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The effect of 1,25 dihydroxyvitamin D₃ treatment on the mRNA levels of β catenin target genes in mice with colonic inactivation of both APC alleles

Marsha DeWitt^{1,3,#}, Robert L. Johnson^{3,4,#}, Paul Snyder^{2,4}, and James C. Fleet^{1,2,3,*}

¹Purdue University Interdisciplinary Life Science Ph.D. program, Purdue University, West Lafayette, IN 47907 USA

²Purdue University Center for Cancer Research, Purdue University, West Lafayette, IN 47907 USA

³ Department of Nutrition Science, Purdue University, West Lafayette, IN 47907 USA

⁴ Department of Comparative Pathobiology, Purdue University, West Lafayette, IN 47907 USA

Abstract

In colon cancer, adenomatous polyposis coli (APC) inactivating gene mutations increase nuclear β -catenin levels and stimulate proliferation. *In vitro*, 1,25 dihydroxyvitamin D (1,25(OH)₂D), suppresses β -catenin-mediated gene transcription by inducing vitamin D receptor (VDR)- β -catenin interactions. We examined whether acute treatment with 1,25(OH)₂D could suppress β -catenin-mediated gene transcription in the hyperplastic colonic lesions of mice with colon-specific deletion of both APC gene alleles (CAC; APC^{580/580}). At four weeks of age, CAC; APC^{580/580} and control mice were injected with vehicle or 1,25(OH)₂D (1 μ g/kg body weight) once a day for three days and then killed six hours after the last injection. mRNA levels of β -catenin target genes were elevated in the colon of CAC; APC^{580/580} mice. 1,25(OH)₂D increased 25 hydroxyvitamin D-24 hydroxylase mRNA levels in the colon of CAC; APC^{580/580} and control mice indicating the treatments activated the VDR. However, 1,25(OH)₂D had no effect on either β -catenin target gene mRNA levels or the proliferation index in CAC; APC^{580/580} or control mice. VDR mRNA and protein levels were lower (–65% and –90%) in the colon of CAC; APC^{580/580} mice compared to control mice, suggesting loss of colon responsiveness to vitamin D. Consistent with this, vitamin D-induced expression of Transient Receptor Potential cation channel, subfamily V, member 6 mRNA was reduced in the colon of CAC; APC^{580/580} mice. Our data show that short term exposure to 1,25(OH)₂D does not suppress colonic β -catenin signaling *in vivo*.

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*To whom correspondence should be addressed. Tel: 1+ 765 494 0302; Fax: 1+ 765 494 0906; fleet@purdue.edu.

#Current addresses: Marsha DeWitt, Department of Dermatology, Indiana University School of Medicine 550 N. University Blvd., Suite 3240, Indianapolis, IN 46202, United States. mdesmet@iupui.edu
Robert Johnson, Eli Lilly and Company, Lilly Corporate Center, 355 East Merrill St., Drop Code 0438, Indianapolis, IN 46225, United States johnson_robert_lee@lilly.com

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Keywords

cancer; hyperplasia; vitamin D; gene expression

Introduction

Studies show that lifestyle factors such as diet can decrease the development of colorectal cancer (1,2). One factor proposed to suppress colorectal cancer development is vitamin D. Epidemiological studies show an inverse association between high serum 25 hydroxyvitamin D (25(OH)D), the marker of vitamin D status, and colon cancer risk (3-8). The anti-cancer effects of vitamin D are mediated through the vitamin D receptor (VDR) which binds to the active form of vitamin D, 1,25 dihydroxyvitamin D (1,25(OH)₂D), and regulates the transcription of genes involved in anti-cancer events (e.g. growth arrest, metastasis, angiogenesis) in colon cancer cells (9). However, an alternate hypothesis to explain vitamin D-mediated growth arrest is that VDR disrupts β -catenin signaling and blocks the Wnt-signaling pathway.

In normal colonic cells, β -catenin is retained in the cytoplasm by interacting with adenomatous polyposis coli (APC) or at the plasma membrane by binding to E-cadherin at adherens junctions. A destruction complex of Axin, APC, and glycogen synthase kinase-3 keeps β -catenin levels low in the nucleus by promoting its proteasomal degradation thereby reducing transcriptional regulation of genes by β -catenin (10,11). Activation of Wnt signaling releases β -catenin from the destruction complex, permits β -catenin nuclear accumulation, and increases binding of β -catenin to the TCF/LEF-1 transcriptional complex to regulate genes controlling cell proliferation (e.g. c-Myc) (12). APC inactivating gene mutations that inhibit β -catenin-APC interactions are found in 85 percent of sporadic colorectal cancers (13).

Several *in vitro* studies have demonstrated that 1,25(OH)₂D inhibits β -catenin transcriptional activity in colon cancer cells (14,15). In addition, siRNA-mediated knockdown of VDR in SW480-ADH cells increased β -catenin-mediated reporter gene activity and inhibited 1,25(OH)₂D-mediated suppression of transcript levels from β -catenin target genes (15). Consistent with this observation, downregulation of VDR gene expression by overexpression of Snail1 or Snail2 blunted 1,25(OH)₂D-mediated suppression of β -catenin transcriptional activity (16,17). In SW480-ADH colon cancer cells, 1,25(OH)₂D treatment induced an interaction between VDR and β -catenin (14,18) which reduced the interaction between β -catenin and TCF-4 necessary for gene transcription (14). In addition, two-hybrid assays show that the VDR AF-2 domain directly interacts with the C terminus of β -catenin (19). The interaction of VDR with β -catenin and the suppression of β -catenin-mediated gene transcription in the presence of 1,25(OH)₂D has also been shown in other colon cancer cell lines with mutant APC genes, such as Caco-2 and HT-29 (20) and in human embryonic kidney cells (HEK-293) (19). However, direct testing of the ability of 1,25(OH)₂D to reduce β catenin-regulated transcript levels *in vivo* has not been reported.

Here we examined whether activating VDR with 1,25(OH)₂D can inhibit β -catenin transcriptional activity in the absence of APC function *in vivo*. To do this, we used the CAC;

APC^{580/580} mouse model we developed in which both APC alleles are inactivated specifically in the epithelial cells of the colon (21). These mice develop dysplastic lesions in the distal colon that are characterized by high levels of nuclear and cytoplasmic β -catenin and increased cellular proliferation. Although β -catenin regulated transcripts are elevated in the distal colonic lesions from these mice, we found that a short course of three 1,25(OH)₂D injections did not decrease expression of known β -catenin targets in either wild type mice or in CAC; APC^{580/580} mice *in vivo*.

Materials and methods

Reagents

Cell culture reagents were obtained from Cambrex (Rockland, ME) and Invitrogen (Carlsbad, CA). Cell culture plastic ware was obtained from BD Biosciences (Sparks, MD). 1,25(OH)₂D was purchased from Enzo Life Science (Farmingdale, NY), dissolved in ethanol, and kept in light-protected vials at -80°C until use. 10% neutral buffered formalin was purchased from VWR (Radnor, PA).

Cell culture

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured as previously described (22). SW480-ADH cells were kindly provided by Dr. José M. González-Sancho (Madrid, Spain). SW480-ADH cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) with L-glutamine and sodium pyruvate supplemented with 1% penicillin/streptomycin and 10% FBS. Cells were maintained at 37°C in a 5% CO₂ – 95% air atmosphere. Caco-2 cells and SW480-ADH were plated in six-well dishes (1.3 × 10⁵ cells per well) and grown to 50% confluent. Cells were treated with vehicle control (0.1% ethanol) or 100 nM 1,25(OH)₂D for 24 h in culture media containing 5% FBS for studies assessing mRNA levels.

Animals

All animal experiments were reviewed and approved by the Purdue Animal Care and Use Committee. Mice received a commercial rodent chow diet (8664, Harlan Tekland, Madison, WI) and water *ad libitum*. APC^{CKO/CKO} mice that contain LoxP sites flanking exon 14 of the APC gene were obtained from the Mouse Model of Human Cancers Consortium (National Cancer Institute in Frederick, MD). When the floxed exon 14 of APC^{CKO/CKO} mice is recombined in the presence of Cre recombinase, the recombined allele is referred to as 580; mice with one recombined allele are referred to as APC^{580/WT} and mice with two recombined alleles are referred to as APC^{580/580}. C57BL/6-Tg(Car1-cre)5Flt (CAC) mice are transgenic mice with large-intestine-specific expression of Cre recombinase driven by a modified carbonic anhydrase I promoter. CAC mice were previously described by our group to drive large-intestine specific recombination of floxed gene alleles (21). Mice with a single APC allele deletion (CAC; APC^{580/WT}) were generated by crossing CAC mice to APC^{CKO/CKO} mice. Mice with two deleted APC alleles (CAC; APC^{580/580}) were generated by crossing CAC; APC^{580/WT} to APC^{CKO/CKO} mice. Cre and APC alleles were genotyped using PCR-based methods. The protocol for Cre genotyping was described previously (21) and the primers APC-Int13F2 (GAGAAACCCTGTCTCGAAAAA) and

APC-Int13R2 (AGTGCTGTTTCTATGAGTCAAC) were used to genotype the APC LoxP allele (430 bp) and the wild-type allele (320 bp). PCR reactions were performed using the cycling conditions: 10 min of 94°C (1 cycle) and 94°C, 30 sec, 58°C, 30 sec and 72°C, 45 sec (30 cycles) followed by 72°C for 5 min.

Experiment 1: At four weeks of age, CAC; APC^{580/580} mice and littermates that lacked the CAC transgene (control mice) were assigned to one of two treatment groups: vehicle (9:1 mix of propylene glycol and ethanol) or 1,25(OH)₂D (1 µg/kg body weight); n=5-16 control mice/group and n=14-20 CAC; APC^{580/580} mice/group. Mice were injected i.p. with 20 µl of 1,25(OH)₂D at 0, 24, and 48 hours and then killed six hours after the last injection. Blood was collected by cardiac puncture and serum was placed at -80°C for analysis of 1,25(OH)₂D levels and serum calcium. Afterwards the distal colon was removed, opened longitudinally, and a digital image was taken to record the gross lesion phenotype. When observed grossly, the distal third of the colon developed raised, hyperproliferative lesions. This segment of the colon was divided into four equal subsamples. Two subsamples were used for RNA isolation and two subsamples were used for histology. The hyperproliferative lesion was represented equally in each subsample. Mucosa scrapings were prepared from each subsample, placed in 1 ml TriReagent, and saved at -80°C for later isolation and analysis of mRNA levels. Samples for histology were fixed in 10% neutral buffered formalin overnight then processed, and embedded in paraffin using standard procedures. Experiment 2: A study was conducted to generate colon tumors for analysis of VDR mRNA levels. Wild-type and CAC; APC^{580/WT} mice received an AIN93G diet (Research Diets, New Brunswick, NJ) and water *ad libitum* from weaning (n=24 per group). At 10 weeks of age, CAC; APC^{580/WT} mice were given 2% (w/v) dextran sulfate sodium (DSS) in their drinking water (MW = 36,000-50,000; MP Biomedicals, LLC) for 5 days. Mice were sacrificed at 20 weeks of age (i.e. 10 wks after the DSS exposure) and distal colonic tumors from CAC; APC^{580/WT} mice and normal mucosa from the distal colon of wild-type mice were harvested and stored in Tri-Reagent at -80°C for later mRNA analysis.

RNA Isolation and Real-Time PCR Analysis

Colonic tissue, tumors, or cultured cells were harvested directly into TriReagent (Molecular Research Center, Inc., Cincinnati, OH), and RNA was isolated and converted to cDNA as previously described (22). cDNA samples were analyzed by real-time PCR analysis using the BioRad SYBR Green supermix (BioRad Laboratories, Hercules, CA). mRNA levels were determined from the threshold cycle (Ct) value and normalized to large P0 ribosomal protein (RPLP0) mRNA levels as previously described (22,23). All primer sets and annealing temperatures are listed in Table I. The cycle conditions for PCR were 95°C, 3 min for one cycle; 40 cycles at 95°C for 10 sec, Annealing temperature for 30 sec, 72°C for 30 sec.

Western blot analysis of VDR in colon extracts

Distal colonic tissue was placed in lysis buffer containing 120 mM NaCl, 0.5% Nonidet P-40, 0.2 mM sodium orthovanadate, 50 mM Tris-HCl pH 8.0, and 1 Protease Inhibitor Cocktail Tablet per 50 ml (Roche Ltd, Basel, Switzerland). Tissue was sonicated for 5 sec on ice using a Fisher model 60 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA).

Samples were centrifuged at $16,300 \times g$ for 10 min at 4°C and the supernatant was saved. 50 μl of extract from each sample ($n=5-6$) was pooled per group. VDR and β -actin protein levels were detected in pooled lysates (50 μg) by methods described previously (22,24). VDR was detected with a mouse-anti-human VDR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-13133) (25). The primary antibody was detected with the horseradish-peroxidase-conjugated goat-anti-mouse IgG light chain specific secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and bands were visualized by enhanced chemiluminescent detection (Thermo Scientific, Rockford, IL). Protein bands were quantified using ImageJ software (<http://rsbweb.nih.gov/ij/>).

Histology and immunohistochemistry

Distal colonic mucosa tissue was prepared and stained with hematoxylin and eosin as described elsewhere (21). Ki-67 immunohistochemistry was done on unstained sections as described elsewhere (21) using a rat monoclonal anti-Ki-67 primary antibody (DakoCytomation; M7249) diluted 1:50. Digital images were collected with an Aperio ScanScope slide scanner (Aperio) using the 20X objective. Between 4 and 22 dysplastic foci within the distal colon were analyzed per mouse ($n=11-13$ mice/group). Dysplastic colonic mucosa was differentiated from normal appearing mucosa and intervening stroma using Genie histology pattern recognition software (Aperio) trained at 20X magnification and 1000 iterations to a mean training accuracy of greater than 90%. The percent Ki-67 positive nuclei, intensity of staining, and nuclear size within the dysplastic foci was determined using the Aperio nuclear IHC quantification algorithm. The detection threshold was optimized manually and a subset of the results was manually verified.

Serum 1,25(OH)₂D metabolite and calcium analysis

Serum 1,25(OH)₂D levels were determined using radio immunoassays (Immunodiagnostic Systems, Scottsdale, AZ). Serum calcium was determined by the QuantiChrom calcium assay kit (BioAssay Systems, Hayward, CA).

Statistical Analysis

All quantitative data are expressed as the mean $\pm$ the standard error of the mean (SEM). Statistical analysis of the data was performed by one or two-way ANOVA followed by Tukey's HSD test ($P < 0.05$) or a 2-tailed t-test as appropriate. If the data were not normally distributed, data were natural log transformed prior to analysis. For data that could not be fit to a normal distribution, a non-parametric one-way ANOVA test was used. P-Values less than 0.05 were considered significant. Our sample size was sufficient to detect a 25% difference in β -catenin target gene transcript levels between control and vitamin D-metabolite treated groups in CAC; APC^{580/580} mice ($\beta = 0.8$, $\alpha = 0.05$).

Results

1,25(OH)₂D treatment reduces mRNA levels of β -catenin target genes in colon cancer cells

We examined whether 1,25(OH)₂D treatment reduces mRNA level of β -catenin target genes in SW480-ADH cells and in Caco-2 cells. Both of these cell lines express the VDR (data not shown) and contain an APC inactivating mutation (26). 1,25(OH)₂D treatment (100 nM, 24

h) significantly up-regulated 25 hydroxyvitamin D-24 hydroxylase (CYP24A1) mRNA expression in both Caco-2 and SW480-ADH cells (Figure 1A) and significantly reduced the mRNA level of four β -catenin target genes (Axin2, TCF-1, Lef-1, and c-Myc) in SW480-ADH cells (Figure 1B). In Caco-2 cells, all β -catenin regulated mRNAs except c-Myc were significantly downregulated in the presence of 1,25(OH)₂D.

The impact of 1,25(OH)₂D injections on CAC; APC^{580/580} mice

The body weight of CAC; APC^{580/580} mice was significantly lower than control mice at harvest. In control mice 1,25(OH)₂D treatment significantly reduced body weight but this effect was not seen in CAC; APC^{580/580} mice (Figure 2A). Grossly, CAC; APC^{580/580} mice developed rectal prolapse at 3-4 weeks of age and areas of mucosal thickening correspond to raised hyperproliferative lesions. Histologically, these lesions are comprised of dysplastic epithelial cells. RT-PCR analysis showed that APC allele deletion increased the mRNA level of five known β -catenin target genes (i.e. c-Myc, MMP-7, TCF-1, LEF-1, and Axin2) in the hyperproliferative lesions of CAC; APC^{580/580} mice (Figure 2B).

Compared to the vehicle group, serum 1,25(OH)₂D levels were significantly increased in both the control and the CAC; APC^{580/580} mice after 1,25(OH)₂D treatment and the increase was higher in CAC; APC^{580/580} mice than in controls (Figure 3A). The elevated serum 1,25(OH)₂D levels were associated with increased serum calcium levels in both groups (control: 10.4±0.27; 1,25(OH)₂D: 13.0±0.38, p<0.05).

CYP24A1 is the most responsive 1,25(OH)₂D target gene (27). As expected, 1,25(OH)₂D injections significantly up-regulated CYP24A1 mRNA expression in both control mice and in CAC; APC^{580/580} mice (Figure 3B). Despite the 53% higher serum 1,25(OH)₂D levels in CAC; APC^{580/580} mice, induction of CYP24 mRNA was comparable in the colons of the two genotype groups. TRPV6 mRNA level was also significantly upregulated by 1,25(OH)₂D treatment but this induction was significantly lower in CAC; APC^{580/580} mice compared to wild-type mice (Figure 3C).

In contrast to what we observed *in vitro*, the mRNA level for the four β -catenin target genes we measured (c-Myc, MMP-7, TCF-1, LEF-1, and Axin2) were not significantly reduced in the distal colonic mucosa of either control mice (data not shown) or CAC; APC^{580/580} mice after treatment with 1,25(OH)₂D (Figure 4). In addition, no significant differences between the vehicle and 1,25(OH)₂D treated mice were found for various indices of cell proliferation in the CAC; APC^{580/580} mice, i.e. percent Ki-67 stained nuclei (42.67 ± 2.73 % and 47.38 ± 2.82 %, respectively), Ki-67 staining intensity (214.0±0.1 and 213.9±0.1, respectively), or nuclear size (41.5±0.9 μ m and 42.2±0.57 μ m, respectively).

CAC; APC^{580/580} mice have lower VDR mRNA and protein levels compared to control mice

VDR mRNA levels in the distal colonic mucosa of CAC; APC^{580/580} mice from Experiment 1 were reduced by 65% compared to control mice. 1,25(OH)₂D injections significantly increased VDR mRNA in CAC; APC^{580/580} mice relative to non-treated CAC; APC^{580/580} mice (Figure 5A). VDR protein levels were reduced by 90% in the

colonic mucosa of CAC; APC^{580/580} mice regardless of the treatment group (Figure 5B). The decline in VDR expression in the colon lesions from CAC; APC^{580/580} mice was accompanied by increased levels of Snail 1 (8.6±2.7 fold, p<0.05) and Snail 2 mRNA (5.5±2.0 fold, p<0.05). In Experiment 2, we found that compared to the normal distal colonic mucosa from wild-type mice, tumors from the distal colon of mice with one deleted APC allele (CAC; APC^{580/WT} mice) also had significantly lower VDR mRNA levels (reduced by 92%, p<0.05) (Figure 5C) and higher levels of Snail 1 mRNA (2.6±0.7 fold, p<0.05).

Discussion

APC mutations are a common initiating event in colon cancer (28) and loss of APC allele heterozygosity is common in human colon tumors (29). Epidemiological and cell culture studies suggest that vitamin D and vitamin D metabolites may inhibit colon cancer development (9) and that vitamin D analogs may be useful therapeutic agents (30). Although the mechanism for this protection is not known with certainty, several *in vitro* studies show that 1,25(OH)₂D-induced growth arrest in colon cancer cells is caused by suppressing the Wnt signaling pathway (14,15,19,20).

Palmer et al. (14) and Larriba et al. (15) previously reported that 100 nM 1,25(OH)₂D treatment reduced the mRNA level of the β -catenin target genes PPAR δ , c-Myc, TCF-1, Axin2, LEF-1, and CD44 in SW480-ADH cells. Consistent with these studies, others have shown that 1,25(OH)₂D treatment significantly reduced expression of a β -catenin responsive reporter gene in breast, kidney, and colon cancer cell lines (14,19,20). Our cell studies confirm these earlier observations; 24 h of treatment with 100 nM 1,25(OH)₂D significantly reduced the mRNA level of four known β -catenin target genes in SW480-ADH cells (Axin2, TCF-1, LEF-1, and c-Myc) and three β -catenin target genes in Caco-2 cells (Axin2, TCF-1, and LEF-1).

One mechanism proposed to explain the inhibitory impact of 1,25(OH)₂D on β -catenin signaling is that a direct protein-protein interaction exists between VDR and β -catenin in the presence of 1,25(OH)₂D (14,18-20). While these findings are interesting, several other mechanisms may explain 1,25(OH)₂D-mediated suppression of β -catenin-target gene expression without the need for a direct interaction of β -catenin and VDR. This includes 1,25(OH)₂D induced expression of the β -catenin-antagonist E-cadherin (14) or the Wnt inhibitor DKK-1 (31) as well as repression of the Wnt agonist DKK-4 (32) or direct regulation of the c-myc gene through VDR binding to the c-myc gene promoter (33).

Our primary goal was to directly test whether the *in vitro* observations demonstrating inhibition of β -catenin-mediated gene activation by 1,25(OH)₂D could be translated into the complex environment of the mouse colon. To do this, mice were injected with pharmacological doses of 1,25(OH)₂D to mimic the high levels of 1,25(OH)₂D (100 nM) used in the cell culture studies conducted by others (14,15,19,20). The 1 μ g/kg body weight dose of 1,25(OH)₂D increased serum 1,25(OH)₂D levels to more than 1500 pmol/L in CAC; APC^{580/580} mice, a level that is >10-fold higher than those normally seen in growing mice. In addition, we used multiple injections of vitamin D metabolites to limit the impact of

metabolic clearance. Since the turnover of the colonic epithelium is 60 h (34), and the proliferation index is over 40% in the colonic lesions from CAC; APC^{580/580} mice (see results), we believe that the multiple injection protocol we used exposed the entire population of cells in the colon crypt to vitamin D metabolites over multiple cell divisions. Finally, to mimic the biology of colon cancer cell lines examined in other studies, we used mice whose colonic epithelium contained high levels of β -catenin signaling due to APC gene deletion. Despite activating VDR with large doses of vitamin D metabolites that stimulate traditional vitamin D target genes (e.g. CYP24A1 and TRPV6) these conditions did not reduce either expression of β -catenin target genes or cellular proliferation in the colon of either control mice or in CAC; APC^{580/580} mice.

Few studies have examined the impact of vitamin D signaling on β -catenin function *in vivo* and none of these studies have attempted to assess acute effects. For example, while a 12 week course of 1,25(OH)₂D treatment (3X per week at 0.33 μ g/kg) reduced total Tcf-1, c-Myc, CD44, and nuclear β -catenin protein levels in the colonic mucosa of Apc^{min/+} mice fed a vitamin D deficient diet (35), this does not demonstrate that the impact of vitamin D is due to a direct interaction between β -catenin and VDR nor does it exclude the possibility that the effects on mRNA levels are an indirect effect of prolonged vitamin D treatment. Similarly, while VDR deletion increased nuclear β -catenin and β -catenin target gene mRNA levels (cyclin D1 and Lef1 mRNA) in aberrant crypt foci (ACF) from 5 month old Apc^{min/+} mice, β -catenin nuclear accumulation was not different in adenomas and carcinomas between Apc^{min/+} mice with different VDR status even though cyclin D1 and Lef1 mRNA levels (reflecting increased β -catenin function) were elevated in carcinomas (15). Finally, Zheng et al. (18) found that the colonic tumors of 4 month old Apc^{min/+}; VDR knockout mice had higher β -catenin levels. However, these mice were not fed the rescue diet that is necessary to prevent hypocalcemia in VDR knockout mice (36) and so these data are confounded.

There could be several reasons why we did not observe inhibition of β -catenin function by vitamin D metabolites in our animal study. First, the earlier data using cells grown in monoculture may not reflect the regulation that occurs *in vivo*. For example, monocultures can be continuously bathed in levels of vitamin D metabolites that are not seen *in vivo* even after injections with pharmacologic levels of 1,25(OH)₂D. In addition, monocultures lack many of interactions and signals experienced by the colonic epithelial cells in their natural environment that can alter their responses to stimuli *in vivo* (e.g. signals from bacteria in the lumen, cell-matrix interactions, and from the stromal environment) (37,38). Second, the length of our treatment may not have been sufficient to influence β catenin target gene expression *in vivo*. However, the 1,25(OH)₂D treatment effectively regulated two traditional vitamin D target genes, CYP24 and TRPV6. In addition, hypercalcemia was already evident at the end of our treatment period and prolonging the treatment period would have negative effects on the health of the mice, thereby confounding the results of the experiment. Regardless, our data do not support the use of 1,25(OH)₂D as a means to suppress β catenin signaling in colon cancer.

Another interesting finding from our study was that VDR mRNA and protein levels were reduced by 70-90% in the colonic lesions from CAC; APC^{580/580} mice compared to normal colonic tissue from control mice. Consistent with a suppression of VDR expression

in early colon lesions, VDR mRNA levels also were reduced in the tumors from the distal colon of CAC; APC^{580/WT} mice compared to normal colonic mucosa. The mechanism accounting for the suppression of VDR levels in colonic epithelial cells after APC allele deletion was likely due to the increased-expression of Snail 1 and 2 that accompanied APC allele loss (see Results and (17,39)). Our data extend findings previously reported by Xu et al. who found that VDR mRNA and protein levels were lower in the normal-appearing colonic mucosa of Apc^{min/+} mice (35) and Anderson et al. who reported that VDR mRNA levels are modestly reduced in human colon tumors (40).

The consequence of lower colonic VDR level is reduced 1,25(OH)₂D mediated gene expression and reduced potential for VDR-mediated inhibition of cancer. Consistent with this hypothesis, we found that despite the fact that CAC; APC^{580/580} mice have serum 1,25(OH)₂D levels that are 59% higher than control mice after 1,25(OH)₂D treatment, vitamin D-induced TRPV6 mRNA levels were significantly lower in the colonic lesions from CAC; APC^{580/580} mice compared to normal colonic mucosa. This suggests it may be more difficult to suppress causing pathways, including β -catenin-mediated gene transcription, when VDR levels are low. Consistent with this, others have reported that 1,25(OH)₂D-mediated growth arrest and VDR- β -catenin interactions are dependent upon VDR in SW480 cells (14,15) and mammary epithelial cells (19) while β -catenin-mediated reporter gene activity was suppressed by 1,25(OH)₂D in Caco-2 and HT-29 cells only when VDR was overexpressed (20).

In contrast to the lower induction of TRPV6 expression we saw in CAC; APC^{580/580} mice, vitamin D-induced CYP24A1 gene expression was not reduced by lower VDR levels in CAC; APC^{580/580} mice. This difference may reflect gene-specific mechanisms of transcriptional regulation. The TRPV6 and CYP24A1 genes are both regulated through VDR-dependent mechanisms. However, the location and activity of the vitamin D response elements is different between the two genes (22,41,42) and vitamin D-dependent CYP24A1 gene expression is enhanced by MAPK and PKC-mediated activation of several transcription factors and co-activators (e.g. MED1, Ets-1, and Sp3 (22,43,44)). In several cell types, including colon cancer cells, increased β -catenin levels can activate MAPK signaling (45). Collectively, this suggests that the APC-deficient lesions in the colon of CAC; APC^{580/580} mice could provide an environment for enhanced CYP24A1 gene expression even when VDR expression is low.

In summary, our data shows that in mice with high β -catenin signaling resulting from APC allele loss, pharmacological doses of 1,25(OH)₂D can activate classical VDR target genes but do not reduce mRNA levels for β -catenin target genes. In addition, we found that the loss of colonic APC activity reduced VDR expression. The implications of our findings are that the use of 1,25(OH)₂D or vitamin D analogs to suppress β -catenin signaling is limited and that tumors with complete loss of APC function may be resistant to vitamin D action. However, our data do not preclude the possibility that vitamin D has anti-cancer actions during earlier stages of colon carcinogenesis or in forms of colon cancer that do not result in complete loss of APC function.

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Highlights

- Colon specific deletion of both APC alleles causes adenomatous colon lesions
- These lesions have elevated colonic beta catenin target gene expression
- 1,25 dihydroxyvitamin D increase colonic expression of classical vitamin D target
- 1,25 dihydroxyvitamin D does not reduce expression of beta catenin target genes
- APC deficient colon lesions have low expression of the vitamin D receptor

Figure 1.

1,25(OH)₂D decreases mRNA levels for β -catenin target genes in Caco-2 and SW480-ADH cells. Proliferating Caco-2 and SW480-ADH cells were treated with 100 nM 1,25(OH)₂D for 24 h. RNA was isolated and analyzed by RT-PCR. Gene expression was normalized to RPLP0 expression. Bars = mean $\pm$ SEM (n=3) **(A)** CYP24A1 mRNA. * Significantly different than EtOH treatment (p < 0.05). **(B)** β -catenin target gene mRNA levels. Bars represent the fold-change in expression following 1,25(OH)₂D treatment. * Significantly reduced by vitamin D treatment (p < 0.05).

Fig. 2.

Impact of 1,25(OH)₂ D treatment on body weight and β -catenin gene targets in CAC; APC^{580/580} mice. Four week old mice were given three daily injections with vehicle or 1,25(OH)₂ D (1 μ g/kg body weight) and killed 6 h after the final injection. Bars = mean $\pm$ SEM (A) Body weight (n= 6-15 mice/group). * = p<0.05 vs vehicle treated wild-type mice. (B) β -catenin target gene mRNA levels in the distal colon of CAC; APC^{580/580} mice. (n=12-15/group). * p < 0.05 vs control mice.

Fig 3.

Impact of 1,25(OH)₂ D treatment on serum 1,25(OH)₂ D and colonic expression of vitamin D target genes in CAC; APC $\Delta 580/\Delta 580$ mice. Four week old mice were given three daily injections with vehicle or 1,25(OH)₂ D (1 μ g/kg body weight) and killed six h after the final injection. Data are expressed as mean $\pm$ SEM. Values with different letter superscripts are significantly different from one another ($p < 0.05$, Tukey's HSD) (A) Serum 1,25(OH)₂D. (n = 10/group). (B) CYP24A1 mRNA and (C) TRPV6 mRNA (n=14-20 mice/group).

Fig. 4.

Impact of 1,25(OH)₂D treatment on β -catenin gene targets and cellular proliferation in CAC; APC^{580/580} mice. Four week old mice were given three daily injections with vehicle or 1,25(OH)₂D (1 μ g/kg body weight) and killed 6 h after the final injection. RNA was isolated from the distal colonic mucosa of CAC; APC^{580/580} mice and RT-PCR was used to measure β -catenin gene targets. Data are expressed as mean $\pm$ SEM (n=14 mice/group).

Fig. 5. VDR mRNA and protein expression in colonic lesions from CAC; APC $\Delta 580/\Delta 580$ or CAC; APC $\Delta 580/\Delta 580$ mice. **(A, B)** Four week old CAC; APC $\Delta 580/\Delta 580$ mice were given three daily injections with vehicle or 1,25(OH)₂ D (1 μ g/kg body weight) and killed six h after the final injection. RNA was isolated from the distal colonic mucosa and RT-PCR was used to measure VDR mRNA **(A)**. Data are expressed as mean $\pm$ SEM (n=5-20 mice/group). Values with different letter superscripts are significantly different from one another (p < 0.05, Tukey's HSD). **(B)** Whole protein extracts from colon were prepared and pooled. Western

blot analysis was conducted for VDR protein levels. β -actin was used as a loading control. (Veh = vehicle; 1,25D = 1,25(OH)₂D) (C) Tumors from the distal colon of 20 wk-old CAC; APC^{580/WT} mice were saved and RNA was isolated and analyzed by RT-PCR for VDR mRNA levels. Bars represent the mean + SEM (n = 24/group), * Significantly different from normal mucosa of wild-type mice (p<0.05).

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Table I

PCR primers used for analysis of mRNA levels.

	Forward ²	Reverse	Ta ³
Human¹			
c-Myc	GTCCTCGGATTCTCTGCTCTC	CCTCATCTTCTTGTCTCCTCTC	58
LEF-1	CTTCCTTGGTGAACGAGTCTG	CTCCTGAGAGGTTTGTGCTTG	56
TCF-1 (46)	CGGGACAGAGGACCATTACAACCTAGATCAAGGAG	CCACCTGCCTCGGCCTGCCAAAGT	61
Axin2 (47)	TTATGCTTTGCACTACGTCCCTCCA	CGCAACATGGTCAACCCTCAGAC	58
CYP24A1	CTCATGCTAAATACCCAGGTG	TCGCTGGCAAAACGCGATGGG	54
RPLP0	GCAATGTTGCCAGTGTCTG	GCCTTGACCTTTTCAGCAA	55
Mouse			
c-Myc (48)	GAAAAGGCCCCCAAGGTAGT	AGAGTTTCGAAGCTGTTCTGA	55
LEF-1 (46)	AGCCAAGGCAGCGACCCAGG	CGGCGCTTGCACTAGACGACAGA	55.8
TCF-1 (46)	CCCCCACAGCACCCCTCCAGAATC	CCAGGTTCAAGGAGTTGTGACGCC	57
MMP-7 (49)	TGTGAGCGCACATCAGTGGGAACA	GCTTTTTTCACGATTGATCCACT	55.8
Axin2 (50)	GAGTAGCGCCGTGTTAGTGACT	CCAGGAAAGTCCGGAAGAGGTATG	54.8
VDR	TACATCCGCTGCCGCCACCCGC	TCAGGAGATCTCATTGCC	55
CYP24A1	CAAACCTGGAAAGCCTATCG	CGCTGCCACTCCTGTCCTT	54
Snail 1	TCTCTAGGCCCTGGCTGCTT	AAAGCACGGTTGCAGTGG	64.0
Snail 2	CTCACCTCGGGAGCATACAG	GAGAAAGGCCACTGGGTAAAG	60.5
RPLP0	AGAAACTGCTGCCTCACATCC	CAATGGTGCCTCTGGAGATT	59

¹ When primer sequences were obtained from previously published studies, the citation is included.

² All primers are written in the 5' to 3' direction

³ Annealing temperature