence in DM with added GEN (40 nM), DPN (50 nM), or DMSO (vehicle control) and treated cells were evaluated for effects on adipocyte differentiation 14 days later. As shown earlier (Fig. 3), GEN reduced adipocyte conversion of MSF cells (Fig. 4A). DPN effects were less robust but paralleled the effects of GEN. Moreover, GEN and DPN comparably reduced lipid accumulation in mature adipocytes (Fig. 4B). DPN showed no effects on *Pparγ* and *Fasn* gene expression (Fig. 4C). However, similar to GEN, DPN modestly increased ($P=0.06$) *Erβ* transcript, with coincident reduction in corresponding protein levels (Fig. 4D). The protein levels of β-actin, as normalizing control, did not differ for DM or DM+DPN-treated cells (data not shown). In the second experiment, we evaluated the specificity of ERβ effects on adipogenesis. MSF cells transfected with *Erβ* siRNAs (and in parallel, non-targeting scrambled siRNAs for controls) were allowed to differentiate into mature adipocytes without or with added GEN (40 nM) and DPN (50 nM). MSF cells transfected with *Erβ* siRNA showed dramatically decreased *Erβ* transcript levels relative to those transfected only with scrambled siRNAs (Fig. 4E). The increase (by 31%) in adipose conversion of MSF cells with *Erβ* siRNAs targeting (relative to scRNA-treatment) (Fig. 4F) was accompanied by a significant increase in *Pparγ* gene expression but no effect on *Fasn* gene expression (Fig. 4G). Co-treatment of MSFs with si*Erβ*+GEN decreased MSF conversion to adipocytes to the level of control (scRNA-treated) cells (Fig. 4F). By contrast, adipose conversion of si*Erβ*-treated MSF cells was not affected by co-treatment with DPN (Fig. 4F).

GEN diminishes mammosphere formation of human breast cancer cells by paracrine signaling

Recent studies have implicated stem/progenitor cells in the initiation and maintenance of breast cancer (Al-Hajj

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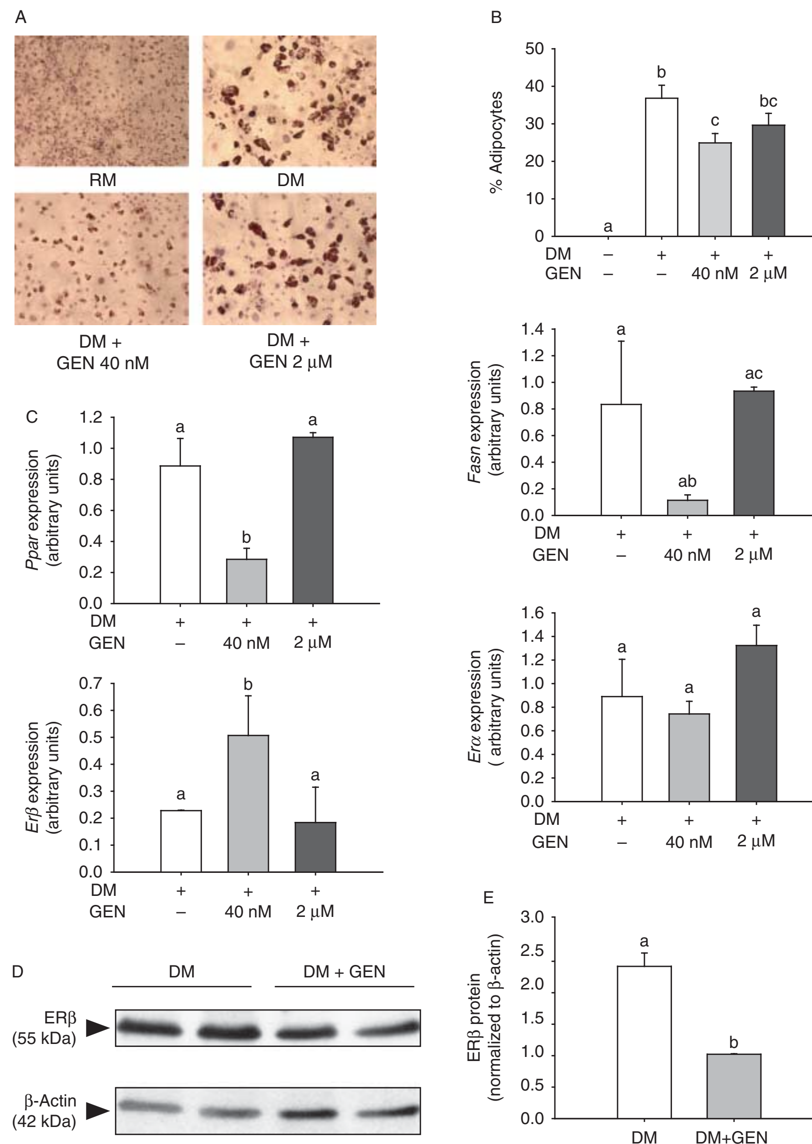

Figure 3
Dose-dependent inhibition of MSF conversion to adipocytes by genistein (GEN). (A) Representative pictures of MSF cells after 14 days of culture in regular (RM) medium and in differentiation medium without (DM) and with 40 nM or 2 µM GEN (DM+GEN) treatments. Cells were stained with Oil Red O. Magnification 100×. (B) The percentage of MSF cells converted to adipocytes, after staining with Oil Red O, was calculated in four microscopic fields/well ($n=4$ wells/treatment group) using Axiovision software. Superscripts with different letters differed at $P \leq 0.05$ by one-way ANOVA. (C) Gene expression in mature adipocytes cultured in DM±GEN (40 nM or 2 µM). Transcript levels were quantified and normalized to the control gene *Tbp*. Results (mean±S.E.M.) shown are representative of two independent experiments, each done in quadruplicate. Superscripts with different letters differed at $P \leq 0.05$ by one-way ANOVA. RM, regular media; DM, differentiation media. (D) Western blots of ERβ protein in mature adipocytes cultured in DM±40 nM GEN. β-Actin was used as normalizing control for protein loading. (E) Graph shows normalized levels of ERβ protein (relative to β-actin) as a function of GEN treatment during MSF differentiation. Data are expressed as mean±S.E.M. from two independent experiments carried out in triplicate. Superscripts with different letters differed at $P \leq 0.05$ by *t*-test.

et al. 2003, Cho *et al.* 2008, Visvader 2009). Further, we (Montales *et al.* 2012) and others (Kakarala *et al.* 2009, Pandey *et al.* 2011) have shown that dietary factors may directly suppress growth of cancer stem-like/progenitor cells *in vitro*, as measured by mammosphere formation. To evaluate whether GEN inhibition of mammary adipocyte differentiation may indirectly influence tumor development by altering stem-like/progenitor cell numbers, mammosphere formation of ER+ human breast cancer MCF-7 cells was determined in the presence of CM from GEN-treated adipocytes (ACM (GEN)) or adipocytes treated with DMSO vehicle (ACM). To exclude the

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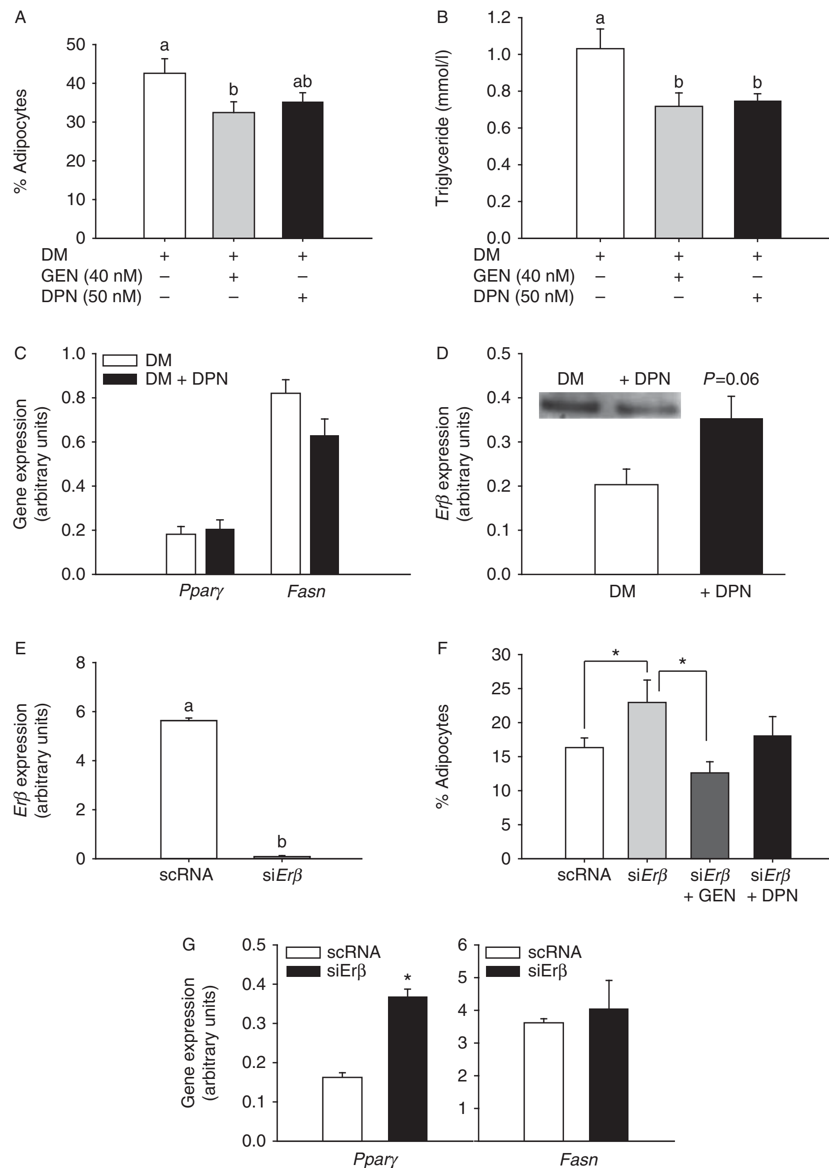

Figure 4
GEN inhibition of MSF conversion to adipocytes involves ERβ signaling. MSF cells were cultured in DM (vehicle control), DM+GEN (40 nM), or DM+DPN (50 nM) for 14 days, and the percentage of MSF cells converted to adipocytes, after staining with Oil Red O, was calculated. (B) Amounts of triglycerides (mmol/l) in mature adipocytes after treatment with DM alone or DM with added GEN or DPN. (C) Expression of *Pparγ* and *Fasn* in MSFs incubated in DM without and with DPN. Transcript levels were quantified by QPCR and normalized to the control gene *Tbp*. For (A, B, and C), results (means ± S.E.M.) shown are representative of two independent experiments, each done in quadruplicate. Superscripts with different letters differed at $P \leq 0.05$, by one -way ANOVA. (D) ERβ transcript and protein levels in MSFs incubated in DM without or with DPN. Transcript levels were quantified by QPCR and normalized to the control gene *Tbp*. Inset, A representative western blot of ERβ protein in DM and DM+DPN-treated cells. β-Actin levels as normalizing control for protein loading did not differ between samples (data not shown). (E) Knockdown of *Erβ* by specific *Erβ* siRNAs in transfected MSFs resulted in the loss of *Erβ* gene expression. Cells were transfected in parallel with non-targeting (scRNA) siRNA. Transcripts were normalized to *Tbp*. Superscripts with different letters differed at $P \leq 0.05$, by t-test. (F) The percentage of MSF cells converted to adipocytes after transfection with scRNA (control) or si*Erβ* siRNA (without or with 40 nM GEN or 50 nM DPN treatments) was measured after staining with Oil Red O. Results (means ± S.E.M.) shown are representative of two independent experiments, each performed in quadruplicate. Means (± S.E.M.) designated with asterisks differed at $P \leq 0.05$. (G) Expression of *Pparγ* and *Fasn* in MSFs transfected with control (scrambled) or *Erβ*-specific siRNAs. Transcript levels were quantified by QPCR and normalized to the control gene *Tbp*. Results (means ± S.E.M.) shown are representative of two independent experiments, each performed in quadruplicate.

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potential effect of adipocyte CM on MCF-7 proliferation that may contribute to changes in mammosphere numbers, MCF-7 cells plated in culture dishes were treated with ACM or ACM (GEN) (diluted 1:1 (vol:vol) ratio with regular MCF-7 culture medium) and assayed for cell viability after 5 days. Adipocyte CM from vehicle-(DMSO) or GEN-treated adipocytes had no effect on MCF-7 cell viability (Fig. 5A). By contrast, there were striking reductions in the number (Fig. 5B) and size (diameter) (Fig. 5C and D) of mammospheres formed at passage 2 (P2) with ACM (GEN), relative to control ACM treatment.

The cytokine IL6, which is synthesized by differentiated adipocytes (Fig. 2E), has been shown to induce the expansion of mammary cancer stem/progenitor cells (Sansone *et al*. 2007, Iliopoulos *et al*. 2011). To determine whether GEN inhibits adipocyte-derived IL6 expression as a way to inhibit mammosphere formation, we evaluated *IL6* transcript levels in GEN-treated adipocytes. Unlike for *Pparγ* and *Fasn* (Fig. 3C), GEN had no effect on *Il6* transcript levels (data not shown). Moreover, IL6 at physiological levels (50 ng/ml) failed to promote mammosphere formation as well as to reverse the previously demonstrated inhibitory effects of 40 nM GEN (Montales *et al*. 2012) on mammosphere-forming ability of MCF-7 cells (Fig. 5E).

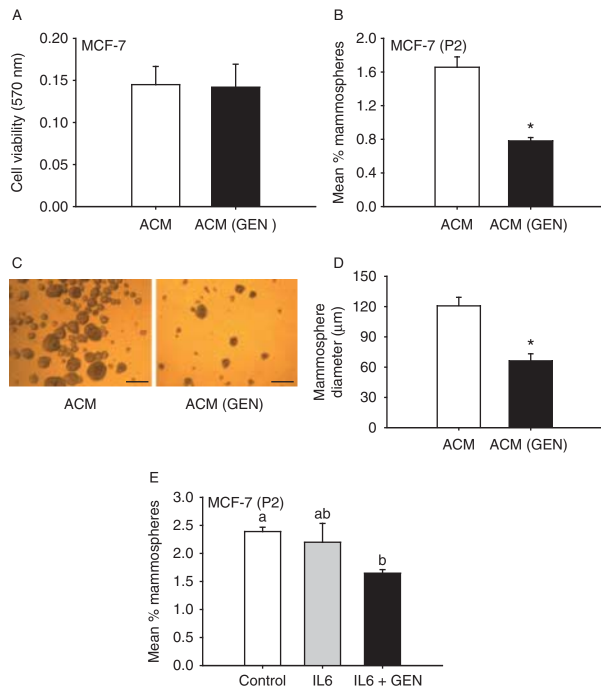

Figure 5
Mammosphere formation of MCF-7 cells cultured in adipose-conditioned medium from mature adipocytes treated with vehicle alone (ACM) or with added GEN (ACM (GEN)) before collection. (A) MCF-7 cells plated in adherent culture plates were incubated with ACM or (ACM (GEN)), and cell proliferation was measured after 5 days by MTT assay. Results (means ± S.E.M.) shown are representative of three independent experiments, each carried out in quadruplicate. (B) MCF-7 cells were seeded using mammosphere-plating medium (1:1 vol:vol ratio with either ACM or ACM (GEN) at a density of 2500 cells/well in non-adherent culture dishes to form primary mammospheres (P1)). P1 mammospheres (collected at day 5 post-plating) were dispersed, plated at a density of 1000 cells/well, and cultured in mammosphere-plating media without further addition of ACM. Mammospheres formed after 7 days (passage 2, P2) were counted. Data presented are for P2, expressed as % mean MFU ± S.E.M. Three independent experiments were performed, each in quadruplicate; shown are representative data. *$P \leq 0.05$, by *t*-test. (C) Representative picture of secondary mammospheres (P2 mammospheres). Scale bar = 100 µm. (D) Mammosphere diameters from P2. A total of 30–50 mammospheres was counted for each treatment group. *Values (means ± S.E.M.) differed at $P \leq 0.05$. (E) MCF-7 cells were cultured in low attachment plates in mammosphere-plating medium with added IL6 (50 ng/ml) or a combination of IL6 (50 ng/ml) and GEN (40 nM). Results (mean % MFU ± S.E.M. of secondary mammospheres (P2)) shown are representative of three independent experiments, each performed in quadruplicate. Values with different letters differed at $P \leq 0.05$, by one-way ANOVA.

Discussion

This study provides new mechanistic insights that may partly explain the favorable association of soy food intake with decreased breast cancer risk in the human population (Wu *et al*. 2008, Hooper *et al*. 2010) and in rodent models of human breast cancer (Jin & MacDonald 2002, Simmen *et al*. 2005, Verheus *et al*. 2007, Wu *et al*. 2008). Using a recently characterized mouse MSF cell line that can differentiate into adipocytes with hormonal treatments, we provide evidence for the specificity of the anti-adipogenic effect of the major soy isoflavone GEN on mammary adipose cells. Our findings based on *in vivo* (mice fed GEN diet) and *in vitro* (MSF conversion to mature adipocytes) models expand previously known effects of GEN on other anatomically distinct adipose depots, which have been correlated with its antitumor effects in relation to obesity's role in breast cancer development (Kim *et al*. 2006, Orgaard & Jensen 2008, Park *et al*. 2008). We report that by inhibiting MSF conversion to adipocytes, GEN at a physiologically relevant concentration (40 nM) can indirectly reduce the expansion of stem-like/progenitor cells that are present as a highly limited population in breast cancer cells and that are considered to initiate and/or maintain mammary tumors (Al-Hajj *et al*. 2003, Cho *et al*. 2008, Visvader 2009). We show that GEN-mediated reduction in adipose conversion of MSF cells involves the marked repression of the transcriptional program of adipogenesis with noted decreases in transcript levels of key adipocyte differentiation and lipogenic factors namely PPARγ, FASN, and KLF9 (Rosen &

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Spiegelman 2000). Finally, we demonstrate that by inducing transcript levels for *Erβ*, which has been shown to mediate inhibition of *Pparγ* transcriptional activity and thus adipogenesis (Foryst-Ludwig *et al.* 2008), GEN alters the trajectory of pre-adipocyte conversion to mature adipocytes. Our present findings of a direct influence of GEN on the adiposity of the mammary fat pad *in vivo* and *in vitro*, together with our previous demonstrations that GEN i) directly enhances mammary epithelial E-cadherin–β-catenin interactions (Su *et al.* 2009), ii) promotes mammary epithelial response to adiponectin (Rahal & Simmen 2011), and iii) inhibits mammosphere expansion (Montales *et al.* 2012), reveal how GEN (as a paradigm for bioactive dietary factors with antitumor effects) may simultaneously target two key mammary compartments (stroma and epithelium) that constitute obligate partners for breast cancer development and progression (Fig. 6).

Our aims in the present study were twofold. First, we wanted to mechanistically evaluate a direct role for GEN on mammary adipocyte differentiation, given our observations in rats (Su *et al.* 2009) that mammary adipocyte cell size is reduced with limited dietary exposure to SPI. Secondly, we sought to delineate how GEN effects on mammary adipocytes may translate to favorable effects on neighboring epithelial subpopulations from which tumors arise. While many bioactive components (Pabona *et al.* 2013) including other isoflavones are present in soy foods, GEN is considered to underlie the major antitumor effects associated with dietary soy intake, partly because it is bioavailable and targets diverse cell types (Verheus *et al.*

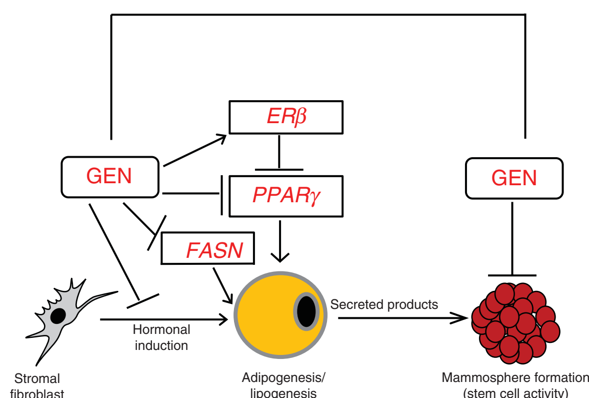

Figure 6
Postulated model for GEN control of mammary adipocyte differentiation. GEN influences *ERβ* (increase; designated →), *PPARγ* (decrease; designated by ⊣), and *FASN* (decrease) gene expression to directly inhibit adipocyte differentiation and to indirectly repress the expansion of mammary cancer stem-like/progenitor cells (measured by mammosphere formation). This model incorporates our previous demonstration of a direct action of GEN on inhibiting mammosphere formation (Montales *et al.* 2012).

2007, Xiao *et al.* 2008, Rahal & Simmen 2011). Our findings that GEN decreases mammary adipocyte cell size *in vivo* and inhibits MSF conversion to adipocytes *in vitro*, the latter resulting in repression of mammosphere formation of human breast cancer cells (a well-accepted surrogate for mammary tumorigenesis), identify GEN as a key mammary anti-adipogenic factor with significant potential for chemoprevention. Moreover, as the negative effect of limited dietary exposure to GEN on mammary adipocyte sizes did not occur simultaneously with observable changes in whole body weights, our results suggest that GEN-associated mammary anti-adipogenic effects *in vivo* may occur early and independent of its metabolic actions on other adipose depots (e.g. increasing systemic insulin sensitivity) for decreasing breast cancer risk. Studies utilizing diet-induced or genetically induced obese mouse models to delineate the temporal response of other fat depots in comparison to mammary adipose cells with dietary intake of GEN or of foods containing this isoflavone may help define the significance of GEN's context-specific anti-adipogenic effects for breast cancer prevention or chemoprevention.

Our findings highlight the participation of ERβ signaling as a mediator of GEN's effects on adipocyte differentiation via a linear pathway involving GEN's induction of *Erβ* expression and consequently of *Pparγ* expression. While GEN displays a higher binding affinity (by 20-fold) for ERβ than for ERα isoform (Kuiper *et al.* 1997) and likely exerts its actions by binding to this ER isoform, our findings suggest that ERβ is not the sole mediator of GEN's inhibition of mammary adipocyte differentiation. We interpret GEN's reversal of siERβ-induced adipocyte differentiation to indicate that GEN does not bind to ERβ to mediate adipogenesis and/or that GEN induction of *Erβ* expression overrides the effects of *Erβ* knockdown under our experimental conditions. Our results showing that with *Erβ* knockdown the selective ERβ agonist DPN tended to inhibit adipocyte differentiation but not as robustly as GEN supports the latter possibility as DPN also increases ERβ expression but not to the same extent as GEN. Moreover, the coincident upregulation of *Pparγ* expression with *Erβ* knockdown, in the absence of comparable effects on FASN, coupled with GEN's effects on FASN suggests that the robust effects of GEN on adipocyte differentiation may be attributed to GEN's cumulative inhibition of ERβ-dependent *Pparγ* gene expression and of ERβ-independent *Fasn* gene expression. The latter suggests other likely mediators of GEN, which can function as a tyrosine kinase inhibitor (Akimoto *et al.* 2001, Gadgeel *et al.* 2009) or via a G-protein-coupled ER (Lin *et al.* 2009).

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Surprisingly, DPN albeit modestly enhancing *Erβ* gene expression did not induce *Pparγ* expression similar to GEN. A possible explanation for this apparent discrepancy may lie in the distinct conformations of the transcriptional complex formed by DPN/ERβ compared with GEN/ERβ, as a consequence of their recruitment of distinct nuclear co-regulators. It is widely acknowledged that the conformation of the nuclear receptor-transcriptional complex dictates the strength of its transcriptional activity and hence target gene expression (Zwart *et al.* 2011). Alternatively, ERβ inhibition of *Pparγ* transcriptional activity may vary with anatomically distinct fat depots as the functional linkage between ERβ and PPARγ was demonstrated only for gonadal fat (Foryst-Ludwig *et al.* 2008) and not mammary adipocytes. The lack of DPN inhibitory effects on *Fasn* gene expression may also contribute to its lesser overall activity on adipocyte differentiation when compared with GEN.

We had previously shown that in the mouse mammary epithelial cell line HC11, which expresses both ER isoforms, ERβ-mediated GEN actions enhanced tumor suppressor PTEN expression (Rahal & Simmen 2011) and promoted membrane-bound E-cadherin interactions with β-catenin to attenuate oncogene Wnt1-stimulated cell proliferation (Su & Simmen 2009). Interestingly, while promotion of ERβ signaling by GEN in HC11 mammary epithelial cells occurred without altering *Erβ* transcription (Rahal & Simmen 2011), GEN effects on inhibiting adipocyte differentiation involve induction of *Erβ* transcript levels. Our findings of increased *Erβ* transcript levels with 40 nM but not 2 μM GEN, coincident with decreased transcript levels for *Pparγ* and *Fasn*, are consistent with a previous report that activation of ERβ downregulates PPARγ transcriptional activity at low (<1 μM) but not at high (>1 μM) GEN doses (Dang *et al.* 2002). While we have no experimental data to address the observed discrepancy in ERβ transcript and protein levels with GEN treatment leading to inhibition of adipocyte differentiation, we suggest that this may be related to increased ERβ protein degradation and/or turnover upon ligand binding and induction of *Erβ* transactivity, as previously reported for ERα. Transactivation of ERα requires estrogen-dependent receptor ubiquination and degradation (Lonard *et al.* 2000). Moreover, the selective ERβ ligand, DPN, elicited the same effects as GEN on ERβ transcript and protein levels.

The present study evaluated the possibility that GEN by directly influencing mammary adipogenesis may indirectly modify epithelial behavior. We herein demonstrated that adipose-CM from mature adipocytes treated with GEN suppressed mammosphere formation of ER+ MCF-7 cells, which have been previously shown to exhibit mammary tumor-initiating properties (Dontu *et al.* 2003, Filmore & Kuperwasser 2008). A relevant question raised by our findings relates to the identity of signaling molecule(s) mediating the observed inhibition of mammosphere-forming activity by adipose-CM from GEN-treated adipocytes. The composition of adipose-CM from control and GEN-treated adipocytes was not compared here and is thus considered a limitation of this study. Nevertheless, we considered IL6 as a candidate paracrine factor as its secretion from adipose stromal cells (Sansone *et al.* 2007) has been shown to promote migration and invasion of breast cancer cells (Walter *et al.* 2009). Moreover, IL6 has been implicated in breast cancer stem cell renewal as well as in the conversion of normal mammary stem cells to breast cancer stem cells (Iliopoulos *et al.* 2009, 2011). Further, its expression increases in anatomically distinct fat depots (Bastard *et al.* 2000, Hoene & Weigert 2008) during adipogenesis, consistent with our demonstration for the MSF cells (Fig. 2D). However, GEN had no effect on IL6 expression in mammary adipocytes. Moreover, IL6 did not induce mammosphere formation and failed to reverse the inhibitory action of GEN on mammosphere-forming ability of MCF-7 cells. Thus, GEN's actions on mammary adipocytes and consequently on epithelial behavior are independent of IL6.

In summary, this study provides the first evidence to our knowledge of dietary factor regulation of MSF conversion to adipocytes and the potential consequence of this regulation on the functional expansion of mammary cancer stem-like/progenitor cells *in vivo*. This study suggests that by inhibiting 'local' adipogenesis in the mammary fat pad, dietary factors may have mammary tumor-preventative effects independent of their effects on body size or adiposity. Further, this study highlights the physiological importance of dietary factors in coordinating multiple local and systemic targets to generate the optimal 'niche' for mammary stem/progenitor cells so as to limit their expansion and subsequent tumor initiation.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-12-0520.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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