

## Genistein Increases Gene Expression by Demethylation of *WNT5a* Promoter in Colon Cancer Cell Line SW1116

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**Abstract.** *Genistein (GEN), one of the soy isoflavones, exhibits protection against colon cancer. The WNT signaling pathway plays a critical role in both normal epithelial regeneration and tumorigenesis in human colon. In this study it was hypothesized that GEN regulates specific genes in the WNT signaling pathway. Colon cancer cell lines DLD-1, SW480, and SW1116 were treated with Novasoy or GEN for 4 days. mRNA levels of several WNT signaling components were analyzed by real-time PCR. Methylation-specific PCR and bisulfite genomic sequencing were used to analyze the methylation status of CpG islands. Both Novasoy and GEN inhibited cell proliferation in all three cell lines. WNT5a mRNA showed a time-dependent induction by Novasoy and GEN in DLD-1 cells but only by GEN in SW1116 cells. Meanwhile, WNT5a CpG island methylation level was decreased in SW1116 by GEN. In conclusion, GEN regulated WNT5a expression, which was accompanied by a decrease in DNA methylation level at the analyzed CpG island.*

Soy contains many bioactive compounds, including isoflavones. Epidemiological studies have suggested that a higher dietary intake of soy products contributes to a lower incidence of colorectal cancer in Asian countries (1, 2). Among various soy isoflavones, genistein (4, 5, 7-trihydroxyisoflavone) (GEN) has been considered a candidate for colon cancer prevention (3-5). *In vitro* data has shown that GEN inhibits the growth of several colon cancer cells, including SW620 and DLD-1 (6-8). The inhibitory effects have been demonstrated to result from both induction of apoptosis and inhibition of proliferation (6). The mechanisms by which GEN inhibits cell growth have been related to various signaling pathways, including the Akt pathway, TGF $\beta$ /SMAD, and

general cell cycle regulation in cancer cells (6, 7, 9). GEN has also been shown to affect the antioxidant pathway and protect cells from oxidant stresses (10). Recently, GEN has been shown to inhibit the growth of both hormone-dependent and hormone-independent cancer cells through interactions with the estrogen and vitamin D receptors (11-13). Regulation by GEN at the transcriptional level includes changes of epigenetic markers such as DNA methylation and histone modifications in several types of cancer and related cancer cell lines (14-17). GEN has been found to alter epigenome patterns in rat and mouse *in vivo* animal models (18-20).

The wingless-int (WNT) signaling pathway is critical for axis formation, cell fate determination, and cell migration (21, 22). The WNT signaling family is composed of a series of secreted glycoproteins with similar and conserved sequences (21, 23). These molecules act through a variety of membrane receptors and function in canonical WNT/ $\beta$ -catenin, PCP, and/or WNT5a/Ca<sup>2+</sup> pathways (24, 25). The WNT pathway is also critical for maintaining epithelial cell differentiation (26, 27). WNT pathways play key roles in colonic stem cell maintenance and epithelial cell proliferation during normal gut development (28-30). On the other hand, aberrant WNT signaling is considered one of the most correlated factors in over 90% of both benign and malignant colorectal tumors (31). Mutated APC,  $\beta$ -catenin, and GSK-3 $\beta$  are three major factors that contribute to aberrant WNT signaling and lead to subsequent transcriptional activation of downstream target genes (25, 32). However, many epigenetic silencing and activating events have been discovered in the WNT pathway that are also related to aberrant WNT signaling, including aberrant expression of *sFRP1*, *DKK1*, and *APC* (33-36).

Recently, it has been reported that dietary GEN interacts with WNT signaling in mammary gland tumor development (37, 38). Therefore the purpose of the present study is to investigate the effect of GEN on WNT pathway regulation in colon cancer development. In this study, it was hypothesized that the effect of soy components on colon cancer might be due to alterations in WNT signaling.

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## Materials and Methods

The colon cancer cell lines DLD-1, SW480, and SW1116 were purchased from ATCC (Manassas, VA, USA). Novasoy and GEN were kindly provided by Dr. William G. Helferich (University of Illinois at Urbana-Champaign, Urbana, IL, USA). Minimum essential medium (MEM) was purchased from SCS Cell Media Facility at the University of Illinois at Urbana-Champaign (Urbana, IL, USA). Unless otherwise mentioned, all general chemicals and laboratory supplies were obtained from Fisher Scientific ([www.fishersci.com](http://www.fishersci.com)). Fetal bovine serum (FBS) and other cell culture media supplements were purchased from Mediatech (Herndon, VA, USA). Cell culture ware was purchased from Sarstedt (Newton, NC, USA). PCR primers for human *WNT3a*, *WNT5a*, *WNT7a*, and *L7a* were synthesized by Invitrogen (Carlsbad, CA, USA).

**Cell culture.** DLD-1, SW480, and SW1116 were maintained in MEM media containing 10% (v/v) FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B. Cells were incubated at 37°C in a 5% CO<sub>2</sub> and 95% air incubator. Novasoy was dissolved at a stock concentration of 200 mg/ml in DMSO. GEN was dissolved at a stock concentration of 75 mmol/l (aglycone equivalent) in DMSO. DLD-1, SW480, or SW1116 cells were plated at 0.5 million per 60 mm culture dish in regular MEM media. Fourteen h later, cells were treated with MEM media containing 0.1% DMSO (v/v), 200 µg/ml Novasoy, or 75 µmol/l GEN, and treatment media were refreshed once after 2 days.

**Quantitative real-time RT-PCR.** On days one, two and four, cells were sampled with TriReagent (Sigma-Aldrich) for total RNA isolated following the manufacturer's instructions. RNA concentrations were determined by 260 nm absorbance. For each sample, cDNA was synthesized from total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a Thermal Cycler 2720 (Applied Biosystems). In each reaction, 2 µg of total RNA were used in a 20 µl volume containing 1× RT buffer, 4 mmol/l dNTPs, 1× RT random primers, and 2.5 U/µl MultiScribe™ Reverse Transcriptase. cDNA synthesis was performed using the following program: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 s. cDNA was then analyzed by two-step real-time PCR using the 7300 real-time PCR system (Applied Biosystems) and detected with SYBR Green I. In each reaction, 25 ng of synthesized cDNA were used in a 25 µl volume containing 12.5 µl SYBR Green master mix (2x, Applied Biosystems) and 0.25 µmol/l of each primer. RT-PCR was performed using the following program: 95°C for 10 min, followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. After PCR, melting curves were acquired stepwise from 55 to 95°C to ensure that a single product was amplified in the reaction. The primers used were as follows: *WNT3a*, sense 5'-GCCAGCCACATGCAC CTCAA-3' and antisense 5'-GCGACCA CCAGCATGCTTCAC-3'; *WNT7a*, sense 5'-CTGGAGGAGAA CATGAAGCTGGAA-3' and antisense 5'-GTGTGGTCCAGCAC GTCTTGGT-3'; *WNT5a*, sense 5'-GACCACATGCAGTACATC GGAGAAG-3' and antisense 5'-TCCACCTCGATGTCGGAA TTG-3'; *L7a*, sense 5'-TTTGG CATTGGACAGGACATCC-3' and antisense 5'-AGCGGGCCAT TTCACAAAG-3'. *L7a* was used as an internal control to normalize raw data. Samples from at least three independent experiments were analyzed and presented.

**Cell growth analysis.** Cells were plated and treated as mentioned above. For each plate, on day four cells were treated with trypsin

and stained with 0.04% (w/v) Trypan Blue solution (Mediatech). Live cell numbers were then counted using a hemocytometer and normalized to those in the DMSO-treated condition. Samples from at least three independent experiments were analyzed and presented.

**Methylation-specific PCR (MSP).** Cells were plated and treated as described above. On day four, cells were scraped with 1× PBS and pelleted by centrifuging. Genomic DNA was isolated with DNeasy Tissue Kit (Qiagen, Valencia, CA, USA), DNA concentration was determined by absorbance at 260 nm. For each sample, 2 µg of total genomic DNA was treated with sodium bisulfite using the EZ Methylation-Gold kit (Zymo Research, Orange, CA, USA). An amount of 5% of final product was then used in each MSP reaction in a 50 µl volume containing 25 µl GoTaq Master Mix (2x, Promega, Madison, WI, USA) and 0.25 µmol/l of each primer. For *WNT5a*, methylated (M) and unmethylated (U) primers were designed to analyze the 5' upstream predicted CpG island between -2500 bp to exon 1 (predicted by <http://www.ebi.ac.uk/emboss/cpgplot/>). For M-primers (-448F/-326R): sense 5'-GTATTTTTTCGGAGAAAAA GTTATGC -3' and antisense 5'-AACCGCGAATTAATATAAACGTC -3'; for U-primers (-449F/-325R): sense 5'-GGTATTTTTTGGAGA AAAAGTTATGTG-3' and antisense 5'-CAACCACAAATTAAT ATAAACATC-3'. (All positions were labeled according to the transcription start site being +1). Generally, the 'C' of a methylated 'CpG' will remain 'C' after bisulfite conversion, and the 'C' of an unmethylated 'CpG' will be converted to 'U' and changed to 'T' after PCR reaction. PCR was performed in a Thermal Cycler 2720 using the following program: 95°C for 10 min; followed by 35 cycles of 95°C for 30 s, the primer Tm for 30 s and 72°C for 1 min; and 72°C for 7 min. Samples from at least three independent experiments were analyzed and presented.

**Methylation-sensitive restriction enzyme PCR (MSREP).** Cells were plated and treated as described above. On day four, cells were scraped with 1× PBS and collected by centrifugation. Genomic DNA was isolated with GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) and quantified by absorbance at 260 nm. For each sample, 160 ng of total genomic DNA was incubated with *HhaI* in each reaction in a 80 µl volume containing 8 µl 10× NEBuffer 4, 0.8 µl 100× BSA, 1 µl 20 U/µl *HhaI* as 'Cut' samples; or incubated without *HhaI* in each reaction in a 80 µl volume containing 8 µl 10× NEBuffer 4, 0.8 µl 100× BSA as 'Uncut' samples (New England Biolabs, MA, USA). The incubations were held at 37°C for 2 h. 'Cut' and 'Uncut' samples were then analyzed by real-time PCR using a 7300 real-time PCR system (Applied Biosystems) and detected with SYBR Green I. In each reaction, 10 ng of processed genomic DNA were used in a 20 µl volume containing 10 µl SYBR Green master mix (2x, Applied Biosystems) and 0.25 µmol/l of each primer. PCR was performed using the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. After PCR, melting curves were acquired stepwise from 55 to 95°C to ensure that a single product was amplified in the reaction. For *WNT5a*, MSREP primers (-461F/-303R) were designed to analyze the 5' upstream predicted CpG island: sense 5'-TGCGTTTTTCAGCGGCATCTC-3' and antisense 5'-CTGCTTCCAGACGCTGATCCTG-3'. For use in the real time PCR analysis, this set of the MSREP primers targeted the CpG sites #5 and #6 within the CpG island. Generally, methylation levels were determined by calculating the ratio of 'Cut' values to 'Uncut' values after the PCR. Samples from at least two independent experiments were analyzed and presented.

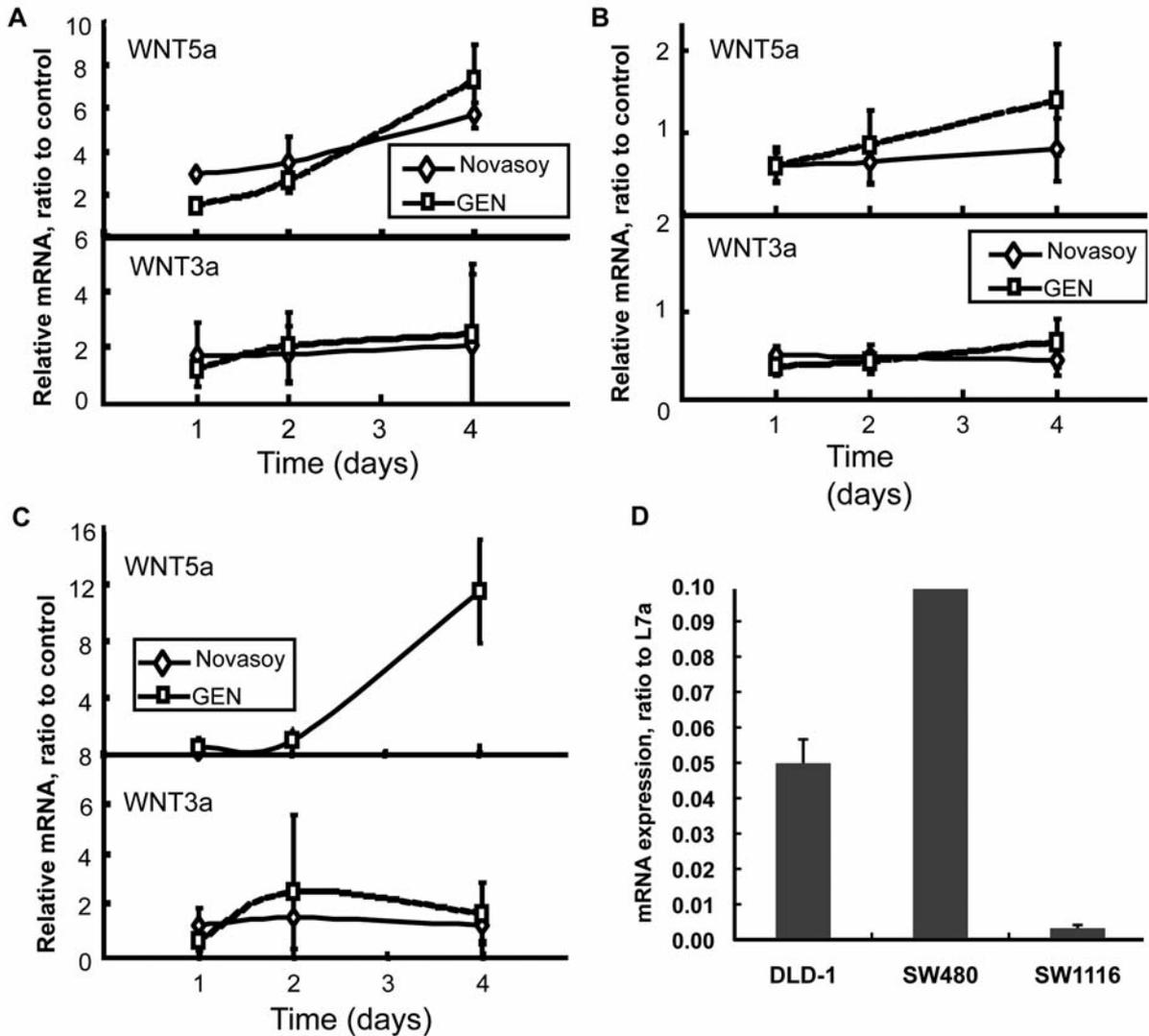


Figure 1. mRNA expression levels of *WNT* signaling molecules in (A) DLD-1, (B) SW480, and (C) SW1116 cell lines. Real-time RT PCR was performed to analyze gene expression. Data were first normalized to *L7a* (internal control), and then to DMSO control. D. Basal *WNT5a* expression in three colon cancer-derived cell lines. Data were normalized to internal control *L7a*. Samples from at least three independent experiments were analyzed and are presented as the mean  $\pm$  S.E.M.

**Bisulfite genomic sequencing (BGS).** Bisulfite sequencing primers were designed for a putative 5' upstream CpG island (predicted by <http://www.ebi.ac.uk/emboss/cpgplot/>) for the *WNT5a* gene. The primers (-500F/-264R) used were: sense 5'-TAATTTGGGGTTGATTTTT GTAGTT-3' and antisense 5'-ATCTCCAACCTCCTCTCTAAATC-3'. A total of 5% of the bisulfite-treated product was amplified following the same methodology as mentioned in the MSP section. PCR products were visualized by ethidium bromide staining after size-fractionation on a 1.0% agarose gel. PCR products were gel purified using Wizard SV Gel and PCR purification system (Promega). Purified DNA fragments were cloned into TOPO vector (Invitrogen). Plasmid DNA was isolated using Wizard Plus SV Miniprep DNA purification system (Promega). Clones were confirmed by restriction enzyme digestion with EcoRI and sequenced at the Biotechnology Center at the University of Illinois at Urbana-Champaign (Urbana, IL, USA).

**Statistical analysis.** Each data point represents the mean and calculated standard deviations from the mean (S.E.M.). *P*-values were calculated using Student's *t*-test and an asterisk indicates  $p < 0.05$ .

## Results

**Responses of *WNT* signals in colon cancer cell lines treated with Novasoy or GEN.** Previous studies have shown that *WNT5a* and *WNT7a* are genes responsive to GEN (46, 51). DLD-1, SW480, or SW1116 cells were incubated in regular MEM media containing Novasoy, GEN or DMSO control. Samples were taken at different points throughout the time course (one, two, or four days). Expression of *WNT5a* was significantly increased in DLD-1 cells after 4

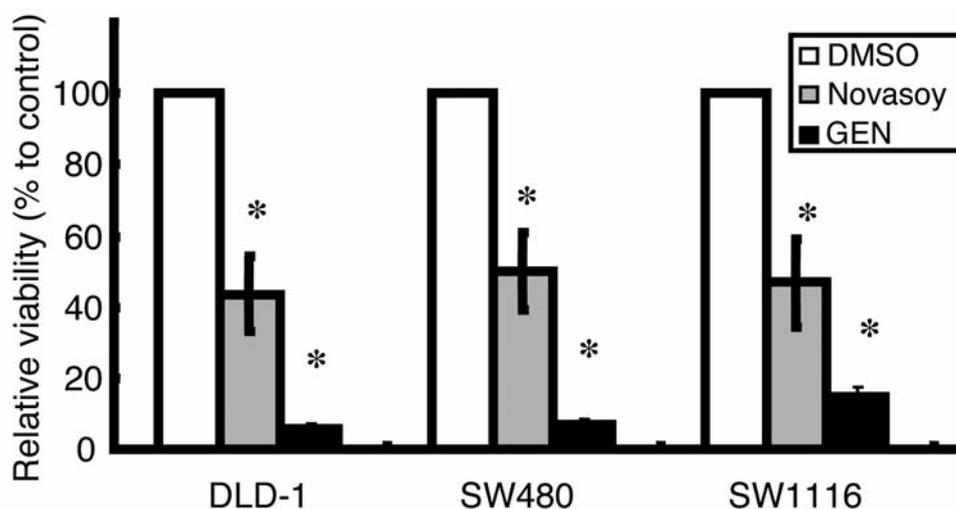


Figure 2. Cancer cell growth was retarded by soy component treatments. On day 4, viable cell numbers from each cancer cell line were counted. Data were presented as the percentage of the DMSO control in each cell line. Samples from at least three independent experiments were analyzed and presented as the mean  $\pm$  S.E.M. Asterisks indicate statistically significant difference at  $p < 0.05$  relative to the DMSO control.

days of treatment with Novasoy or GEN ( $p < 0.05$ ) when compared to DMSO (Figure 1A). In SW1116, GEN induced the highest response of *WNT5a* expression (Figure 1C,  $p < 0.05$ ). The treatments did not show any effect on *WNT5a* expression in SW480 cells (Figure 1B). The treatments did not have any effects on either *WNT7a* (data not shown) or *WNT3a* expression in any of the cell lines. It is worth noting that SW1116 had the lowest *WNT5a* level among the three cell lines when the basal mRNA expression was examined without any soy treatment (Figure 1D).

*Both Novasoy and GEN treatments inhibited cell proliferation in colon cancer cell lines.* DLD-1, SW480, and SW1116 cells were incubated in regular MEM media containing Novasoy, GEN, or DMSO control. Cell numbers were counted on day four. Both Novasoy and GEN significantly decreased cell numbers in all three colon cancer cell lines compared to DMSO. GEN showed a much stronger inhibition on cell growth than Novasoy on day four (Figure 2).

*Demethylation of WNT5a in SW1116.* Previous studies have shown that *WNT5a* is differentially methylated in colon cancer cell lines (55). The DNA methylation status of *WNT5a* in response to GEN treatment was therefore examined in this study. DLD-1, SW480, or SW1116 cells were incubated in regular MEM media containing Novasoy, GEN, or DMSO control. Samples were taken at day four. Methylation-selective PCR was performed using primers that selectively amplify methylated vs. unmethylated genomic DNA after bisulfite conversion (Figure 3A). Gel electrophoresis showed

that the band from the unmethylated state was significantly increased in SW1116 cells treated with GEN compared to DMSO on day four (U, Figure 3B). There was no detectable signal from DLD-1 or SW480 cells for the methylated PCR (Figure 3B).

*Methylation-sensitive restriction enzyme PCR (MSREP) data confirmed the demethylation effect of GEN on WNT5a in SW1116.* Genomic DNA was digested using *HhaI* and quantified by real-time PCR using primers covering CpG #5 and #6 as shown in Figure 3A. Methylation levels were calculated as the ratio of PCR product from 'Cut' samples to the 'Uncut' samples. GEN significantly reduced *WNT5a* methylation level in SW1116 (Figure 3C).

*Bisulfite genomic sequencing (BGS) indicated reduced methylation in the promoter region of WNT5a in SW1116.* The DNA methylation level was further examined by bisulfite sequencing of the promoter region for *WNT5a* (Figure 3A: BGS indicates the primer locations for the bisulfite sequencing). Each individual CpG was analyzed after bisulfite conversion of the genomic DNA and subsequent PCR amplification and cloning. Results in DLD-1 and SW480 showed that there was little DNA methylation in the targeted region and that there was no difference in DNA methylation between cells treated with GEN or DMSO for four days (Figure 4A). On the other hand, SW1116 cells showed ~20% CpG methylation in this region at the *WNT5a* promoter (Figure 4B). Furthermore, GEN treatment significantly reduced the methylation level in SW1116 cells (Figure 4B).

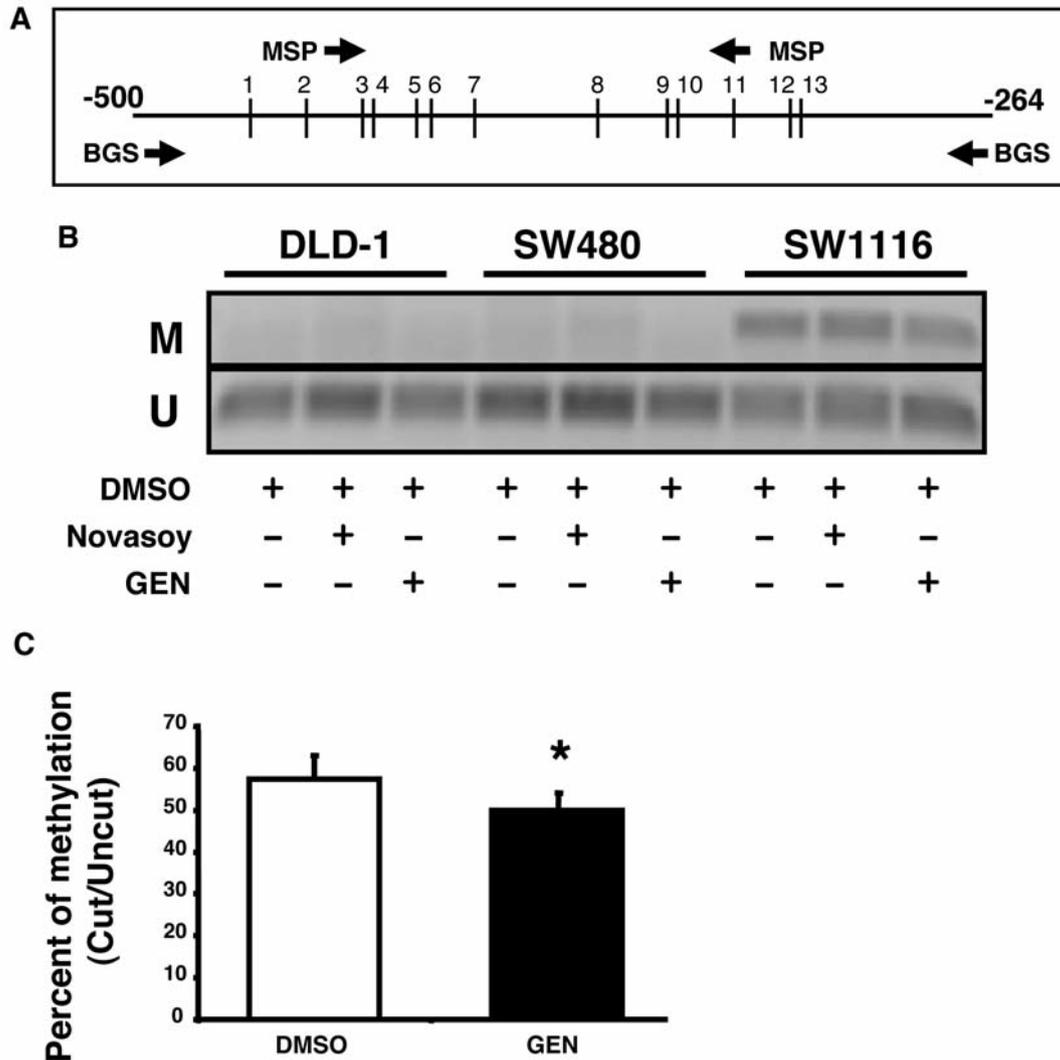


Figure 3. *WNT5a* promoter CpG methylation changes induced by soy component treatments in the three cell lines. **A**: Schematic drawing of the predicted CpG island at the *WNT5a* promoter (-500 to -264). Vertical lines with numbers represent individual CpG sites within the CpG island. Black arrows indicate primer locations and directions for methylation selective PCR (MSP) and bisulfite genomic sequencing (BGS). **B**: Methylation-specific PCR (MSP) of the *WNT5a* promoter. MSP was performed using genomic DNA treated with sodium bisulfite and then amplified by PCR with methylation-specific (M) or unmethylation-specific (U) primers. Methylated and unmethylated products were visualized by ethidium bromide staining on an agarose gel and quantified. **C**: Methylation-sensitive restriction enzyme PCR (MSREP) of *WNT5a* in SW1116. Genomic DNA was digested using *Hha*I and quantified by real-time PCR using primers covering CpG # 5 and #6. Methylation levels were calculated as the ratio of PCR product from 'Cut' samples to the 'Uncut' samples. Samples from at least three independent experiments were analyzed and presented. Each bar represents the mean  $\pm$  S.E.M. Asterisks indicate statistically significant difference at  $p < 0.05$  relative to the DMSO control.

## Discussion

The purpose of this study was not only to examine the effects of GEN on the WNT signaling pathway in the human colon cancer cell lines DLD-1, SW480, and SW1116, but also to investigate the potential mode of action by which GEN inhibits cell proliferation. This study showed that: (i) GEN treatment selectively induced *WNT5a* expression in specific colon cancer cell lines; (ii) the DNA methylation level at the

*WNT5a* promoter is variable in different colon cancer cell lines; and (iii) in colon cancer cell line SW1116, GEN treatment specifically induced demethylation of the *WNT5a* promoter CpG island, effectively reducing the methylation level of *WNT5a* in SW1116.

*WNT5a* can act through different membrane receptors and participate in both canonical and non-canonical WNT pathways (25, 39, 40). The multiplicity of its interactions results in the diverse, and sometimes contradictory, roles of

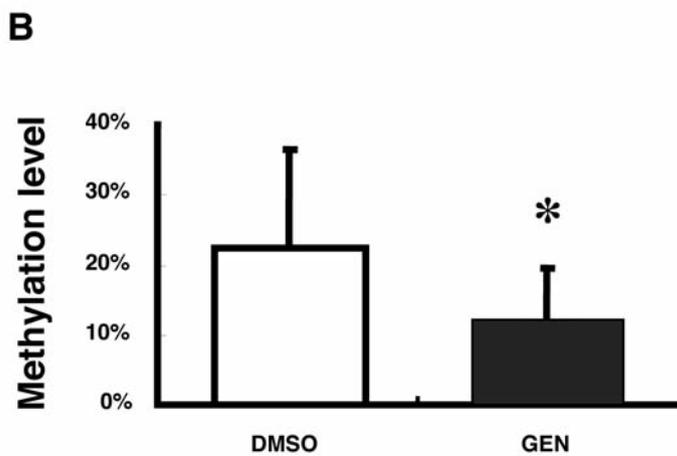
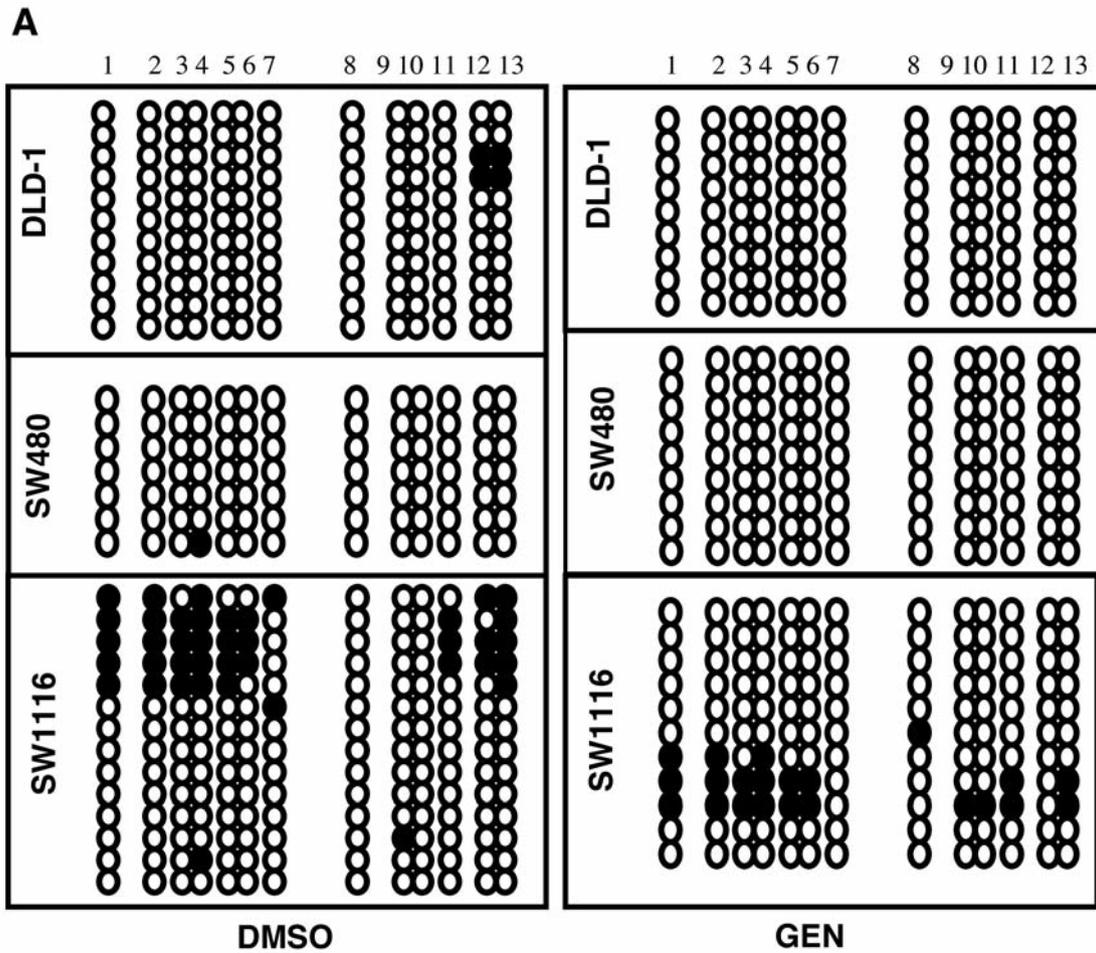


Figure 4. Bisulfite genomic sequencing of *WNT5a* upstream CpG island. A: PCR was performed using genomic DNA treated with sodium bisulfite and BGS primers. PCR products were cloned and sequenced. Each open circle represents an unmethylated CpG while a filled circle represents a methylated CpG. Each row of circles represents an individual clone used for sequencing. Each CpG is numbered according to descriptions in Figure 3A. B: Quantification of BGS results in the SW1116 cell line. Samples from at least two independent experiments were analyzed and data are presented as the mean±S.E.M. Asterisks indicate statistically significant difference at  $p<0.05$  relative to the DMSO control.

*WNT5a* in cancer development (41). In colon cancer, it is reported that lower *WNT5a* expression is closely correlated with poor prognosis in patients (42). Interestingly, in the present study it was obvious that in SW1116, a cell line derived from an early-stage tumor (43), *WNT5a* showed the lowest expression compared to other more advanced tumor cell lines. With the novel finding that *WNT5a* mRNA expression was up-regulated by GEN in this early-stage colon cancer cell line, further studies should focus on the targeted up-regulation of *WNT5a* by GEN as the potential mechanism by which GEN inhibits colon cancer development.

From the present study, it is clear that soy isoflavones significantly inhibit growth of various colon cancer cell lines. Moreover, GEN alone is more active than the complex isoflavone Novasoy. Although at 200 ppm Novasoy contains the same amount of GEN (75  $\mu\text{mol/l}$ ) as the GEN treatment alone, the combination of isoflavones was not as effective as GEN alone in growth inhibition of the colon cancer cells. This might be due to potential interactions between GEN and other isoflavones such as daidzein (7, 44). A similar observation was made previously in an *in vivo* animal model for mammary epithelial cells, where dietary soy protein isolates with a combination of isoflavones did not induce the same response from the WNT signaling pathway as GEN did alone (37). Apparently, the modest growth inhibition and the differential *WNT5a* expression pattern caused by this combination of isoflavones may represent an entirely different mechanism that regulates colon cancer development.

This is the first study focusing on GEN and its regulation of the WNT signaling pathway in colon cancer cells. It has been shown from previous studies that GEN interacts with many signaling pathways, including NF- $\kappa$ B and Akt, and that it reduces *WNT7a* mRNA expression in Ishikawa endometrial adenocarcinoma cells (10, 37, 45). It has been reported that in the rat mammary gland epithelium, dietary GEN induced gene expression of a WNT signaling antagonist, sFRP2, and reduced *WNT5a* expression {Su, 2007 #446}. The same group later reported that in mammary epithelial cells, GEN treatment, through interaction with E-cadherin, attenuated both WNT signaling and cell proliferation (38). In the current study, GEN appeared to have complex effects on *WNT5a* signaling in a cell line-specific manner. SW1116 was established from a tumor that was categorized as early stage according to Duke's classification, before the modified TNM staging system took effect (43). *WNT5a* expression was up-regulated in this early-stage cell line. On the other hand, *WNT5a* was not affected by GEN in SW480, a cell line established from a more advanced tumor. The differential effect should be studied further to ascertain the potential benefit of soy GEN on prevention of colon cancer in its early stages.

Previous studies have suggested several candidate mechanisms by which GEN prevents cancer, one of which is

*via* interactions with several signaling pathways (10, 46) and resulting downstream cellular events, such as transcriptional activation and posttranslational modification (47, 48). Recently, it has been shown in various cancer types that GEN induces gene expression of critical factors by reducing DNA methylation levels, leading to reactivation of tumor suppressor genes (49-53).

The current results support the notion that GEN serves as a natural demethylation agent and that it is specifically effective on colon cancer cells from early-stage colon cancer. The GEN treatment affected the DNA methylation of *WNT5a*. It has been shown that *WNT5a* down-regulation is correlated with hypermethylation of its promoter in human colon cancer patients (54, 55). The present study further showed that down-regulation by promoter hypermethylation occurs in cell lines from earlier stages of colon cancer but not in cell lines from later stages. This suggests possibilities for further research into the development of early intervention strategies that target *WNT5a* silencing by DNA methylation.

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None of the Authors have any conflict of interest.

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