Inhibition of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway decrease DNA methylation in colon cancer cells

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Abstract

The extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK-MAPK) pathway is a critical intermediary for cell proliferation, differentiation and survival. In the human colon cancer cell line SW1116, treatment with the DNA methyltransferase1 (Dnmt1) inhibitor 5-aza-2’-deoxycytidine (5-aza-dC) or the ERK-MAPK inhibitors PD98059 or rottlerin, or transient transfection with the MAP/ERK kinase (MEK)1/2 siRNA downregulates Dnmt1 and proliferating cell nuclear antigen (PCNA) levels. In this report, we found that drug treatment or siRNA transfection of SW1116 cells induced promoter demethylation of the p16^{INK4A} and p21^{WAF1} genes, which up-regulated their mRNA and protein expression levels. Flow cytometry revealed that rottlerin treatment induced cell cycle arrest at phase G1 ($P < 0.05$). Thus, the ERK-MAPK inhibitor treatment or siRNA-mediated knockdown of ERK-MAPK decreases DNA methylation via down-regulating Dnmt1 expression and other unknown mediator(s) in SW1116 colon cancer cells.

Colon cancer is one of the most commonly occurring tumors and the leading cause of cancer-death worldwide. Cells derived from the colon cancer cell line SW1116 are frequently used in molecular biological experiments (1-3). Mitogen-activated protein kinases (MAPks) are serine-threonine kinases that are activated by phosphorylation in response to a wide array of extracellular stimuli(4,5). The extracellular signal-regulated kinase (ERK)-MAPK pathway is a critical pathway for cell proliferation, differentiation and survival. Activation of ERK1 and ERK2 (ERK 1/2) in this pathway modulate a wide variety of cellular activities via the regulation of several transcription factors. The ability of the ERK-MAPK pathway to promote cell growth by activation of Cyclin D is counterbalanced by the concomitant production of the cyclin-dependent kinase inhibitor p21^{WAF1}. Moderate activation of the pathway leads to cell proliferation, while hyperactivation results in p21^{WAF1}-mediated growth arrest. In addition, induction of the cell cycle inhibitory INK4 proteins, including p16^{INK4A}, is mediated by the Ras/Raf/MEK/ERK pathway (6). Several lines of evidence have indicated that overexpression and activation of ERK-MAPK plays an important role in colon cancer progression, and that may be a useful molecular target for colon cancer therapy (7).

To date, 11 protein kinase C (PKC) isoenzymes have been identified (8). They have been implicated in the regulation of cell growth and differentiation, as well as in apoptosis and tumor progression (9). Although PKC-δ is the most thoroughly studied member in the PKC subfamily, many questions remain to be answered about its substrate-specific activities (10).

Aberrant DNA methylation is now considered as an important epigenetic alteration which is intimately involved in the initiation and development of colon cancer in both mouse and human tumor model systems (11,12). In particular, hypermethylation in the promoters of tumor suppressor genes correlates with the loss of expression of these genes in cancer cell lines and primary tumors (13,14). In recent years, several reports have shown that two ERK-MAPK inhibitors, procainamide and hydralazine, produce hypomethylation in T cell lines (4,15-17). These observations have led to efforts to use these drugs to demethylate tumor suppressor genes in cancer cells. However, the mechanism whereby these ERK-MAPK pathway inhibitors affect DNA methylation that silences gene transcription in cancer, particularly in human colon cancer, is poorly understood. Furthermore, the effect of the PKC-δ inhibitor rottlerin(18) on tumor-related gene regulation and the cell cycle in human colon cancer cell lines is unknown.

The aim of this study was to further elucidate the etiological association of the ERK-MAPK pathway and methylation in human colon cancer, and to investigate the extent of the loss of MAP/ERK kinases (MEK)1 and 2 and the effects of ERK signaling on cell proliferation and cycling. A colon cancer cell line, SW1116, was incubated with the MEK1/2 inhibitor PD98059.
(2-(2'-amino-3'-methoxyphenyl) oxanaphthalen-4-one, a MEK inhibitor) and the PKC-δ inhibitor rottlerin, and transiently transfected with the selective ablation of MEK subtypes by RNA interference (RNAi). The influence of these drugs or transfections on DNA methylation, subsequent expression of tumor suppressor genes and the cell cycle, were also assessed. We also investigated the mediating mechanisms of ERK-MAPK on DNA methylation.

**Experimental Procedures**

**Cell culture** - The colon cancer-derived cell line SW1116 was maintained by serial passages in RPMI 1640 containing 10% heat-inactivated FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cultures were incubated at 37°C using standard tissue culture incubators as previously described (11). A total of 10⁶ cells were plated per 100-mm dish.

**Treatment with PD 98059, rottlerin and 5-aza-dC** - PD98059 and rottlerin were purchased from Promega (Madison, WI, USA) and from Sigma-Aldrich (St. Louis, MO, USA), respectively. The inhibitors were dissolved in DMSO and used at a final concentration of 50 μM and 20 μM, respectively. ERK-MAPK inhibitor treatments were performed on serum-starved SW1116 cells before stimulation with 50 ng/ml of phorbol myristate acetate (PMA) for 15 min. To assess the effect of these inhibitors on methylation and gene expression, the cells were exposed to different concentrations, 2 μM, 5 μM and 10 μM of 5-aza-dC (Sigma) for 24 h and 72 h as the controls for ERK-MAPK inhibitor treatment. Cells treated with DMSO for 24 h were used as a vehicle controls. DNA and RNA were harvested at various time points throughout the treatments.

**RNAi and transient transfections** - MEK1/2 expression was inhibited with reagents from commercial siRNA kits according to the manufacturer’s instructions. The SignalSilence™ MEK1 kit and SignalSilence™ MEK2 kit (Cell Signaling Technology, Danvers, MA, USA) included human MEK1 siRNA, MEK2 siRNA, control siRNA (Fluorescein Conjugate) and siRNA transfection reagent. Briefly, SW1116 were seeded in 12-well plates in medium containing 10% serum at a density that would allow cells to reach 50% confluence on the day of transfection. The transient transfections were performed using 100 nM of each siRNA duplex. For the double knock-down, 100 nM of MEK1 and MEK2 siRNA were used. These siRNAs were complexed with transfection reagent, in serum-free and antibiotic-free culture fluid for 5 h at 37°C. After the transfection reagents were applied, the cellular media were replaced with serum-containing maintenance media and the cells were incubated for 48 h. All experiments were repeated at least three times. The transfection efficiency was determined by fluorescein conjugated non-specific siRNA-transfected cells using a fluorescence microscope (Olympus, Japan). Cells were incubated for 5 min at room temperature with lysis buffer (K₂HPO₄, Triton), and lysates were assayed for luciferase activity. The protein concentrations were determined with a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL), and luciferase activity was quantitated by luciferase assays (Promega, Madison, WI) using a luminometer. The selective silencing of the appropriate MEK isozymes was confirmed by Western blotting with MEK1 and MEK2 specific antibodies.

**Cell Viability Assays** - The SW1116 cells were seeds onto 24-well plates with 2000 cells/well, cultured for 24 h, and transfected with the indicated siRNAs. The cells were then treated with the ERK-MAPK inhibitors or 5-aza-dC. Cell proliferation was measured using the methyl thiazolyl blue tetrazolium bromide (MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetra-zolium, Calbiochem, San Diego, CA, USA) colorimetric dye assay in quadruplicate. At each time point, the SW1116 cells were incubated with 0.3 ml MTT dye (1 mg/ml in serum-free media) for 2 h at 37°C. Adding 200μl of 0.04 N HCl in isopropyl alcohol produced the color, the absorbance of which was monitored at 570 nM.

**Real-time RT-PCR for mRNA expression of tumor-suppressor genes** - To evaluate the
efficiency of treatment with MAPK inhibitors or Dnmt inhibitor, and transfection with MEK1/2 siRNA, mRNA transcription levels of Dnmts, p16\textsuperscript{INK4A} and p21\textsuperscript{WAF1} were detected using real-time RT-PCR. Total RNA was isolated with a commercial kit (Trizol®) according to the manufacturer’s instructions (Invitrogen/Gibco BRL, Carlsbad, CA, USA). Reverse transcription reactions using 5 μg of total RNA in a final reaction volume of 20 μl were performed with Superscript II reverse transcriptase (Invitrogen Life Technologies). Relative quantitation data was obtained using the comparative Ct method with the ABI PRISM 7700 Sequence Detection System (software version 1.6) according to the manufacturer’s protocol. Primers and fluorogenic probes were provided by Shenyou Company (Shanghai). The sequences of the probes and the primers, as well as the PCR reaction conditions, are shown in Table 1. Real-time PCR was also performed with the primers and probe for β-actin to normalize each of the extracts for amplifiable human DNA. Results were expressed as the ratio of copies of target genes to β-actin. The Ct values were measured, and the average Ct of triplicate samples was calculated. Significant alteration of mRNA expression was defined as a 3-fold difference in the expression level after treatment relative to that before treatment(19).

**MSP and Bisulfite sequencing of p16\textsuperscript{INK4A} and p21\textsuperscript{WAF1} promoters-** Methylation-specific PCR (MSP) was performed in CpG-rich regions of the p16\textsuperscript{INK4A} and p21\textsuperscript{WAF1} gene promoters in order to monitor changes in the DNA methylation of tumor suppressor genes due to drug treatments or transfection. Bisulfite modification protocols were adapted as described by Xiong and Laird (20). Genomic DNA treated with bisulfite was amplified with p16\textsuperscript{INK4A} and p21\textsuperscript{WAF1} promoter specific primers (Table 2). The primers were designed without CpG dinucleotides to enable both methylated and unmethylated alleles to be amplified.

There were 100 ng of bisulfite-treated DNA, 0.1 mM dNTPs, 2.0 mM MgCl\textsubscript{2}, and 0.5 μM primers in a 50 μl PCR reaction. The PCR product was directly loaded onto 3% agarose gels and electrophoresed. The gel was stained with ethidium bromide and directly visualized under UV illumination. Wild-type p16\textsuperscript{INK4A} and p21\textsuperscript{WAF1} primers were used to verify that complete conversion of the DNA had occurred in the bisulfite reaction. A positive control for complete methylation was also amplified.

To further identify the methylation pattern within the CpG islands of the p16\textsuperscript{INK4A} and p21\textsuperscript{WAF1} promoter in SW1116 cells, bisulfite sequencing were performed. DNA was treated with bisulfite as described above. Primer sequences of p16\textsuperscript{INK4A} and p21\textsuperscript{WAF1}, annealing temperatures, and expected PCR product sizes are summarized in Table 2. PCR products were sequenced using ABI PRISM 3730 sequencer.

**Determination of Dnmt1, p21\textsuperscript{WAF1}, PCNA and phospho-ERK-MAPKs using Western blotting -** Western blot assays were performed to examine the phosphorylation of ERK-MAPK molecules and Dnmt1, p21\textsuperscript{WAF1}, and PCNA protein expression. Whole cell extracts were prepared from both treated and mock-treated, or transfected and mock-transfected SW1116 cells by a previously described method(21). After electrophoresis, 400 μg of proteins were electro-eluted at 120 V onto a PVDF membrane (Invitrogen). Primary antibodies raised against phosphorylated ERK1/2, pan-ERK1, pan-ERK2, phospho-MEK1/2, pan-MEK1/2, anti-MEK1, anti-MEK2 and phospho-PKC-δ were purchased from Cell Signaling Technology (Beverly, MA). Pan-PKC-δ was obtained from Biosource International (Camarillo, CA, USA), that only recognizes the activated phosphorylated form of ERK1/2 by Western blot analysis. Antibody anti-Dnmt1 was got from New England BioLabs (Newton, MA), antibodies anti-p21\textsuperscript{WAF1} and anti-PCNA were purchased from Sigma (St. Louis, MO.). Antibody against β-actin (Sigma) was used as a control for protein input.

The densities of the Western blotting bands in each lane were normalized to the amount of total protein (for Dnmt1, PCNA or p21\textsuperscript{WAF1}) or total MAPKs as determined by the density of the β-actin band; or pan-MAPKs. For example, if the β-actin band was 30,000 units (pixels of brightness), then Dnmt1 was normalized by the...
following calculation: 30,000/ (density of β-actin) × (density of Dnmt1). The Western blotting analysis was repeated at least three times (21).

**Flow cytometric (FCM) detection of cell cycle progression** - Cell cycle analysis was carried out by FCM (22). In brief, approximately 1 × 10^8 cells were removed from treated and mock-treated cultures at specified time points. The cells were washed twice with PBS and fixed in ice-cold ethanol for 1 h. The samples were then concentrated by removing the ethanol and exposure to 1% (v/v) Triton X-100 (Sigma, USA) and 0.01% RNase (mg/ml, Sigma) for 10 min at 37°C. Cellular DNA was stained in the dark with 0.05% propidium iodide for 20 min at 4°C. Cell cycle distributions were determined using a flow cytometer (Model FACSCALIBAR, B.D., USA). The data obtained from 10,000 cells was analyzed with the MultiCycle software package (Phoenix, San Diego, CA, USA).

**Statistics** - Data were representative of at least three independent experiments performed in triplicate, and are presented as the means ± standard deviation. Comparisons between groups were made using the Student’s paired t test. Relationships were analyzed by Fisher’s exact test using SAS 6.12 software. A value of P < 0.05 was taken to indicate a significant difference between the mean values.

**Results**

Both ERK-MAPK inhibitors and siRNA transfection suppress colon cancer cell growth

We first examined the antiproliferative effects of ERK-MAPK inhibitors on the colon cancer cells. Methyl thiazolyl blue tetrazolium bromide (MTT) assays showed that treatment of SW1116 cells with the PKC-δ inhibitor rottlerin resulted in a significant inhibition of growth starting from 24 h at 20 μmol/L. Growth inhibition was also apparent at a low dose, 2.5 μmol/L, after 72 h (Fig 1A). Dose-dependent studies using MTT tests further demonstrated that after 72 h of treatment, the MEK1/2 inhibitor PD98059 suppressed cell growth at intermediate concentrations (50 μmol/L) (Fig 1B).

Our results indicated that RNAi-induced MEK1 or MEK2 deficiencies inhibited SW1116 cell growth, which declined maximally 24 h after transfection. This suppression lasted for another 12 h, and almost recovered at 48 h post-transfection. As shown in Figure 1C, the degree of growth inhibition caused by siMEK1 was higher than that caused by siMEK2, while the growth inhibition was significantly different with both MEK1/2-deficient (siMEK1/2) at 24 h post-transfection (P< 0.01)).

**PD98059 or rottlerin treatment down-regulate phosphorylation of ERK-MAPK**

Cells were incubated with ERK-MAPK inhibitors, and cell lysates were analyzed for MAPKs phosphorylation by Western blot analysis. Total ERK1/2 protein levels were not decreased in PD98059- and rottlerin-treated lysates compared with the vehicle controls. However, PD98059 and rottlerin treatment did decrease the PMA-stimulated phosphorylation of MEK1/2 and ERK1/2 in SW1116 cells (middle and upper panels, Fig 2). In contrast to the inhibition exerted by PD98059, rottlerin also significantly down-regulated the phosphorylation of PKC-δ (lower panel, Fig 2).

**The depletion effect of MEK1 and MEK2 by RNAi**

We used siRNA to selectively reduce the expression levels of MEK1 and MEK2, which are major subunits of the ERK-MAPK pathway. With the high transfection efficiency of siRNA (data not shown), Western blot analysis revealed that the MEK1/2 protein levels in SW1116 cells transfected with the MEK siRNAs singly and together were reduced to 36% ± 1.02%, 34% ± 0.98% and 9% ± 0.76%, respectively. Control SW1116 cells transfected with nonsilencing siRNA in the three experiments produced normal MEK1 and MEK2 levels (Fig. 3A).

The effect of depleting MEK and ERK activities by suppressing MEK1/2 was assessed in Western blots using antibodies specifically recognizing phosphorylated (and thus activated) MEK1/2 and ERK1/2. Both knockout
experiments resulted in almost a total loss of pan- and phospho-MEK levels, while the phospho-ERK protein levels were analyzed as readouts for MEK activity upon MEK1/2 knock-down and the results showed that phospho-ERK was also descent (Figures 3B and 3C).

**ERK-MAPK inhibitors and siRNA transfection repress PCNA and Dnmt1, but not Dnmt3a and Dnmt3b**

Western blotting was also performed to examine the effects of ERK-MAPK inhibitors on proliferating cell nuclear antigen (PCNA) and DNA methyltransferase1 (Dnmt1). As shown in Fig 4, rottlerin ($P < 0.01$) and PD98059 ($P < 0.05$) significantly down-regulated PCNA expression (Fig 4), and also induced a decrease in Dnmt1 protein expression ($P < 0.05$) that was similar to previous results obtained with cells treated by the Dnmt inhibitor 5-aza-dC ($P < 0.01$) (Fig 4).

Blocking the ERK-MAPK pathway inhibited SW1116 cell growth, as assessed by measuring PCNA and Dnmt levels. PCNA is a cell proliferation marker gene, and Dnmt1 plays an important role in maintaining DNA methylation patterns via complicated networks including signaling pathways and transcriptional factors relating to cell differentiation or carcinogenesis. Thus, we analyzed the effects of MEK1 and MEK2 depletion on PCNA and Dnmt1 protein levels $24\text{ h}$ following cellular transfections. Upon depletion of either MEK1 or MEK2 alone, the PCNA in protein levels decreased along with cell viability and proliferation. Moreover, the protein expression of PCNA was significantly decreased in the double knock down of MEK1/2. Unexpectedly, our data indicated that the protein of Dnmt1 expression decreased concordantly with cell proliferation inhibition and PCNA (Fig 4).

Furthermore, Dnmt3a and Dnmt3b are essential for de novo methylation. To clarify the effects of Dnmt 3a and Dnmt 3b with blocking the ERK-MAPK pathway, we examined their expressions by Real-time RT-PCR (Figure 5). Our results indicated that ERK-MAPK inhibitors and siRNA transfection failed to affect the expression level of Dnmt3a and Dnmt3b.

**ERK-MAPK inhibitors and siRNA transfection up-regulate p16$^{INK4A}$ and p21$^{WAF1}$**

In this study, inhibiting the ERK-MAPK pathway induced cell cycle arrest. However, members of the cyclin-dependent kinase inhibitor (CDKI) family, including p21$^{WAF1}$, p16$^{INK4A}$ and p27$^{KIP1}$, inhibit a wide range of cyclin CDK complexes involved in G1 and S phase progression. Thus, real-time PCR was used to ascertain whether inhibiting or blocking the ERK-MAPK pathway exerts any effects on colon cancer-related tumor suppressor genes. The assay was also used to clarify whether PKC-δ and MEK1/2 signaling would interact with the methylation-selective tumor-related genes. We found that both PD98059 and rottlerin increased p16$^{INK4A}$ and p21$^{WAF1}$ transcriptional levels, which is consistent with data from samples treated with 5-aza-dC (Fig 5). The results of MEK1/2 depletion also showed that p16$^{INK4A}$ and p21$^{WAF1}$ transcriptional levels increased. Meanwhile, Western blot analysis indicated that PD98059, rottlerin and MEK1/2 siRNA induced p21$^{WAF1}$ protein expression. (Figure 4).

**ERK-MAPK inhibitors treatment or siRNA transient transfection induce demethylation of p16$^{INK4A}$ and p21$^{WAF1}$**

To confirm that ERK-MAPK inhibition directly affect the methylation of p16$^{INK4A}$ and p21$^{WAF1}$ promoters, the methylation status following treatments with ERK-MAPK inhibitors was determined using MSP. Bisulfite treatment converted cytosine residues in the genomic DNA to uracil, which were amplified as thymine during the subsequent PCR. As shown in Fig 6A and Fig 6C, primers that specifically amplified either the methylated or unmethylated form of the p16$^{INK4A}$ promoter produced 150-bp methylated bands from mock-treated SW1116 cells, indicating that the p16$^{INK4A}$ promoter was methylated in these alleles. The methylated bands of p16$^{INK4A}$ gene from cells treated with 5-aza-dC (10 $\mu$m) or ERK-MAPK inhibitors (50 $\mu$m of PD98059 and 20 $\mu$m of rottlerin), MEK1/2 siRNA (100 nM) for 24 h were consistently less intense than the products of mock-treated cells. Thus, there
was more 151-bp unmethylated product, than the corresponding methylated product, in 5-aza-dC or MAPK inhibitor-treated cells. These data indicated that untreated SW1116 cells were methylated at some CpG sites in most alleles. This finding also excluded the possibility that successful amplification could be attributed to incomplete bisulfite conversion.

We also examined the methylation state of p21WAF1 gene. As shown in Fig 6B and Fig 6D, the methylated band was predominant in mock-treated or -transfection control cells. Similar to the results obtained with 5-aza-dC, rottlerin treatment and MEK1/2 depletion increased the unmethylated band and decreased the methylated band of the p21WAF1 promoter. Thus, PD98059 inhibitors favored the demethylation of p21WAF1.

Sequencing analysis had the similar changes as MSP. As shown in Fig 7, CpGs of p16INK4A and p21WAF1 promoters in mock-treated SW1116 cells showed multiple cytosines due to the resistance of methylated cytosines within CpG dinucleotides to bisulfite conversion. However, cells treated with 5-aza-dC or ERK-MAPK inhibitors (PD98059 and rottlerin), MEK1/2 siRNA transfection showed numerous thymidines due to sodium bisulfite conversion of unmethylated cytosines to uracil.

Finally, the correlation between promoter methylation status and reactivation of expression of p16INK4A and p21WAF1 genes treated with ERK-MAPK inhibitors or transfected with MEK1/2 siRNA was investigated. We found that increased p16INK4A and p21WAF1 mRNA expression were significantly associated with promoter demethylation.

**ERK-MAPK inhibition arrest the cell cycle**

To further study the signaling pathways involved in ERK-MAPK inhibition induced cell cycle arrest, the effects of PD98059, rottlerin and MEK1/2 siRNA on p16INK4A - or p21WAF1-mediated G1-phase arrest were evaluated. As illustrated in Fig 8, pretreatment of SW1116 cells with rottlerin and MEK1/2 siRNA blocked the cell cycle in G1 phase (P < 0.05). Interestingly, PD98059 caused another cell cycle perturbation---- S-phase arrest (23) (P< 0.05). This observation is consistent with up-regulation of p16INK4A and p21WAF1 mRNA, suggesting that the ERK-MAPK pathways are involved in this process and rottlerin partially induced G1 arrest. Overall, we consistently observed a relationship between p16INK4A and p21WAF1 expression and cell cycle arrest in PD98059-, rottlerin-treated cells or MEK1/2 RNAi transfected cells.

**Discussion**

Deregulation of the cell cycle commonly contributes to tumorigenesis in human colon cancer. The ERK-MAPK pathway is necessary for cell proliferation (7,24), and activation of this signaling pathway has been shown to be important in intestinal epithelial differentiation (25). There is also a growing body of evidence suggesting that activation of the ERK-MAPK pathway is involved in the pathogenesis, progression and oncogenic behavior of human colon cancer (7,26).

Regarding the relationship between ERK-MAPK signaling pathway and DNA methylation, Richardson and colleagues have suggested that Dnmt is decreased by inhibiting signaling through the ras-MAPK pathway in human T cells(15). The T cells of lupus patients show decreased Dnmt mRNA and enzyme activity, resulting in DNA hypomethylation(4). In contrast, relatively little is known about mechanisms that the inhibition of MAPK use to induce the expression of tumor suppressor genes, and no data are yet available on the relationship among ERK-MAPK, Dnmt1 and DNA methylation in human cancer cells, including those of colon cancer.

Herein, we demonstrate that imbalanced MEK1/2 signaling leads to changes in the amplitude and duration of ERK-MAPK activity. Consequently, p16INK4A and p21WAF1 levels are altered, and cell cycle progression is perturbed. siMEKs deplete these target proteins almost completely, but the residual unaffected MEK1/2 molecules remain functional, retaining a low level of constant ERK activation which considered as base line.

Methylation of 5’CpG islands in tumor suppressor genes and elevated Dnmt levels were also prevalent features of human neoplasia. In the present study, we found that hindering
ERK-MAPK activity by inhibiting or blocking MEK1/2 via RNAi transfection down-regulated Dnmt1 mRNA and protein expression levels in a similar fashion as the Dnmt inhibitor in SW1116 cells.

ERK-MAPK inhibitors, particularly rottlerin, resulted in the demethylation of p16\(^{INK4A}\) and p21\(^{WAF1}\). Moreover, PD98059, rottlerin and MEK siRNA increased the expression of p16\(^{INK4A}\) and p21\(^{WAF1}\). Taken together, these experiments provide direct evidence linking endogenous ERK activation to DNA methylation in a colon cancer cell line. We believe that these differences are because the MEK inhibitor and siMEKs induced the expression of p16\(^{INK4A}\) and p21\(^{WAF1}\), arrested cell cycle progression and inhibited cell proliferation following a decreased activity of the Dnmt inhibitor gene promoter.

The findings of several studies have suggested that PKC-\(\delta\) is involved in the regulation of cell growth and differentiation, and that it plays a role in apoptosis and tumor development (27). However, the effects of PKC-\(\delta\) on tumor suppressor gene expression are poorly understood. Little is known about the biological function of PKC-\(\delta\) as it relates to methylation. The data from this report show that rottlerin, a PKC-\(\delta\)-specific inhibitor, decreases Dnmt1 expression and increases p16\(^{INK4A}\) and p21\(^{WAF1}\) expression, while PD98059 up-regulate significantly p16\(^{INK4A}\) and partially up-regulate p21\(^{WAF1}\) when compared with vehicle controls. These results provide further support for the hypothesis that PKC-\(\delta\) and might also including MEK activation plays an important role in the hypermethylation of tumor-suppressor genes and the pathogenesis of colon cancer. Comparison of rottlerin-treated to mock-treated cells further indicated that rottlerin treatment produced a notable partial reversal of the transformed phenotype.

Cell differentiation requires the proper balance of activation and inhibition of the ERK-MAPK signaling molecules. FCM analysis showed that rottlerin treatment, MEK2 or MEK1/2 siRNA induced the cell cycle arrest in the G1 phase. The findings that ERK-MAPK signal transduction pathway inhibitors affect cell cycle regulation in SW1116 cell line are consistent with the view that ERK-MAPK signaling may regulate DNA methylation. These results are also the first indication that rottlerin plays a role in p16\(^{INK4A}\)-or p21\(^{WAF1}\)-induced cell cycle arrest. Interestingly, PD98059 and MEK1 siRNA arrest cell cycle in S phase but not G1 phase in SW1116 cell line. In fact, PD98059 inhibits MEK1 only but not including MEK2 (28,29) and the loss of MEK2 or ERK2 caused cell cycle arrest at G1(30). PD98059 affect cell transition from S to G2 phase in undifferentiated Caco-2(31,32), and induce cell cycle arrest of the G1 phase in humans colon cancer cell line SW620 but not in HCT116(32). The transient transfection of MEK1/2 siRNAs is sufficient to provide the control of cell cycle entry via the ERK response genes p21\(^{WAF1}\) and p16\(^{INK4A}\), but fails to induce a sustained expression of p21\(^{WAF1}\) and growth arrest.

The question of how ERK-MAPK inhibitors or MEK1/2 siRNA down-regulate DNA methylation merits further attention. Dnmt1 and other mediator(s) are involved in this process. In previous reports, PCNA has been shown to interact with p21\(^{WAF1}\)(33,34) and PCNA-labeling indices were significantly higher in methylated prostate cancer samples than in unmethylated samples (\(P = 0.048\))(35). Moreover, the observation that PCNA can bind to Dnmt1 is consistent with the view that maintenance of the methylation pattern in the genome is also dependent upon PCNA and the relative levels of p21\(^{WAF1}\) and Dnmt1(36). Accordingly, PCNA can only be co-immunoprecipitated with Dnmt1 from transformed cells. In addition, when DNA damage occurs, p21\(^{WAF1}\) colocalises with PCNA at repair sites and can effectively prevent methylation of damaged DNA by precluding Dnmt1 interaction with PCNA, but only in non-transformed cells(36,37). Furthermore, Williams and colleagues demonstrated that complete loss of caveolin-1 results in PCNA over-expression that is accompanied by p21\(^{WAF1}\) down-regulation and basal hyperactivation of ERK 1/2(38). However, there are not the sufficient evidences that indicated PCNA is involved in the process of inhibiting ERK-MAPK pathway decrease DNA methylation.

Data from the present experiment however
showed that ERK-MAPK inhibitors treatment or MEK1/2 siRNA transfection induce the re-expression of p21WAF1 through decreasing Dnmt1 and demethylation. Recent reports from several laboratories have revealed that ERK pathway signaling can increase p21WAF1 protein levels (39). In contrast, MAPK suppresses p21WAF1 expression in SW 480 cells (40) and COLO-205 cells (41), but induces S-phase arrest in DLD-1 cells (42). These observations suggest that ERK pathway signaling may have differing effects in different cell types, and thus that the consequences of activating the ERK-MAPK pathway may vary with cell type.

It has become clear that there are complicated interactions among ERK-MAPK pathway constituents, DNA methylation and the expression of p21WAF1 in SW1116 cells.

In light of the present data considered in the context of previous findings, we can hypothesize that Dnmt1 but not Dnmt3a or Dnmt3b expression is decreased by rottlerin, PD98059 treatment and MEK1/2 depletion due to an ERK-MAPK pathway-dependent inhibition of other mediator(s) (unknown mediators might be include PCNA). Ultimately these findings may provide a novel strategy for colon cancer therapy.
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Conflict of interest:

We declare no conflict of interest.
Abbreviations list

5-aza-dC, 5-aza-2'-deoxycytidine; ERK-MAPK, extracellular signal-regulated kinase-Mitogen-activated protein kinases; RNAi, RNA-mediated interference; siRNA, small interference RNA; FCM, Flow cytometry; MEK, MAP/ERK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium; MSP, methyl-specific PCR; PKC, protein kinase C; Dnmt1, DNA methyltransferase 1; RT-PCR, reverse-transcription polymerase chain reaction.

Figure Legends

Figure 1. ERK-MAPK inhibition suppress the cell growth on SW1116 cell line. SW1116 cells were seeded in a 96-well plate until subconfluent. Viable cells were determined by MTT assay. Three independent time and dose courses were performed in triplicate. Cells were treated with PD98059 (A) or rottlerin (B), or transient transfected with MEK1/2 siRNA(C). *P < 0.05; **P < 0.01.

Figure 2. ERK-MAPK inhibitors down-regulate the phosphorylation of MAPKs. Cells were lysed, and equal amounts of protein were analyzed by Western blot analysis using antibodies against phospho-ERK1/2 or pan-ERK (upper panel), phospho-MEK1/2 or pan-MEK 1/2 (middle panel) and phospho-PKC-δ or pan- PKC-δ (lower panel). The density of the Western blot bands were normalized to the total amount of MAPK protein as determined by the density of the band in Western blotting for pan-ERK1/2, pan-MEK1/2 or pan-PKC-δ. The data shown are representative of three replicate experiments. Cells were treated for 72 h with 50 μM of PD98059 or 20 μM of rottlerin.

Figure 3. siRNA-mediated inhibition of MEK1/2 resulted in different MAPK activation characteristics. SW1116 cells were transiently transfected with the indicated siRNAs, and protein lysates were prepared at the indicated times after transfection. A, knockdown efficiency was assayed by Western blots using subtype-specific antibodies. The samples were obtained 24 h after transfection. B, to assay for MEK activation in siRNA-transfected cells, Western blots using anti-phosphoMEK, and anti-panMEK were employed. β-actin was used for the loading control. C, to assay for ERK activation, Western blots using anti-phosphoERK and anti-panERK were employed. β-actin was used for the loading control. Quantification of the target protein bands relative to β-actin control is shown below the blots.

Figure 4. ERK-MAPK inhibitors or siRNAs down-regulate PCNA, Dnmt1 and up-regulate p21WAF1 protein expression. The density of Western blot bands were normalized to the amount of total protein as determined by the density of the β-actin band. The data shown are representative of three replicate experiments. Cells were treated for 72 h with 10 μM of 5-aza-dC, 50 μM of PD98059 or 20 μM of rottlerin, or transfection with siRNAs after 24 h.

Figure 5. ERK-MAPK inhibitors or siRNAs down-regulate Dnmt1 and failed to effect the expression of Dnmt3a and Dnmt3b. ERK-MAPK inhibitors or siRNAs mediated knockdown of MEK1/2 up-regulate the transcription of p16INK4A and p21WAF1 in SW1116 cells. Results are expressed in arbitrary units relative to the mock control, and represent the mean ± SEM of two independent experiments. *P < 0.05; **P < 0.01. The data shown are representative of three replicate real-time RT-PCR experiments. Cells were treated for 72 h with 10 μM of 5-aza-dC, 50 μM of PD98059 or 20 μM of rottlerin, or transfection with siRNAs after 24 h.
Figure 6. MAPK inhibitors induce demethylation of the p16$^{\text{INK4A}}$ (A/C) and p21$^{\text{WAF1}}$ (B/D) promoters. MSP was performed on DNA from SW116 cells with primers designed to specifically detect methylated and unmethylated promoter regions. U, unmethylated-MSP; M, methylated-MSP. The data shown are representative of three replicate MSP experiments. Cells were treated for 72 h with 10 μM of 5-aza-dC, 50 μM of PD98059 or 20 μM of rottlerin, or transfection with siRNAs after 24 h.

Figure 7 Bisulfite sequencing chromatogram of p16$^{\text{INK4A}}$ and p21$^{\text{WAF1}}$. CpGs of p16$^{\text{INK4A}}$ and p21$^{\text{WAF1}}$ promoters in PBS-treated SW1116 cells shows multiple cytosines due to the resistance of methylated cytosines within CpG dinucleotides to bisulfite conversion, whereas, cells treated with 5-aza-dC or ERK-MAPK inhibitors (PD98059 and rottlerin), MEK1/2 siRNA transfection show numerous thymidines due to sodium bisulfite conversion of unmethylated cytosines to uracil. A, chromatogram of p16$^{\text{INK4A}}$; B, chromatogram of p21$^{\text{WAF1}}$.

Figure 8. FCM analysis of SW1116 cells. Rottlerin induced cell cycle arrest at phases G1 to S, PD98059 caused a cell cycle perturbation---- S-phase arrest. The number in the frame represents the mean ± SEM of three independent experiments (cells from mock treatment (or alcohol control) vs those from drug treatment). *$P<0.05$ vs. control, Fisher’s exact test. Cells were treated with: A, DMSO only (vehicle control); B, 50μM of PD98059 for 24 hours; C, 20μM of rottlerin for 24 hours; D, transfection with 100 nM of MEK1/2 siRNAs after 24 h.
### Table 1. Sequences of primers and probes for real-time PCR

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<th>Gene</th>
<th>Primer (forward) (5'→3')</th>
<th>Primer (reverse) (5'→3')</th>
<th>Probe</th>
<th>GenBank Number</th>
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### Table 2. Sequences of primers for MSP and Bisulfite genomic sequence

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Figure 2

![Figure 2 Image]

- Phospho-Erk1
- Phospho-Erk2
- pan Erk1 (44kDa)
- pan Erk2 (42kDa)

Phospho-Erk1/2

- Phospho-MEK 1/2
- pan MEK 1/2 (45kDa)

Phospho-MEK1/2

- Phospho-PKC δ
- pan PKC δ (78kDa)

Phospho-PKCδ
Figure 3

A

- control siRNA
- MEK1 siRNA
- MEK2 siRNA
- MEK1
- MEK2
- β-actin (43kDa)

B

- control siRNA
- MEK1 siRNA
- MEK2 siRNA
- phospho-MEK1/2
- pan-MEK1/2
- β-actin (43kDa)

C

- control siRNA
- MEK1 siRNA
- MEK2 siRNA
- phospho-ERK1
- phospho-ERK2
- pan-ERK1 (44kDa)
- pan-ERK2 (42kDa)
- β-actin (43kDa)
Figure 4
Figure 6

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A

B

C

D

200bp 100bp

200bp 100bp

200bp 100bp

200bp 100bp

+   | -   | -   | -   | Control siRNA
-   | +   | -   | +   | MEK1 siRNA
-   | -   | +   | +   | MEK2 siRNA

M   | U   | M   | U   | M   | U   | M   | U   | U   |

M   | U   | M   | U   | M   | U   | M   | U   | U   |

M   | U   | M   | U   | M   | U   | M   | U   | U   |
Figure 7

A  p16 wt

PBS

PBS + 5-aza-dC

DMSO + PMA + PD98059

DMSO + PMA + Rottlerin

siMEK1/2

B  p21 wt

PBS

PBS + 5-aza-dC

DMSO + PMA + PD98059

DMSO + PMA + Rottlerin

siMEK1/2
Figure 8

A: G0/G1: 46.8%, S: 37.9%, G2/M: 15.3%

B: G0/G1: 57.8%, S: 39.3%, G2/M: 2.9%

C: G0/G1: 71.9%, S: 25.4%, G2/M: 1.7%

D: G0/G1: 69.4%, S: 21.7%, G2/M: 8.9%
Inhibition of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway decrease DNA methylation in colon cancer cells
Rong Lu, Xia Wang, Zhao-Fei Chen, Dan-Feng Sun, Xiao-Qing Tian and Jing-Yuan Fang

J. Biol. Chem. published online February 16, 2007

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