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Original Article

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Maternal soybean genistein on prevention of later-life breast cancer through inherited epigenetic regulations

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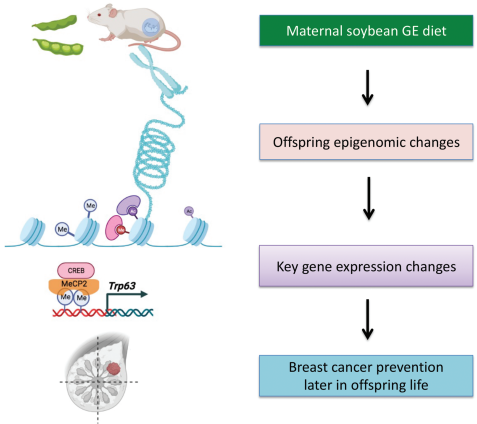
Abstract

Breast cancer has strong developmental origins and maternal nutrition composition may influence later-life breast cancer risk in the offspring. Our study focused on a bioactive dietary component, genistein (GE) enriched in soybean products, to investigate specific timing of maternal GE exposure that may influence preventive efficacy of GE on offspring breast cancer later in life, and to explore the potential epigenetic mechanisms. Our results indicate a time-dependent effect of maternal GE exposure on early-life breast cancer development in offspring mice. Through integrated transcriptome and methylome analyses, we identified several candidate genes showing significantly differential gene expression and DNA methylation changes. We further found maternal long-term GE treatment can induce inherited epigenetic landmark changes in a candidate tumor suppressor gene, *Trp63*, resulting in transcriptional activation of *Trp63* and induction of the downstream target genes. Our results suggest that maternal long-term exposure to soybean GE may influence early-life epigenetic reprogramming processes, which may contribute to its temporal preventive effects on breast cancer in the offspring. This study provides important mechanistic insights into an appropriate maternal administration of soybean products on prevention of breast cancer later in offspring life.

Graphical Abstract



Abbreviations: DEG, Differentially expressed gene; DML, Differentially methylated loci; DMR, Differentially methylated region; GE, Genistein; RRBS, Reduced representation bisulfite sequencing.

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Introduction

Evidence has shown that many chronic human diseases and disorders have developmental origins (1). Early-life events including maternal diets or nutrition are believed to have profound impacts on offspring health and disease outcomes, and epigenetic mechanisms may serve as a link between maternal exposure and disease formation in adulthood (2–7). Epigenetics defines a variety of biological processes that influence gene expression leading to heritable changes in gene activity or phenotypes without changing DNA sequence. Individualized epigenome initiates during early development, through which unique epigenetic landmarks are established via epigenetic reprogramming processes (8). DNA methylation is the most important epigenetic reprogramming event during early development in mammals, which involves the addition of a methyl group to the 5-position of the cytosine ring in CpG dinucleotides often in the regulatory region of a gene such as the promotor. These unique epigenetic signatures will be maintained throughout the lifespan and can even be transmitted to subsequent generations through germline-mediated epigenetic inheritance, providing faithful gene transcription regulation machinery throughout generations (9).

Unlike the genome, the epigenome is particularly susceptible to dysregulation and sensitive to environmental stimuli during early development. Maternal nutrition status is considered one of the most important environmental factors during early development that can significantly influence the fetal epigenome partially due to the fact that maternal diets are the exclusive nutritional supply during prenatal, gestational, and neonatal periods for the offspring. Nevertheless, the vulnerability to maternal diets during the critical developmental stage provides an excellent opportunity to reverse dysregulated epigenetic profiles that may lead to a beneficial health outcome later in life.

Breast cancer is the most common type of cancer in women in the United States. It has become increasingly apparent that the origin of breast cancer development can retrospect to early maternal and fetal lifestyles (10). Thus, maternal exposure to certain diets with epigenetic modulating properties could affect epigenetic reprogramming processes leading to permanent changes in offspring epigenetic fingerprints that alter the susceptibility to breast cancer later in life. Among the candidate diets with potential epigenetic regulatory properties, soybean diets and their derived bioactive isoflavones such as genistein (GE) attract extensive interest. Epidemiological studies indicate a strong correlation of soybean intake with a lower rate of breast cancer incidence and recurrence in Asian women (11,12). Various studies have demonstrated that soybean GE is considered as a safe and potent chemopreventive agent against breast cancer *in vitro* and *in* vivo (13). In addition, GE is considered as an important dietary epigenetic modulator that affects key gene expression through epigenetic mechanisms (14–18). However, the preventive effects of soybean GE on later-life breast cancer seem largely dependent on the timing of exposure during the lifetime. For example, epidemiological and animal studies have shown that early-life intake of soybean products exhibits a better preventive effect against breast cancer than adulthood consumption (19–21).

In the current studies, we seek direct evidence that development of breast cancer may originate from a fetal environment and the critical timing of maternal nutrition exposure may influence the disease outcome through epigenetic mechanisms. Our results showed that the preventive outcome for maternal GE was explicitly dependent on exposure timing, and temporal epigenetic changes controlled important gene expression that are central to the efficacy of maternal GE on breast cancer prevention later in life. This study provides important insights into an appropriate temporal administration of soybean-based botanical diets or bioactive compounds that can maximize their beneficial effects leading to improved human health.

Materials and methods

Animal models

Two transgenic mouse models, C3(1)-SV40 Tag (FVB-Tg(C3-1-TAg)cJeg/JegJ) (SV40) and FVB/N-Tg(MMTVneu)202Mu (Her2/neu), were purchased from the Jackson Laboratory and colonized in our laboratory. The female mice of these models can spontaneously develop estrogen receptor (ER)-negative mammary tumors and the median tumor latency is around 16 wks for SV40 and 30 wks for Her2/neu mice, respectively (22). Mice were housed in the Animal Resource Facility at the University of Alabama at Birmingham (UAB) and were maintained in conventional housing conditions (12-h dark/12-h light cycle, 24 ± 2°C temperatures, and 50 ± 10% humidity).

Dietary treatments

Female mice (7–10 mice/group) were administered either control (phytoestrogen-free modified AIN-93G diet; TestDiet, St. Louis, MO) or GE diet (modified AIN-93G diet supplemented with 250 mg genistein per kg diet; TestDiet) *ad libitum*. Dietary information was provided in Supplementary Data 1, available at *Carcinogenesis* online. Dams were mated at 12 weeks of age and assumed to be pregnant when a vaginal plug was expelled. Male mice were separated after conception. Pups were weaned at postnatal days 28 (PD28). Due to different litters in different groups, offspring animal numbers in each treatment groups varied around 25 female offspring/group. Two maternal exposure windows as well as a postnatal treatment plan were designed and delineated in Figure 1A.

1). Control: The control group was administered control diet in both mothers and offspring.
2). Maternal short-term GE treatment (ST-GE): Maternal GE dietary administration began from the first day of conception until weaning. The weaned pups were maintained on control diet throughout their lifespan.
3). Maternal long-term GE treatment (LT-GE): Maternal GE dietary administration began in early life from 4 weeks of age in mothers until PD28. The weaned offspring mice were maintained on control diet throughout their lifespan.
4). Postnatal GE treatment (Post-GE): Mice were fed GE diet postnatally from weaning.

Tumor evaluation and collection

We used tumor latency as a primary outcome and tumor weight as the secondary outcome. Tumor incidence was measured weekly. The experiment was terminated when the control mice reached 100% tumor rate or the mean tumor diameter in individual animal exceeded 1.0 cm. At the endpoint, the mammary tumors were collected, weighed, and used for

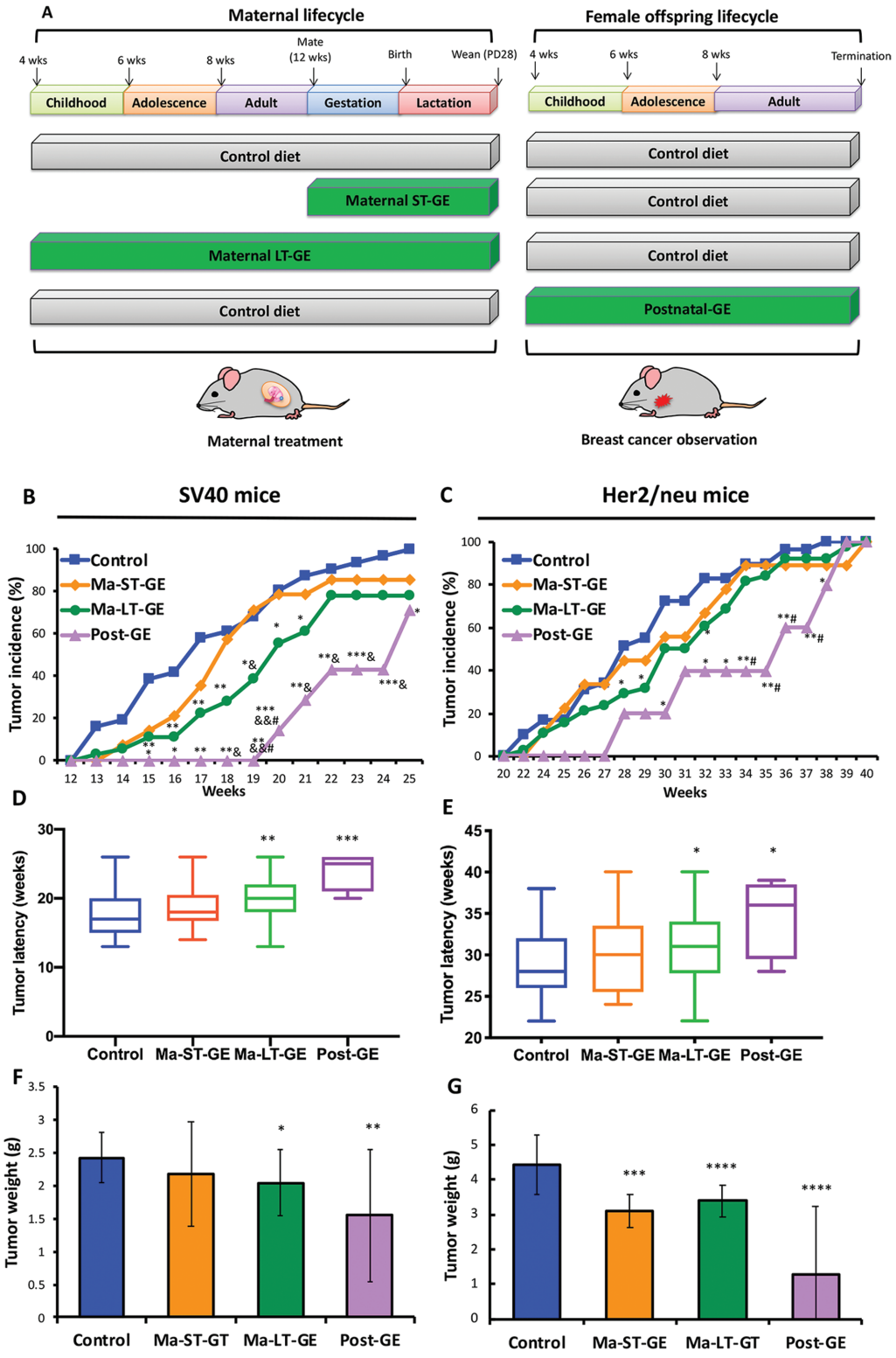

Figure 1. Breast tumor growth in female offspring under different exposure timings of GE administration. (A) Schematic representation of animal study for maternal GE intervention. The upper bar represents mouse life stages in the mother and female offspring. Female SV40 and Her2/neu transgenic mice were administered GE diet (250 mg/kg) under different exposure windows: 1) Control: mice were fed *ad libitum* with the control diet; 2) Maternal short-term GE (Ma-ST-GE): mice were administered GE diet from the first day of conception at 12 wks until weaning; 3) Maternal long-term GE (Ma-LT-GE): mice were administered GE diet from early life childhood (4 wks) until weaning; and 4) Postnatal GE (Post-GE): mice were fed GE diet postnatally from 4 wks of age until termination of the experiment. Offspring mice were weaned at 4 wks of age (PD28) and maintained on the control or GE diet throughout their lifespan until termination of the experiment. Tumor growth parameters were monitored and recorded weekly. (B-G) Breast tumor growth in female SV40 (left panel) or Her2/neu (right panel) offspring mice. B and C, tumor incidence; D and E, median tumor latency; F and G, tumor weight. Columns, mean; Bars, SD; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, significantly different from the control group; &, significantly different from the Ma-ST-GE group; # significantly different from Ma-LT-GE group.

further analyses. All experiments and procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at UAB (Animal Project Numbers: 20653, 20671).

RNA sequencing (RNA-Seq) analysis

Total RNAs were extracted from mammary tumors of mice offspring in each treatment group using standard protocol. RNA-Seq analysis was performed in RNA samples from control and maternal LT-GE treatment groups (3–4 mice/group). RNA quantity control, library preparation, RNA-Seq reaction preparation, sequence alignment, and annotation were used standard pipelines as described previously (6). We utilized the DESeq platform to evaluate differentially expressed gene (DEGs) by estimation of fold change and dispersion of sequence data (23). The false discovery rate (FDR) < 0.05 served as the cutoff for differential expression between the two groups. The ratio values or fold changes for each gene were calculated by the difference of the average $\log_2$ expression value between different groups (positive value refers to upregulation and vice versa). For downstream analysis, we used an online tool, DAVID (Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov/) to identify functional biological modules and pathways from DEG lists. Gene expression heatmaps and volcano plots were generated using the Cluster and Java Tree View applications.

Reduced representation bisulfite sequencing (RRBS) analysis

Genomic DNAs were extracted from the mammary tumors of mice offspring using DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD). RRBS analysis was performed with DNA samples from same set of tumor samples for RNA-Seq analysis to minimize any biological variation. A total of 1 μg of genomic DNA was treated by *Msp*I restriction enzyme digestion and undergone bisulfite conversion. Bisulfite-converted DNA libraries were produced and amplified, and single-end reads were sequenced on an Illumina HiSeq-2500 following library quality control. RRBS data analysis was performed using a standard pipeline (24) including the assessment of the read qualities through FastQC, trimming process via TrimGalore and alignment the bisulfite reads to UCSC Genome Browser mouse GRCm38/mm10 reference sequence using Bismark (25). Post alignment and Bismark supplementary methylation extractor script were used to extract the methylation call for every single methylation locus analysis. Bismark coverage output dataset for each sample was utilized for downstream statistical analysis. Reads were filtered based on coverage, with a cutoff of at least three reads per site, and normalized for coverage before analysis.

To detect differential methylation status between the control and maternal LT-GE treatment groups, DSS (Dispersion Shrinkage for Sequencing Data) statistical test (26) was conducted at each CpG site to determine differentially methylated loci (DML) followed by differentially methylated regions (DMRs). We performed smoothing to quantify DMRs on a set of specified thresholds between the groups of samples including minimum number CpG sites (3 CpGs) and minimum length (30 bps) required for DMR determination. The threshold for DMR calling was set to < 0.1. Differential methylation was defined as a sliding linear model correct with *P*-value < 0.05.

Integrated analysis of RNA-Seq and RRBS assays

To further elucidate the potential correlation between gene transcription and DNA methylation, we integrated transcriptomic and methylomic data by merging the significant DEGs and DML-correlated genes using ggplot package in R (version 3.6.1). In order to visualize the relationship between DEGs and DML, scatter plot with $\log_2$ (fold change) and methylation difference was generated. For integrated genes overlapping with DMRs, the significance of the correlation between DMR and gene transcription was calculated by the Spearman correlation test and the cutoff for significance was set to 0.05.

Validation of DNA methylation status by Targeted NextGen Bisulfite Sequencing (tNGBS)

Six identified candidate genes (*Trp63*, *Cyclin D1*, *Cyclin A1*, *Myc*, *Rarb*, *Keratin 18*) were screened for methylation percentage in various regulatory regions by a commercially available company (EpigenDX, Hopkinton, MA). The list of gene structure, the assay design, and tNGBS procedure were provided in Supplementary Data 2, available at *Carcinogenesis* online.

Validation of gene expression by quantitative real-time RT-PCR and western blot analysis

The identifed target genes included *transformation related protein 63* (*Trp63*), *Myc*, *Cyclin D1*, *Cyclin A1*, *Twist1*, *Keratin 18*, *inhibitor of DNA binding 1* (*Id1*), *retinoic acid receptor beta* (*Rarb*), *progesterone receptor* (*Pgr*), *snail family zinc finger 2* (*Snai2*), *secreted frizzled-related protein* (*Sfrp1*) and *stratifin* (*Sfn*) were validated gene expression by real-time RT-PCR and/or western blot analysis followed by standard protocols. Detailed procedures were provided in Supplementary Data 3, available at *Carcinogenesis* online.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with the SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's protocol. Detailed ChIP procedure and specific primer sequences were provided in Supplementary Data 3, available at *Carcinogenesis* online.

Cell culture and treatment

Normal human mammary epithelial cells (HMECs) were obtained from Lonza (Basel, Switzerland). Precancer cells were established from normal HMECs that were stably transfected with *SV40* and human telomerase reverse transcriptase, *hTERT* (27). Human triple-negative breast cancer (TNBC) cell lines, MDA-MB-157, and MDA-MB-231, were purchased from ATCC (Manassas, VA). Detailed procedures for cell culture, treatment, MTT assay, and apoptosis analysis were provided in Supplementary Data 3, available at *Carcinogenesis* online.

Statistical analysis

Other than RNA-Seq and RRBS analyses that have been handled by biostatisticians, statistical significance between the values of control and treatment groups was evaluated by one-way ANOVA followed by Tukey's test for multiple comparisons via GraphPad Prism 8.00 version. Statistical significance between the numbers of subjects was evaluated by Chi-square

and Fisher's exact test. Values were presented as mean ± SD (standard deviation) and $p < 0.05$ was considered statistically significant.

Results

Timing of maternal GE exposure influenced the preventive outcome on later-life breast tumor development

To determine the effects of maternal soybean GE on prevention of later-life breast cancer, we used SV40 and Her2/neu transgenic mouse models that can spontaneously develop mammary tumors driven by overexpressed oncogenes (22). We administered maternal dietary regimen by using formulated GE chow diet at the concentration of 250 mg GE/kg diet. This concentration is equivalent to a human being with 60 kg body weight consuming 120 mg of soybean isoflavone/per day (28), which can be achieved by taking 3 cups of boiled soybean indicating this concentration is physiologically relevant for future human study. We also tested the potential influences of this maternal GE dietary regimen on general health indexes and mammary gland development in the offspring (Supplementary Data 3, available at *Carcinogenesis* online). Our results showed that there were no negative effects on the above-mentioned factors suggesting the maternal GE is safe to use during pregnancy and shows no harm to mothers and their offspring.

To explore the optimal exposure timing of soybean GE, two maternal dietary regimens, LT-GE and ST-GE, as well as a postnatal GE treatment were employed (Figure 1A). Our results showed that maternal early-life exposure to dietary GE (LT-GE) led to a prominent inhibition of breast cancer incidence in mice offspring of SV40 (left panel) and Her2/neu (right panel) transgenic mouse models, whereas maternal short-term exposure to GE (ST-GE) during pregnancy showed less protective effects compared to the control (Figure 1B,C). Furthermore, maternal LT-GE but not ST-GE significantly extended tumor latency in both tested models (Figure 1D,E), suggesting the exposure window for dietary GE during the mother's lifetime is a key factor that determines the outcome of breast cancer development in the offspring. In addition, maternal LT-GE treatment significantly reduced tumor weight in both mouse models although this effect was more obvious in Her2/neu model (Figure 1F,G). Postnatal-GE treatment from early-life that resembled treatment strategy in mothers exhibited the most prominent preventive and inhibitory effects leading to decreased tumor incidence, delayed tumor latency, and reduced tumor weight. Interestingly, maternal LT-GE resulted in similar chemoprevention effects in the offspring as seen in the mice that were subjected to direct GE exposure (postnatal-GE), suggesting these protective effects from maternal diet can transmit and persist in the next generation. Because the impacts of maternal LT-GE were more promising in SV40 than Her2/neu mice, we therefore conducted the subsequent genome-wide analyses in SV40 mice using maternal LT-GE as an optimal maternal GE dietary regimen.

Maternal LT-GE led to genome-wide alterations in offspring transcriptome

To further determine the underpinning mechanisms, we next sought to test transcriptomic changes by RNA-Seq analysis to identify target genes in regulation of the maternal LT-GE mediated early breast cancer prevention. The hierarchical cluster showed DEGs in the mammary tumors of SV40 offspring between the control and maternal LT-GE treatment groups (Figure 2A). Among a total of 13637 top hit genes, we found 1333 genes significantly differentially expressed. Further comparison identified 391 genes with a $\log_2$ fold change above 2 followed by a stringent statistical analysis (FDR < 0.01) as illustrated as green dots in the ovals of the volcano plot in Figure 2B. Gene function analysis via DAVID revealed multiple signaling pathways have been regulated by the maternal LT-GE treatment (Figure 2C). These DEGs were ubiquitously involved in key molecular pathways during early development and oncogenic transition, which indicates a potential role of maternal GE in regulation of specific regulatory pathways that may contribute to its induced transplacental breast cancer prevention effects.

Maternal LT-GE altered genome-wide DNA methylation profiles in the offspring

Given the importance of DNA methylation during early development, we next used RRBS assay to study the impact of maternal LT-GE diet on genome-wide DNA methylation profile changes that may contribute to the outcomes of breast cancer later in life. Among 58011 sequenced CpG loci, we identified 4327 DML that showed significantly differential methylation profiles between control and maternal LT-GE groups, in which 2297 loci (53.1%) were hypermethylated and 2030 loci (46.9%) were hypomethylated (Figure 2D) in response to maternal LT-GE treatment. These DML were found evenly distributed across the genome and chromosomal regions (Figure 2E). Further smoothing approach identified 139 dynamic DMRs (Figure 2F), among which 92 DMRs (66.2%) lost methylation and 47 DMRs (33.8%) gained methylation after maternal GE treatment. We also compared the location distribution of DML and DMRs throughout the genome. We found that the distribution patterns of DML and DMRs were similar, in which the majority of altered loci and regions were located in intronic, intergenic, and exonic regions (Figure 2G). There were about 4.37% of DML and 8.03% of DMRs located in promoter and upstream of mRNA (5'UTR) regions, where DNA methylation plays major roles in regulation of gene transcriptional activities. Focusing on these dynamic DMRs, we performed gene functional analysis by DAVID and found that most of the DMR-affiliated genes that were heavily enriched or loss of DNA methylation after maternal GE treatment involved in key molecular events such as development, differentiation and transcriptional regulation, et al. (Figure 2H). These results suggest that DNA methylation regulation may play an important role in maternal GE diet-induced chemoprevention effects on breast cancer. The original and processed RNA-Seq and RRBS data can be retrieved through an online data repository, Gene Expression Omnibus (GEO), with assigned GEO accession number GSE194163.

Integrated analyses identified key genes involved in maternal LT-GE induced chemoprevention effects on offspring breast cancer

DNA methylation plays a vital role in regulating gene transcription. We performed integrative analyses to explore potential correlations between maternal LT-GE-induced DNA methylation and transcriptional response. Through bioinformatic

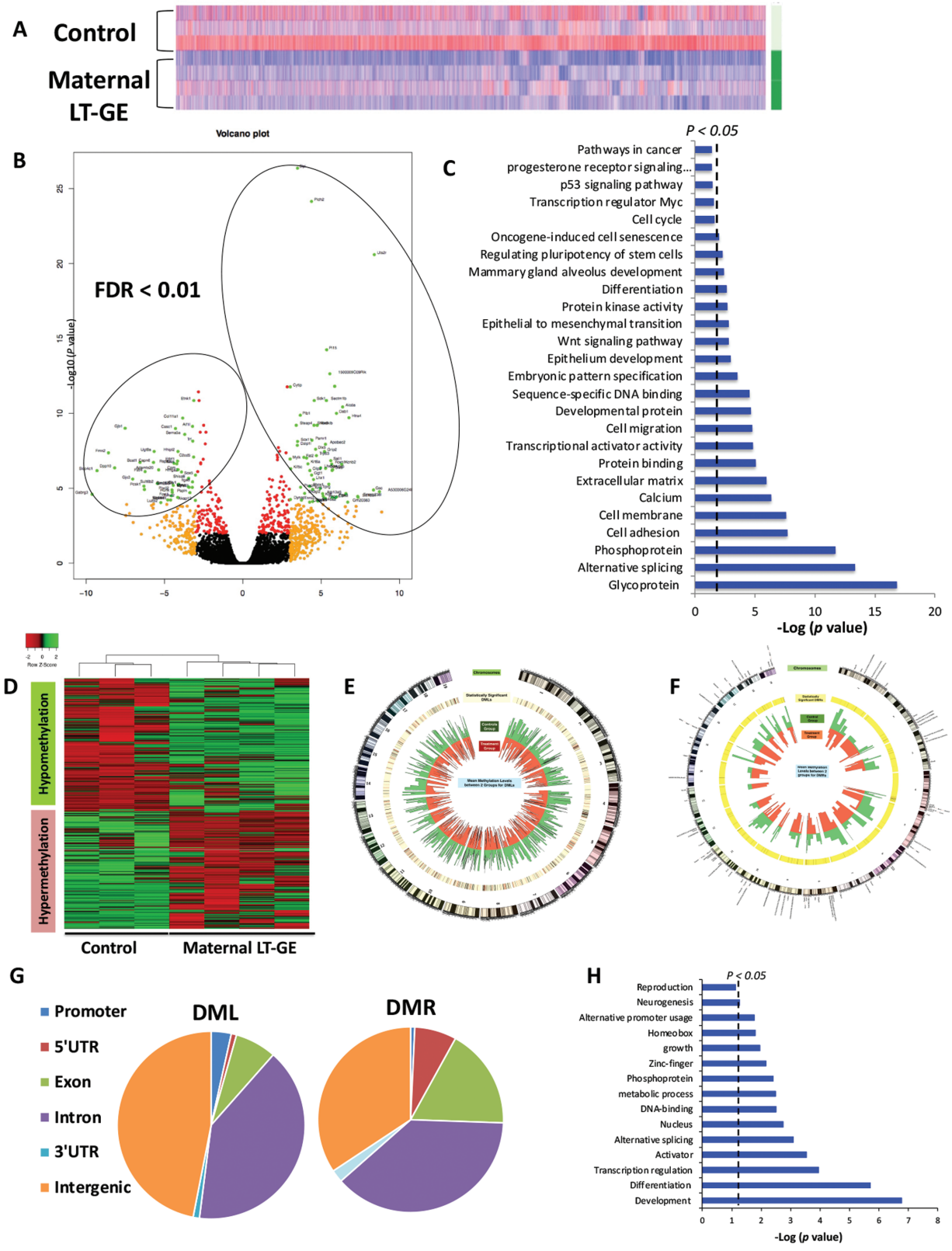

Figure 2. Maternal LT-GE impacted transcriptomic and methylomic profiles in offspring breast tumors. (A-C) Transcriptomic profiling by RNA-Seq analysis in SV40 offspring breast tumors with maternal LT-GE treatment. (A) Hierarchical cluster analysis demonstrated DEGs. Columns indicate individual mRNA expression values and rows correspond to different treatment groups. (B) Volcano plot showed $\log_2$ fold changes and statistical significance of the annotated DEGs between control and maternal LT-GE treatment. The green spots in oval shapes indicate the most significant DEGs with FDR < 0.01 and $\log_2$ fold change > 2. (C) Gene function and ontology analysis by DAVID. Y axis shows multiple signaling and regulatory pathways that have been significantly regulated by the maternal LT-GE treatment. Dotted line represented a threshold with significance ($P < 0.05$). (D-H) Genome-wide DNA methylation profiling by RRBS analysis in control and maternal LT-GE treated SV40 offspring breast tumors. (D) Heatmap result showed significantly DML with treatment (columns) and differentially methylated CpG sites (rows). Hypermethylated loci are shown in red and hypomethylated loci in green in response to maternal LT-GE. (E and F) Genome-wide distribution of DML (E) and DMRs (F): Bands from the outside to inside represent chromosomes (gray), significant DML or DML (yellow), control (green) and maternal LT-GE (orange). (G) Location distribution of DML and DMRs. (H) DMR-affiliated gene function and ontology analysis by DAVID.

integrative analysis among 1333 DEGs and 4327 DML, we were able to identify 589 annotated genes that have shown significantly differential expression and contained significantly methylated loci simultaneously (Figure 3A). We incorporated these genes with key breast cancer-related reference genes (Human Breast Cancer RT^2 Profiler, Qiagen) as well as essential pathway genes, and eventually identified 12 target genes including *Trp63*, *Cyclin A1*, *Cyclin D1*, *Myc*, *Rarb*, *Id1*, *Keratin 18*, *Pgr*, *Sfn*, *Sfrp1*, *Twist 1* and *Snai2* showing significantly differential expression and/or methylation changes for further validation studies (29-44, Table 1). Among them, a tumor suppressor gene, *Trp63*, exhibited the most significant changes in gene expression and DNA methylation (orange dots in Figure 3A). We believe that regulation of these genes may be sensitive to timing of maternal GE exposure leading to epigenetic-mediated transcriptional changes and altered health outcome transmitted to the next generation.

Validation analyses of specific gene expression and methylation changes

To confirm the transcriptomic results by RNA-Seq, we first validated gene expression of the 12 identified target genes by real-time quantitative RT-PCR. We found that the mRNA expression patterns in most target genes were consistent with the RNA-Seq results except for *Pgr* and *Id1* genes (Figure 3B and Table 1). For example, maternal LT-GE treatment can significantly increase several key tumor suppressor gene expressions such as *Trp63*, *Rarb*, *Sfn*, *Sfrp1*, and *Cyclin A1*, whereas decrease tumor promoting genes such as *Myc*, *Cyclin D1* and *Twist1*. These gene expression changes may contribute to maternal GE-induced long-term beneficial effects on prevention of breast cancer in the offspring.

We believe major epigenetic mechanisms such as DNA methylation may play critical roles in regulation of these gene expressions. We then investigated specific DNA methylation status in the regulatory regions of 6 candidate genes including *Trp63*, *Cyclin D1*, *Cyclin A1*, *Myc*, *Rarb*, and *Keratin 18*, which expression validations have shown positively aligned with RNA-Seq results, and were reported as epigenetic-controlled genes (30,31,33,35,36,38). As shown in Figure 3C and the summary in Table 1, we observed that the average DNA methylation rate in the 5'-upstream region of *Cyclin D1* and *Myc* genes was below 1% regardless of control or treatment, indicating persistent DNA hypomethylation status in these gene promoters. Interestingly, a number of tested CpG sites in both *Cyclin A1* and *Rarb* genes showed significantly decreased DNA methylation levels in the promoter regions in

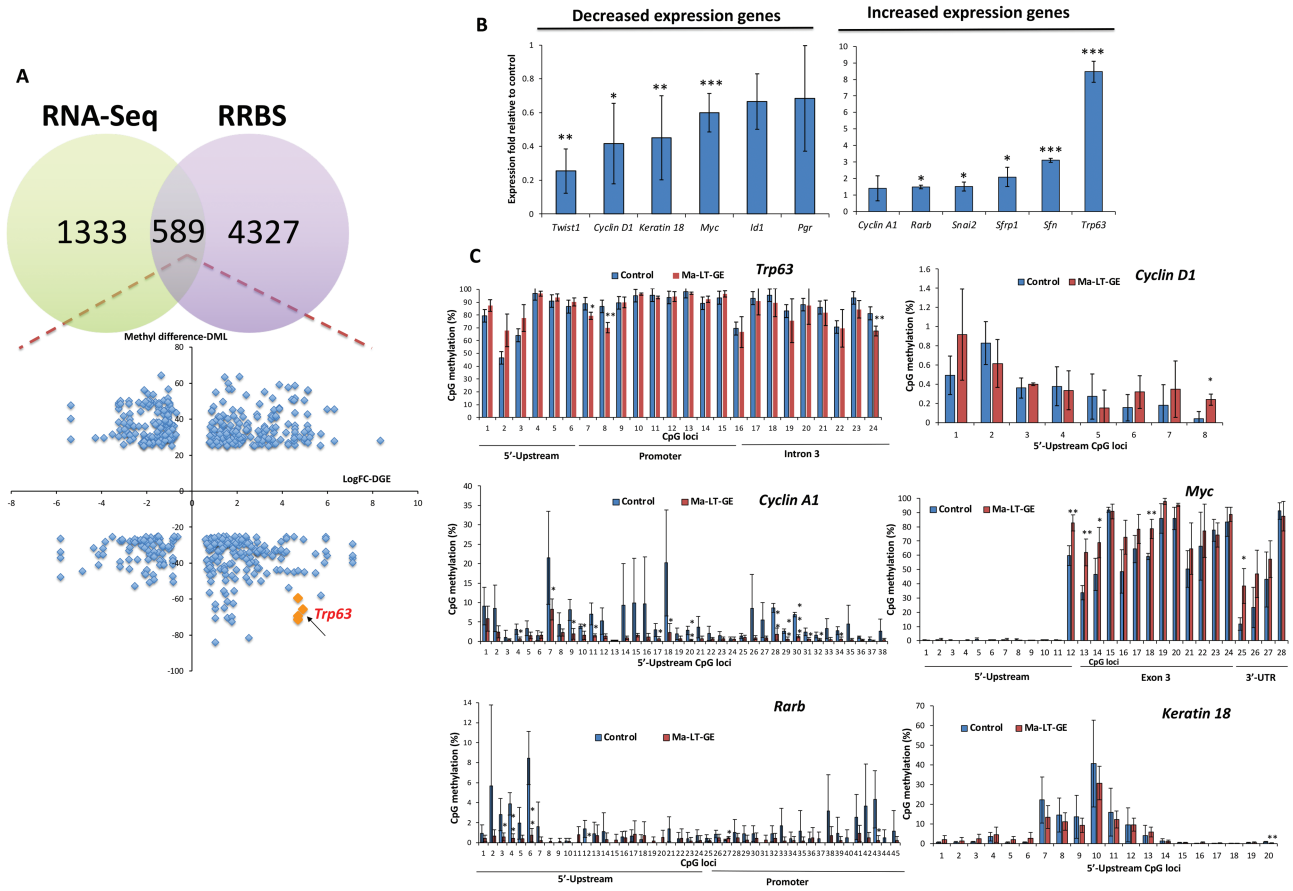

Figure 3. Validation analyses of candidate gene expression and methylation changes. (A) Integrative analysis by combining RNA-Seq and RRBS results. Scatter plots showed 589 genes that have shown significantly differential expression and contained significantly methylated loci simultaneously. X axis shows $\log_2$ fold change of DEGs (positive-upregulation, negative-downregulation). Y axis shows methylation difference (positive-hypermethylation, negative-hypomethylation). Orange dots indicate *Trp63* gene. (B) Quantitative real-time RT-PCR was performed to measure candidate genes. Data were in three biological repeats from three independent experiments and were normalized to *GAPDH* and calibrated to the levels in control samples as 1. (C) Specific DNA methylation in the regulatory gene regions was determined by tNGBS for selected target genes including *Trp63*, *Cyclin D1*, *Cyclin A1*, *Myc*, *Rarb* and *Keratin 18*. Percentage of methylation levels was calculated by dividing the number of methylated reads by the total number of reads. Columns, mean; Bars, SD; *, $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from control.

Table 1. Identified key genes showed significantly differential expression and/or methylation changes in response to maternal LT-GE treatment

Gene	Function in cancer	Epigenetic regulation	RNA-Seq	Real-time RT-PCR	RRBS	tNGBS
Trp63	Tumor suppressor gene,*p53*family member (29)	Modulation of *TAp63*methylation status by JMJD3-demethylase activity (30)	DEG, significant increase (26.3 fold)	Significant increase (8.47 fold)	DML, DMR, hypomethylation	Locus-specific demethylation in the promoter and intron 3
Myc	Oncogene	Key transcriptional factor that frequently participates in epigenetic regulation of target gene expression (31)	DEG, significant decrease (0.43 fold)	Significant decrease (0.6 fold)	No DML/DMR detected	DNA hypomethylation in the promoter (<1% methylation rate; hypermethylation in majority of tested loci in exon 3 and 3′-UTR
Cyclin D1	Tumor promoting gene, regulates cell cycle (32)	Epigenetic modifications such as DNA methylation and histone acetylation involve in *Cyclin D1* transcriptional regulation (33).	DEG, significant decrease (0.22 fold)	Significant decrease (0.42 fold)	No DML/DMR detected	DNA hypomethylation in the promoter (<1% methylation rate)
Cyclin A1	A p53-induced tumor suppressor gene that mediates apoptosis, G2/M cell cycle arrest (34)	DNA methylation play a role in regulation of *Cyclin A1* gene expression (35)	DEG, significant increase (35.3 fold)	Increase (NS)	No DML/DMR detected	Promoter demethylation in majority of tested loci
Rarb	Tumor suppressor gene,a member of the thyroid-steroid hormone receptor superfamily (36)	Aberrant DNA hypermethylation and histone modifications of *Rarb* is associated with cancer progression (36)	DEG, significant increase (2.91 fold)	Significant increase (1.48 fold)	No DML/DMR detected	Promoter demethylation in majority of tested loci
Keratin 18	Tumor prognostic indicator, high expression was associated with poor prognosis (37)	Aberrant *Keratin 18* expression is associated with promoter methylation (38)	DEG, significant decrease (0.44 fold)	Significant decrease (0.45 fold)	DML, hypermethylation	Mixed DNA methylation changes in the promoter
Id1	Oncogene, overexpressed in many types of cancer (39)	Promoter hypermethylation leads to silenced *Id1* gene expression (39)	DEG, significant increase (2.85 fold)	Decrease (NS)	DML, hypermethylation	ND
Pgr	Important ER-regulated gene, driver of early breast cancer progression	Epigenetic mechanisms play a role in *Pgr* transcriptional regulation (40).	DEG, significant increase (7.78 fold)	Decrease (NS)	No DML/DMR detected	ND
Sfn	P53-regulated inhibitor of cell cycle progression, tumor suppressor gene (41)	Frequent epigenetic silenced *Sfn* in several types of cancers (41)	DEG, significant increase (2.85 fold)	Significant increase (3.1-fold)	No DML/DMR detected	ND
Sfrp1	Negative regulator of the Wnt pathway, loss of expression in breast cancer (42)	Epigenetic mechanisms play a role in *Sfrp1* expression regulation (42).	DEG, significant increase (2.39 fold)	Significant increase (2.09 fold)	No DML/DMR detected	ND
Twist 1	Transcriptional factor, tumor promoting gene (43)	Frequently participates in epigenetic regulation of target gene expression (43)	DEG, significant decrease (0.17 fold)	Significant decrease (0.25 fold)	DML, hypomethylation	ND
Snai2	Overexpression is correlated with poor clinical outcome	Epigenetic mechanisms play a role in *Snai2* expression regulation (44).	DEG, significant increase (4.2 fold)	Significant increase (1.51 fold)	No DML/DMR detected	ND

DEG, differentially expressed gene; DML, differentially methylated loci; DMR, differentially methylated region; NS, not significant; ND, not detected.

response to maternal LT-GE treatment, which may contribute to increased expression of these genes. There were mixed DNA methylation status changes in the promoter of *Keratin 18* gene, indicating different mechanisms other than epigenetics may participate in control of *Keratin 18* gene expression. Different from other tested genes, *Trp63* gene maintained a relatively high level of DNA methylation in both promoter and intronic regions (>50%). However, we observed that DNA methylation levels in several specific loci of the promoter and intronic regions were significantly decreased in response to maternal LT-GE. We assume that these hypomethylated DNA loci may play a role in regulating transcriptional activation of *Trp63*. We next focused to explore how maternal GE influenced DNA methylation profiles and how DNA methylation alterations led to gene expression changes throughout different generations by using *Trp63* gene, a key tumor suppressor gene, to test our hypothesis in the following studies.

p63-regulated signaling pathways may contribute to maternal LT-GE diet-induced breast cancer prevention in mice offspring

To determine whether *Trp63* gene expression may change correspondingly in response to different timing of maternal exposure strategies, we evaluated *Trp63* gene expression and found the increased expression patterns of *Trp63* gene in mammary tumors were consistent with preventive effects of these treatment strategies against breast cancer (Figure 4A). These results indicate *Trp63* gene expression plays an important role in controlling soybean GE exposure timing-dependent breast cancer prevention efficacy. However, these changes were not observed in normal mammary glands suggesting a unique regulatory machinery may be involved in *Trp63* transcriptional regulation during breast tumorigenesis.

As *Trp53* gene suppresses tumor formation through induction of cell cycle arrest and apoptosis, we next deciphered whether *Trp63* gene as a homologous gene of *Trp53* may demonstrate similar functions. As illustrated in Figure 4B,C, our results showed that upregulation of p63 was accompanied by significant decreases of Cyclin D1 and inactivated Rb (phosphorylated Rb, pRb) as well as upregulation of p27 as a negative cell cycle regulator that may contribute to halting cell cycle progression in offspring tumors. In addition, maternal LT-GE can also trigger apoptosis-related signaling pathways by significantly decreasing the apoptosis inhibitor, Bcl-2, but increasing pro-apoptotic protein, Bak, leading to apoptotic cascade (increased cleaved caspases 3).

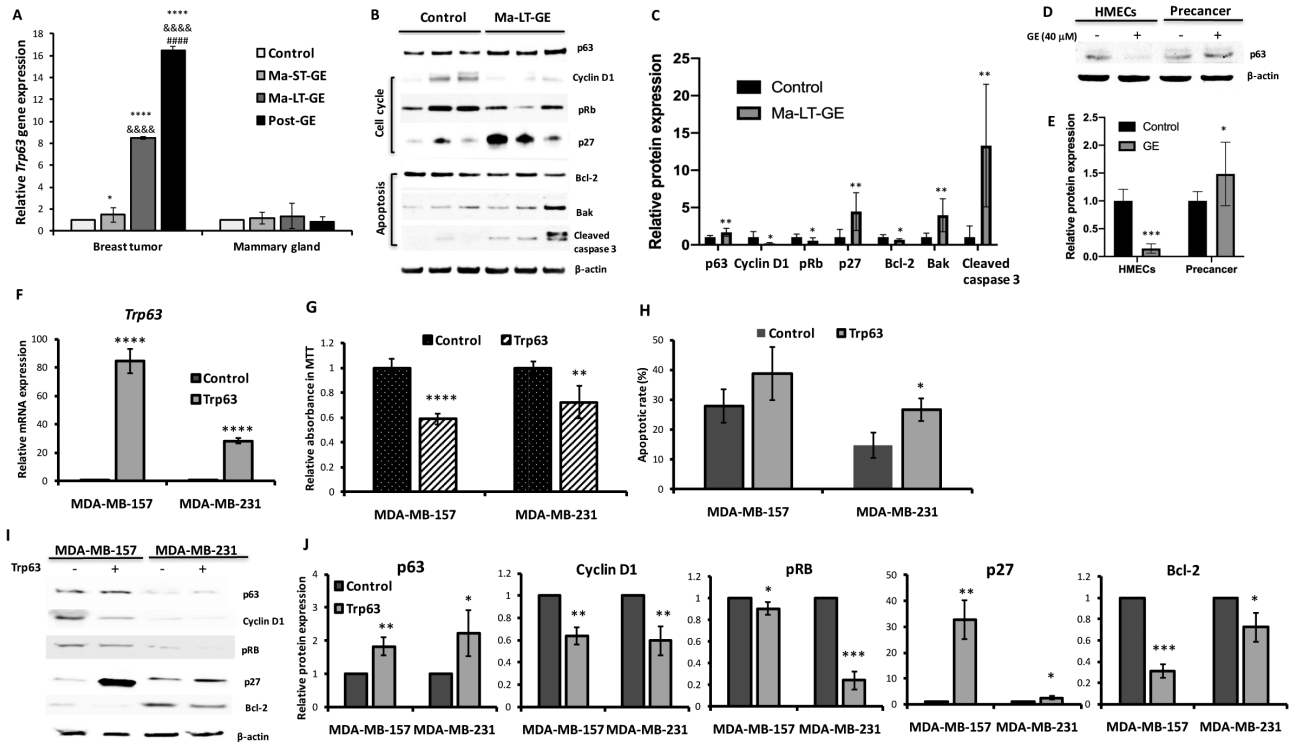

Figure 4. *Trp63* gene expression and its regulated signaling pathways. (A) *Trp63* gene transcriptional levels were determined by real-time RT-PCR in mammary tumors and normal mammary glands from control, maternal ST-GE, maternal LT-GE and postnatal-GE treatment. (B) Protein levels including p63, cell cycle-related proteins such as Cyclin D1, pRb and p27, and apoptosis-related proteins (Bcl-2, Bak and Cleaved caspase 3) were analyzed by western blot from 3 randomly selective animals in either control or maternal LT-GE groups. (C) Histogram showed quantified protein levels in mouse tumors. (D) p63 protein levels in normal HMECs or HMECs-derived precancer cells in response to GE treatment. (E) Quantification of p63 protein in HMECs and precancer cells. (F) *Trp63* mRNA expression by quantitative real-time RT-PCR in human TNBC cell lines, MDA-MB-157 and MDA-MB-231, after transient transfection with either Trp63 expression vector (Trp63) or empty plasmid (control). (G) Cell proliferation in transfected TNBC cells by MTT assay. (H) Apoptosis assay after 72 h transfection. (I) Protein expression of p63, Cyclin D1, pRb, p27 and Bcl-2 in control or Trp63-transfected TNBC cells. (J) Quantification of protein expression in transfected TNBC cells. All protein levels were normalized to β-actin as an internal control and calibrated to control or untreated group as 1. Representative photographs of the cropped blots from the experiments were repeated three times. Columns, mean; Bars, SD; * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$, **** $P < 0.0001$, significantly different from control; &, significantly different from the Ma-ST-GE group; # significantly different from Ma-LT-GE group.

p63 is a master regulator for epidermal development, epithelial cell maintenance, and tumorigenesis (29,45). We next inquired whether direct exposure to GE may affect p63 expression in normal human mammary epithelial cells (HMECs) and HMECs-derived precancer cells (27). Interestingly, GE treatment resulted in opposite effects in regulating p63 expression in normal or precancerous cells, whereby p63 expression was significantly decreased in normal HMECs but increased in HMECs-derived precancer cells (Figure 4D,E), suggesting that p63 plays distinct roles during different progressive stages of breast cancer.

To further determine the role of *Trp63* activation in human breast cancer cells, we overexpressed *Trp63* gene in two human triple-negative breast cancer (TNBC) cell lines, MDA-MB-157 and MDA-MB-231, by transient transfection with full-length of Trp63 ORF. After 72 h transfection, the transcriptional level of *Trp63* was dramatically increased by 84.3- and 28.1-fold in both MDA-MB-157 and MDA-MB-231 cells, respectively (Figure 4F). This resulted in subsequent cell proliferation inhibition (Figure 4G) and apoptotic response (Figure 4H) in transfected TNBC cells. We next assessed whether overexpressed *Trp63* can induce cell cycle arrest- and apoptosis-related gene expression changes that may be responsible for these phenotypic consequences. As shown in Figure 4I,J, increased p63 can significantly decrease Cyclin D1, pRb, and Bcl-2 but increase p27 expression at the protein level, leading to growth inhibition and apoptosis in human breast cancer cells. These results further validated that *Trp63* activation is critical to halt breast neoplastic progression.

Maternal LT-GE induced epigenetic inheritance in the *Trp63* gene

It is presumable that maternal soy exposure during critical developmental stages may alter DNA methylome in the offspring, which may in turn influence breast cancer risk later in life. We therefore tested our hypothesis by comparing DNA methylation alterations of *Trp63* gene between the postnatal-GE and maternal LT-GE groups to determine potential hereditable epigenetic landmarks that could be transmitted from the mothers to the offspring. Targeted DNA methylation analysis revealed that a persistent loss of DNA methylation occurred at certain defined loci in the promoter region (ADS9384) and intron 3 (ADS9388) of *Trp63* gene in the breast tumors from the mothers directly exposed to GE diet (postnatal-GE) and their offspring (maternal LT-GE) (Figure 5A). Importantly, our results, for the first time, provide direct evidence demonstrating that maternal dietary soybean GE may induce locus-specific epigenetic inheritance in the offspring, which may contribute to its breast cancer prevention potential later in life.

Maternal LT-GE induced DNA methylation inheritance changes influenced recruitment of transcriptional factors to the *Trp63* promoter

To further determine the underlying mechanisms regarding how the specific methylation inheritance alterations influence *Trp63* gene transcriptional activities, we performed ChIP assay to study binding ability changes of two important transcriptional factors, *CREB1* and *MeCP2*, which binding activities have been frequently influenced by methylation status of the binding motif (46,47). We designed specific primers targeting the *Trp63* gene regulatory regions including the promoter and intron 3 regions that have shown inherited hypomethylation in response to maternal LT-GE treatment (Figure 5B). As illustrated in Figure 5C, our results showed that maternal LT-GE treatment can significantly increase recruitment of transcriptional activator, *CREB1*, to the gene regulatory regions, especially in the P2 (promoter) and P3 (intron 3) regions containing a cAMP response element (cre), which may contribute to its induced transcriptional activation of *Trp63*. Interestingly, the binding ability of *MeCP2* increased concomitantly. As *MeCP2* normally binds to methylated CpGs leading to transcriptional repression, our finding suggests that *MeCP2* binding may also induce transcriptional activation when co-occupied with *CREB1*. These changes were not observed in breast tumor with postnatal GE treatment. We further evaluated histone modification changes in the *Trp63* promoter by detecting two important chromatin marks, trimethyl-H3K4 and trimethyl-H3K27 in the P2 region. Our results showed that postnatally direct exposure to GE diet significantly increased enrichments of the transcriptional activator, trimethyl-H3K4, but decreased the transcriptional repressor, trimethyl-H3K27, in the *Trp63* promoter (Figure 5C, right panel). However, maternal LT-GE did not induce significant changes in these chromatin marks.

As summarized in Figure 5D, we speculate that: 1) DNA hypermethylation and histone modifications such as enriched trimethyl-H3K27 and loss of trimethyl-H3K4 in the *Trp63* promoter lead to *Trp63* transcriptional suppression that contributes to breast tumor development; 2) postnatal GE can induce DNA hypomethylation and histone modification changes such as increased trimethyl-H3K4 but decreased trimethyl-H3K27 in the *Trp63* promoter leading to *Trp63* transcriptional activation and tumor inhibition; 3) maternal LT-GE can induce heritable locus-specific hypomethylation in the specific regulatory regions of the *Trp63* gene that facilitates the binding of *MeCP2* to the unmethylated CpGs, which in return increases recruitment of transcriptional activator such as *CREB1* to the *Trp63* promoter region leading to subsequent transcriptional activation and tumor suppression.

Discussion

Breast cancer has strong inherited tendency and development of breast cancer can be influenced by maternal exposure to certain environmental factors such as nutrition or dietary components (3,10). Genistein (GE), a natural isoflavone enriched in soybean products such as soy milk, soy protein, and tofu, has been proposed to associate with a lower rate of breast cancer in Asian women who consume soybean foods as their traditional diets (11,12). More importantly, soybean GE is believed to be a safe and potent dietary chemopreventive agent against breast cancer through, at least in part, regulation of epigenetic mechanisms such as DNA methylation or histone modifications (14–18). The current studies provided strong evidence suggesting that exposure window of maternal dietary GE is a key factor that determines the outcome of breast cancer later in life. Most importantly, for the first time, we discovered that maternal LT-GE can induce epigenetic inheritance in key tumor-related genes, which may contribute to maternal LT-GE-induced breast cancer prevention potential later in offspring life.

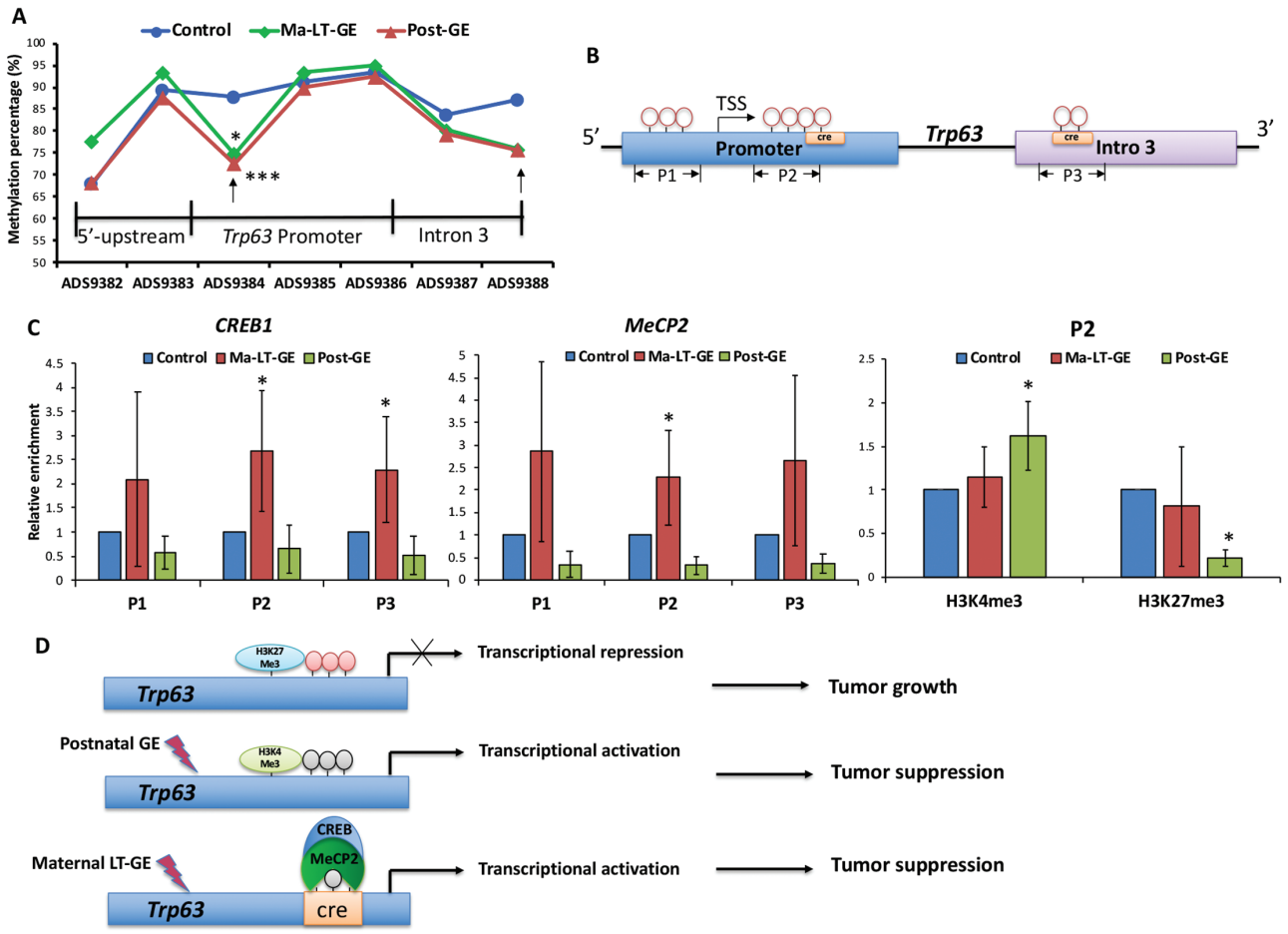

Figure 5. Exposure timing-dependent epigenetic regulation of *Trp63* expression. (A) Maternal LT-GE induced DNA methylation inheritance in the *Trp63* gene. Methylation changes in the regulatory regions of the *Trp63* gene were evaluated in breast tumors by tNGBS. Arrows point coincident loci-specific methylation loss in the promoter and intron 3 regions in response to postnatal-GE (mother) or maternal LT-GE treatment (offspring) suggesting a potential epigenetic inheritance. (B) An illustration indicates specific primers, P1, P2 and P3, target the *Trp63* promoter and intron 3 regions. Circle represents CpG sites and orange box indicates *CREB1* binding site, cre. TSS, transcription start site. (C) ChIP assays were performed to determine binding ability changes of *CREB1* (left) and *MeCP2* (middle) as well as enrichment changes of histone methylation marks (right, P2 region), trimethyl-H3K4 and trimethyl-H3K27, in the *Trp63* regulatory regions in response to maternal LT-GE and postnatal-GE treatments. The histogram shows relative enrichment as the ratio of the immunoprecipitated DNA to input DNA was calibrated to the levels in control samples via real-time PCR. Results were in three biological repeats from three independent experiments. Columns, mean; Bars, SD; $*P < 0.05$, $***P < 0.001$, significantly different from control. (D) Schematic illustration proposes working scenarios of epigenetic regulation of *Trp63* expression under different timings of GE exposure. In control mice, DNA hypermethylation (red circles) and enriched chromatin repressor, trimethyl-H3K27, in the *Trp63* promoter lead to *Trp63* transcriptional suppression that contributes to breast tumor development; postnatal GE (direct exposure such as mother) can induce DNA hypomethylation (gray circles) and increased chromatin activator, trimethyl-H3K4, but decreased chromatin suppressor, trimethyl-H3K27, in the *Trp63* promoter leading to *Trp63* transcriptional activation and tumor inhibition; in maternal LT-GE group, inherited locus-specific hypomethylation loci (gray circles) can facilitate the binding of *MeCP2* to the unmethylated CpGs, which in return increase recruitment of transcriptional activator such as *CREB1* to the *Trp63* promoter region leading to *Trp63* transcriptional activation and tumor inhibition.

Accumulating evidence indicates the timing for soybean intake is critical to determine its chemopreventive effects on breast cancer and early-life exposure seems to maximize this beneficial outcome (19,20). Soybean products are known to have transplacental effects. Several studies show that maternal consumption of dietary GE leads to beneficial health outcomes in mice offspring such as reduced risk in developing obesity, cardiovascular diseases, and breast cancer (18,21,48). However, the preventive efficacy can be affected by exposure time during maternal life (21). Importantly, for the first time, our results demonstrated that maternal LT-GE beginning from the mother's early lifetime exhibited more prominent protective effects than maternal ST-GE starting from gestation, confirming that exposure window of maternal dietary GE determines the preventive outcome of breast cancer in the offspring adult life. As maternal LT-GE and ST-GE treatments differentiate the role of maternal early-life exposure by including the period of germline development, it implies that the exposure window, even before conception, may be important in influencing the chemopreventive efficacy of soybean GE against breast tumorigenesis. Similar chemoprevention effects were observed when long-term soybean GE was directly administered from early life postnatally, suggesting these protective effects due to maternal diet can transmit to the offspring and persist throughout generations. The preventive effects of maternal GE in mice offspring serve as a plausible explanation for the lower incidence of breast cancers in Asian women, where the GE-rich soy products are their traditional daily diet throughout generations (11,12). In addition, the descendants of Asian immigrants, who have become "westernized" in terms of their diets, are still

showing low incidence in developing breast cancer compared to American women. This indicates that a heritable phenotypic change against breast cancer is carried on to the progenies likely mediated by an altered epigenome in the absence of GE exposure, especially those imprinted epigenetic hallmarks in germ cells that can be inherited throughout multi-generations (3,9).

Our previous studies showed that soybean GE can exhibit its tumor suppressing effects through regulation of key tumor-related gene expression mediated by epigenetic mechanisms (14–17). As individualized epigenome establishes during early developmental stage, maternal nutrition or diets with epigenetic modulatory property such as soybean isoflavone GE may influence early-life epigenetic reprogramming processes leading to altered susceptibility to certain diseases in the offspring (3). As expected, our studies showed that maternal LT-GE led to significant differential gene expression and genome-wide DNA methylation profile changes in the breast tumors of mice progenies. We were able to identify several key regulatory genes showing significant differential gene expression and/or methylation changes in response to maternal LT-GE treatment. These genes have been well studied as tumor- and development-related genes that may influence molecular proceeding of breast cancer initiation through epigenetic regulation (29-44, Table 1). After validation, we finalized *Trp63* as our candidate gene that showed the most dramatic gene expression in accordance with methylation changes.

The *Trp63* gene has been identified as one of the most ancient members of the p53 family as they share a high sequence and structural homology (29), which leads to a speculation that p63 proteins would function as tumor suppressors similar to p53. Coincidentally, our results indicate that maternal LT-GE can induce a significant increment of p63 gene expression accompanied by activation of p53-stimulated downstream signaling pathways such as cell cycle arrest and apoptosis, contributing to its cancer prevention effects in SV40 mice offspring. A number of studies suggests that loss of overall p63 expression is frequently seen in more aggressive and metastatic tumors (29,45,49). Our gain-of-function studies validated the importance of p63 reactivation on suppression of aggressive human TNBC cells through inhibition of cell cycle and induction of apoptosis, further indicating that maternal LT-GE induced p63 reactivation is major mechanistic event contributing to its chemopreventive effects later in life.

We found maternal LT-GE induced p63 upregulation was accompanied with loci-specific DNA demethylation in the *Trp63* promoter and intronic regions of the offspring tumors. Likewise, similar patterns of gene expression and DNA methylation were observed in the mice directly exposed to soybean GE from early life, indicating critical timing exposure to maternal nutrition may induce inherited epigenetic landmarks and their-mediated key gene expression changes leading to altered health outcome transmitted to the next generation. Further studies confirm that DNA methylation changes in loci-specific regulatory regions of the *Trp63* gene by maternal LT-GE lead to an increased binding of a transcriptional factor, *CREB1*, through a *MeCP2*-mediated transcriptional activation. Interestingly, postnatally direct exposure to soybean resulted in transcriptional activation of *Trp63* more likely through influencing dynamics of histone modification patterns. In contrast to previous studies that *MeCP2* functions as a transcriptional repressor by binding methylated CpG dinucleotides and recruiting transcriptional repressors, a key finding from our study is that *MeCP2* could act as a transcriptional activator when bound to less methylated CpG. This assumption mirrors findings by Chahrour et al., indicating that *MeCP2* may function as both an activator and repressor of transcription during development of neurological disease (50). Our results indicate that, on top of the inherited epigenetic landmarks, diverse mechanisms may be involved in *Trp63* transcriptional activation under a time-dependent exposure manner in response to dietary soybean GE during maternal lifespan, although similar phenotypic changes are induced.

Evidence on early-life disease programming provides promising prevention approaches that center on maternal dietary intervention. In this study, we elucidated the temporal effects of maternal soybean diet on later-life breast cancer prevention and explored potential epigenetic mechanisms. This profound protection may persist throughout the lifespan of the individual and even across the generations to the offspring and grand-offspring mediated through inherited epigenetic hallmarks in the germline and somatic cells. The concentration of GE used in our study is safe and physiologically achievable, and the duration of treatment represents a persistent eating habit that is commonly seen in Asian populations. Thus, our study provides important mechanistic insights into an appropriate maternal administration of soybean-based botanical compounds that will eventually benefit health welfare in both mothers and their children, especially the women who are at a higher risk of developing aggressive forms of breast cancer.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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Conflict of Interest Statement

The authors declare no potential conflicts of interest.

Data Availability

Statements: The genomic data are available in a public repository, Gene Expression Omnibus (GEO), and can be accessed using GEO accession number GSE194163.

References

1. Bateson, P. et al. (2004) Developmental plasticity and human health. *Nature*, 430, 419–421.
2. Robillard, J.E., et al. (2006) Influence of early life events on health and diseases. *Trans. Am. Clin. Climatol. Assoc.,* 117, 313–319; discussion 319; discussion 319-20.
3. Li, Y., et al. (2014) Impact of epigenetic dietary compounds on transgenerational prevention of human diseases. *AAPS J.*, 16, 27–36.
4. Li, S., et al. (2019) Prenatal epigenetics diets play protective roles against environmental pollution. *Clin. Epigenetics.*, 11, 82.
5. Li, S., et al. (2020) Maternal epigenetic regulation contributes to prevention of estrogen receptor-negative mammary cancer with broccoli sprout consumption. *Cancer Prev. Res. (Phila).*, 13, 449–462.
6. Li, Y., et al. (2018) Temporal efficacy of a sulforaphane-based broccoli sprout diet in prevention of breast cancer through modulation of epigenetic mechanisms. *Cancer Prev. Res. (Phila).*, 11, 451–464.
7. Li, Y. (2018) Epigenetic mechanisms link maternal diets and gut microbiome to obesity in the offspring. *Front. Genet.*, 9, 342.
8. Li, E. (2002) Chromatin modification and epigenetic reprogramming in mammalian development. *Nat. Rev. Genet.*, 3, 662-73.
9. Heard, E. et al. (2014) Transgenerational epigenetic inheritance: myths and mechanisms. *Cell*, 157, 95–109.
10. Hilakivi-Clarke, L. et al. (2006) Fetal origins of breast cancer. *Trends Endocrinol. Metab.*, 17, 340–348.
11. Messina, M. (2016) Soy and health update: evaluation of the clinical and epidemiologic literature. *Nutrients*, 8, 754.
12. Chen, M. et al. (2014) Association between soy isoflavone intake and breast cancer risk for pre- and post-menopausal women: a meta-analysis of epidemiological studies. *PLoS One*, 9, e89288.
13. Barnes, S. (1995) Effect of genistein on in vitro and in vivo models of cancer. *J. Nutr.*, 125, 777S–783S.
14. Li, Y. et al. (2010) Impact on DNA methylation in cancer prevention and therapy by bioactive dietary components. *Curr. Med. Chem.*, 17, 2141–2151.
15. Li, Y. et al. (2009) Genistein depletes telomerase activity through cross-talk between genetic and epigenetic mechanisms. *Int. J. Cancer.*, 125, 286–296.
16. Li, Y. et al. (2013) Epigenetic reactivation of estrogen receptor-α (ERα) by genistein enhances hormonal therapy sensitivity in ERα-negative breast cancer. *Mol. Cancer.*, 12, 9.
17. Li, Y. et al. (2013) Epigenetic regulation of multiple tumor-related genes leads to suppression of breast tumorigenesis by dietary genistein. *PLoS One.*, 8, e54369.
18. Dolinoy, D.C. et al. (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ. Health Perspect.*, 114, 567–572.
19. Messina, M. et al. (2009) Early intake appears to be the key to the proposed protective effects of soy intake against breast cancer. *Nutr. Cancer.*, 61, 792–798.
20. Shu, X.O. et al. (2001) Soyfood intake during adolescence and subsequent risk of breast cancer among Chinese women. *Cancer Epidemiol. Biomarkers Prev.*, 10, 483–488.
21. Warri, A. et al. (2008) The role of early life genistein exposures in modifying breast cancer risk. *Br. J. Cancer*, 98, 1485–1493.
22. Sharpless, N.E. et al. (2006) The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat. Rev. Drug Discov.*, 5, 741–754.
23. Love, M.I. et al. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.*, 15, 550.
24. Gu, H. et al. (2011) Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. *Nat. Protoc.*, 6, 468–481.
25. Krueger, F. et al. (2011) Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics*, 27, 1571–1572.
26. Wu, H. et al. (2013) A new shrinkage estimator for dispersion improves differential expression detection in RNA-seq data. *Biostatistics*, 14, 232–243.
27. DeAngelis, J.T. et al. (2011) 2D difference gel electrophoresis analysis of different time points during the course of neoplastic transformation of human mammary epithelial cells. *J. Proteome Res.*, 10, 447–458.
28. Reagan-Shaw, S. et al. (2008) Dose translation from animal to human studies revisited. *FASEB J.*, 22, 659–661.
29. Melino, G. (2011) p63 is a suppressor of tumorigenesis and metastasis interacting with mutant p53. *Cell Death Differ.*, 18, 1487–1499.
30. Fonseca, M.B. et al. (2012) TAp63γ demethylation regulates protein stability and cellular distribution during neural stem cell differentiation. *PLoS One*, 7, e52417.
31. Poole, C.J. et al (2017) MYC-master regulator of the cancer epigenome and transcriptome. *Genes (Basel)*, 8, 142.
32. Barnes, D.M. et al. (1998) Cyclin D1 in breast cancer. *Breast Cancer Res. Treat.*, 52, 1–15.
33. Guo, Z.Y. et al. (2011) The elements of human cyclin D1 promoter and regulation involved. *Clin Epigenetics*, 2, 63–76.
34. Rivera, A. et al. (2006) Cyclin A1 is a p53-induced gene that mediates apoptosis, G2/M arrest, and mitotic catastrophe in renal, ovarian, and lung carcinoma cells. *Cell. Mol. Life Sci.*, 63, 1425–1439.
35. Müller, C. et al. (2000) Methylation of the cyclin A1 promoter correlates with gene silencing in somatic cell lines, while tissue-specific expression of cyclin A1 is methylation independent. *Mol. Cell. Biol.*, 20, 3316–3329.
36. Moison, C. et al. (2014) Synergistic chromatin repression of the tumor suppressor gene RARB in human prostate cancers. *Epigenetics*, 9, 477–482.
37. Zhang, J. et al. (2019) KRT18 is correlated with the malignant status and acts as an oncogene in colorectal cancer. *Biosci. Rep.*, 39¸ BSR20190884.
38. Kwan, R. et al. (2015) Absence of keratins 8 and 18 in rodent epithelial cell lines associates with keratin gene mutation and DNA methylation: Cell line selective effects on cell invasion. *Exp. Cell Res.*, 335, 12–22.
39. Ke, J. et al. (2018) Inhibitor of DNA binding proteins: implications in human cancer progression and metastasis. *Am J Transl Res*, 10, 3887–3910.
40. Li, Y. et al. (2020) Loss of progesterone receptor through epigenetic regulation is associated with poor prognosis in solid tumors. *Am J Cancer Res*, 10, 1827–1843.
41. Lodygin, D. et al. (2005) The role of epigenetic inactivation of 14-3-3sigma in human cancer. *Cell Res.*, 15, 237–246.
42. Baharudin, R. et al. (2020) Epigenetics of SFRP1: The Dual Roles in Human Cancers. *Cancers (Basel)*, 12, 445.
43. Xu, Y. et al. (2017) Twist1 promotes breast cancer invasion and metastasis by silencing Foxa1 expression. *Oncogene*, 36, 1157–1166.
44. Sundararajan, V. et al. (2019) SNAI1 recruits HDAC1 to suppress SNAI2 transcription during epithelial to mesenchymal transition. *Sci. Rep.*, 9, 8295.
45. Soares, E. et al. (2018) Master regulatory role of p63 in epidermal development and disease. *Cell. Mol. Life Sci.*, 75, 1179–1190.
46. Sunahori, K. et al. (2009) Methylation status of CpG islands flanking a cAMP response element motif on the protein phosphatase 2Ac alpha promoter determines CREB binding and activity. *J. Immunol.*, 182, 1500–1508.
47. Chen, L. et al. (2015) MeCP2 binds to non-CG methylated DNA as neurons mature, influencing transcription and the timing of onset for Rett syndrome. *Proc. Natl. Acad. Sci. U.S.A.*, 112, 5509–5514.
48. Bonacasa, B. et al. (2011) Impact of dietary soy isoflavones in pregnancy on fetal programming of endothelial function in offspring. *Microcirculation*, 18, 270–285.
49. Buckley, N.E. et al. (2011) The DeltaNp63 proteins are key allies of BRCA1 in the prevention of basal-like breast cancer. *Cancer Res.*, 71, 1933–1944.
50. Chahrour, M. et al. (2008) MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science*, 320, 1224–1229.