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# G-protein-coupled receptor for short-chain fatty acids suppresses colon cancer

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**GPR43 is a G-protein-coupled receptor for short-chain fatty acids (SCFAs). Expression of GPR43 is detected in hematopoietic tissues and the large intestine. SCFAs are derived from bacterial fermentation and metabolism of undigested dietary fibers and have been recognized for their cancer prevention activities in the colon. The role of SCFAs, particularly butyrate, in colon cancer therapy has been extensively studied, and its tumor suppressive functions are believed to be due to their intracellular actions, notably inhibition of histone deacetylase. In our study, we show that SCFAs also exert their antitumor effects *via* receptor GPR43 and that GPR43 is frequently lost in colon cancer cells. Immunohistostaining revealed that GPR43 immunoreactivity was high in normal colon tissues ($N = 31$) but was markedly reduced or completely lost in most colorectal adenocarcinoma tissues ($N = 70$) and their corresponding lymph node metastatic adenocarcinomas ($N = 38$). RT-PCR analysis detected the presence of full length GPR43 mRNA in only one (HT-29) of nine established human colon cancer cell lines. Restoration of GPR43 expression in HCT8 human colonic adenocarcinoma cells induced G0/G1 cell cycle arrest and activated caspases, leading to increased apoptotic cell death after propionate/butyrate treatment. Restored GPR43 expression, coupled with propionate treatment, induced an upregulation of p21 and a decrease in the levels of cyclin D3 and cyclin-dependent kinases (CDKs) 1 and 2, while the CDK4 and CDK6 levels remained unchanged. Our results suggest that GPR43 functions as a tumor suppressor by mediating SCFA-induced cell proliferation inhibition and apoptotic cell death in colon cancer.**



Colorectal cancer is the third most common cancer in the United States.[1] A combination of genetic factors and environmental causes including radiation, chemical carcinogens and diet contribute to tumorigenesis in the colon.[2] In the past several decades, progress has been made in identifying genetic mutations in colon carcinogenesis.[3,4] A lesser understood, but widely implicated, risk factor for cancer is dietary intake. SCFAs are major byproducts of the bacterial fermentation and metabolism of undigested dietary fiber in the human large intestine and colon.[5] SCFAs, such as butyrate and propionate, are reported as antitumor agents that induce differentiation, growth arrest and apoptosis in colon cancer cells.[6–11] The antitumor effect of SCFAs is strongly supported by epidemiological studies showing that a diet rich in fiber is associated with a decreased incidence and growth of colon cancer.[12]

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In the colonic lumen, the molar ratio among the three major SCFAs (which constitute ~90% of the SCFAs generated in the lumen) is ~60:25:15 for acetate (2C): propionate (3C):butyrate (4C), which may vary depending upon a spectrum of factors.[13] In addition to butyrate, which has been shown to inhibit colon cancer growth, propionate was shown to inhibit HT-29 colon adenocarcinoma cell growth.[14] Propionate was also shown to inhibit cell growth and trigger apoptosis in other colorectal carcinoma cells.[15,16] The data suggest that SCFAs guard against colon carcinogenesis.

Recently, SCFAs were reported as ligands for orphan G-protein-coupled receptor GPR41 and *GPR43*[17] SCFAs activate GPR41 and GPR43 in a dose dependent and specific manner.[17,18] GPR41 is strongly expressed in the adipocytes and is involved in leptin production when stimulated with propionate.[17,19,20] GPR43 expression has been mainly detected in hematopoietic tissues, with the highest levels in leukocyte populations, indicating that if is likely involved in immune responses after infections, as bacteria produce SCFAs.[17–19,21] Aside from the immune system, GPR43 has been shown to be present in both human colon[22] and rat distal ileum and colon.[23] The tissue-restricted expression pattern suggests that GPR43 plays a role in the normal development or functions of the colon tissue, but it is unknown whether GPR43 is involved in abnormalities in colon including tumorigenesis.

So far, the tumor suppressive function of propionate/butyrate has been mainly focused on their intracellular action.

GPR43, which is a G-protein-coupled receptor for SCFAs, has been reported to be expressed in the colon and intestine.[22,23] Herein, we focus on studying the role of GPR43 in SCFA-mediated colon cancer suppression. We found that GPR43 expression was markedly reduced and, in some cases, completely lost in colon adenocarcinomas and in established colon cancer cell lines. Restoration of GPR43 sensitized HCT8 cells toward propionate/butyrate-induced G0/G1 cell cycle arrest and apoptosis. Thereby, we report a novel approach through which propionate/butyrate exert their antitumor effects *via* GPR43.

## Material and Methods

### Materials

Human colon cancer cell lines CACO-2, HCT116, SW480 and SW620 cells were purchased from American Type Culture Collection. HCT8 was kindly provided by Dr. Deliang Cao while HT-29, CBS, FET and MOSER were kindly gifted by Dr. Subhas Chakrabarty. A human colon normal or cancerous tissue array was purchased from Biomax. FBS was purchased from Atlanta Biologicals. Rabbit polyclonal anti-G-protein-coupled receptor GPR43 was purchased from Novus Biologicals. Myc and Flag antibodies were purchased from OriGene Technologies. MEK1, cyclin D3, CDK1, CDK2 and PCNA antibodies were purchased from BD Transduction Laboratories. p27 and Rip antibodies were from Santa Cruz Biotechnology. Bcl-2 and p53 antibodies were from Calbiochem. Antibody against β-actin was from Sigma-Aldrich. All other antibodies were purchased from Cell Signaling Technology. PCR reagents and MTS solution were from Promega. ORF Clones of Homo sapiens free fatty acid receptor 2 (GPR43), inserted in either pCMV6 Entry (Myc/Flag tagged) (denoted as GPR43) or pCMV6-AC-GFP vector (denoted as GPR43GFP), were purchased from OriGene Technologies.

### Cell culture and restoration of GPR43 expression

HCT8, HCT116, HT-29, SW480, SW620, CBS, FET and MOSER cells were grown in a RPMI 1640 medium supplemented with 10% FBS and antibiotics–antimycotics (100 units/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotericin B) in a humidified incubator under an atmosphere containing 5% $CO_2$ at 37°C. CACO-2 cells were grown in a Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and antibiotics–antimycotics. Cells were transfected with GPR43 overexpression constructs using DNAfectin™ liposome transfection reagent (Applied Biological Materials).

### Immunohistochemistry

To confirm the specificity of GPR43 antibody, HCT8 cells were transfected with Myc/Flag tagged GPR43 expression construct. Immunocytostaining was performed against MYC and GPR43 antibody simultaneously. We found that Myc tagged GPR43 could be detected by GPR43 antibody (Supporting Information Figure 1). Cancerous tissue array immunostaining against GPR43 was conducted as described.[24] The staining was observed with a BX41 system microscope (Olympus, Center Valley, PA). The *H*-score indicated GPR43 staining intensity was calculated as described.[24]

### GPR43 promoter activity measurement

*GPR43* promoter reporter (S104949, chr19+:40631513–40632611) was purchased from SwitchGear Genomics. The promoter construct was cotransfected with a Rellina luciferase construct into target colon cancer cells. The promoter activities were measured using the Promega Dual-Luciferase (R) Reporter (DLR[TM]) Assay System.

### Treatment with inhibitors of DNA methylation and histone deacetylation

HCT8, HT-29, SW480 and Caco-2 cells (50–60% confluence) were treated with 5′-azadeoxycytidine (1 μmol/l), which inhibits DNA methyltransferase, or trichostatin A (0.5 μmol/l), which inhibits HDAC, or both or vehicle only for 72 hr.[25] Fresh complete medium containing the drugs was replaced every day during treatment.

### Reverse transcription-polymerase chain reaction

The expression of *GPR43* at the mRNA level in colon cancer cells was evaluated by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cell cultures with an RNeasy Mini Kit (QIAGEN) and treated with RNAse-free DNAse. RT was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega Corporation). Full-length GPR43 was amplified by RT-PCR with primers: GPR43 (993 bp), 5′-ATGCTGCCGG ACTGGAAGAG-3′/3′-CTACTCTGTAGTGAAGTCCG-5′. *p16 INK4A* was amplified by RT-PCR with primers: 5′-CCTCGTGCTGATGCTACTGA-3′/3′-TTCTTTCAATCGGGG ATGTC-5′ or 5′-GAGGATTTGAGGGACAGGGT-3′/3′-CAT CATCATGACCTGGATCG-5′. *p21 Waf1/Cip1* was amplified by RT-PCR with primers: 5′-AGGCACTCAGAGGAGGT GAG-3′/3′-ACAAGTGGGGAGGAGGAAGT-5′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control for normalization.

### Western blotting

Western blotting was conducted as described.[24] In brief, HCT8 cells with GPR43 or control vector expression subjected to different treatment were collected with RIPA buffer (Sigma, R0278) combined with protease inhibitor cocktail (Sigma, S8820). Equal amount of protein was applied to SDS-PAGE gels. The immunoblots were visualized by Odyssey Infrared Imaging System (LI-COR Biosciences). Because there is no commercially available GPR43 antibody that is suitable for Western blotting detection, the exogenously expressed GPR43 was detected by either a Myc or Flag antibody.

### cAMP assays

cAMP assays were performed using cAMP-Glo Assay (Promega) following the protocol of the manufacturer. HCT8 cells

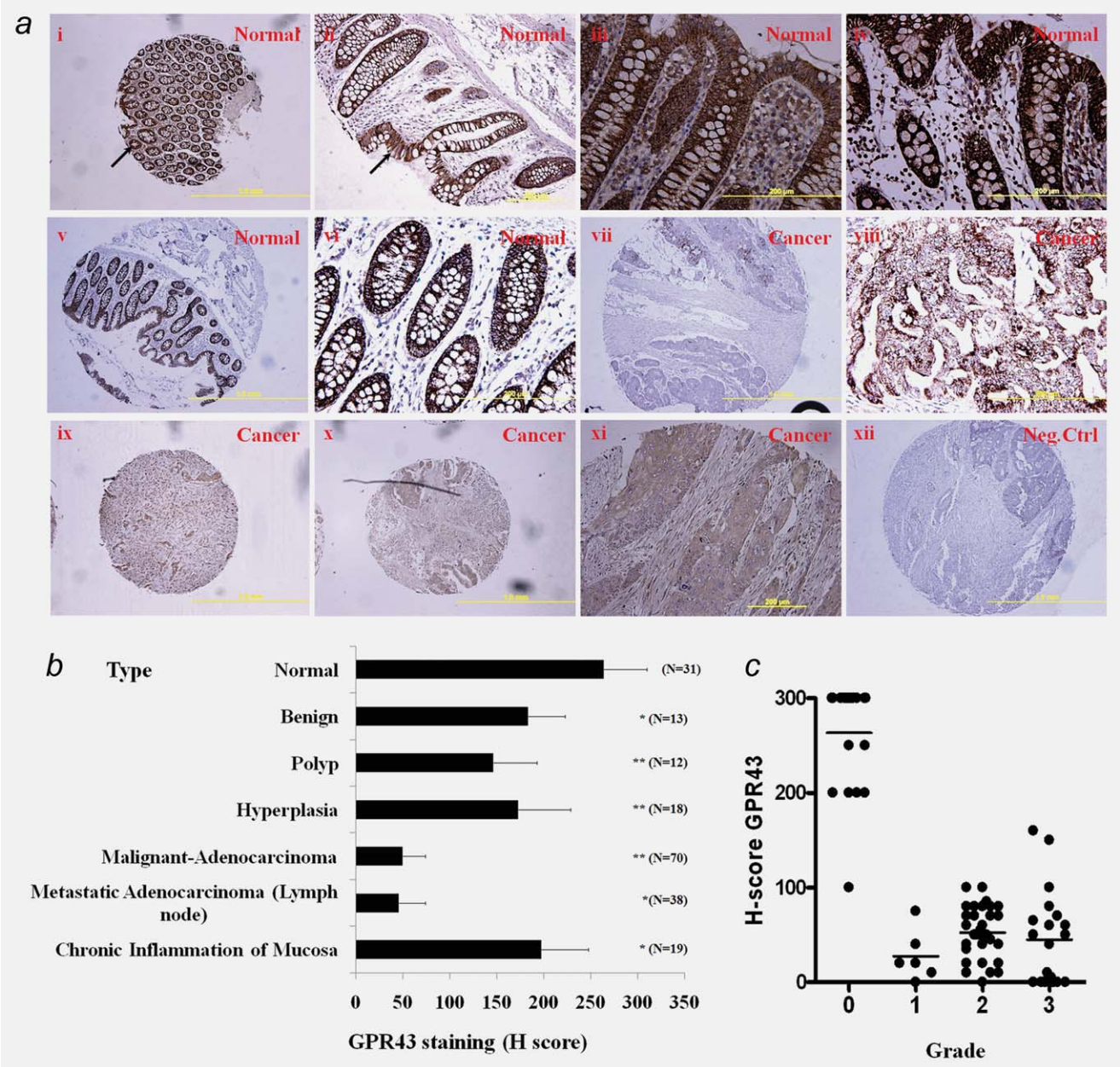


**Figure 1.** Reduction of GPR43 expression within human colon cancers. (*a*) Immunohistostaining of GPR43 in human colon tissues. (i–vi) Micrographies of four representative tissue samples from normal colon tissue; (vii–xi) micrographies of five representative tissue samples from tubular adenocarcinoma (vii and viii), colon lymph node metastatic adenocarcinoma (ix) and colon adenocarcinoma (x and xi). Brown GPR43 staining. Tumor sections incubated with nonspecific rabbit IgG were used as negative control (xii). (i, v, vii, ix, x and xii, Bar = 1.0 mm; ii, iii, iv, vi, viii and xi, Bar = 200 μM). (*b*, *c*) The bar graphs show expression levels (*H*-score) of GPR43 in a colon tissues array categorized either according to tissue type (*b*) or pathology diagnosis tissue grade (*c*). (*b*) The bars represent the mean ± SE. $*p < 0.05$; $**p < 0.01$, when compared with the normal colon group. (*c*) The Grades 1–3 in pathology diagnosis a equivalent to well-differentiated, moderately differentiated or poorly differentiated tissues, respectively, under a microscope.

transfected with GPR43 and control vector were seeded into 96-well plates (density of $2 \times 10^4$ per well). Twenty-four hours after seeding, the cells were treated with 3 mM sodium propionate and 10 μM forskolin dissolved in induction buffer for the indicated time at 37°C. The intracellular levels of cAMP were detected using the detection solution provided in the kit after cell lysis. The luminescence values were measured by Luminoskan Ascent from Thermo Scientific.

### Flow cytometry analysis of the cell cycle

HCT8 cells with GPR43 expression restored or the a vector control were harvested, washed twice with 1× PBS,

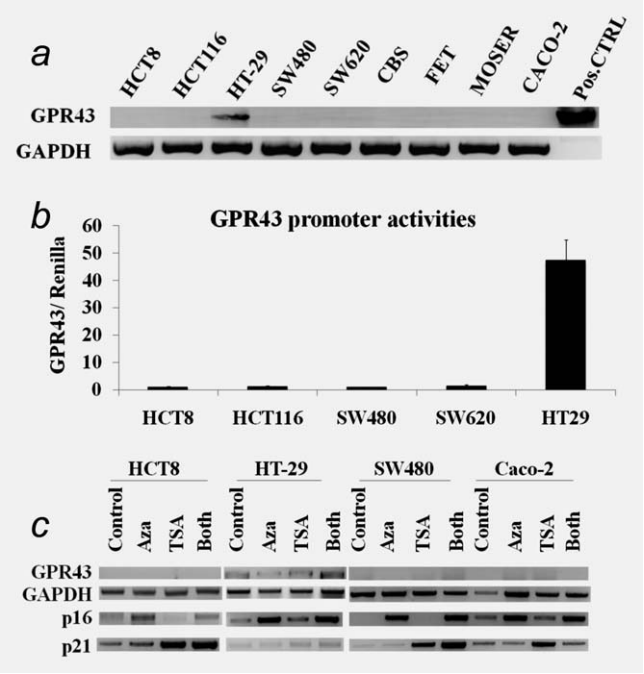


**Figure 2.** Characterization of *GPR43* expression in colon cancer cell lines. (*a*) RT-PCR detection of *GPR43* at the mRNA level. A plasmid of *GPR43* was used as a positive control. The data represent at least three repeated experiments. (*b*) *GPR43* promoter activators were evaluated in five colon cancer cell lines. (*c*) HCT8, HT-29, SW480 and Caco-2 cells were treated with either vehicle ethanol (control), Aza or TSA either individually or in combination (both). The expression levels of GPR43, p16 and p21 were examined by RT-PCR.

resuspended in 1 ml of fixative solution (30% PBS/70% methanol) and incubated at 4°C for 1 h. Cells were stained with 0.5 ml of PI/RNase staining solution (0.1% Triton X-100, 0.2 mg/ml DNase–RNase A, and 20 μg/ml propidium iodide) and incubated at room temperature for 30 min in the dark before flow cytometric analysis.

### Colony formation assay

HCT8 cells with or without GPR43 expression restored were plated in 12-well plates (400 cells per well) with the indicated treatments. Two weeks later, colonies were fixed with 100% methanol and stained with Hematoxylin (Zymed, Carlsbad, CA), and images were taken by using a BX41 system microscope (Olympus, Center Valley, PA). Colonies with a diameter >0.05 mm were enumerated. The results were presented as the mean number of colonies derived from averaged values of six repeats.

### Isolation of plasma membrane-bound, ectopically expressed GPR43

HCT8 cells transfected with the GPR43 expression construct were grown to 90–95% confluence. Plasma membrane proteins were biotinylated and isolated with the use of a commercially available kit (Cell Surface Protein Biotinylation and Purification Kit; Pierce Biotechnology). Proteins were then immunoblotted as described.

### Statistical analysis

Student's *t*-test was used for statistical analysis between two groups, unless otherwise indicated. A *p*-value, less than 0.05, was considered significant. For statistical analysis of GPR43 immunoreactivities in colon specimens, nonparametric tests were used as previously described.[24] The IC50s of propionate or butyrate toward colon cancer cells were fitted and analyzed by IDBS XLfit5.

## Results

### Reduction of GPR43 expression within human colon cancers

To determine the potential role of GPR43 in colon cancer, we first evaluated GPR43 expression in normal human colon tissues and human colorectal cancer tissues by immunohistochemistry using a validated antibody (Supporting Information Figure 1). GPR43 immunoreactivities were evident in nearly all normal colon tissue samples (Fig. 1*a*, i–vi). In contrast, GPR43 immunoreactivity was markedly reduced, or even completely lost in colon cancer (Fig. 1*a*, vii–xi). In normal colon glands, GPR43 immunoreactivities were present at the surface of the differentiated apical epithelium (Fig. 1*a*, ii–iv and vi). The GPR43 immunoreactivities were scored and analyzed according to the pathology diagnosis parameters. As shown in Figure 1*b*, when compared to its expression in normal colon tissues ($n = 31$), GPR43 expression was reduced by ~80% in human colon malignant adenocarcinoma tissues ($n = 70$). In colon hyperplasia ($n = 18$), polyp ($n = 12$) and benign colon cancer ($n = 13$), GPR43 expression was reduced moderately. In terms of pathology diagnosis tissue grade, there was a rapid loss of GPR43 expression in colon carcinoma tissues, starting at Grade 1 and maintained in Grades 2 and 3 (Fig. 1*c*). These results suggest that GPR43 expression level is markedly reduced in colon adenocarcinomas.

### Loss of GPR43 expression in colon cancer cells is caused by mechanisms other than histone deacetylation or promoter hypermethylation

Next, we investigated GPR43 expression in human colon cancer cell lines. The receptor expression was detected in only one colon cancer cell line (HT-29) through RT-PCR analyses (Fig. 2*a*). The high level of *GPR43* expression in HT-29 cells was corroborated by the fact its *GPR43* promoter activity was ~ 40 folds higher than in other cell lines (Fig. 2*b*). Transcriptional repression or silencing of a gene can be caused by epigenetic changes, especially promoter hypermethylation or chromatin compaction due to histone deacetylation (HDAC) or both.[25–27] To determine whether these mechanisms may explain, at least partially, the loss of *GPR43* expression in colon cancer cells, we treated HCT8, SW480 and Caco-2 cells with trichostatin A, which inhibits HDAC,[26,28] or 5′-

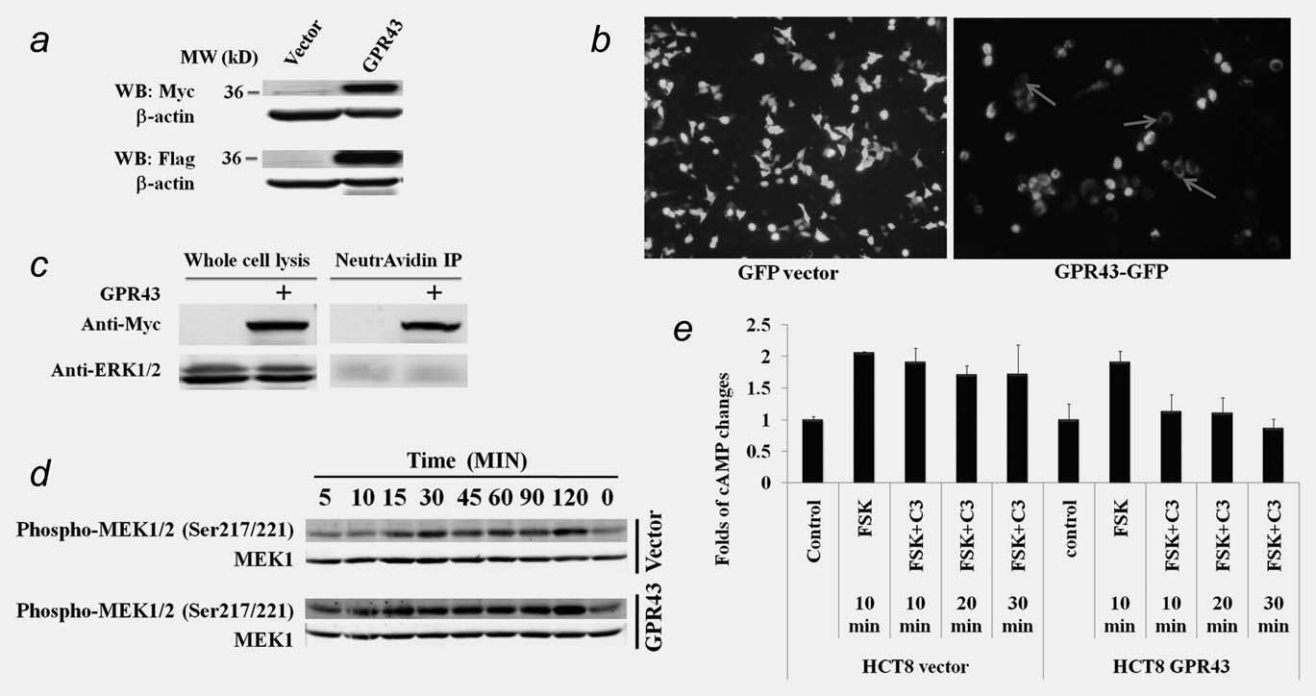


**Figure 3.** Restoration of functional GPR43 in an established human colon cancer cell line. (*a*) Expression of GPR43 as a fusion protein with Myc-Flag tag in HCT8 cells. Top/bottom panel, Western blot against Myc/Flag antibody, respectively. (*b*) HCT8 cells were transfected with either GFP control vector (left) or GPR43GFP (right). (*c*) Detection of plasma membrane bound GPR43. The surface protein was immunoprecipitated with neutravidin and probed with Myc antibody. Endogenous cytoplasmic protein (ERK), which was not immunoprecipitated under the same conditions, was used a negative control. Restoration of GPR43 expression sensitized propionate-induced MEK phosphorylation (*d*) and cAMP depletion (*e*). HCT8 transfected with either GPR43 or a control vector were treated with 3 mM propionate for different times as indicated. For cAMP measurement, the bars represent the mean ± SE ($n = 3$).

azadeoxycytidine, which inhibits DNA methyltransferase,[27] or both. We also applied above treatment to HT-29 cells to determine whether they would alter the level of GPR43 expression. As shown in Figure 2*c*, none of these treatments was able to restore *GPR43* expression. In contrast, 5′-azadeoxycytidine was effective in upregulating the mRNA level of cyclin-dependent kinase inhibitor *p16*, whose promoter is suppressed by hypermethylation, while TSA was able to increase the transcription of cyclin-dependent kinase inhibitor *p21* in colon cancer cells.[29] Together, these data suggest that mechanisms other than hypermethylation and HDAC are responsible for the silencing of the *GPR43* gene in colon cancer cells.

### Restoration of functional GPR43 in an established human colon cancer cell line

To investigate a potential role of GPR43 expression, or lack of expression, in colon cancer, we attempted to restore its expression. As shown in Figure 3*a*, transfection of GPR43 expression construct led to a strong expression that peaked about 1 day after transfection. To study the cellular localization of exogenously expressed GPR43, we transfected HCT8 cells with GFP fused GPR43. As shown in Figure 3*b* and Supporting Information Figure 2, GPR43 was localized onto the cell membrane and displayed ring like structure while some portion of GPR43 was retained within cytosol. In contrast, a GFP control vector was evenly distributed across the whole cells and did not show any cellular localization specificity. The cell membrane bound GPR43 was further confirmed by a biotinylation assay (Fig. 3*c*).

To evaluate the functional activity of exogenously expressed GPR43, we treated HCT8 cells with the GPR43 agonist propionate. HCT8 cells with GPR43 expression became more sensitized toward propionate-induced MEK1 phosphorylation and cellular cAMP level reduction,[19] compared with those cells transfected with empty vector (Figs. 3*d* and 3*e*). Taken together, the results confirmed that the restored GPR43 functions to initiate cell signaling elicited by its agonist.

### Role of GPR43 in propionate/butyrate-induced colon cancer apoptosis

SCFAs, particularly propionate and butyrate, are well known for their preventive effects toward colon cancer.[6,9,15] In agreement with these reports, propionate and butyrate exhibited cytotoxicities toward the colon cancer cell line HCT8. The IC50 value of propionate was 5 mM (HCT8) while butyrate was more cytotoxic with an IC50 of 2 mM (Supporting Information Figure 3).

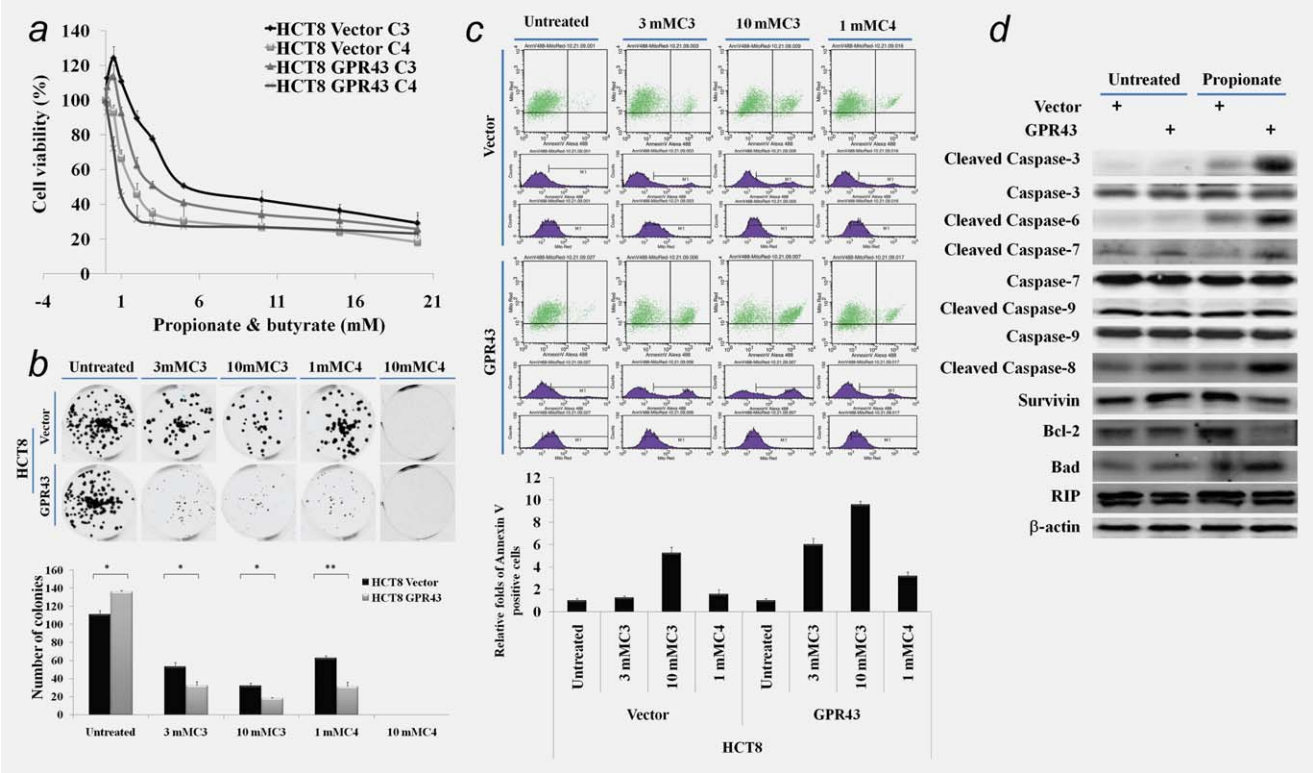


**Figure 4.** Role of GPR43 in propionate/butyrate-induced colon cancer apoptosis. (*a*) HCT8 cells were transfected with GPR43 or a control vector. Twenty-four hours after the transfection, the cytotoxic effects of propionate and butyrate were examined through an MTS assay 48 hr after the indicated treatments. The bars represent the mean ± SE ($n = 4$). The potentiation of cell death was also indicated by clonogenic survival (*b*) and phosphatidylserine (PS) based annexin V staining (*c*). (*d*) Effects of GPR43 expression restoration on caspases and antiapoptotic protein regulation during propionate (3 mM)-induced apoptotic-cell death after 48-hr treatment. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Next, we examined the effects of GPR43 expression on colon cancer cell survival during propionate/butyrate treatment. As shown in Figure 4*a*, HCT8 cells with GPR43 expression became more sensitized toward propionate or butyrate-caused cell death. The IC50 of propionate after a 48-hr treatment decreased from 5 to 2 mM while the IC50 of butyrate was reduced from 2 to 0.8 mM (Fig. 4*a*). The increased sensitivity was further supported by the reduced clonogenic survival for HCT8 cells with GPR43 expression (Fig. 4*b*). Importantly, the size of the colonies was substantially smaller when GPR43 was expressed. To further characterize the increased cell death, phosphatidylserine (PS)-based annexin V staining was performed to assess the number of cells undergoing apoptosis (Fig. 4*c*). Forty-eight hours after the initiation of treatment, GPR43 expression resulted in a marked increase in annexin V positive cancer cells compared with those cells transfected with control vector.

As an independent approach to reflect cancer cell apoptosis, caspase family members that might function during apoptosis were examined by immunoblots (Fig. 4*d*). We observed increased activation of initiator caspases 6, 7 and 8, and executioner caspase 3 in HCT8 cells with GPR43/propionate treatment (Fig. 4*d*). In addition, the expression of various antiapoptotic proteins such as Bcl-2 and Survivin was reduced during GPR43/propionate-induced apoptosis. Furthermore, we also examined several proapoptotic proteins such as Bad and receptor-interacting protein (RIP). We found Bad was upregulated in GPR43/propionate-induced apoptosis in HCT8 cells, while the RIP level remained unchanged. Increased sensitization toward propionate/butyrate-induced cell death under GPR43 restoration was also observed in SW480 cells, which were established from a primary adenocarcinoma of the colon of a male (data not shown). Altogether, the results suggest GPR43 expression potentiated propionate or butyrate-induced apoptotic cell death.

### Role of GPR43 in propionate/butyrate-induced inhibition of colon cancer proliferation

In addition to triggering apoptosis in colorectal tumor cells, propionate and butyrate have been reported to inhibit colon cancer proliferation through G0/G1phase cell cycle arrest.[14,15] In our study, we observed through the clonogenic survival assay that the size of clones derived from HCT8 cells with GPR43 expression was substantially smaller than those cells transfected with a control vector (Fig. 4*b*), which suggested

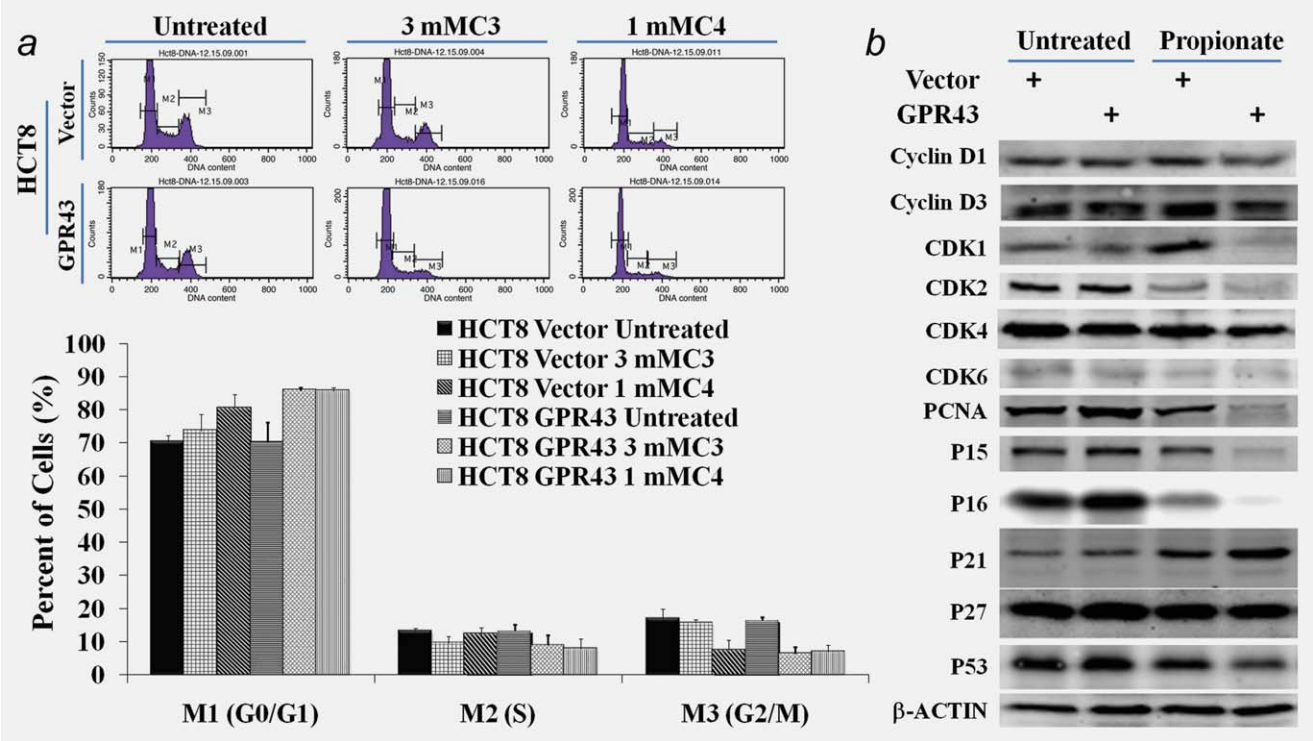


**Figure 5.** Role of GPR43 in propionate/butyrate-induced inhibition of colon cancer proliferation. (*a*) HCT8 cells were transfected with GPR43 or a control vector. Twenty-four hours after the transfection, the cells were treated with propionate and butyrate at the indicated concentrations for 48 hr. The percentage of cells in separate cell phases was shown, and the bars represent the mean ± SE ($n = 3$). (*b*) Expression of a number of cell cycle regulators associated with cell cycle arrest in colon cancer HCT8 cells following GPR43/propionate (3 mM) treatment for 48 hr. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

pronounced proliferation inhibition in HCT8 cells subjected to GPR43/SCFAs treatment. The reduced cell proliferation was associated with an increase in the G0/G1 phase arrest with a concomitant decrease in the proportion of cells in the S and $G_2$/M phase (Fig. 5*a*).

Next, we investigated the effect of GPR43/propionate treatment on the level of several G1 phase-related proteins. Particularly, we focused on D-type cyclins (the key regulator of G1-S progression) and D-type cyclins related CDKs, p21 Waf1/Cip1 (the main inactivator of the cyclin D and CDK2/4/6 complex assembly and activity), and p53 (the most important regulator of Waf1/Cip1 transcription). We found that propionate downregulated cyclin D1/3 expression more significantly in HCT8 cells with GPR43 expression than those transfected with a control vector (Fig. 5*b*). We also observed a dramatic reduction of CDK1 and CDK2 in HCT8 cells with GPR43/propionate treatment, whereas neither CDK4 nor CDK6 was significantly changed. In addition to cell cyclins and CDKs, proliferating cell nuclear antigen (PCNA), a well-accepted marker of proliferation, was reduced in HCT8 cells with GPR43/propionate treatment.

To investigate the mechanisms by which D-type cyclin related CDKs were downregulated, we examined the levels of CDK inhibitors (CKIs). So far, two classes of CKIs have been defined: the INK proteins, which block activation of D cyclin-containing complexes,[30] and the Cip/Kip proteins, which target D-, E- and A-containing complexes.[31] In the INK family, we found that both the p15 and p16 levels were reduced in HCT8 cells with GPR43/propionate treatment; while in Cip/Kip family, we found that p21 expression was dramatically increased in HCT8 cells with GPR43/propionate treatment. Another member of Cip/Kip proteins, p27, was slightly decreased at 48 hr. Finally, we found that in contrast to elevated p21 expression, p53 was reduced in HCT8 cells with GPR43/propionate treatment (Fig. 5*b*). The results indicate that the molecular events involved in GPR43-mediated colon cancer cell cycle arrest during propionate treatment include the downregulation of PCNA, cyclin D3, CDK1 and CDK2, and the stimulation of p21 expression in a p53-independent manner. Taken together, the data provide evidence that GPR43 may be an inducer of apoptosis and a negative regulator of cell cycle progression in colon cancer cells. They also partially explain why it is advantageous for colon cancer cells to suppress its expression.

## Discussion

SCFAs are well-known antiproliferative agents for colon cancer cells. They are generated by microbial fermentation of resistant starches and indigestible fiber in the hindgut by anaerobic gut flora. SCFAs are expected to enter colon cancer cells

to exert growth inhibitory effects. Sellin *et al.* demonstrated that SCFAs are rapidly absorbed by colorectal epithelial colonocyte and that transporters that provide luminal protons stimulate SCFA absorption.[32] GPR43 is a recently identified G-protein-coupled receptor for SCFAs.[17] In our study, we demonstrate that GPR43 restoration potentiates the antitumor effects of propionate and butyrate in human colon cancer.

We found that GPR43 expression is frequently lost in cancerous colon tissues. Previously, GPR43 has been detected in both the human colon[22] and in the rat distal ileum and colon.[23] Immunohistochemistry data from studies by Karaki *et al.* have shown that GPR43 was detected at the enteroendocrine (in rat distal ileum and colon)[23] and enterocytes (human colon).[22] Enterocytes, together with, Goblet cells, enteroendocrine cells, and Paneth cells make up the four principal cell types of the small intestinal epithelium. In agreement with these findings, we found that GPR43 was abundantly expressed in normal human colon mucosa tissue, with the highest expression of GPR43 observed at surface differentiated epithelium as well as in the basal cells at the bottom of the crypt (Fig. 1). In sharp contrast, in malignant colon adenocarcinoma tissues, GPR43 immunoreactivities were dramatically reduced by 80%. We also found that colon hyperplasia and benign colon tumor still retain about 65% of GPR43 expression compared with normal tissues (Fig. 1*b*). The expression patterns of GPR43 suggest that the loss of GPR43 is related to the transition from a benign stage to a malignant carcinoma. The results suggest that a loss of GPR43 expression may contribute to colon cancer development and progression.

Among all SCFAs, propionate is the most potent agonist toward GPR43.[17,19] Propionate is one of the major SCFAs produced by fermentation, accounting for ~25% of total SCFAs in colonic fluid.[13] The $EC_{50}$ of propionate toward GPR43 is ~4.85 mmol/l. In humans, although the usual concentration of propionate in circulation is too low (~4–5 μmol/l) to activate the receptor, propionate reaches local concentrations as high as 2–15 mmol/l in the terminal ileum and 60–130 mmol/l in the proximal colon.[33,34] In addition, the fact that GPR43 is expressed in the human colon[22] suggests that the ability of propionate to inhibit colorectal cancer cell proliferation and trigger apoptosis may also be mediated extracellularly *via* the receptor.

One clue to GPR43 function(s) comes from the analyses of the effects of its potent ligands on colon cancer cell proliferation and survival.[14–16,35] Herein, we found that restoration of GPR43 expression enabled the HCT8 cells to become more sensitized toward propionate/butyrate-induced apoptotic cell death (Figs. 4*a*–4*c*). This was accompanied by elevated activation of initiator caspases 6, 7 and 8, and executioner caspase 3 (Fig. 4*d*). Several antiapoptotic proteins of the inhibitor of apoptosis (IAP) family such as Bcl-2 and Survivin, whose overexpression leads to resistance to apoptosis caused by various apoptotic stimuli in colon cancer cells,[36] were reduced precipitously during GPR43/propionate-induced apoptosis. We also examined the death receptor related RIP level. RIP is critically involved in tumor necrosis factor receptor-1 (TNF-R1)-induced NF-kappa B activation, and overexpression of RIP has been shown to induce cell death in transfected cells.[37] However, we found that RIP remained constant during GPR43/propionate-induced apoptosis. This indicates that RIP may not be involved in GPR43/propionate-induced apoptosis.

Propionate and butyrate have been reported to inhibit colon cancer proliferation through G0/G1phase cell cycle arrest.[14,15] In our study, we observed that the GPR43 restoration potentiated propionate/butyrate's inhibitory effects toward colon cancer proliferation. Cell cycle progression has been shown to be modulated by the fluctuation of various cyclins/CDKs and corresponding regulators known as cyclin kinase inhibitors. Among the Cip/Kip family of CDK inhibitors, the tumor suppressor protein p21 (Waf1/Cip1) acts as a key inhibitor of cell cycle progression. In association with CDK2 complexes, it serves to inhibit kinase activity and block progression through G1/S.[38] It may also enhance assembly and activity in complexes of CDK4 or CDK6 and cyclin D.[39] The carboxy-terminal region of p21 is sufficient to bind and inhibit PCNA, a subunit of DNA polymerase, and may coordinate DNA replication with cell cycle progression.[40] In our study, we found that p21 was dramatically increased concomitant with GPR43/propionate-induced cell cycle arrest (Fig. 5*b*). Therefore, it is highly possible that p21 is involved in GPR43/propionate-induced cyclin D3, CDK1 and CDK2 downregulation and resultant G1/G0 cell cycle arrest. Upregulation of p21 by tumor suppressor p53 in response to DNA-damaging stresses is well established and is thought to be an integral part of the p53-mediated growth-arrest pathway.[41] However, p21 expression has been showed to be upregulated independently of p53 as well.[42] Here, we show that expression of p53 is reduced in GPR43/propionate-induced cell cycle arrest (Fig. 5*b*). Therefore, regarding the mechanism of action through which GPR43/propionate upregulates p21waf1/cip1 expression, the present results seem to suggest a p53-independent pathway. In addition, contrary to upregulation of p21 expression during GPR43/propionate-induced cell cycle arrest, we found two members of INK family of CDK inhibitors p15 and p16 were downregulated (Fig. 5*b*). p15 and p16 have been shown to be able to specifically inhibit cyclin D/CDK4/6 complexes.[43,44] As shown in Figure 5*b*, in contrast to the significant downregulation of CDK1/2, we observed only slight changes of CDK4/6 during GPR43/propionate treatment. Based on these results, it is possible that p15 and p16 are not involved in GPR43/propionate-induced cell cycle arrest.

In addition to investigating the role of GPR43 restoration caused sensitization of colon cancer cells toward propionate treatment *in vitro*, we have attempted to demonstrate an effect of GPR43 restoration on tumor xenograft formation. We have tried to establish HCT8 sublines that stably express

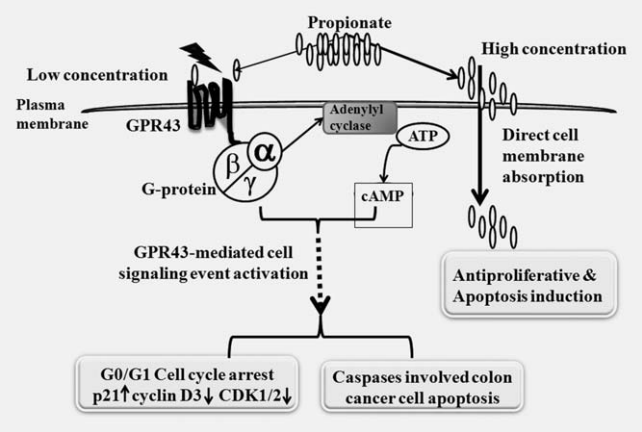


**Figure 6.** Schematic representation of the proposed role of GPR43 as a functional tumor suppressor in colon cancer. The bacterial fermentation product of dietary fiber propionate inhibits colon cancer cell proliferation and induces apoptotic cell death by two approaches: one through direct cell membrane absorption and the other one through GPR43 activation of multiple cellular signaling events, leading to growth inhibition and apoptosis by influencing several key cell cycle regulators and caspases involved cell apoptosis activation.

GPR43. However, the efforts are hampered by the growth disadvantages presented by the HCT8 cells with GPR43 expression restored. Currently, we are exploring alternatives such as tet-inducible system to restore GPR43 expression. Clearly much more studies are needed to determine whether GPR43 restoration can be a feasible approach to prevent or treat colon cancer. Nevertheless, we are excited about the possibility of GPR43 as a possible effector in colon cancer prevention and treatment.

In our study, RT-PCR analysis detected the presence of full length *GPR43* mRNA in only one (HT-29) of nine established human colon cancer cell lines. However, despite that fact that GPR43 restoration potentiated propionate-induced cell death and cell cycle arrest, we have not observed significant difference between HT-29 (GPR43 expressing) and HCT8 (non-GPR43-expresing) cells regarding their sensitivity toward propionate-induced biologic effects (cell proliferation and apoptosis). One possibility is that HT-29 cells may develop adaptive strategies to counteract the GPR43 mediated cell deaths. It might also result from the different genetic background of the two cell lines as HCT-8 is an ileocecal colorectal adenocarcinoma derived from a male while HT-29 is a colorectal adenocarcinoma from a female.

It should also be noted that ectopically restored GPR43 may not necessarily reflect the physiological levels of GPR43 expression. Currently we do not have detailed understanding how GPR43 expression is regulated and physiological levels of GPR43. It is likely GPR43 expression can vary under different physiological conditions. By restoring GPR43 expression, we observed a novel function of GPR43 in colon cancer suppression in the presence of it ligands. Realizing the potential tumor suppressing activity of GPR43, we attempted to restore the endogenous *GPR43* in colon cancer cells. The loss of GPR43 may be due to epigenetic silencing. However, we did not find evidences suggesting that the loss of GPR43 expression in colon cancer cells is caused by either HDAC-induced chromatin over-compaction or promoter (gene) hypermethylation. Further efforts are needed to explore how *GPR43* is silenced in colon cancer and whether *GPR43* can be restored to augment the antitumor activities of SCFAs in colon.

In summary, GPR43 behaves as a functional tumor suppressor in the colon. It is abundantly expressed in normal colon tissues, moderately reduced in colon hyperplasia and benign colon tumors, but downregulated precipitously in colon carcinoma cells. Restoration of GPR43 in colon cancer cells retards growth and increases apoptosis concomitantly in the presence of propionate. The growth inhibition is strictly related to a persistent block of the cells in the G0/G1 phase of cell cycle and to alterations in some cell cycle-related proteins. In addition, several members of caspases are involved during GPR43/propionate-induced apoptotic cell death. Therefore, the major microbial fermentation product propionate in the human colon is able to inhibit colon cancer cell proliferation and trigger apoptosis by two independent approaches: one through direct entry into the cells as suggested in the literature, and the other through GPR43 activation of cellular signaling events, leading to growth inhibition and apoptosis induction (Fig. 6).

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