Genistein Affects Histone Modifications on Dickkopf-Related Protein 1 (DKK1) Gene in SW480 Human Colon Cancer Cell Line

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Abstract
Genistein (GEN) is a plant-derived isoflavone and can block uncontrolled cell growth in colon cancer by inhibiting the WNT signaling pathway. This study aimed to test the hypothesis that the enhanced gene expression of the WNT signaling pathway antagonist, DKK1, by genistein treatment is associated with epigenetic modifications of the gene in colon cancer cells. Genistein treatment induced a concentration-dependent G2 phase arrest in the human colon cancer cell line SW480 and reduced cell proliferation. Results from several other human colon cancer cell lines confirmed the growth inhibitory effects of genistein. Overexpression of DKK1 confirmed its involvement in growth inhibition. Knockdown of DKK1 expression by siRNA slightly induced cell growth. DKK1 gene expression was increased by genistein in SW480 and HCT15 cells. DNA methylation at the DKK1 promoter was not affected by genistein treatment in all the cell lines tested. On the other hand, genistein induced histone H3 acetylation of the DKK1 promoter region in SW480 and HCT15 cells. This indicates that increased histone acetylation is associated with the genistein-induced DKK1 expression. The association between histone acetylation and DKK1 gene expression is confirmed by the histone deacetylase inhibitor trichostatin A (TSA) treatment. In conclusion, genistein treatment decreases cell growth and proliferation in colon cancer cell lines. The effect is associated with the increased DKK1 expression through the induction of histone acetylation at the DKK1 promoter region.

Introduction
Soy contains various bioactive components, which have received much attention in their potential ability to reduce cancer risk [1,2]. Epidemiological studies showed that consuming higher levels of dietary soy products contributes to the lower incidence of colorectal cancer in Asian countries [3,4,5]. Specifically, genistein (4, 5, 7-trihydroxy-isoflavone), a natural isoflavone abundant in soy, has been shown to reduce colorectal cancer risk [6,7]. These studies provide strong evidence for the need to further investigate the mechanisms behind genistein’s anticancer potential.

In cell culture studies, genistein has been reported to alter cell physiology in several colon cancer cell lines. A recent study performed by Fan et al. identified that colon cancer cells had changed morphology, including chromatin condensation and nuclear fragmentation after genistein treatment [8]. In addition, higher concentrations of genistein of >10 μmol/L significantly induced inhibition of cell proliferation and DNA fragmentation in a human colon cell line [9]. Moreover, in colon cancer cell lines Caco-2 and SW620, cell death was induced by soybean extract treatment [10]. Therefore, genistein is becoming a promising compound in colon cancer prevention and treatment. In the present study, we have further explored the antitumor properties of genistein by testing cell cycle progression and cell proliferation in several colorectal cancer cells in response to treatment with increasing concentrations of genistein.

Various pathways and mechanisms have been proposed to be responsible for genistein’s ability to reduce cancer risk. IGF-IR signaling, the AKT pathway and cell growth regulation are associated with the antitumor effects of genistein [9,11]. Additionally, genistein also possesses antioxidant properties by mimicking estrogen via estrogen receptor-mediated phosphorylation of ERK1/2 and activation of the NFkB signaling pathway [12]. Further reports from recent studies in animals indicated that genistein inhibited hormone-dependent or -independent cancer cells by regulating interactions between vitamin D and estrogen receptor [13,14,15]. Genistein regulates gene transcription in various cancer cell lines by epigenetic regulations, e.g. DNA methylation and histone modifications [16,17,18]. Genistein alters the DNA methylation of various genes in rat and mouse models [19,20]. However, the mechanisms behind genistein’s role in cell proliferation or apoptosis during carcinogenesis remains poorly understood.

The Wingless-int (WNT) signaling pathway comprises a large number of growth factors that are involved in organogenesis, proliferation, regeneration, cell fate determination and cell-cell adhesion [21,22]. WNT proteins bind to Frizzled receptors (FRZ) and low-density lipoprotein receptor-related protein (LRP) co-receptors, causing cytosolic β-catenin stabilization and accumulation. Accordingly, nuclear β-catenin increases and complexes with TCF/LEF transcription factors, leading to the increased tran-
scription of target genes, including cyclin D1 [23]. Aberrant WNT signaling is one of the contributors for the transition from normal colonic epithelium to malignant tumor cells [24,25]. Genistein was recently reported to suppress WNT signaling in colon cancer cell lines [26,27], which provides one potential mechanism for genistein’s anticancer capacity.

One of the key regulators of the WNT signaling pathway is its antagonist Dickkopf 1 (DKK1). DKK1 prevents β-catenin-mediated signal transduction by binding to LRP and Kremen proteins, promoting cells differentiation and apoptosis [28,29,30,31,32]. The silencing or loss of DKK1 has been documented in various diseases. In colorectal cancer silenced DKK1 expression is tightly associated with microsatellite instability of tumor subtypes [33]. It is reported that the tumor suppressing capacity of DKK1 is repressed by the hypermethylation of its promoter in the advanced stages of colorectal cancer [34]. In human renal carcinoma cells, chemically inducing histone acetylation at the DKK1 promoter resulted in the re-activation of DKK1 expression, demonstrating that in addition to DNA methylation, histone tail modifications might also contribute to the transcriptional control of critical genes related to cancer development [35].

In the present study, we investigated the anticancer properties of genistein by identifying its effects on cancer cell physiology and examining the mechanistic basis of DKK1 activation. In particular, this study is among the first to link genistein-mediated epigenetic modifications of DKK1 to the anticancer properties of genistein in colorectal cancer.

Results

Genistein Treatment Selectively Induced DKK1 Gene Expression but did not Alter DKK1 Promoter Methylation Patterns

To investigate the correlation between DNA methylation at the DKK1 promoter CpG island and the effect of genistein treatment on DKK1 expression, real-time RT-PCR was performed to measure DKK1 mRNA expression and methylation specific polymerase chain reaction (MSP) and bisulfite sequencing (BSF) of the DKK1 promoter region were performed to examine the promoter methylation status (Fig. 1). First, DKK1 gene expression was analyzed in available colon cancer cell lines to investigate the effects from genistein treatment. Among the cell lines tested, SW480 and HCT15 cells showed induction of DKK1 gene expression by genistein (Fig. 1A). Analysis of the DNA methylation at the DKK1 promoter region confirmed that DKK1 gene expression in general is inversely related to the methylation level of the region tested (~159/+109, Fig. 1B, 1C, and 1D). Specifically, RKO, SW48, and DLD-1 cells exhibited very high, if not complete, methylation of the region (Fig. 1C, and 1D). This hypermethylation is inversely related to the level of DKK1 gene expression as shown in Fig. 1A. For the DKK1-expressing cell lines HCT15, HT29, and SW480, there is minimal DNA methylation observed (Fig. 1C and 1D). More importantly, genistein treatment did not alter the DNA methylation of the DKK1 promoter region in any cell lines tested, regardless of the level of DNA methylation in the region (Fig. 1C and 1D).

Genistein Dose- and Time-dependently Induced DKK1 Gene Expression

Real-time RT-PCR was performed to measure DKK1 mRNA expression levels in response to different genistein doses and exposure times. Overall, DKK1 expression was increased by genistein in a dose-dependent manner (Fig. 2A). After 2 d of genistein treatment, DKK1 mRNA expression was 3-fold and 8-fold higher following the 50 and 75 μmol/L genistein treatments respectively, when compared to the no-genistein control (p<0.05, Fig. 2A). The mRNA level of DKK1 in SW480 cells was also increased by genistein treatment (75 μmol/L) in a time-dependent manner (Fig. 2B) and reached a >10-fold induction at d 4 compared to the level in the DMSO control.

The induction of DKK1 was confirmed by measuring its protein expression level in SW480 cells (Fig. 2C). DKK1 protein expression showed a 3-fold induction by 75 μmol/L of genistein treatment in SW480 cells following a 4-d treatment when compared to the control.

Genistein Inhibited Cell Cycle Progression in SW480 Cells

To investigate the effect of genistein on cell cycle progression of SW480 cells, the DNA content of SW480 cells was measured by flow cytometry analysis. Histograms showed that the population of cells at G1 was markedly decreased by 75 μmol/L of genistein treatment compared to the no-genistein control, with a decrease of cells in the S phase (Fig. 3A). On the other hand, genistein treatment resulted in an accumulation of cells at G2/M phase in SW480 cells.

The effects of genistein on cell cycle progression were further analyzed by treatment of cells with genistein in concentrations from 1 to 75 μmol/L. Percentage of cells in different phases was calculated as the ratio of gated cells to the total cell population. Genistein concentrations above 5 μmol/L increased the percentage of cells at G2/M phase significantly (p<0.05) with the highest accumulation observed at 75 μmol/L of genistein (p<0.05, Fig. 3B). The results demonstrate that the percentage of cells in the G1 phase was abolished following 75 μmol/L of genistein treatment (Fig. 3B). The percentage of S phase cells was significantly reduced (p<0.05) following 50 and 75 μmol/L genistein treatments after a transient increase by 25 μmol/L genistein when compared to the no-genistein control. Taken together, cell cycle progression in SW480 cells was regulated by genistein in a dose-dependent manner.

Genistein Inhibited Cell Proliferation in SW480 and Several Other Colon Cancer Cell Lines

Cell proliferation was tested by the WST-1 assay, which showed that genistein treatments decreased the cell numbers of SW480 cells in a dose-dependent manner (Fig. 3C). At genistein concentrations above 15 μmol/L cell numbers were significantly decreased when compared to the no-genistein control (p<0.05). This anti-proliferative effect of genistein was confirmed in several colon cancer cell lines (Figure S1). The AnnexinV-propidium iodide assay showed no change by any of the genistein treatments, indicating that genistein does not affect the apoptosis rate (data not shown). These results indicate that growth-suppressing effect of genistein is generalizable to the common human colon cancer cell lines.

Cyclin D1 mRNA Expression Decreased following Genistein Treatment in SW480 Cells

Cell cycle control genes p21, Cyclin D1 and c-MYC are highly correlated to the regulation of cell growth. Based on the data presented above, cell cycle arrest occurred in G2 in SW480 cells by genistein treatment. To further investigate the effects of genistein on the cell cycle progression, the mRNA expression of cell cycle control genes p21, Cyclin D1 and c-MYC were measured by real-time RT-PCR. Our results showed that the expression of Cyclin D1 was decreased significantly (p<0.05) by 50 and
75 μmol/L of genistein compared to control (Fig. 4, p<0.05). The expression of p21 and c-MYC was not affected by any of the genistein treatments. The decreased expression of Cyclin D1 is in agreement with the cell cycle data from flow cytometry showing that cell cycle progression was inhibited by genistein treatment.

Overexpression of DKK1 Inhibited Cell Cycle Progression and Cell Proliferation in SW480 Cells

To investigate the potential role of DKK1 in cell cycle progression, SW480 cells were transfected with a pCMV-XL5 plasmid containing human DKK1. Overexpression of DKK1 was confirmed by RT-PCR analysis of mRNA and western blot analysis of protein (Fig. 5A and 5B). Analysis of Cyclin D1 expression showed that overexpression of DKK1 induced similar repression of the gene expression as the genistein treatment (Fig. 5A). Overexpression of DKK1 significantly increased the percentage of cells in the G2/M phase when compared to the vector control (p<0.05, Fig. 5C), although to a less extent compared to the genistein treatment. Meanwhile, the percentage of cells in the G1 phase was significantly decreased in cells
overexpressing DKK1 (p<0.05), and there was no significant change in the number of cells in the S phase. Results from the WST-1 assay showed that overexpression of DKK1 decreased the proliferation of SW480 cells, although to a much less extent compared to the genistein treatment (p<0.05, Fig. 5D). Overall, the overexpression of DKK1 produced similar effects on inhibiting cell progression and cell proliferation as did the genistein treatment, suggesting a role of DKK1 in these cellular events.

**Knockdown of DKK1 in SW480 Cells Partially Reversed the Changes of Cellular Profile Caused by Genistein Treatment**

To investigate whether the alteration of cellular properties of SW480 caused by genistein is dependent on the DKK1 gene expression, siRNA targeting DKK1 was transfected into SW480 cells followed by genistein treatment. Real-time RT-PCR showed that siRNA effectively reduced DKK1 expression in SW480 in both control and genistein treatments (p<0.05, Fig. 5E). Moreover, the reduction of Cyclin D1 mRNA expression by genistein treatment as observed in non-specific siRNA-treated cells (N/S si) was abolished by the knockdown of DKK1 (DKK1 si, Fig. 5E). Meanwhile, although the growth-inhibitory effect from genistein is observed in both N/S siRNA and DKK1 siRNA groups, knockdown of DKK1 significantly increased the proliferation of SW480 cells compared to that of control siRNA in genistein treatment (p<0.05, DKK1 siRNA vs N/S siRNA in genistein treatments, Fig. 5F).

**Genistein Induced Histone Acetylation within the DKK1 Gene in SW480 and HCT15 Cells**

ChIP was performed to analyze the chromatin structure at the promoter (+96/+28), coding (+635/+742), and 5’ upstream control regions (−2781/−2713) of the DKK1 gene (Fig. 6A). The 5’ upstream control region was used as a negative control to show the relative inactive region during transcription. DLD-1 cells were used as a negative control to show the chromatin status when the DKK1 gene expression is silenced and not responsive to genistein treatment. Normal rabbit IgG was used as an internal control antibody and any DNA binding similar to the binding in the control IgG was considered to be non-specific. Increased RNA polymerase II (PolII) binding at the DKK1 promoter region indicated increased transcription of the DKK1 gene in SW480 cells in response to genistein (p<0.05, Fig. 6B). In contrast, there was minimal PolII binding within the DKK1 gene in DLD-1 cells, confirming the silenced transcription of the gene in DLD-1 cells (Fig. 6B).

To determine whether the increased transcription level of the DKK1 gene in response to genistein was modulated by alterations of chromatin structure, antibodies against acetylated, methylated, or phosphorylated histones were used in the ChIP assay. Histone modifications at the three representative regions of the DKK1 gene were first examined in SW480 cells after the genistein treatment, using DLD-1 cells as the negative control. Increases in acetylated histone H3 at the promoter and coding regions of DKK1 were observed in SW480 cells after genistein treatment (p<0.05, Fig. 6B, H3Ac), with no significant acetylation of histones in DLD-1 cells before or after genistein treatment (Fig. 6B). The abundance of the other acetylated histone tested, the acetylated histone H4, was not affected by genistein treatment in either cell lines (H4Ac).

Although there was a much higher level of dimethyl histone H3 lysine 4 in SW480 cells compared to DLD-1 cells, genistein treatment did not affect the methylation status of this histone H3 residue (Fig. 6B, H3K4Me2). Phosphorylation of histone H3 at serine 10 was also investigated and the result showed that there was a modest decrease at various regions of the DKK1 gene by genistein treatment in all cell lines tested (p<0.05, Fig. 6B, H3S10p). In summary, the active transcription of the DKK1 gene was associated with the increased histone H3 acetylation at its gene region, which likely resulted in the recruitment of PolII to its promoter.

**DKK1 mRNA Expression was Induced by Histone Deacetylase (HDAC) Inhibitor in SW480 Cells**

TSA is a histone deacetylase (HDAC) inhibitor that interacts with most of the HDAC family members and induces acetylation of histones. To confirm the effects of genistein-induced histone acetylation on DKK1 transcription, we treated SW480 cells with a series of TSA concentrations and compared the results to genistein treatment. The mRNA expression of DKK1 was increased significantly by the TSA treatment in a dose-dependent manner, similar to the result observed from genistein treatment (p<0.05, Fig. 7).

**Discussion**

The present study intended to identify the potential mechanisms of the antitumor effects of genistein. We observed that both mRNA and protein levels of DKK1 were upregulated by genistein treatment. Our results demonstrated that genistein treatment induced cell cycle arrest and inhibited cell proliferation in SW480 colon cancer cells. This was later confirmed in several other colon cancer cell lines. Both overexpression and knockdown of DKK1 confirmed the involvement of DKK1 in the inhibition of cell cycle progression and cell proliferation. Our result demonstrated that increased DKK1 expression might be one of the reasons that caused the cell cycle arrest and inhibition of cell proliferation by genistein treatment. Furthermore, comparison between the DKK1-expressing cells including HCT15, HT29 and SW480, and the DKK1-repressed cells including RKO, SW48 and DLD-1 showed that gene transcription of DKK1 is inversely related to its promoter methylation status. Genistein does not affect DNA methylation of the DKK1 promoter. On the other hand and more importantly, we have made the observation that genistein induces histone acetylation within the DKK1 gene and this is correlated with the activation of its gene transcription.
The anticancer roles of genistein, including promotion of apoptosis, inhibition of cell cycle progression, cell proliferation and invasion, the blockage of angiogenesis and metastasis in various types of cancers have been well documented [7,36,37,38]. The molecular mechanisms behind these anticancer functions of genistein include modulations of cell cycle inhibitors, the regulation of apoptosis-related genes, as well as the inhibition of IGF-IR and PI3K/AKT signaling [11,39,40,41]. Work by our

Figure 3. Effects of genistein treatment on cell physiology in SW480. SW480 cells were treated with various concentrations of genistein for 2 d before sample collection. A) Representative DNA histograms of genistein treatment of 0 and 75 μmol/L by flow cytometry. Y-axis represents the relative DNA content and x-axis shows cell cycle stages. B) Cell cycle analysis of genistein treated cells. The percentage of cells in G1 (○), S (▲) or G2/M (■) is shown. Data represent the means ± SEM of three independent experiments. C) WST-1 proliferation assay. WST-1 signals from each well were read at 450 nm for absorbance against a reference wavelength of 630 nm. Absorbance was converted to actual cell numbers using a standard generated by serial dilutions of a known number of cells. Three independent cell samples were analyzed and presented as the mean ± SEM. Asterisks (*) indicate statistical significance compared to 0 μmol/L of genistein (p<0.05).

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group has shown that genistein inhibited another critical pathway in colon cancer development, the WNT/β-catenin pathway, by interfering with the promoter methylation of specific genes. Specifically, genistein induced WNT5a expression by decreasing methylation within the gene’s promoter region [27]. Genistein also inhibited the WNT/β-catenin pathway by activating another WNT antagonist, sFRP2, by demethylating CpG islands within the gene [26]. The current study expanded the knowledge to include DKK1 as another factor in WNT signaling that mediates the response from genistein treatment. As a repressor of cancer cell growth, DKK1 is a main antagonist that interferes with the WNT pathway and downregulates the expression of the downstream target genes including Cyclin D1 [28,42,43].

The expression of DKK1 was silenced by promoter hypermethylation in the advanced stage of colon cancer (Dukes’ C and D) [34]. In DLD-1 cell line from Dukes’ C, a more advanced stage of colon cancer, demethylation of the DKK1 gene by 5-aza-2'-deoxycytidine, a DNMT inhibitor, re-activated DKK1 expression [34,44]. Since genistein has been shown to activate genes through CpG demethylation [19], we first tested the possibility of DKK1

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**Figure 4.** mRNA expressions of p21, Cyclin D1 and c-MYC by genistein treatments. SW480 cells were treated with 0, 1, 5, 15, 25, 50 or 75 μmol/L of genistein for 2 d. The mRNA expression levels of p21, Cyclin D1 and c-MYC were measured by RT-PCR. L7a was used as the internal control. Three independent experiments were analyzed and presented as the mean±SEM. Asterisks (*) indicate statistical significance compared to Control, 0 μmol/L genistein (p<0.05).

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Figure 5. Overexpression and silencing of DKK1 in SW480 cells. pCMV vector containing human DKK1 gene was used to transfect cells before sample collection for analysis. Empty pCMV vector was used as the control for the overexpression experiments. Three independent cell samples were analyzed and presented as the mean ± SEM. A) mRNA expression of DKK1 and Cyclin D1 in regular and DKK1-transfected SW480 cells. The mRNA expression was analyzed by RT-PCR. Data were normalized to internal control L7a. B) DKK1 protein expression in regular cells and transfected SW480 cells.
cells. Whole cell protein extracts were collected from transfected SW480 cells and western blot analysis of DKK1 was performed as described in materials and methods. A representative blot is shown and the quantification represents the mean ± SEM from 3 independent dishes. Actin was used as the loading control. C) Cell cycle analysis of regular and DKK1-transfected SW480 cells. Result was obtained by flow cytometry. Y-axis represents % of gated cells and x-axis shows different cell cycle stages, including G1, S and G2/M. D) WST-1 proliferation assay in vector- and DKK1-transfected SW480 cells. WST-1 signals were converted to actual cell numbers using a standard generated by serial dilutions of a known number of cells. Data were normalized to control for genistein treatment and vector for DKK1 transfection, respectively. Asterisks (*) indicate statistical significance compared with the respective control group (genistein to control; DKK1 to vector; p < 0.05). For the knockdown of DKK1, siRNA duplexes against human DKK1 gene were transfected into SW480 cells. Scrambled sequences were used as the control siRNA. E) mRNA expression of DKK1 and Cyclin D1 in control siRNA (N/ S si) and DKK1 siRNA (DKK1 si)-treated SW480 cells by control and genistein treatments. The mRNA expression was analyzed by RT-PCR. Data were normalized to internal control L7a. F) WST-1 proliferation assay in control siRNA and DKK1 siRNA SW480 cells. WST-1 signals were converted to actual cell numbers using a standard generated by serial dilutions of a known number of cells. Asterisks (*) indicate statistical significance compared to Control 0 μmol/L genistein (p < 0.05). The bracket indicates statistical difference between the N/S si and DKK1 si groups treated with genistein (p = 0.002).

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Cell Culture and Treatments

The human colon cancer cell lines SW480, DLD-1, HCT15, HT29, RKO and SW48 were purchased from ATCC (Manassas, VA). Minimum essential medium (MEM) was manufactured by the Cell Media Facility at the University of Illinois. Unless otherwise mentioned, all general chemicals and laboratory supplies were obtained from Fisher Scientific (www.fishersci. com). Cell culture ware was purchased from Sarstedt (Newton, NC). SW480 and DLD-1 cells were cultured in MEM, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (ABAM) at 37°C in a humid incubator with 5% CO2. All experiments were performed using cells with 2–6 passages.

For testing the effects of genistein dosage, SW480 cells were treated in triplicate with genistein-containing media of 0, 1, 5, 15, 25, 50 or 75 μmol/L for 2 to 4 d, and cell samples were collected for mRNA expression, cell cycle and cell proliferation analysis. For western blot, DNA methylation, and ChIP analysis, cells were treated with 75 μmol/L of genistein (GEN75).

Quantitative Real-time PCR

Following genistein treatment, cells were harvested in TRI-Reagen (Sigma-Aldrich, St Louis, MO) according to the manufacturer’s instructions. mRNA concentration was measured by SmartSpec Plus spectrophotometer (BioRad laboratories Inc, Irvine, CA) at 260 nm. Total mRNA (2 μg) was used for cDNA synthesis using High Capacity cDNA Reverse transcription Kit (Applied Biosystems, Foster City, CA) in a DNA 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). The program was as follows: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 s. After the reaction, 25 ng of samples were used for quantitative real-time PCR and gene expression levels were determined using
2x Perfecta SYBR Green fast master mix (Quanta BioSciences, www.vwr.com) in a 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Primer sequences for the experiment were designed using Vector NTI software (Invitrogen Corporation, Carlsbad, CA) and primers were synthesized by Integrated DNA Technologies (www.idtdna.com). The sequences of the primers used are as follows: L7a, forward, 5’-TTTGGCATTGGACAGGACATCC-3’ and reverse, 5’-AGCGGGGCCATTTCACAAAG-3’; DKK1, forward, 5’-GATCATAGCACCTTGAGTGGA-3’ and reverse, 5’-GCCACAGTCTGTGACCGG-3’.
3' p21 forward, 5'-CCCGCAGCTGTGCGCTAATG-3' and reverse, 5'-CTCGGTGACAAAGTCGAAGTC-3'; c-MYC forward, 5'-GCTCCTGGCAAAAGGTCAGAGTCT-3' and reverse, 5'-ACCAGTGGGCTGTGAGGAGGTT-3' and Cyclin D1, forward, 5'-CGCCCTCGGTGTCCTACTTCAA-3' and reverse, 5'-GTGGCGACGATCTTCCGCAT-3'. The reaction was as follows: 95°C for 15 min to activate Taq polymerase followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. After amplification, dissociation curves were acquired by stepwise increases from 55°C to 95°C to ensure a specific product was amplified in the reaction. Standard curves with slope of -3.30±0.20 and R²≥0.99 were accepted. Human L7a was used as an internal control to normalize the raw data.

Western Blotting Analysis

After treatments, cells were lysed in 500 μL lysis buffer (0.125 mol/L Tris–HCl, pH 6.8, 1% SDS, 0.04% bromophenol blue, and 20% glycerol, v/v) with 1x protease inhibitor (Roche applied sciences, Indianapolis, IN) and phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich, St. Louis, MO). Total protein was size-fractionated on a 12% Tris–HCl polyacrylamide gel and transferred onto a PVDF membrane (BioRad laboratories Inc, Irvine, CA) at 0.3 A. The PVDF membrane was incubated with blocking solution containing 10% (w/v) nonfat dry milk (NFDM), 20 mmol/L Tris–HCl, pH 7.6, 137 mmol/L NaCl, and 0.1% (v/v) Tween-20 for 1 h at room temperature. A rabbit polyclonal antibody against DKK1 (sc-25516, Santa Cruz, CA) was diluted to 1:1,000 in the blocking solution with 10% NFDM and incubated with the membrane for 3 h at room temperature. Subsequently, the membrane was washed with blocking solution containing 5% NFDM for 5×5 min. A goat anti-rabbit HRP-conjugated secondary antibody was diluted to 1:10,000 in the blocking solution containing 5% NFDM and incubated with the membrane for 1 h at room temperature. After washing with 5×5 min in the blocking solution containing 1% (w/v) NFDM, the membrane was exposed to the enhanced chemiluminescence reagent Super Signal West Dura (Thermo Fisher Scientific, Rockford, IL). Signals from the membrane were detected and quantified by ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA). Actin (sc-1616-R, Santa Cruz, CA) was used as a loading control.

Cell Cycle Analysis by Flow Cytometry

At the end of the 2-d genistein treatment as described above, cells were trypsinized, centrifuged at 1000×g, and re-suspended in 0.5 mL 1×PBS. For fixation, 0.5 mL 100% ice-cold ethanol was added to each sample and incubated for a minimum of 20 min. After centrifugation at 1000×g for 6 min, ethanol was decanted and 0.5 mL 1× PBS containing 50 μg/mL Propidium Iodide (PI) and 100 μg/mL RNase A was added to the pellet and mixed. Samples were incubated at room temperature in the dark for a minimum of 20 min before analysis by BD LSR II flow cytometer (BD Bioscience, Bedford, MA). Data were analyzed using FCS 3.0 software.

Cell Proliferation Analysis Using WST-1 Assay

At the end of the 2-d genistein treatment as described above, 10 μL cell proliferation reagent, WST-1 (Roche applied science, Indianapolis, IN) was added to each well of the 96-well plate and incubated for 4 h. At the same time, a serial dilution of cells were mixed with 10 μL WST-1 reagents and aliquoted to the unused wells of the same plate as the standards for converting the absorbance readings to cell numbers. The plate was shaken thoroughly for 1 min. The absorbance of samples was read at 450 nm against a reference wavelength of 630 nm in a microplate reader (ELX800, BioTek, Winooski, VT).

DKK1 Overexpression

SW480 cells were cultured as described above and transfected with either the control pCMV vector or DKK1-containing pCMV (OriGene, Rockville, MD). The empty pCMV vector was used as a transfection control. After transfection with Superfect Transfection Reagent (Qiagen, Valencia, CA), cells were replenished with fresh MEM and incubated for 48 h before sample collection.

Figure 7. DKK1 mRNA expression after TSA treatment. DKK1 mRNA level was analyzed in SW480 cells treated with 50, 100 or 200 nmol/L of TSA (TSA50, TSA100, and TSA200, respectively) for 1 d. Genistein treatment was done as previously described. mRNA expression level was analyzed using RT-PCR. Three independent cell samples were analyzed and presented as the mean±SEM. Values with different letters differ (p<0.05). doi:10.1371/journal.pone.0040955.g007
DKK1 Knockdown

SW480 cells were plated at 0.5 × 10⁶ per well in 6-well plate or 3000 per well in 96-well plates and incubated overnight at 37°C with 5% CO₂. At the end of 2-d genistein treatment as described above, transfection was performed. At the time of transfection, 300 nmol/L of siRNA against human DKK1 (sir, Integrated DNA Technologies) was prepared in 200 μL serum-free media (SEF). The siRNA solution was incubated with 7 μL DharmaFect#2 (in 200 μL SEF, Dharmacon, www.fishersci.com) at room temperature for 20 min to form the transfection complex. Each well of the 6-well plates and the 96-well plates was replenished with 1.6 mL and 80 μL antibiotic-free MEM including DMSO or genistein (final concentration of genistein is 75 μmol/L), respectively before the transfection complex was added (400 μL/well for 6-well plates and 20 μL/well for 96-well plates). The transected cells were incubated at 37°C for 2-d for subsequent mRNA, protein, cell cycle and cell proliferation analysis, siRNA duplex for DKK1 used is a combination of three duplexes as follows: 5'- rGrCrGrGrGrArUrArCrArGrArArUrGrArUrGrArUrGrGrArUrCrArGrUrCrGrGrArArGrArGrArG- 3' (HSC.RNAI.N012242.12.1), 5'- ArGrGrGrGrGrArGrArGrArArArArArGrArGrArG- 3' and 5'- rArUrUrGrGrArGrArGrGrArArAr- 3' (HSC.RNAI.N012242.12.2); 5'- rGrUrArUrCrArCrArCrArCrArCrArCrArGrArUrGrArUrGrArUrGrArUrArUrAr UrArUrArUrArUrArUrArUrArUrArUrArUrArUrArUrArUrArUrArUrArUrArUrG- 3' (HSC.RNAI.N012242.12.3., Integrated DNA Technologies).

Methylation Specific PCR (MSP)

HCT15, HT29, RKO and SW48 cells were plated and treated as mentioned above. On d 2 of genistein treatment, cells were scraped and collected by centrifugation. Genomic DNA was isolated from the cell lines using GenElute genomic DNA Kit (Sigma-Aldrich, St. Louis, MO) and DNA concentration was measured by 260 nm absorbance. Total genomic DNA (2 μg) was treated with sodium bisulfite reagent using the EZ Methylation-Gold kit (Zymo Research, Orange, CA). In each MSP reaction, 10 ng of final product was used in a 20 μL volume containing 10 μL 2x Perfecta SYBR Green fast master mix (Quanta BioSciences, www.qbi.com) and 0.25 μmol/L of each primer in a 7300 real-time PCR system (Applied Biosystems, Foster City, CA). For human DKK1, one pair of methylated (M) primers (MSP DKK1 M -60~+73) and one pair of unmethylated (U) primers covering the same region (MSP DKK1 U -62~+73) were used to analyze DNA methylation at the 5’ proximal region within the CpG island predicted by CpGPlot (www.ebi.ac.uk/emboss/cpgplot/). Methylation level is calculated as the percentage of M/(M+U). The primers used in MSP are: MSP-M, forward, 5'TTAAGGGGTCCGAATTTC-3, reverse, 5' TCGAATCATCAAAATACCCGCAT-3; and MSP-U, forward, 5'TTTTAAAGGGGTCCGAATTTC-3, reverse, 5'TTGAACTACAAAATACCCGAAAAT-3. Bisulfite Sequencing

Genomic DNA was isolated from SW480 and DLD-1 cells using DNeasy Tissue Kit (Qiagen, Valencia, CA). Bisulfite conversion was performed with 1.4 μg of total genomic DNA by EZ Methylation-Gold kit (Zymo Research, Orange, CA) according to the manufacturer’s instructions. After purification, 200 ng of converted DNA was used as a template for each PCR reaction. Promoter CpG island for DKK1 was predicted as being located at -223 to +122 (http://www.ebi.ac.uk/emboss/cpgplot/) and primers were designed to amplify the -159 to +109 region (forward, 5’-GTTGAAGTGTTAAAGGGTTTTT-3 and reverse, 5’-GTTTTACAAATCCCTTACCAAG-3). The amplions from the PCR were cloned using TOPO-PCR cloning kit (Invitrogen, Carlsbad, CA) and sequenced at the Biotechnology Center at the University of Illinois (Urbana, IL). The C in a CpG is unmethylated when sequencing result shows a “C” and methylated when sequencing result shows a “T”.

Chromatin Immunoprecipitation (ChIP)

ChIP analysis was performed according to a modified protocol [51]. SW480 and DLD-1 cells were treated with 75 μmol/L genistein solution for 4 d before harvest. Cross-linking was performed using 1% formaldehyde for 10 min at room temperature. Nuclei were collected after nuclei swelling buffer treatment (5 mmol/L PIPES pH 8.0, 85 mmol/L KCl, 0.5% NP40) and lysed in SDS lysis buffer (50 mmol/L Tris-HCl pH 3.1, 10 mmol/L EDTA, 1% SDS) containing protease and phosphatase inhibitor cocktails. The chromatin was sonicated using a Sonic Dismembrator (model F100, Fisher Scientific, Pittsburgh, PA) with power set at 5 on ice for 5 bursts of 40 s with 2 min cooling between each burst. The average length of sonicated chromatin was measured by agarose gel electrophoresis to be ~500 bp. Cell debris was removed by centrifugation at 13,000 × g for 10 min at 4°C. Sheared chromatin was diluted to 10 mL with ChIP dilution buffer. One mL of diluted chromatin was incubated overnight at 4°C with 2 μg of each antibody. The chromatin-antibody complex was precipitated with 60 μL of 50% slurry of pre-blocked protein G-agarose beads (Millipore, Billerica, MA) for 2 h. A normal rabbit IgG was used as a negative control. Supernatant from the normal rabbit IgG was saved as the input DNA for each sample. The protein G-agarose beads were washed sequentially with 1 mL each of the following solutions: low salt (20 mmol/L pH 8.0 Tris-HCl, 0.1% SDS, 2 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100), high salt (20 mmol/L pH 8.0 Tris-HCl, 0.1% SDS, 2 mmol/L EDTA, 500 mmol/L NaCl, 1% Triton X-100), LiCl (10 mmol/L pH 8.0 Tris-HCl, 0.25 mmol/L LiCl, 1% NP40, 1 mmol/L EDTA, 1% sodium deoxycholate) and twice TE (pH 8.0). Antibody/protein/DNA complexes were eluted by 2×150 μL elution buffer (1% SDS and 50 mmol/L NaHCO₃) at 37°C for 15 min each. Reverse cross-linking was performed with 20 μL of 5 mol/L NaCl and 1 μg of RNase A at 65°C for 5 h. Chromatin DNA was purified using QiaPrep miniprep kit (Qiagen, Valencia, CA) after proteinase K digestion. Immunoprecipitated DNA was detected by real time RT-PCR using primers targeting different regions of the DKK1 gene: promoter region: forward, 5’-GGCTTTGTTGTGTCCTCCCTCCAAAG-3 and reverse, 5’-GGACCCCCGGCTGCTTATA-3; coding region, forward, 5’-GATCATGACCTCTGAGGGG-3 and reverse, 5’-GACAGCATCTGTAGACCCCG-3 and 5’- upstream control region, forward, 5’-ACTGGCTGTTGAGAAATG-3 and reverse, 5’-TCATTGGCAAAAGATGTC-3. The antibodies used are as follows: from Millipore, acetylated histone H3 (H3Ac, 06-599), acetylated histone H4 (H4Ac, 06-866), dimethyl-histone H3 lysine 4 (H3K4me2, 07-030); from Santa Cruz Biotechnologies, normal rabbit IgG (IgG, sc-2027), RNA polymerase II (PolII, sc-899) and phosphorylated histone H3 serine 10 (H3S10p, sc-8656).

Trichostatin A (TSA) Treatment

SW480 cells were plated at 0.2 × 10⁶ per dish in a 60 mm culture dish in regular MEM containing 10% FBS and 1% ABAM. After overnight culture, cells were treated with culture media containing 50, 100 or 200 mmol/L TSA for 1 or 2 d. Total
mRNA was isolated and mRNA expression was analyzed by real time RT-PCR as described above.

**Statistical Analysis**

Unpaired two-tailed Student’s t test was performed for mRNA and protein expression, cell proliferation and cell cycle analyses, and Methylation Specific PCR. Two-way ANOVA using LSMeans was performed for data from ChiP (SAS Institute Inc., Cary, NC). One-way ANOVA and Tukey test was applied to compare the significance in levels among different groups in Fig. 4F and Fig. 7. Data are presented as means ± SEM. Statistical significance was set as p<0.05.

**Supporting Information**

**Figure S1** Cell proliferation in colon cancer cells. WST-1 proliferation assay was performed in DLD-1, HCT15, HT29, RKO and SW48 cells. WST-1 signals were converted to actual cell numbers using a standard generated by serial dilutions of a known number of cells. Data were normalized to respective control for each treatment. Y-axis represents relative cell number (% to control). Asterisks (*) indicate statistical significance compared with the control group of the same cell line (p<0.05). (TIFF)

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**Author Contributions**

Conceived and designed the experiments: HC. Performed the experiments: HW QL HC. Analyzed the data: HW HC. Contributed reagents/materials/analysis tools: QL HW. Wrote the paper: HW HC.

**References**

1. Cross HS, Kallay E, Farhan H, Weiland T, Manhardt T (2003) Regulation of extracellular vitamin D metabolism as a tool for colon and prostate cancer prevention. Recent Results Cancer Res 164: 413–5: 413–425.

2. Kennedy AR (1995) The evidence for soybean products as cancer preventive agents. J Nutr 125: 7388–7438.

3. Yang G, Sha XD, Li H, Chow WH, Cai H, et al. (2009) Prospective cohort study of soy food intake and colorectal cancer risk in women. Am J Clin Nutr 89: 577–583.

4. Spector D, Anthony M, Alexander D, Arab L (2003) Soy consumption and colorectal cancer. Nutr Cancer 47: 1–12.

5. Oba S, Nagata C, Shimizu N, Shimizu H, Kametani M, et al. (2007) Soy product consumption and the risk of colorectal cancer: a prospective study in Takayama, Japan. Nutr Cancer 57: 151–157.

6. Mesina M, Benmink M (1999) Soy foods, isoflavones and risk of colon cancer: a review of in vitro and in vivo data. Baillieres Clin Endocrinol Metab 12: 707–728.

7. Sarkar FH, Li Y (2003) Soy isoflavones and cancer prevention. Cancer Invest 21: 744–755.

8. Fan YZ, Li GH, Wang YH, Ren QY, Shi HJ (2010) Effect[s of genistein on colon cancer cells in vitro and in vivo and its mechanism of action]. Zhonghua ZhongLiu Za Zhi 32: 4–9.

9. Linsalata M, Russo F, Notarnicola M, Guerra V, Cavallini A, et al. (2005) Effects of genistein on the polyamine metabolism and cell growth in DLD-1 human colon cancer cells. Nutr Cancer 52: 84–93.

10. Zhu Q, Meisinger J, Van Thiell DH, Zhang Y, Moharban S (2002) Effects of soybean extract on morphology and survival of Caco-2, SW620, and HT-29 human colon cancer cells. Nutr Cancer 42: 131–140.

11. Kim EJ, Shin HK, Park JH (2005) Genistein inhibits insulin-like growth factor-I receptor signaling in HT-29 human colon cancer cells: a possible mechanism of the growth inhibitory effect of Genistein. J Med Food 8: 431–438.

12. Borras C, Gambini J, Gomez-Cabreria MC, Saurt J, Pallardo FV, et al. (2006) Genistein, a soy isoflavone, up-regulates expression of antioxidant genes: involvement of estrogen receptors, ERK1/2, and NFkappaB. FASEB J 20: 2136–2138.

13. Biers G, Rajna E, Manhardt T, Gerdenitsch W, Kallay E, et al. (2007) Gender-specific modulation of markers for premalignancy by nutritional soy and calcium in the mouse colon. J Nutr 137: 2118S–2138S.

14. Cross HS, Lipkin M, Kallay E (2006) Nutrients regulate the colonic vitamin D system in mice: relevance for human colon malignancy. J Nutr 136: 561–564.

15. Kallay E, Adlercreutz H, Farhan H, Lechner D, Bajna E, et al. (2002) Phytoestrogens regulate vitamin D metabolism in the mouse colon: relevance for colon tumor prevention and therapy. J Nutr 132: 2490S–2493S.

16. Majid S, Kikuno N, Nelles J, Noonan E, Tanaka Y, et al. (2008) Genistein mediated histone acetylation and demethylation activates tumor suppressor genes in prostate cancer cells. Int J Cancer 123: 352–560.

17. Day JK, Baner AM, DeSorboes C, Zhang Y, Kim BE, et al. (2002) Genistein alters methylation patterns in mice. J Nutr 132: 24198S–24218S.

18. Kikuno N, Shiina H, Urakami S, Kawamoto K, Hirata H, et al. (2008) Genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. Environ Health Perspect 116: 567–572.

19. Logun CY, Nuse R (2004) The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20: 781–810.

20. Cadigan KM, Nuse R (1997) Wnt signaling: a common theme in animal development. Genes Dev 11: 3286–3305.

21. Huo HH, Cheng SF, Chen LM, Liu JY, Chu CH, et al. (2006) Over-expressed estrogen receptor-alpha upregulates STXN-Alpha gene expression and down-regulates beta-catenin signaling activity to induce the apoptosis and inhibit proliferation of LoVo colon cancer cells. Mol Cell Biochem 289: 101–109.

22. Clevers H (2006) Wnt/beta-catenin signaling in development and disease. Cell 127: 469–480.

23. Clevers H, Badille B, et al. (2006) EphB/EphrinB receptors and Wnt signaling in colorectal cancer. Cancer Res 66: 2–5.

24. Zhang Y, Chen H (2011) Genistein attenuates WNT signaling by up-regulating FR2p1 in a human colon cancer cell line. Exp Biol Med (Maywood) 236: 714–722.

25. Tang Z, Chen H (2010) Genistein increases gene expression by demethylation of WNT5a promoter in colon cancer cell line SW1116. Anticancer Res 30: 4537–4545.

26. Balico A, Liu G, Yaniv A, Gazit A, Aaronson SA (2001) Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRPI6/ Arrow. Nat Cell Biol 3: 683–696.

27. Aguilera O, Pena C, Garcia JM, Larriba MJ, Ordonez-Moran P, et al. (2007) The Wnt antagonist DICKKOP1 gene is induced by 1alpha,25-dihydroxyvitamin D3 associated to the differentiation of human colon cancer cells. Carcinogenesis 28: 1077–1084.

28. Mao B, Wu W, Davidson G, Markhold J, Li M, et al. (2002) Keenem proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. Nature 417: 664–667.

29. Mao B, Wu W, Li Y, Hoppe D, Stannick P, et al. (2001) LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature 411: 321–325.

30. Semenov MV, Tanai K, Brott BK, Kuhl M, Sokol S, et al. (2001) Head inducer Dickkopf1 is a ligand for Wnt coreceptor LRPI6. Curr Biol 11: 951–961.

31. Ravindranath MH, Muthugounder S, Presser N, Viswanathan S (2004) Antiangiogenic therapeutic potential of soy isoflavone, genistein. Adv Exp Med Biol 546: 121–165.

32. Zhou JR, Mykherje P, Gugger ET, Tanaka T, Blackburn GL, et al. (1998) Inhibition of murine bladder tumorigenesis by isoferolones via alterations in the cell cycle, apoptosis, and angiogenesis. Cancer Res 58: 5231–5238.

33. Sarkar FH, Li Y (2002) Mechanisms of cancer chemoprevention by soy isoferolone genistein. Cancer Metastasis Rev 21: 265–280.

34. Singh AV, Franke AA, Blackburn GL, Zhou JR (2006) Soy phytochemicals prevent orthotopic growth and metastasis of bladder cancer in mice by
alterations of cancer cell proliferation and apoptosis and tumor angiogenesis. Cancer Res 66: 1851–1858.

40. Zhou JR, Yu L, Zhong Y, Naur RL, Franke AA, et al. (2002) Inhibition of orthotopic growth and metastasis of androgen-sensitive human prostate tumors in mice by bioactive soybean components. Prostate 53: 143–153.

41. Choi YH, Zhang L, Lee VH, Park KY (1996) Genistein-induced G2/M arrest is associated with the inhibition of cyclin B1 and the induction of p21 in human breast carcinoma cells. Int J Oncol 13: 391–396.

42. Gonzalez-Sancho JM, Aguilera O, Garcia JM, Pendas-Franco N, Pena C, et al. (2005) The Wnt antagonist DICKKOPF-1 gene is a downstream target of beta-catenin/TCF and is downregulated in human colon cancer. Oncogene 24: 1098–1103.

43. Moors M, Bose R, Johansson-Haque K, Edoff K, Okret S, et al. (2012) Dickkopf 1 mediates glucocorticoid-induced changes in human neural progenitor cell proliferation and differentiation. Toxicol Sci 125: 488–493.

44. Mikata R, Yokomuka O, Fuku K, Inazeki F, Arai M, et al. (2006) Analysis of genes upregulated by the demethylating agent 5-aza-2’-deoxycytidine in gastric cancer cell lines. Int J Cancer 119: 1616–1622.

45. Majid S, Dar AA, Ahmad AE, Hirata H, Kawakami K, et al. (2009) BTG3 tumor suppressor gene promoter demethylation, histone modification and cell cycle arrest by genistein in renal cancer. Carcinogenesis 30: 662–670.

46. Majid S, Dar AA, Shahryari V, Hirata H, Ahmad A, et al. (2010) Genistein reverses hypermethylation and induces active histone modifications in tumor suppressor gene B-Cell translocation gene 3 in prostate cancer. Cancer 116: 66–76.

47. Lee J, Yoon YS, Chung JH (2008) Epigenetic silencing of the Wnt antagonist DICKKOPF-1 in cervical cancer cell lines. Gynecol Oncol 109: 270–274.

48. Sikandar S, Dixon D, Shen X, Li Z, Besterman J, et al. (2010) The class I HDAC inhibitor MGCD0103 induces cell cycle arrest and apoptosis in colon cancer initiating cells by upregulating Dickkopf-1 and non-canonical Wnt signaling. Oncotarget 1: 596–605.

49. Santos-Rosa H, Schneider R, Bannister AJ, Sherriff J, Bernstein BE, et al. (2002) Active genes are tri-methylated at K4 of histone H3. Nature 419: 407–411.

50. Mahadevan LC, Willis AG, Barratt MJ (1991) Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadacin acid, and protein synthesis inhibitors. Cell 65: 775–783.

51. Chen H, Pan YX, Dudenhausen EE, Kilberg MS (2004) Amino acid deprivation induces the transcription rate of the human asparagine synthetase gene through a timed program of expression and promoter binding of nutrient-responsive basic region/leucine zipper transcription factors as well as localized histone acetylation. J Biol Chem 279: 50829–50839.