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Implications for Breast Cancer Prevention

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14. ABSTRACT Mammary stem cells (MaSC) are required for tissue homeostasis and epithelial expansion during development; however, dysregulation of MaSC self-renewal such as by Wnt1 overexpression leads to neoplastic transformation. To date, in vivo dietary regulation of MaSC and its relation to breast cancer has not been evaluated. Therefore, I proposed to investigate the effect of lifetime intake of soy protein (SPI) diet relative to the control casein (CAS) diet on mammary stem cell (MaSC) population and its correlation to tumor formation in MMTV-Wnt1-Transgenic (Wnt1-Tg) female mice. Tumor incidence was lower in SPI fed Wnt1-Tg mice relative to those fed CAS (48.3% vs.73.5%; p<0.05). Fluorescence activated cell sorting analysis of freshly isolated MECs revealed that the MaSC-enriched subpopulation (CD29hiCD24+) was decreased by 50% in pre-neoplastic mammary glands of Tg mice fed SPI. Further, the tumor initiating population (Thy1+CD24+) was also decreased by SPI diet. I am in the process of confirming SPI effects on self-renewal by transplantation assay, known to be the 'gold standard' to measure MaSC activity. Our findings provide the first in vivoreport of dietary effects on MaSC population and its association to breast cancer outcome.					
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INTRODUCTION

Epidemiological studies demonstrate an inverse association between high consumption of soy foods and breast cancer risk among Asian women whose early intake of soy products is 10 to 20 times higher than their American counterparts (1). This association was not related to classical risks for breast cancer such as mammographic density and estrogen levels, suggesting other mechanisms are involved, yet to be discovered. We have shown that dietary exposure to soy protein isolate (SPI) protects against breast cancer in rats partly due to upregulation of tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (PTEN) and inhibition of the oncogenic Wnt signaling pathway (2-5). Breast cancer is thought to originate from a population of stem and progenitor cells that are relatively undifferentiated and long-lived/immortal, which, in turn, facilitate long-term accumulation of oncogenic mutations and susceptibility to transformation (6). Ectopic expression of Wnt1 in the mammary gland of female mice results in heterogeneous mammary tumors relevant to human disease (7). A linkage between PTEN and Wnt signaling pathways in mammary tumorigenesis is suggested by the findings that PTEN deficiency results in decreased latency of tumor formation in MMTV-Wnt1 transgenic mice (Wnt1-Tg) (7). Stem cell renewal is a tightly regulated process, dysregulation of which can lead to neoplastic transformation. The normal mouse mammary stem cell population (CD29^{hi}CD24⁺) is expanded in pre-neoplastic mammary glands of Wnt1-Tg female mice suggesting that dysregulated Wnt signaling mediates tumorigenesis in part by favoring the conversion of normal to cancer stem cells (8). Indeed, cancers arising from Wnt1-Tg mice were shown to originate from cancer stem cells (CD24⁺Thy1⁺) (9). Therefore, ***I hypothesize that dietary soy protein isolate protects against Wnt1-induced mammary tumorigenesis by up-regulating PTEN signaling and decreasing normal mammary stem cell population, thus preventing the conversion of normal stem cells to cancer stem (tumor initiating) cells.***

BODY

My main goal is to explore novel mechanisms of dietary prevention of breast cancer linking the PTEN and Wnt signaling pathways on the regulation of mammary stem cell fate. Wnt1 is a common integration site for mouse mammary tumor virus (MMTV) (7) and its overexpression in the mammary gland of MMTV-Wnt1-Transgenic (Wnt1-Tg) female mice causes spontaneous mammary tumors from a small population of cancer stem cells (CD24⁺Thy1.1⁺) (9), probably due to an expansion of normal stem cells (CD29^{hi}CD24⁺) (8). Therefore, Wnt1-Tg mice serve as an excellent model to study PTEN-associated genes, regulated by dietary factors, on mammary stem cell self-renewal and tumorigenesis *in vivo*, with strong relevance to human disease. The study is composed of two Specific Aims. Aim 1 will identify PTEN-dependent signaling pathways involving anti-proliferative and pro-differentiation-associated genes which may underlie mammary tumor protection by dietary soy/genistein (GEN), using genome-wide expression profiling of mammary epithelial cells (MECs) exposed to GEN in the presence or absence of PTEN short hairpin (sh)-RNA. I expect to identify a number of differentiation associated genes to be up-regulated by GEN in MECs, an effect that will be lost in the presence of PTEN knockdown. Aim 2 evaluates the effect of diet on the population and self-renewal potential of mammary cancer stem cells by *in vitro* mammosphere assay. Cell populations enriched for mammary stem cells (MaSCs) (CD29^{hi}CD24⁺) and tumor initiating cells (Thy1⁺CD24⁺) will be analyzed from pre-neoplastic mammary tissue of Wnt1-Tg mice fed casein (CAS), soy protein isolate (SPI), and GEN by using Fluorescent Activated Cell Sorting

(FACS). I expect to see a decreased population of cancer stem cells in the mammary glands of Wnt1-Tg mice fed SPI and GEN relative to the control CAS diet, consistent with the predicted decreased tumor incidence for these diets.

In the first aim, I evaluated the hypothesis that *soy isoflavone GEN mediates the crosstalk between the tumor suppressors PTEN and p53 in human mammary epithelial cells (MECs) to promote cell differentiation and cell cycle arrest by increasing the nuclear PTEN pool*. This now published work (3) describes the linear pathway by which a GEN-mediated increase in PTEN nuclear localization initiates an autoregulatory loop involving PTEN-dependent increases in p53 nuclear localization, PTEN/p53 physical association, PTEN/p53 co-recruitment to the PTEN promoter region, and p53 transactivation of PTEN promoter activity. The findings go beyond nutrition and breast cancer. I provided the first direct evidence that PTEN can regulate its own expression in MECs by using siRNA and ChIP assay.

To address Aim 2, I investigated SPI effects relative to the control CAS diet, on mammary tumor development in MMTV-Wnt1-Transgenic (Wnt1-Tg) female mice and on the mammary stem cell (SC) population in pre-neoplastic Wnt1-Tg female mice. From gestation day 4 (GD 4), dams were fed the control CAS diet, composed of casein as the sole protein source. Female Wnt1-Tg pups continued on CAS diet until weaned at PND 21. At weaning, female Wnt1-Tg mice were assigned to different semipurified isocaloric diets according to the American Institute of Nutrition (AIN-93G) formulation. The diets are 1) CAS diet, casein is the sole protein source, 2) SPI diet, soy protein isolate as sole protein source containing 430 mg of total isoflavones/kg diet, including 276 mg/kg genistein and 132 mg/kg daidzein, and 3) GEN diet, made of casein with addition of aglycone GEN. Mice were fed these diets throughout the study. Mammary tumor latency, incidence and multiplicity were carefully observed and recorded. As shown in **Figure 1A**, tumor incidence at 8 months of age of Wnt1-Tg mice fed SPI (n=30) was lower than those fed CAS (46.7% vs. 73.5%; $p < 0.05$) (n=34). Interestingly, tumor latency in SPI-fed Wnt1-Tg mice was shorter than for the CAS-fed group (4.65 vs. 5.88 months; $P < 0.05$) (**Figure 1B**). Tumor growth and weight was similar in both groups (**Figure 1C and 1D**).

Next, I evaluated SPI effects relative to CAS, on the MaSCs in pre-neoplastic mammary glands of post-natal day 75 (PND75) Wnt1-Tg mice. The percentage of MaSCs was quantified by FACS of freshly isolated mammary epithelial cells (MECs) based on their expression of mouse mammary SC markers namely CD29 and CD24 within the Lineage negative (Lin⁻) population (CD45⁻, TER119⁻, CD31⁻). FACS analysis revealed that the MaSC-enriched subpopulation (CD29^{hi}CD24⁺) was decreased by 50% in pre-neoplastic mammary glands of Wnt1-Tg mice fed SPI (**Figure 2A**), without affecting the luminal (CD29^{low}CD24⁺) or stromal populations (**Figure 2B**). Further, we showed that the tumor initiating/cancer stem cell population (Thy1⁺CD24⁺), which comprises a small percentage in Wnt1-Tg mice fed control CAS diet (~1 %), was also decreased (by 2-fold) in mammary glands of SPI-fed Wnt1-Tg mice (**Figure 2C**). Our findings suggest that targeting of MaSC (CD29^{hi}CD24⁺) by SPI diet in a neoplastic environment such as Wnt1 overexpression could inhibit conversion of normal to cancer stem cells (Thy1⁺CD24⁺) and thus provide a rationale for the observed mammary tumor protective effects (**Figure 1A**).

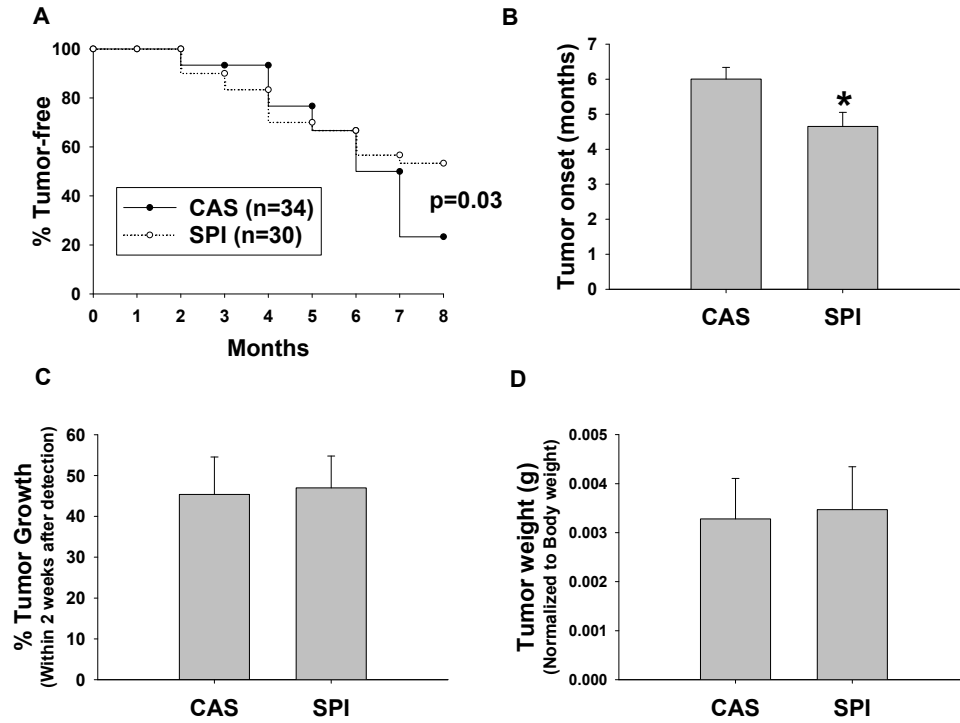


Figure 1. Soy protein isolate (SPI) protects against Wnt1-induced breast cancer. (A) Percentage of tumor-free Wnt1-Tg female mice fed casein (CAS; n=34) or SPI (n=30). (B) Tumor latency for Wnt1-Tg mice that developed tumors. * $P < 0.05$ for SPI relative to CAS. SPI diet had no effect on tumor growth (C) or weight (D).

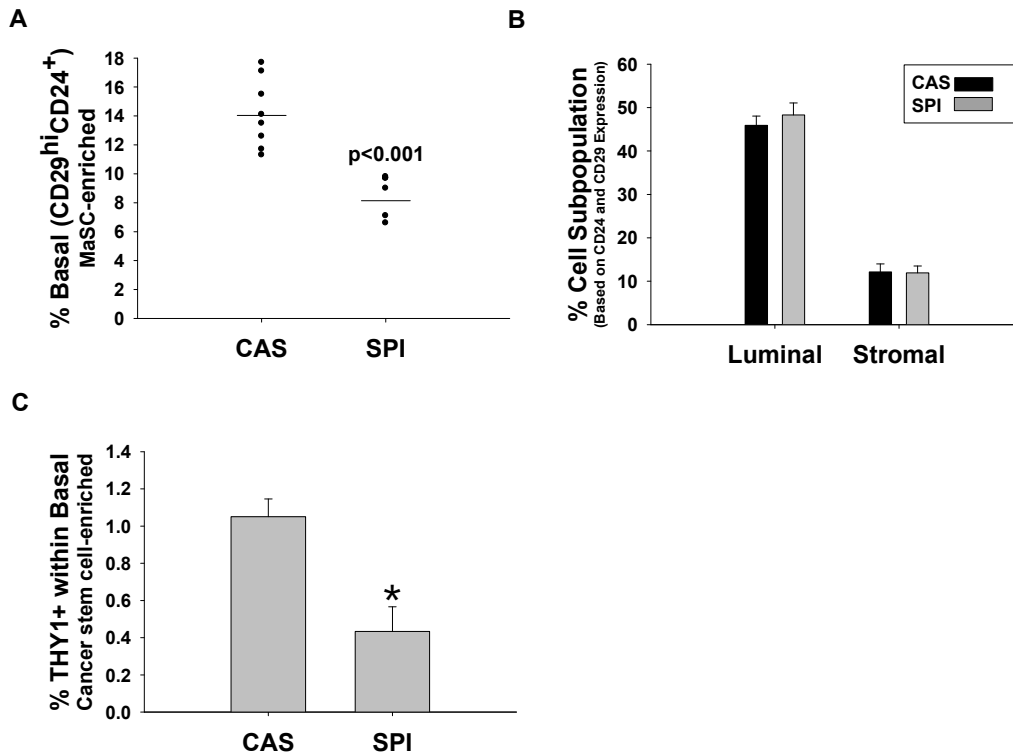


Figure 2. Soy protein inhibits MaSC expansion and cancer stem cell conversion in Wnt1-Tg mice. (A) SPI decreased MaSC-enriched population. (B) Diet had no effect on luminal or stromal populations. (C) SPI reduced the cancer stem cell population in pre-neoplastic mammary tissue of Wnt1-Tg mice. Data shown is from at least 5-7 independent experiments. * $P < 0.05$ for SPI relative to CAS.

Several studies, including our own, have suggested that soy isoflavone genistein (GEN) is partly responsible for breast cancer protective effects of soy diet (2-5). Therefore, we analyzed whether GEN can recapitulate SPI effects on tumor protection and stem cell regulation in Wnt1-Tg mice. As shown in **Figure 3A**, post-wean dietary intake of GEN protects against spontaneous mammary tumor development in Wnt1-Tg mice, similar to effects observed with SPI (**Figure 1**). Although GEN did not alter tumor growth (**Figure 3C**), there was a trend for reduced tumor weight in tumors from GEN-fed mice (**Figure 3D**). To confirm whether tumor protective effects of GEN involve regulation of MaSCs, we examined the effect of GEN on MaSC and tumor initiating cells in pre-neoplastic mammary tissue of Wnt1-Tg mice as a possible mechanism for tumor protection. Dietary intake of GEN inhibited the MaSC-enriched (CD29^{hi}CD24⁺) and cancer stem cell-enriched (Thy1⁺CD24⁺) populations (**Figure 4A and 4B**) in Wnt1-Tg mice prior to tumor development, suggesting that by targeting MaSC and preventing their conversion to cancer/tumor initiating cells, GEN is able to confer protection against breast cancer.

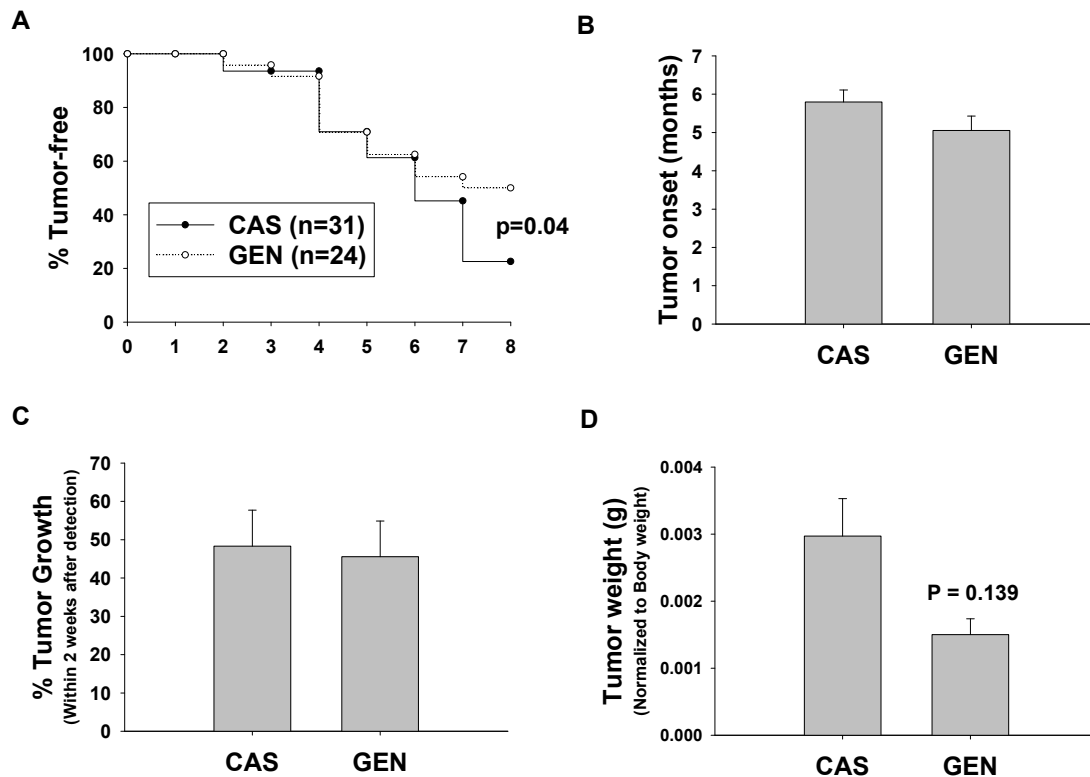


Figure 3. Major soy isoflavone genistein (GEN) protects against mammary tumorigenesis in Wnt1-Tg mice (A) Percentage of tumor-free Wnt1-Tg female mice fed casein (CAS; n=34) or GEN (n=24). GEN had no effect on tumor latency (B) or growth (C). (D) Tumors from GEN-fed mice tended to have lower weight.

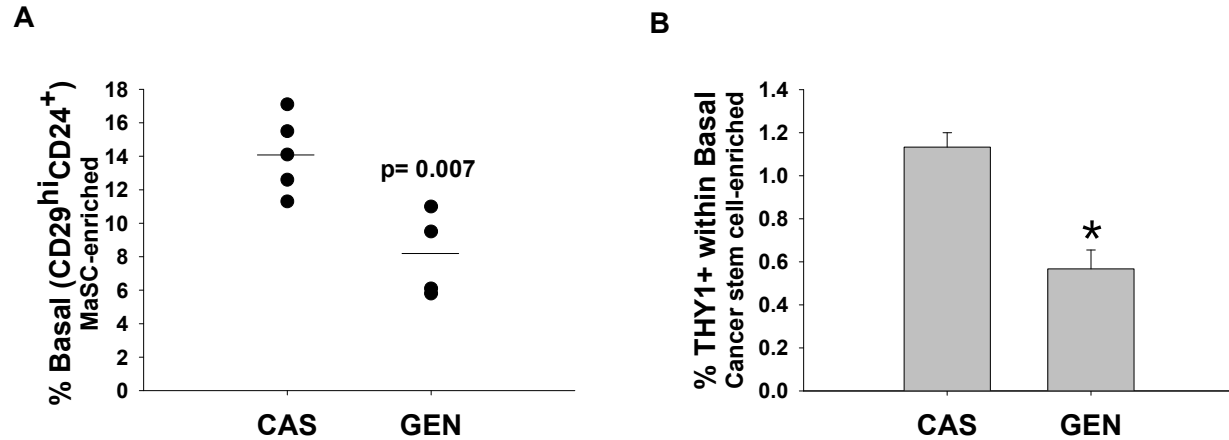


Figure 4. GEN inhibits MaSC-enriched and cancer stem population in pre-neoplastic mammary glands of Wnt1-Tg mice. (A) MaSC-enriched (CD29^{hi}CD24⁺) population is decreased (2 fold) by GEN. (B) GEN also decreases the number of Thy1+ cancer stem like cells or tumor initiating cells. Data shown is from 5 independent experiments; * $P < 0.05$ relative to CAS.

By definition, MaSCs have the ability to self-renew and undergo multilineage differentiation into different cell types of epithelial tree, mainly luminal epithelial (ductal and alveolar) and myoepithelial cells (6). Self-renewal is an intrinsic property of stem cells that allow their maintenance and propagation. A mammosphere assay has been developed based on the ability of a small population of mammary stem/progenitor cells to grow in suspension and form spheres while the majority of cells which lack self-renewal ability sink to the bottom of plate and die (10). Primary passage of mammospheres mostly contains cells that form single colonies of either luminal or myoepithelial cells, suggesting that they are mainly composed of restricted progenitor cells (11). However, serial passage of primary mammospheres selects for cells able to form mixed colonies (luminal and myoepithelial), indicating an enrichment for early progenitor and stem-like cells. Therefore, we have used the mammosphere assay as a surrogate assay to test the effect of diet on self-renewal of MECs from pre-neoplastic mammary glands of Wnt1-Tg mice fed CAS and SPI diets. As shown in **Figure 5A**, MECs from SPI-fed and CAS-fed Wnt1-Tg mice had similar numbers of primary mammospheres (passage 1; P1), suggesting that diet has minimal effect on the progenitor population, which also agrees with the FACS data where diet did not change the luminal (CD29^{low}CD24⁺) population (**Figure 2B**). Serial passage of primary spheres into secondary spheres (Passage 2; P2), revealed that MECs from SPI-fed Wnt1-Tg mice formed lower number of secondary spheres (**Figure 5B**) compared to CAS group, indicating SPI targeting of self-renewal in MaSC, which is supported by decreased percentage of MaSC-enriched population (CD29^{hi}CD24⁺) in freshly isolated MECs (**Figure 2A**). In conclusion, we were able to provide the first evidence for *in vivo* dietary regulation of MaSC by soy protein/GEN diet using FACS analysis and mammosphere assay. We are in the process of collecting RNA from secondary mammospheres formed by MECs from CAS, SPI and GEN-fed Wnt1-Tg mice for gene expression profiling analysis to identify pathways underlying dietary inhibition of self-renewal.

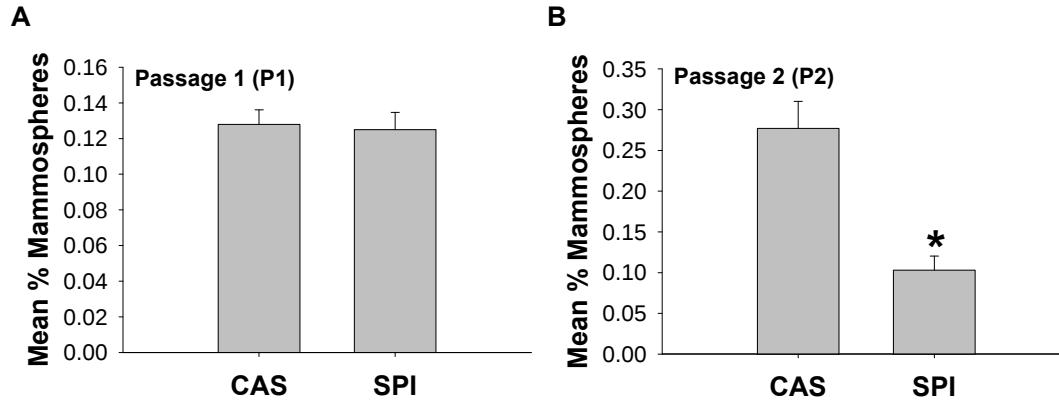
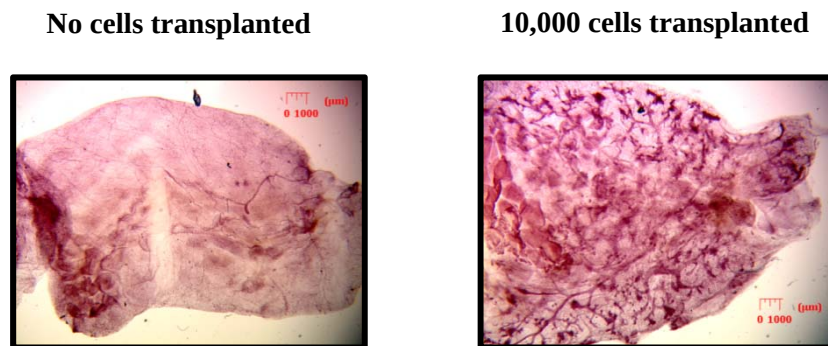


Figure 5. MECs from SPI-fed Wnt1-Tg mice have lower self-renewal potential. Freshly isolated MECs from pre-neoplastic PND75 Wnt1-Tg mice fed CAS or SPI were grown in suspension and allowed to form primary mammospheres (P1). After 5 days of culture, primary mammospheres were dissociated and passaged to form secondary mammospheres (P2). Manual counting of mammosphere forming units (MFUs) were done at day 5 and day 7 of primary and secondary passage, respectively. (A) SPI diet had minimal effect on primary mammosphere formation. (B) MECs from SPI-fed Wnt1-Tg mice have lower ability to form secondary mammospheres, suggesting decreased self-renewal of stem/early progenitor cells. Data represent the mean % MFUs $\pm$ SEM of two independent experiments ($n=16$ per each experiment); * $P<0.05$ relative to CAS.

Transplantation of mammary epithelial cells or mammary tissue into cleared fat pad of weaning mice generates a functional epithelial tree within 8 weeks (12). The transplantation assay developed by De Ome and colleagues is the ‘gold standard’ to test the self-renewal and thus regenerative capacity of MaSC (13). I have started transplantation of MECs from CAS- and SPI-fed Wnt1-Tg mice into cleared fat pads of syngeneic mice as a proof of concept for dietary regulation of MaSC with *in vivo* regenerative capacity. My preliminary experiments show that MECs from a CAS-fed mouse are able to generate a complete epithelial tree upon transplantation into cleared fat pad of syngeneic mice (**Figure 6; Right**), while the non-transplanted fat pad had no outgrowth (**Figure 6; Left**). Ongoing experiments using MECs from CAS and SPI Wnt1-Tg mice transplanted into the same mice (CAS on right inguinal mammary gland; SPI on left inguinal mammary gland) will confirm the dietary regulation of MaSC activity (**Figures 2A and 4A**). To mechanistically identify molecular pathways that are responsible for dietary (SPI/GEN) effects on MaSC activity, I have collected RNA from sorted MaSC-enriched population from CAS and SPI fed mice to be used for gene array using a Gene Chip Mouse Genome 430 2.0 Array (4 chips per dietary group) (Affymetrix, Santa Clara, CA).

Figure 6



STATISTICAL METHODS

Data shown for dietary regulation of MaSC (CD29^{hi}CD24⁺) and cancer stem cells (Thy1⁺CD24⁺) by FACS analysis of MECs from pre-malignant (non-tumor) mammary tissue of Wnt1-Tg mice represents the average of at least three independent experiments, with MECs pooled from 5-7 mice per experiment, per diet (CAS or SPI). Data is presented as mean $\pm$ standard error of the mean (SEM) for each subpopulation analyzed. Statistical analysis was performed using SigmaStat software package version 3.2 (SPSS, Chicago, IL). Statistical significance between the treatment diet groups, based on P values ≤ 0.05 , was determined using one-way ANOVA followed by Tukey's post hoc analysis. For mammary tumor incidence, mice (n=24-34)/dietary group were used to allow a significant minimum decrease of ~20% to be detected with 80% power using the one-sided alpha level Chi-square test.

KEY RESEARCH ACCOMPLISHMENTS

- Generated evidence showing that SPI diet can decrease both the population of normal MaSCs (**Figure 2A**) and cancer stem cells (**Figure 2C**); supporting our hypothesis that SPI diet prevents the conversion of normal to cancer stem cells and thus tumor initiation (**Figure 1A**).
- Confirmed SPI inhibition of self-renewal of MaSC using mammosphere assay (**Figure 5**).
- Showed that soy isoflavone GEN (**Figure 3A and 3D**) can recapitulate the inhibitory effects of SPI on Wnt1-induced mammary tumor formation *in vivo* (**Figure 1A**).
- Determined that dietary GEN (**Figure 4**) is in part responsible for control of MaSC expansion by SPI diet (**Figure 2A**) and cancer stem cell conversion (**Figure 2C**).
- Therefore, these data provides the first direct link between dietary regulation of mammary stem cells and tumor protection *in vivo*.

SIGNIFICANCE

Mammary stem cells (MaSC) are required for tissue homeostasis and epithelial expansion during development; however, dysregulation of MaSC self-renewal such as by Wnt1 overexpression leads to neoplastic transformation. To date, *in vivo* dietary regulation of MaSC and its relation to breast cancer has not been evaluated. The main goal of my current Department of Defense (DOD)-funded study is to determine whether dietary regulation of MaSC and tumor initiating cells in the mammary tissue can have implications to breast cancer development. Our findings on inhibition of MaSC expansion and cancer stem cell conversion by dietary SPI/GEN in an oncogenic environment (e.g., overexpression of Wnt1 oncogene) and their correlation with lower tumor incidence provide novel insights, not previously explored, on the disparity in breast cancer among Asian women and their Western counterparts. This is particularly important to design better dietary guidelines early in life and manage children born with predisposition to breast cancer.

REPORTABLE OUTCOMES

- First author publication in *Endocrinology* journal (15).
- President's Choice - Publication of the Month, October 2011, Arkansas Children's Hospital Research Institute. **Rahal OM**, Simmen RC. Paracrine-acting adiponectin promotes mammary epithelial differentiation and synergizes with genistein to enhance transcriptional response to estrogen receptor β signaling. *Endocrinology*. 2011 Sep;152(9):3409-21. Epub 2011 Jun 28.
- Poster Contest Finalist – Era of Hope Conference, August 2-5, 2011, Orlando Florida.
- Travel Award - University of Arkansas for Medical Sciences Graduate School for attendance at the 2011 Era of Hope Conference (Orlando, Florida)

CONCLUSION

Our group has identified the up-regulation of the tumor suppressor PTEN and the inhibition of Wnt signaling pathway as mechanisms underlying the breast cancer preventive effects of soy foods (2-5). Both PTEN and Wnt pathways are involved in MaSC regulation and breast cancer (3, 8). We provided the first link for regulation of aberrant MaSC expansion by SPI/GEN diet and decreased mammary tumor formation. My study is highly relevant to our goal of furthering current understanding of the etiology of breast cancer, given extensive evidence for the cancer stem cell hypothesis which suggests that mammary cancer stem cells are not only the origin of breast cancer but also the cause of recurrence and death due to metastasis in affected patients. My studies elucidating the mechanisms for early prevention of breast cancer by diet/dietary factors have great potential to significantly influence public health policies to alleviate the increasing economic, financial, and emotional burdens of breast cancer and other diseases caused by poor nutrition.

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APPENDICES

APPENDIX 1: *Endocrinology* research article (2011) (added at the end of the report)

APPENDIX 2: Abstract submitted to the 2012 Experimental Biology Meeting. April 21-25, 2012. San Diego, CA.

Maternal blueberry diet suppresses Wnt1-induced mammary tumor progression in offspring

Authors: Omar M. Rahal, John Mark Pabona, Leah Hennings, Ronald L. Prior, Thomas Kelly, Ahmed Al-Dwairi, Frank A. Simmen, and Rosalia C.M. Simmen

Affiliations: Arkansas Children's Nutrition Center and University of Arkansas for Medical Sciences

Despite the well-accepted notion of peri-natal origins of adult diseases, the factors and regulatory mechanisms underlying breast cancer development remain unclear. Diet is a highly modifiable determinant of breast cancer risk, and the effects of the *in utero* nutritional environment persist beyond fetal life. We investigated whether *in utero*/lactational exposure to blueberry (BB) via maternal diet alters the trajectory of Wnt1-induced mammary tumorigenesis in offspring. Wnt1 transgenic mice were exposed to maternal diets of casein (CAS; n=33) or blueberry-supplemented CAS (3% BB; n=28) from gestation day 4 until post-natal day 21. Offspring were then weaned to CAS and mammary tumor development was followed until age 8 months. While tumor incidence and latency were similar for both groups, tumor weight (by 2-fold, p=0.034) and growth rate (by 60%; p=0.008) were reduced in offspring of BB- versus CAS-fed dams. Tumors from the BB group had higher expression of tumor suppressors PTEN and E-cadherin and lower cyclin D1 and pro-apoptotic Bcl2 levels. Transcript levels for DNA methylation enzymes DNMT1 and EZH2 were higher in BB tumors. Serum levels of insulin and of leptin/adiponectin ratio were lower for tumor-bearing BB than CAS offspring at sac. Our findings support a role for nutritional epigenetics in adult breast cancer outcome.

Funding: USDA-CRIS (RCMS), DOD-BCRP (OMR) and NIH-NCI (FAS).

APPENDIX 3: Abstract submitted to the 2011 Era of Hope Conference. August 2-5, 2011.
Orlando, FL. (*Poster Contest Finalist*)

BC093685-2620

**REGULATION OF MAMMARY STEM CELL POPULATION WITH DIETARY
INTAKE OF SOY PROTEIN ISOLATE REVEALS NOVEL MECHANISMS FOR DIET-
MEDIATED CONTROL OF MAMMARY TUMORIGENESIS**

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Breast cancer risk is highly modified by environmental factors including diet. Previously, we showed that dietary intake of soy protein isolate (SPI) decreased mammary tumor incidence and increased mammary tumor latency in rats relative to those fed a control casein (CAS) diet when exposed to the chemical carcinogen NMU. Mammary tumor preventive effects by SPI were associated with upregulation of the tumor suppressor PTEN and downregulation of the oncogenic Wnt-signaling components in mammary epithelial cells (MECs) leading to enhanced differentiation. Given that breast cancer is considered to be initiated by stem cells (SCs) with tumorigenic potential, termed cancer stem cells (CSCs), and mammary overexpression of Wnt-1 in mice causes spontaneous breast tumors due to the expansion of mammary CSCs, we hypothesized that diet may alter the mammary SC population to effect mammary tumor prevention. Here, we investigated SPI effects relative to CAS, on mammary tumor development in MMTV-Wnt1-Transgenic (Wnt1-Tg) female mice and on the mammary SC population in virgin wild-type (WT) and pre-neoplastic Wnt1-Tg female mice. Tumor incidence at 8 months of age of Wnt1-Tg mice fed SPI (n=30) post weaning was lower than in those fed CAS (48.3% vs. 73.5%; $p < 0.05$) (n=34). Interestingly, tumor latency in SPI-fed Wnt1-Tg mice was shorter than for the CAS-fed group (4.65 vs. 5.88 months; $P < 0.05$). Tumor weight and growth rate were similar for the diet groups. To evaluate SPI effects relative to CAS, on mammary SC population, epithelial cells from mammary tissues were isolated from Wnt1-Tg (PND75) mice. The percentage of mammary SCs was quantified by fluorescence-activated cell sorting analysis of MECs based on their expression of mouse mammary SC markers (CD29 and CD24) within the lineage negative (Lin-) population (CD45-, TER119-, CD31-). The Lin-CD29^{hi}CD24^{hi} subpopulation in MECs was decreased by 50% in Wnt1-Tg mice fed SPI post weaning relative to those fed CAS, decreasing the likelihood of mutations that convert normal to CSCs and could explain the protective effects of SPI on tumor incidence. Interestingly, the SC population was expanded by 2-fold in MECs of WT mice fed SPI relative to the CAS group, which could be beneficial for mammary gland development and tissue homeostasis. Our findings provide the first report of dietary effects on the SC population in MECs in vivo. The dichotomy of SPI effects on tumor outcome in mammary tissues with dysregulated Wnt signaling may be related to the loss of the complex regulatory grid between PTEN and Wnt/b-catenin pathways, both of which control SC fate. The possibility that diet can influence tumor progression at the level of the SC population suggests the important contribution of nutrition to the etiology of breast cancer and to the early management of breast health. *This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-10-1-0047, the U.S. Department of Agriculture, and Agricultural Research Service.*

APPENDIX 4: Co-authored abstract submitted to the 2011 Era of Hope Conference. August 2-5, 2011. Orlando, FL.

Targeting of Mammary Stem Cells by Dietary Factors in Breast Cancer Prevention

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Breast cancer is the most common malignancy of women in the Western world, with ~50,000 of those afflicted dying annually from the disease. Although many risk factors are associated with the development and progression of breast cancer, diet/nutrition constitutes a highly modifiable risk. Breast cancer is increasingly acknowledged to be initiated by mutations in a limited population of undifferentiated cells termed stem cells that 'sit' at the top of the mammary epithelial hierarchy. Over-expansion of the stem cell population can alter the balance of cell proliferation and differentiation and thus, increase the number of mutated cells that can initiate and maintain tumors which eventually metastasize. Novel strategies to decrease the over-expansion and promote the elimination of tumor-initiating cells are warranted for the effective treatment and prevention of breast cancer. Our studies test the hypothesis that dietary factors confer protection from breast cancer by preventing the expansion of stem/progenitor cells with tumorigenic potential. We established female mice transgenic for the oncogene Wnt-1 (Wnt-Tg), which develop spontaneous mammary tumors by 6-8 months of age, as a model system for dietary prevention of mammary tumor formation. Mice were fed American Institute of Nutrition-based isocaloric diets that differed only by protein source, namely Casein (CAS) and soy protein isolate (SPI). SPI was used as a paradigm for healthy foods, given the epidemiological linkage of decreased breast cancer incidence in women with high consumption of soy-rich foods. Lifetime dietary exposure to SPI beginning at pre-puberty resulted in lower mammary tumor incidence ($P<0.05$) at 8 months of age in Wnt-Tg females (48.3%; $n=30$), relative to those fed the control CAS diet (73.5%; $n=34$). Analyses of the mammary epithelial stem cell population by flow cytometry, using established mouse mammary stem cell markers CD29 and CD24 ($CD29^{hi}CD24^{+}$) within the Lineage-negative population indicated decreased number of stem cells (by 50%; $P<0.05$) in mammary glands of Wnt-Tg mice fed SPI. The mammosphere-forming efficiency of mammary epithelial cells isolated from mammary glands of SPI-fed mice was similarly lower (by 2-fold; $P<0.05$) than in those of mice fed CAS. To identify dietary component(s) that target stem cells to inhibit mammary tumor formation, we evaluated the ability of the major soy isoflavone genistein (GEN), a phytoestrogen, to inhibit mammosphere formation in two human breast cancer cell lines, namely the estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB231. Cells plated in ultralow attachment plates formed mammospheres at a frequency of 1-2% within 5 days of seeding. GEN at physiological doses ($40\text{ nM}>2\text{ }\mu\text{M}$) decreased the number of mammosphere-forming units in both cell lines, relative to medium alone. Our studies demonstrate a functional (inverse) connection between exposure to a 'healthy' diet during early life and abundance of mammary stem cells, providing strong support for healthy dietary strategies in young children to decrease incidence of breast cancer during

adulthood. Further, our work established the formation of mammospheres as a promising diagnostic tool for evaluating dietary factors with mammary tumor-inhibiting potential, which may help improve the efficacy of traditional chemotherapy.

Funding Support: DoD-BCRP-W81XWH-08-0548 (RCMS), USDA-CRIS 6251-5100002-06S (RCMS), and DoD-BCRP Predoctoral fellowship W81XWH-10-1-0047 (OR).

APPENDIX 5: Co-authored abstract presented at the 2011 Experimental Biology Meeting, April 9-13, 2011. Washington, DC (*Accepted for Oral Presentation*)

Repression of Mammosphere Formation in Breast Cancer Cells by Soy Isoflavone Genistein and Blueberry Polyphenols

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Epidemiological evidence implicates diets rich in fruits and vegetables in breast cancer prevention due to their phytochemical components, yet mechanisms underlying their presumed anti-tumor activities are not well-understood. A small population of mammary epithelial cells, termed cancer stem cells (CSC), may be responsible for initiating and sustaining tumor development. To evaluate dietary components that selectively target CSC and thus, provide mammary tumor protection, we utilized the estrogen receptor-positive MCF-7 and estrogen receptor-negative MDA-MB231 human breast cancer cell lines. Within 5 days of culture, both cell lines formed mammospheres at a frequency (1-2%) consistent with a subset of the cell population exhibiting stem cell-like characteristics. The soy isoflavone genistein dose-dependently decreased (40 nM > 2 μ M; by 2-3-fold) mammosphere numbers from both cell lines, relative to medium alone. A mixture of phenolic acids that include hippuric acid, ferrulic acid and 3-hydroxycinnamic acid, based on concentrations found in sera of rats fed diets containing 10% blueberry similarly inhibited (by 2-fold) mammosphere formation in MDA-MB231 but not in MCF-7 cells. The adipokine leptin and the inflammatory cytokine interleukin-6 had no activity in these cells. Findings suggest that dietary factors may target cancer cells with stem-like properties in the prevention of breast cancer.

Grant Funding Source: USDA-CRIS 6251-51000-005-02S; Department of Defense Breast Cancer Research Program 0810548 (RCMS) and W81XWH-10-1-0047 (OR)

APPENDIX 6: Co-authored abstract presented at the 2011 Experimental Biology Meeting. April 9-13, 2011. Washington, DC (*Accepted for Oral Presentation*)

Soy peptide lunasin induces PTEN-mediated apoptosis in human breast cancer cells

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The tumor suppressor PTEN inhibits the AKT signaling pathway whose unrestrained activity underlies many human malignancies. Previously we showed that dietary intake of soy protein isolate (SPI) enhanced PTEN expression in mammary tissue of rats with lower NMU-induced mammary tumor incidence relative to those fed casein-based diet. While epidemiological studies corroborate the breast cancer protective effects of soy, specifically of the major soy isoflavone genistein (GEN), the identity of other bioactive soy components remains relatively unknown. Here we evaluated the effects of lunasin, a soybean peptide previously detected in sera of rats and humans consuming soy-rich diets, on PTEN-mediated apoptosis of the mammary carcinoma cell line MCF-7. Lunasin (2 μ M >50 nM) increased PTEN expression and nuclear localization (by 2.5-fold); enhanced PTEN-mediated cellular apoptosis (by 10-15-fold); and altered levels of p53 (increased) and p21WAF1 (decreased) transcripts (P<0.05). GEN (2 μ M >20 nM) elicited similar effects as lunasin on PTEN expression and PTEN-mediated apoptosis in MCF-7 cells. Lunasin and GEN are known to regulate core histone acetylation by which PTEN promoter activity is similarly controlled. Findings suggest that activation of PTEN expression by bioactive soy components, possibly via epigenetic mechanisms may underlie breast cancer protection. [USDA-CRIS; Department of Defense BCRP]

Paracrine-Acting Adiponectin Promotes Mammary Epithelial Differentiation and Synergizes with Genistein to Enhance Transcriptional Response to Estrogen Receptor β Signaling

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Mammary stromal adipocytes constitute an active site for the synthesis of the adipokine, adiponectin (APN) that may influence the mammary epithelial microenvironment. The relationship between "local," mammary tissue-derived APN and breast cancer risk is poorly understood. Here, we identify a novel mechanism of APN-mediated signaling that influences mammary epithelial cell proliferation, differentiation, and apoptosis to modify breast cancer risk. We demonstrate that early dietary exposure to soy protein isolate induced mammary tissue APN production without corresponding effects on systemic APN levels. In estrogen receptor (ER)-negative MCF-10A cells, recombinant APN promoted lobuloalveolar differentiation by inhibiting oncogenic signal transducer and activator of transcription 3 activity. In ER-positive HC11 cells, recombinant APN increased ER β expression, inhibited cell proliferation, and induced apoptosis. Using the estrogen-responsive 4X-estrogen response element promoter-reporter construct to assess ER transactivation and small interfering RNA targeting of ER α and ER β , we show that APN synergized with the soy phytoestrogen genistein to promote ER β signaling in the presence of estrogen (17 β -estradiol) and ER β -specific agonist 2,3-bis(4-hydroxyphenyl)-propionitrile and to oppose ER α signaling in the presence of the ER α -specific agonist 4,4',4'-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol. The enhancement of ER β signaling with APN + genistein cotreatments was associated with induction of apoptosis, increased expression of proapoptotic/prodifferentiation genes (*Bad*, *p53*, and *Pten*), and decreased antiapoptotic (*Bcl2* and *survivin*) transcript levels. Our results suggest that mammary-derived APN can influence adjacent epithelial function by ER-dependent and ER-independent mechanisms that are consistent with reduction of breast cancer risk and suggest local APN induction by dietary factors as a targeted approach for promotion of breast health. (*Endocrinology* 152: 0000–0000, 2011)

Obesity (assessed by body mass index) increases the risk of breast cancer in postmenopausal women by 30–50% (1–3), in part due to aromatization of androstenedione to estrone by adipose tissue and subsequent conversion to the active hormone estradiol. Further, obese

women at diagnosis are more likely to have higher grade and poor prognosis regardless of their menopausal status (4–6). The adipose tissue produces and secretes at least 50 different polypeptide hormones termed adipokines that can act in an endocrine, paracrine, or autocrine manner,

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Abbreviations: APN, Adiponectin; APNR, APN receptor; CAS, casein; CSS, charcoal-stripped serum; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; E₂, 17 β -estradiol; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response element; 4xERE-TK-Luc, 4xERE-TK-Luciferase; GEN, genistein; MEC, mammary epithelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PND, postnatal day; PPT, 4,4',4'-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol; PTEN, phosphatase and tensin homolog deleted on chromosome ten; QPCR, quantitative real-time PCR; scRNA, scrambled RNA; siRNA, small interfering RNA; SPI, soy protein isolate; STAT3, signal transducer and activator of transcription 3; TK, thymidine kinase.

thus changing the notion of this tissue from a simple fat depot into a very active endocrine organ (7, 8). Unlike most adipokines, serum adiponectin (APN) level is lower in obese individuals compared with normal weight or lean subjects (9) and is considered to be a link between obesity and breast cancer (10–14).

APN effects are mediated through two types of receptors: APN receptor (APNR)1, which is expressed ubiquitously and has higher affinity for the low molecular weight APN trimer, and APNR2, expressed mainly in the liver and has similar affinity for the low and high molecular weight forms of APN (15). Physiological doses of APN inhibit cell proliferation and/or induce apoptosis of both estrogen receptor (ER)-negative (MDA-MD231) and positive (T47D and MCF-7) breast cancer cell lines in a cell type-specific manner (16–18). A recent study using mouse mammary tumor virus-polyomavirus middle T antigen transgenic mice with decreased APN expression demonstrated that *in vivo* APN haploinsufficiency facilitates mammary tumorigenesis by down-regulation of tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) activity and activation of phosphatidylinositol 3 kinase/AKT signaling (19).

A role for estrogen in the etiology of breast cancer is supported by increased risk of the disease during conditions of prolonged estrogen exposure, such as early menarche, late menopause, late first full-term pregnancy, and nulliparity (20). Biological effects of estrogen are mediated mainly by two members of the nuclear receptor superfamily, ER α and ER β . These ER isoforms can form homo- or heterodimers and, via the classical pathway, bind to estrogen response elements (ERE) in target genes or, through nonclassical pathways, interact with other transcription factors (21). Both receptors are coexpressed in approximately 70% of breast tumors, and although ER α is associated with cell proliferation and ER β with antiproliferative effects, the exact role of ER β in breast cancer remains controversial (22–24). However, ER α /ER β ratio is higher in breast tumors compared with normal tissue due to loss of ER β expression during tumor progression (25–27), suggesting a tumor suppressor role for ER β (28–33). Genomic and proteomic expression analysis of breast cancer cells indicate that when both ER are coexpressed, ER β inhibits the overall proliferative/survival actions of ER α (34–43).

The soy isoflavone genistein (GEN) is considered to partly mediate the protective effects of soy-rich diets against breast cancer (44, 45) by its preferential activation of ER β signaling, as shown by enhanced recruitment of steroid receptor coactivator more strongly to ER β in the presence of GEN (40, 46). In breast cancer cells expressing both ER, GEN inhibits the proliferative actions of ER α by

increasing the expression of a number of ER β -mediated proteins involved in apoptosis, cell cycle, motility, and lipid metabolism (47). Further, APN induces the expression of both ER in malignant mammary epithelial cells (MEC) (48), suggesting that APN may exert its antitumor effects by regulating the direction of ER α and/or ER β signaling. However, whether APN similarly functions in normal (nontumorigenic) MEC to influence ER α /ER β cross talk and, more importantly, whether this function of APN is coregulated by GEN have not been determined.

Here, we evaluated the hypothesis that dietary induction of APN synthesis and/or secretion by mammary stromal adipocytes leads to enhancement of ER β signaling on neighboring MEC by paracrine-acting APN. We show that lifetime dietary exposure of weaning and young adult female rats to soy protein isolate (SPI) increased APN protein levels in mammary tissue without parallel effects on systemic APN levels. Using nonmalignant human (MCF-10A) and mouse (HC11) MEC, we demonstrate that recombinant APN can enhance differentiation of ER-negative MCF-10A cells by its suppression of basal signal transducer and activator of transcription 3 (STAT3) activity and promote apoptosis and differentiation of ER-positive HC11 cells by its activation of ER β signaling in cooperation with the ER β -specific ligand GEN. Further, using specific agonists to ER α [4,4',4''-(4-propyl-(1*H*)-pyrazole-1,3,5-triyl)trisphenol (PPT)] and ER β [2,3-bis(4-hydroxyphenyl)-propionitrile (DPN)] along with small interfering RNA (siRNA) technologies targeting either receptor isoform, we define novel synergistic roles for APN and GEN in promoting ER α /ER β cross talk in MEC.

Materials and Methods

Animals and diets

All animal experiments were carried out under protocols approved by The University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. Time-mated Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were individually housed in polycarbonate cages under conditions of 24 C, 40% humidity, and a 12-h light, 12-h dark cycle. At gestation d 4, dams were randomly assigned to one of two semipurified isocaloric diets containing either casein (CAS) (New Zealand Milk Products, Santa Rosa, CA) or SPI (Solae, St. Louis, MO) as sole protein source and formulated following the American Institute of Nutrition-93G guidelines (49), except that corn oil was substituted for soybean oil. SPI contains the isoflavones GEN (216 ± 2 mg/kg) and daidzein (160 ± 6 mg/kg) as aglycone equivalents. Animals were provided food and water *ad libitum*. At delivery, all pups from dams of the same diet groups were pooled, and 10 pups (five per sex) were randomly assigned to each dam to nurse. Female pups were weaned at postnatal day (PND)21 to the same diets as their dams and were fed this diet throughout the study. The inguinal mammary glands (number 4)

were collected at PND21 and at PND50 ($n = 5$ female offspring per PND) and processed for Western blot analyses as described below.

Cell culture and treatments

The mouse MEC line HC11 (kindly provided by Jeffrey M. Rosen (Baylor College of Medicine, Houston, TX) and the human nontumorigenic MEC line MCF-10A (American Type Culture Collection, Manassas, VA) were maintained in growth medium at 37°C in a 5% CO₂ incubator as previously described (50, 51). Phenol red-free media supplemented with charcoal-stripped serum (CSS) was used for serum starvation (0.5% CSS) and treatments (2.5% CSS). Recombinant mouse or human APN (R&D Systems, Inc., Minneapolis, MN) dissolved in PBS was used at 8 µg/ml and GEN (Sigma Chemical Co., St. Louis, MO) dissolved in dimethylsulfoxide was used at 40 nM. Treatments with PBS and dimethylsulfoxide served as negative controls.

Serum APN levels

The concentrations of APN in sera of CAS- and SPI-fed rats collected at PND21 and PND50 were measured using a rat APN ELISA kit (Linco Research, St. Charles, MO). The assay sensitivity was 0.15 ng/ml, and intra- and interassay variations were less than 8.5%.

Antibodies and Western blot analysis

Whole-cell extracts were prepared and immunoblotted following previously described protocols (50, 52). Antimouse APN (Abcam, Inc., Cambridge, MA), antiphospho-STAT3 Tyr705 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-STAT3 (Santa Cruz Biotechnology, Inc.) antibodies were each used at 1:1000 dilution. Anti- α -tubulin (Santa Cruz Biotechnology, Inc.) and anti- β -actin (Sigma Chemical Co.) antibodies at 1:2000 dilutions were used as normalizing controls for protein loading. Blots were stripped with Restore Western blot stripping buffer (Pierce Biotechnology, Rockford, IL) before reprobing with additional antibodies. Immunoreactive signals were visualized using Amersham ECL Plus (GE Healthcare Life Sciences, Piscataway, NJ) and quantified using the Bio-Rad molecular analyst detection system and Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Quantitative real-time PCR (QPCR)

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad Laboratories). QPCR was carried out using the SYBR Green Supermix (Bio-Rad Laboratories) and ABI Prism 7000 Detection System (Applied Biosystems, Foster City, CA). Primers (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>) for PCR were designed to span introns using Primer Express software (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The expression of each target mRNA was calibrated to a standard curve generated using pooled cDNA stocks and normalized to that of TATA-box binding protein (*Tbp*).

Cell viability and numbers

The number of viable cells was evaluated using a cell proliferation assay kit [3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-

nyltetrazolium bromide (MTT); American Type Culture Collection] according to the manufacturer's instructions. Cells (20,000 cells/well) were seeded in 96-well plates and treated with APN (8 µg/ml) or vehicle (PBS) every 2 d for 6 d. Absorbance values (570 nm) reflect the ability of metabolically active cells to reduce the yellow tetrazolium MTT salts into a purple precipitate. Viable cell numbers were determined under the same treatment conditions by Trypan blue exclusion method (INC Biomedicals, Inc., Aurora, OH). Each experiment was conducted in quadruplicate and repeated twice.

Acini morphogenesis assay

MCF-10A cells were seeded on a layer of Matrigel (BD Biosciences, San Jose, CA) in eight-well chamber slides and allowed to form acini as previously described (52). Culture medium containing 2% charcoal-stripped horse serum and 5 ng/ml epidermal growth factor (EGF) without (vehicle alone) or with added APN (8 µg/ml) was refreshed every 4 d. At least 80 acini were counted from five random areas per chamber ($\times 20$ objective), with four chambers for each treatment group. Acini number and diameter were assessed at d 12 of culture using a phase contrast microscope (Carl Zeiss AG, Oberkochen, Germany) ($\times 20$ objective). Confocal images of 4',6-diamidino-2-phenylindole-stained acini were collected on a Zeiss LSM510 confocal microscope ($\times 20$ objective).

Fluorescence-activated cell sorting

Untreated and treated HC11 cells were harvested with trypsin, washed with ice-cold PBS, and fixed with ice-cold 70% ethanol. After propidium iodide (10 µg/ml) staining, at least 10,000 cells were analyzed using a Becton Dickinson FACSCalibur (BD Biosciences). Histograms were generated with the CellQuest software program (BD Biosciences).

Transient transfection and luciferase assays

The 4xERE-thymidine kinase (TK)-Luciferase reporter construct was generously provided by Benita S. Katzenellenbogen (University of Illinois, Urbana-Champaign, IL). HC11 cells were cotransfected with the reporter plasmid or empty vector (pGL3B) (each added at 0.5 µg/well) and with *Renilla*-Luciferase construct (50 ng/well) using Lipofectamine 2000 (Invitrogen), as previously described (52, 53). Incubation with 17 β -estradiol (E₂) (10 nM), DPN (40 nM), PPT (40 nM), or GEN (40 nM) was carried out without or with APN (8 µg/ml) pretreatment for 24 h. Cells were lysed in lysis buffer (Promega, Madison, WI) and quantitative determination of luciferase activity used a Dual-Luciferase Reporter Assay System (Promega) and a MLX Microplate Luminometer (Dynex Technologies, Chantilly, VA). ERE-Luc activity was normalized to that of *Renilla* luciferase, which served as an internal control for transfection efficiency. Data are presented as means $\pm$ SEM from at least three independent experiments, with each experiment performed in quadruplicates.

Apoptosis assay

HC11 cells were seeded in white-walled 96-well plates (15,000 cells/well) and treated with E₂ (10 nM) in the presence or absence of GEN (40 nM) and/or APN (8 µg/ml) for 72 h. Cell apoptosis was determined by quantifying caspase-3 and caspase-7 activity using the luminometric Caspase-Glo 3/7 assay

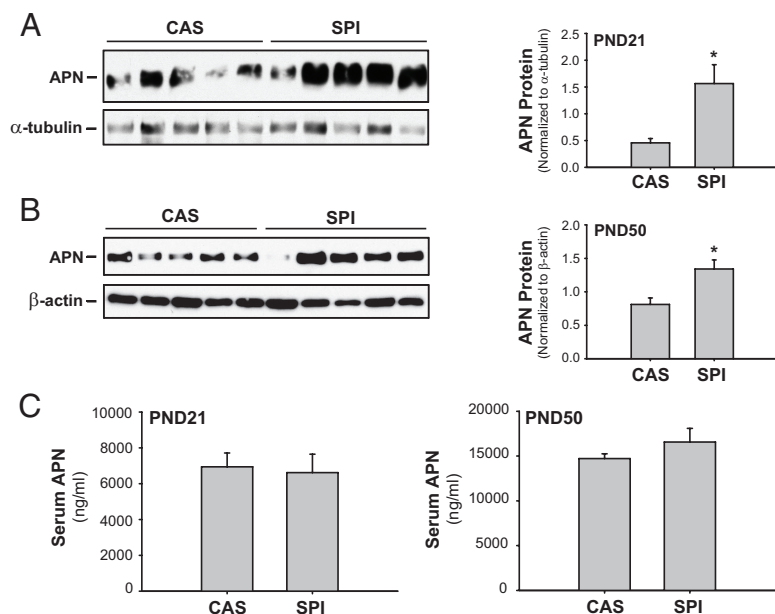


FIG. 1. Dietary SPI induces local APN protein expression in mammary tissue. A and B, *left*, Western blot analysis of APN protein in mammary tissue of female rat offspring at PND21 (A) or PND50 (B) exposed to CAS or SPI diets. *Each lane* represents an individual animal and contains 50 μ g of total protein. *Right*, Immunoreactive bands were quantified by densitometry and values normalized to those of loading control α -tubulin or β -actin, respectively, and are presented as histograms; *, $P < 0.05$ relative to CAS. C, Serum APN levels were quantified in weanling (PND21) or young adult (PND50) rats using a rat APN ELISA kit as described in *Materials and Methods* ($n = 7$ rats per diet, per PND).

kit (Promega) following the manufacturer's protocol and a MLX Microplate Luminometer (Dynex Technologies).

Data analysis

Computer-assisted statistical analyses were performed using the StatView 5.0 program for Windows. Data were analyzed by Student's t test, one-way ANOVA, or two-way ANOVA. Differences between means in two-way ANOVA were further analyzed by Tukey's test. A value of $P < 0.05$ was considered significant.

Results

Dietary SPI increases mammary APN expression

To date, most studies linking APN to obesity-related breast cancer are based on systemic APN levels; hence, the relationship between mammary APN expression and breast cancer occurrence has not been fully determined. Here, we examined whether early exposure to dietary SPI that promoted mammary epithelial differentiation *in vivo* (54) and conferred protection from chemical-induced mammary tumor formation in female rat offspring (55, 56) is associated with higher APN production in mammary adipocytes. Mammary tissues from female rats exposed to dietary SPI or the control diet CAS beginning at gestation d 4 until tissue collection at PND21 and at

PND50 were assessed for APN expression by Western blotting. Levels of APN protein (molecular mass 30 kDa) were higher in mammary tissues of weanling and young adult rats exposed to SPI than to CAS diets (Fig. 1, A and B). Serum APN levels increased with age (PND50 > PND21) (Fig. 1C) and were within the reported physiological range for humans and mice (57). Despite the lower body weights of PND21 and PND50 rats exposed to dietary SPI when compared with the control group (54), there was no comparable increase in systemic APN levels with diet, as was shown for mammary tissue (Fig. 1, A–C). These data provide the first *in vivo* evidence for dietary regulation of “local” mammary APN expression and suggest that mammary adipose-derived APN may be a mediator of the mammary tumor protective effects of dietary SPI.

APN inhibits mammary epithelial proliferation and promotes cellular apoptosis

The tumor protective actions of APN on breast epithelium are likely mediated at multiple levels, including proliferation, apoptosis, insulin sensitivity, growth factor sequestration, recruitment of proinflammatory cytokines, and angiogenesis (58). To mechanistically dissect the functional outcomes of greater mammary adipocyte APN production/secretion with SPI dietary exposure (Fig. 1, A and B) on neighboring epithelial cells, mouse nontumorigenic MEC HC11 were treated with recombinant mouse APN (8 μ g/ml) and assessed for cell proliferation/viability and apoptosis status relative to control (vehicle only treated) cells. Cells administered growth media without added EGF for the same period as APN (no EGF, Fig. 2A) served as positive control in these experiments. APN treatment for 24 or 48 h decreased cell viability, as measured by the MTT assay, relative to control cells (Fig. 2A). Consistent with the MTT assay, APN treatment decreased cell numbers ($0.64 \pm 0.03 \times 10^6$) relative to control group ($0.83 \pm 0.02 \times 10^6$; $P < 0.05$) (Fig. 2B). Fluorescence-activated cell sorting analysis of cells treated with APN for 12 h showed an increase in the percentage of cells in the sub- G_0 (apoptotic) phase, with no changes noted at other cell cycle stages (Fig. 2, C and D). The higher apoptotic status with APN treatment was correlated with an early, although transient, decrease (compare 6 and 24 h) in transcript levels for survivin (Fig. 2E), the antiapoptotic protein normally up-regulated in human breast cancer (59), relative to untreated cells. APN did not alter the expression

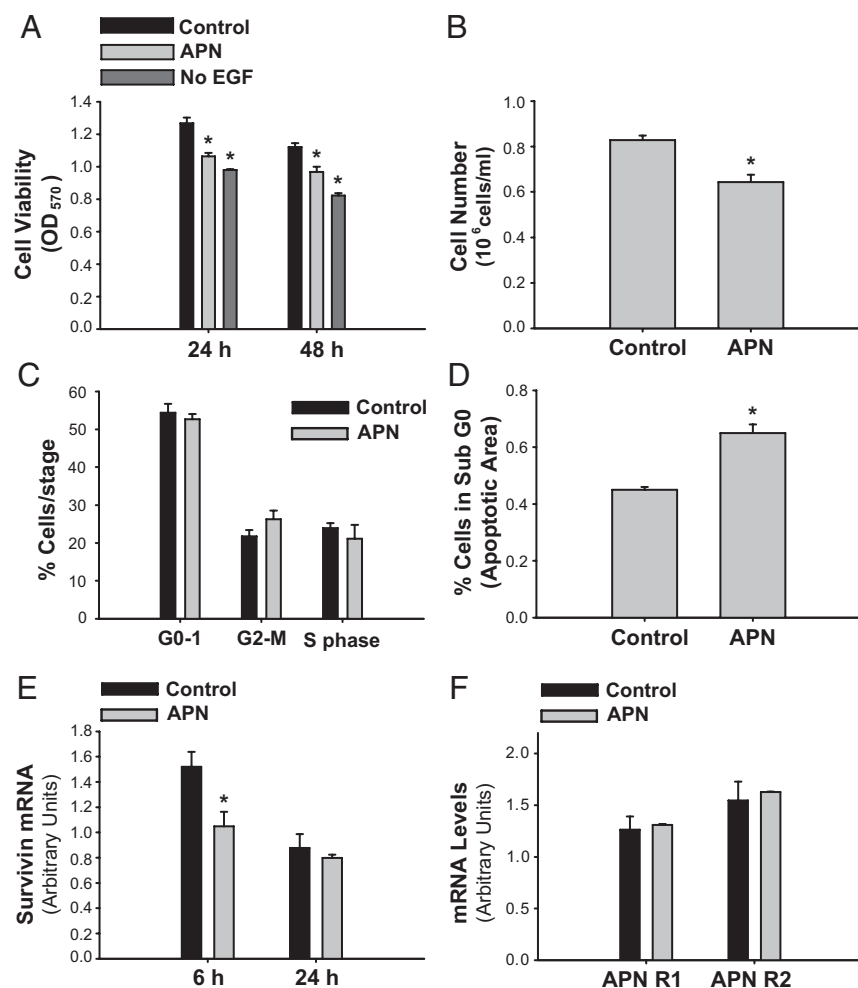


FIG. 2. APN inhibits proliferation and promotes apoptosis of ER-positive HC11 cells. A, HC11 cells treated with APN (8 μ g/ml) for 24 or 48 h had decreased cell proliferation compared with control cells (vehicle treated) as measured by the MTT assay. Cells incubated in medium without added EGF for the same duration served as a positive control for decreased cell growth. B, Cells were seeded in 100-mm dishes and treated in same manner as for MTT assay. Viable cells were quantified by the trypan blue exclusion method after 12 h of treatment using a hemocytometer. C and D, Control and APN-treated cells were analyzed by fluorescence-activated cell sorting. E and F, Transcript levels of the antiapoptotic protein survivin and of APNR1 and APNR2 were quantified by QPCR and normalized to *Tbp*. Results are mean $\pm$ SEM from two independent experiments performed in triplicate; *, $P < 0.05$ relative to control.

of its receptors APNR1 and APNR2 (Fig. 2F), which have been shown to mediate its pleiotropic actions in target cells (15).

APN enhances mammary epithelial differentiation

To further address APN regulation of mammary epithelial function as an underlying mechanism for mammary tumor protection, APN effects on differentiation were assessed in two MEC lines using distinct outcomes. Expression of β -casein mRNA levels, a marker of mammary epithelial differentiation, was evaluated in the ER-positive HC11 cells treated with recombinant mouse APN or vehicle, in the presence of ovine prolactin (5 μ g/ml; ovine

prolactin-21, AFP-10692C) for 48 h. As shown in Fig. 3A, APN up-regulated β -casein mRNA levels by 6.2-fold ($P = 0.006$) relative to control (vehicle) cells. In the ER-negative, nontumorigenic human MEC MCF-10A plated on Matrigel-coated chamber slides, APN promoted the formation of acini structures with hollow lumen (Fig. 3, B and C), resembling the morphogenesis of the mammary gland during pregnancy (60). The shift toward the formation of larger acini [40–90 ($\times 10^2$) μ m² range] from the smaller-sized structures [<10 ($\times 10^2$) μ m² range] was observed as early as d 6 (data not shown) and persisted through d 12 of APN treatment (Fig. 3, B–D), indicating induction by APN of early lobuloalveolar differentiation. These results suggest that APN promotes the differentiation of nontumor MEC, irrespective of ER status.

In a previous study using genome-wide profiling of mammary tissue of weanling rats exposed to CAS or SPI via maternal diet (54), we found that expression levels of the transcription factor STAT3 were attenuated with dietary exposure to SPI, relative to CAS. Given the reported inhibition by APN of STAT3 signaling (61), the constitutive activation (measured as tyrosine phosphorylation) of STAT3 in breast cancer (62), and findings that promotion of tumor cell survival is partly mediated by activated STAT3 through up-regulation of the antiapoptotic protein survivin (59, 62), we evaluated whether enhanced differentiation of MEC HC11 and MCF-10A induced by APN is mediated by APN inhibition of STAT3 signaling. Western blot analyses showed comparable levels of total STAT3 protein (using anti-STAT3 antibodies) in control and APN-treated HC11 and MCF-10A cells (Fig. 3, E and F). However, whereas HC11 cells demonstrated undetectable/low activated STAT3 levels (anti-pSTAT3 immunoreactivity) with or without APN treatment (Fig. 3E), MCF-10A cells showed robust STAT3 activity that was significantly attenuated by APN (Fig. 3F). These results suggest that context-dependent signaling mechanisms may underlie the biological response of MEC to the protective effects (*e.g.* increased differentiation) of APN.

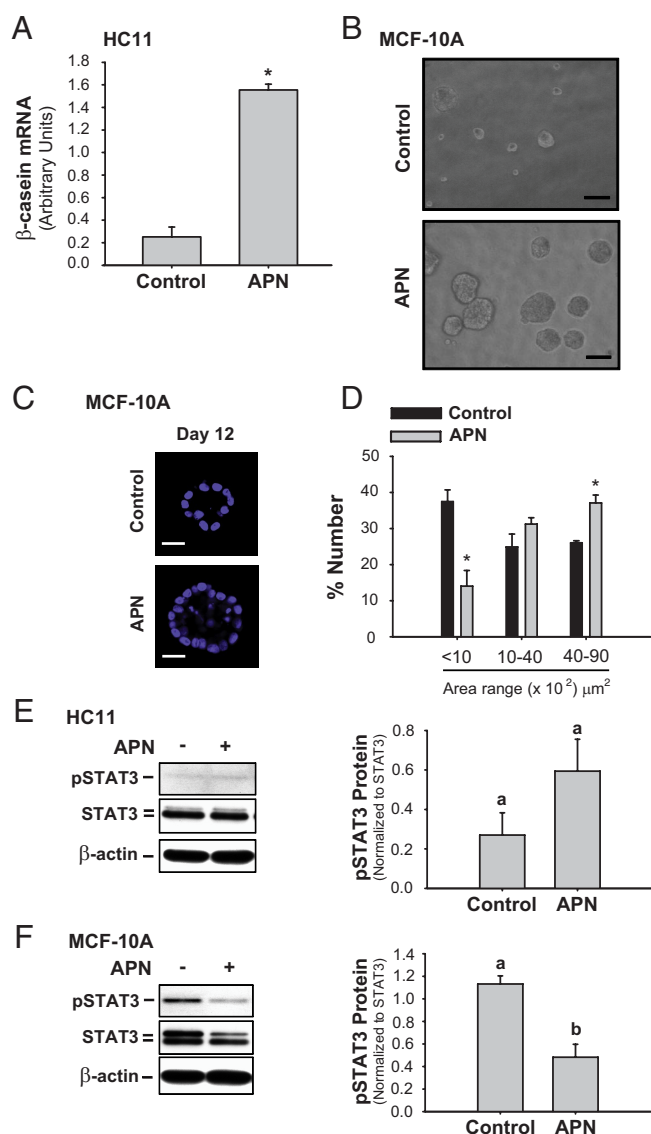


FIG. 3. APN enhances differentiation of MEC. **A**, Gene expression of the mammary gland differentiation marker β -CAS was quantified in HC11 cells exposed to prolactin for 48 h and then treated with APN (8 μ g/ml) or vehicle for 24 h. **B–D**, MCF-10A cells were plated on a layer of Matrigel and allowed to form acini with hollow lumens as described in *Materials and Methods*. **B**, Phase contrast images of acini treated with vehicle (control) or APN. Scale bar, 50 μ m. **C**, Z-sections of 4',6-diamidino-2-phenylindole-stained acini showing hollow lumen and morphology at d 12 of culture were recorded by confocal imaging. Scale bar, 20 μ m. **D**, The numbers of acini were counted at d 12 of mammary acini morphogenesis from five randomly chosen areas per chamber with four chambers per treatment group. Results are mean $\pm$ SEM from two independent experiments; *, $P < 0.05$ relative to control. **E** and **F**, HC11 or MCF-10A cells were treated with mouse or human recombinant APN for 18 h, respectively, and whole-cell extracts were prepared. Protein levels of phosphorylated STAT3 (pSTAT3) (upper panel), total STAT3 (middle panel), and β -actin (lower panel) were analyzed in cell lysates (20 μ g of total protein) by Western blotting. Immunoreactive bands were quantified by densitometric scanning. Values normalized to those of loading control β -actin are presented as histograms (right panel). Representative blots from three independent experiments with similar results are shown; *, $P < 0.05$ relative to control.

APN promotes ER β transcriptional activity

In light of earlier findings suggesting an inverse estrogen (E_2)/APN connection in human breast cancer cells (63), we addressed the participation of ER on APN signaling in the nonmalignant, ER-positive HC11 cells. We first evaluated whether APN altered the expression levels of ER α and ER β isoforms in these cells at the mRNA and protein levels, by QPCR and Western immunoblottings, respectively. Cells treated with APN for 6 h showed lower ER α (2-fold) and higher ER β (2-fold) transcript levels, relative to control (vehicle treated) cells (Fig. 4A). A more robust effect ($\sim$ 10-fold) of APN on ER β protein expression was observed when compared with vehicle-treated cells, in contrast to the lack of effect noted on ER α protein levels (Fig. 4, B and C). On a per protein basis, however, expression levels of ER α were greater than those of ER β in these cells. Thus, APN may promote ER β signaling in HC11 cells by increasing ER β relative to ER α gene and protein expression.

To evaluate the functional consequence of the preferential induction by APN of ER β relative to ER α expression in mammary epithelium, the transcriptional response of HC11 cells to ER β agonists DPN and GEN (20-fold higher affinity for ER β than for ER α) (40) were compared with those of E_2 (binds ER α and ER β with similar affinities) and ER α -specific agonist PPT, in control and APN-treated cells. Cells were transfected with the estrogen-responsive 4xERE-TK-Luciferase (4xERE-TK-Luc) construct as a reporter for ER transactivation. In cells transfected with the 4xERE-TK-Luc plasmid, treatment with E_2 and with PPT resulted in similar (2-fold) increases in Luc promoter activity, relative to untreated cells (Fig. 4D). GEN elicited a modest, although significant, increase in Luc reporter activity relative to nontreated cells; the magnitude of the increase was lower than that obtained with E_2 and PPT treatments (Fig. 4D). DPN-treated cells displayed Luc reporter activity that, although not statistically significant, was numerically higher to those of untreated cells. Because both ligand-activated ER isoforms are known to bind the ER recognition sequence in the 4xERE-TK-Luc construct with comparable affinities, results are consistent with the predominant expression of ER α relative to ER β in non-APN-treated HC11 cells (Fig. 4, B and C). Interestingly, APN treatment had no effect on the magnitude of ER-mediated transcriptional responses individually elicited by E_2 , DPN, or GEN (which all bind ER β) (Fig. 4D), despite APN induction of ER β expression levels in these cells (Fig. 4, A–C). These collective data suggest that APN actions may be manifest only under specific cellular contexts, possibly when both ER are functionally active (*i.e.* ligand activated) or under conditions

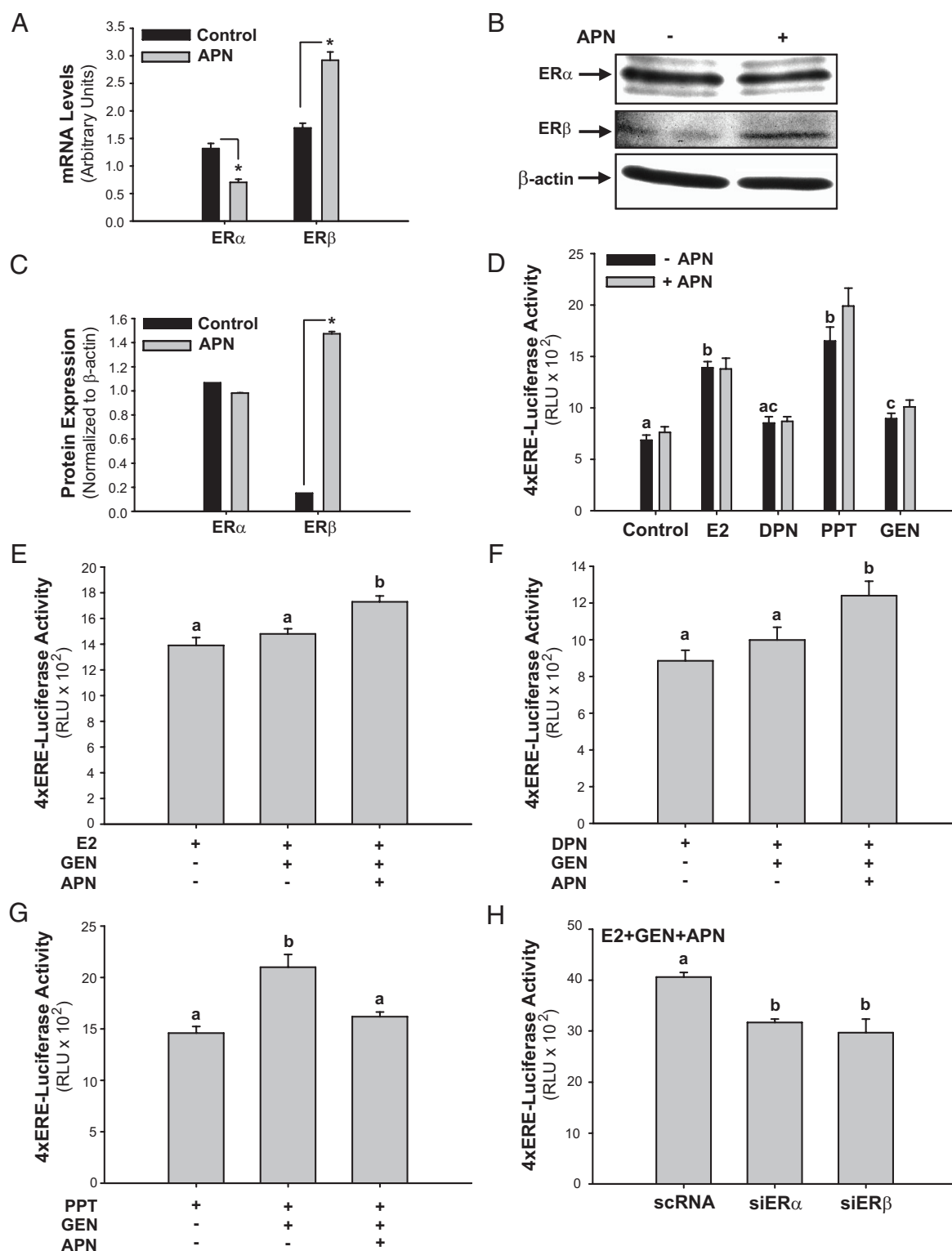


FIG. 4. APN induces ER β expression and synergizes with GEN to promote ER β transcriptional activity. **A**, Transcript levels of ER α and ER β were quantified by QPCR in HC11 cells treated with APN (8 μ g/ml) or vehicle (control) for 6 h. **B**, Western blot analysis of ER α and ER β in lysates from cells treated as in **A**. Each lane contains 20 μ g of total protein. **C**, Protein levels in **B** were quantified by densitometry and normalized to β -actin; *, $P < 0.05$ relative to control. **D–H**, HC11 cells were transfected with 0.5 μ g of 4xERE-TK-Luc promoter/reporter construct in the presence or absence of control siRNA (scRNA) or siRNA targeting ER α or ER β and treated with APN and specific ER ligands, alone and in combination. **D**, Cells were treated with vehicle (control), E₂, DPN, PPT, or GEN in the presence or absence of APN (8 μ g/ml) for 24 h, and lysates were subsequently analyzed for luciferase activity. Effects of GEN and APN on the transcriptional response of E₂ (**E**), DPN (**F**), and PPT (**G**) were analyzed. **H**, Effects of knockdown of ER α and ER β by respective siRNA on Luciferase reporter activity in cells treated with E₂ + GEN + APN were evaluated, relative to nontargeting (scRNA) siRNA. Values are means $\pm$ SEM from at least three independent experiments performed in triplicate. Means with different letters differed from control (**D–G**) or nontargeting scRNA (**H**) at $P < 0.05$. RLU, Relative luminescence unit.

requiring the availability of ER-specific ligands (*e.g.* GEN for ER β) that can recruit specific coactivators and/or repressors (46).

GEN synergizes with APN to influence ligand-activated ER β transcriptional activity

We have previously shown that GEN at physiologically relevant doses (40 nM, 2 μ M) elicited increased differentiation, decreased proliferation, and promoted apoptosis of MEC *in vitro*, consistent with it being a major bioactive component of soy foods with breast cancer protective effects (50–52, 64). Our findings, thus far, suggest that *in vivo*, mammary stromal adipocytes and neighboring epithelial cells constitute coordinate targets of GEN action. To examine whether stromal adipocyte-derived APN promotes GEN action in MEC by enhancing GEN activation of ER β signaling, we evaluated whether APN influences the transcriptional activity of GEN in the presence of the physiologically relevant ligand E₂ and compared these effects with those elicited with specific ER β (DPN) and ER α (PPT) agonists. HC11 cells were treated with E₂ (10 nM), DPN (40 nM), or PPT (40 nM) in the presence or absence of APN (8 μ g/ml) and/or GEN (40 nM), and 24 h later, 4xERE-Luc activity was analyzed. GEN in the absence of APN had no effect on basal ERE-TK-Luc activity of E₂ (Fig. 4E). Similar for E₂, GEN had no significant effect on DPN-activated promoter activity in the absence of APN (Fig. 4F). By contrast, GEN increased PPT-activated transcriptional responses in non-APN-treated cells (Fig. 4G). Interestingly, APN cotreatment with GEN significantly influenced these cells' transcriptional responses to all ligands (Fig. 4, E–G). In particular, APN enhanced Luc promoter activity in GEN + E₂-treated (Fig. 4E) and GEN + DPN-treated (Fig. 4F) cells while decreasing this activity in PPT + GEN-treated cells to PPT-alone levels (Fig. 4G).

Given that GEN preferentially binds ER β , the above findings are consistent with GEN + APN promoting ER β signaling by increasing ER β homodimer (with DPN) or ER α /ER β heterodimer (with E₂) transcriptional activities at the expense of ER α homodimer-mediated (with PPT) transactivity. To address this, ER α or ER β expression was knocked down in APN + GEN + E₂-treated cells transfected with 4xERE-TK-Luc plasmid, using a pool of siRNA targeting each ER isoform. A decrease by approximately 25% in 4xERE-TK-Luc promoter activity was observed with ER α siRNA or ER β siRNA added at equivalent concentrations (50 nM), relative to cells treated with nontargeting siRNA [scrambled RNA (scRNA), 50 nM] (Fig. 4H). We determined 50 nM as an optimal dose with effective knockdown ($\sim$ 70%) at the mRNA level (data not shown). Interestingly, the magnitude of the decrease in

promoter activity achieved by targeting either ER α or ER β with respective siRNA was comparable with the extent of promoter activity induction in APN + GEN + E₂-treated cells, when compared with those treated with E₂ + GEN in the absence of APN (Fig. 4E).

APN/GEN promotion of ER β signaling is associated with up-regulation of proapoptotic and prodifferentiation gene expression

To determine whether APN promotion of GEN-mediated ER β signaling may underlie the observed *in vivo* enhancement of MEC differentiation associated with exposure to SPI or GEN-supplemented diets, leading to mammary tumor protection (50–52, 55, 56, 64), we determined the expression of select proapoptotic (*Bad*, *p53*), antiapoptotic (*Bcl2*, *survivin*), and prodifferentiation/proapoptotic (*Pten*) genes in APN + GEN-treated cells cotreated with either E₂, DPN, or PPT. Because APN induced ER β signaling in the absence (Fig. 4, A–C) or presence of E₂ (Fig. 4E), and in view of previous findings showing enhancement of cell proliferation by E₂ upon blockage of ER β in HC11 cells (65), the activity of proapoptotic proteins caspase-3 and caspase-7 was initially evaluated in HC11 cells treated with E₂ alone, E₂ + GEN, and E₂ + GEN in the presence of APN, for 72 h. APN + E₂ + GEN cotreatments enhanced caspase-3/7 activity as measured by luminescence, compared with treatment with E₂ or E₂ + GEN (Fig. 5A). Further, cells cotreated with APN + GEN + E₂ showed increased *Pten* and decreased *survivin* expression, with no comparable changes in *Bad*, *Bcl2*, and *p53* transcript levels, when compared with non-APN-treated controls (Fig. 5B). Cells cotreated with DPN in the presence of GEN + APN elicited changes in gene expression levels consistent with APN + GEN inhibition of ER α signaling and promotion of ER β -mediated transcriptional responses (Fig. 5C). In particular, transcript levels for *Bad*, *p53*, and *Pten* were increased, whereas those for *Bcl2* and *survivin* were attenuated with DPN + GEN + APN cotreatments. The transcript levels of *Bad* and *p53* were increased, and those for *Bcl2* were decreased, with no changes in *Pten*, or *survivin* expression levels, with PPT + GEN in the presence of APN, relative to without APN (Fig. 5D). Taken together, our results show that the concerted actions of APN and GEN in the promotion of ER β signaling under physiological E₂ levels may underlie the contribution of adiposity/diet interactions to influence mammary epithelial differentiation and apoptosis for breast cancer prevention.

Discussion

In this study, we present a novel mechanism for dietary regulation of ER β signaling in MEC involving the adipo-

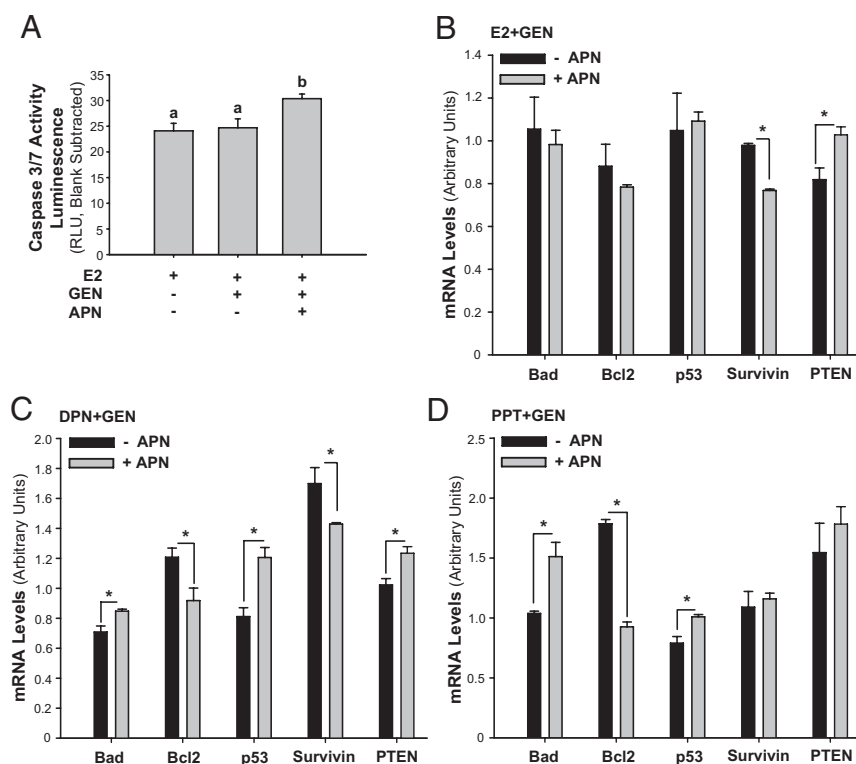


FIG. 5. Synergistic actions of APN and GEN enhance apoptosis in MEC. A, HC11 cells were treated with E₂ (10 nM) in the presence or absence of GEN (40 nM) and/or APN (8 μ g/ml) for 72 h and subsequently assessed for caspase-3/7 activity, as described under *Materials and Methods*. Means with different letters differed from control at $P < 0.05$. Representative graph from two independent experiments, each performed in quadruplicate is shown. B–D, Gene expression of proapoptotic, differentiation-related (Bad, p53, and PTEN), and antiapoptotic (Bcl2, survivin) proteins was quantified by QPCR in cells treated with E₂ + GEN (B), DPN + GEN (C), and PPT + GEN (D) in the presence (+APN) or absence (–APN) of APN for 24 h. *Tbp* was used as a normalizing control; *, $P < 0.05$ relative to (–) APN group. Bad, Bcl2-Associated agonist of cell death; p53, tumor suppressor p53; Bcl2, B-cell leukemia/lymphoma 2; RLU, relative luminescence unit.

kinase APN, whose attenuated expression with higher adiposity is associated with increased breast cancer risk. We provide evidence to support a model (Fig. 6) in which dietary induction of APN protein levels in the mammary stromal adipocytes induces ER β expression in MEC to facilitate synergistic activation of ER β signaling by the ER β -selective ligand GEN in concert with the physiologically relevant ligand E₂, to inhibit proliferation, enhance differentiation, and promote apoptosis of MEC for breast cancer prevention. We further show that in MEC lacking functional ER, simulating those in prepuberty or postmenopausal conditions, APN functions in an ER-independent manner by inhibiting STAT3 activation, an event associated with increased epithelial differentiation as measured by mammary acini formation. Together, our findings provide a linear pathway by which diet-regulated mammary adipocyte APN and, hence, mammary adiposity status may modify the prepubertal/peripubertal and perimenopausal/postmenopausal mammary epithelium

to achieve enhanced differentiation and integrate the local stromal adipocyte environment in orchestrating ER-dependent and ER-independent mechanisms to promote neighboring epithelial resistance to tumorigenic agents (66).

A major novel finding of the present study is the robust local induction of APN protein in mammary tissue by dietary intake of soy protein at peripuberty, a critical stage of mammary gland development. Although decreased serum APN levels are associated with increased risk and aggressiveness of breast cancer (12–14), a detailed role for local mammary tissue-derived APN in the promotion of breast cancer has not been fully established. Our current and previous (54) analyses of mammary glands of peripubertal rats from dams consuming SPI diets at amounts modeling the regular intake of soy-rich foods by the Asian population demonstrate the impact of early maternal diet on the adiposity of the developing mammary gland. Further, our identification of APN as a diet-regulated mammary adipocyte-secreted protein that mediates differentiation events in normal (nontumorigenic) ER-positive (HC11) and ER-negative (MCF-10A) MEC has important implications for the suggested intrauterine origins of breast cancer risk. In support of the latter,

recent findings implicate a role for maternal obesity in the programming of APN signaling in offspring (67) and for systemic APN in children as a presumptive determinant of obesity-related diseases in later adult life (68–70).

Another novel finding from this study is the demonstration of APN/ER cross talk, leading to the promotion of ER β transcriptional activation in normal MEC. Although a connection between APN and ER signaling in breast cancer cells has been previously raised (63), the current study takes a further step by addressing the influence of the dietary bioactive factor GEN in APN/ER cross talk in normal (nonbreast cancer) epithelial cells. We showed that APN in the presence of GEN modified the direction of genomic ER signaling by two mechanisms. First, APN preferentially increased ER β isoform expression in HC11 cells, resulting in a higher ratio of ER β to ER α at the levels of transcript and protein. Second, APN in concert with GEN altered the transcriptional responses of HC11 cells

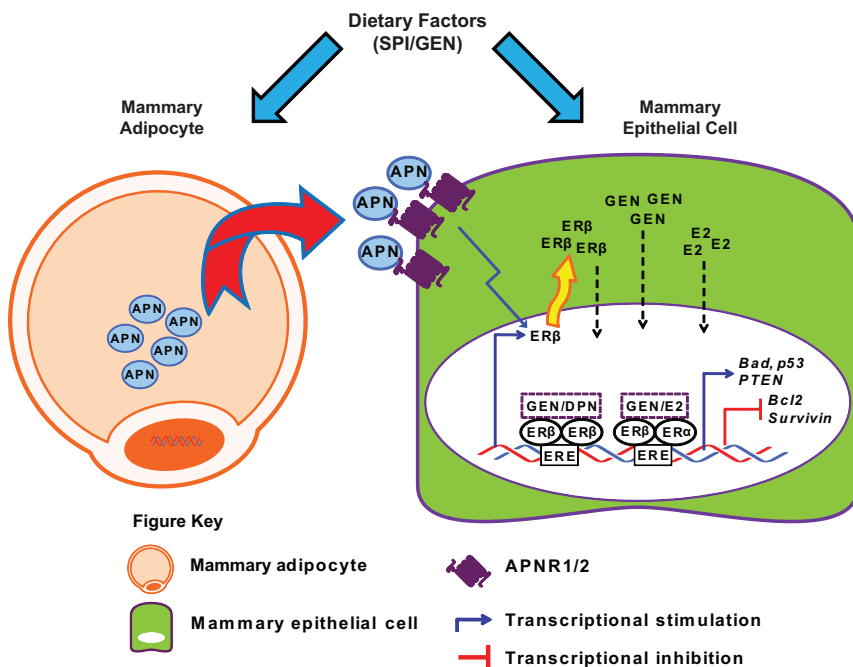


FIG. 6. Proposed model for synergistic actions of mammary adipocyte-derived APN and GEN on ER α/β cross talk to influence MEC proliferation, apoptosis, and differentiation. Dual targeting of mammary stromal adipocytes and MEC by dietary factors (e.g. soy isoflavone GEN) results in paracrine signaling between these two compartments. Dietary factor (SPI and/or GEN) induction of APN production by the mammary adipocyte initiates APN paracrine action on neighboring MEC expressing APNR, to induce ER β expression and ER β transcriptional activity in synergy with the ER β -selective ligand GEN. Biological outcomes of this signaling include inhibition of ER α -mediated cell proliferation and enhancement of differentiation and apoptosis (up-regulation of *Bad*, *p53*, *PTEN*; down-regulation of *Bcl2*, *survivin*), all of which are hallmarks of decreased breast cancer risk.

to E₂ and pure ER α and ER β agonists. With E₂, which normally induces formation of ER α homodimers and ER α/β heterodimers, and with PPT, which induces solely ER α homodimers, the presence of APN + GEN promoted the formation of ER α/β heterodimers and/or ER β homodimers at the expense of ER α homodimers, thus enhancing GEN-mediated ER β activation. Although we did not quantify the magnitude of the shift from ER α to ER β signaling with APN + GEN cotreatments, the functional outcome of increased ER β signaling was manifested as higher expression of proapoptotic and prodifferentiation genes, both of which are hallmarks of decreased breast cancer risk. The demonstration that *Pten* expression was similarly induced by APN + GEN in concert with E₂ or DPN but not PPT suggests *Pten* as a gene target of ER β transactivation in MEC. Further, the induction of *Bad*, *Bcl2*, and *p53* expression by APN + GEN + DPN and the inhibition of their respective expression by APN + GEN + PPT reflect the opposing effects of ER β on ER α transcriptional activity. These findings are consistent with ER β attenuation of ER α -mediated transcriptional activation (34–42, 43) and the negative consequence of ER β signaling on ER α -enhanced epithelial proliferation (65). Importantly, these results support the notion that mammary adiposity (using APN

as a measure) regulates the balance of ER α vs. ER β signaling in the presence of selective estrogen modulators, to alter MEC phenotype. Although it is also possible that APN action may occur via non-classical (ligand independent) mechanisms involving MAPK-induced ER phosphorylation (71), we believe that this is unlikely, because APN effects on induction of ER β transcriptional activity occurred only in synergy with the selective ER β ligand GEN. Our findings that APN elicited a decrease in ER α transcript but not protein levels raise the possibility of distinct transcriptional and posttranslational regulation by APN of ER proteins; however, to our knowledge, this has not been previously reported.

An important feature of APN that was additionally demonstrated in the present study is the ability of this adipokine to induce mammary epithelial differentiation by a mechanism independent of ER signaling. Specifically, in ER-negative MCF-10A cells, APN promotion of epithelial differentiation was associated with its inhibition of activated STAT3 signaling as measured by decreased levels of phosphorylated

STAT3. These results are consistent with previous studies documenting multiple proproliferation signaling cascades blocked by APN (58) and underscore the broad implications of dysfunctional adipose tissue on numerous metabolic, inflammatory, and chronic diseases. Further, given that the lack of ER signaling simulates that of prepuberty and postmenopausal status in women, these findings provide a mechanism by which adiposity can influence breast cancer risk.

In summary, we identified dietary regulation of mammary-specific APN production to occur at an early developmental window, and which may guide the direction of ligand-activated ER signaling in neighboring epithelial cells (Fig. 6). This model predicts that maintenance of dysfunctional adipose tissue elicited by an obesogenic state will have significant deleterious consequences to mammary breast health beginning at puberty. Our results also raise the intriguing (although yet untested) possibility that increased exposure to environmental agents with distinct selective ER modulator activities in the face of the obesity pandemic among children and young adults (72) may underlie, in part, the rising incidence of breast cancer worldwide.

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