Regulation of VDR Expression in Apc-Mutant Mice, Human Colon Cancers and Adenomas

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Abstract

One variable that may affect the ability of vitamin D to reduce colon cancer risk is the expression of its high-affinity receptor, VDR. Here, we show that vitamin D does not reduce tumor formation in Apc1414/14 mice and that VDR expression is lost in the majority of the colon tumor cells. The extent of VDR loss corresponded inversely to the level of β-catenin nuclear localization and could be observed in early lesions composed of just a few crypts. Analysis of reported VDR regulators showed that the repressing class I histone deacetylases (HDAC) were significantly elevated in the tumors (up to 4-fold), whereas the VDR-activating retinoid X receptors (RXR) were downregulated (~50%). Expression of the Slug repressor was also increased, but was found primarily in stromal cells. Analysis of epigenetically active compounds on colon cell lines and intestinal organoids showed that HDAC inhibitors were particularly adept at stimulating VDR expression. Treatment of tumor-bearing Apc1414/14 mice with the HDAC inhibitor panobinostat increased VDR expression in the tumors and normal mucosa. The RXR agonist bexarotene failed to activate VDR expression, indicating that RXR ligands were not limiting. Analysis of human microarray data indicated that VDR mRNA is frequently downregulated in colon adenomas, which correlated positively with RXR expression and inversely with HDAC 2 and 8 expression. Human adenomas showed variable VDR protein expression levels, both between and within individual lesions. Determining the mechanisms of VDR regulation in colon neoplasms may significantly enhance our ability to use vitamin D as a cancer prevention agent. Cancer Prev Res; 8(5); 387–99. ©2015 AACR.

Introduction

Colon cancer is the third most common type of cancer in the United States and accounts annually for about 11% of all cancer-related deaths (CDC and American Cancer Society; refs. 1, 2). Although early detection and polyp removal through screening colonoscopy has offered significant benefit (3), colon cancer continues to take a serious toll on the U.S. population. Identifying dietary agents and supplements that reduce the risk of colon cancer development could be a powerful accomplishment to screening colonoscopy; high-risk individuals presenting multiple or advanced colon lesions could use chemopreventive agents to reduce the risk of “interval” cancers that develop in between examinations. The power of chemoprevention as a tool for colon cancer prevention is strongly supported by the finding that a selected chemopreventive agent combination, difluoromethylornithine (DFMO) and NSAID, can reduce polyp occurrence by up to 90% (4). The success of this trial was based, in part, on the identification of a complementary pair of agents. Ideally, chemo-preventive agents and agent combinations would be devised that treat molecular deficiencies and/or suppress aberrant growth regulatory pathways promoting cancer development in an individual (5). It is therefore important to understand the activities and limitations of individual agents, and to identify complementary agent combinations that provide more complete protection.

Vitamin D has long been suspected to reduce the risk of colon cancer development. Initial positive results came from geographical correlation studies showing an inverse relationship between sunlight exposure and colorectal cancer-related death rates (6). Subsequent studies identified an interaction between high dietary or plasma vitamin D levels with a reduced colon cancer risk (7–9). Although some observational studies have not detected protection by vitamin D (10, 11), there is sufficient positive data to consider vitamin D as a potential chemopreventive agent. Preclinical experiments likewise generated some positive data. Vitamin D was first tested in carcinogen-induced rodent colon cancer models (MNU, MNNG and DMH rat models) where significant vitamin D protection was reported (12–17). Vitamin D protection has also been observed in a diet-induced model of colon cancer; sporadic colon tumors induced by a Western-style diet high in fat and low in vitamin D, calcium, and folate can be suppressed by increasing vitamin D and calcium (18–20). However, preclinical models also revealed some limitations. Belleli and colleagues (16) reported a significant reduction in vitamin D receptor (VDR) activity within the colonic mucosa 10 weeks after DMH treatment, suggesting that the ability of vitamin D to elicit protection might become diminished under some circumstances. Evidence of tumor protection has been observed in the ApcMin+/, but the degree of protection has been found to vary depending upon the experimental conditions. In one study, tumor frequency was not significantly affected, but tumor burden was decreased when...
animals received 1,25-dihydroxyvitamin D₃ injections while on an AIN-93G diet (21, 22). More substantial protection was observed when animals on a vitamin D-deficient diet were given injections of 1,25-dihydroxyvitamin D₃ (23). The limitations of vitamin D protection are also evident in a number of human intervention trials. In the Women’s Health Initiative polyp prevention trial, total vitamin D intake was not found to significantly reduce the risk of recurrent adenomas (24). In a large placebo-controlled trial in which postmenopausal women received a daily vitamin D and calcium supplement for 7 years, the incidence of invasive colorectal cancer did not differ significantly between the supplementation group and the placebo group (25). These conflicting preclinical and clinical data prompted us to test the effect of vitamin D supplementation on the Apc¹⁴/+ mouse model, because this model generates a truncated APCR protein similar to that which occurs in human cancers (26). In addition, we incorporated vitamin D into the diet at levels that approximated the high and low range of typical human consumption. Finally, we assessed the expression of VDR and other important regulatory proteins that control VDR expression.

Much of the present data point to the high-affinity VDR as a critical mediator of vitamin D protection. Zheng and colleagues (27) tested this directly and found that Apc₆¹⁴/+ mice on a VDR-null background developed larger tumors than wild-type controls. Cancer suppression by VDR may be mediated, in part, by binding and inhibiting β-catenin (27). Numerous reports have investigated VDR expression at different stages of colon cancer development with a number of studies finding that VDR expression is frequently lost in advanced lesions. Initial studies of colon cancer cell lines showed that well-differentiated cell lines tend to maintain higher levels of VDR expression relative to poorly differentiated lines with a greater metastatic potential (28). Studies of patient-derived colorectal carcinoma tissue extracts initially generated conflicting results (28–32), but later studies using histologic approaches have generally shown VDR expression to be lost in metastatic cancers (33, 34). The loss of VDR expression in advanced cancers is linked to the epithelial-to-mesenchymal transition (EMT) and appears to be mediated by expression of the SNAIL1 and SLUG transcription factors (35–37). A connection between colonic inflammation and VDR repression has also been uncovered, suggesting a possible link between long-standing ulcerative colitis and increased colonic cancer risk. VDR expression was found to be significantly lower in inflamed colonic mucosa (38), with the greatest reduction observed in long-standing ulcerative colitis patients at the highest risk of colon cancer (38). Complementary results have been obtained in a mouse model of inflammation-promoted colon cancer. Mice treated with azoxymethane (AOM) and DSS show reduced VDR expression in inflamed mucosa, and ultimately develop tumors with reduced VDR expression (39). The mechanism underlying the VDR expression changes in this model is not entirely clear, but may involve SNAIL1 and SLUG expression during the acute inflammatory phase.

Here, we show that VDR expression and vitamin D responsiveness is dramatically downregulated in colon tumors in the Apc¹⁴/+ mouse, and that reduced VDR expression is observed even in very small lesions. VDR downregulation in this model appears to be driven, in part, by increased histone deacetylase (HDAC) expression, as VDR expression can be stimulated by treatment with the HDAC inhibitor, panobinostat (40). Our data suggest that cancer prevention by vitamin D might be limited to early stages of cancer development, and that vitamin D intervention alone might not be sufficient to slow the growth and progression of initiated lesions. However, our data also suggest that agents that stimulate VDR expression may effectively enhance and extend the protective effects of vitamin D.

Materials and Methods

Mice

Male and female Apc¹⁴/+ mice were fed modified AIN-93G diet containing low (250 IU/kg) or high (2,500 IU/kg) vitamin D₃ (Harlan Laboratories) from 5 to 16 weeks of age. Calcium levels were maintained at 0.5% for both diets. Body weight was measured once per week, with no significant weight changes between the groups observed. For drug treatments, mice on the high vitamin D₃ diet were injected intraperitoneally (i.p.) with four doses of panobinostat (10 mg/kg) or 5% dextrose (vehicle control) over 3 consecutive days (once/day, twice/day, and once/day) during the last week of the experiment (40). Mice on the high vitamin D₃ diet were also treated with four doses of bexarotene (50 mg/kg) or sesame oil (vehicle control) by gavage over 3 consecutive days (once/day; ref. 41). Mice were sacrificed 2 to 3 hours after the last dose. Mice were maintained in a temperature-controlled, light-cycled room and allowed free access to drinking water and the diet. Animal experiments were conducted after approval of the Center for Comparative Medicine (CCM), University of Connecticut Health Center (Farmington, CT).

Tumor incidence and multiplicity

Mice were sacrificed at 16 weeks of age for tumor scoring and histologic analyses as described previously (42). The small intestine and colon were harvested and flushed with ice-cold PBS and excised longitudinally. Some of the colon tumors were snap-frozen in liquid nitrogen. Remaining tissues were fixed flat in 10% neutral buffered formalin solution for overnight and stored in 70% ethanol. The tissues were stained with 0.2% methyl blue and the number and size of the tumors were scored under a dissecting microscope.

Immunohistochemistry

Small intestine and colon were paraffin-embedded and sectioned at a 5-μm thickness. Tissue sections were deparaffinized, antigen retrieved in 10 mmol/L sodium citrate and incubated with 3% hydrogen peroxide for 20 minutes at room temperature. Sections were blocked with 10% goat serum and incubated overnight at 4°C with anti-VDR (1:2,000; Santa Cruz Biotechnology), anti-β-catenin (1:2,000; Sigma-Aldrich) or anti-Slug (1:50; Cell Signaling Technology). Sections were then incubated with secondary antibody for 30 minutes at room temperature, followed by signal detection using DAB solution (Vector Laboratories Inc.). Tissues were counterstained with hematoxylin. No primary antibody controls were run for all antibodies. The specificity of the VDR-D-6 mouse monoclonal antibody was previously reported (28). The β-catenin antibody has been reported to show the expected patterns of stabilization and cellular translocation during cancer development in Apc-mutant mouse models (42).

RNA analysis

Isolated colon tissue and tumors were placed in 1 ml of TRIzol reagent (Invitrogen) and homogenized. Chloroform extraction and precipitation was performed as described by Invitrogen.
was quantified with a Thermo Scientific NanoDrop 8000 Spectrophotometer. Two micrograms of RNA was reverse transcribed using the Applied Biosystems High Capacity cDNA Reverse Transcription reagents and analyzed using TaqMan assays (Life Technologies) on a Applied Biosystems 7500 Fast Real-time PCR System. ACTB (actin-beta gene) was used as an internal control for the RNA quantification. The Applied Biosystems 7500 Standard Real-time PCR System software was used to analyze the date. The comparative \( \Delta \Delta C_t \) method was used to determine the relative gene expression levels. Statistical analysis was performed using Excel and GraphPad Prism software.

Cell culture

HT29 and HCT116 colon cancer cell lines were obtained from the American Type Culture Collection and cultured in McCoy 5A medium with 10% fetal bovine serum, nonessential amino acids, and antibiotic/antimycotic (Life Technologies). Cell lines from the ATCC are authenticated. YAMC and IMCE cells were a gift from Dr. Whitehead (who established them; Vanderbilt University) and were maintained in RPMI medium as previously described (44, 45).

Establishment and analysis of intestinal organoids

\( Apc^{Min/+} \) ESCs were maintained and differentiated into intestinal organoids using 30% Wnt3a conditioned media as previously described (46, 47). Organoids were transferred to a 96-well microwell nontreated flat-bottomed dish (Thermo Scientific) containing WntCM media with compounds obtained from the Cayman Epigenetics Screening Library (Cayman Chemical Company). The final concentration of each compound was 25 \( \mu \)mol/L. Organoids were collected for gene expression analysis after 48 hours. Following cell lysis, mRNA was captured on poly-T RNA capture plates as previously described (47). Preamplification was carried out with 5 \( \mu \)L of cDNA, 5 \( \mu \)L of 0.2 \( \times \) TaqMan primer pool, and 10 \( \mu \)L of TaKaRa PCR mix (6.3-\( \mu \)L nuclease-free \( H_2O \), 2-\( \mu \)L 10 \( \times \) Buffer, 1-6-\( \mu \)L dNTP mix, 0.1-\( \mu \)L TaKaRa Taq: TaKaRa-Bio) per reaction, using an initial temperature cycle of 95\( ^\circ \)C/3 minutes, 55\( ^\circ \)C/2 minutes, 72\( ^\circ \)C/2 minutes, followed by 15 cycles of 95\( ^\circ \)C/15 seconds, 60\( ^\circ \)C/2 minutes, 72\( ^\circ \)C/2 minutes. Amplified cDNA was diluted at 1:20 in nuclease-free water for further quantitative PCR (qPCR) analysis. qPCR was carried out in 384-well optical PCR plates using the 7900HT thermal cycler (all from Life Technologies). Resulting data were analyzed by Applied Biosystems RQ Manager 1.2, Microsoft Excel, and SAS JMP.

Microarray data analysis

The Gene Expression Omnibus (GEO) dataset GDS2947 was downloaded from NCBI and analyzed using Excel and GraphPad Prism software (48). The Pearson correlation coefficient and \( P \) values were determined to assess the potential relationships between VDR and the class I HDACs (HDACs 1, 2, 3 and 8), RXRA, and SNAI2/SLUG. Significance was set for \( P < 0.05 \).

Statistical analysis

Bulk RNA data were analyzed using a Student \( t \) test for comparing two treatment groups, or by using an analysis of variance test (ANOVA) when comparing more than two groups. A Tukey
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Dietary vitamin D and tumor protection

The Apc<sup>min/+</sup> mouse model harbors a frameshift mutation within exon 14, which generates a truncated protein of a size similar to that frequently observed in both familial adenomatous polyposis (FAP) patients and sporadic colon cancers (26). We determined whether colon and small intestinal tumor formation in these animals could be suppressed by the level of dietary vitamin D. The vitamin D3 level in defined mouse diets is 1,000 IU/kg diet (as set by the National Research Council). For our studies, 250 IU/kg was selected for the low dose, which is suboptimal for VDR target gene activation, but sufficient to maintain animal bone growth and general health (49). A 10-fold higher level of 2,500 IU was used to model a “supplemented” diet that would be used to achieve cancer prevention. However, as shown in Fig. 1A and B, no difference in the number of small intestinal tumors was observed, nor did high levels of vitamin D have any impact on the size of small intestinal tumors formed. In the colon, where the vast majority of human APC-initiated tumors develop, there was a trend toward reduction in tumor frequency and load, but this effect did not reach significance (Fig. 1C and D). Similar findings were obtained when these vitamin D levels were tested on a more promotional dietary background with higher fat and load, but this effect did not reach significance (Fig. 1C and D). A post-hoc test was used to determine the significance of differences between multiple groups, with P < 0.05 considered significant.

**Results**

**Dietary vitamin D and tumor protection**

To determine whether VDR expression was reduced at an early stage of tumor development, the tissue was examined for very small lesions showing aberrant β-catenin stabilization and nuclear localization (arising from Apc loss of heterozygosity; ref. 51). Figure 3 shows a single crypt with increased levels of β-catenin stabilization and nuclear localization (Fig. 3H, white arrowheads). This single crypt also showed reduced VDR expression (Fig. 3G, white arrowheads), indicating that VDR expression can be lost at a very early stage of colon tumor development. Ten single crypt lesions were observed in the study group, and only one maintained VDR expression at normal levels (90% VDR-positive cells). The very early loss of VDR expression is consistent with the inability of vitamin D supplementation to reduce tumor incidence in this model.

**VDR loss in colon tumors**

Given the lack of protection from tumor development by a high vitamin D diet observed in Fig. 1, we determined the VDR expression status in colon tumors and adjacent normal tissue of mice on the low and high vitamin D diets. The data shown in Fig. 2A indicate that VDR mRNA expression was reduced approximately 5-fold in tumors relative to normal tissue. In addition, the high vitamin D diet stimulated VDR expression in normal tissue but not in the tumors. The VDR-target gene Cdh1 showed a similar pattern of expression, being vitamin D responsive in the normal tissue, but not in the tumors (Fig. 2B; ref. 50). Analysis of VDR protein expression by immunohistochemistry (IHC) reflected the change in mRNA expression; normal regions of the tissue stained intensely for nuclear VDR, whereas large regions of the tumor were negative (Fig. 3A). These findings are consistent with a model in which loss of VDR expression limits the ability of vitamin D to control tumor formation and growth.

Although large regions of the tumor were VDR negative, a closer analysis of the staining revealed that clusters of cells within the tumors retained VDR expression (Fig. 3B). Another example of VDR staining is shown in Fig. 3C, where a series of adenomatous colon crypts express VDR at their base. A higher magnification view shows that the VDR expression in these adenomatous crypts is variable (Fig. 3E), with some crypts showing VDR-expressing cells (double arrow) and others being VDR-negative (white arrowheads). Because the extent of β-catenin nuclear localization can vary between adenomatous crypts, and antagonism between VDR and β-catenin has been reported, a serial section of tissue was analyzed for β-catenin nuclear localization (Fig. 3D and F). An inverse association was observed between VDR expression and β-catenin nuclear localization in the majority of the adenomatous crypts examined, with coexpression apparent in relatively few lesions (less than 5%). Potential mechanisms underlying this inverse association are discussed below.

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**Figure 2.** Relative gene expression in normal and tumor tissue of mice on the low (250 IU) and high (2,500 IU) vitamin D3 diet. RNA was isolated from exophytic tumors and adjacent normal tissue at the 16-week time point. The relative expression of VDR (A) and Cdh1 (B) was determined using TaqMan assays. A significant reduction of VDR (P < 0.0001) and Cdh1 (P < 0.01) expression was observed in tumors. In addition, a vitamin D only significantly increased expression of these genes in normal tissue (P < 0.001). A total of 7 and 5 mice, respectively, were examined in the low and high vitamin D groups.

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Expression of VDR regulators in Apc<sup>14+/−</sup> tumors

VDR expression is tightly regulated by a complex promoter and enhancer element. Several transcriptional regulatory proteins have been demonstrated to affect VDR expression under a number of different physiologic contexts, so we investigated the potential role of these factors in the loss of VDR expression in tumors. Colon cancer cells have been reported to lose VDR expression as the result of increased Snail and Slug expression (35). These transcription factors can be expressed in colon cancer cells following the EMT. The Snail and Slug transcription factors bind to an E-box element within the VDR promoter and inhibit its expression through the recruitment of transcriptional corepressors. Although Snail1 expression was unchanged in the tumors, Slug was increased approximately 2-fold (Fig. 4A). Because Slug is normally expressed in stromal cells, an IHC was performed to determine whether tumor epithelial cells within the lesion were expressing Slug. As shown in Fig. 4B, Slug staining was limited to stromal cells positioned between the adenomatous crypts of the tumor. The decreased VDR expression in the tumor cells can therefore not be directly accounted for by an increased expression of Snail1 or Slug, although it is possible that the stromal cells may indirectly affect VDR expression in the tumors.

Other negative regulators of VDR include the class I HDACs, which are commonly overexpressed in human and mouse colon tumors (52–56). As shown in Fig. 4C, all of the class I HDACs were increased in expression in the Apc<sup>14+/−</sup> tumors. Conversely, all three retinoid X receptor (RXR) genes, which are positive regulators of VDR expression that interact with the VDR promoter and activate its expression, were downregulated (Fig. 4D). These data suggest that an increase in HDAC expression and/or a decrease in RXR expression may contribute to the reduction in VDR expression observed in the Apc<sup>14+/−</sup> tumor cells.

Reactivation of VDR expression by HDAC inhibitors

Given the association between increased HDAC expression and VDR repression, we assessed the ability of HDAC inhibitors to stimulate VDR expression in cancer cells. We first examined a number of cell culture models for their responsiveness to HDAC inhibition. In Fig. 5A and B, HCT116 and HT29 cells were treated with SAHA and assayed for VDR expression. VDR was activated in HCT116 cells by SAHA, indicating that HDACs limit VDR expression in this human cell line. The level of VDR activation was found to be comparable with p21, a gene that has been well documented for its regulation by HDACs in colon cancer cells (57, 58). In contrast, VDR expression in HT29 cells was not activated by SAHA, even though p21 was responsive (Fig. 5A). One possible explanation for the lack of VDR activation in HT29 cells is that the VDR promoter in these cells is subjected to higher level of epigenetic repression, such as DNA methylation. To determine whether DNA methylation was limiting VDR expression in HT29 cells, cells were treated with 5-aza-deoxycytidine for 48 hours. As shown in Fig. 5B, 5-aza-deoxycytidine was able to activate VDR expression in HT29 cells, consistent with VDR silencing through DNA methylation in this cell line. (A longer 96-hour treatment with 5-aza-2′-deoxycytidine did not...
increase VDR expression in HCT116 cells. Finally, to assess the role of HDACs in VDR regulation is mouse colon cells, we tested the ability of panobinostat to activate VDR expression in conditionally immortalized YAMC (Apc<sup>+/−</sup>) and IMCE (Apc<sup>−/−</sup>) cells (44, 45). Panobinostat readily activated the expression of VDR in both cell lines, consistent with the role of HDACs in regulating VDR expression in colon oncogenic cells.

Given that HDACs are overexpressed in Apc<sup>−/−</sup> tumors (Fig. 4C), and that VDR expression can be reactivated in some colon cancer cell lines with HDAC inhibitors, the effect of HDAC inhibition on VDR expression in vivo was tested. Panobinostat was chosen for these studies, given its broad spectrum of activity and favorable pharmacokinetic properties (relative to other hydroxamic acid HDAC inhibitors; ref. 40). Four doses were administered, each spaced approximately 12 hours apart (Fig. 6A). Approximately 3 hours after the last dose, animals were euthanized and tumor and normal tissues were obtained. As shown in Fig. 6B, VDR expression was increased in the tumors by panobinostat. We next compared the activation of VDR with that of p21, a gene that is readily activated in colon cancer cells by HDAC inhibitors (Fig. 5). The degree of VDR activation was comparable (albeit slightly lower) with p21. Finally, we tested the response of adjacent normal tissue to panobinostat treatment. VDR expression in normal tissue was also responsive to panobinostat, and was in fact more responsive than p21 within the normal tissue. These findings are consistent with HDACs contributing to the suppression of VDR in colon tumors and adjacent epithelium.

Histone acetylation frequently works in combination with other chromatin modifications to enforce epigenetic gene regulation. We therefore tested a panel of agents that target a variety of epigenetic modifiers, including HDACs, methyltransferases, and demethylases, on intestinal organoids generated from Apc<sup>−/−</sup> ESCs. (Intestinal organoids were used here because they maintain a similar structural arrangement as normal tissue, and we have developed approaches that facilitate the medium throughput analysis of gene expression changes in this system (47)). Whereas p21 expression was responsive to many of the epigenetically active compounds, VDR was selectively and robustly activated only in response to HDAC inhibitors (Fig. 7). As reported in other cell systems, the HDAC inhibitors also suppressed expression of the Wnt target gene Myc, whereas the effect of other epigenetic agents on Myc expression was more variable.

Given that RXR expression is reduced in Apc<sup>−/−</sup> tumors, we also determined whether the pharmacologic RXR agonist bexarotene may activate VDR expression in these tumors. Bexarotene was provided to the animals through gavage for 3 days, and 3 hours after the last dose, tissue was prepared and assessed for VDR expression. Bexarotene did not activate VDR expression, in tumors nor in normal tissue (Supplementary Fig. S2). Although this result does not exclude the potential contribution of RXR downregulation to the reduced VDR expression in the colon tumors, it appears that the availability of RXR agonists does not limit VDR expression in this model.

Relationship between VDR expression and transcriptional regulators in human adenomas

Taking advantage of the NCBI GEO database, we used a published microarray dataset comparing human adenomas and adjacent normal tissue to determine whether VDR mRNA expression was reduced in the adenomas (48). As shown in Fig. 8A, 29 out of 32 matched normal-adenoma pairs showed lower levels of VDR expression in the adenomas. The reduction in VDR expression was inversely associated with the expression of the class I HDACs, all of which were significantly increased in the adenomas. To further examine the relationship between VDR expression and potential regulatory proteins, a correlation analysis was
vitamin D may prevent colon cancer (59 high-risk patients. Epidemiologic studies have suggested that complement to colonoscopic screening efforts, particularly in the risk of colon cancer development could provide a powerful concentration tested (< 0.01). C, YAMC and IMCE cells were treated with the indicated levels of panobinostat for 24 hours. The expression of VDR mRNA was determined. Increased mRNA expression was determined to be significant for both panobinostat concentrations tested (*, P < 0.007).}

Figure 5.
Epigenetic regulation of VDR in colon derived cell lines. A and B, HCT116 and HT29 cells were treated with 10 μmol/L SAHA or 5 μmol/L 5-azaacytidine for 48 hours. RNA was then prepared and analyzed for VDR and p21 expression. Significant changes are indicated by asterisks (P < 0.01). C, YAMC and IMCE cells were treated with the indicated levels of panobinostat for 24 hours. The expression of VDR mRNA was determined. Increased mRNA expression was determined to be significant for both panobinostat concentrations tested (*, P < 0.007).

performed. As shown in Fig. 8B, a significant inverse correlation between VDR expression and HDACs 2 and 8 was observed. This association was less significant for HDAC1 (P = 0.003) and nonsignificant for HDAC3 (P = 0.074). These data suggest that the increased expression of a subset of HDACs may contribute to VDR repression in human adenosas. As found in the ApcΔ14/+ tumors, RXRA expression was significantly reduced in human adenosas, and this reduction was correlated with VDR expression, suggesting a potential coregulation of these two nuclear receptors. Finally, the expression of SNAIL2/SLUG was lower in adenomas than normal tissue, and showed a positive correlation with VDR expression. This result argues against a role of SNAI2/SLUG in VDR repression in human adenosas.

We also examined the expression of VDR in human adenosas by IHC. Representative examples of tubular adenosas are shown in Fig. 9A and B. Close analysis of cells within individual lesions showed variable levels of VDR expression, and included cells with high to no detectable VDR expression. In this regard, the human adenosas are similar to the lesions formed in the ApcΔ14/+ mouse, although the VDR-positive cells in the mouse tumors were more likely to be found in restricted cell clusters. Implications of these findings with regard to cellular growth control and cancer prevention by vitamin D are discussed below.

Discussion
The effective utilization of chemopreventive agents to reduce the risk of colon cancer development could provide a powerful complement to colonoscopic screening efforts, particularly in high-risk patients. Epidemiologic studies have suggested that vitamin D may prevent colon cancer (59–64). However, intervention trials in patient cohorts supplemented with vitamin D have generated ambiguous results (25). On the other hand, preclinical studies in mouse colon cancer models have shown moderate cancer protection. For example, in ApcΔ101/+ mice, three weekly injections of 1,25-dihydroxyvitamin D3 supplementation did not reduce tumor incidence, but reduced tumor burden by almost half. In a study intended to optimize vitamin D as a dietary chemopreventive agent, we first attempted to demonstrate vitamin D protection in the ApcΔ14/+ mouse model (26). Tumors formed in this model are genetically similar to those formed in FAP patients (26), and carry Apc truncation mutations like the majority of sporadic cancers. Interestingly we found no significant protection in ApcΔ14/+ mice, either in the small intestine or in the colon. Upon examination of normal mucosa and neoplastic tissue, we found an extensive loss of VDR expression in tumors formed in these animals. Previous work has shown that VDR expression can be reduced within inflamed colonic epithelium and in neoplastic lesions associated with ulcerated mucosa (39). Our findings here show that the loss of VDR expression can occur even in the absence of inflammation. The lack of colon cancer protection afforded by vitamin D in this model may therefore be related to the lack of VDR expression in the lesions.

Examination of the VDR expression pattern in the ApcΔ14/+ tumors showed a number of interesting features. First, even though the majority of cancer cells within the tumor did not express VDR, a subpopulation of cells expressed VDR at levels similar to those found in normal crypts. Lineage tracing and transcription profiling experiments have revealed that colon cancers are composed of multiple cell types with varying proliferative capacities and differentiation profiles (65, 66). Although it is not clear how VDR expression relates to the different cell types found in the tumors, it is likely that this cell heterogeneity is contributing to the variability in expression. Examination of human adenosas likewise showed variability in VDR expression within individual lesions. However, unlike the tumors formed in ApcΔ14/+ mice, where VDR-expressing cells were clustered within the lesion,
VDR-expressing cells in human adenomas were intermingled among nonexpressing cells. The relationship between the expressing and nonexpressing cells within the mouse colon lesions and human adenomas is presently unclear, but identifying these cell types could provide insight into the chemopreventive potential of vitamin D. Another interesting feature of VDR expression in the Apc$^{14/+}$ tumors is that VDR expression in adenomatous crypts is inversely associated with β-catenin nuclear localization. Although the basis of this inverse relationship is unclear, there are reports of VDR and β-catenin competition for nuclear binding sites, raising the possibility that this inverse expression may be mechanistically linked (67). Specifically, VDR has been reported to compete with β-catenin for binding to the Lef1 transcription factor, suggesting that VDR might reduce the nuclear accumulation of β-catenin by blocking its binding to nuclear Lef1 (67). Finally, a loss of VDR expression could be observed in very early lesions in the mouse, suggesting that a decrease in VDR expression occurs soon after Apc loss and β-catenin stabilization.

The loss of VDR expression in human colon cancers has previously been reported to occur after a lesion has undergone an EMT (35, 68–70). For these advanced cancers, VDR expression is frequently repressed through the actions of the SNAIL transcription factors (SNAIL1 and SNAIL2/SLUG), which are transcription regulatory proteins central to EMT enforcement. However, mouse colon cancer models typically undergo a “truncated EMT” in which vimentin expression increases, E-cadherin decreases, but SNAIL expression is not activated (71). Consistent with these reports, we did not observe increased Snail1 or Slug expression in the epithelial cells of the colon tumors of Apc$^{14/+}$ mice, although Slug expression was increased within the stroma. These findings indicate that some other mechanism is involved in repressing VDR expression. Our analysis of VDR and SNAIL expression in human colon adenomas is likewise inconsistent with SNAIL repressing VDR expression in these lesions. Whether the mechanism of VDR repression is similar in Apc$^{14/+}$ mice and human adenomas remains to be determined.

VDR expression is regulated by a number of different promoter response elements and transcription factors, so any one of a number of regulatory pathways may be responsible for the decreased VDR expression observed in the lesions. One pathway of interest is a positive feedback loop in which VDR–RXR complexes activate VDR transcription by binding vitamin D response elements (VDREs) in the VDR promoter (72). Our data suggest that this positive feedback loop is shut down in Apc$^{14/+}$ tumors, because vitamin D supplementation did not activate VDR expression in the tumors. There are reports that RXR and RXR agonists can regulate VDR expression (72, 73). Consistent with this possibility, we found that RXXR expression was downregulated in the tumors of Apc$^{14/+}$ mice (specifically RXRA and RXRG). RXXR downregulation has been reported in other mouse colon cancer models, and appears to be mediated by promoter methylation (74). However, we were unable to activate VDR expression with the synthetic RXR agonist bexarotene, suggesting that the availability of RXR ligands is not limiting VDR expression in Apc$^{14/+}$ mice.

Another frequent molecular aberration in both mouse and human colon tumors that may be related to VDR repression is the increased expression of HDACs. HDACs catalyze the deacetylation of histone proteins, which can reduce the expression of target promoters. The class I nuclear HDACs (HDACs 1, 2, 3, and 8) are frequently increased in expression in colon cancer cells and we found a similar upregulation in the Apc$^{14/+}$ mouse model and in human adenomas (55). The increased expression of the HDACs may be related to hyperactivation of Wnt signaling in the tumors resulting from mutations to Apc. For example, HDAC2 is activated by the Myc transcription factor in response to mutations within the Apc gene and associated β-catenin stabilization (55). Studies have shown that HDACs primarily regulate the expression level of active genes, or genes poised for activation (75). VDR appears to fall into this category because its expression in both normal and tumor tissue of Apc$^{14/+}$ mice was stimulated by the HDAC inhibitor panobinostat. Increased HDAC expression in the mouse tumors may therefore contribute to the reduced level of VDR expression received four i.p. injections of panobinostat over a 3-day period (10 mg/kg, spaced approximately 12 hours apart). Control animals received injections of 5% dextrose as the vehicle control. Three hours after the final injection, colon tumors and adjacent normal tissue was obtained and assayed for VDR and p21 expression. B and C, VDR and p21 expression was activated by panobinostat in the tumors (B) and the adjacent normal tissue (C). VDR and p21 increases were significant in the tumors, and VDR activation was significant in the normal tissue (** P < 0.01; *** P < 0.001). Five animals were in each group.
expression. HDACs are, however, unlikely to be entirely responsible for VDR repression in the tumors, because panobinostat did not completely restore VDR expression to normal levels. HDAC inhibitors were, however, generally capable of stimulating VDR expression in some human cancer cell lines, as well as conditionally immortalized mouse colon cell lines and a mouse intestinal organoid system. In one colon cancer cell line, VDR appeared to be repressed through DNA methylation, indicating that other mechanisms for VDR silencing are also possible.

The finding that HDAC inhibitors activate VDR expression in tumors and normal tissue raises the possibility that HDAC inhibitory compounds may be capable of enhancing cancer chemoprevention by vitamin D. In one report, the selective HDAC2 inhibitor, OSU-HDAC42, reduced tumor formation in the large and small intestine of ApcMin/+ mice by 26% and 44%, respectively (76), and it would be of interest to know whether vitamin D supplementation might further reduce tumor formation. Although it is not feasible to use pharmacologic HDAC inhibitors as chemopreventive agents, it has been reported that a number of natural compounds and dietary factors have intrinsic HDAC inhibitory activity, including fiber-derived short chain fatty acids and isothiocyanates derived from cruciferous vegetables (58, 77–79). Although agents with HDAC inhibitory activity have been found to be protective in mouse colon cancer models, the contribution of VDR to this protection has thus far been untested. It is not clear which dietary compounds might be best suited for stimulating VDR expression in humans, or whether agents targeting other regulatory proteins might further promote expression.

In this regard, recently developed intestinal organoid-based high-throughput gene analysis approaches may be useful because a wide variety of compounds and compound combinations can be readily analyzed in this system (47). Whether vitamin D is effective for reducing colon cancer risk in human populations is unclear. Observational and interventional studies have generated conflicting data on this topic (25, 59, 61, 64). However, a recent analysis of serum vitamin D levels and colon cancer development from the Nurses’ Health Study and the Health Professionals’ Follow-up Study found that individuals with the highest serum vitamin D levels had approximately half the risk of developing colon cancer relative to the lowest quintile (80). Interestingly, VDR expression in the resulting cancers was lost at the same rate, regardless of serum vitamin D levels. Although there are a number of potential explanations for this finding, it appears that there are a number of mechanisms by which cancer suppression by vitamin D can be overcome. ApcD14/+ mice appear to model VDR repression mediated through the increased expression of HDACs. These mice could therefore be useful for identifying compounds that alleviate HDAC-mediated repression. Understanding how vitamin D and VDR expression affects colon cancer development could improve the way in which...
vitamin D is used in the clinic as a cancer chemopreventive agent. Given that the benefits and risks associated with vitamin D are well understood, it may turn out to be a highly effective cancer-preventive agent when used in the proper dietary context (e.g., under conditions that suppress VDR silencing) and targeted to the appropriate patient population.

Figure 8.
VDR and class I HDAC expression levels in human adenomas and adjacent normal colonic tissue. A, data from the NCBI GEO database (GDS2947) were downloaded and plotted to show VDR and HDAC expression in the matched samples. A significant downregulation in VDR expression was observed in the adenomas, which is in contrast to the upregulation of the class I HDACs \( P < 0.0001 \). B, VDR expression negatively correlated with HDAC2 and HDAC8 expression, and positively correlated with RXRA and SNAI2/SLUG in human adenomas. Correlations were significant as determined by Pearson, with the \( P \) values indicated on the graphs.

Figure 9.
Representative IHC images of human adenomas stained for VDR expression. Two independent adenomas (A and B) showing VDR-expressing and nonexpressing cells within a lesion.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: C. Giardina, B. Brenner, D.W. Rosenberg
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Nakanishi, A. Khan, B. Brenner, D.W. Rosenberg
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References


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