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Breast cancer risk is highly modifiable by diet; however, mechanisms underlying dietary protection against mammary tumorigenesis remain poorly understood. A proportion of breast carcinomas is associated with deregulation of β-catenin stability and amplification of c-Myc expression. We recently showed that dietary exposure to the soy isoflavone genistein (Gen) inhibited Wnt transduction in rat mammary epithelial cells in vivo. Here, we explored the role of Gen on cell adhesion protein, E-cadherin, expression to downregulate β-catenin proto-oncogene function. In mammary glands of female rats exposed to dietary Gen, E-cadherin and β-catenin protein levels were increased, concurrent with higher β-casein gene expression. In HC11 mouse mammary epithelial cells, Gen diminished basal and Wnt-1-induced cell proliferation and attenuated Wnt-1 targets c-Myc and Cyclin D1 expression. Whereas, Gen had no effect on E-cadherin transcript levels, the abundance of membrane E-cadherin protein and of E-cadherin–β-catenin adhesion complex was increased by Gen, attendant with downregulation of Wnt-1-induced free β-catenin accumulation in cytosol. Gen inhibition of Wnt-induced c-Myc expression was mimicked by an estrogen receptor (ER)-β-specific but not ER-α-specific agonist and was attenuated with loss of ER-β expression, concordant with decreased E-cadherin expression. E-cadherin small-interfering RNA targeting eliminated Gen inhibition of Wnt-stimulated c-Myc expression and promoted Gen induction of basal c-Myc transcript levels and subsequent proliferation. Our studies identify E-cadherin as a Gen cellular target and demonstrate that the dichotomy in mammary epithelial response to Gen may be a function of cellular E-cadherin expression.

Introduction

Breast cancer is a complex disease with multiple histopathologies that arise from genetic and epigenetic modifications of genes involved in growth control, DNA repair, apoptosis and differentiation (1). The likelihood of developing breast cancer at some time in a woman’s life is approximately one in eight (12%), with an estimated 180 000 new cases and 50 000 deaths reported annually in the USA alone (2). There is increasing acceptance based on epidemiological studies that breast cancer has its origin during early development, and this can be influenced by nutrition (3,4). The lower incidence of breast cancer in Asia (by 4-fold) relative to that in Western countries (5) has been linked to high consumption of soy-rich foods by Asian women. In two recently published studies of soy consumption by Japanese and Dutch women, increased plasma concentration of the major soy isoflavone genistein (Gen) due to higher dietary intake of soy foods was associated with reduced breast cancer risk (6,7). Such findings have triggered a number of clinical trials (http://www.clinicaltrials.gov/) on the antitumor properties of soy and Gen. Nevertheless, conflicting data due to variations in dose, timing, route, frequency and physiologic contexts of exposure have precluded definitive demonstration of a positive relationship between breast cancer risk and chemoprevention by soy and soy bioactive components in humans and animal models (3,8).

Work by our group and others have shown that lifetime exposure to soy-rich foods containing Gen as well as Gen-supplemented diets protect rodent models from chemically induced mammary carcinogenesis by multiple mechanisms (9–12) involving signaling pathways that target genes deregulated in human breast cancers. Using gene microarray profiling, our laboratory identified key components of the Wnt pathway that are altered in mammary epithelial cells by dietary exposure to Gen (13). In particular, the expression of Wnt ligand 5A and Wnt downstream target Cyclin D1 was decreased, whereas that of the extracellular Wnt receptor antagonist secreted frizzled-related protein-2 was increased by Gen (13). In the mouse, Wnt signaling is a key event not only in normal mammary gland development (14) but also in mammary tumorigenesis (15). However, in humans, mutations in Wnt components are rarely seen in breast cancer, with the exception of metaplastic breast carcinoma (16). In contrast, β-catenin protein pools are stabilized in >50% of breast carcinomas (17) due to mutations in β-catenin itself or to defective pathways involved in its proteasomal degradation (16). Moreover, the β-catenin downstream target gene c-Myc is amplified in at least 15% of breast cancers (18). Further, the epithelial cell–cell adhesion protein E-cadherin, which sequesters β-catenin in the membrane, preventing its cytosolic accumulation, subsequent nuclear import and inappropriate transcriptional activation of target genes (19), is also found to be largely absent or diminished in lobular breast carcinomas and infiltrating luminal and ductal carcinomas (20). Thus, cellular transformation by deregulation of β-catenin stability may be inhibited by increasing the expression and/or activity of E-cadherin and members of the adenomatous polyposis coli (APC) degradation complex.

To address the inhibition of β-catenin proliferative activity as a potential mechanism by which Gen confers protection from breast cancer, we evaluated whether this dietary factor promotes E-cadherin signaling in the mouse mammary epithelial cell line HC11. These non-malignant cells contain an intact E-cadherin protein, express estrogen receptor (ER)-α and -β isoforms and have been extensively characterized for β-catenin signaling (21–23). We found that Gen increases E-cadherin expression through an ER-β-mediated pathway, upregulates E-cadherin–β-catenin cell adhesion complex formation and decreases Wnt-induced cytosolic and nuclear β-catenin accumulation and transcription of proliferation-associated Cyclin D1 and c-Myc genes. Further, we show that the epithelial cell growth phenotype elicited by Gen is dependent on membrane E-cadherin expression levels. These findings suggest that Gen regulation of E-cadherin signaling may constitute an important determinant of the transcriptional response of mammary epithelial cells to Gen and may be exploited for the prevention and treatment of breast cancer.

Materials and methods

Animals and tissue collection

Animals were maintained in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals, following procedures approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee. Female time-mated Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) were housed individually in polycarbonate cages. Rats at gestation day 4 were randomly assigned to one of two semipurified isocaloric diets made according to the American Institute of Nutrition-93G formulation (24), with corn oil substituting for soybean oil and containing as sole protein source either casein (CAS; New Zealand Milk Products, Santa Rosa, CA) or CAS supplemented with Gen in the aglycone form (Gen, 250 mg/kg feed; Sigma Chemical Co., St Louis, MO). Animals were provided food and water ad libitum. At delivery, all pups from dams of the same diet groups were...
poooled and 10 pups (five per sex) were randomly assigned to each dam for suckling. Female pups were weaned at postnatal day 21 to the same diet as their dams and were fed this diet throughout the study. At post-natal day 50, female pups were killed and the abdominal mammary gland (number four) was removed. A portion of the right gland was immediately homogenized in Trizol (Invitrogen, Carlsbad, CA) and frozen in liquid nitrogen for RNA analyses, whereas the rest of the tissue was frozen at −80°C for protein analyses by western blot.

**Cell culture and treatments**

The mouse mammary epithelial cell line HC11 (kindly provided by Dr Jeffrey M. Rosen, Baylor College of Medicine) was maintained in growth medium containing RPMI-1640 (Sigma–Aldrich, St. Louis, MO) supplemented with 10% (vol/vol) bovine calf serum (Invitrogen), 10 ng/ml epidermal growth factor (Invitrogen), 5 μg/ml insulin and 50 μg/ml gentamicin (Sigma–Aldrich) in 5% CO2; 95% air at 37°C. Prior to all treatments, cells were subjected to a 24 h serum starvation (0.5% charcoal- stripped bovine calf serum growth medium). HC11 cells were treated with Gen (40 nM, Sigma–Aldrich) for 6 h with and without added recombinant human Wnt-1 protein (Invitrogen) or lithium chloride (LiCl) (Sigma) at concentrations described under each experiment. For knock down of the CDH1 gene encoding E-cadherin and the Esr2 gene encoding ER-β, a pool of four double-stranded small-interfering RNAs (siRNAs) targeting mouse CDH1 or mouse Esr2 (on Target plus Reagent; Thermo Fisher Scientific, Waltham, MA) were used at 25 and 50 nM final concentration, respectively. Control siRNAs (scrambled) (on Target plus Reagent) were used at the same concentrations in parallel studies. The siRNAs were introduced by Lipofectamine 2000 (Invitrogen) in GibcoOPTI-MEM I reduced serum medium (Invitrogen). Forty-eight hours later, cells were treated with Gen or Wnt-1 and harvested 6 h thereafter. Each treatment group had three to four replicates.

3-(4,5-Dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay and cell counts

HC11 cells were seeded at a density of 1 × 10^4 cells per well onto 96-well plates and allowed to adhere overnight followed by 24 h serum starvation. Cells were treated for 6 days with Gen (40 nM) in the presence or absence of Wnt-1 protein (20 ng/ml), with medium replenished every 48 h. Metabolically active cells were quantified following the manufacturer’s instructions [3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay kit; American Type Culture Collection, Manassas, VA]. Cell numbers were determined under the same treatment conditions by using Trypan Blue staining. In cells transfected with siRNAs, treatments with Gen (40 nM) were carried out 48 h after transfection with and without Wnt-1 (20 ng/ml), and 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay was conducted 24 h later. Each treatment condition was evaluated in triplicates.

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol Reagent (Invitrogen). Integrity of total RNA was monitored on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). One microgram of total RNA was reverse transcribed using iScript reagent (Bio-Rad, Hercules, CA). Primers were designed from separate mRNAs (scrambled) (on Target plus Reagent) were used at the same concentrations in parallel studies. The siRNAs were introduced by Lipofectamine 2000 (Invitrogen) in GibcoOPTI-MEM I reduced serum medium (Invitrogen). Forty-eight hours later, cells were treated with Gen or Wnt-1 and harvested 6 h thereafter. Each treatment group had three to four replicates.

**Immunoblotting and immunoprecipitation**

HC11 cells were seeded on sterile 22 mm glass cover slides at a density of 3.0 × 10^5 cells per well and grown overnight. Cells were treated with vehicle or Gen (40 nM) in the presence or absence of LiCl (25 mM) or Wnt-1 protein (10 ng/ml) for 24 h. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized in 0.25% Triton– phosphate-buffered saline washes after each step. Cells were incubated in signal enhancer solution (Image-iT®; Invitrogen) for 30 min and then overnight at 4°C with anti-β-catenin rabbit polyclonal antibody (1:250 dilution; Cell Signaling) or anti-portal antibody (1:250 dilution; Cell Signaling). Cells were incubated with the biotinylated secondary anti-rabbit antibody (Vectastain elite ABC kit) for 30 min at room temperature and subsequently, with rhodamine (tetramethylrhodamine isothiocyanate)- conjugated streptavidin (1:250 dilution) (Jackson ImmunoResearch Laboratories, West Grove, PA) for another 30 min at room temperature, with 1× phosphate-buffered saline washes before and after each step. Cells were mounted using Vectashield mounting medium with 4 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Fluorescence images were acquired using a Zeiss AXIOVERT 200M inverted microscope (Carl Zeiss AG, Oberkochen, Germany) at ×400 magnification.

**Statistical analysis**

Two or three independent experiments were conducted for each study, with each experiment performed in triplicate or quadruplicate. Data were presented as means ± SEMs, relative to the control (vehicle) treatment. Statistical analysis was performed using SigmaStat software 3.5 (SPSS, Chicago, Illinois), and significance between treatment groups (P < 0.05) was determined using one-way analysis of variance followed by Tukey’s post-hoc analysis or Student’s t-test.

**Results**

**Increased mammary expression of E-cadherin with dietary Gen**

In a previous study (13), we showed that in rat mammary glands of post-natal day 50 rats fed Gen diet, immunoreactive E-cadherin and β-catenin were predominantly localized to ductal epithelial cell membranes. The levels of expression of these proteins in mammary tissues as a function of diet were quantified by western blots. E-cadherin protein was increased by 2-fold in the Gen, relative to the CAS group (Figure 1A), in the absence of similar effects on E-cadherin transcript levels (data not shown). E-cadherin upregulation with Gen was associated with increased expression of β-catenin and the mammary...
differentiation-associated gene β-casein (Figure 1B and C). The amounts of phosphorylated β-catenin, representing the β-catenin pool targeted for ubiquination/degradation (17), did not differ between the diet groups (Figure 1A).

Gen increases levels of membrane E-cadherin and E-cadherin–β-catenin cell adhesion complex

To determine Gen effects on E-cadherin and β-catenin expression levels leading to increased epithelial differentiation in vivo, we confirmed the positive correlation between dietary exposure to Gen and levels of E-cadherin in mammary tissues using non-malignant mouse mammary epithelial cells in vitro. HC11 cells were treated for 6 h with vehicle or Gen (40 nM), the latter dose corresponding to that found in sera of humans regularly consuming soy-rich foods (7). E-cadherin expression was increased in membrane fractions of Gen-treated relative to vehicle-treated cells (Figure 2A). There was no corresponding increase in E-cadherin transcript levels at all tested time points (0.5, 1, 3 and 6 h; data not shown), concordant with that found in mammary tissues in vivo. Non-nuclear proteins (membrane/cytosol fraction) from HC11 cells treated with Gen also showed increased levels of E-cadherin and β-catenin, relative to vehicle-treated cells (Figure 2B).

Corresponding nuclear fractions had very low to undetectable β-catenin in both control and Gen-treated cells (data not shown). To determine if the increase in β-catenin and E-cadherin levels with Gen reflects increased cell adhesion complex formation between these proteins, the non-nuclear fractions from control and Gen-treated cells were subjected to immunoprecipitation with anti-E-cadherin antibody or control IgG. Immunoprecipitates were then analyzed by western blot using anti-β-catenin and anti-E-cadherin antibodies. Gen-treated cells showed higher levels of β-catenin protein that was precipitated by anti-E-cadherin antibody than control cells (immunoblotting: β-catenin Ab; Figure 2C). The same trend was seen for E-cadherin using anti-E-cadherin antibody (immunoblotting: E-cadherin Ab; Figure 2C). Control IgG did not immunoprecipitate either protein (data not shown).

Gen inhibits basal and Wnt-1-induced mammary epithelial cell proliferation, through an ER-β-dependent pathway

Activated Wnt signaling mediated by β-catenin is known to promote cell growth and inhibit differentiation (26), whereas Gen is considered to induce growth arrest to favor cells to differentiate (9–11). HC11 cells treated with Gen (40 nM) showed a dose-dependent decrease in viability over a 6 day period (Figure 3A). When challenged with Wnt-1,
cells demonstrated a dose-dependent increase in viability, which was inhibited by Gen at low (10 and 20 ng/ml), but not at high (50 and 100 ng/ml) added Wnt-1 (Figure 3A). Consistent with the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay, Wnt-1 (20 ng/ml) increased cell numbers (10.04 ± 0.47 × 10^4) relative to the control (untreated) group (8.37 ± 0.54 × 10^4) (P < 0.05). Cells treated with Wnt-1 in the presence or absence of Gen for 6 h were evaluated for expression of the canonical Wnt/β-catenin signaling proliferative target genes c-Myc and Cyclin D1 by quantitative real-time polymerase chain reaction. The patterns of c-Myc and Cyclin D1 expression with Wnt-1 in the absence or presence of Gen were consistent with the inhibitory effects of Gen on Wnt-1-induced cell proliferation/viability at a low Wnt-1 dose (10 ng/ml) (Figure 3B). Gen effects were abolished (50 ng/ml) or reversed (100 ng/ml) at higher concentrations of added Wnt-1 (Figure 3C).

Gen exhibits a 20-fold higher affinity for ER-β than for ER-α (27). To determine if Gen inhibition of Wnt-1 activation is mediated by both or either isoforms, the specific ER-α agonist propyl pyrazole triol (40 nM) (28) and ER-β agonist diarylpropionitrile (40 nM) (29) were evaluated in parallel with Gen, for effects on Wnt induction of c-Myc expression. Propyl pyrazole triol alone induced c-Myc gene

**Fig. 2.** Gen increases membrane E-cadherin levels and promotes membrane E-cadherin–β-catenin complex formation. (A) E-cadherin protein levels in membrane fractions of HC11 cells treated with Gen or vehicle alone (Control), analyzed by western blots. Each lane (20 μg protein) represents an individual sample. Loading control is a <30 kDa protein detected by Ponceau staining of western blot. (B) E-cadherin and β-catenin protein levels in non-nuclear (cytosolic + membrane) compartments of HC11 cells with or without added Gen, analyzed by western blots. Graphs show the relative expression levels (ADU, normalized to α-tubulin) for each protein as a function of treatment. *P < 0.05 relative to control group. (C) Non-nuclear (cytosolic + membrane) proteins were immunoprecipitated with E-cadherin antibody, and the resultant precipitates were analyzed for levels of immunoprecipitated β-catenin and E-cadherin, respectively, by western blots. Graph (ADU) shows the levels of immunoprecipitated proteins with E-cadherin antibody in cells with or without Gen treatment. *P < 0.05 relative to control group.
expression and its effects were not altered by Wnt (Figure 4A). In contrast, diarylpropionitrile, similar to Gen, did not affect basal and inhibited Wnt-1-induced c-Myc expression levels (Figure 4A). To confirm the role of ER-β in mediating Gen actions, the expression of Esr2 encoding ER-β was knocked down by siRNA in cells treated or not with Gen (40 nM). Esr2 expression was not affected by Gen, whereas a pool of Esr2 siRNAs attenuated Esr2 expression by at least 50% (Figure 4B). Decreased Esr2 levels attenuated Gen induction of E-cadherin expression, but had no effect on basal E-cadherin levels (Figure 4C). There was a corresponding loss of Gen inhibition of β-catenin transactivation in cells treated with Wnt-1, with decreased Esr2 expression (Figure 4D). In particular, the expression ratios of β-catenin targets Cyclin D1 and c-Myc were increased in Wnt + Gen-treated cells relative to Gen-treated cells alone, in the presence of siRNAs to Esr2 when compared with non-specific (scr) siRNAs (Figure 4D).

**Gen inhibits non-membrane β-catenin protein accumulation**

We analyzed the levels of β-catenin in membrane and cytosolic fractions of HC11 cells treated with vehicle, Wnt-1 (20 ng/ml) and LiCl (25 mM), in the absence or presence of Gen (40 nM) by immunofluorescence (Figure 5A) and western blotting (Figure 5B and C). LiCl inhibits glycogen synthase kinase 3β (GSK3β)-mediated β-catenin ubiquitination and subsequent degradation and was used here to determine if repression by Gen of Wnt/β-catenin signaling partly occurs through activation of GSK3β function. Consistent with previous data (Figure 2, above), Gen increased membrane E-cadherin levels, and this induction was not affected by Wnt-1 or LiCl cotreatments (Figure 5B). Wnt-1 and LiCl alone had no effect on E-cadherin levels. Cells treated with Wnt-1 or LiCl showed significant accumulation of β-catenin in cytosol, when compared with the control or Gen-treated groups (Figure 5A and C). Gen increased membrane levels of β-catenin and blocked Wnt-1 inhibition of β-catenin membrane localization (Figure 5A). Gen similarly inhibited LiCl-mediated accumulation of cytosolic β-catenin, although this was not readily apparent in western blots (Figure 5C).

**Loss of E-cadherin abolishes Gen inhibition of Wnt-1 activation**

To directly assess whether the observed Gen-mediated increase in E-cadherin protein is required for Gen inhibition of β-catenin activation cascade, CDH1 gene encoding E-cadherin protein was knocked down using CDH1 siRNA. Initial studies with increasing doses of CDH1 siRNA (10, 25 and 50 nM) established 25 nM as an optimal dose for effective knockdown. Using CDH1 siRNA at 25 nM, ~50% knockdown of CDH1 messenger RNA was achieved in the absence of effects on c-Myc gene expression, relative to the scr siRNA-treated cells (Figure 6A). The decrease in CDH1 expression with siRNA targeting was similarly observed at the level of the protein in both basal and Gen-treated cells and was accompanied by a corresponding decrease in β-catenin (Figure 6B). The scr and CDH1 siRNA-transfected cells were incubated in medium without and with added Wnt-1 (20 ng/ml), and c-Myc expression was evaluated. In cells transfected with scr siRNA, Gen had no effect on basal and abolished Wnt-1-induced c-Myc gene expression (Figure 6C), confirming an earlier result (Figure 2). In contrast, in cells with attenuated E-cadherin expression (CDH1 siRNA-treated cells), Gen increased basal c-Myc transcript levels and lost its inhibitory effect on Wnt-1-induced expression of this oncogene (Figure 6C). CDH1 siRNA similarly altered the effects of Gen on basal and Wnt-1-induced cell viability (measured 24 h post-Gen treatment), relative to cells transfected with scr siRNA (Figure 6D).

**Discussion**

The present study presents a novel mechanism by which the soy isoflavone Gen may confer protection against breast cancer. Using the HC11 mouse mammary epithelial cell line in vitro to mechanistically dissect the contribution of dietary Gen in inhibiting mammary tumorigenesis in vivo (12,30), we show that Gen at low doses (nanomolar range): (i) inhibited basal and Wnt-1-induced mammary epithelial proliferation; (ii) suppressed Wnt-1-induced nuclear accumulation of β-catenin; (iii) upregulated E-cadherin protein expression and promoted formation of the E-cadherin–β-catenin cell adhesion complex and (iv) elicited the above through ER-β- and
Fig. 4. Gen increases E-cadherin protein levels and inhibits Wnt-1/β-catenin transactivity via ERβ. (A) Effects of the ER-α-specific agonist propyl pyrazole triol (PPT) (40 nM) and of the ER-β-specific agonist diarylpropionitrile (DPN) (40 nM), relative to Gen, on c-Myc expression in the presence and absence of Wnt-1 treatment. (B) Levels of Esr2 transcripts (relative to those of vehicle-treated cells in the presence of scr siRNAs; value of 1) in control and Gen-treated cells upon transfection with scr (control) siRNAs or siRNAs targeting Esr2. (C) E-cadherin protein levels in membrane fractions of HC11 cells treated with Gen (+) or vehicle alone (−), in the presence of scr (control) siRNAs or siRNAs targeting Esr2. Each lane (20 µg protein) represents an individual sample. Loading control is α-tubulin. (D) Ratio of Cyclin D1 and c-Myc transcript levels in cells treated with Wnt + Gen relative to Wnt-only treated cells transfected with either scr (control) siRNAs or siRNAs targeting Esr2. Data were expressed as mean ± SEM from three individual experiments. Subscripts with different letters differed at P < 0.05.

Fig. 5. Gen inhibits β-catenin protein accumulation. (A) HC11 cells under different treatment conditions were incubated with anti-β-catenin antibody. Nuclei (DAPI, 4′,6-diamidino-2-phenylindole), β-catenin (rhodamine) and localization of β-catenin in cellular compartments (merge) were visualized by fluorescence microscopy. Bar = 20 µm; magnification = ×400. (B) Membrane fractions from HC11 cells treated as indicated were evaluated for presence of immunoreactive E-cadherin or β-catenin by western blots. Loading control is a 30 kDa protein detected by Ponceau staining of western blot. (C) Cytosolic fractions from HC11 cells were treated as indicated and evaluated for expression of immunoreactive β-catenin by western blot. Loading control is α-tubulin. For (B) and (C), a representative blot is shown from two independent experiments. Each lane was loaded with 20 µg total protein.
E-cadherin-mediated transduction pathways. Our findings that Gen regulates E-cadherin signaling in mammary epithelial cells have important implications for mammary tumorigenesis and cancer prevention. First, given that loss of E-cadherin is a critical step in epithelial–mesenchymal transition, leading to tumor progression (31), infiltrating ductal carcinomas are associated with decreased E-cadherin expression (20) and the pathogenesis of human breast cancers is correlated with $\beta$-catenin stabilization (17) and amplification of $\beta$-catenin-T-cell factor (TCF)/lymphoid enhancer factor (LEF) target genes Cyclin D1 (17) and c-Myc (18), our results suggest that mammary tumor progression may be alleviated by dietary Gen intake. Second, the recent report that E-cadherin controls a complex transcriptional network, partly independent of $\beta$-catenin function (32), suggests wide-ranging possibilities for Gen to directly or indirectly influence molecular pathways underlying tumor initiation and growth through regulation of E-cadherin expression levels. Finally, given that the antiproliferative response of mammary epithelial cells to Gen appears to be dependent on functional E-cadherin, with attenuated expression of the latter resulting in Gen promotion, rather than inhibition of cell growth, our findings argue for the limited application of therapeutic strategies utilizing dietary intake of this isoflavone to the early stages of human breast cancers, when E-cadherin expression is still relatively intact. Our data further indicate that Gen regulation of E-cadherin function may constitute a common mechanism by which non-protein dietary factors confer tumor protection since epigallocatechin-3-gallate, a polyphenol component of green tea (33), and Vitamin D3 (34) both prevented E-cadherin loss and progression of tumorigenesis in experimental models.

Our previous work suggested that dietary Gen disrupts the Wnt-signaling pathway, leading to decreased expression of Cyclin D1 in mammary epithelial cells of young adult rats relative to those fed CAS diet (13). In the present study, we show that this Gen effect occurs at the level of E-cadherin signaling since upregulation of E-cadherin protein expression in vivo and in vitro similarly resulted in increased $\beta$-catenin association with E-cadherin, inhibition of $\beta$-catenin proliferative effects and promotion of epithelial differentiation. It is noteworthy that the in vitro effects elicited by Gen using the ‘non-malignant’ mammary epithelial HC11 cells were achieved at a dose (40 nM) that is well within the physiological concentration seen by cells in vivo (35,36). This is in contrast to previously reported studies that use high levels (>10 $\mu$M) of Gen to mimic in vivo effects (11). Thus, the in vitro system described here will be useful to further explore the physiological relevance of E-cadherin-mediated signaling pathways regulated by Gen and other dietary factors displaying anti-mammary tumor effects (37,38).

In normal cells, E-cadherin plays critical roles in epithelial cell adhesion, differentiation and maintenance of epithelial polarity. Loss of E-cadherin constitutes an early event in epithelial–mesenchymal transition, eventually leading to high-grade neoplasia and metastasis (31). In the present study, Gen induction of membrane E-cadherin suppressed cell proliferation and inhibited transcriptional activation of $\beta$-catenin downstream targets Cyclin D1 and c-Myc, by sequestering $\beta$-catenin in a membrane complex, thereby preventing its accumulation in the cytoplasm and eventual nuclear import. Activation of Wnt signaling predominantly occurs by increasing cytoplasmic $\beta$-catenin stability through inhibition of the APC–axin–GSK3$\beta$ complex

![Fig. 6. E-cadherin is required for the antiproliferative effects of Gen. (A) siRNA targeting of CDH1 decreased E-cadherin but not c-Myc transcript levels. *P < 0.05 relative to scr (control) siRNA-treated group. (B) siRNA targeting of CDH1 decreased E-cadherin and $\beta$-catenin protein levels in transfected cells treated or not with Gen (40 nM). Each lane (20 $\mu$g total protein) represents an individual sample. Loading control is $\alpha$-tubulin. (C) c-Myc expression in HC11 cells treated or not with Wnt-1 (20 ng/ml) in the presence of CDH1 siRNA or scr siRNA, analyzed by quantitative real-time polymerase chain reaction. Results are mean ± SEM of three independent experiments. (D) HC11 cells treated as indicated were evaluated for cell viability by the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay. Superscripts with different letters differed at $P < 0.05$.](image-url)
formation (26). Our findings that membrane localization of β-catenin was inhibited by Wnt-1 and LiCl and that Gen partially reversed this effect, suggest that downstream signaling components of Wnt may block the functional physical associations of E-cadherin and β-catenin, perhaps by modifications in the structures of these proteins through tyrosine phosphorylation. Consistent with this postulate, tyrosine kinases have been reported to phosphorylate β-catenin, resulting in its dissociation from E-cadherin (39). Moreover, Gen is a specific inhibitor of tyrosine protein kinase (40), albeit at much higher levels (micromolar range) than used here, and Wnt-1 has been shown to transactivate Erβ1, a tyrosine kinase (22).

The dependence of Gen on E-cadherin signaling to mediate its inhibitory effects on β-catenin transcriptional activity is supported by the CDH1 targeting studies. Knock down of E-cadherin transformed Gen to a promoter of basal c-Myc expression and resulted in the loss of its ability to inhibit Wnt activation. These results may partly explain the reported conflicting protumorigenic and antitumorigenic effects of Gen (41–43) and suggest that mammary epithelial cells with an intact E-cadherin signaling system may respond to the biological effects of Gen distinct from those of cells with loss of or diminished expression of this epithelial adhesion molecule. These findings raise provocative questions on the potential role of diet in altering the prognosis of existing tumorigenic breast cells. Further, these data indicate that Gen can repress and activate distinct sets of target genes, with the ultimate outcome on the differentiation phenotype being determined by the ability of these gene products to interact with E-cadherin and/or its downstream transcriptional pathways.

Relative to control cells, Gen increased E-cadherin protein levels in the presence or absence of CDH1 siRNA, suggesting that Gen regulation of E-cadherin expression predominantly occurs post-transcriptionally through various mechanisms including inhibition of E-cadherin internalization, shedding and degradation (44,45). To determine how Gen may post-transcriptionally regulate E-cadherin expression, we evaluated the expression levels of caveolin-1 and an ubiquitin E3 ligase, Siah2, transcripts in control and Gen-treated HC11 cells. Caveolin-1 promotes the stabilization of cell–cell adhesion complex and inhibits β-catenin-TCP/LEF-dependent transcription (46). On the other hand, Siah2 is upregulated by estrogen/ERα signaling (47), and thus may be involved in the degradation of E-cadherin. We found no effects of Gen on the expression of these two transcripts (data not shown), albeit this does not preclude Gen effects on the levels of the respective proteins.

We show here that the ability of Gen to regulate E-cadherin expression and activity leading to the repression of Wnt-signaling cascade is mediated by ER-β since Gen effects were exclusively mimicked by the ER-β-specific agonist diapropionitrile, and attenuation of ER-β expression resulted in the loss of Gen induction of E-cadherin levels and of Gen repression of Wnt-1-induced β-catenin transactivity. Our results agree with previous reports that loss of ER-β expression in the lactating mammary gland in vivo (48) and in HC11 cells in vitro (49) is associated with decreased membrane E-cadherin protein levels. In contrast, the ER-α-specific agonist propyl pyrazole triol increased c-Myc expression and acted as a mitogen, similar to Wnt-1, albeit cotreatments of HC11 cells with both had no additive or synergistic proliferative effects. Interestingly, we found that in two normal mammary epithelial cell lines that lack ER expression, namely MCF-10A and human mammary epithelial cells (Invitrogen), Gen treatment failed to induce E-cadherin expression (data not shown), consistent with Gen effects requiring functional ER-β. Taken together, these observations support an endocrine role of Gen in suppressing the etiology and progression of breast cancer through maintenance of the differentiated epithelial phenotype.

Based on the present results, we suggest that Gen promotion of mammary epithelial cell differentiation to confer protection from breast cancer involves its post-transcriptional regulation of E-cadherin expression, leading to sequestration of β-catenin in the membrane and inhibition of β-catenin transcriptional activity. Although several oncogenes and tumor suppressor genes have now been reported to be modulated by Gen (9,43,50,51), this study provides the first evidence for E-cadherin as a cellular Gen target. Moreover, our findings suggest the potential utility of E-cadherin and, by association, its downstream targets as predictive markers for identifying differences in diet-induced regulation of molecular and cellular events in mammary epithelial cells with distinct E-cadherin expression. Elucidation of these mechanisms will facilitate the development of diet-based preventative strategies to decrease breast cancer risk.

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**References**

Genistein and E-cadherin signaling in mammary epithelial cells


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