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Award Number: W81XWH-08-1-0548

TITLE: Diet, Stem Cells, and Breast Cancer Prevention

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CONTRACTING ORGANIZATION: Arkansas Children's Hospital Research Institute, Little Rock, AR 72202-4610

REPORT DATE: January 2011

TYPE OF REPORT: FINAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-01-2011	FINAL	1 Sep 2008 - 31 Dec 2010

4. TITLE AND SUBTITLE
Diet, Stem Cells and Breast Cancer Prevention

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0548

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Rosalia C.M. Simmen

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Arkansas Children's Hospital Research
Institute, Little Rock, AR, 72202-4610

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and
Materiel Command, Fort Detrick, MD

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT-Breast cancer is the most common malignancy of women in the Western world. Many risk factors are associated with the development and progression of breast cancer; however, diet/nutrition constitutes a highly modifiable risk. Breast cancer is considered to be initiated by mutations in a limited population of undifferentiated cells termed mammary stem cells (MaSCs) 'sitting' at the top of the mammary epithelial hierarchy. Over-expansion of the stem cell population leads to increased numbers of mutated MaSCs that initiate and maintain tumors that can metastasize. Novel strategies to decrease the over-expansion and promote the elimination of tumor-initiating cells are warranted for effective prevention and treatment of breast cancer. Our studies test the hypothesis that dietary factors confer protection from breast cancer by preventing the expansion of MaSCs with tumorigenic potential. We established female mice transgenic for the oncogene Wnt-1 (Wnt-Tg mice), which develop spontaneous mammary tumors by 5-6 months of age, as a model for dietary prevention of mammary tumor formation. Mice were fed AIN-93G based isocaloric diets that differed only by protein source, namely control Casein (CAS) and Soy Protein Isolate (SPI). SPI was used as paradigm for healthy foods. We found that lifetime dietary exposure to SPI beginning at post-weaning lowered tumor incidence in Wnt-Tg mice (48.3%) relative to those fed the control diet (73.5%; $P < 0.05$). Importantly, SPI-fed Tg mice had undetectable 'tumorigenic' MaSC population and lower numbers of normal MaSCs, relative to CAS-fed Tg mice at postnatal day 75. Our studies established a functional connection between diet and abundance of MaSCs for breast cancer prevention.

15. SUBJECT TERMS
Diet, nutrition, stem cells, Wnt-transgenic, breast cancer, mammary gland

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	30	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Breast cancer is the most common malignancy of women in the Western world, with ~50,000 of those afflicted dying from the disease annually in the United States alone. Although many risk factors are associated with the development and progression of breast cancer, diet/nutrition constitutes a highly modifiable risk. The presence of a limited population of undifferentiated cells termed mammary stem cells (MaSCs) that 'sit' at the top of the mammary epithelial hierarchy and which give rise to distinct epithelial compartments with specific functions is now well-supported by landmark studies that also provided well-characterized surface markers for MaSC isolation in human and mouse mammary epithelium. While MaSCs are normally involved in mammary tissue homeostatic renewal processes, the intriguing concept that breast cancer maybe initiated by mutations in these cells has recently gained much ground. MaSC renewal is tightly regulated; thus, overexpansion of this population may lead to increased accumulation of mutated cells that can initiate and maintain tumors which eventually metastasize. Given that mutated MaSCs maybe the key to the etiology of breast cancer, a greater understanding of how they arise and novel strategies to limit their self-renewal capacity are warranted for effective disease prevention and treatment.

BODY

The major objective of the current studies is to establish the role of diet in the regulation of cancer stem cells leading to the primary prevention of breast cancer. The linkage of diet and stem cells in mammary tumor development initiated by aberrant Wnt signaling, the latter a major contributor to stem cell expansion, was addressed by using mammary tumor virus (MMTV)-Wnt-1-transgenic mice (Tg). The study has two Specific Aims. **Aim 1** seeks to establish the mammary tumor-prone Tg female mice as model to evaluate the protective effects of soy-based diets against Wnt-induced mammary tumors. The prediction is that soy protein isolate (SPI), relative to control diet Casein (CAS), will significantly decrease the incidence of mammary tumors and the occurrence of malignant tumors in adult females. **Aim 2** will examine if accumulation of the mammary stem/progenitor cell population associated with increased Wnt signaling in heterozygous Tg females is decreased with dietary intake of SPI, relative to CAS. The prediction is that the tumor stem cell population will be lower in SPI-fed relative to CAS-fed mice.

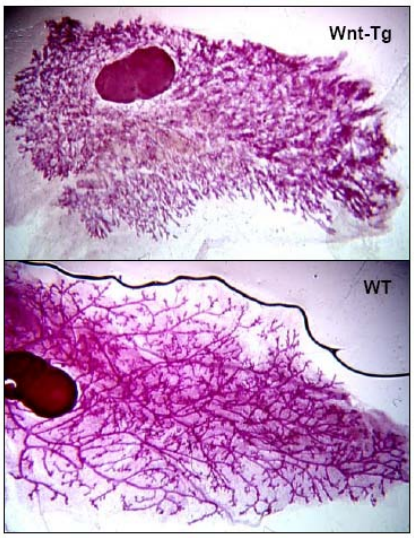

Figure 1. *Mammary ductal morphogenesis in Wnt-1-Tg mice compared to Wildtype mice. Whole mount staining was done on mammary glands harvested at PND50 mice fed the SPI diet beginning at weaning.*

To address **Aim 1**, female Wnt-Tg mice at weaning [postnatal day (PND) 21] were randomly assigned to 1 of 2 semi-purified AIN93G-based isocaloric diets that differed only by protein source, namely CAS and SPI. Mice (n=34 for CAS; n=30 for SPI) were given *ad libitum* access to food and water. Mice were monitored for development of tumors by palpation starting at 10 weeks of age, and the age of initial appearance of tumors and initial tumor volume (measured by caliper) were recorded. Mice were necropsied two weeks after the initial appearance of a tumor to determine tumor growth rate.

Tumor volume was recorded at tumor collection, and tumor pathology was scored by a board-certified pathologist (Dr. Leah Hennings) at the Histology Core Laboratory, Department of Pathology of the University of Arkansas for Medical Sciences.

Mice expressing *Wnt-1* under the control of the MMTV promoter develop extensive hyperplasias of the mammary gland. **Figure 1** shows whole mounts of mammary glands harvested from virgin wildtype (WT) and Wnt-Tg mice of the same age (PND100) fed the SPI diet. While the WT mice exhibited normal mammary ductal morphogenesis, Tg mice displayed mammary gland hyperplasia with excessive ductal side-branching.

Tumor incidence in control CAS-fed Tg mice was 73.5% (n=34) while that for SPI-fed Tg mice (n=30) was 48.3%; $P<0.05$ by Fisher's Exact test). Tg mice fed CAS developed tumors within 5-6 months of age (5.88±0.32 months). SPI-fed Tg counterparts developed tumors earlier at 4.64±0.44 months ($P<0.05$, relative to CAS). Diet did not alter the rate of tumor growth, with CAS (81.63±9.16%) and SPI (83.33±2.95%) showing the same percentage increase in tumor volume 2 weeks after initial tumor detection. Finally, histopathological analyses of tumors from mice fed either CAS or SPI indicated tumors with comparable morphologic features (papillary adenocarcinoma, solid carcinoma with adenosquamous features). Collectively, these studies indicated that dietary intake of SPI relative to control diet CAS, is mammary tumor-protective in the Wnt-Tg mouse model of breast cancer. SPI diet reduced time of tumor onset, suggesting SPI effects on tumor progression, without affecting tumor size and tumor pathology.

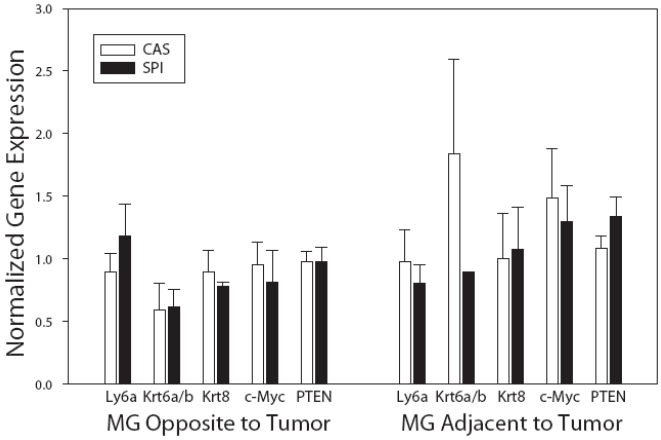

Figure 2. *Gene expression in mammary tissues opposite and adjacent to tumors of Wnt-1-transgenic mice fed either CAS or SPI beginning at weaning. n=6 mice/diet group.*

To further evaluate how SPI may promote tumor progression coincident with reducing tumor initiation (i.e., lower tumor incidence relative to CAS diet), we evaluated dietary effects on the expression phenotype of a subset of genes in mammary tissues opposite and adjacent to sites of mammary tumors. PTEN and c-myc represent genes whose expression are altered during the development of tumors in Wnt-Tg mice, whereas Ly6a (Stem cell antigen, Sca-1), Keratin 6a/b (Krt6a/b), and Keratin 8 (Krt8) are considered markers of stem/progenitor cells. **Figure 2** shows that diet had no effect on the expression levels of all genes in mammary tissues in either location relative to the tumor. These results suggest that dietary effects on the expression of these genes may occur at an earlier stage during mammary tumor development.

The above findings indicated that dietary SPI is tumor-protective in the mouse model of breast cancer initiated by dysregulation of the Wnt signaling pathway. The mammary tumor protective effect of dietary SPI intake in Tg mice recapitulated that observed in the NMU- and DMBA-models of rat mammary carcinogenesis, where we previously observed 19-26% protection comparable to the 25% found for Wnt-Tg mice in the present study. Since dysregulation of Wnt signaling is observed in human breast cancer and given that Wnt-initiated tumor stem cell markers are well-characterized, our results establish the Wnt-Tg mice as an excellent model for the study of stem cell/diet interaction (**Publications 1, 2, 3**).

In **Aim 2**, we examined whether SPI diet with mammary tumor-protective effects altered the frequency of the mammary stem cell (MaSC) population, relative to CAS diet. To address this, we first evaluated the effects of diet (CAS vs. SPI) on the percent of normal MaSC (relative to total input cells) in mammary glands of young adult (PND100) wild-type (WT) mice and of pre-neoplastic (PND75) Wnt-Tg mice. The well-characterized mouse mammary stem cells markers CD29 and CD24 ($CD29^{hi}CD24^{+}$) within the Lineage-negative population were used to quantify the abundance of the normal (non-tumor) mammary stem cell population by fluorescence activated cell sorting. In 5 independent experiments, with each experiment using 3-4 PND100 WT mice fed either CAS or SPI diets beginning at weaning (PND21), we found a higher percentage of the $CD29^{hi}CD24^{+}$ population (by 2.4-fold) in mammary glands of mice fed SPI than in those fed CAS. These results are consistent with the function of stem cells in tissue homeostatic renewal processes. In PND75 Tg mice, however, the percent of the $CD29^{hi}CD24^{+}$ population was lower (by 1.9-fold) in 5 of 10 independent experiments (n=3-4 mice fed either CAS or SPI per experiment), with the rest (5 of 10) showing no difference (SPI/CAS ratio=0.91). The decrease in the population of MaSC with SPI diet in the tumorigenic environment of oncogene Wnt-overexpression is in line with the notion that the protective effects of diet may involve controlling the expansion of stem cells that can undergo mutations. We next evaluated the percent of tumorigenic MaSC population in mammary glands of PND75 Tg (hyperplastic) mice using the stem cell-surface markers Thy1 and CD24 ($Thy1^{+}CD24^{+}$) within the Lineage-negative population. We found that in 6 of 6 independent experiments, $Thy1^{+}CD24^{+}Lin^{-}$ population was detected in mammary epithelial cells of Tg mice fed CAS (range of 0.1 to 1.3%); in contrast, the same cell population was not detected in isolated mammary epithelial cells in 3 of 6 Tg mice fed SPI. Results identify the MaSC population as a target of dietary factors and suggest that dietary protective

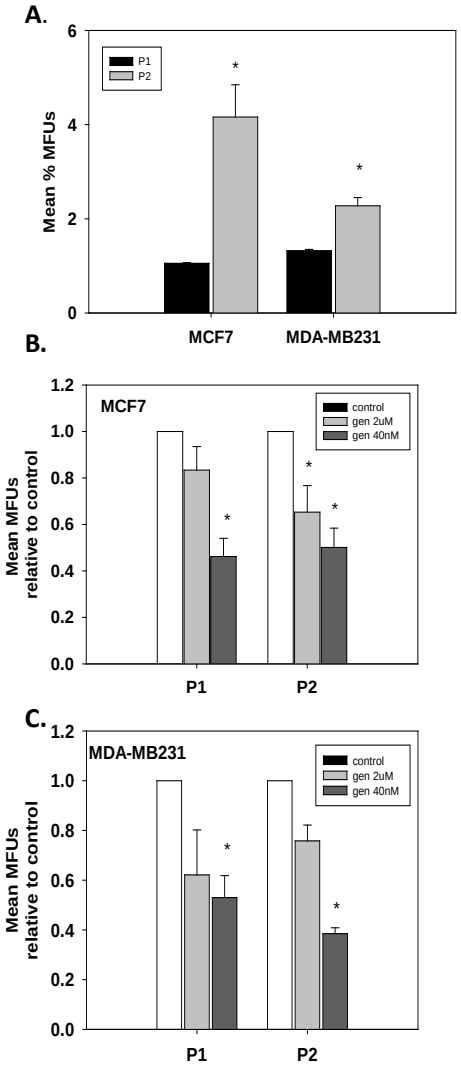

Figure 3. *Mammosphere formation assay. A) Enrichment of MFUs in human mammary epithelial cells. B) Effects of GEN on relative numbers of MFUs in MCF-7 cells. C) Effects of GEN on relative numbers of MFUs in MDA-MB231 cells.*

effects may be elicited by limiting the MaSC population within a tumorigenic environment that can give rise to mammary tumors (**Publication 1, 3**). These exciting and novel data are currently being prepared for publication;a preliminary report of the above findings was presented at the recent San Antonio Breast Cancer Symposium in December 2010 (**Publication 3**).

Based on the results obtained, we have initiated additional experiments to further understand the mechanisms underlying the protective effects of dietary factors on the MaSC population. Using two human breast cancer cell lines, namely the estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB231, we evaluated the effects of the phytoestrogen genistein (GEN), the major isoflavone component of soy foods, on the ability of these malignant cells to form mammospheres *in vitro*. The mammosphere formation assay is an indirect test of self-renewal and is considered a measure of the presence of a sub-population of epithelial cells that has the ability to 'seed tumors'. Cells plated in ultralow attachment plates formed mammospheres (mammosphere forming units, MFU) at a frequency of 1-2% within 5 days of seeding. The mammospheres collected at the first passage (P1) were enriched in the second passage P2 for both cell lines (**Fig. 3A**), confirming the presence of a cell sub-population with the ability to self-renew. Treatment with GEN only on day 1 of initial plating decreased the number of MFUs in both cell lines, relative to medium (control) alone, at both passages (P1, P2). Importantly, the lower, more physiologically relevant dose of GEN (40 nM) elicited a greater inhibitory effect than the higher (2μM) dose (**Fig. 3B, C**). These results indicate that GEN may target ER-positive and ER-negative breast cancer cells with stem-like properties, suggesting the therapeutic relevance of diet and dietary factors in the elimination of tumor-initiating cells (**Publications 4, 5**).

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that the Wnt-Tg mouse model of mammary carcinogenesis is a relevant model for investigating mammary tumor protection by diet
- Demonstrated that SPI, the major component of soy foods (and soy milk formula) is mammary tumor protective at adulthood, when consumed beginning at pre-puberty, indicating the significant influence of early (healthy) nutrition on mammary cancer risk
- Demonstrated that the mammary stem cell population exists and that its frequency can be influenced by diet and dietary factors as shown in *in vivo* (Wnt-Tg mice) and *in vitro* (human breast cancer cell lines) models
- Provided strong support for the functional (positive) association between a healthy diet and numbers (frequency) of normal mammary stem cells
- Established a functional (negative) association between a healthy diet and 'tumorigenic' mammary stem cell numbers
- Established the relevance of mammosphere-forming units (MFUs) *in vitro* as a promising diagnostic tool for evaluating dietary factors with mammary tumor-inhibiting potential

REPORTABLE OUTCOME

- Five scientific presentations in national meetings (Abstracts listed below) describing studies on the mechanistic linkage between diet and breast cancer risk were supported, in part, by the award.
- Three publications, one now published in J Nutritional Biochemistry (listed below) and two currently in preparation were supported, in part, by the award.
- Omar Rahal, PhD student in the PI's research team was successfully awarded a pre-doctoral fellowship from the Department of Defense Breast Cancer Research Program based on preliminary studies conducted as part of this award.
- PI presented two invited seminars detailing aspects of the studies conducted as part of the award: 1) Arkansas Biosciences Research Institute Symposium (Arkansas State University at Jonesboro, September 2009); and 2) Yeungnam University Molecular Biology and Biotechnology Symposium (Korea, November 2009).

CONCLUSIONS

Our project tested the novel concept that cancer stem/progenitor cells in mammary tissues are targets of bioactive dietary factors: We found that diet and dietary factors may confer protection from breast cancer by preventing the expansion of this unique cell population with tumorigenic potential. Our previous studies have demonstrated that bioactive components of soy foods (e.g., GEN) alter PTEN and E-cadherin/Wnt signaling pathways in mammary epithelial cells, consistent with their mammary tumor protective effects. Given that PTEN and E-cadherin/Wnt signaling regulate stem/progenitor cell survival and renewal, our work provides a new paradigm on targets and actions of dietary factors for breast cancer prevention (**Publications 3, 4, 6**). Further studies confirming an inverse functional association between diets known to be protective against breast cancer in the human population and the abundance of cancer-initiating (stem) cells will lead to novel dietary strategies for the prevention and treatment of breast and other types of cancers, to reduce tumor growth.

PERSONNEL (Supported partly by DoD-BCRP Grant Award)

1. John Mark P. Pabona, M.D., Postdoctoral Fellow (entire period of grant funding)
2. Omar Rahal, M.Sc., Ph.D. student (October 2008 to November 2009)
3. Rosalia C.M. Simmen, Ph.D., Professor (PI)

APPENDICES (Publications supported by DoD-BCRP Grant Award)

1. Simmen RCM, Su Y, Pabona JMP, Rahal O, Simmons C, Hennings L. 2009. Early effects of dietary soy and genistein in rodent models of mammary tumorigenesis. FASEB J (Abstract), Annual Meeting of Experimental Biology, New Orleans.
2. Rahal, O.M. and Simmen, R.C.M. 2009. Induction of PTEN/p53 crosstalk in mammary epithelial cells: a novel mechanism of breast cancer prevention by the dietary factor genistein.

Cancer Res (Suppl. 3, pp. 695S -696S): Abstract, Annual Meeting of the San Antonio Breast Cancer Symposium 2009

3. Rahal. O.M., Pabona, J.M.P., Su, Y., Fox SR, Hennings, L., Rogers, T., Nagarajan, S. and Simmen, R.C.M. 2010. Expansion of mammary stem cell population with dietary intake of soy protein isolate reveals novel mechanisms for diet-mediated control of mammary tumorigenesis. Cancer Res (Suppl. 4): Abstract, Annual Meeting of the San Antonio Breast Cancer Symposium 2010.
4. Montales M T, Rahal O, Rogers T, Kang J, Wu X, Simmen R CM. 2011. Repression of Mammosphere Formation in Breast Cancer Cells by Soy Isoflavone Genistein and Blueberry Polyphenols. FASEB J (Abstract, Annual Meeting of the Experimental Biology 2011)
5. Pabona JMP, Dave B, Rahal O, de Lumen BO, de Mejia E, Simmen RCM. 2011. Soy Peptide Lunasin Induces PTEN-mediated Apoptosis in Human Breast Cancer Cells. FASEB J (Abstract, Annual Meeting of the Experimental Biology 2011)
6. Su Y, Shankar K, Rahal, O, Simmen R CM. 2011. Bidirectional signaling of mammary epithelium and stroma: implications for breast cancer-preventive actions of dietary factors. J Nutr Biochem (In press).

APPENDIX (Publications Listed in Chronological Order)

Publication 1: Abstract Presented at the Experimental Biology Meeting 2009, New Orleans

Early Effects of Dietary Soy and Genistein in Rodent Models of Mammary Tumorigenesis.

Rosalia CM Simmen[1,2], Ying Su[1,2], John Mark P Pabona[1], Omar Rahal[2,3], Christian Simmons[1], Leah Hennings[1,4]. [1]Physiology & Biophysics, [2]Interdisciplinary Biomedical Sciences, and [4]Pathology, University of Arkansas for Medical Sciences, and [2]Arkansas Children's Nutrition Center, Little Rock, AR 72202.

The risk of breast cancer is highly modifiable by diet. Breast cancer may have its origins during early mammary development, thus, the increasing popularity of soy food consumption among pregnant and breast-feeding women and early exposure to soy protein and bioactive components through soy infant formula could have significant implications on adult incidence of this disease. Since soy protein isolate (SPI) and genistein (GEN) diets decreased chemically-induced tumor incidence in adult female rats, dietary effects on genetic pathways underlying mammary tumorigenesis were evaluated. In rat mammary epithelial cells, SPI and GEN, relative to casein diet increased tumor suppressor PTEN and E-cadherin expression; these effects were recapitulated *in vitro* by GEN. Dietary SPI also decreased lipogenic gene expression in rat mammary stromal adipocytes *in vivo*, which was mimicked by GEN in 3T3-L1 adipocytes *in vitro*. Since Wnt signaling perturbation alters the epithelial hierarchy, MMTV-Wnt1 mice were investigated for dietary SPI and GEN effects on mammary progenitor cell population during disease development. Female mice at weaning were assigned to CAS, SPI- or GEN-based diets and mammary tumor incidence was monitored. Diet-mediated changes in mammary transcriptional programs and in epithelial subpopulations may underlie protection from developing mammary lesions.
USDA-CRIS-6251-5100002-06S; DOD-BCRP.

Publication 2: Abstract Presented at the San Antonio Breast Cancer Symposium, December 2009

Induction of PTEN-p53 crosstalk in mammary epithelial cells: a novel mechanism of breast cancer prevention by the dietary factor genistein. Omar M Rahal, MS[1, 3] and Rosalia CM Simmen, PhD[1,2, 3].[1]Interdisciplinary Biomedical Sciences, University of Arkansas for Medical Sciences, Little Rock, United States; [2]Physiology and Biophysics, University of Arkansas for Medical Sciences and [3]Arkansas Children's Nutrition Center, Little Rock, AR, United States, 72202

Consumption of soy foods either at an early age or for lifetime has been associated with reduced risk for developing breast cancer in humans and in animal models. However, this association continues to be controversial and the precise mechanisms for protection remain elusive. Among the soy products, the isoflavone genistein (GEN) has been widely suggested to confer mammary tumor protection. Previously we demonstrated the increased expression of tumor suppressors PTEN and p53 in mammary epithelial cells (MECs) isolated from young adult female rats fed dietary soy protein isolate (SPI) or casein (CAS) supplemented with GEN, when compared to MECs from rats fed the control (CAS) diet. Since NMU-administered rats fed SPI had reduced tumor incidence and increased tumor latency than those fed CAS, PTEN and p53 likely mediate the observed tumor resistance with SPI *in vivo*. We hypothesized that GEN induction of PTEN and p53 in MECs results in the formation of a PTEN/p53 functional complex to negatively regulate breast cancer development. Here, we used the human non-tumorigenic, ER-negative mammary epithelial cell line, MCF-10A, as an *in vitro* system to mechanistically dissect ER-independent actions of GEN involving PTEN and p53. GEN (40 nM, 2μM) augmented PTEN and p53 expression in treated relative to control cells. GEN also induced nuclear co-localization and physical association of PTEN and p53. To test a functional consequence of GEN-induced PTEN/p53 cross-talk on mammary epithelial phenotype, we analyzed GEN effects on cell cycle progression and acini formation in 3D cultures. Our results showed attenuated cell proliferation and lower cyclin D1 and pleiotrophin transcript levels in GEN-treated cells, which were abrogated by small interfering RNA to PTEN, indicating PTEN-dependence. Using FACS analysis, we showed that GEN induced cell cycle arrest at G_0-G_1 phase. Treatment with GEN promoted early acini formation of MECs grown in Matrigel, which temporally coincided with PTEN-dependent suppression of p21 and p27 transcript levels. Further analyses of GEN effects on MECs demonstrated induction by GEN of PTEN promoter-luc reporter activity as measured by dual-luciferase assay. Interestingly, treatment with siRNA to either PTEN or p53 reduced basal and GEN-induced PTEN promoter activity. Given that p53 binds to the PTEN promoter, our results suggest a feed-forward cycle in which dietary factor (GEN) induction of nuclear PTEN leads to PTEN promotion of its own signaling. By maintaining a stable pool of nuclear p53 to boost its transcription, PTEN ensures its continuous expression in MECs to favor cell differentiation. These data elucidate a novel mechanism by which dietary factors with PTEN-inducing activity may attenuate breast cancer risk and development. Funding by USDA-CRIS 6251-5100002-06S and the Department of Defense Breast Cancer Program (0810548).

Publication 3: Abstract Presented at the San Antonio Breast Cancer Symposium, December 2010

Expansion of Mammary Stem Cell Population with Dietary Intake of Soy Protein Isolate Reveals Novel Mechanisms for Diet-Mediated Control of Mammary Tumorigenesis. *Rahal O, Pabona JMP, Su Y, Fox SR, Hennings L, Rogers T, Nagarajan S, Simmen RCM. Arkansas Children's Nutrition Center and University of Arkansas for Medical Sciences, Little Rock, AR*

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 up-regulation of the tumor suppressor PTEN and down-regulation 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 SCs with tumorigenic potential, termed cancer stem cells (CSCs), and mammary over-expression 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-Wnt 1-Transgenic (Tg) female mice and on the mammary SC population in virgin wildtype (WT) and pre-neoplastic Tg female mice. Tumor incidence at 8 months of age of Tg mice fed SPI (n=32) was lower than those fed CAS (51.6% vs.71%; p=0.08) (n=33). Interestingly, tumor latency in SPI-fed Tg mice was shorter than for the CAS-fed group (4.4 vs. 5.6 months; $P<0.05$). Tumor growth rate was similar for the diet groups. To evaluate SPI effects relative to CAS, on mammary SC population, epithelial cells from mammary tissues were isolated from WT (PND 100) and 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 expanded by two-fold in WT mice fed SPI post-weaning relative to those fed CAS. Similarly, the SC population was increased by 1.5-fold in MECs of Tg mice fed SPI relative to the CAS group. Mammary glands of WT mice exposed to SPI had higher levels of tumor suppressor PTEN and E-cadherin proteins at puberty (PND35) and at adulthood (PND50) and lower β-catenin protein expression at PND50, over those of the CAS group. 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 maybe related to the loss of the complex regulatory grid between PTEN and Wnt/β-catenin pathways, both of which control stem cell 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. Supported by USDA- ARS and Department of Defense Breast Cancer Research Program.

Publication 4: To be presented at the Experimental Biology Meeting 2011, Washington DC

Repression of Mammosphere Formation in Breast Cancer Cells by Soy Isoflavone Genistein and Blueberry Polyphenols. Maria Theresa Montales[1,2], Omar Rahal[1,3], Theodore Rogers[1], Jie Kang[1], Xianli Wu[1] and Rosalia CM Simmen[1,2]. [1]Arkansas Children's Nutrition Center, [2]Physiology & Biophysics and [3]Interdisciplinary Biomedical Sciences, University of Arkansas for Medical Sciences, Little Rock, AR.

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 (ER)-positive MCF-7 and ER-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 μM; 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. By contrast, leptin and interleukin-6 had no activity in these cells. Results suggest that dietary factors may selectively target ER-positive and ER-negative 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

Publication 5: To be presented at the Experimental Biology Meeting 2011, Washington DC

Soy Peptide Lunasin Induces PTEN-mediated Apoptosis in Human Breast Cancer Cells. John Mark P Pabona[1,2], Bhuvanesh Dave[3], Omar Rahal[1,2], Ben O de Lumen[4], Elvira de Mejia[5], Rosalia CM Simmen[1,2]. [1]Arkansas Children's Nutrition Center, [2]University of Arkansas for Medical Sciences, Little Rock, AR,[3]The Methodist Hospital Research Institute, Houston, TX, [4]University of California, Berkeley, CA,[5]University of Illinois, Urbana, IL

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 μM >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 $p21^{WAF1}$ (decreased) transcripts ($P<0.05$). GEN (2 μM >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]

Publication 6: Journal of Nutritional Biochemistry (In press)-attached

Available online at www.sciencedirect.com

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Journal of Nutritional Biochemistry xx (2010) xxx–xxx

Journal of Nutritional Biochemistry

Bidirectional signaling of mammary epithelium and stroma: implications for breast cancer—preventive actions of dietary factors

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Received 26 April 2010; accepted 18 September 2010

Abstract

The mammary gland is composed of two major cellular compartments: a highly dynamic epithelium that undergoes cycles of proliferation, differentiation and apoptosis in response to local and endocrine signals and the underlying stroma comprised of fibroblasts, endothelial cells and adipocytes, which collectively form the mammary fat pad. Breast cancer originates from subversions of normal growth regulatory pathways in mammary epithelial cells due to genetic mutations and epigenetic modifications in tumor suppressors, oncogenes and DNA repair genes. Diet is considered a highly modifiable determinant of breast cancer risk; thus, considerable efforts are focused on understanding how certain dietary factors may promote resistance of mammary epithelial cells to growth dysregulation. The recent indications that stromal cells contribute to the maintenance of the mammary epithelial 'niche' and the increasing appreciation for adipose tissue as an endocrine organ with a complex secretome have led to the novel paradigm that the mammary stromal compartment is itself a relevant target of bioactive dietary factors. In this review, we address the potential influence of dietary factors on mammary epithelial–stromal bidirectional signaling to provide mechanistic insights into how dietary factors may promote early mammary epithelial differentiation to decrease adult breast cancer risk.

Keywords: Mammary gland; Epithelium; Adipocyte; Diet; Breast cancer; Obesity

1. Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer deaths among women in the United States. In 2009 alone, more than 190,000 new cases of invasive breast cancer were reported, which accounted for ~25% of all cancers among women in the United States [1]. Similar to all cancers, breast cancer is a genetic and epigenetic disease with diverse histopathological and clinical outcomes [2]. Although the major reasons for breast cancer deaths are complications arising from metastasis, the natural history of breast cancer involves progression through defined molecular, pathological and clinical stages [3,4]. The widely accepted view of breast tumor progression, known as linear progression [5], assumes the gradual transition of breast lesions from premalignant, hyperplastic states into ductal carcinoma *in situ*, invasive carcinoma and, finally, metastatic disease [6]. Recent clinical studies demonstrating heterogeneity in tumors from breast cancer patients now suggest that the linear progression model maybe overly simplistic [7,8]. In the more recently described diversity evolution model [9], the constant selection pressures provided by numerous environmental cues or therapeutic interventions are posited to lead to the high clonal diversity found in tumors as well as the drug resistance that may develop during treatment [10].

The mammary gland is comprised of myoepithelial and luminal epithelial cells embedded in a complex stromal matrix ('mammary fat pad') comprised predominantly of fibroblasts, adipocytes and macrophages (Fig. 1). The prevailing concept in the field is that the discrete mammary epithelial subtypes and neighboring stromal cells arise, respectively, from the asymmetric division of epithelial and mesenchymal cells of origin ('stem cells') and the subsequent differentiation of lineage-committed progenitor cells [11,12]. Emerging data on mammary stem cells have raised the possibility that this epithelial subpopulation 'sitting at the top' of the mammary epithelial hierarchy serves as initial target of oncogenic agents [11].

The transformation of normal mammary epithelial cells to malignancy is manifested as aberrant growth and survival responses to extracellular signals. The latter include those derived from the endocrine milieu, as well as from the stroma, whose physical proximity to epithelial cells allows for dynamic paracrine regulation and the integration of signals from circulating hormones and growth actors [13,14]. In a recent review, Arendt et al. [15] detailed the

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doi:10.1016/j.jnutbio.2010.09.008

complex local and systemic contributions of the stromal compartment to normal mammary development and to malignant breast development. Molecular and phenotypic changes within the stroma affect their interactions with neighboring cells, resulting in a microenvironment that can be supportive of epithelial progression to malignancy [16–18]. The distinct molecular signatures displayed by enriched populations of stromal cells underlying epithelial cell populations from normal breast tissue and invasive cancer [19,20] provide a convincing molecular rationale for the stromal compartment as instrumental to tumor progression. Increased understanding of the contribution of underlying stroma to breast cancer, predominantly an epithelial cell phenomenon, provides exciting potential for manipulating the mammary stromal compartment in the development of therapy [15,21]. Given the emerging evidence for dietary contribution to breast cancer risk [22] through diet-mediated regulation of mammary epithelial differentiation, proliferation and apoptosis [23–27] coupled with the recognition that mammary fate and ductal development are controlled to a large extent by mammary fibroblastic and adipocyte mesenchyme [15], the prospect that diet-associated components may equally influence mammary stromal biology to influence the course of differentiation or neoplastic growth of the mammary epithelium is not far-fetched.

The invitation to write this minireview was prompted by our findings that mammary stromal adipocytes are early biological targets of dietary factors, specifically of the major isoflavone genistein (GEN) *in vivo* [27]. In that report, we showed that limited exposure (i.e., *in utero* and lactational only) of female rat offspring to a maternal diet containing soy protein isolate (SPI) as major protein source resulted in mammary stromal adipocyte-specific genomic changes (e.g., lipogenic gene expression) coincident with increased differentiation of mammary tissues that were distinct from those exposed to the control diet with casein as the major protein source. Further, we showed that the functional consequence of SPI-mediated adipocyte metabolic changes on neighboring mammary epithelium *in vivo* can be recapitulated by GEN *in vitro* through direct actions on differentiated 3T3-L1 adipocytes, a function likely related to their increased secretion of the adipokine adiponectin with GEN treatment [27]. Little is known of the gene pathways and mechanisms by which specific dietary factors may target the stromal compartment to promote breast health. We begin this review by highlighting seminal information on cell signaling mechanisms underlying mammary tumor protection by dietary factors. Next, we describe how mammary stromal remodeling has been implicated in underlying epithelial biology, with a focus on the emerging links between mammary adiposity and mammary ductal development as an indication of adipose-directed signaling. Finally, we discuss recently described, albeit limited, information on stromal-localized molecular targets of dietary factors, which may serve as paracrine mediators of dietary factor action on mammary epithelial cells.

2. Dietary factors and mammary epithelial targets in breast cancer protection

The incidence of breast cancer is high in the United States [1], with an increasing trend noted globally [28], yet strategies addressing its prevention remain extremely limited. Indeed, the current emphasis on the clinical management and treatment of breast cancer dramatically contrasts with the inadequacy of efforts directed toward disease prevention. In addition, there is reluctance among the general populace to embrace the concept that nutrition and lifestyle constitute highly modifiable risk factors for the prevention of breast cancer. In part, this may be due to the oftentimes conflicting reports, based largely on epidemiological studies, of the protective health benefits of specific diets. For example, high dietary fat intake, especially high polyunsaturated fatty acids, has been linked to the promotion of breast cancer in animal models [29,30] but currently not in humans [31,32]. On the other hand, saturated fat consumption is linked to breast cancer in women, but this has not been conclusively demonstrated in animal studies [33]. Similarly, dietary vitamin A, carotenoid and Vitamin D intake has been individually shown to prevent breast cancer in a number of human and animal studies, although a unifying outcome remains lacking [34,35]. The differences in physiological status of human subjects (prepubertal and post-pubertal; premenopausal and postmenopausal), source of dietary factors (from foods or supplements) as well as varying doses and 'developmental window' of dietary exposure in the many studies described in the literature [22,32,36] had preempted conclusive indications of the breast cancer-preventive benefits of consumption of any dietary factor. While studies with animal models and cell lines have been faulted for their simplistic approach toward understanding dietary prevention of breast cancer susceptibility, given the heterogeneity of the human population, these models have been invaluable in providing mechanistic insights regarding the contributions of specific bioactive components to breast cancer risk.

Efforts to understand the mechanisms underlying the breast cancer-preventive effects of dietary factors have focused on their

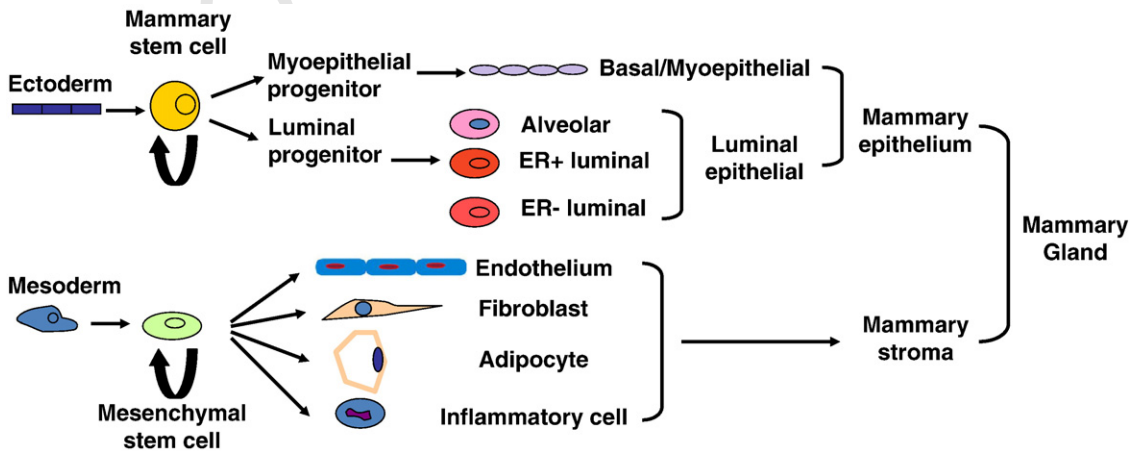

Fig. 1. The origin and lineage of the different cell types in the mammary gland. The mammary epithelium (luminal and myoepithelial) is embedded in the complex stromal matrix (also designated mammary fat pad) composed predominantly of fibroblasts, adipocytes and immune cells. The complexity of the mammary gland is a function of its distinct constituent cell types, which are subject to different endocrine and local regulation and which exhibit diverse functions. ER+ve, estrogen receptor positive; ER−ve, estrogen receptor negative.

biological and genomic consequences on mammary epithelial cells, where breast cancer arises. In particular, curcumin from turmeric [37], resveratrol from grape [38], capsaicin from chili pepper [39], flavonoids such as hesperetin and naringenin in citrus fruits and tomatoes [40], isoflavones (e.g., GEN, daidzein) from legumes and red clover [41,42] and epigallocatechin-3-gallate from green tea [43] have been demonstrated to provide different levels of preventive effects in rodent and cell culture models. An extensive discussion of the literature on the numerous mechanisms reported to underlie dietary prevention of breast cancer is beyond the scope of this current review, given the excellent recent reviews on this subject [44–48]. Suffice it to say that common mechanisms of actions have emerged: these include carcinogen activation/detoxification by metabolic enzymes, increased antioxidant and anti-inflammatory effects, induction of cell cycle arrest and inhibition of cell proliferation, decreased cell survival, enhancement of differentiation, increased expression and functional activation of various genes and corresponding proteins that are involved in DNA damage repair, tumor suppression and angiogenesis and down-regulation of oncogenes. Importantly, while the signaling pathways affected by various dietary factors in mammary epithelial cells are numerous, these pathways are interrelated, not mutually exclusive and as expected, utilize similar sets of genes previously elaborated in other tumor types [49].

Global gene expression profiling of mammary epithelial cells and subsequent functional annotation of gene expression changes have proven to be an effective tool for the discovery of novel pathways mediating dietary factor protection of mammary tumorigenesis. In studies from our laboratory using Affymetrix GeneChip microarrays [50], we showed a very low percentage of epithelial genes (~0.5% of 14,000 genes evaluated) whose expression is altered by exposure to either SPI or GEN diet beginning *in utero* to early adult stage (postnatal day 50), relative to control casein diet. The functional association of these identified genes with signaling pathways involved in immune response, protein and carbohydrate metabolism, growth regulation and stem cell niche (e.g., Wnt and Notch pathways) has provided invaluable insights into important targets of SPI-associated bioactive components and, in particular, GEN to induce epithelial changes for increased resistance to carcinogenic agents [51,52]. Indeed, our independent identification of the tumor suppressor *PTEN* [53] and of E-cadherin/Wnt/β-catenin signaling [54] as molecular pathways influenced by dietary exposure to SPI and GEN *in vivo* and by GEN *in vitro* has been bolstered by the recently elaborated linkage between these two signaling pathways in the regulation of normal and malignant mammary stem/progenitor cells *in vivo* and *in vitro* [55]. Similar support has been provided by other published studies, including those for epigallocatechin-3-gallate [56], phytoestrogens [57] and polyunsaturated fatty acids [58]. Taken together, the cellular pathways mediating dietary factor actions in the context of mammary epithelial growth regulation implicate their collective opposing actions on the expression and/or activity of tumor suppressors and oncogenes and their respective downstream targets.

3. Mammary stromal signaling in breast cancer prevention

How does the mammary stroma compartment potentiate resistance of its neighboring preneoplastic cells to tumor-initiating events? Much insight has emerged from studies on carcinoma-associated stromal fibroblasts, which can transdifferentiate into myofibroblasts and which have been demonstrated to promote primary tumor growth in human xenograft models when compared to noncancerous stromas [19,20]. The altered activity of tumor-associated stromal fibroblastic cells was associated with genetic and epigenetic alterations in specific gene subsets including that of the tumor suppressor *p53*, leading to increased expression of growth factors, cytokines and extracellular matrix components and which, by paracrine signaling, promoted neoangiogenesis and epithelial-to-mesenchymal transition in neighboring cells [19,59]. In an elegant recent study by Trimboli et al. [60], the conditional inactivation of the tumor suppressor *PTEN* in stromal fibroblasts of mouse mammary glands was shown to promote the initiation, progression and malignant transformation of mammary epithelium. *PTEN* loss was linked to increased extracellular matrix component deposition and innate immune infiltration, two key events associated with tumor malignancy and with activation of Ras, JNK and Akt growth-regulatory pathways [60]. This and similar studies [61–63] strongly support the notion that altered signaling in the tumor stroma, in this case, stromal fibroblasts, elicits aberrant epithelial growth regulation, leading to tumor manifestation.

Adipocytes constitute a significant component of the mammary stromal compartment and, similar to fibroblasts, are considered essential for mammary tumor growth and survival. While the mouse mammary fat pad consists primarily of adipocytes, this is not the case for the human mammary gland, where the developing mammary epithelium is closely sheathed by stromal fibroblasts. Nevertheless, the proximity of adipocytes to the epithelium and their high secretome activity [64,65] suggest significant influence. Indeed, the findings that (1) obesity, a disorder arising from altered gene–nutrient interactions, is a risk factor for breast cancer development [66], (2) diet-induced obesity in mice results in enlarged mammary glands and suppression of normal ductal development [67], and (3) adipose tissue from obese human subjects synthesize high and low levels of the adipokines leptin and adiponectin, respectively [68,69], which display opposing effects (promotion by leptin; inhibition by adiponectin) on mammary epithelial proliferation and which have been associated with regulation of mammary tumor development in mice [70], provide strong support for the influence of mammary adipocytes on breast cancer progression.

Interestingly, despite the increasing focus on obesity and nutrition/diet as major determinants of mammary epithelial oncogenesis, the connection between dietary factors with putative mammary tumor-protective effects and normal mammary adipose tissue biology has not been directly demonstrated. Two studies have recently appeared that highlight this association, albeit indirectly. Cho et al. [71] reported that the polyphenol (−)-catechin, among the many polyphenols present in green tea, enhanced the expression and secretion of adiponectin in 3T3-L1 adipocytes *in vitro*. The increase in adiponectin secretion by (−)-catechin was accompanied by increased insulin-dependent glucose uptake in differentiated adipocytes and decreased expression of the transcription factor Kruppel-like 7, which inhibits adiponectin expression [71]. While these *in vitro* findings did not directly address the consequence(s) of (−)-catechin promotion of adiponectin expression and secretion on mammary epithelial growth regulation, they are consistent with previous indications that green tea extracts have antiobesogenic activity [72] and inhibit mammary tumor initiation and progression in animal models of breast cancer [73]. In the second study by our group [27], we incorporated *in vivo* and *in vitro* strategies to link genomic and functional consequences in rat mammary glands upon *in utero*/lactational exposure to dietary SPI with paracrine signals from GEN-treated 3T3-L1 adipocytes to induce mammary epithelial differentiation. While our studies did not identify the paracrine signal(s) mediating the enhanced differentiation of mammary epithelial cells, we posited that one likely candidate is adiponectin, given the increased secretion of this adipokine in differentiated adipocytes treated with GEN at physiological doses [27]. Preliminary findings provide support to the latter, based on the higher adiponectin protein levels in the mammary glands of young adult female rat offspring exposed to SPI following the above dietary regimen, in the absence of changes in systemic levels of this adipokine (O. Rahal and R.C.M. Simmen, unpublished observations). Given that early only and

lifelong exposure to soy-enriched diets are mammary tumor-preventive in rodent models of carcinogenesis [52,74], findings that were borne out by epidemiological studies [75], the 'chicken-or-the-egg" question as to which mammary compartment (stromal or epithelial) is initially targeted by dietary factors to achieve the final outcome of increased mammary epithelial differentiation for decreased sensitivity to oncogenic agents, may constitute a fruitful direction for future investigation.

While the aforementioned studies investigated aspects of dietary influences on lipogenic and adipogenic regulators in the mammary adipocyte, mechanisms for dietary regulation at the level of adipocyte differentiation are also plausible. A great deal of our understanding of the molecular basis of adipocyte differentiation has been gained from studies of clonal fibroblastic preadipocyte cell lines (3T3-L1, 3T3-442A) and *ex vivo* studies of stromal vascular cells isolated from animals [76,77]. Committed preadipocytes, upon hormonal induction *in vitro* and via elusive *in vivo* signals, begin the differentiation program involving CREB-mediated phosphorylation of the transcription factor CAAAT-enhancer binding protein-β [77–79], followed by mitotic clonal expansion and activation of CAAAT-enhancer binding protein-α and peroxisome proliferator-activated receptor (PPAR)-γ. These, along with the sterol regulatory element binding protein-1c, transactivate a number of adipocyte-specific genes that maintain the adipocyte phenotype [80,81]. Throughout life, adipose tissue mass is regulated by a balance between formation (via hypertrophy of existing adipocytes and hyperplasia) and lipolysis. While the molecular events underlying adipocyte differentiation from precursor cells have been extensively studied, the precise origins of the adipose tissue *in vivo* are still poorly understood. In this context, two important recent advances in our understanding are noteworthy. First, using novel PPAR-γ reporter mouse strains (PPAR-γ-Rosa26 reporter and PPAR-γ-TRE-H2B-GFP) where endogenous PPAR-γ promoter leads to indelible marking of daughter cells with LacZ or GFP, Tang et al. [82], performed cell lineage tracing experiments. These elegant studies revealed that most adipocytes reside in the mural cell compartment in close to the adipose vasculature and are already committed to an adipocyte fate *in utero* or early postnatal life. The second major advance in this area has been the identification of early adipocyte progenitor cells in the adipose tissue using flow cytometry. Using fluorescence-activated cell sorting, Rodeheffer et al. [83] identified cells that are $Lin^{-}CD29^{+}CD34^{+}Sca1^{+}CD24^{+}$ residing in the adipose tissue and that likely represent early adipocyte precursors since they can reconstitute a normal adipose tissue when injected into 'fat-less' lipodystrophic mice. It should be noted that the origin of adipocytes in the mammary fat pad has not been examined to date. In light of these studies, it is important to begin to address whether diet/dietary factor-associated cancer protection may be linked with altered commitment/differentiation of mammary preadipocytes.

4. Dietary factors and candidate mammary stromal targets for breast cancer prevention

While there is a paucity of information to directly link the targeting of specific mammary stromal cell types by known dietary factors to neighboring mammary epithelial growth regulation, a few candidate mediators have emerged. The most relevant are the adipokines adiponectin and leptin, which, because of their mammary adipocyte source, demonstrated regulation of mammary epithelial proliferation, differentiation and apoptosis though distinct mechanisms [70,84–86], and the negative and positive association of their expression levels, respectively, with breast cancer risk and adiposity [87–89]. *In vitro*, the isoflavone GEN has been shown to enhance secretion (hence, availability as endocrine/paracrine signals) of adiponectin [27] and to inhibit that of leptin [90]. The bioactive component chitosan from edible mushrooms, which was found to demonstrate antiobesogenic activity in rats [91], similarly reduced visceral adipose tissue leptin levels in mice consuming chitosan-supplemented diet [92]. Further, the short-chain fatty acid propionic acid, which is produced by the colonic fermentation of dietary fiber known to be preventive for the development of obesity [93], was shown to increase leptin messenger RNA expression and corresponding protein secretion, in the absence of coincident effects on adiponectin, in human omental and subcutaneous adipose tissue explants [94]. While the increased secretion of leptin by propionic acid appears counterintuitive to its antiobesity and, by extension, anticipated antimammary tumorigenic effects, this was accompanied by the reduced expression of the proinflammatory factor adipokine resistin, suggesting that the repertoire of adipokines presented to target cells may predict the final growth/proliferative outcome. In this regard, a recent study has shown significantly elevated plasma resistin levels in patients with breast cancer relative to those without disease [95], consistent with the link between inflammation and breast cancer risk.

Our group's approach to mechanistically address the directional signaling from stromal to epithelial cells initiated by bioactive dietary factor targeting of mammary fat pad involves (1) defining the *in vivo* measures of mammary epithelial and stromal differentiation upon early dietary SPI exposure and (2) recapitulating these responses in nontumorigenic mammary epithelial cells exposed to conditioned medium from differentiated 3T3-L1 adipocyte treated with GEN *in vitro* [27]. While our experiments constitute proof of concept, there are caveats that require further scrutiny. Our studies did not unequivocally identify GEN-specific gene targets in stromal fibroblasts and adipocytes distinct from those of epithelial cells, since the gene expression analyses were carried out using whole mammary tissues. Moreover, the biological and molecular outcomes observed *in vitro* with GEN precluded the contribution of other SPI-associated bioactive components, which may elicit more direct effects than could be attributed to GEN alone. Finally, it was not possible to demonstrate the converse directional signaling (i.e., from epithelial to stromal compartment) that may equally underlie mammary tumor prevention. In support of the existence of epithelial-to-stromal dialog, it was shown that during the development of breast cancer, the stromal compartment responded to signals from tumorigenic cells, leading to a more 'reactive' stroma and amplification of the tumorigenic state [96]. Additional studies using isolated adipocytes and fibroblastic cells derived from mammary fat pad or *in vivo* sampling of mammary fat pad followed by proteomic analyses [65,97], as a function of whole diets and purified bioactive components, will provide a 'glimpse' of the mammary secretome and presumably regulators of mammary stromal mediated epithelial changes.

The elegant study by Lam et al. [70] demonstrating the precise role of adiponectin in mammary carcinogenesis can serve as a paradigm for mechanistically elucidating the role of adipocyte-specific gene targets of diet and dietary factors on mammary tumor prevention. In that study, MMTV-polyomavirus middle T-antigen transgenic mice with reduced adiponectin expression were generated to test the effects of adiponectin haploinsufficiency on the promotion of mammary tumors. Similar kinds of studies could be performed to test the function of candidate mammary adipocyte genes that are identified from gene expression analyses of tissues from rodent models under different dietary programs. In this regard, the recent report on the characterization of a 5.4-kb adiponectin promoter/5′ regulatory region that confers adipocyte-specific expression of target genes may provide an avenue for studying gene function in the context of bidirectional signaling in the mammary gland [98]. While it is unknown whether mammary adipose tissue exhibits specialized responses to extracellular signals or displays gene expression patterns distinct from retroperitoneal (subcutaneous) adipose tissue, an earlier study showed that the lipid composition in adipose tissue of virgin rat mammary glands resemble that of the retroperitoneal adipose [99].

5. Concluding remarks

The notion that the mammary fat pad is a direct target of bioactive dietary factors for mammary tumor protection is not difficult to envision, given that in any biological system, nothing stands alone. It is perhaps paradoxical that studies to address this remain relatively limited and the concept that bidirectional signaling within the mammary microenvironment for breast cancer prevention remains an intriguing observation. While the stromal compartment is not the main target of carcinogens [100], the possibility that a very early event upon carcinogenic insult is the sensing by stromal cells of 'something amiss' in adjacent epithelial cells is not unlikely. If this is the case, the identification of mammary fibroblast- and adipocyte-specific 'early' molecular targets by bioactive components in model systems may eventually provide biomarkers for the very early stages of the disease. The recent characterization of a mammary stromal fibroblastic cell line from mice that can differentiate to a preadipocyte lineage [101] in coculture studies with nontumorigenic or tumorigenic mammary epithelial cells will enable a proof-of-principle evaluation of the epithelial/stromal adipocyte dialog and associated mediators.

The findings that mammary stroma can reprogram testicular and neural stem cells to produce progeny committed to a mammary epithelial cell fate [102,103] and that a precancerous mammary stem cell may be programmed to become breast cancer [104] suggest the possibility that direct dietary factor effects on mammary stroma may alter stem cell behavior to inhibit neoplastic transformation. Thus, while mammary stem cells may constitute direct targets of bioactive dietary components as recently suggested by the report that curcumin added *in vitro* can induce mammosphere-forming ability in normal and malignant breast cells [105], a dual effect of dietary factors on mesenchymal and epithelial stem cells is also likely.

Further, dietary factors may directly influence the stem cell compartment in mammary stroma at the levels of the preadipocyte pool and the number of multipotent stem cells that enter the adipocyte lineage. The effects of obesity, high fat diets and other dietary factors on mammary preadipocyte populations remain unknown. It has been suggested that the inability of a particular adipose depot to expand may be causative in the accumulation of hypertrophic adipocytes and a predisposing factor in metabolic disease. Hence, it is possible that certain diets or dietary factors may mediate indirect beneficial actions on mammary epithelial cells via their modulation of preadipocyte commitment and/or differentiation of new mammary adipocytes. A recent report that *in utero* exposure to the environmental agent tributylin induced multipotent stem cells to differentiate into adipocytes provides strong support to this possibility [106].

Finally, while the contribution of inflammatory/immune cells found in mammary stroma is not included in the present review, their relevance as dietary factor targets to mediate epithelial proliferation and differentiation cannot be ignored, given that local inflammation associated with solid tumors is partly a consequence of immune cells in the tumor stroma [107]. Indeed, we observed that immune-related genes constitute major targets of dietary exposure to SPI and GEN in mammary epithelial cells of young adult rats [50]. The down-regulated expression of epithelial genes involved in antigen presentation, antigen processing and inflammation, including that of interleukin 17β, a homolog of interleukin 17, which is linked to neutrophil chemotaxis, suggests the possibility of similar specific targeting of immune cells localized

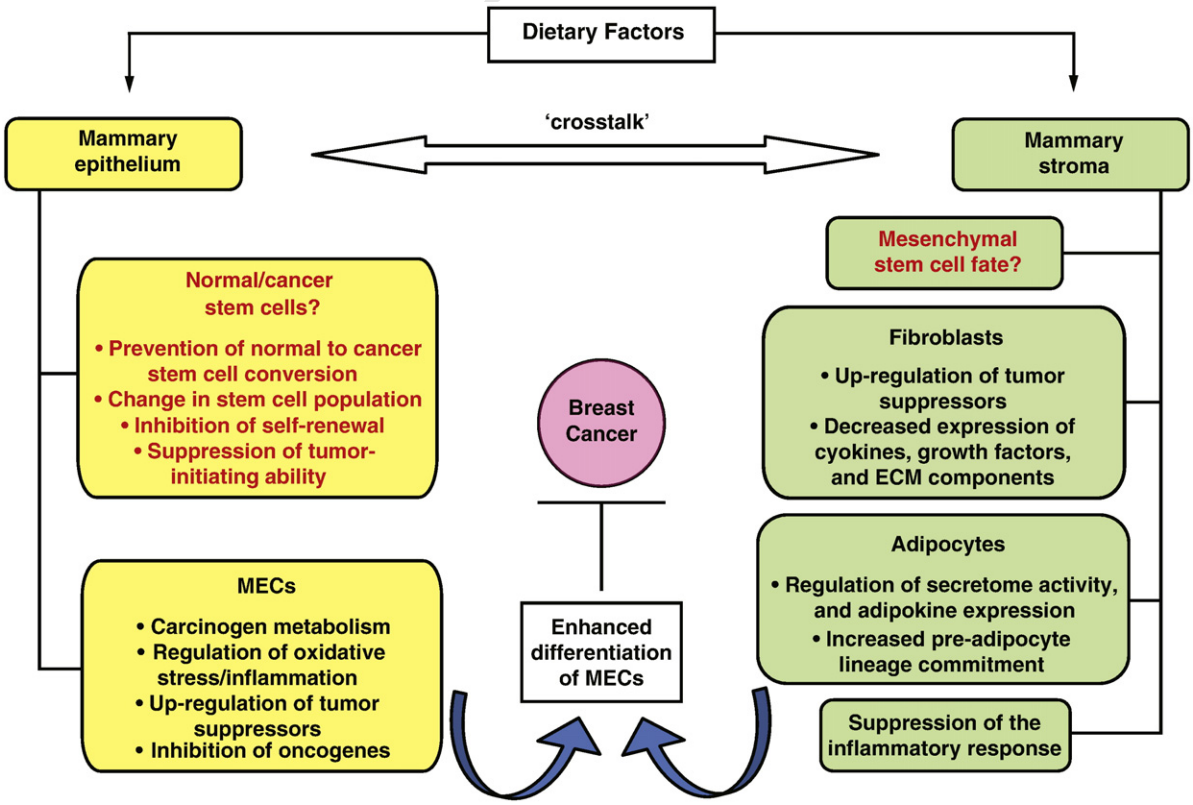

Fig. 2. A proposed model of cellular processes regulated by dietary factors in mammary epithelial and stromal compartments for breast cancer protection. The bidirectional arrows indicate an ongoing dialog between the mammary compartments. Mammary epithelial and mesenchymal stem cells are considered to represent cells of origin for each compartment. The composite actions of each mammary cell type result in the enhanced differentiation and, hence, increased resistance of mammary epithelial cells to carcinogenic insults, leading to decreased breast cancer risk.

to stroma and is consistent with promotion by the immune microenvironment of tumor progression [107].

In summary, bidirectional signaling between mammary stroma and epithelial cells promoted by bioactive dietary components constitutes a relevant biological event for mammary tumor prevention (Fig. 2). Thus, it is essential that, in future studies where dietary factor effects are described for mammary tumor prevention, their contributions to the phenotype and molecular profiles of mammary stromal fibroblasts and adipocytes are investigated coincident with those of neighboring epithelium. Gaining a better understanding of the complex interrelationships among the different mammary compartments in response to environmental ('dietary') cues may expand nutritional strategies for breast cancer prevention and therapeutic interventions.

Acknowledgements

We thank Dr. Frank A. Simmen (UAMS) for critical comments on the manuscript. Work described from Dr. R.C.M. Simmen's laboratory was supported in part, by grants from the USDA-CRIS 6251-5100002-06S, Department of Defense Breast Cancer Research Program (CDMRP W81XWH-08-0548), the Fashion Footwear Association of New York (FFANY/QVC) and the Arkansas Children's Hospital Research Institute/Children's University Medical Group Program.

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Journal of Nutritional Biochemistry xx (2010) xxx–xxx

Journal of Nutritional Biochemistry

Bidirectional signaling of mammary epithelium and stroma: implications for breast cancer—preventive actions of dietary factors

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Received 26 April 2010; accepted 18 September 2010

Abstract

The mammary gland is composed of two major cellular compartments: a highly dynamic epithelium that undergoes cycles of proliferation, differentiation and apoptosis in response to local and endocrine signals and the underlying stroma comprised of fibroblasts, endothelial cells and adipocytes, which collectively form the mammary fat pad. Breast cancer originates from subversions of normal growth regulatory pathways in mammary epithelial cells due to genetic mutations and epigenetic modifications in tumor suppressors, oncogenes and DNA repair genes. Diet is considered a highly modifiable determinant of breast cancer risk; thus, considerable efforts are focused on understanding how certain dietary factors may promote resistance of mammary epithelial cells to growth dysregulation. The recent indications that stromal cells contribute to the maintenance of the mammary epithelial 'niche' and the increasing appreciation for adipose tissue as an endocrine organ with a complex secretome have led to the novel paradigm that the mammary stromal compartment is itself a relevant target of bioactive dietary factors. In this review, we address the potential influence of dietary factors on mammary epithelial–stromal bidirectional signaling to provide mechanistic insights into how dietary factors may promote early mammary epithelial differentiation to decrease adult breast cancer risk.

Keywords: Mammary gland; Epithelium; Adipocyte; Diet; Breast cancer; Obesity

1. Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer deaths among women in the United States. In 2009 alone, more than 190,000 new cases of invasive breast cancer were reported, which accounted for ~25% of all cancers among women in the United States [1]. Similar to all cancers, breast cancer is a genetic and epigenetic disease with diverse histopathological and clinical outcomes [2]. Although the major reasons for breast cancer deaths are complications arising from metastasis, the natural history of breast cancer involves progression through defined molecular, pathological and clinical stages [3,4]. The widely accepted view of breast tumor progression, known as linear progression [5], assumes the gradual transition of breast lesions from premalignant, hyperplastic states into ductal carcinoma *in situ*, invasive carcinoma and, finally, metastatic disease [6]. Recent clinical studies demonstrating heterogeneity in tumors from breast cancer patients now suggest that the linear progression model maybe overly simplistic [7,8]. In the more recently described diversity evolution model [9], the constant selection pressures provided by numerous environmental cues or therapeutic interventions are posited to lead to the high clonal diversity found in tumors as well as the drug resistance that may develop during treatment [10].

The mammary gland is comprised of myoepithelial and luminal epithelial cells embedded in a complex stromal matrix ('mammary fat pad') comprised predominantly of fibroblasts, adipocytes and macrophages (Fig. 1). The prevailing concept in the field is that the discrete mammary epithelial subtypes and neighboring stromal cells arise, respectively, from the asymmetric division of epithelial and mesenchymal cells of origin ('stem cells') and the subsequent differentiation of lineage-committed progenitor cells [11,12]. Emerging data on mammary stem cells have raised the possibility that this epithelial subpopulation 'sitting at the top' of the mammary epithelial hierarchy serves as initial target of oncogenic agents [11].

The transformation of normal mammary epithelial cells to malignancy is manifested as aberrant growth and survival responses to extracellular signals. The latter include those derived from the endocrine milieu, as well as from the stroma, whose physical proximity to epithelial cells allows for dynamic paracrine regulation and the integration of signals from circulating hormones and growth actors [13,14]. In a recent review, Arendt et al. [15] detailed the

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doi:10.1016/j.jnutbio.2010.09.008

complex local and systemic contributions of the stromal compartment to normal mammary development and to malignant breast development. Molecular and phenotypic changes within the stroma affect their interactions with neighboring cells, resulting in a microenvironment that can be supportive of epithelial progression to malignancy [16–18]. The distinct molecular signatures displayed by enriched populations of stromal cells underlying epithelial cell populations from normal breast tissue and invasive cancer [19,20] provide a convincing molecular rationale for the stromal compartment as instrumental to tumor progression. Increased understanding of the contribution of underlying stroma to breast cancer, predominantly an epithelial cell phenomenon, provides exciting potential for manipulating the mammary stromal compartment in the development of therapy [15,21]. Given the emerging evidence for dietary contribution to breast cancer risk [22] through diet-mediated regulation of mammary epithelial differentiation, proliferation and apoptosis [23–27] coupled with the recognition that mammary fate and ductal development are controlled to a large extent by mammary fibroblastic and adipocyte mesenchyme [15], the prospect that diet-associated components may equally influence mammary stromal biology to influence the course of differentiation or neoplastic growth of the mammary epithelium is not far-fetched.

The invitation to write this minireview was prompted by our findings that mammary stromal adipocytes are early biological targets of dietary factors, specifically of the major isoflavone genistein (GEN) *in vivo* [27]. In that report, we showed that limited exposure (i.e., *in utero* and lactational only) of female rat offspring to a maternal diet containing soy protein isolate (SPI) as major protein source resulted in mammary stromal adipocyte-specific genomic changes (e.g., lipogenic gene expression) coincident with increased differentiation of mammary tissues that were distinct from those exposed to the control diet with casein as the major protein source. Further, we showed that the functional consequence of SPI-mediated adipocyte metabolic changes on neighboring mammary epithelium *in vivo* can be recapitulated by GEN *in vitro* through direct actions on differentiated 3T3-L1 adipocytes, a function likely related to their increased secretion of the adipokine adiponectin with GEN treatment [27]. Little is known of the gene pathways and mechanisms by which specific dietary factors may target the stromal compartment to promote breast health. We begin this review by highlighting seminal information on cell signaling mechanisms underlying mammary tumor protection by dietary factors. Next, we describe how mammary stromal remodeling has been implicated in underlying epithelial biology, with a focus on the emerging links between mammary adiposity and mammary ductal development as an indication of adipose-directed signaling. Finally, we discuss recently described, albeit limited, information on stromal-localized molecular targets of dietary factors, which may serve as paracrine mediators of dietary factor action on mammary epithelial cells.

2. Dietary factors and mammary epithelial targets in breast cancer protection

The incidence of breast cancer is high in the United States [1], with an increasing trend noted globally [28], yet strategies addressing its prevention remain extremely limited. Indeed, the current emphasis on the clinical management and treatment of breast cancer dramatically contrasts with the inadequacy of efforts directed toward disease prevention. In addition, there is reluctance among the general populace to embrace the concept that nutrition and lifestyle constitute highly modifiable risk factors for the prevention of breast cancer. In part, this may be due to the oftentimes conflicting reports, based largely on epidemiological studies, of the protective health benefits of specific diets. For example, high dietary fat intake, especially high polyunsaturated fatty acids, has been linked to the promotion of breast cancer in animal models [29,30] but currently not in humans [31,32]. On the other hand, saturated fat consumption is linked to breast cancer in women, but this has not been conclusively demonstrated in animal studies [33]. Similarly, dietary vitamin A, carotenoid and Vitamin D intake has been individually shown to prevent breast cancer in a number of human and animal studies, although a unifying outcome remains lacking [34,35]. The differences in physiological status of human subjects (prepubertal and post-pubertal; premenopausal and postmenopausal), source of dietary factors (from foods or supplements) as well as varying doses and 'developmental window' of dietary exposure in the many studies described in the literature [22,32,36] had preempted conclusive indications of the breast cancer-preventive benefits of consumption of any dietary factor. While studies with animal models and cell lines have been faulted for their simplistic approach toward understanding dietary prevention of breast cancer susceptibility, given the heterogeneity of the human population, these models have been invaluable in providing mechanistic insights regarding the contributions of specific bioactive components to breast cancer risk.

Efforts to understand the mechanisms underlying the breast cancer-preventive effects of dietary factors have focused on their

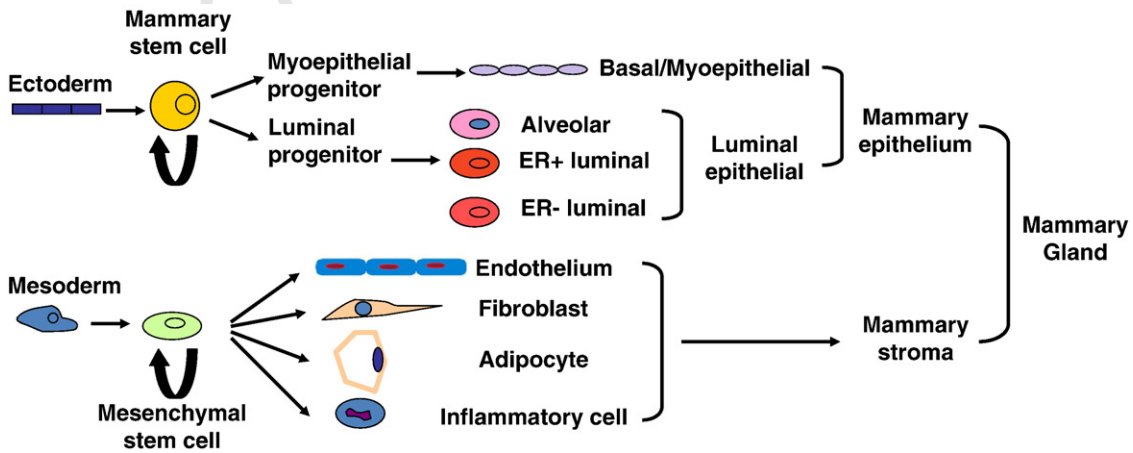

Fig. 1. The origin and lineage of the different cell types in the mammary gland. The mammary epithelium (luminal and myoepithelial) is embedded in the complex stromal matrix (also designated mammary fat pad) composed predominantly of fibroblasts, adipocytes and immune cells. The complexity of the mammary gland is a function of its distinct constituent cell types, which are subject to different endocrine and local regulation and which exhibit diverse functions. ER+ve, estrogen receptor positive; ER−ve, estrogen receptor negative.

biological and genomic consequences on mammary epithelial cells, where breast cancer arises. In particular, curcumin from turmeric [37], resveratrol from grape [38], capsaicin from chili pepper [39], flavonoids such as hesperetin and naringenin in citrus fruits and tomatoes [40], isoflavones (e.g., GEN, daidzein) from legumes and red clover [41,42] and epigallocatechin-3-gallate from green tea [43] have been demonstrated to provide different levels of preventive effects in rodent and cell culture models. An extensive discussion of the literature on the numerous mechanisms reported to underlie dietary prevention of breast cancer is beyond the scope of this current review, given the excellent recent reviews on this subject [44–48]. Suffice it to say that common mechanisms of actions have emerged: these include carcinogen activation/detoxification by metabolic enzymes, increased antioxidant and anti-inflammatory effects, induction of cell cycle arrest and inhibition of cell proliferation, decreased cell survival, enhancement of differentiation, increased expression and functional activation of various genes and corresponding proteins that are involved in DNA damage repair, tumor suppression and angiogenesis and down-regulation of oncogenes. Importantly, while the signaling pathways affected by various dietary factors in mammary epithelial cells are numerous, these pathways are interrelated, not mutually exclusive and as expected, utilize similar sets of genes previously elaborated in other tumor types [49].

Global gene expression profiling of mammary epithelial cells and subsequent functional annotation of gene expression changes have proven to be an effective tool for the discovery of novel pathways mediating dietary factor protection of mammary tumorigenesis. In studies from our laboratory using Affymetrix GeneChip microarrays [50], we showed a very low percentage of epithelial genes (~0.5% of 14,000 genes evaluated) whose expression is altered by exposure to either SPI or GEN diet beginning *in utero* to early adult stage (postnatal day 50), relative to control casein diet. The functional association of these identified genes with signaling pathways involved in immune response, protein and carbohydrate metabolism, growth regulation and stem cell niche (e.g., Wnt and Notch pathways) has provided invaluable insights into important targets of SPI-associated bioactive components and, in particular, GEN to induce epithelial changes for increased resistance to carcinogenic agents [51,52]. Indeed, our independent identification of the tumor suppressor *PTEN* [53] and of E-cadherin/Wnt/β-catenin signaling [54] as molecular pathways influenced by dietary exposure to SPI and GEN *in vivo* and by GEN *in vitro* has been bolstered by the recently elaborated linkage between these two signaling pathways in the regulation of normal and malignant mammary stem/progenitor cells *in vivo* and *in vitro* [55]. Similar support has been provided by other published studies, including those for epigallocatechin-3-gallate [56], phytoestrogens [57] and polyunsaturated fatty acids [58]. Taken together, the cellular pathways mediating dietary factor actions in the context of mammary epithelial growth regulation implicate their collective opposing actions on the expression and/or activity of tumor suppressors and oncogenes and their respective downstream targets.

3. Mammary stromal signaling in breast cancer prevention

How does the mammary stroma compartment potentiate resistance of its neighboring preneoplastic cells to tumor-initiating events? Much insight has emerged from studies on carcinoma-associated stromal fibroblasts, which can transdifferentiate into myofibroblasts and which have been demonstrated to promote primary tumor growth in human xenograft models when compared to noncancerous stromas [19,20]. The altered activity of tumor-associated stromal fibroblastic cells was associated with genetic and epigenetic alterations in specific gene subsets including that of the tumor suppressor *p53*, leading to increased expression of growth factors, cytokines and extracellular matrix components and which, by paracrine signaling, promoted neoangiogenesis and epithelial-to-mesenchymal transition in neighboring cells [19,59]. In an elegant recent study by Trimboli et al. [60], the conditional inactivation of the tumor suppressor *PTEN* in stromal fibroblasts of mouse mammary glands was shown to promote the initiation, progression and malignant transformation of mammary epithelium. *PTEN* loss was linked to increased extracellular matrix component deposition and innate immune infiltration, two key events associated with tumor malignancy and with activation of Ras, JNK and Akt growth-regulatory pathways [60]. This and similar studies [61–63] strongly support the notion that altered signaling in the tumor stroma, in this case, stromal fibroblasts, elicits aberrant epithelial growth regulation, leading to tumor manifestation.

Adipocytes constitute a significant component of the mammary stromal compartment and, similar to fibroblasts, are considered essential for mammary tumor growth and survival. While the mouse mammary fat pad consists primarily of adipocytes, this is not the case for the human mammary gland, where the developing mammary epithelium is closely sheathed by stromal fibroblasts. Nevertheless, the proximity of adipocytes to the epithelium and their high secretome activity [64,65] suggest significant influence. Indeed, the findings that (1) obesity, a disorder arising from altered gene–nutrient interactions, is a risk factor for breast cancer development [66], (2) diet-induced obesity in mice results in enlarged mammary glands and suppression of normal ductal development [67], and (3) adipose tissue from obese human subjects synthesize high and low levels of the adipokines leptin and adiponectin, respectively [68,69], which display opposing effects (promotion by leptin; inhibition by adiponectin) on mammary epithelial proliferation and which have been associated with regulation of mammary tumor development in mice [70], provide strong support for the influence of mammary adipocytes on breast cancer progression.

Interestingly, despite the increasing focus on obesity and nutrition/diet as major determinants of mammary epithelial oncogenesis, the connection between dietary factors with putative mammary tumor-protective effects and normal mammary adipose tissue biology has not been directly demonstrated. Two studies have recently appeared that highlight this association, albeit indirectly. Cho et al. [71] reported that the polyphenol (−)-catechin, among the many polyphenols present in green tea, enhanced the expression and secretion of adiponectin in 3T3-L1 adipocytes *in vitro*. The increase in adiponectin secretion by (−)-catechin was accompanied by increased insulin-dependent glucose uptake in differentiated adipocytes and decreased expression of the transcription factor Kruppel-like 7, which inhibits adiponectin expression [71]. While these *in vitro* findings did not directly address the consequence(s) of (−)-catechin promotion of adiponectin expression and secretion on mammary epithelial growth regulation, they are consistent with previous indications that green tea extracts have antiobesogenic activity [72] and inhibit mammary tumor initiation and progression in animal models of breast cancer [73]. In the second study by our group [27], we incorporated *in vivo* and *in vitro* strategies to link genomic and functional consequences in rat mammary glands upon *in utero*/lactational exposure to dietary SPI with paracrine signals from GEN-treated 3T3-L1 adipocytes to induce mammary epithelial differentiation. While our studies did not identify the paracrine signal(s) mediating the enhanced differentiation of mammary epithelial cells, we posited that one likely candidate is adiponectin, given the increased secretion of this adipokine in differentiated adipocytes treated with GEN at physiological doses [27]. Preliminary findings provide support to the latter, based on the higher adiponectin protein levels in the mammary glands of young adult female rat offspring exposed to SPI following the above dietary regimen, in the absence of changes in systemic levels of this adipokine (O. Rahal and R.C.M. Simmen, unpublished observations). Given that early only and

lifelong exposure to soy-enriched diets are mammary tumor-preventive in rodent models of carcinogenesis [52,74], findings that were borne out by epidemiological studies [75], the 'chicken-or-the-egg" question as to which mammary compartment (stromal or epithelial) is initially targeted by dietary factors to achieve the final outcome of increased mammary epithelial differentiation for decreased sensitivity to oncogenic agents, may constitute a fruitful direction for future investigation.

While the aforementioned studies investigated aspects of dietary influences on lipogenic and adipogenic regulators in the mammary adipocyte, mechanisms for dietary regulation at the level of adipocyte differentiation are also plausible. A great deal of our understanding of the molecular basis of adipocyte differentiation has been gained from studies of clonal fibroblastic preadipocyte cell lines (3T3-L1, 3T3-442A) and *ex vivo* studies of stromal vascular cells isolated from animals [76,77]. Committed preadipocytes, upon hormonal induction *in vitro* and via elusive *in vivo* signals, begin the differentiation program involving CREB-mediated phosphorylation of the transcription factor CAAAT-enhancer binding protein-β [77–79], followed by mitotic clonal expansion and activation of CAAAT-enhancer binding protein-α and peroxisome proliferator-activated receptor (PPAR)-γ. These, along with the sterol regulatory element binding protein-1c, transactivate a number of adipocyte-specific genes that maintain the adipocyte phenotype [80,81]. Throughout life, adipose tissue mass is regulated by a balance between formation (via hypertrophy of existing adipocytes and hyperplasia) and lipolysis. While the molecular events underlying adipocyte differentiation from precursor cells have been extensively studied, the precise origins of the adipose tissue *in vivo* are still poorly understood. In this context, two important recent advances in our understanding are noteworthy. First, using novel PPAR-γ reporter mouse strains (PPAR-γ-Rosa26 reporter and PPAR-γ-TRE-H2B-GFP) where endogenous PPAR-γ promoter leads to indelible marking of daughter cells with LacZ or GFP, Tang et al. [82], performed cell lineage tracing experiments. These elegant studies revealed that most adipocytes reside in the mural cell compartment in close to the adipose vasculature and are already committed to an adipocyte fate *in utero* or early postnatal life. The second major advance in this area has been the identification of early adipocyte progenitor cells in the adipose tissue using flow cytometry. Using fluorescence-activated cell sorting, Rodeheffer et al. [83] identified cells that are $Lin^{-}CD29^{+}CD34^{+}Sca1^{+}CD24^{+}$ residing in the adipose tissue and that likely represent early adipocyte precursors since they can reconstitute a normal adipose tissue when injected into 'fat-less' lipodystrophic mice. It should be noted that the origin of adipocytes in the mammary fat pad has not been examined to date. In light of these studies, it is important to begin to address whether diet/dietary factor-associated cancer protection may be linked with altered commitment/differentiation of mammary preadipocytes.

4. Dietary factors and candidate mammary stromal targets for breast cancer prevention

While there is a paucity of information to directly link the targeting of specific mammary stromal cell types by known dietary factors to neighboring mammary epithelial growth regulation, a few candidate mediators have emerged. The most relevant are the adipokines adiponectin and leptin, which, because of their mammary adipocyte source, demonstrated regulation of mammary epithelial proliferation, differentiation and apoptosis though distinct mechanisms [70,84–86], and the negative and positive association of their expression levels, respectively, with breast cancer risk and adiposity [87–89]. *In vitro*, the isoflavone GEN has been shown to enhance secretion (hence, availability as endocrine/paracrine signals) of adiponectin [27] and to inhibit that of leptin [90]. The bioactive component chitosan from edible mushrooms, which was found to demonstrate antiobesogenic activity in rats [91], similarly reduced visceral adipose tissue leptin levels in mice consuming chitosan-supplemented diet [92]. Further, the short-chain fatty acid propionic acid, which is produced by the colonic fermentation of dietary fiber known to be preventive for the development of obesity [93], was shown to increase leptin messenger RNA expression and corresponding protein secretion, in the absence of coincident effects on adiponectin, in human omental and subcutaneous adipose tissue explants [94]. While the increased secretion of leptin by propionic acid appears counterintuitive to its antiobesity and, by extension, anticipated antimammary tumorigenic effects, this was accompanied by the reduced expression of the proinflammatory factor adipokine resistin, suggesting that the repertoire of adipokines presented to target cells may predict the final growth/proliferative outcome. In this regard, a recent study has shown significantly elevated plasma resistin levels in patients with breast cancer relative to those without disease [95], consistent with the link between inflammation and breast cancer risk.

Our group's approach to mechanistically address the directional signaling from stromal to epithelial cells initiated by bioactive dietary factor targeting of mammary fat pad involves (1) defining the *in vivo* measures of mammary epithelial and stromal differentiation upon early dietary SPI exposure and (2) recapitulating these responses in nontumorigenic mammary epithelial cells exposed to conditioned medium from differentiated 3T3-L1 adipocyte treated with GEN *in vitro* [27]. While our experiments constitute proof of concept, there are caveats that require further scrutiny. Our studies did not unequivocally identify GEN-specific gene targets in stromal fibroblasts and adipocytes distinct from those of epithelial cells, since the gene expression analyses were carried out using whole mammary tissues. Moreover, the biological and molecular outcomes observed *in vitro* with GEN precluded the contribution of other SPI-associated bioactive components, which may elicit more direct effects than could be attributed to GEN alone. Finally, it was not possible to demonstrate the converse directional signaling (i.e., from epithelial to stromal compartment) that may equally underlie mammary tumor prevention. In support of the existence of epithelial-to-stromal dialog, it was shown that during the development of breast cancer, the stromal compartment responded to signals from tumorigenic cells, leading to a more 'reactive' stroma and amplification of the tumorigenic state [96]. Additional studies using isolated adipocytes and fibroblastic cells derived from mammary fat pad or *in vivo* sampling of mammary fat pad followed by proteomic analyses [65,97], as a function of whole diets and purified bioactive components, will provide a 'glimpse' of the mammary secretome and presumably regulators of mammary stromal mediated epithelial changes.

The elegant study by Lam et al. [70] demonstrating the precise role of adiponectin in mammary carcinogenesis can serve as a paradigm for mechanistically elucidating the role of adipocyte-specific gene targets of diet and dietary factors on mammary tumor prevention. In that study, MMTV-polyomavirus middle T-antigen transgenic mice with reduced adiponectin expression were generated to test the effects of adiponectin haploinsufficiency on the promotion of mammary tumors. Similar kinds of studies could be performed to test the function of candidate mammary adipocyte genes that are identified from gene expression analyses of tissues from rodent models under different dietary programs. In this regard, the recent report on the characterization of a 5.4-kb adiponectin promoter/5′ regulatory region that confers adipocyte-specific expression of target genes may provide an avenue for studying gene function in the context of bidirectional signaling in the mammary gland [98]. While it is unknown whether mammary adipose tissue exhibits specialized responses to extracellular signals or displays gene expression patterns distinct from retroperitoneal (subcutaneous) adipose tissue, an earlier study showed that the lipid composition in adipose tissue of virgin rat mammary glands resemble that of the retroperitoneal adipose [99].

5. Concluding remarks

The notion that the mammary fat pad is a direct target of bioactive dietary factors for mammary tumor protection is not difficult to envision, given that in any biological system, nothing stands alone. It is perhaps paradoxical that studies to address this remain relatively limited and the concept that bidirectional signaling within the mammary microenvironment for breast cancer prevention remains an intriguing observation. While the stromal compartment is not the main target of carcinogens [100], the possibility that a very early event upon carcinogenic insult is the sensing by stromal cells of 'something amiss' in adjacent epithelial cells is not unlikely. If this is the case, the identification of mammary fibroblast- and adipocyte-specific 'early' molecular targets by bioactive components in model systems may eventually provide biomarkers for the very early stages of the disease. The recent characterization of a mammary stromal fibroblastic cell line from mice that can differentiate to a preadipocyte lineage [101] in coculture studies with nontumorigenic or tumorigenic mammary epithelial cells will enable a proof-of-principle evaluation of the epithelial/stromal adipocyte dialog and associated mediators.

The findings that mammary stroma can reprogram testicular and neural stem cells to produce progeny committed to a mammary epithelial cell fate [102,103] and that a precancerous mammary stem cell may be programmed to become breast cancer [104] suggest the possibility that direct dietary factor effects on mammary stroma may alter stem cell behavior to inhibit neoplastic transformation. Thus, while mammary stem cells may constitute direct targets of bioactive dietary components as recently suggested by the report that curcumin added *in vitro* can induce mammosphere-forming ability in normal and malignant breast cells [105], a dual effect of dietary factors on mesenchymal and epithelial stem cells is also likely.

Further, dietary factors may directly influence the stem cell compartment in mammary stroma at the levels of the preadipocyte pool and the number of multipotent stem cells that enter the adipocyte lineage. The effects of obesity, high fat diets and other dietary factors on mammary preadipocyte populations remain unknown. It has been suggested that the inability of a particular adipose depot to expand may be causative in the accumulation of hypertrophic adipocytes and a predisposing factor in metabolic disease. Hence, it is possible that certain diets or dietary factors may mediate indirect beneficial actions on mammary epithelial cells via their modulation of preadipocyte commitment and/or differentiation of new mammary adipocytes. A recent report that *in utero* exposure to the environmental agent tributylin induced multipotent stem cells to differentiate into adipocytes provides strong support to this possibility [106].

Finally, while the contribution of inflammatory/immune cells found in mammary stroma is not included in the present review, their relevance as dietary factor targets to mediate epithelial proliferation and differentiation cannot be ignored, given that local inflammation associated with solid tumors is partly a consequence of immune cells in the tumor stroma [107]. Indeed, we observed that immune-related genes constitute major targets of dietary exposure to SPI and GEN in mammary epithelial cells of young adult rats [50]. The down-regulated expression of epithelial genes involved in antigen presentation, antigen processing and inflammation, including that of interleukin 17β, a homolog of interleukin 17, which is linked to neutrophil chemotaxis, suggests the possibility of similar specific targeting of immune cells localized

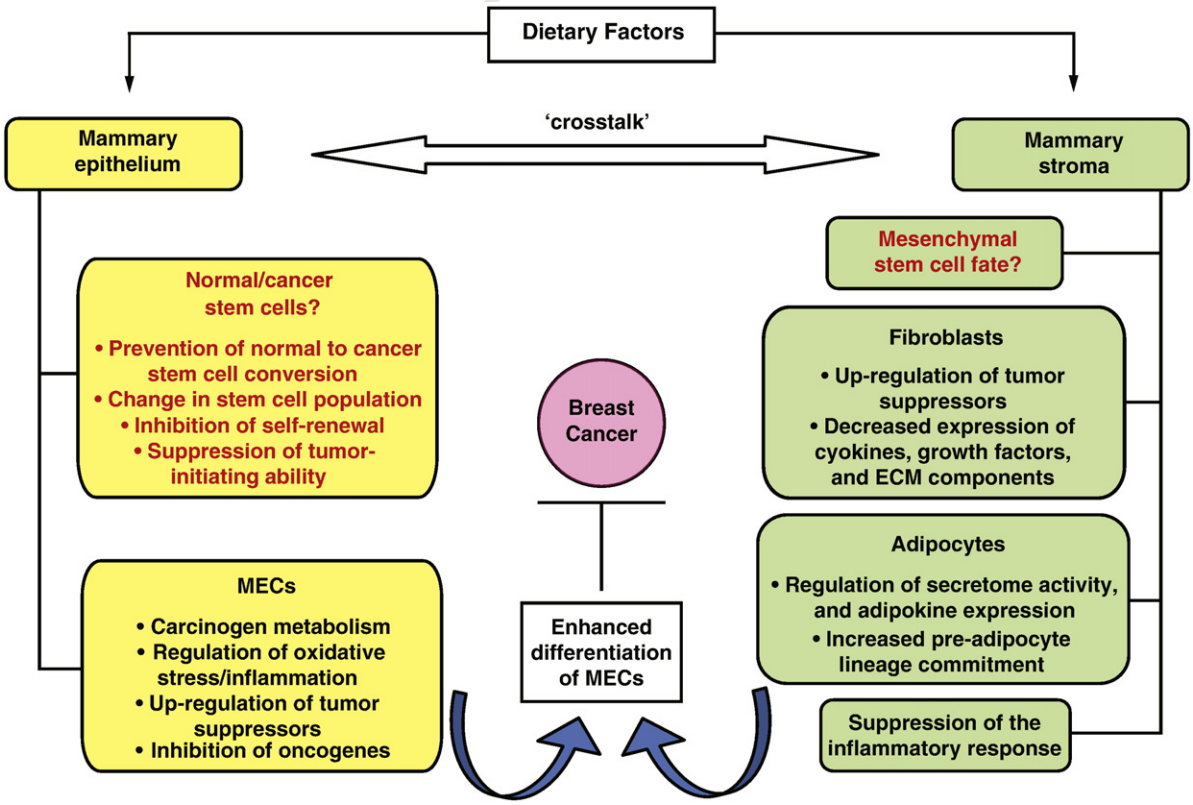

Fig. 2. A proposed model of cellular processes regulated by dietary factors in mammary epithelial and stromal compartments for breast cancer protection. The bidirectional arrows indicate an ongoing dialog between the mammary compartments. Mammary epithelial and mesenchymal stem cells are considered to represent cells of origin for each compartment. The composite actions of each mammary cell type result in the enhanced differentiation and, hence, increased resistance of mammary epithelial cells to carcinogenic insults, leading to decreased breast cancer risk.

to stroma and is consistent with promotion by the immune microenvironment of tumor progression [107].

In summary, bidirectional signaling between mammary stroma and epithelial cells promoted by bioactive dietary components constitutes a relevant biological event for mammary tumor prevention (Fig. 2). Thus, it is essential that, in future studies where dietary factor effects are described for mammary tumor prevention, their contributions to the phenotype and molecular profiles of mammary stromal fibroblasts and adipocytes are investigated coincident with those of neighboring epithelium. Gaining a better understanding of the complex interrelationships among the different mammary compartments in response to environmental ('dietary') cues may expand nutritional strategies for breast cancer prevention and therapeutic interventions.

Acknowledgements

We thank Dr. Frank A. Simmen (UAMS) for critical comments on the manuscript. Work described from Dr. R.C.M. Simmen's laboratory was supported in part, by grants from the USDA-CRIS 6251-5100002-06S, Department of Defense Breast Cancer Research Program (CDMRP W81XWH-08-0548), the Fashion Footwear Association of New York (FFANY/QVC) and the Arkansas Children's Hospital Research Institute/Children's University Medical Group Program.

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