Repression of 15-Hydroxyprostaglandin Dehydrogenase Involves Histone Deacetylase 2 and Snail in Colorectal Cancer

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Abstract

Prostaglandin E₂ (PGE₂) promotes cancer progression by modulating proliferation, apoptosis, angiogenesis, and the immune response. Enzymatic degradation of PGE₂ involves the NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). Recent reports have shown a marked diminution of 15-PGDH expression in colorectal carcinomas (CRC). We report here that treatment of CRC cells with histone deacetylase (HDAC) inhibitors, including sodium butyrate and valproic acid, induces 15-PGDH expression. Additionally, we show that pretreatment of CRC cells with HDAC inhibitors can block epidermal growth factor–mediated or Snail-mediated transcriptional repression of 15-PGDH. We show an interaction between Snail and HDAC2 and the binding of HDAC2 to the 15-PGDH promoter. In vivo, we observe increased Hdac2 expression in Apc-deficient mouse adenomas, which inversely correlated with loss of 15-Pgdh expression. Finally, in human colon cancers, elevated HDAC expression correlated with down-regulation of 15-PGDH. These data suggest that class 1 HDACs, specifically HDAC2, and the transcriptional repressor Snail play a central role in the suppression of 15-PGDH expression. These results also provide a cyclooxygenase-2–independent mechanism to explain increased PGE₂ levels that contribute to progression of CRC. [Cancer Res 2008;68(22):9331–7]

Introduction

The development of colorectal cancer (CRC) involves the accumulation of genetic alterations and epigenomic changes that affect cell growth, cell death, and the tumor microenvironment. Aberrant Wnt, Ras, and cyclooxygenase-2 (COX-2) signaling pathways have been shown to play major roles in the progression of colon cancer and other solid malignancies. Evidence of a positive relationship between COX-2 expression and human CRC was first reported in 1994 (1). Several subsequent studies confirmed that COX-2 expression is elevated in approximately 50% of adenomas and 80% of adenocarcinomas (2, 3). One promising group of compounds for prevention of CRC is nonsteroidal anti-inflammatory drugs that primarily target COX enzymes (COX-1 and COX-2). Both of these enzymes convert arachidonic acid into an endoperoxide intermediate that is further metabolized to five structurally related prostaglandins, including PGE₂, PGD₂, PGF₂α, PGL₂, and thromboxane A₂. However, recent clinical trial results indicate that prolonged use of high dosages of some COX-2–selective inhibitors is associated with unacceptable cardiovascular side effects, although these drugs were extremely effective in preventing polyp recurrence in humans.

A preponderance of data implicates prostaglandin E₂ (PGE₂), the most abundant prostaglandin in CRCs (4), as a significant mediator in cancer progression. COX-2–derived PGE₂ is known to promote tumor-associated neovascularization and inhibition of cell death and to stimulate cell proliferation and motility (5). Treatment of in vivo rodent CRC models with PGE₂ increases cell proliferation and confers a survival advantage on epithelial cells of the gastrointestinal tract (6, 7). For example, Wang and colleagues (8) recently reported that PGE₂ treatment of Apc Min/− mice increased the size and number of intestinal adenomas, especially those in the large intestine. Although steady-state tissue levels of PGE₂ depend on relative rates of biosynthesis and breakdown, virtually all reports examining the role of PGE₂ in physiology and disease have focused solely on COX-dependent formation of this bioactive lipid. A plausible complementary pathway yielding increased local levels of PGE₂ in cancer involves reduced degradation of PGE₂ by NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH).

Human 15-PGDH (encoded by HPGD) gene is located on chromosome 4 and encodes a 29-kDa protein that catalyzes the rate-limiting step of prostaglandin catabolism via oxidation of the 15(S)-hydroxyl group of prostaglandins to yield inactive 15-keto metabolites (9, 10). Genetic deletion of 15-Pgdh in mice leads to increased tissue levels of PGE₂ (11). Whereas prior studies on the distribution and activity of 15-PGDH have focused primarily on parturition and uterine biology, recent data suggest that 15-PGDH plays a role in carcinogenesis (12, 13), with data suggesting that 15-PGDH behaves as a tumor suppressor in lung, breast, and colon cancers (14–18). Additional support for this hypothesis was recently reported using an animal model, in which examination of gastrointestinal tract of 15-Pgdh−/− mice crossed Apc Min/− mice showed that genetic ablation of 15-Pgdh resulted in a 7.6-fold increase in colon tumors arising in these mice (19). Taken together, these reports strongly support the hypothesis that 15-PGDH plays an important role as a tumor suppressor gene in the prevention of carcinogenesis.

Previously, we observed repression of 15-PGDH expression in a subset of human CRCs and CRC cells (15). Furthermore, we reported evidence suggesting that one mechanism of 15-PGDH repression occurs through epidermal growth factor (EGF) induction of the transcriptional repressor Snail to regulate 15-PGDH expression (15, 17). Specifically, EGF can induce Snail, which binds E-box elements (CANNTG) found within the 15-PGDH promoter to
repress transcription (17). In the present study, we further examined the epigenetic regulation of 15-PGDH by histone deacetylases (HDAC) in CRC cells to obtain a better understanding of the underlying mechanism(s) involved. Specifically, our data suggest that HDACs interact with Snail at the 15-PGDH promoter to aid in transcriptional repression of this gene. We show that multiple HDAC inhibitors, including sodium butyrate (NaB) and valproic acid (VPA), induce 15-PGDH expression in CRC cells. Additionally, we show that pretreatment of CRC cells with HDAC inhibitors can block EGF- or Snail-mediated transcriptional repression of 15-PGDH. Chromatin immunoprecipitation (ChIP) assays examining the 15-PGDH promoter in CRC cells shows loss of HDAC2 binding after treatment with a HDAC inhibitor. Furthermore, we observed increased expression of Hdac2 in Apc-deficient mouse adenomas, which inversely correlates with loss of 15-PGDH expression in these polyps. Finally, in human colon cancers, elevated HDAC expression correlates well with down-regulation of 15-PGDH.

Materials and Methods

Reagents and cell lines. VPA, NaB, 5-aza-2‘-deoxycytidine (Aza-dC), and EGF were purchased from Sigma. CRC cell lines HT-29 and LS-174T were maintained in McCoy’s 5A, whereas HEK 293T cell lines were grown in DMEM. Medium was supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin. Cells were maintained in 5% CO2 atmosphere at 37°C.

Quantitative real-time PCR. Total cellular RNA was isolated from cell lines with Trizol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA for each RNA sample was synthesized using a SuperScript III First-Strand Synthesis System (Invitrogen). PCR contained iQ SYBR Green Supermix (Bio-Rad), 50 ng of each primer, and 5 μL of 1:500 diluted reverse transcriptase template in a 25 μL reaction volume. Oligonucleotides for GAPDH and 15-PGDH were used as previously described (17). The quantitative real-time PCR for each treatment was done in triplicate, and the average Ct was determined. The relative gene expression was normalized to GAPDH and calculated using the 2-ΔΔt method.

RNA interference. A short hairpin RNA retroviral expression system, pRetroSuper (20), was used to knock down Snail expression as previously described (17).

Immunoblotting. Analysis of whole-cell lysates was completed as previously described (17). The following antibodies were used: 15-PGDH (1:2,000; Novus Biologicals), β-actin (1:2,500; Sigma), hemagglutinin (HA; 1:1,000; Covance Research Products, Inc.), HDAC2 (1:1,000; Santa Cruz Biotechnology), and DNTM1 (1:500; Abcam). Membranes were treated with horseradish peroxidase–conjugated secondary antibodies and developed using chemiluminescent detection reagent (Amersham).

Transient transfection and reporter gene analysis. Transfection and analysis of a 2.4-kb 15-PGDH promoter luciferase construct in CRC cells was completed as previously described (17).

Immunohistochemistry. Paraffin sections of adult ApcMin/+ intestine were dewaxed, rehydrated, and incubated overnight at 4°C using an antibody against acetyl-histone H3 (1:100; Upstate). Negative controls received no antibody. The Vectastain avidin-biotin complex method peroxidase system (Vector Laboratories) was used for immunodetection.

Animals. C57BL/6 and C57BL/6-ApcMin/+ mice were obtained from The Jackson Laboratory. The mice were housed and fed with standard mouse diet in the Animal Care Facility according to NIH and institutional guidelines for laboratory animals.

Human colorectal tissue samples. Human colorectal tumor specimens were obtained from surgical resections, with Vanderbilt Internal Board approval as previously described (15).

ChIP assay. CRC cells were cultured with either NaB, Aza-dC, or a control vehicle, and ChIP assay was conducted with HDAC2 antibodies using a previously described ChIP assay protocol (21). Primers for the 15-PGDH promoter were 5'-GGTAGGCTACCAGCGGCTCT-3' and 5'- GTTCCCATCTCGTAATCAGTGG-3'.

Statistical analysis. The data are expressed as the mean ± SE. Statistical significance was determined by Student’s t test. All tests were two sided and value of P < 0.03 was considered statistically significant. Spearman correlation coefficient was used to estimate the association between two continuous measurements.

Results

HDAC inhibitors increase expression of 15-PGDH. To further understand the mechanisms responsible for regulating 15-PGDH expression in CRC cells, we determined whether HDACs are involved in the transcriptional repression of 15-PGDH. To investigate this process, we examined multiple CRC cell lines, including HT-29 and LS-174T.

![Figure 1. HDAC inhibitors increase 15-PGDH expression in CRC cells. A and B, 15-PGDH transcripts were quantitated by real-time PCR in HT-29 cells (data for LS-174T cells not shown) treated with increasing concentrations of either NaB or VPA for 24 h. The relative number of 15-PGDH transcripts for each treatment was normalized to GAPDH. The fold induction of the relative levels of 15-PGDH transcripts when compared with control or NaB or VPA treatments are shown on top of each column. C and D, HT-29 cell lysates (50 mg) treated as shown above each lane were separated by 4% to 20% SDS-PAGE; immunoblot probed with antibodies to the proteins shown at right. β-Actin was used as a loading control. Data are averages of three independent experiments with similar findings. Columns, mean; bars, SE.](cancerres.aacrjournals.org)
We determined whether treatment of CRC cells with HDAC inhibitors, VPA and NaB, could alter 15-PGDH expression compared with mock-treated cells. As shown in Fig. 1A, treatment of CRC cells for 24 h with increasing doses of NaB, which is known to inhibit HDAC activity, induces increased levels of 15-PGDH transcripts as observed by quantitative real-time PCR (5 mmol/L NaB; P = 0.049). A similar increase in 15-PGDH protein expression was also observed after NaB treatment of CRC cells (Fig. 1C). In addition, we observed that stimulation of CRC cells with VPA for 24 h increased both 15-PGDH transcript levels and 15-PGDH protein expression in a dose-dependent manner (Fig. 1B and D, respectively). Cells treated with 5 mmol/L VPA displayed a 3.8-fold increase in 15-PGDH expression (P = 0.048).

Inhibition of HDACs blocks EGF- and Snail-mediated transcriptional repression of 15-PGDH. Previously, we reported that the 5′-untranslated region of 15-PGDH contains three Snail binding sites, referred to as E-box elements (CANNTG), in the proximal promoter and established that EGF treatment and/or expression of a wild-type Snail expression construct can reduce 15-PGDH expression in CRC cells (17). Therefore, we sought to determine whether inhibition of HDAC activity could block the ability of EGF or Snail expression to down-regulate 15-PGDH expression in CRC cells.

In accordance with our previous report (17), we observed that EGF treatment of CRC cell lines resulted in reduced 15-PGDH promoter activity (Fig. 2A and B). Exposure of CRC cells to NaB in the absence of EGF resulted in approximately a 4.7-fold increase of 15-PGDH promoter activity (P = 0.0024; Fig. 2A). Interestingly, pretreatment of CRC cells with EGF, before addition of the HDAC inhibitor NaB, is insufficient to completely ablate the increase of 15-PGDH promoter activity observed following treatment with NaB alone (Fig. 2A). Similarly, addition of VPA to CRC cells also resulted in a significant 7.1-fold increase in 15-PGDH promoter activity (P = 0.048; Fig. 2B), and we observed that pretreatment of CRC cells with EGF did not inhibit the ability of VPA to induce 15-PGDH promoter activity (Fig. 2B).

We next examined whether Snail expression can reduce 15-PGDH expression and determined if Snail interaction with HDACs are required to regulate this process. Addition of a wild-type Snail expression construct to CRC cells repressed 15-PGDH luciferase promoter activity (Fig. 2C and D); however, in cells also treated with 5 mmol/L NaB or 5 mmol/L VPA, we did not observe repression of 15-PGDH promoter activity even in the presence of Snail overexpression (Fig. 2C and D). Instead, we observed that treatment of cells with HDAC inhibitors and Snail resulted in a significant increase in 15-PGDH promoter activity (Fig. 2C and D).

Taken together, we find that Snail may require HDACs to repress 15-PGDH promoter activity and that HDAC inhibitors can inhibit repression of 15-PGDH expression mediated by EGF and Snail in CRC cells.

Interaction of Snail and HDAC2 at the 15-PGDH promoter. Examination of the 15-PGDH promoter reveals the presence of multiple transcription factor binding sites, including multiple Sp1 and Sp3 sites, which are known to involve binding of HDACs (22). Published reports suggest that the transcriptional repressor Snail and other related transcriptional repressors can interact with HDACs, including HDAC1 and HDAC2, to repress transcription of target genes (23, 24). To confirm these reports, we transfected a wild-type Snail expression construct containing a HA tag in HEK
293T cells to detect possible interactions between Snail and HDACs using immunoprecipitation techniques. As shown in Fig. 3A, we observe that HA-tagged Snail is able to coprecipitate with HDAC2, whereas a Snail-HDAC2 complex is not observed in untransfected control cells. In addition, we observed interactions of Snail with HDAC1 by coimmunoprecipitation in HA-Snail–transfected HEK 293T cells (data not shown).

Next, we determined whether HDAC2 binds to the 15-PGDH promoter in CRC cells. Using the ChIP assay, our data show that in mock-treated CRC cells, HDAC2 binds to the 15-PGDH promoter, whereas NaB treatment for 24 hours results in reduced HDAC2 binding (Fig. 3B). Quantitative analysis of DNA from these ChIP assays also shows decreased HDAC2 binding in NaB-treated samples compared with mock-treated controls (Fig. 3C). Taken together, our data suggest that HDAC2 can interact with Snail and that HDAC2 can bind to the 15-PGDH promoter, and the resulting HDAC2-Snail complex is involved in changes in 15-PGDH expression in vitro.

HDAC expression inversely correlates with 15-PGDH expression in vivo. To corroborate our in vitro findings, we analyzed expression and localization of HDACs and 15-PGDH in vivo by using mice that harbor a nonsense Apc mutation and spontaneously develop adenomas. Examination of 15-Pgdh expression by in situ hybridization shows that 15-Pgdh expression is observed in the well-differentiated villi in wild-type mice, whereas 15-Pgdh expression is absent in adenomas taken from ApcMin/+ mice (Fig. 4A; ref. 17). Examination of Hdac2 expression in wild-type mice shows moderate levels of expression in the proliferative crypt compartment (Fig. 4A), and increased Hdac2 expression seems to be present in adenomas taken from ApcMin/+ mice (Fig. 4A). Comparison of the localization of gene expression shows that Hdac2 expression inversely correlates with 15-Pgdh expression in this animal model. We next quantified 15-Pgdh and Hdac2 expression in matched normal and polyp tissue taken from 15-week-old ApcMin/+ mice by quantitative real-time PCR (Fig. 4B). These data support our previous observation wherein we observe reduced 15-Pgdh expression in adenomas from ApcMin/+ mice (17), whereas Hdac2 expression seems to be reciprocally elevated in these adenomas (Fig. 4A and B).

To examine the ability of HDAC inhibitors to disrupt HDAC expression in vivo, we treated ApcMin/+ mice (n = 9) with VPA (400 mg/kg) or vehicle for 4 weeks. Consistent with our model and one previous report (25), we observe that VPA treatment inhibits Hdac2 expression and nearly eliminates tumor burden in ApcMin/+ mice (Supplementary Fig. S1A and B). Immunohistochemical analysis of acetyl-histone H3 confirmed that VPA was absorbed appropriately (Supplementary Fig. S1C). Thus, VPA inhibits HDAC2 expression in vivo and should be studied further for its ability to repress tumorigenesis in the intestine.

Based on our findings that Hdac2 expression is inversely correlated with 15-Pgdh expression in adenomas in ApcMin/+ mice, we hypothesize that a similar relationship might exist in matched human normal and tumor samples. We have previously observed that 15-PGDH expression is significantly down-regulated in most CRCs (15, 17). Subsequent examination of HDAC2 expression from a subset of matched normal and tumor samples shows that HDAC2 expression increases 3-fold on average in CRCs (Fig. 4C). Notably, we observe that independent, pairwise comparison in 11 normal and tumor samples shows that 15-PGDH and HDAC2 are reciprocally expressed within both normal and tumor samples (Fig. 4D). In addition, we observed that other class I HDACs displayed a similar reciprocal expression pattern with 15-PGDH expression in matched normal and tumor samples (Supplementary Fig. S2A and B; Supplementary Table S1).

Epigenetic regulation of 15-PGDH expression by HDAC inhibitors and demethylating agents. Epigenetic repression of tumor suppressor genes by increased methylation often disrupts differentiation of epithelial and hematopoietic cells and can promote cancer progression. Recently, it has been suggested that the 15-PGDH promoter is methylated in ~ 30% of primary breast tumors and one breast cancer cell line (16). To begin to address whether methylation of the 15-PGDH promoter also occurs in CRCs, we carried out preliminary experiments to determine whether treatment of CRC cells with Aza-dC, a known demethylating agent, could restore 15-PGDH expression in CRC cells. Our preliminary findings indicate that after 72 hours of Aza-dC treatment in CRC cells, we observed an increase in 15-PGDH protein expression compared with vehicle-treated cells (Fig. 5A), whereas Aza-dC treatment decreased expression of DNA methyltransferase 1 (Fig. 5A). Interestingly, when CRC cells are treated with both Aza-dC for 72 hours and NaB for the last 24 hours, we
found an additive increase in 15-PGDH protein expression compared with only NaB treatment for 24 hours (Fig. 5B). Taken together, our observations support the hypothesis that the 15-PGDH promoter may be hypermethylated in CRCs; however, further experiments are necessary to identify specific CpG islands that are methylated.

Discussion

Our observations suggest that down-regulation of 15-PGDH expression can occur by several epigenetic mechanisms in CRCs. Here, we identify a role for HDACs in the repression of the PGE2 catabolic enzyme, 15-PGDH, in both CRC cells and primary CRC samples. A previous report by Tong and colleagues (26) suggests that treatment of a lung cancer cell line, A549, with the HDAC inhibitors scriptaid and NaB can restore both 15-PGDH expression and activity.

Here, we suggest that the mechanism of transcriptional silencing through histone deacetylation also occurs in CRC cells and may involve HDACs, specifically HDAC2, along with additional factors, such as the transcriptional repressor Snail, to repress 15-PGDH expression. Several reports suggest that transcriptional factors, including Slug and Snail, can interact with histone deacetylases to form repressor complexes for gene transcription (23, 24). For example, it has been shown that Snail can form a repressor complex to inhibit E-cadherin (encoded by CDH1) expression through recruitment of the corepressor SIN3A, HDAC1, and HDAC2 and that Snail requires a HDAC2 interaction to repress E-cadherin (23). Treatment of cells with a HDAC inhibitor is sufficient to block the repressor effect observed with Snail (23). In addition, Tripathi and colleagues (24) and Bailey and colleagues (27) have shown that the tumor suppressor gene BRCA2 contains several E-box elements and that the Snail family member Slug is involved in silencing BRCA2 transcription. ChIP assays suggest that Slug mediates its action by recruiting HDAC1 to the silencer E-box (24, 27). Taken together, these findings are consistent with the idea that the Snail family of transcriptional repressors may act in concert with HDACs in regulating expression of tumor suppressor genes and promoting carcinogenesis.

Previous studies examining class I HDAC expression in CRCs have shown that HDAC1, HDAC2, and HDAC3 expression is
The regulation of cell cycle (p21Cip1/Waf1 and cyclins) and apoptosis affecting changes in expression of certain genes involved in HDAC inhibition on gene expression seem to be highly selective by models. HDAC inhibition results in the accumulation of acetylated and induce differentiation and/or apoptosis in a variety of tumor structurally diverse group of compounds that inhibit proliferation agents for the treatment of solid malignancies. They represent a in inhibit gene expression.

in which Snail and HDAC2 bind to the 15-PGDH promoter to mechanism for 15-PGDH repression during colon carcinogenesis previously reported a similar reciprocal relationship between CRCs and intestinal polyps agree with these previous observations. We show here that 15-PGDH is reciprocally expressed compared with HDAC2 in these samples. Interestingly, we have previously reported a similar reciprocal relationship between 15-PGDH and Snail expression (17). Taken together with our in vitro results showing that both Snail (17) and HDAC2 can be localized to the 15-PGDH promoter, our data suggest a potential mechanism for 15-PGDH repression during colon carcinogenesis in which Snail and HDAC2 bind to the 15-PGDH promoter to inhibit gene expression.

HDACs are emerging as an exciting new class of anticancer agents for the treatment of solid malignancies. They represent a structurally diverse group of compounds that inhibit proliferation and induce differentiation and/or apoptosis in a variety of tumor models. HDAC inhibition results in the accumulation of acetylated nuclear histones in both tumor and normal tissues. The effects of HDAC inhibitors on gene expression seem to be highly selective by affecting changes in expression of certain genes involved in regulation of cell cycle (p21Cip1/Waf1 and cyclins) and apoptosis elevated in tumor samples (25, 28, 29). For example, immuno-histochemical analysis of 57 human colon cancers showed elevated HDAC2 expression in 82% of tumor samples (25). Furthermore, HDAC2 levels were also elevated in intestinal polyps from Apc\textsuperscript{Min/+} mice compared with normal tissue (25). Our \textit{in vivo} results examining HDAC2 expression in both human CRCs and intestinal polyps agree with these previous observations. We show here that 15-PGDH is reciprocally expressed compared with HDAC2 in these samples. Interestingly, we have previously reported a similar reciprocal relationship between 15-PGDH and Snail expression (17). Taken together with our \textit{in vitro} results showing that both Snail (17) and HDAC2 can be localized to the 15-PGDH promoter, our data suggest a potential mechanism for 15-PGDH repression during colon carcinogenesis in which Snail and HDAC2 bind to the 15-PGDH promoter to inhibit gene expression.

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In summary, our data suggest that HDACs, including HDAC2, and the transcriptional repressor Snail may play a significant role in suppressing 15-PGDH expression and provide an alternative COX-2–independent mechanism responsible for increased PGE2 levels in the tumor microenvironment and thus promote progression of CRC.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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