Review

Effects of 1α,25-Dihydroxyvitamin D_3 in Human Colon Cancer Cells

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Abstract. *Colorectal cancer is a major health problem worldwide. Epidemiological studies and work on experimental animals strongly suggest a protective effect of 1α,25-dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$) against colon neoplasia. 1,25(OH)$_2$D$_3$ is a pleiotropic hormone that has multiple actions in the organism. By binding to the widely expressed high affinity vitamin D receptor (VDR) it regulates the transcription rate of many genes. Other non-genomic effects of 1,25(OH)$_2$D$_3$ also appear to modulate the physiology of numerous cell types. Human normal and cancer colon epithelial cells express VDR and the key enzymes involved in 1,25(OH)$_2$D$_3$ synthesis and degradation and are, thus, responsive to the hormone. 1,25(OH)$_2$D$_3$ inhibits proliferation, induces differentiation and sometimes the apoptosis of human colon cancer cells. A great variety of mechanisms and signaling pathways are involved. Since VDR mediates most, if not all, 1,25(OH)$_2$D$_3$ actions, the control of VDR expression is a crucial aspect of 1,25(OH)$_2$D$_3$ biology. Here, the molecular mechanisms underlying the actions of 1,25(OH)$_2$D$_3$ are reviewed and the repression of the VDR gene by the transcription factor SNAIL in human colon cancer cells is discussed. Understanding these mechanisms may provide the basis for the potential use of this hormone and its non-hypercalcemic derivatives in the prevention and treatment of colon cancer.*

Colon Cancer and Vitamin D

Colorectal cancer (CRC) is a major cause of cancer death worldwide and there is no satisfactory therapy when surgery is not curative (1). CRC is strongly related to social and geographic parameters, as developed countries account for over 65% of all patients. The risk of contracting CRC increases rapidly when people migrate from low- to high-risk countries, suggesting that local environmental exposure influences susceptibility. Only a small proportion (between 5 and 10%) of CRC cases are attributable to the cancer syndromes of familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), while the majority are considered sporadic (2). In addition, sporadic cases may have a strong component of familial aggregation, the genetic basis of which is unknown.

Fifteen years ago, Fearon and Vogelstein (3) proposed a genetic model involving the alteration of several oncogenes and tumor suppressor genes, which describes the transition from healthy colonic epithelia through increasingly dysplastic adenoma to malignant cancer (suppressor pathway). Later studies have basically confirmed this model and also added new genes and proposed others, still uncharacterized, as complementary driving forces for this neoplasia (4). Thus, alterations in the Wnt/β-catenin pathway (in *Adenomatous Polyposis Coli*/*APC*, *CTNNB1*/*β-catenin* or *AXIN2* genes) invariably seem to occur at initial stages, while mutations in *K-RAS*, *B-RAF* or the transforming growth factor (TGF)-β signaling pathway confer additional malignant features to adenoma cells. Adenomas progress to carcinomas *in situ* and malignancy

Abbreviations: 1,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; 25(OH)D$_3$, 25-hydroxyvitamin D$_3$; APC, *Adenomatous Polyposis Coli*; CIN, chromosomal instability; CRC, colorectal cancer; DAG, diacylglycerol; EGF, epidermal growth factor; FAP, familial adenomatous polyposis; FGF, fibroblast growth factor; HNPCC, hereditary nonpolyposis colorectal cancer; IGF, insulin-like growth factor; IP3, inositol 1,4,5-triphosphate; LCA, lithocholic acid; MIN, microsatellite instability; MMR, mismatch repair; PKC, protein kinase C; TCF, T-cell factor; TGF, transforming growth factor; VDR, vitamin D receptor.

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coincides with inactivation of the *TP53* gene in around 50% of tumors (5).

Two forms of genetic instability contribute to CRC progression: chromosomal instability (CIN) and microsatellite instability (MIN). Mutations in the Wnt/β-catenin pathway gene *APC* and in *TP53* contribute to the CIN phenotype (5, 6) characterized by allelic loss and aneuploidy (7). Epigenetic silencing of mismatch repair (MMR) genes or, less frequently, somatic mutations in these genes, are responsible for approximately 15% of the sporadic tumors that present the MIN phenotype (mutator pathway) (8-10). The familial syndromes FAP and HNPCC are good models for CIN and MIN tumors, respectively.

Vitamin D is obtained from the diet, dietary supplements and, mainly, the conversion of 7-dehydrocholesterol to vitamin D_3 by the action of solar UV-B radiation (280-320 nm) in the skin. Vitamin D_3 is hydroxylated in the liver to 25-hydroxyvitamin D_3 ($25(OH)D_3$) by vitamin D_3 25-hydroxylase (product of the *CYP27A1* gene). Subsequent hydroxylation by 25-hydroxyvitamin D_3 1α-hydroxylase (product of the *CYP27B1* gene) renders 1α,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$, calcitriol), the most active metabolite of vitamin D.

$1,25(OH)_2D_3$ is a pleiotropic hormone. In addition to its classic regulatory effects on calcium and phosphate metabolism and bone biology, it has antiproliferative, pro-apoptotic and pro-differentiation effects. These so-called novel actions suggest anticancer activity. To avoid the toxic hypercalcemic effects of high-dose treatments with $1,25(OH)_2D_3$, many derivatives generically termed deltanoids have been synthesized. $1,25(OH)_2D_3$ and the deltanoids exert their actions mainly *via* their high affinity receptor (vitamin D receptor, VDR) through a complex network of genomic (transcriptional and post-transcriptional) and also non-genomic mechanisms, which are partially coincident in the various cells and tissues studied (11).

Epidemiological studies combining multiple sources of vitamin D or examining serum $25(OH)D_3$ associate an above-average vitamin D intake and serum metabolite concentrations with a significant reduction in the incidence of CRC. However, if only dietary vitamin D is considered, inverse correlations are found only occasionally. Dietary sources may comprise only part of the total vitamin D needed, with supplements and solar radiation providing the balance (12).

Studies in animal models have also suggested that a high-fat diet, with low levels of vitamin D, increases the risk of CRC (13). In long-term studies, wild-type mice fed with a Western-style diet (high fat and phosphate and low vitamin D and calcium content) showed hyperproliferation in colonic epithelial cells in the absence of carcinogen exposure (14-16). Short periods (12 weeks) on this diet induced colon-crypt hyperplasia (17). Furthermore, these effects were suppressed when the Western-style diet was supplemented with calcium and vitamin D, suggesting that hyper-proliferation could be prevented by increasing the dietary calcium and vitamin D (13). The Western-style diet was also tested in the mutant Apc^{min} mouse, a model of intestinal carcinogenesis that develops multiple neoplasias throughout the intestinal tract soon after birth. The mice carried a truncated *Apc* allele with a nonsense mutation in exon 15 (*Apc1638*). The numerous polyps that developed in normal conditions (standard AIN-76A diet) were increased by the Western-style diet and their survival diminished (18). Apc^{min} mice were also treated with $1,25(OH)_2D_3$. After 10 weeks of treatment, the tumor number was not affected, although there was a significant decrease in the total tumor load (sum of all polyp areas) over the entire gastrointestinal tract (46% reduction).

In summary, vitamin D has protective effects against CRC and other types of cancer, particularly in association with calcium. Intense research into the molecular basis of this action has been undertaken.

Expression of VDR and Vitamin D_3 Metabolic Enzymes in Normal and Cancer Colon Cells

Tissue responsiveness to $1,25(OH)_2D_3$ depends mainly on the expression levels of VDR and the hydroxylases regulating $1,25(OH)_2D_3$ synthesis and degradation. Numerous studies have demonstrated variable levels of VDR expression in normal epithelial and colon cancer cells. VDR expression is low in normal colon tissue, mainly in differentiated luminal crypt cells (19). In mice, VDR expression is higher in the proximal than in the distal colon (20).

One of the first studies of VDR expression in colon cancer cell lines (CaCo-2) showed that it was higher when the cells were confluent and differentiated in culture (21). Subsequent studies corroborated that VDR is associated with a high degree of cell differentiation (22, 23). The comparison between human malignant colonic tissue and normal mucosa from the same patient revealed significantly higher VDR expression in tumoral than in normal tissue (23-25). However, VDR expression is only enhanced in low-grade tumor tissue, whereas in advanced carcinomas it decreases or disappears (19, 25-28). This causes ligand unresponsiveness and, possibly, failure of therapy with vitamin D analogs. It also suggests that colon cancer cells express VDR as long as they retain a certain level of differentiation (29). Concordantly, a high level of VDR expression is associated with a favorable prognosis in CRC (25, 30).

To examine the functional role of VDR in colon, *vdr*-deficient mice have been analyzed. These mice display hyperproliferation and increased DNA damage, mainly in the colon *descendens* (20). Although they do not have high

rates of spontaneous colon cancer, the results implicate $1,25(OH)_2D_3$ action in the prevention of hyperproliferation and oxidative DNA damage, at least in the distal colon, while normal growth conditions of mucosal cells are maintained (20).

In addition, molecular variants (polymorphisms) of the *VDR* gene may influence the development of colon cancer. Further studies are, however, needed to evaluate the association of these variants with diet and lifestyle factors to clarify the impact of *VDR* gene polymorphisms on cancer etiology (31).

25-Hydroxyvitamin D_3 1α-hydroxylase and 25-hydroxyvitamin D_3 24-hydroxylase (which converts $1,25(OH)_2D_3$ to less active compounds; encoded by the *CYP24* gene) are expressed in the kidney and several other cell types. Low levels of both enzymes are expressed in the colon (32, 33). $1,25(OH)_2D_3$ regulates its own synthesis and degradation through the induction of the *CYP24* and the repression of the *CYP27B1* genes (11). In normal mouse colon, *CYP27B1* mRNA expression is similar in the proximal and distal colon, whereas *CYP24* expression is higher in the proximal (34). Furthermore, as with VDR, the expression of 25-hydroxyvitamin D_3 1α-hydroxylase is higher at early stages of colon tumor progression than in normal mucosa or in undifferentiated tumors (27, 33, 35). Up-regulation of both VDR and *CYP27B1* can be considered intrinsic tumor-suppressive functions. *CYP24* expression is higher in tumors than in adjacent normal tissue and much higher in poorly-differentiated cancers (35). *CYP27B1* expression is significantly down-regulated in *vdr* knock-out mice, probably due to enhanced proliferation (20).

$1,25(OH)_2D_3$ synthesis can be regulated by dietary consumption of soybean, which is rich in phytoestrogens. These products increased *CYP27B1* and reduced *CYP24* expressions in the mouse colon, which resulted in high $1,25(OH)_2D_3$ levels and enhanced protection against CRC (28, 36). This may explain the low incidence of prostate, breast and colon cancer in people who consume a typical Asian diet, which contains high amounts of soybean products (genistein and other phytoestrogens). It may also explain the lower incidence of CRC in women, due to their higher estrogenic background (29, 37).

Non-genomic and Genomic Effects of $1,25(OH)_2D_3$ in Colon Cancer Cells

$1,25(OH)_2D_3$ initiates biological responses either by inducing rapid non-genomic effects (seconds-minutes to hours) or *via* regulatory actions at the transcriptional level called genomic effects (hours to days) (38). Non-genomic effects include the opening of voltage-gated Ca^{2+} and Cl^- channels and changes in the activity of certain enzymes (kinases, phospholipases).

$1,25(OH)_2D_3$ is a flexible molecule that rotates about its 6,7 single carbon bond, which can generate a large array of ligand shapes ranging from 6-s-*cis* (6C) to the open and extended 6-s-*trans* (6T) form (38). Remarkably, the 6C configuration favors activation of a non-genomic pathway by binding to a putative membrane receptor (VDR or other unknown receptors) (39-42). Recent data, showing that VDR is present in caveolae-enriched intestinal plasma membranes, suggest that it may mediate at least some non-genomic effects (43).

Binding of $1,25(OH)_2D_3$ to the membrane surface receptor may activate second messenger systems. Studies with freshly-isolated rat colonocytes and human CaCo-2 cells have revealed a rapid pathway mediated *via* the phosphoinositide (PI) transduction system, which requires the presence of the plasma membrane receptor. $1,25(OH)_2D_3$ rapidly stimulated the hydrolysis of membrane PI, generating the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (44, 45). Subsequently, the intracellular calcium (Ca^{2+}) concentration was increased due to IP3-mediated release of intracellular Ca^{2+} stores and to Ca^{2+} influx into the cell through the opening of the receptor-mediated Ca^{2+} channel, as happens in other cell types (46, 47). Due to the rise in Ca^{2+} concentration and DAG, two isoforms of protein kinase C (PKCα and PKCβ2) were activated in rat colonocytes (39), whereas only PKCα was activated in CaCo-2 cells (48). Later studies from the same group found that $1,25(OH)_2D_3$ activated the tyrosine kinase c-Src in the basolateral membranes of colonocytes by a heterotrimeric guanine nucleotide binding protein (G-protein)-dependent mechanism, with subsequent activation of PI-specific phospholipase C-γ, responsible for hydrolysis of PI (49). Furthermore, $1,25(OH)_2D_3$ also activated phosphatidylcholine-phospholipase D (PLD) in CaCo-2 cells in a concentration-dependent manner. PLD stimulation occurred by both PKC-dependent and -independent mechanisms. The products of PLD have been associated with regulation of cell proliferation and differentiation among other cellular functions (50, 51).

Recently, a mouse model has been established that expresses a mutant VDR with an intact hormone-binding domain, but lacking the first zinc finger necessary for DNA binding, which abrogates the genomic and non-genomic functions of $1,25(OH)_2D_3$. These data indicate that VDR might mediate the non-genomic actions of $1,25(OH)_2D_3$ (52). In conclusion, the study of non-genomic responses is still in its developmental phase and their relationship to $1,25(OH)_2D_3$ anticancer activity is still unclear.

The 6T $1,25(OH)_2D_3$ configuration preferentially mediates genomic responses (38). These are better understood and their generation is homologous to that of classic steroid hormones. The receptor-hormone complex, localized in the nucleus, acts as a transcription factor and

binds to specific sequences, termed vitamin D response elements (VDRE), in the promoter region of its target genes, thus modulating their expression (11, 53, 54). Many genes are responsive to $1,25(OH)_2D_3$, but not all contain the VDRE consensus sequence, which suggests their regulation might be indirect, a consequence of the cascade of events induced by $1,25(OH)_2D_3$ (55).

Several studies examined the change in gene expression profiles associated with $1,25(OH)_2D_3$ treatment in human colon adenocarcinoma cell lines. In a subpopulation of SW480 cells (SW480-ADH) that express VDR, $1,25(OH)_2D_3$ induced E-cadherin gene (*CDH1*) expression and epithelial differentiation (56). Transcriptome studies performed in SW480-ADH cells using oligonucleotide microarrays revealed that $1,25(OH)_2D_3$ changed the RNA expression levels (≥3.5-fold) of numerous genes, including many involved in transcription, cell adhesion, DNA synthesis, apoptosis, redox status and intracellular signaling. Some of these results were validated by Northern and Western blotting or immunofluorescence analysis, including *c-JUN, JUNB, ZNF-44/KOX7,* G_0S2, plectin, filamin and the putative tumor suppressor genes *NES-1* and protease M (57). The gene expression patterns regulated in colon SW480-ADH cells have similarities with those found in $1,25(OH)_2D_3$- or deltanoid EB1089-treated head and neck cancer cells (58, 59). In LS-174T colon cancer cells, which lack E-cadherin and do not differentiate in response to $1,25(OH)_2D_3$, the expression profile served as a control, not only to evaluate the correlation between gene expression and phenotype changes caused by $1,25(OH)_2D_3$, but also to estimate the contribution of E-cadherin to the gene expression profile in SW480-ADH cells. The number of expression changes found in LS-174T was lower than in SW480-ADH and the comparison between the two cell lines revealed that only four genes (protease M, bilirubin UDP-glucuronosyltransferase isoenzyme 2, *CYP24* and *ZFAB*) regulated by $1,25(OH)_2D_3$ in SW480-ADH cells were also regulated in LS-174T cells (57).

In well-differentiated CaCo-2 cells, the gene expression profile induced by $1,25(OH)_2D_3$ was also studied using oligonucleotide microarrays. Only twelve genes exhibited significant changes in expression, three of which had already been identified in other cell systems (*CYP24, JUNB* and amphiregulin) and the remaining nine (*e.g.*, Gem, *TIG1,* ceruloplasmin, sorcin) were validated as $1,25(OH)_2D_3$ targets by RT-PCR. Many of these genes could be involved in the antiproliferative action of $1,25(OH)_2D_3$ (60).

Regulation of Human Colon Cancer Cell Proliferation, Survival and Differentiation by $1,25(OH)_2D_3$

The anticancer activity of $1,25(OH)_2D_3$ in colon cancer cells stems mainly from the inhibition of proliferation and the induction of apoptosis and differentiation. These actions can operate in combination, the predominant effect varying from one cell to another (11, 55, 61, 62). The growth-inhibitory action of $1,25(OH)_2D_3$ has been observed in many human colon cancer cell lines and also in cultured primary human colon adenoma- and carcinoma-derived cells (22, 56, 63-65). The antitumor activity of certain $1,25(OH)_2D_3$ analogs has been analyzed in xenograft models. EB1089 showed activity against the growth of tumors generated by LoVo and SW480-ADH cells in immunosuppressed mice (26, 66) and Ro25-6760 and Paricalcitol showed activity against the growth of those generated by HT-29 cells (67, 68). Furthermore, $1,25(OH)_2D_3$ reduced the rate of crypt cell production of colonic tissue taken from patients with FAP (69).

Some of the antimitotic actions of $1,25(OH)_2D_3$ and its analogs are mediated by the induction of G0/G1 cell-cycle arrest as a result of the up-regulation of the cyclin-dependent kinase (CDK) inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (70-72). The mechanisms underlying these regulatory effects are different. While the $p21^{WAF1/CIP1}$ gene promoter has a VDRE and its expression is induced by $1,25(OH)_2D_3$ in many cell types (11, 73), the induction of the $p27^{KIP1}$ gene, which lacks VDRE, is mediated by the transcription factors NF-Y and Sp1 as well as by protein stabilization (74, 75). $1,25(OH)_2D_3$ also induced the expression of growth-arrest and DNA damage 45α (GADD45α) protein, which is involved in cell cycle arrest after DNA damage and is required for the maintenance of genomic stability (11, 57). Furthermore, $1,25(OH)_2D_3$ regulates many other genes related to proliferation, including *c-MYC, c-FOS* and *c-JUN* (55, 61, 62).

In addition to the effects on cell-cycle-regulatory proteins, $1,25(OH)_2D_3$ exerts its antiproliferative action by interfering with certain signaling pathways that control epithelial cell growth. The first one described was that of epidermal growth factor (EGF), which induces proliferation of colon epithelial cells. This effect was counteracted by $1,25(OH)_2D_3$ in primary cultures of human colon adenocarcinoma cells and in CaCo-2 cells. $1,25(OH)_2D_3$ reduced EGF receptor (EGFR) expression and also decreased the amount of membrane EGFR, promoting its ligand-induced internalization (76). However, EGF, in turn, down-regulates VDR expression in CaCo-2 cells. Therefore, activation of the EGF pathway could allow colon carcinoma cells to escape from the antitumoral action of $1,25(OH)_2D_3$ (77, 78).

Colorectal adenomas overexpress insulin-like growth factor (IGF)-II, which acts as a mitogen and a survival agent. Accordingly, HT-29 adenocarcinoma colon cells secrete IGF-II, which stimulates their growth (79). $1,25(OH)_2D_3$ and certain analogs inhibit IGF-II secretion and increase the production of IGF-binding protein-6, which negatively modulates IGF-II signaling (57, 80).

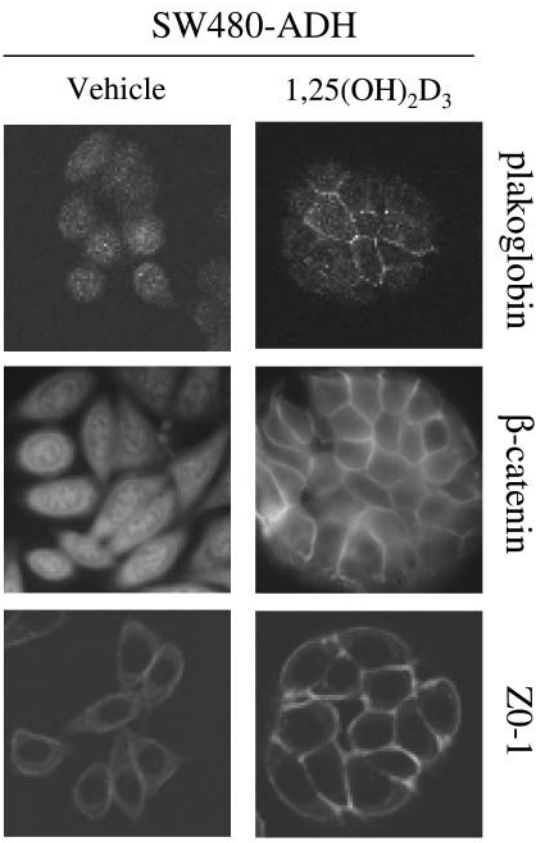

Figure 1. *1,25(OH)$_2$D$_3$ induces relocalization of junctional proteins from the nucleus to the plasma membrane adhesion structures. Immunofluorescence analysis of ZO-1, β-catenin and plakoglobin expression in SW480-ADH cells treated or not with 10^{-7} M 1,25(OH)$_2$D$_3$ for 48 h.*

Moreover, 1,25(OH)$_2$D$_3$ induces type II IGF receptor (IGFR-II), which also blocks this pathway as it accelerates IGF-II degradation (81). Therefore, interference with the IGF-II signaling pathway may contribute to the anticancer action of 1,25(OH)$_2$D$_3$.

The growth of normal colon epithelial cells is inhibited by TGF-β. However, most human colon cancer cells are resistant to the action of TGF-β and progression from colonic adenoma to carcinoma is accompanied by resistance to TGF-β (82). It has been reported that 1,25(OH)$_2$D$_3$ sensitized SW480 and CaCo-2 cells to the growth inhibitory action of TGF-β. 1,25(OH)$_2$D$_3$ induces the expression of type I TGF-β receptor and also that of IGFR-II, which facilitates the activation of the TGF-β precursor (57, 81). In addition, SMAD3, a TGF-β signaling downstream protein, binds to SRC-1 and acts as a co-activator of VDR and, therefore, cooperates in the induction of 1,25(OH)$_2$D$_3$ target genes (83).

In addition to cell cycle inhibition, 1,25(OH)$_2$D$_3$ and its analogs induced apoptosis in colon cancer cells (70, 84, 85). Apoptosis induction by 1,25(OH)$_2$D$_3$ follows the regulation of genes that control death pathways. 1,25(OH)$_2$D$_3$ and its analogs up-regulate the pro-apoptotic protein BAK (85) and promote the release of the anti-apoptotic protein BAG-1 from the nucleus (86). However, the effect of 1,25(OH)$_2$D$_3$ on the expression of other pro-apoptotic (BAX) or anti-apoptotic (BCL-2, BCL-X_L) proteins differed from one cell line to another (57, 85). The apoptosis induced by 1,25(OH)$_2$D$_3$ and its analogs did not require an intact *TP53* tumor suppressor gene (61, 85). This would allow the use of 1,25(OH)$_2$D$_3$ analogs for cancer treatment independently of the tumor *TP53* status. This is especially appropriate in CRC, in which many tumors present alterations in *TP53*.

The antiproliferative action of 1,25(OH)$_2$D$_3$ and its analogs is commonly linked to stimulation of cell differentiation. 1,25(OH)$_2$D$_3$ induces strong enterocytic differentiation of CaCo-2 and HT-29 cells. Treated cells present a prominent brush-border membrane with high activity of brush-border-associated enzymes, such as alkaline phosphatase (22, 64, 72, 87). In addition, 1,25(OH)$_2$D$_3$ increases the number of intermediate filaments, desmosomes and microvilli (64, 88). Alkaline phosphatase activity, widely considered as a colon differentiation marker, was also induced by 1,25(OH)$_2$D$_3$ in primary cultured colon carcinoma cells and in other colon cancer cell lines (65, 85). Recently, our group found that 1,25(OH)$_2$D$_3$ and several analogs promoted epithelial differentiation in SW480-ADH cells, a subpopulation of SW480 colon adenocarcinoma cells that express VDR (Figures 1 and 3B) (56). This differentiation is associated with the induction of E-cadherin expression, the main component of adherent junctions and responsible for the maintenance of the epithelial phenotype. In addition, 1,25(OH)$_2$D$_3$ induced the expression of the components of tight junctions occludin, *Zonula occludens* (ZO)-1, ZO-2, and also that of vinculin, which is located in tight junctions, adherent junctions and focal adhesion plaques. 1,25(OH)$_2$D$_3$ also promotes the translocation of β-catenin, plakoglobin and ZO-1 from the nucleus to the plasma membrane (Figure 1). The effect of 1,25(OH)$_2$D$_3$ on E-cadherin expression has also been observed in other colon cancer cell lines, such us CaCo-2, HT-29 and SW1417 (56).

In addition, combined exposure of HT-29 cells to 1,25(OH)$_2$D$_3$ and butyrate enhances differentiation and cell-growth arrest. Cells induced to differentiate by this means maintain the differentiated phenotype long after both compounds have been removed (89). Furthermore, 1,25(OH)$_2$D$_3$ and butyrate synergistically induced $p21^{WAF1/CIP1}$ expression and alkaline phosphatase activity in CaCo-2 cells (90). The authors showed that this effect was mediated by butyrate-induced overexpression of VDR. Therefore, a combination of both compounds may be a useful approach for CRC prevention and treatment.

VDR functions as a receptor for the secondary bile acid lithocholic acid (LCA) (91, 92). A high-fat diet leads to colon LCA accumulation, which induces DNA damage and inhibits DNA repair enzymes in colonic cells. Accordingly, LCA promotes colon cancer in experimental animals and high levels of LCA have been found in CRC patients (93). Several mechanisms may protect colon cells from LCA. CYP3A-dependent hydroxylation and Sult2a2-dependent sulfation increase the water solubility and facilitate the elimination of LCA. Furthermore, the multidrug resistance-associated protein 3 (MRP3) is localized on the basolateral face of enterocytes and mediates LCA release into the bloodstream (94). Activation of VDR by LCA or $1,25(OH)_2D_3$ transcriptionally induces *CYP3A, Sult2a2* and *MRP3* expression in a feed-back mechanism that resulted in colon LCA elimination (91, 92, 94-96). Therefore, these coordinated mechanisms for LCA detoxification could partly explain the protective action of $1,25(OH)_2D_3$ against CRC.

In summary, the effects and mechanism of action of $1,25(OH)_2D_3$ in colon cancer cells are well established and support a beneficial role of its analogs in CRC prevention and treatment.

$1,25(OH)_2D_3$ Antagonizes Wnt/β-catenin Signaling Pathway

Wnt proteins are a family of secreted signaling factors with multiple functions in development and homeostasis (97, 98). Some of them act *via* the well-characterized canonical Wnt signaling pathway (also known as the Wnt/β-catenin pathway). Activation of this pathway is initiated by binding of Wnt proteins to cell surface receptors composed of a member of the Frizzled protein family and one of the LDL receptor-related proteins, LRP-5 or LRP-6 (99, 100). Signaling from Wnt receptors proceeds through the proteins Dishevelled and Axin, leading to inactivation of a cytoplasmic complex containing the APC protein and glycogen synthase kinase (GSK)-3β. This enzyme catalyzes the phosphorylation of β-catenin required for its degradation by the proteasome (97). Canonical Wnt signaling thus induces stabilization of cytosolic β-catenin. A fraction of β-catenin then enters the nucleus, binds transcription factors, such as those of the TCF/LEF family, and modulates the transcription of target genes that promote proliferation and invasiveness (101). Wnt proteins can also signal through other (non-canonical) pathways unrelated to β-catenin that involve Rho, c-Jun N-terminal kinase or Ca^{2+}, but their putative relationship to cancer is unknown.

Wnt signals have been implicated in the proliferation of intestinal epithelial progenitor cells (102, 103). Proliferative progenitor cells locate at the bottom of the intestinal crypts and accumulate nuclear β-catenin, probably due to Wnt signals from the surrounding stroma that maintain the proliferative status of this compartment. As these cells move upward they are committed to differentiate and β-catenin is no longer detected in their nuclei. The requirement of an active canonical Wnt pathway to ensure proliferation of progenitor cells is evident, since mutation of the intestinal-specific TCF/LEF family member TCF4 induces loss of proliferative compartments in the small intestine (104). Moreover, targeted expression of the soluble Wnt inhibitor Dickkopf-1 in mice reduces epithelial proliferation, coinciding with the loss of crypts (105).

Constitutive activation of Wnt/β-catenin signaling occurs in nearly all colorectal tumors due to mutations in either the *APC* gene or, less frequently, *CTNNB1/β-catenin* or *AXIN2* (4). Activation of this pathway is an early, if not initiating, event in colonic tumorigenesis, and mutational activation of canonical Wnt signaling may be the principal driver of intestinal tumors (4). Adenoma cells thus represent the transformed counterparts of crypt progenitor cells.

Results from our group have demonstrated that $1,25(OH)_2D_3$ and several non-hypercalcemic analogs can antagonize canonical Wnt signaling in human colorectal cancer cells (56). In SW480-ADH cells, $1,25(OH)_2D_3$ inhibits the transcriptional activity of β-catenin by two mechanisms (Figure 2). First, it rapidly increases the amount of VDR bound to β-catenin, thus reducing the interaction between β-catenin and TCF4. Therefore, $1,25(OH)_2D_3$ modulates TCF/LEF target genes in an opposing way to that of β-catenin. Second, the reduction of β-catenin transcriptional activity caused by $1,25(OH)_2D_3$ is accompanied by the nuclear export of β-catenin and its relocalization to the plasma membrane, which is concomitant to E-cadherin protein expression (Figures 1 and 2). In some cells, such as LS-174T, this effect is independent of E-cadherin expression. These results indicate that $1,25(OH)_2D_3$ down-regulates the Wnt/β-catenin signaling pathway, which may control the phenotype of colon epithelial cells and may thus be used in CRC prevention.

Upon β-catenin stabilization in colon cancer cells due to its own mutation or that of APC, binding to VDR may buffer its stimulatory action on TCF4 target genes, a protective effect which can be lost along with VDR expression during malignant progression. Additionally, data obtained by our group suggest that nuclear β-catenin might transiently potentiate VDR transcriptional activity before β-catenin moves out of the nucleus and/or VDR is extinguished (56).

Inhibition of Wnt/β-catenin signaling by other ligand-activated nuclear hormone receptors has been described. Retinoic acid (RA) decreases the activity of β-catenin/TCF complexes in breast cancer cells. β-catenin interacts with the RA receptor (RAR) in a retinoid-dependent manner and RAR competes with TCF for β-catenin binding (106). In

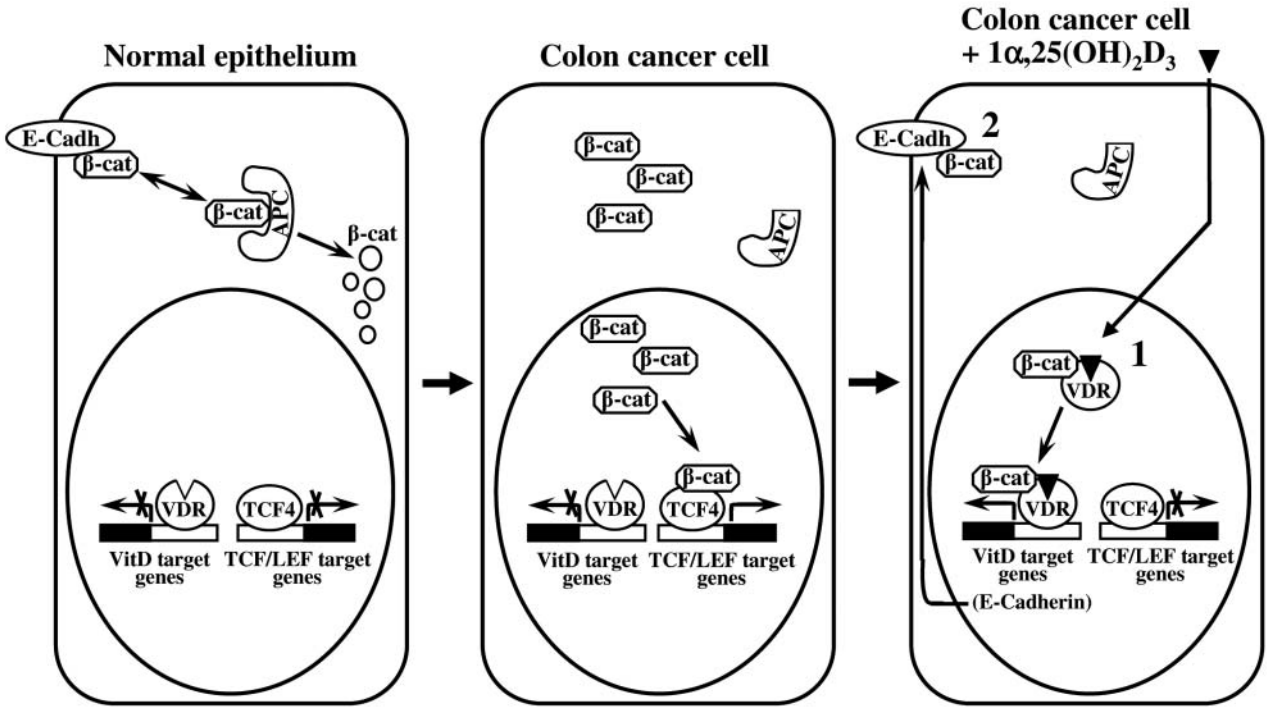

Figure 2. *1,25(OH)$_2$D$_3$ antagonizes the Wnt/β-catenin signaling pathway. Scheme of the mechanisms of inhibition of Wnt/β-catenin signaling by 1,25(OH)$_2$D$_3$. (Left) Cytosolic and nuclear β-catenin are not detected in normal colon epithelial cells due to APC-mediated degradation by the proteasome. β-Catenin remains bound to E-cadherin. In this situation β-catenin target genes are silent. (Middle) APC mutation (frequently truncation) or E-cadherin loss impedes β-catenin phosphorylation by GSK-3β and subsequent degradation. β-Catenin then accumulates in the cytosol and nucleus, where it binds TCF/LEF and induces many genes. (Right) 1,25(OH)$_2$D$_3$ treatment of colon cancer cells induces rapid binding of liganded VDR to β-catenin and so inhibits the formation of β-catenin-TCF/LEF complexes and induction of their target genes. In addition, ligand-activated VDR regulates numerous genes including CDH1 encoding E-cadherin protein. E-cadherin sequesters β-catenin at the plasma membrane adherens junctions causing its progressive disappearance from the nucleus and the long-lasting blockade of β-catenin-TCF/LEF target genes.*

addition, the androgen receptor (AR) can repress β-catenin/TCF-mediated transcription in both prostate cancer and colon cancer cells (107). A specific protein-protein interaction occurs between β-catenin and AR and the amount of β-catenin in complex with AR is increased by androgen. The ligand-binding domain of AR and the amino-terminus combined with the first armadillo repeats of β-catenin are necessary for this interaction (108, 109). Competition with TCF also occurs. A role for AR in the translocation of β-catenin to the nucleus has also been suggested (110). As in the case of VDR, β-catenin serves as a coactivator for both AR and RAR (106, 108).

Moreover, the peroxisome proliferator-activated receptor (PPAR)γ and Wnt/β-catenin have opposite effects in pre-adipocyte differentiation. Activated PPARγ inhibits β-catenin expression at a post-translational level through a mechanism that involves the proteasome and is APC-independent (111, 112).

Shah and colleagues have proposed that interaction with the p300 co-regulator underlies the trans-repression of Wnt/β-catenin signaling by nuclear receptors and their ligands (113). They showed that the C- and N-terminal trans-activating domains of β-catenin and the activation function domains (AF-1, ligand-independent, and/or AF-2, ligand-dependent) of nuclear hormone receptors were required for trans-repression. This suggests that the trans-repressive effects might be a result of interaction with a co-activator common to both nuclear receptors and β-catenin. Inhibition of the histone acetyltransferase p300 by mutant E1A repressed β-catenin activity to the same extent as RA/RAR, whereas overexpression of p300 did not affect basal β-catenin activity, although it completely prevented RA/RAR-mediated trans-repression (113).

SNAIL Represses VDR Expression and 1,25(OH)$_2$D$_3$ Action

The presence of functional VDR is required for the cellular response to 1,25(OH)$_2$D$_3$, and a relationship between VDR levels and growth inhibition has been

proposed in colon cancer (23, 114). Thus, the absence of VDR is associated with colonic hyperproliferation (20). Therefore, variation in VDR levels is an important regulatory mechanism of the action of $1,25(OH)_2D_3$ (115). It should be emphasized that understanding the regulation of VDR expression may also be clinically useful in cancer treatment, as tumor response to $1,25(OH)_2D_3$ analogs may be limited by low levels of VDR expression, such as those found in advanced colon cancer.

The *VDR* gene structure is complex and various groups have studied the transcriptional mechanisms by which VDR abundance is regulated. The transcription factors Wilm's tumor suppressor, Zeb-1, Cdx-2 and Sp1 induce VDR expression (115). Recently, our group reported that the transcription factor SNAIL repressed VDR expression in human colon cancer, suggesting a role of SNAIL in the down-regulation of VDR observed in advanced colon tumors (26).

SNAIL is a zinc-finger transcription factor involved in processes that imply cell movement during embryonic development and tumor progression (116). SNAIL over-expression has been observed in colon (26, 117), as well as in gastric, melanoma, breast, hepatocellular and synovial cancers (118-122). High SNAIL expression leads to the acquisition of fibroblastic properties by epithelial tumoral cells (epithelial-to-mesenchymal transition) that result in the development of undifferentiated and invasive tumors (116). The main event of this process is the repression of the invasion suppressor E-cadherin gene by the binding of SNAIL to three E-boxes in the E-cadherin promoter (123, 124). SNAIL represses the expression of many other epithelial genes, such as occludin, several claudins, *ZO-1*, cytokeratin 18 and *MUC-1*. At the same time it induces fibronectin, vimentin and certain metalloproteinases that contribute to the mesenchymal phenotype (116, 125). Besides inducing the epithelial-to-mesenchymal transition, SNAIL decreases cell proliferation and confers resistance to apoptosis (126, 127).

We observed an inverse correlation between *VDR* and *SNAIL* RNA levels in a series of human colon cancer cell lines (unpublished data). This led us to clone and analyze the first 600 nucleotides of the human *VDR* gene promoter, searching for putative SNAIL binding sites. We found three E-boxes within this fragment of the promoter and verified the binding of SNAIL to all of them (26). Overexpression of SNAIL in human colon cancer cells repressed VDR expression and blocked the regulation of $1,25(OH)_2D_3$ target genes and the pro-differentiation effects (Figure 3). Moreover, SNAIL inhibited the antitumoral action of EB1089 in xenografted mice. The study of *SNAIL* and *VDR* levels in normal and tumoral samples from biopsies of colon cancer patients showed that tumoral overexpression of *SNAIL* correlated with the down-regulation of *VDR* (26).

SNAIL up-regulation is linked to the acquisition of invasive properties and metastatic potential by tumors and so is considered to be a marker of tumor malignancy (116). Therefore, it is probable that the down-regulation of VDR expression observed in high-grade tumors could be due to SNAIL. Our data support the need to analyze the tumor levels of VDR and/or SNAIL in order to select patients for therapy with $1,25(OH)_2D_3$ analogs. Patients with low-grade colon tumors (which may be positive for VDR expression and negative for SNAIL) are preferential candidates for this therapy (128).

The mechanisms that govern the induction of SNAIL expression in tumoral cells are beginning to be identified. The secreted proteins FGF, EGF, TGF-β and Wnt have

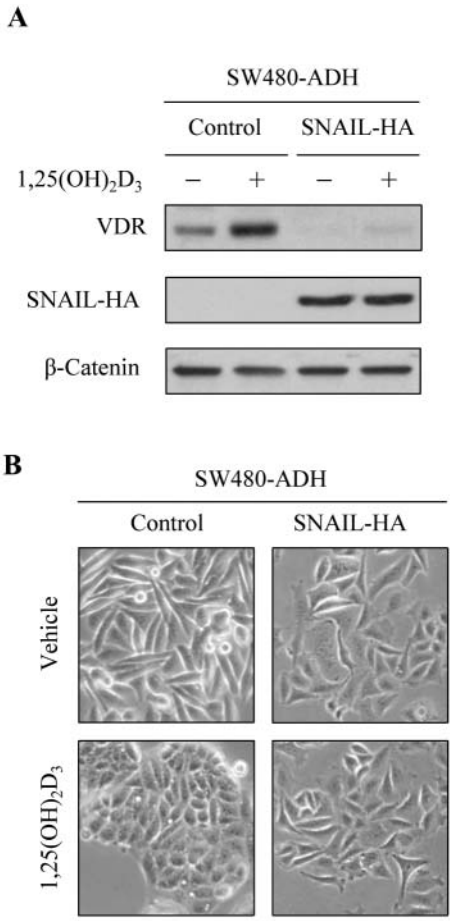

Figure 3. *SNAIL inhibits VDR expression and blocks the pro-differentiation action of $1,25(OH)_2D_3$ on human SW480-ADH colon cancer cells. (A) Western blot analysis showing the reduced expression of VDR in SW480-ADH cells expressing SNAIL (SNAIL-HA) as compared to mock-infected cells (Control). These cell lines were generated using recombinant retroviruses as reported (26). (B) Phase contrast micrographs of Control and SNAIL-HA SW480-ADH cells treated or not with 10^{-7} M $1,25(OH)_2D_3$ for 48 h, showing that SNAIL blocked the induction of an adhesive epithelial phenotype by $1,25(OH)_2D_3$.*

been established as promoters of SNAIL expression (116, 129). On the basis of our data, the induction of SNAIL by these or other mechanisms would lead to VDR down-regulation and the blockade of $1,25(OH)_2D_3$ action.

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