Maternal Epigenetic Regulation Contributes to Prevention of Estrogen Receptor–negative Mammary Cancer with Broccoli Sprout Consumption

Shizhao Li[1], Min Chen[2], Huixin Wu[1], Yuanyuan Li[3], and Trygve O. Tollefsbol[1,4,5,6,7]

ABSTRACT

Cruciferous vegetables have been of special interest due to the rich presence of bioactive compounds such as sulforaphane which show promising potential on cancer prevention and therapy as an epigenetic dietary strategy. Abnormal epigenetic alteration as one of the primary contributors to tumor development is closely related to breast cancer initiation and progression. In the present study, we investigated the effect of dietary broccoli sprouts (BSp), a common cruciferous vegetable, on prevention of estrogen receptor (ER)-negative mammary tumors at three different temporal exposure windows using a spontaneous breast cancer mouse model. Our findings indicate that maternal BSp treatment exhibited profound inhibitory and preventive effects on mammary cancer formation in the nontreated mouse offspring. The BSp diet administered to adult mice also showed suppressive effects on mammary cancer but was not as profound as the maternal BSp preventive effects. Moreover, such protective effects were linked with differentially expressed tumor- and epigenetic-related genes, as well as altered global histone acetylation, DNA methylation, and DNA hydroxymethylation levels. We also found that the expression changes of tumor-related genes were associated with the levels of histone methylation of H3K4 and H3K9 in the gene promoter regions. In addition, BSp-enriched sulforaphane was shown to increase protein expression of tumor suppressor genes such as p16 and p53 and inhibit the protein levels of Bmi1, DNA methyltransferases, and histone deacetylases in ERα-negative breast cancer cell lines. Collectively, these results suggest that maternal exposure to key phytochemicals may contribute to ER-negative mammary tumor prevention in their offspring through epigenetic regulations.

Introduction

Breast cancer is a frequently diagnosed cancer and one of the leading causes of cancer-related death among women worldwide. Like other cancers, breast cancer involves complicated etiologic disorders that may be related to an interaction between environmental factors (endogenous and exogenous) and the genetically susceptible host. Tumor initiation and progression involve both genetic mutation and posttranslational changes (1), the latter of which epigenetic alterations is known as one of the most important regulatory patterns. Epigenetics refer to mitotically or meiotically heritable changes in gene expression without a change in the DNA sequence (2), among which DNA methylation and covalent histone modifications are the most important epigenetic regulations that have been well studied.

It is well recognized that both genetic and epigenetic perturbations are provoking causes of breast carcinogenesis (3), and dysregulation of epigenetic modification of genes related to proliferation, metastasis as well as apoptosis has been implicated in breast tumorigenesis. Because epigenetic changes are potentially reversible, promising novel therapeutic approaches targeting epigenetic regulations are being developed for various cancers. In fact, some DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors have been used in cancer pharmacologic therapies based on a deeper understanding of the characteristics and epigenomic dysregulation implicated in tumorigenesis (4).

Of great interest is that certain bioactive compounds of the so-called "epigenetics diet" (5) have been shown to modify the epigenome through inhibiting HDACs and/or DNMTs as well as modulating microRNA (miRNA), leading to beneficial health outcomes including cancer prevention. Such dietary components include, but are not limited to, isothiocyanates in broccoli (*Brassica oleracea L. var. italica*), genistein in soybean, resveratrol in grape, epigallocatechin-3-gallate in green tea, and

[1]Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama. [2]Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, Alabama. [3]Department of Obstetrics, Gynecology and Women's Health, University of Missouri, Columbia, Missouri. [4]Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama. [5]Nutrition Obesity Research Center, University of Alabama at Birmingham, Birmingham, Alabama. [6]Comprehensive Center for Healthy Aging, University of Alabama at Birmingham, Birmingham, Alabama. [7]Comprehensive Diabetes Center, University of Alabama at Birmingham, Birmingham, Alabama.

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S. Li and M. Chen contributed equally to this article.

Corresponding Authors: Trygve O. Tollefsbol, University of Alabama at Birmingham, 175 CH, Birmingham, AL 35294-1170. Phone: 20-5934-4573; Fax: 120-5975-6097; E-mail: trygve@uab.edu; and Yuanyuan Li, ylgrk@missouri.edu

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ascorbic acid in fruits. Cruciferous vegetables such as broccoli sprouts, cauliflower, kale, and cabbage have gained recognition as nutraceutical foods due to the rich presence of health-promoting phytochemicals such as glucosinolates, vitamins, polyphenols, and minerals leading to their preventive potential against multiple human diseases including cancers (6). Notably, Cruciferae contain substantial quantities of isothiocyanates, for example, sulforaphane (SFN), glucoraphanin, and 4-methylsulfinylbutyl isothiocyanate, which are acting as potent inducers of phase II antioxidant enzymes against carcinogenesis (7). A growing body of studies has demonstrated that an important cruciferous vegetable, broccoli sprouts (BSp), and their derived phytochemical extracts exert potent chemopreventive effects on numerous cancers (8, 9). Our previous studies also showed that BSp supplementation can result in breast tumor suppression *in vitro* and *in vivo* through inhibition of HDACs and/or DNMTs activities (10).

Although the supplemental forms, pharmacologically achievable concentrations, pharmacokinetics, and molecular mechanisms through which cruciferous vegetable-derived diets lead to beneficial effects in prevention of multiple human diseases have been reported, there is an urgent need to address the optimal exposure windows of this type of diet on prevention of cancers including breast cancer. Both the Developmental Origins of Health and Disease (DOHaD) and the Fetal Basis of Adult Disease (FEBAD) emphasize the key roles of environmental cues, including diets and nutrients, at critical periods on developmental reprogramming and plasticity (2). An interesting study indicates that maternal dietary supplementation with indole-3-carbinol (I3C), another compound derived from cruciferous vegetables, provides greater chemoprotection effect for the fetus against transplacental carcinogenesis (11). In addition, compared with other nationalities, early-life consumption of cabbage showed a lower incidence of breast cancer in the Polish (12). Accumulating evidence now shows that early life, especially prenatal, dietary administration is associated with persistent epigenomic, gene regulation, and metabolic changes in the offspring, thus impacting the health condition of children and adults through nutritional programming (11–13).

We hypothesized that the maternal/prenatal stage could be a potential critical window for BSp or other epigenetic-modulating dietary administration against mammary cancer. Different from conventional dietary intervention modes through direct exposure within the pathogenic stages, we extended our studies to novel maternal interventions throughout different developmental stages in the offspring. Nutritional exposure during these stages may intervene in early ontogenesis and subsequently lead to different mammary tumor susceptibility later in life (14). The present study, therefore, is an endeavor to seek appropriate nutritional exposure during early-life stages by the use of epigenetic modifying dietary components for human breast cancer prevention. Our findings indicate in part that maternal, especially gestation and lactation, dietary BSp consumption can impact tumorigenesis through epigenetic regulation leading to improved protection of the offspring from ER-negative mammary cancer later in life.

Materials and Methods

Animal experiment

Mouse model

We used the wild-type (WT) Her2/neu [FVB/N-Tg (MMTVneu)202Mul], a spontaneous mammary tumor mouse model, in this study. The female mice of this model can develop focal hyperplastic and dysplastic mammary tumors with the expression of the WT *Her2/neu* gene, which has also been deeply implicated in human breast cancer genesis. The Her2/neu mice develop ER-negative mammary tumors at early age (∼20 weeks; median latency is around 30 weeks; ref. 15). The breeder mice (4 weeks of age) were obtained from the Jackson Laboratory and bred from 10 weeks of age to obtain adequate colonies for follow-up experiments. A standard PCR analysis with tail DNA of mice at 3 weeks of age was used to identify the Tag genotypes according to a previous study (16). All the mice were housed in the Animal Resource Facility of the University of Alabama at Birmingham and were maintained under the following conditions: 12-hour dark/12-hour light cycle, 24 ± 2°C temperatures, and 50 ± 10% humidity. We used an online power and sample size calculator (http://powerandsamplesize.com/) to determine the power and sample size by two-proportion comparison (13).

Dietary

A customized BSp diet (TestDiet) was prepared with AIN-93G diet base and adjusted for macronutrient content as used previously (13). The modified AIN-93G contained 26% (w/w) BSp, which was obtained from Natural Sprout Company. Detailed dietary ingredients and nutritional profile are provided in Supplementary data (Supplementary Fig. S1). To provide maximum protection against possible changes, diets were stored in airtight containers and were kept away from light under refrigeration (2°C for up to 6 months and −20°C for long-term life).

Experimental designs

Pregestational maternal BSp treatment: Her2/neu female mice (10 mice) were fed the BSp diet from weaning (3 weeks of age) to 10 weeks of age. The treated female mice were then mated with nontreated Her2/neu male mice and were assumed to be pregnant when a vaginal plug was expelled. Pregnant mice were treated with control AIN-93G diet from their conception throughout gestation and the lactation period. The offspring were weaned at postnatal 21 days of age (PD21). 21 female offspring were selected and randomly assigned into 3 cages. These mice were maintained with control AIN-93G diet until the termination of the experiment. Mammary tumor observation and tissue samples collection were only performed with the female offspring; (**Fig. 1**).

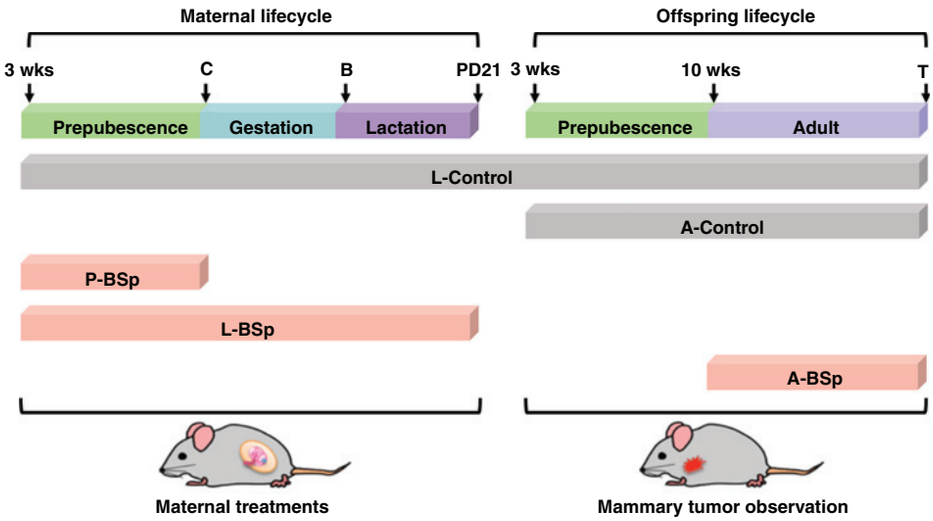

Figure 1.

Schematic representation of experimental design for BSp dietary treatments. The top bar represents different life stages in the maternal and female offspring lifecycle. Transgenic mice (Her2/neu) were administered the BSp diet under different exposure time points: (i) control: female mice were fed *ad libitum* with the control AIN-93G diet throughout the study; (ii) pregestational maternal BSp treatment (P-BSp): The BSp diet was gi-ven to the mother from postnatal 3 weeks of age until mating (10 wks); (iii) long-term maternal BSp treatment (L-BSp): the BSp diet was given to the mother from postnatal 3 weeks of age until the weaning of their offspring (PD21); (iv) postnatal adult BSp treatment (A-BSp): The BSp diet was given to female offspring from 10 weeks of age until the termination of the experiment. Mice were mated at 10 weeks of age followed by 3 weeks of gestation. Offspring were weaned at 3 weeks of age. Two age-matched controls were applied in this study. The long-term control (L-Control) was used in the P-BSp and the L-BSp treatments. The adult control (A-Control) was used as the control of the A-BSp group. In the P-BSp and L-BSp groups, weaned female offspring were maintained on the control AIN-93G diet until the termination of the experiment and monitored for tumor growth weekly. For the A-BSp treatment, female mice were born to a mother administered with the control AIN-93G diet and monitored for tumor growth weekly after weaning. C, conception or gestation day 0; B, birth; PD21, postnatal day 21; 10 wks, onset of adulthood; T, termination of the experiment. The experiments were terminated when all of the control mice developed tumors and had an average tumor diameter exceeding 1.0 cm.

Long-term maternal BSp treatment: Her2/neu female mice (10 mice) were fed the BSp diet from weaning (3 weeks of age). The treated female mice were mated with nontreated Her2/neu male mice at 10 weeks of age and were assumed to be pregnant when a vaginal plug was expelled. Pregnant mice were sequentially treated with the BSp diet throughout their gestation and lactation periods. Offspring were weaned at postnatal 21 days of age (PD21). 21 female offspring were selected and randomly assigned into 3 cages. These mice were maintained on the control AIN-93G diet until the end of the experiment. Breast tumor observation and tissue samples collection were only performed with the female offspring (**Fig. 1**).

Adult BSp treatment: Her2/neu female mice (14 mice) were treated with the BSp diet from 10 weeks of age which was continued until termination (**Fig. 1**).

Control: Her2/neu female mice were fed *ad libitum* with the control AIN-93G diet throughout the study. Two age-matched controls were applied in this study. The long-term control (L-Control) was used in the pregestational maternal BSp (P-BSp) and the long-term maternal BSp (L-BSp) treatments. The adult control (A-Control) was used as the control of the A-BSp group.

Tumor observation and sampling

The Her2/neu mouse model can develop mammary tumors at early age (around 20 weeks of age). Tumor size and latency were measured and recorded weekly. Tumor volume was determined by the following formula: tumor volume (cm^3) = 0.523 × [length (cm) × $width^2$ (cm^2); ref. 17]. The experiments were terminated when all of the control mice developed tumors and had an average tumor diameter exceeding 1.0 cm. The mammary tumors were then excised, weighed, and preserved in liquid nitrogen for follow-up analysis. Animal procedures were reviewed and approved by the UAB Institutional Animal Care and Use Committee (IACUC; Animal Project Numbers: 10088 and 20653). All mouse-related operations were carried out in accordance with the guidelines of the IACUC at UAB.

Cell culture and treatment

We chose two ERα-negative breast cancer cell lines, MDA-MB-157 and MDA-MB-231, for *in vitro* validation in this study. MCF10A, an immortalized noncancerous human mammary epithelial cell line, was used as a noncancerous control. All three cell lines were purchased from ATCC and enough vials of the first three generations of each cell line were prepared once purchased and preserved in liquid nitrogen for cell line renewal every 2 months or less. Short tandem repeat analysis was used to authenticate these cell lines and the most recent authentication was performed in May, 2018. All three cell lines were routinely screened to confirm *Mycoplasma*-negative status. MDA-MB-157 and MDA-MB-231 cells were grown in DMEM (Corning) supplemented with 10% FBS (R&D

Systems) and 1% penicillin/streptomycin (Corning). The MCF10A cells were cultured in DMEM/F12 medium (Corning) with 5% donor horse serum (R&D Systems), 20 ng/mL EGF (PeproTech), 0.5 mg/mL hydrocortisone (Sigma), 100 ng/mL cholera toxin (Sigma), 10 μg/mL insulin (Sigma), and 1% penicillin/streptomycin (Corning). Cells were maintained in an incubator at 5% CO_2 with controlled temperature (37°C) and humidity. After seeding, cells were allowed 24 hours to adhere to the plate after which they were treated with 5 μmol/L concentration of SFN for a total of 3 days. DMSO was used as a vehicle control and the culture medium in all treatments were replaced with fresh medium every 24 hours for the duration of the experiment.

MTT analysis

3-(4,5-dinethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess cells viability after treating with SFN. Appropriate cells were seeded in 96-well plates and were allowed 24 hours to adhere to the plate after which they were treated with the indicated concentration of SFN (0, 5, 10, 15, 20, and 25 μmol/L) for a total of 72 hours. After the treatments, MTT was added to the culture medium to achieve a final concentration of 1 mg/mL followed by 3.5-hour incubation at 37°C until purple insoluble formazan were viable. 100 μL DMSO was used to dissolve these purple precipitates at room temperature with gentle shaking on a rocking platform for about 15 minutes. The absorbance was measured at 570 nm using a microplate reader (Epoch model, Biotek).

Real-time PCR for mRNA quantification

The total RNA was extracted from tumor samples using TRIzol LS Reagent (Invitrogen) according to the manufacturer's protocol. The total RNA was quantified using a NanoDropTM One Spectrophotometer (Thermo Fisher Scientific) with the optical density (OD) value at 260 nm, and the purity was assessed by determining the OD260/OD280 ratio as well as formaldehyde–agarose gel electrophoresis. The cDNA was synthesized with an iScript cDNA Synthesis Kit (Bio-Rad) according to the protocols provided by the manufacturer. All cDNA samples were stored at −20°C for further analysis. Gene (*β-actin, p16, p53, PTEN, Bmi1, Tert, Dnmt1, Dnmt3a, Hdac1*) expressions were performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Briefly, a 10-μL PCR mixture was prepared as follows: 5 μL of SsoAdvanced Universal SYBR Green Supermix (2×), 1 μL of forward primer (10 μmol/L), 1 μL of reverse primer (10 μmol/L), 1 μL of cDNA, and 2 μL of nuclease-free water. Primers for real-time PCR were synthesized by the Integrated DNA Technologies, and detailed information of primers is listed in Supplementary data (Supplementary Table S1). The real-time PCR reaction was conducted on a Bio-Rad CFX Connect Real-time System programmed as follows: 94°C for 4 minutes, 35 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes. All samples were assessed in triplicate and *β-actin* was selected as a housekeeping gene and used for correction computing. The average cycle threshold (C_t) values were used for quantification using the $2^{-\Delta\Delta C_t}$ method (18).

Western blotting analysis

For tissue samples, the total protein from approximately 50 mg of frozen mammary tumor samples was extracted using Tissue Protein Extraction Reagent (T-PER, Thermo Fisher Scientific) according to the manufacturer's protocol. For cell lysis, protein samples were prepared according to the protocol used in the telomerase PCR ELISA (TRAP) Kit (Roche Applied Science). The Bradford Assay was applied to measure the protein concentrations. 4%–15% NuPAGE Tris-HCl precast gels (Invitrogen) were used for electrophoresis. After separation, the proteins were transferred onto nitrocellulose membranes. Membranes were then probed with different antibodies to p16, p53, PTEN, Bmi1, Tert, Dnmt1, Dnmt3a, and Hdac1, respectively. β-actin was used as the loading control for each membrane. Detailed information of the primary antibodies used in the Western blotting analysis is listed in Supplementary data (Supplementary Table S3). Immunoreactive bands were visualized using Clarity Max Western ECL Blotting Substrates (Bio-Rad) and documented by ChemiDoc XRS+ System (Bio-Rad). The protein expression levels were quantified using the Image J software.

Histone acetyltransferase and DNMT activity assay

Nuclear protein of mammary tumor samples was extracted using the EpiQuik Nuclear Extraction Kit (EpiGentek) according to the manufacturer's protocol. Nuclear protein was then used for histone acetyltransferase and DNMT enzymatic activity determination utilizing the EpiQuik HDAC Activity/Inhibition Assay Kit (EpiGentek) and EpiQuik DNMT Activity/Inhibition Assay Ultra Kit (EpiGentek), respectively. The manufacturer's protocols were followed accordingly.

Global DNA methylation and hydroxymethylation analysis

DNA extracts of mammary tumor tissues were prepared using DNeasy Blood & Tissue Kits (Qiagen) and the manufacturer's procedure was followed. The NanoDrop One Spectrophotometer (Thermo Fisher Scientific) was used to quantify DNA yields and quality. The Global DNA methylation status was specifically indicated with the levels of 5-methylcytosine (5-mC) in total DNA and was measured by the MethylFlash Methylated DNA 5-mC Quantification Kit (Colorimetic) from EpiGentek. In addition, the MethylFlash Hydroxymethylated DNA 5-hmC Quantification Kit (Colorimetric), which was also purchased from EpiGentek, was used to quantify global hydroxymethylation status in total DNA samples.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed with the Simple ChIP Plus Enzymatic Chromatin IP Kit (Magnetic Beads; Cell Signaling Technology) according

to the manufacturer's protocol. Briefly, snap-frozen mouse mammary tumor tissues were cross-linked with a 1.5% final concentration of formaldehyde. Cross-linked chromatin was digested by micrococcal nuclease followed by moderate sonication. Trimethyl-Histone H3 (Lys4; H3K4) antibody (Cell Signaling Technology) and trimethyl-Histone H3 (Lys9; H3K9) antibody (Cell Signaling Technology) were used for immunoprecipitation of desired DNA. Protein G magnetic beads were applied to adsorb and elute target chromatin. Desired DNA was purified after the reversal of cross-links. The databases including Cistrome Data Browser (http://cistrome.org/db/#/), ENCODE (https://www.encodeproject.org/), and PubMed (https://www.ncbi.nlm.nih.gov/pubmed) were used to search for referable ChIP-seq studies. Obtained GSE ID numbers from published studies were then used for displaying detailed information in Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). We further used University of California, Santa Cruz Genome Browser (https://genome.ucsc.edu/) to visualize selected datasets and modification information in promoter regions of the indicated genes in this study. DNA sequences of selected regions in the gene promoters were used for designing ChIP-qPCR primers using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Obtained primers were first tested for their quality with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) followed by RT-qPCR verification. Quantitative PCR was then performed to determine the histone modification status in the promoter regions of *p16, p53, PTEN, Bmi1*, and *Tert* genes. We also used PROMO (version 8.3; http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) to identify putative transcription factor binding sites of these amplified sequences in the gene promoters (Supplementary Fig. S2). Briefly, a 10 μL of reaction system was employed as follows: 5 μL of SsoAdvanced Universal SYBR Green Supermix (2×), 1 μL of forward primer (10 μmol/L), 1 μL of reverse primer (10 μmol/L), 2 μL of DNA obtained from immunoprecipitation, and 1 μL of nuclease-free water. Primer sequences used were all obtained from the Integrated DNA Technologies and listed in Supplementary Table S2. The real-time PCR reaction was performed as follows: 95°C for 15 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 30 seconds. All samples were run in triplicate, and a fold enrichment method was used for ChIP-qPCR data analysis (19).

Statistical analyses

Statistical analyses were performed using SPSS version 24.0 (SPSS Inc.). The significance of tumor incidence was evaluated using χ^2 test. All other experiments with two experimental groups were analyzed with two-tailed Student *t* test. In experiments with three treatment groups, one-way ANOVA with Tukey posttest was used. Results were presented as means ± SE from at least three separate experiments. The results were considered statistically significant when **, $P < 0.01$ and *, $P < 0.05$.

Results

Maternal BSp treatments were more effective in preventing ER-negative mammary tumor development compared with adult BSp treatment

We used the Her2/neu transgenic mouse model in our study that has been applied in numerous mammary cancer studies (13, 15). This model can develop ER-negative mammary tumors that are driven by overexpression of the *Her2/neu* oncogene. As the activated *Her2/neu* gene is very common in human malignant breast tumors, the Her2/neu transgenic mouse represents an excellent model to study human breast cancer (20). In addition, the BSp concentration (26%) used in this study is equivalent to a daily intake of 234 g BSp for an adult (60 kg) human (21). This concentration of BSp is therefore physiologically available and practical. Our preliminary data revealed that dietary BSp showed no negative effects on maternal and offspring health as well as mammary gland development, indicating its safe application in all three exposure windows in this study. This dietary botanical also had no effects on the Her2/neu transgenic transoncoprotein or its phosphorylation state.

To explore an optimal exposure window and effectiveness for dietary BSp supplementation on ER-negative mammary tumor prevention, we employed three different dietary treatment periods (P-BSp, L-BSp, and A-BSp administrations, illustrated in **Fig. 1**), which represented conventional human eating habits in the present study.

We introduced the A-BSp group as a parallel comparison to investigate the impacts of direct BSp diet consumption on mammary tumor development within one generation. Although the effects were not as profound as observed in the two maternal BSp treatments, the A-BSp group led to significantly decreased tumor incidence after 27 weeks of age (**Fig. 2A** and **B**). In the P-BSp group, in which BSp diets were administered in maternal prepubescence, we found that the BSp diet can prominently suppress tumor growth, decrease tumor incidence, and delay tumor latency in the offspring (**Fig. 2C–F**), as well as in the L-BSp group, in which dams were fed the BSp diet from weaning throughout their prepubescence, gestation, and lactation periods (**Fig. 2C–F**). Notably, the long-term maternal BSp group had a longer tumor latency (**Fig. 2E**) and the pregestational group displayed a stronger inhibiting effect on tumor growth (**Fig. 2D**).

Maternal BSp treatments resulted in expression changes in multiple tumor- and epigenetic-related genes

To explore the molecular mechanisms by which maternal/adult dietary BSp administration suppressed ER-negative mammary tumor growth, we first evaluated the gene and protein expression levels of several tumor suppressor genes [*p16, p53,* and phosphatase and tensin homolog (*PTEN*)], tumor-promoting genes [B lymphoma Mo-MLV insertion region 1 homolog (*Bmi1*) and telomerase reverse transcriptase (*Tert*)] as well as key epigenetics modification-related genes (*Dnmt1, Dnmt3a,* and *Hdac1*) in the mammary tumor samples

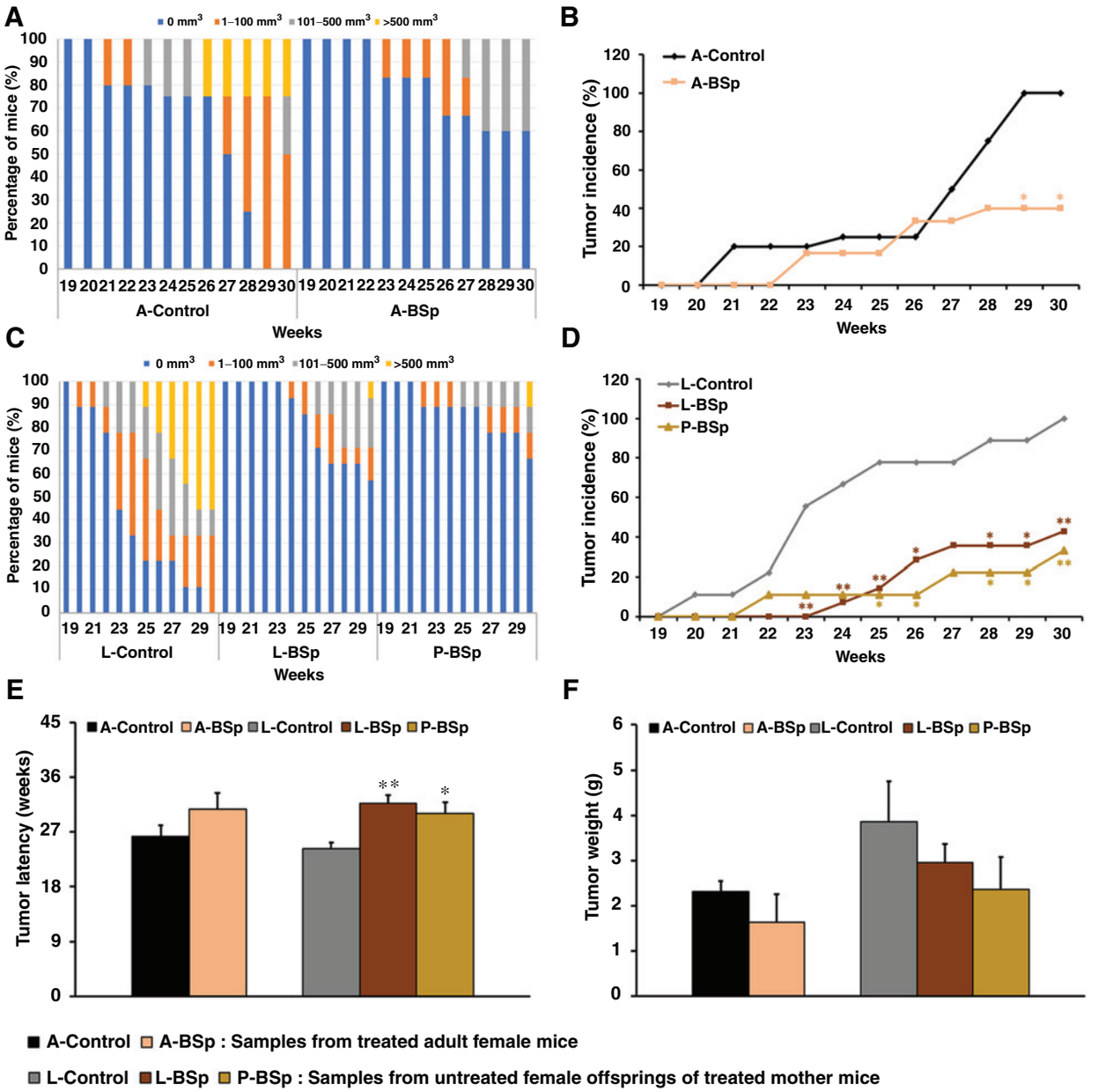

Figure 2.

Tumor growth in female progeny with adult BSp (A-BSp), pregestational maternal BSp (P-BSp), and long-term maternal BSp (L-BSp) dietary administrations. Female Her2/neu transgenic mice were administered either regular control (AIN-93G) diet or 26% BSp diet from 10 weeks of age until termination of the experiment (A-BSp), from postnatal 3 weeks of age until prior to mating (10 wks; P-BSp), or until the weaning of their offspring (3 wks; L-BSp). Weaned female offspring were maintained on the control diet throughout their lifespan until termination of the experiment and monitored for tumor growth weekly. The adult control (A-Control) was applied as the control in A-BSp treatment group. The long-term control (L-Control) was used as the control in P-BSp and L-BSp treatments. **A** and **C,** tumor growth volume; **B** and **D,** tumor incidence; **E,** tumor latency; **F,** tumor weight. Columns, mean; Bars, SD; *, $P < 0.05$; **, $P < 0.01$, significantly different from the control group.

derived from maternal BSp-treated offspring mice at termination (T; **Fig. 1**).

The results indicated that both P-BSp and L-BSp treatments significantly increased transcription levels of *p16, p53,* and *PTEN* (**Fig. 3A**) as well as their protein levels (**Fig. 3B** and **C**) compared with the control group in the offspring tumors. Interestingly, compared with the P-BSp treatment, all three tumor suppressor genes showed higher expression levels at both gene and protein levels in the L-BSp group. We did not observe significant expression

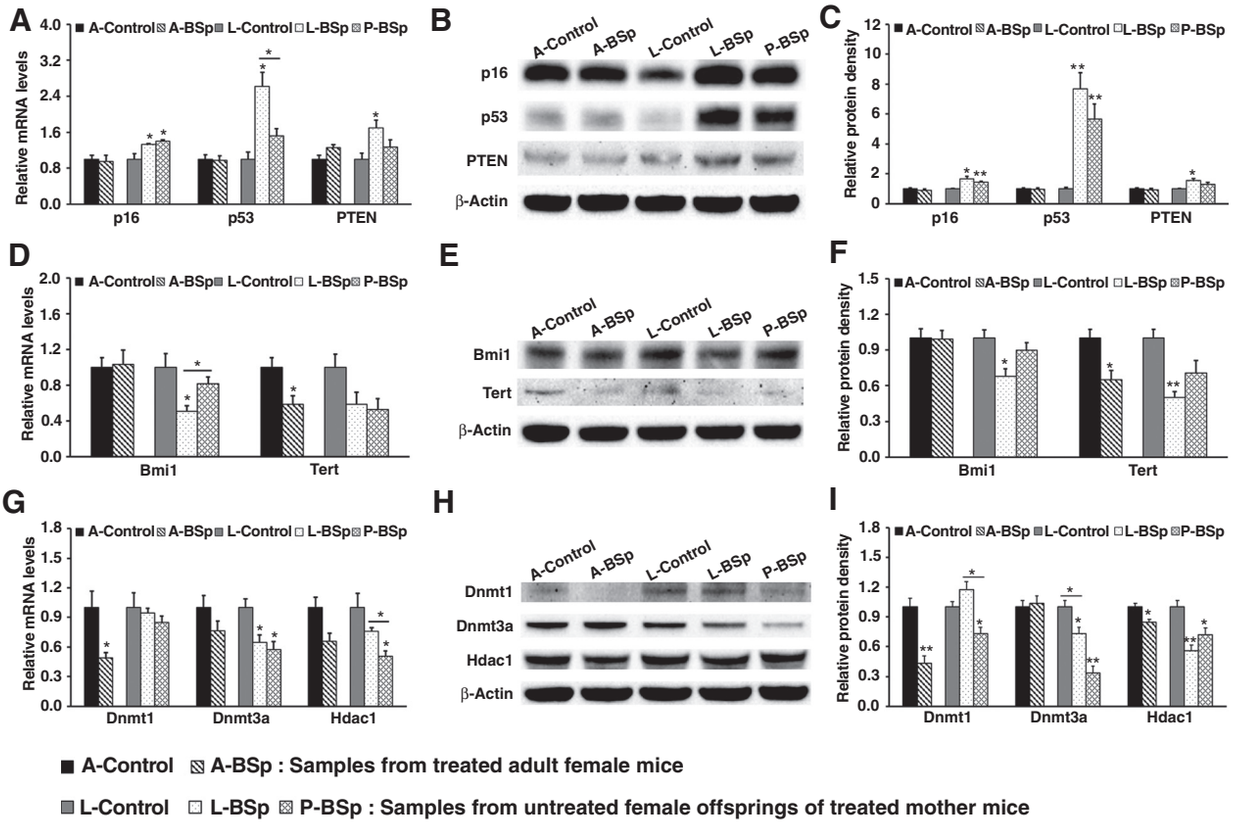

Figure 3.

Expression changes of tumor suppressor, tumor-promoting, and key epigenetics modification-related genes in the mammary tumors of the mouse offspring. Real-time PCR and Western blot analysis were performed to measure expression changes of *p16, p53, PTEN, Bmi1, Tert, Dnmt1, Dnmt3a*, and *Hdac1* in mammary tumor samples derived from the maternal pregestational BSp (P-BSp) treatment group, the maternal long-term BSp (L-BSp) treatment group, and the adult BSp (A-BSp) treatment group. **A**, **D,** and **G,** Relative gene transcription levels of the target genes in the mammary tumors. **B**, **E,** and **H,** Western blot analysis showing protein expression changes of the target proteins in the mammary tumor. *β-actin* was used as a housekeeping control gene and its antibody was used to correct loading error. Representative bands were selected from experiments repeated three times. **C**, **F,** and **I,** Quantification of the protein levels. Data were acquired from three independent experiments and were normalized to β-actin and calibrated to levels in the control group samples. Columns, mean; bars, SE; *, $P < 0.05$; **, $P < 0.01$, significantly different from the control group or between indicated groups.

changes of tumor suppressor genes in the A-BSp group (**Fig. 3A–C**).

Simultaneously, maternal BSp diets also induced significantly decreased expression of *Bmi1* and *Tert* (**Fig. 3D**), important tumor-promoting genes, in the mammary tumors of the offspring. In addition, protein expression levels of these two genes were consistent with their transcription levels (**Fig. 3E** and **F**). Like the expression patterns in tumor suppressor genes, both *Bmi1* and *Tert* revealed stronger inhibition effects in the L-BSp group, indicating that dietary BSp may exhibit better preventive effects on development of mammary tumor in the offspring when the dams are exposed to a long-term window. In addition, we found that the *Tert* gene also showed significant decreases in both transcription and protein expression levels in the A-BSp treatment group (**Fig. 3D–F**).

To further investigate the underlying epigenetic mechanisms, we also assessed the expression changes of several important epigenetic-modulatory enzymes including Dnmt1 and Dnmt3a and Hdac1 in mammary tumors of Her2/neu mouse offspring. Our results showed that both maternal BSp dietary exposures induced significantly decreased transcriptional levels of *Dnmt1, Dnmt3a,* and *Hdac1* (**Fig. 3G**), as well as their protein levels (**Fig. 3H** and **I**). In the adult group, the BSp diet also significantly decreased the expression of *Dnmt1* and *Hdac1* at both the gene and protein levels; however, this diet did not detectably affect *Dnmt3a* gene expression (**Fig. 3G–I**) in this treatment group. These results collectively suggest important roles of the BSp diet in inhibition of DNMTs and HDACs in mammary tumorigenesis.

Maternal BSp treatments may influence histone acetylation, global DNA methylation, and hydroxymethylation status via regulating of HDAC and DNMT

In an effort to further understand the epigenetic mechanisms underlying our observations, we also sought to determine enzymatic changes of known epigenetic modifiers, HDACs and DNMTs, in mammary tumors of the mouse

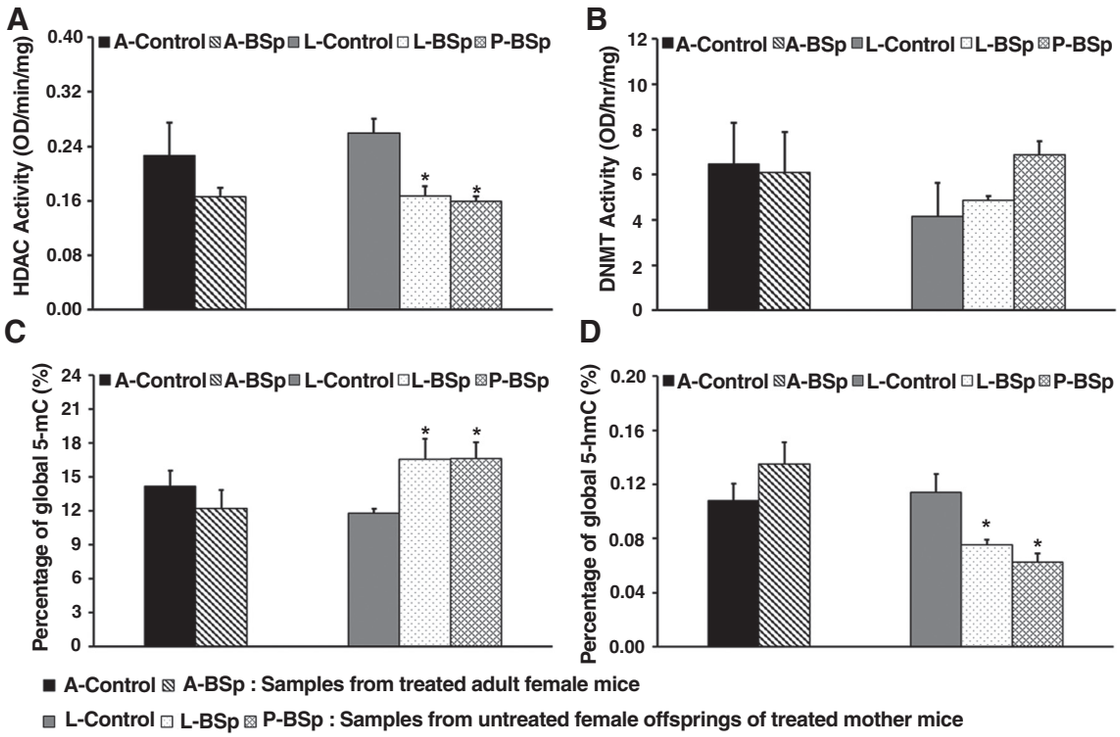

Figure 4.

HDAC and DNMT activity, Global DNA methylation and DNA hydroxymethylation levels in the ER-negative mammary tumors. Global DNA methylation levels were represented by global 5-mC percentage in genomic DNA. Global DNA hydroxymethylation levels were indicated by genomic 5-hmC content in total DNA. **A,** Histone deacetylases activity. **B,** DNA methyltransferases activity. **C,** Global 5-mC percentage in genomic DNA. **D,** Global 5-hmC percentage in genomic DNA. Results were in triplicate from three randomly selected mouse mammary tumors from each treatment group. Columns, mean; bars, SE; *, $P < 0.05$, significantly different from the control group.

offspring. In **Fig. 4**, we demonstrated significant decreases in enzymatic activity of HDACs in both maternal BSp treatments (**Fig. 4A**) indicating that the maternal BSp diet may influence HDACs activity in ER-negative mammary tumors of the offspring. It is noted that DNMTs activity did not show remarkable changes in the P-BSp and L-BSp groups compared with the control group (**Fig. 4B**). We also did not observe significant change of both HDACs and DNMTs in the A-BSp group (**Fig. 4A** and **B**).

Because the DNMTs enzyme activities were not consistent with the gene expression changes of *Dnmt1* and *Dnmt3a*, we further determined global DNA methylation levels represented by global 5-mC percentage in genomic DNA, and DNA hydroxymethylation levels indicated by genomic 5-hmC content in total DNA, in the offspring mammary tumors. Surprisingly, maternal BSp diets induced significant increases of global 5-mC percentage in the genomic DNA derived from the mammary tumor samples of the offspring (**Fig. 4C**). Consistently, we observed decreased global 5-hmC percentage, which usually paired up with global 5-mC for detection, in the genomic DNA of maternal BSp-treated offspring tumors (**Fig. 4D**). The A-BSp treatment had no effect on global DNA methylation and DNA hydroxymethylation levels in the mammary tumors (**Fig. 4C** and **D**).

Differentially expressed tumor-specific genes in maternal BSp-treated mice may be regulated by histone methylation at their promoters

Histone modifications are one of the most important epigenetic mechanisms that are involved in gene regulation. Studies have shown that certain BSp-derived bioactive compounds have strong abilities in influencing histone modification (22, 23). We therefore conducted further investigation to explore whether the BSp diet can elicit any regulation on histone remodeling that may contribute to differential expression of tumor-specific genes. ChIP-qPCR was performed to analyze histone modification status in the promoter regions of tumor suppressor genes (*p16, p53,* and *PTEN*) and tumor-promoting genes (*Bmi1* and *Tert*) by detecting two important chromatin markers: H3K4me3, a transcriptional active marker, and H3K9me3, a chromatin repressor. As shown in **Fig. 5**, we found that maternal BSp diets can significantly decrease enrichment of H3K9me3 in the promoters of *p16, p53,* and *PTEN* (**Figs. 5A**, **6B**, and **C**). The A-BSp group showed no effect on the enrichment of H3K9me3 in these gene promoters (**Figs. 5A**, **6B**, and **C**). In the L-BSp group, especially, H3K9me3 had significantly lower enrichment in the promoter regions of all three tumor suppressor genes compared with the P-BSp group (**Fig. 5A–C**). Significant enrichment changes of

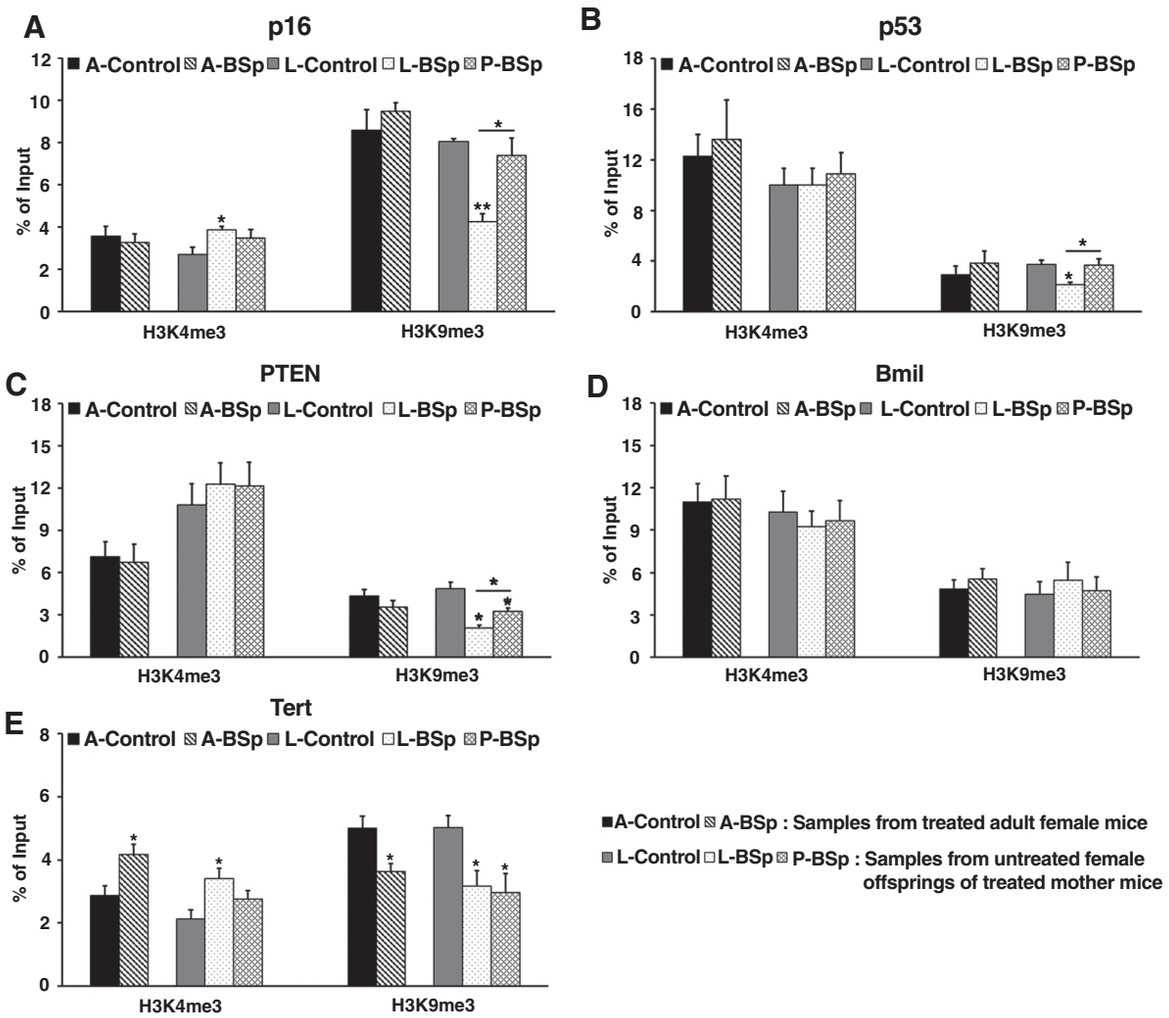

Figure 5.

Histone modification status at promoter regions of tumor suppressor and tumor-promoting genes in the ER-negative mammary tumors. **A,** H3K4me3 and H3K9me3 enrichment at the gene promoter region of *p16*; **B,** H3K4me3 and H3K9me3 enrichment at the gene promoter region of *p53*; **C,** H3K4me3 and H3K9me3 enrichment at the promoter region of *PTEN*; **D,** H3K4me3 and H3K9me3 enrichment at the gene promoter region of *Bmi1*; **E,** H3K4me3 and H3K9me3 enrichment at the gene promoter region of *Tert*. The histogram shows the amount of immunoprecipitated DNA expressed as a percentage of the total input DNA. Results were in triplicate from three randomly selected mouse mammary tumors from each treatment group. Columns, mean; bars, SE; *, $P < 0.05$; **, $P < 0.01$, significantly different from the control group.

H3K4me3 at tumor suppressor gene promoters were not detected except for a prominent increase at the *p16* promoter region in the L-BSp group (**Fig. 5A**).

We found that dietary BSp had no effect on enrichment change of H3K4me3 and H3K9me3 at the *Bmi1* promoter (**Fig. 5D**), indicating that unidentified mechanisms or pathways other than epigenetics may be involved in *Bmi1* gene expression in maternal BSp-treated mice. Interestingly, dietary BSp elevated the binding of H3K4me3, while decreasing the binding of H3K9me3 in the *Tert* promoter (**Fig. 5E**), suggesting its expression may be simultaneously regulated by promoter H3K4me3 and H3K9me3 modifications.

BSp-enriched SFN reduced cellular viability and affected the expression of multiple tumor- and epigenetic-related genes in ERα-negative breast cancer cells

To further test mechanistic insights of dietary BSp on prevention of breast cancer later in life, we evaluated the effects of SFN, one of the most enriched and important functional ingredients in BSp, to validate BSp functions on prevention of breast cancer *in vitro* for this study. MTT assay demonstrated the direct and dose-dependent inhibitory effects of indicated concentration of SFN (0, 5, 10, 15, 20, and 25 μmol/L) on the viability of two ERα-negative breast cancer cell lines, MDA-MB-231 and MDA-MB-157 cells (Supplementary Fig. S3). We chose to use 5 μmol/L concentration of SFN that did not show

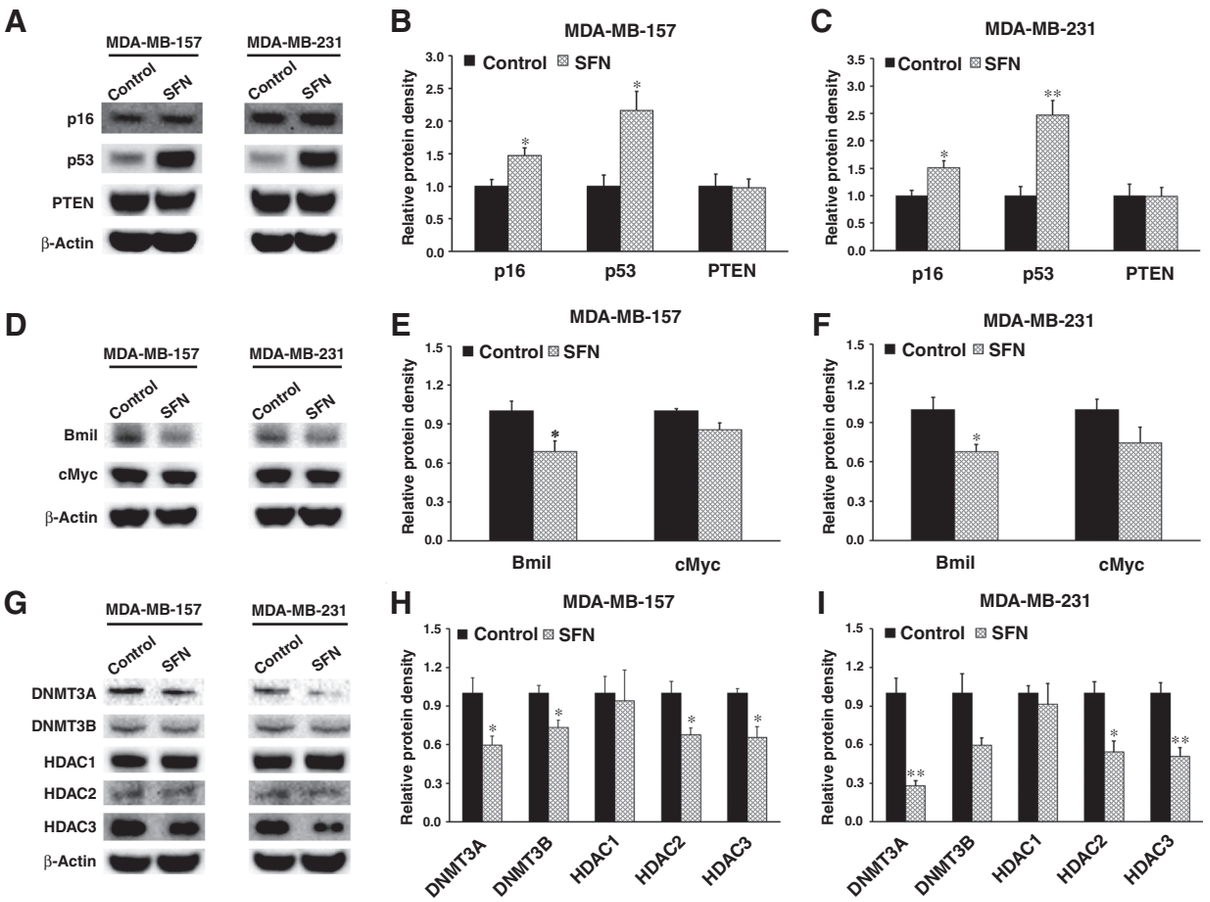

Figure 6.

Expression changes of tumor suppressor, tumor-promoting, and key epigenetics modification-related genes in ERα-negative breast cancer cell lines. Western blot analysis was performed to measure expression changes of *p16, p53, PTEN, Bmi1, cMyc, DNMT3A, DNMT3B, HDAC1, HDAC2,* and *HDAC3* in SFN-treated MDA-MB-157 and MDA-MB-231 cell lines. **A**, **D,** and **G,** Western blot analysis showing protein expression changes of the target proteins in MDA-MB-157 and MDA-MB-231 cells. *β-actin* was used as a housekeeping control gene and its antibody was used to correct loading error. Representative bands were selected from experiments repeated three times. **B**, **E,** and **H,** Quantification of the protein levels in treated MDA-MB-157 cells. **C**, **F,** and **I,** Quantification of the protein levels in treated MDA-MB-231 cells. Data were acquired from three independent experiments and were normalized to β-actin and calibrated to levels in the control group samples. Columns, mean; bars, SE; *, $P < 0.05$; **, $P < 0.01$, significantly different from the control group or between indicated groups.

toxicity to noncancerous MCF10A cells to explore underlying molecular mechanisms by which SFN reduces cellular viability in breast cancer cells (**Fig. 6**).

We first evaluated the protein expression levels of several tumor-related genes including tumor suppressor genes such as *p16, p53,* and *PTEN*, and tumor-promoting genes such as *Bmi1* and *cMyc*. Our results showed that SFN significantly upregulated p16 and p53 protein levels (**Fig. 6A–C**). Simultaneously, the protein level of Bmi1 was found significantly downregulated in SFN-treated breast cancer cells (**Fig. 6D–F**). However, SFN treatment did not affect the expression of PTEN and cMyc in MDA-MB-157 and MDA-MB-231 cell lines.

To determine whether epigenetic modulations were involved in these effects, we further detected expression levels of several epigenetic regulatory genes involved in DNA methylation such as DNMT3A, DNMT3B, and histone modification such as HDAC1-3, respectively. In **Fig. 6G–I**, we found that SFN can significantly downregulate protein expressions of DNMT3A, DNMT3B, HDAC2, and HDAC3 in breast cancer cells. These results were consistent with previous studies indicating inhibitory effects of SFN on processes of DNA methylation and histone acetylation. However, we did not observe significant changes of HDAC1 in both cancer cell lines and any expression change of DNMT3B in the MDA-MB-231 cell line at protein levels. These results indicate that dietary BSp exhibits its preventive effects on breast cancer through SFN-mediated epigenetic regulation of key tumor-related genes.

Discussion

Studies have reported that several mechanisms may contribute to preventive or therapeutic effects of cruciferous vegetable-derived phytochemicals on numerous cancers including regulation of phase II detoxification enzymes (24),

induction of apoptosis (25), and cell-cycle arrest (26), as well as regulation of certain signal pathways, such as a MYC-WWP1 inhibitory pathway targeting PTEN (1), induction of Nrf2-ARE–mediated transcription (27), and suppression of TLR4-NFkB–mediated signaling pathway involved in the inflammatory response (28). However, the investigations of different efficacies of these botanicals consumed at different ages of life remain lacking. To explore an applicable exposure window for dietary BSp supplementation on the prevention of ER-negative mammary tumor, we first employed two maternal BSp administrations: pregestational (exposure from maternal weaning to their sexual maturity) and long-term (beginning after maternal weaning until the weaning of their offspring) maternal treatments, which represent common human eating habits. The result showed that both maternal BSp treatments led to suppressed tumor growth, decreased tumor incidence, and delayed tumor latency. An adult BSp diet group was also introduced as a parallel comparison, which exhibited protective effects likewise, but was not as profound as the maternal BSp preventive effects. These results suggest that maternal/prenatal stages may be key windows of bioactive dietary impacts for potential chemoprevention effects on ER-negative mammary tumors. The preimplantation embryo and germ cells during development are most susceptible to certain environmental factors, including diets and nutrition, because the epigenome in those cells is undergoing dramatic elimination and reconstruction (2). Strikingly, in the present study, the long-term maternal treatment showed a longer tumor latency compared with the pregestational group, revealing a likelihood that the perinatal stage may be particularly critical for breast tumor initiation and progression later in life. On the other hand, the impact of the maternal treatment duration could be an important factor that influences mammary tumorigenesis because the long-term maternal BSp group with a longer treatment time (lasting 13 weeks) shows stronger preventive effect compared with the pregestational BSp treatment (lasting 7 weeks). Our recent study has also found that postnatal early-life BSp treatment with longer treatment time displayed more preventive effects on mammary tumorigenesis compared with the postnatal adult BSp group (13).

As loss-of-function of tumor suppressor genes and/or gain-of-function of oncogenes resulting from aberrant genetic and epigenetic regulations are regnant driving forces underlying tumorigenesis (29), we then tested important tumor-related gene expression, including *p16, p53, PTEN, Bmi1,* and *Tert*, in tumor samples derived from all maternal dietary BSp-treated mice. Briefly, *p16*, an inhibitor of cyclin-dependent kinases, can decelerate the cell cycle by prohibiting progression from phases G to S, therefore it acts as a tumor suppressor (30). The *p53* gene is frequently mutated (>50%) in cancers, indicating its crucial role in tumorigenesis suppression, and the increased expression of *p53* may seem a solution for tumor therapy or prevention (31). *PTEN* is another tumor suppressor gene frequently mutated, deleted, or silenced in multiple carcinomas, and it is haploinsufficient in its tumor suppressive function (32). *Bmi1* has been reported as an oncogene, and its overexpression seems to play key roles in several types of cancer, including breast cancer (33). *TERT* is a catalytic subunit of telomerase, which is essential for telomere maintenance. As activation of *TERT* is associated with malignancy and normal somatic cells do not express *TERT*, it is considered as a potential therapeutic target against cancer (34). In the present study, we found that the gene expression, including mRNA and protein levels, of all three tumor suppressor genes, *p16, p53,* and *PTEN*, as well as two tumor-promoting genes, *Bmi1* and *Tert*, positively correlated with the effects of maternal and adult BSp administration on ER-negative mammary cancer. These findings suggest that prenatal BSp consumption can dramatically suppress mammary tumorigenesis through upregulating key tumor suppressor genes, while downregulating the expression of certain tumor-promoting genes.

Because increasing investigation emphasizes the roles of epigenetic regulation in tumorigenesis as well as cancer prevention and therapy, we speculate that epigenetic-related mechanisms or pathways may also be involved in the expression of these tumor-related genes with respect to their differential expression in response to dietary BSp administration. BSp, a representative cruciferous vegetable, is considered as a key component of the "epigenetics diet" that can reverse epigenetic dysregulation especially through regulation of HDACs, resulting in beneficial health outcomes such as cancer prevention (5, 13). Histone acetylation leads to an open chromatin structure that facilitates the binding of transcription factors to specific gene loci, whereas HDACs participate in transcription repression through removal of an acetyl group from lysine residues. This may lead to condensed chromatin and a loss of transcription of regulatory genes responsible for the tumor suppression processes, such as apoptosis and cell-cycle arrest, leading to tumorigenesis (27). Overexpression of HDACs is common hallmarks of human cancers (35). Thus, suppression of HDACs is considered as an important strategy for cancer prevention and/or therapy. Consistent with the fact that SFN, phenylhexyl isothiocyanate (PHI), 3,3′-diindolylmethane (DIM), phenethyl isothiocyanate (PEITC), and some other abundant bioactive compounds in the BSp diet are potent HDAC inhibitors (22, 23, 36, 37), we also found that the BSp diet significantly reduced the expression of Hdac1 and inhibited enzymatic activity of HDACs.

Strikingly, although both maternal and adult BSp treatments downregulated *Dnmts* gene expression, we did not find detectable changes in total DNMT enzymatic activity in this study. This suggests unidentified mechanisms, such as posttranslational modification, may be implicated in DNA methylation regulation. We therefore assessed global DNA methylation status and DNA hydroxymethylation in genomic DNA, to further explore the cause for the inconsistency between gene expression and enzymatic activity of the DNMTs. Surprisingly, maternal BSp treatment significantly elevated the percentage of genomic 5-mC, which is also consistent with reduced 5-hmC content in genomic DNA derived from maternal BSp-treated

offspring tumors. 5-hmC is one of the DNA oxidation demethylation intermediates of 5-mC (38) and a novel epigenetic marker of most, if not all, types of human cancer (39, 40). Global hypomethylation of genomic DNA is a hallmark of most cancer genomes, promoting genomic instability, structural abnormalities in chromosomes, and cell transformation (41). Increased genomic DNA methylation levels in this study may be another indication of enhanced mammary tumor suppressive effect. In addition, our recent study also revealed an elevated genomic 5-mC content in total DNA derived from breast cancer cell lines (MCF-7 and MDA-MB-231) once treated with SFN or combined with withaferin A, another epigenetic-regulating dietary compound (42).

To further understand the potential epigenetic mechanisms involving differential expression of tumor-related genes, we also determined the status of two important histone modification markers, H3K4me3 (active) and H3K9me3 (inactive), in the promoter regions of these genes. Covalent modifications on the tail domains of histone proteins are important gene regulations. H3K4me3, one of the least abundant histone modifications, is highly enriched at active promoters and positively correlated with transcription (43). The enrichment of H3K9me3, another common histone modification in chromatin, to the opposite, can decrease chromatin accessibility leading to transcription inhibition (44). Intriguingly, the expression changes of all three tumor suppressor genes were found only negatively correlated with the enrichment of H3K9me3 in their promoters; however, no significant change, except *p16* in long-term BSp group, was found for H3K4me3 enrichment. This suggests that inactive histone modifications may play more important roles in the regulation of these tumor suppressor genes than active markers with respect to maternal BSp-induced early chemoprevention effects. As to *Bmi1*, we did not find a relationship between its expression and two chromatin markers in its promoter, indicating other pathways or mechanisms other than epigenetics may be involved in this process.

It is known that hypermethylation in the *TERT* promoter paradoxically is positively correlated with its transcription, which is opposite to the regulation of most other genes (45). Interestingly, in this study, we also found that the reduced expression of Tert was associated with increased enrichment of H3K4me3 along with decreased enrichment of H3K9me3 in its promoter, demonstrating an exceptional relationship of its expression with histone markers in the gene promoter region. A study conducted by Zhang and colleagues showed that the depletion of SIRT1 in hepatocellular carcinoma cells led to substantial reduction in *TERT* expression via decreasing H3K9me3 in the *TERT* gene promoter (46). Another study also revealed that dietary botanical-induced *TERT* inhibition in breast cancer cell lines is associated with elevated enrichment of active promoter chromatin markers (acetyl-H3, acetyl-H3K9, and acetyH4) as well as a decreasing of H3K9me3 in *TERT* regulatory regions (45).

SFN, a naturally occurring isothiocyanate in BSp, is found to be a highly promising chemoprevention agent against a variety of cancers. Most of the SFNs in BSp are synthesized and stored as relatively stable precursors, known as glucoraphanin. Once the BSp are ingested and fully transited to the gastrointestinal system, glucoraphanin is hydrolyzed by endogenous myrosinase released from chewed or otherwise damaged plant cells and become bioavailable as SFN (47). A small amount of myrosinase presented in the microbial flora in the lower intestine is also involved in partial digestion of glucoraphanin. As SFN is not only a potent inducer of phase II enzymes, but also a well-documented HDAC inhibitor, we chose SFN to validate the effects of BSp on mammary tumor inhibition *in vitro*. We found that SFN alone can increase protein expression of tumor suppressor genes, p16 and p53, and decrease the protein levels of tumor-promoting genes such as Bmi1 in ERα-negative breast cancer cell lines. Meanwhile, SFN was found to decrease expression of DNMTs and HDACs indicating BSp may inhibit breast cancer cell growth through regulation of epigenetic mechanisms. Taken together, SFN could be a critical ingredient in BSp that contributes to BSP-induced suppression of mammary tumorigenesis partially through reversal of aberrant epigenetic markers. Notably, the key gene expression patterns were similar in maternal long-term BSp-treated offspring tumors and direct SFN-treated breast cancer cells suggesting the protective effects are persistent from the mothers to the offspring through, at least in part, SFN-mediated epigenetic regulations on key regulatory genes. It is presumable that maternal BSp influences the fetal epigenetic reprogramming through SFN during early development, which may in turn lead to a beneficial outcome later in life, for example, reduced risk of breast cancer.

Importantly, the concentrations of BSp (26%) used in this study are equivalent to a daily intake of about 234 g BSp for an adult human (60 kg) and about 119 g BSp for a child (20 kg), which is considered pharmacologically achievable and has translational potential (10, 21). In addition, the ER-negative mammary tumor mouse model used in the present study resembles human pathogenesis (20), thus allowing assessment of the initiation and progression of spontaneous breast cancers. Moreover, we extended our exploration into maternal/prenatal stages regarding appropriate exposure windows of bioactive compounds, primarily considering the potential magnifying effect of the perturbation during critical periods of ontogenesis and epigenomic reprogramming, as well as the transplacental effect of epigenetic-regulating diets on breast cancer prevention. Collectively, our study proposes new translational strategies for breast cancer prevention in humans.

It should be pointed out that investigations of maternal dietary supplementation targeting chronic diseases, including cancer, in the following generation are still in the exploratory stage because epigenetic implication in fetal adaptation to nutritional intervention is still in need of a comprehensive understanding (2). The general epigenome, especially DNA methylation and histone modification patterns in mammals, is

established during gametogenesis and early embryogenesis, offering a key window with developmental plasticity. The epigenetic dysregulation triggered by anomalotrophy during these sensitive periods, including early postnatal life, of individual development can persist across the life course leading to altered disease susceptibility later in life. However, Shorey and colleagues found that both SFN-rich broccoli sprout powders and purified SFN can enhance a carcinogen, dibenzo[*def, p*]chrysene, induced morbidity and tumorigenesis of the offspring when they were incorporated into the maternal diet, from gestation day 9 to postnatal day 21, at 10% wt/wt and 400 ppm, respectively (48). Nevertheless, I3C, which is contained with small quantities in BSp, can act as both an inhibitor or a promoter of tumor formation in animals, depending upon different tumor types and the timing of administration (49). These studies provide further evidence that exposure windows are crucial to maternal nutritional intervention against chronic diseases. Thus, in-depth understanding of the relationships between dynamic changes and sensibility of the epigenome in early life, dietary properties, and disease susceptibly may lead to considerable progress in prenatal dietary strategies for cancer prevention.

In summary, maternal/prenatal BSp administration can profoundly suppress ER-negative mammary tumorigenesis in the offspring via modulating histone acetylation and DNA methylation status, as well as the expression of tumor-related genes, which were shown to be regulated by histone methylation in their promoter regions. Importantly, our results also revealed that the perinatal period may be particularly critical for mammary tumor initiation and progression later in life and could be a potential target stage for nutritional intervention. This study may provide implications into new strategies with respect to appropriate administration windows of key diets for coping with early breast tumorigenesis in humans and additional future studies may now be directed toward specific compounds of these temporally based diets as well.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Li, Y. Li, T.O. Tollefsbol
Development of methodology: T.O. Tollefsbol
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Li, M. Chen, H. Wu, Y. Li, T.O. Tollefsbol
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Li, M. Chen, H. Wu, Y. Li
Writing, review, and/or revision of the manuscript: S. Li, M. Chen, H. Wu, Y. Li, T.O. Tollefsbol
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Li, M. Chen, Y. Li, T.O. Tollefsbol
Study supervision: T.O. Tollefsbol

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