DNA methylation and histone modifications of Wnt genes by genistein during colon cancer development

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This study aims to elucidate the epigenetic mechanisms by which genistein (GEN) maintains a normal level of Wnt genes during colon cancer development. We have reported that soy protein isolate (SPI) and GEN repressed WNT signaling, correlating with the reduction of pre-neoplastic lesions in rat colon. We hypothesized that SPI and GEN induced epigenetic modifications on Sfrp2, Sfrp5 and Wnt5a genes, suppressing their gene expression induced by azoxymethane (AOM), a chemical carcinogen, to the similar level as that of pre-AOM period. We identified that in the post-AOM period, histone H3 acetylation (H3Ac) was downregulated by SPI and GEN at the promoter region of Sfrp2, Sfrp5 and Wnt5a, which paralleled with the reduced binding of RNA polymerase II. Nuclear level of histone deacetylase 3 was enhanced by SPI and GEN. The diets suppressed the trimethylation of histone H3 Lysine 9 (H3K9me3) and the phosphorylation of histone H3 Serine 10 (H3S10P). Methylation of the specific region of Sfrp2, Sfrp5 and Wnt5a genes was increased by SPI and GEN, which was inversely correlated with the reduction of gene expression. Bisulfite sequencing further confirmed that dietary GEN induced DNA methylation at CpG island of the promoter region of Sfrp5. Importantly, this region includes a fragment that had decreased H3Ac. Here, we present a potential epigenetic mechanism by which dietary GEN controls the responses of WNT genes during carcinogen induction, which involves DNA methylation, histone modifications and their interactions at the regulatory region of gene.

Introduction

Epigenetic modulations such as DNA methylation and histone modifications closely orchestrate the transcriptional activity of genes, and specific epigenetic structures are required for maintaining the normal function of cells (1). Insufficient control of the epigenetic modifications activates oncogenes and deactivates tumor suppressor genes, leading to the development of various cancers (2). Colon cancer has recently been reported to occur through the accumulation of aberrant DNA methylation (3) and disruption of histone codes (4). Abnormal DNA methylation was detected in the normal-appearing mucosa at the early stage of colon carcinogenesis (3). Global hypomethylation results in chromosome instability and activation of oncogenes (5–8). Changes of the methylation intensity of the promoter-CpG islands, which are regions rich in CpG dinucleotide, alter the expression level of key tumor suppressor genes (9).

In addition to DNA methylation, histone modifications are considered as critical regulators in mediating gene activity as well. Acetylation, methylation and phosphorylation at specific residues of histones such as Lys 4, Lys 9 and Ser 10 provide chemical driving forces for chromatin configuration, preventing or allowing the initiation of gene transcription (10,11). Increased activities of histone deacetylases such as HDAC3 in the nucleus are associated with the reduction of histone acetylation (12). Moreover, the crosstalk between DNA methylation and histone modifications has been of great interest recently. It is been shown that methylation of histone H3K9 can direct DNA methylation (13). On the other hand, DNA methylation could also affect histone methylation (14,15). It has been demonstrated that nucleosomes composed of acetylated histones assemble unmethylated (UM) DNA, whereby the addition of methyl groups on the identical DNA sequences correlates with the recruitment of non-acetylated histones (16).

Wnt signaling pathway plays a crucial role in normal colon epithelium development as well as in tumorigenic steps (17,18). During normal development, Wnt signaling is regulated and maintained at a normal level (19,20). Aberrant Wnt signaling has been proposed to promote colon carcinogenesis and is associated with over 90% of colorectal cancer cases (21,22). There is increasing evidence demonstrating that epigenetic modifications of Wnt-related genes correlate with the dysregulation of Wnt signaling during colon cancer development. For instance, hypermethylation on the promoter regions of secreted frizzled receptor proteins reduced gene expression of the Wnt antagonists and caused constitutive Wnt signaling during colorectal cancer progression (22). Moreover, surveillance of Wnt signaling by Wnt inhibitors was diminished through altered histone modifications (23).

Our group has shown previously that genistein (GEN) increased the expression of Wnt signaling molecules, such as sFRP2 and WNT5a, by demethylation of CpG within their promoters in colon cancer cells (24,25). We also reported that GEN induced the expression of Dickkopf-related protein-1 by increasing histone H3 acetylation (H3Ac) at promoter region of the gene (26). Only a few in vivo studies have been conducted to elucidate dietary GEN-induced epigenetic modifications and related changes in gene expression. One study has shown gene-specific changes in DNA methylation in mice fed a GEN-containing diet (27). Another evidence that GEN affects DNA methylation was that maternal exposure to dietary GEN altered the epigenome of offspring in viable yellow agouti (A/Y/a) mice. GEN induced hypermethylation of CpG in the viable yellow agouti (A/Y/a) mice and decreased expression level of the Agouti gene (28). No in vivo study has been reported regarding dietary GEN-induced histone modifications in regulating gene expression.

Dietary intake and epigenetic remodeling closely interact with each other and the proper maintenance of the epigenome is critical in normal development. Thereby, we conducted this study to extend our knowledge of the epigenetic modifications by which dietary GEN maintained a normal expression profile of Wnt signals, Sfrp2, Sfrp5 and Wnt5a, in the azoxymethane (AOM)-induced colon neoplasia in rat (29). In the previous study, we observed that the expression level of Sfrp2, Sfrp5 and Wnt5a, as well as the protein level of β-catenin, the hallmark of Wnt signaling activity, was maintained by dietary GEN in the post-AOM period. Importantly, the suppression of these Wnt signals was closely correlated with the decreased frequency of total aberrant crypt foci (ACF) in the colon. These results indicated that rats fed a life-long GEN-containing diet were less affected by a carcinogen load, which probably occurred through maintaining a normal Wnt signaling by dietary GEN. Here, we demonstrated that dietary GEN maintained the expression of Sfrp2, Sfrp5 and Wnt5a at a low level in the colon epithelium of carcinogen-injected rats through regulating the DNA methylation and decreasing the H3Ac methylation at histone H3K9 and phosphorylation at histone H3S10 within the regulatory region of genes. Moreover, we have identified the potential crosstalk between DNA methylation and histone modifications at the CpG-rich region within the transcriptional regulatory region of Sfrp5.

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; ChIP, chromatin immunoprecipitation; CTL, control; GEN, genistein; H3Ac, histone H3 acetylation; H3K9me3, trimethylation of histone H3 Lysine 9; H3S10P, phosphorylation of histone H3 Serine 10; MSP, methylation-selective PCR; Pol II, polymerase II; SPI, soy protein isolate; UM, unmethylated.
Materials and methods

Detailed description of Materials and methods are included in the Supplementary Methods, available at Carcinogenesis Online.

Animal and diets

Timed-pregnant Sprague-Dawley rats (Charles River Laboratories) were fed one of three isocaloric diets throughout gestation and lactation periods. The diets were AIN-93G diet formula based: (i) control (CTL; casein protein), (ii) soy protein isolate (SPI; NutroBio.com. contains 140 mg/kg GEN aglycone equivalent) and (iii) GEN (CTL plus 140 mg/kg GEN). Male pups were weaned at day 24 and maintained on the same diet as their mother until killed. Pre-AOM sampling of colon samples occurred when the offspring were 7 weeks old (CTL, n = 6; SPI and GEN, n = 8). The rest of the rats were injected with AOM (15 mg/kg body wt) at 7 and 8 weeks of age. Post-AOM sampling of colon samples occurred 6 weeks later. Colon tissues were collected for all subsequent analyses. All procedures were approved by the institutional animal care and use committee at the University of Illinois at Urbana-Champaign.

RNA isolation

Scraped colon epithelia were ground in liquid nitrogen and total RNA was isolated using TRI reagent (Sigma–Aldrich). Complementary DNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Quantitative PCR analysis

Real-time PCR was conducted for gene expression analysis, methylation-specific PCR analysis and chromatin immunoprecipitation (ChIP) assay using Fast SYBR Green master mix (Quanta) in a 7300 thermal cycler (Applied Biosystems). The primers used in this study are listed in the Supplementary Tables 1 and 2, available at Carcinogenesis Online.

ChIP analysis

ChIP analysis was performed following a modified protocol (30). Briefly, 100 mg frozen descending colon epithelial tissue from each individual animal was lysed, sonicated and incubated with antibodies against proteins of interest (Supplementary Table 3, available at Carcinogenesis Online). Normal rabbit IgG was used as the negative control. Immunoprecipitated chromatin was analyzed by real-time PCR using specific primer sets (Supplementary Table 1, available at Carcinogenesis Online). The binding of RNA polymerase II (Pol II) at the coding region of housekeeping gene L7a was tested as the positive control (Supplementary Figure 1, available at Carcinogenesis Online).

Methylation-selective PCR

Genomic DNA was isolated from the colon tissues using DNeasy Tissue Kit (Qiagen). Bisulfite conversion was conducted according to the manufacturer’s instructions (ZymoResearch) (25). UM primers (Supplementary Table 2, available at Carcinogenesis Online) were used in PCR reactions to analyze the two regions of interest (UM1 and UM2) and one control region (UM3) for each gene.

Bisulfite sequencing

Primers were designed for a putative 5′ upstream CpG island of Sfrp5 (Sfrp5 BSF in Supplementary Table 2, available at Carcinogenesis Online). Genomic DNA from three rats in either CTL or GEN was used for bisulfite sequencing. Bisulfite-converted template was amplified by PCR using the specific primers designed for the genomic regions of interest and cloned subsequently. Ten individual clones from each group were verified and sequenced at the Keck Biotechnology Center at the University of Illinois at Urbana-Champaign.

Protein isolation and immunoblotting

Nuclear protein was extracted from 100 mg of scraped colon epithelia and used in western blot analysis for HDAC3 protein. A nuclear protein lamin A was used as the loading control (29).

Statistical analysis

Results were reported as mean ± standard error of the mean (SEM). Differences were considered significant at P < 0.05 unless otherwise mentioned. Regression analysis was performed to examine the possible relationships between the intensity of unmethylation and the expression level of the gene. Linear relationship was considered significant at P < 0.05 (R program, http://www.r-project.org/).

Results

DNA methylation status of a specific CpG-rich region was correlated with the gene expression of Sfrp2, Sfrp5 and Wnt5a UM CpGs at the regulatory region of a gene often represent an actively transcribing status of the gene (31). We analyzed the relationship between the intensity of UM CpG measured by methylation-selective PCR (MSP)
at two regions and the level of gene expression of Sfrp2, Sfrp5 and Wnt5a genes in the post-AOM period using regression analysis. We identified a specific region of Sfrp2 and Wnt5a gene that displayed a significant correlation between level of UM CpG and that of gene expression of the two genes (Figure 1; +102/+183 of Sfrp2, P = 0.013; +61/+128 of Wnt5a, P = 0.031). Unmethylation intensity of a specific region of Sfrp5 gene tended to be correlated with the expression of gene (Figure 1; −380/−295 of Sfrp5, P = 0.056). Alterations on DNA methylation at the above-identified regions were further analyzed to examine the effects of dietary GEN.

Dietary GEN increased methylation of the specific CpG-rich region of Sfrp2, Sfrp5 and Wnt5a in the post-AOM period.

To test whether dietary GEN suppressed the response of gene expression to a carcinogen load by affecting DNA methylation, the above-identified CpG region of each gene was tested (Figure 2). In the post-AOM period, a significant decrease of unmethylation was observed at +102/+183 of Sfrp2 in the GEN group (Figure 2A). Although AOM injection increased unmethylation at −380/−295 of Sfrp5 (P < 0.05), SPI and GEN resist this demethylation by AOM, resulting in a significant lower level of unmethylation compared with CTL in the post-AOM period (Figure 2B). The +61/+128 region of Wnt5a did not show significant change of unmethylation in the SPI or GEN group compared to the CTL group (Figure 2C).

Using the intensity of methylation of Sfrp2, Sfrp5 and Wnt5a at the specified region as an indicator, we were able to discriminate the pattern of methylation of Sfrp2, Sfrp5 and Wnt5a in the SPI and GEN groups from the CTL group in the post-AOM period using heatmap analysis (Figure 2D). Overall, SPI and GEN group displayed a trend of increased methylation in the post-AOM period within the indicated region of each gene, which was in agreement with the overall suppression of the gene expression by these two diets.

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**Fig. 2.** Change of DNA methylation of Sfrp2, Sfrp5 and Wnt5a in rat descending colons. Intensity of unmethylation at promoter of each gene was presented (A: Sfrp2, B: Sfrp5 and C: Wnt5a). Samples were collected from rats fed control (CTL, n_{pre-AOM} = 6; n_{post-AOM} = 8), SPI (SPI, n_{pre-AOM} = 8; n_{post-AOM} = 8) and GEN (GEN, n_{pre-AOM} = 8; n_{post-AOM} = 8) diets. Data were shown as ratio of unmethylation intensity of the region of interest to that of the region of control and presented as means ± SEM. *, significant difference compared with CTL in post-AOM, P < 0.05. ≠, mean values of CTL that are significantly different compared with the CTL in pre-AOM, P < 0.05. (D) Heatmap of the intensity of unmethylation at +102/+183 of Sfrp2, −380/−295 of Sfrp5 and +61/+128 of Wnt5a. Samples from four rats in each group were analyzed and presented. Each square represents the methylation level of the gene from an individual rat. The data are scaled to 0 as the mean and ±1 as the standard deviation. Color intensity indicates level of methylation.
Dietary GEN suppressed binding of Pol II at the promoter of Sfrp2, Sfrp5 and Wnt5a in the post-AOM period

To determine whether dietary GEN prevents AOM-induced gene expression by affecting transcriptional activity of the gene, we tested the binding of RNA Pol II at promoter regions of Sfrp2, Sfrp5 and Wnt5a (Figure 3, bars on primary y-axis). Our results showed that AOM induced the binding of Pol II significantly. SPI and GEN decreased the binding of Pol II at the promoter of Sfrp2, Sfrp5 and Wnt5a in the post-AOM period. There was a strong interaction ($P < 0.01$) between dietary treatment and AOM induction for all three genes, indicating that dietary GEN affected the binding of Pol II differently in the pre- and post-AOM period. Without AOM induction, the Pol II binding at promoter region of these genes were the same among treatment groups. However, after AOM induction, comparing to the upregulated binding of Pol II by AOM, SPI and GEN were able to maintain the Pol II binding to the similar level as that of the pre-AOM period. Gene expression tested by reverse transcription–PCR analysis for each gene was also shown as the filled circles on the secondary y-axis (Figure 3). The intensity of Pol II binding was closely related to the level of gene expression (Figure 3). This indicated that the reduction of gene expression by dietary GEN was caused by the decreased transcriptional activity.

Dietary GEN reduced H3Ac at the promoter of Sfrp2, Sfrp5 and Wnt5a in the post-AOM period

To investigate the overall landscape of histone tail modifications at the regulatory regions of genes, we examined the combinations of different types and sites of modifications on histone tails. The ones that showed significant change by AOM induction and/or by diets were included in the discussion. We examined the effect of dietary GEN on histone acetylation by testing the level of histone acetylation (H3Ac and H4Ac) at the promoter regions of Sfrp2, Sfrp5 and Wnt5a. AOM treatment caused 2- to 8-fold increases in H3Ac in the control groups in the three gene tested (Figure 4, $P < 0.05$). Although dietary GEN did not alter the level of H4Ac in either pre- or post-AOM period (data not shown), H3Ac level at the promoters of Sfrp2, Sfrp5 and Wnt5a was downregulated to about 50% by SPI and GEN diets in the post-AOM period (Figure 4, left panel; $P < 0.05$). Furthermore, dietary GEN reversed the AOM-induced H3Ac at the promoter of Sfrp2 to the pre-AOM level in the control group. There is a strong interaction between dietary treatment and AOM induction for H3Ac on Sfrp5 and Wnt5a ($P < 0.01$), indicating that the diet affected H3Ac differently in the pre- and post-AOM period. Therefore, dietary GEN affected the expression of Wnt genes by decreasing the acetylation of lysine residues at histone H3 at the specific regions of the genes.

Dietary GEN repressed methylation of lysine 9 and phosphorylation of serine 10 of histone H3 at the promoter of Sfrp2, Sfrp5 and Wnt5a in the post-AOM period

We tested the effect of dietary GEN on methylation (trimethyl lysine 9, H3K9Me3) and phosphorylation (serine 10, H3S10P) of histone H3. The two neighboring histone modifications have been shown to interact with each other, leading to activation or inactivation of gene expression (32). AOM injection induced 2- to 4-fold increase for H3K9Me3 and H3S10P at the promoter of Sfrp2, Sfrp5 and Wnt5a in the control group (Figure 4, middle and right panels; $P < 0.05$). Increased intensity of these histone modifications at the promoter region of the genes paralleled the increased gene expression observed in the post-AOM control. In the pre-AOM period, there was no dietary effect on H3K9Me3 on the genes examined. Binding of H3S10P on the three genes was increased significantly by GEN but not SPI in the pre-AOM period ($P < 0.05$). After AOM injection, H3K9Me3 and H3S10P were both decreased by SPI and GEN compared with CTL at the promoter of Sfrp2 and Sfrp5 ($P < 0.05$), to the levels similar as the pre-AOM groups. At the same time, GEN but not SPI reduced the level of H3K9Me3 and H3S10P at promoter of Wnt5a. Statistical analysis showed a strong interaction ($P < 0.01$) between dietary treatment and AOM induction at all three genes, indicating that the diet affected the level of methylated H3K9 and phosphorylated H3S10 in the pre- and post-AOM period differently.
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Dietary GEN induced the nuclear level of HDAC3 protein in the post-AOM period

To identify the potential regulators of histone acetylation, nuclear level of HDAC3 protein was examined. The level of HDAC3 was increased by ~3-fold in SPI and GEN in the post-AOM period (Figure 5; *P* < 0.05). There was a strong interaction (*P* = 0.016) between dietary treatment and AOM induction, suggesting that the effect of dietary treatment on HDAC3 was different between pre- and post-AOM period. This indicates that SPI and GEN diets suppressed the H3Ac by upregulating HDAC3 in the nuclei of colon epithelial cells.

Dietary GEN induced an increase of CpG methylation and a decrease of H3Ac at the same region of Sfrp5 promoter after AOM induction

We further examined dietary GEN-induced methylation at individual CpG site within the CpG-rich region at Sfrp5 promoter (~314/+137; Figure 6) using bisulfite sequencing. The region mapped by bisulfite sequencing overlapped with the one (~380/-295) that had a decreased level of unmethylation in the post-AOM SPI and GEN group detected by MSP assay (Figure 2B). Comparing with an average of 12.5% methylation out of total 20 CpGs within the bisulfite sequencing-covered region in CTL group, GEN had an average methylation of 16.5%. This confirmed the increase of DNA methylation by GEN diet by MSP analysis. Importantly, further analysis showed that the CpG methylation of −162/−74 within the sequenced region had an average of 27.5% in the GEN group, which was 90% higher compared with CTL (15%, *P* = 0.05). Notably, the −162/−74 region also had a 3-fold decrease of H3Ac by GEN (Figure 4, box highlighted in Figure 6).

**Discussion**

This study provides the first evidence that epigenetic remodeling is critical for the repression of Wnt signaling by GEN during the early pre-neoplastic development in colon. We report that DNA methylation of the proximal regions on selected genes of the Wnt pathway is closely related to the decreased gene expression. Alterations of histone configurations at these critical regions were also associated with the regulation of gene expression by dietary GEN. Importantly, investigations on the specific promoter region of the Sfrp5 gene revealed a strong association between increased DNA methylation and decreased acetylation, methylation and phosphorylation of the histone H3. Overall, this study provides the identification of critical
Epigenetic remodeling by genistein in rat colon

Epigenetic markers on the regulation of gene expression during the early stage of neoplastic development in colon.

Studies have suggested that the higher level of consumption of soy isoflavones in Asian countries contributes to the lower incidence of colon cancer compared with the western countries (33). It was reported that the serum concentration of rats fed a diet containing 100 p.p.m. GEN was similar to that of human subjects consuming a traditional Asian diet (34). Rats fed GEN diets (75 or 150 mg/kg) in both pre- and post-AOM induction periods had decreased ACF (35). Another study reported that feeding soy (182 mg/kg of GEN) from gestation to 26 weeks of age reduced AOM-induced colon tumor burden and size in rats (36). On the other hand, it was reported that old female F344 rats fed with soy diet (1200 mg/kg of GEN) 1 week prior to AOM injection were not protected against the formation of ACF (37). Therefore, in this study, rats were fed a moderate level of dietary GEN (140 mg/kg), which was relevant to an Asian diet, throughout life. This level of exposure is also easily achieved using the standard AIN93 diet when soy protein is used as the sole protein source (20%). Using this model, we have previously reported that this level of dietary GEN maintained the WNT/β-catenin signaling at a low level, therefore suppressed the formation of ACF in the descending colon of rats during carcinogen induction (29). In that publication, it is shown that the β-catenin signaling was greatly induced after the carcinogen load. But SPI and GEN diet kept β-catenin expression at the pre-induction level, indicating that the protection from dietary GEN against ACF formation was mediated through the maintenance of the proper β-catenin signaling (29). As the nuclear β-catenin increased in the control group after AOM injection, the expression of Sfrp5 and Wnt5a was also significantly induced. On the contrary, SPI and GEN maintained the expression of these three genes and the nuclear β-catenin at the similar level as that of pre-AOM period, suggesting a promising role of dietary GEN in maintaining colon health by preventing the induction of WNT/β-catenin signaling by a carcinogen load.

Many lines of evidence have shown that GEN treatment modulates gene expression through mediating chromatin configurations or DNA methylation of gene in cancer cells. In this study, we focused on elucidating the mechanism of how dietary GEN mediated and kept the expression level of Sfrp5, Sfrp5 and Wnt5a low in the descending colon after AOM injection by examining the alterations of epigenetic structures of these genes. It is shown that the changing patterns of Pol II association at the promoter region of Sfrp5 and Wnt5a were in accordance with that of the gene expression, indicating that the regulation of gene expression of Sfrp5 and Wnt5a by GEN occurred at the transcriptional level. It has been reported that GEN increases acetylation of histone tails and upregulates the expression of tumor suppressor genes in prostate

Fig. 5. Nuclear HDAC3 abundance in rat descending colon. (A) Representative blots of HDAC3 and lamin A from western blot analysis. (B) Quantification of western blot analysis. Lamin A served as the loading control for nuclear extracts. Samples from three rats in each group were analyzed and data were presented as the means ± SEM. *, mean values that are significantly different compared with CTL counterpart within the same time period, P < 0.05.

Fig. 6. DNA methylation at Sfrp5 promoter in rat descending colon after AOM induction. Bisulfite sequencing was performed to detect the change of DNA methylation at the proposed CpG island of Sfrp5 (~314/+137). Each CpG site is underlined in the tested region (top panel). Ten clones from each dietary group were sequenced and presented (bottom panel). Open circles represent unmethylated CpG sites and filled circles represent methylated CpG sites. Each row of circles represents an individual clone used for sequencing. Framed circles indicate the region (~162/−74) that overlaps with ChIP assay.

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cancer cells (38,39). Our results showed that the decreased level of H3Ac at promoters of Sfrp2, Sfrp5 and Wnt5a in the post-AOM period paralleled with the downregulation of the gene expression. As one of the major nuclear histone deacetylases, HDAC3 has been shown to be more susceptible to dietary factors such as sulforaphane than other HDACs in colon cancer cells (40). We, therefore, tested the effect of dietary GEN on the nuclear HDAC3 protein abundance. Our result showed that in accordance with the decreased H3Ac, dietary GEN upregulated the nuclear level of HDAC3 in the post-AOM period, suggesting that the increase of nuclear HDAC3 by dietary GEN caused a reduction of H3Ac, leading to a decreased gene expression.

In addition to modulating histone acetylation, GEN has also been shown to reduce the methylation of H3K9 and restore the gene expression in prostate cancer cells (41). In this study, we observed the similar downregulation of the methylation of H3K9 by GEN at the promoter of the selected genes in rat colon in the post-AOM period. The fact that dietary GEN decreased the gene expression of Sfrp2, Sfrp5 and Wnt5a at the same time of the downregulation of the H3K9Me3 clearly demonstrated the complex interactions among the histone tail residues in the cancer development model in vivo. Phosphorylation of histone H3S10 has been used as a marker for mitotic fraction of cell cycle progression in cancer (42). Decrease of the phosphorylation at H3S10 was observed in breast cancer cells after GEN treatment, and it contributed to the reduced cell cycle progression into mitosis. In this study, we observed 2-3 folds of increase in phosphorylated H3S10 by AOM induction, demonstrating the tumorigenic potential of this carcinogen. Interestingly, our result showed that the binding of H3S10P at promoter region of Sfrp2, Sfrp5 and Wnt5a was increased by GEN but not SPI in the pre-AOM period. Further studies are needed to compare the role of H3S10P in modulating gene expression at the regulatory region of gene under normal and carcinogenic conditions. Although it has been reported that the phosphorylation of H3S10 suppressed the methylation of neighboring H3K9 (43), we observed a simultaneous reduction of both modifications by dietary GEN at the promoters of transcriptionally repressed genes. Overall, our results on the interactions of selected histone tail modifications by diet during carcinogenesis illustrated the critical involvement of all histone modifications as well as the complex interactions among them. Further studies are needed to decipher the interactions among these epigenetic markers, which will lead to a better understanding of the regulation of oncogenes at epigenetic level.

Changes of DNA methylation at the regulatory region of gene with rich CpG content have profound influence on gene expression (44). It is well known that during cancer development, hypomethylation leads to oncogene activation (45). In this study, we have shown that the increased methylation at the promoter region of Sfrp2, Sfrp5 and Wnt5a was correlated with the reduced expression of these genes, suggesting that the GEN-induced methylation might be responsible for the change of gene expression. Importantly, the heatmap generated from our data analysis indicates a potential use of the methylation panel of Sfrp2, Sfrp5 and Wnt5a as a molecular marker to discriminate and identify early abnormalities during colon cancer development.

As the two major epigenome regulators, DNA methylation and histone modifications do not only work independently but instead cross-talk with each other, leading to proper configurations of chromatin structures for the initiation of transcription (39). Besides confirming the results that GEN increased the methylation of CpG at the promoter region of Sfrp5 by MSP assay, bisulfite sequencing provided the first clue of the association between DNA methylation and histone modifications on transcriptional regulations mediated by dietary GEN. Importantantly, higher methylation at the region that had decreased level of H3Ac, H3K9Me3 and H3S10P by dietary GEN was observed in GEN group, suggesting a combined effect on the regulation of Sfrp5 expression by dietary GEN. Future studies are needed to provide mechanistic explanations to GEN-mediated interactions between DNA methylation and histone modifications.

Genome-wide DNA methylation patterns are erased and re-established during early embryonic development (46). It is known that early exposure to GEN or other estrogen-like chemicals can alter DNA methylation, which persists into adulthood, affecting the physiologic development of offspring (28,47). The experiment reported in this study was designed to investigate the effect of life-long exposure including pre- and post-natal periods, as well as pre- and post-AOM, to SPI and GEN on the epigenetic patterns of Wnt genes. We have observed the significant epigenetic modifications as well as the persistence of these effects well into adult life in this model. This study provided a foundation for further investigations on the early stages of life where the epigenetic programming occurs and also for investigations on the mechanisms for the passing down of the epigenetic information to later life.

In summary, we presented the first evidence that dietary GEN maintains a cell’s normal Wnt signaling in the colon epithelium of carcinogen-injected rats through regulating the epigenetic structures of Wnt genes. The results elucidated that dietary GEN maintained the expression of Wnt genes after carcinogen exposure through the regulation of DNA methylation and histone modifications at the regulatory regions of the genes, suggesting a promising role of dietary GEN in attenuating neoplastic development at epigenetic level.

Supplementary material
Supplementary Figure 1 and Tables 1–3 can be found at http://carcin.oxfordjournals.org/

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