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Review

Vitamin D and Wnt/β-catenin Pathway in Colon Cancer: Role and Regulation of *DICKKOPF* Genes

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Abstract. *Colorectal cancer is a major health problem worldwide. Aberrant activation of the Wingless-type mouse mammary tumour virus integration site family (Wnt)/β-catenin signalling pathway due to mutation of adenomatous polyposis coli (APC), β-catenin (CTNNB1) or AXIN genes is the most common and initial alteration in sporadic colorectal tumours. Numerous epidemiological and experimental studies have indicated a protective action of vitamin D against colorectal cancer. Previous work has demonstrated that the most active vitamin D metabolite, 1α,25-dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$) inhibits β-catenin transcriptional activity by promoting vitamin D receptor (VDR) binding to β-catenin and the induction of E-cadherin expression. Recently, 1,25(OH)$_2$D$_3$ has been shown to distinctly regulate two genes encoding the extracellular Wnt inhibitors DICKKOPF-1 and DICKKOPF-4 (DKK-1, DKK-4). By an indirect transcriptional mechanism, 1,25(OH)$_2$D$_3$ increases the expression of DKK-1 RNA and protein, which acts as a tumour suppressor in human colon cancer cells harbouring endogenous mutations in the Wnt/β-catenin pathway. In contrast, 1,25(OH)$_2$D$_3$ represses DKK-4 transcription by inducing direct VDR binding to its promoter. Unexpectedly, DKK-4 is a target of the Wnt/β-catenin pathway and is up-regulated in colorectal tumours, and it has been shown to increase cell migration and invasion and to promote a proangiogenic phenotype. Together, these results show that 1,25(OH)$_2$D$_3$ exerts a complex set of regulatory actions leading to the inhibition of the Wnt/β-catenin pathway in colon cancer cells that is in line with its protective effect against this neoplasia.*

Abbreviations: 1,25(OH)$_2$D$_3$, 1α,25-Dihydroxyvitamin D$_3$; 25(OH)D$_3$, 25-hydroxyvitamin D$_3$; APC, adenomatous polyposis coli; DKK, DICKKOPF; FAP, familial adenomatous polyposis; LEF/TCF, lymphoid enhancer factor/T-cell factor; LRP, low density lipoprotein receptor-related protein; VDR, vitamin D receptor.

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Vitamin D and Colon Cancer – Brief Overview

Colorectal cancer is the second most frequent malignancy and the second leading cause of death from cancer in Europe, with 412,900 cases diagnosed and 207,400 deaths in 2006. By sex, it constitutes the second most frequent tumour in women after breast cancer and the third in men after lung and prostate tumours (1).

There is strong evidence supporting the hypothesis that vitamin D may reduce the risk of colorectal cancer (2). It is now becoming clear that adult vitamin D deficiency is endemic and epidemiological data suggest a link between UV-B exposure or vitamin D deficiency and cancer (3). Several studies have recently revealed an inverse relationship between 25-hydroxyvitamin D$_3$ (calcidiol, 25(OH)D$_3$) levels and colorectal cancer mortality (4-7), showing that the improvement of vitamin D status may reduce the risk and the incidence of cancer (8-10). The involvement of calcium in this effect is unclear.

The majority of the pleiotropic actions of 1α,25-dihydroxyvitamin D$_3$ (calcitriol, 1,25(OH)$_2$D$_3$), the most active vitamin D metabolite, are mediated by its nuclear receptor (VDR), a ligand-regulated transcription factor and a member of the nuclear receptors superfamily, that binds to specific sequences (vitamin D response elements, VDRE) in its target genes and modulates their expression (11, 12). VDR is expressed in nearly all human tissues and although initially considered to be exclusively nuclear, is now believed to shuttle constantly between the nucleus and the cytoplasm upon ligand activation.

In addition to its classical actions on the normal development and mineralization of a healthy skeleton, $1,25(OH)_2D_3$ suppresses tumour progression by restraining cell proliferation and inducing cell differentiation and apoptosis in a large variety of tumour cells, including cells of the intestine (13-16). The predominant effect of $1,25(OH)_2D_3$, be it pro-differentiative, anti-proliferative or pro-apoptotic, depends largely on the differentiation status, the VDR expression level and the cancer cell type (17). Briefly, cell-cycle arrest may result from the induction of cyclin-dependent kinase inhibitors such as $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ and the repression of cyclin D1, or direct induction of alpha growth arrest and DNA-damage-inducible α (*GADD45α*), whereas the inhibition of B-cell CLL/lymphoma 2 (*BCL2*) and the activation of BCL2-associated X (*BAX*) and BCL2-antagonist/killer (*BAK*) contribute to the apoptosis sensitization (reviewed in (12, 14)).

High VDR expression has been reported to be associated with a favourable prognosis in colorectal cancer (18, 19). However, VDR expression is lost during tumour dedifferentiation, which correlates with the up-regulation of SNAIL1, a transcriptional repressor of *VDR* (20, 21). This may help to explain the loss of responsiveness to the antitumour effects of $1,25(OH)_2D_3$ and its analogues *in vitro* and *in vivo*, and therefore be used as an indicator of patients who are unlikely to respond to this therapy (reviewed in (22)).

Many genes are regulated by $1,25(OH)_2D_3$ either directly, through VDR binding to their regulatory regions, or indirectly, *via* intermediate genes, or by affecting other pathways such as Wnt/β-catenin (see below). The blockade of β-catenin transcriptional activity and the induction of E-cadherin, a major contributor to intercellular adhesion that is lost in the adenoma to carcinoma transition, must be important for the phenotypic change of tumour cells towards a normal epithelial phenotype induced by $1,25(OH)_2D_3$ (13, 23).

The Wnt/β-catenin Signalling Pathway

The Wnts comprise a large family of highly conserved growth factors that are responsible for important developmental and homeostatic processes throughout the animal kingdom (24). Secreted Wnt proteins may bind to a plethora of potential Wnt membrane receptors which include Frizzleds, low density lipoprotein receptor-related proteins (LRPs), RYK receptor-like tyrosine kinase (RYK)/Derailed, retinoid-related orphan receptor (Ror)-2, and FRL1/Cripto, and elicit different types of intracellular responses. In the best understood Wnt/β-catenin or Wnt canonical signalling pathway, Wnt binding to Frizzled and LRP5/6 co-receptors induces β-catenin protein stabilization and entry into the nucleus where it modulates the transcription of target genes (Figure 1). In the absence of Wnt

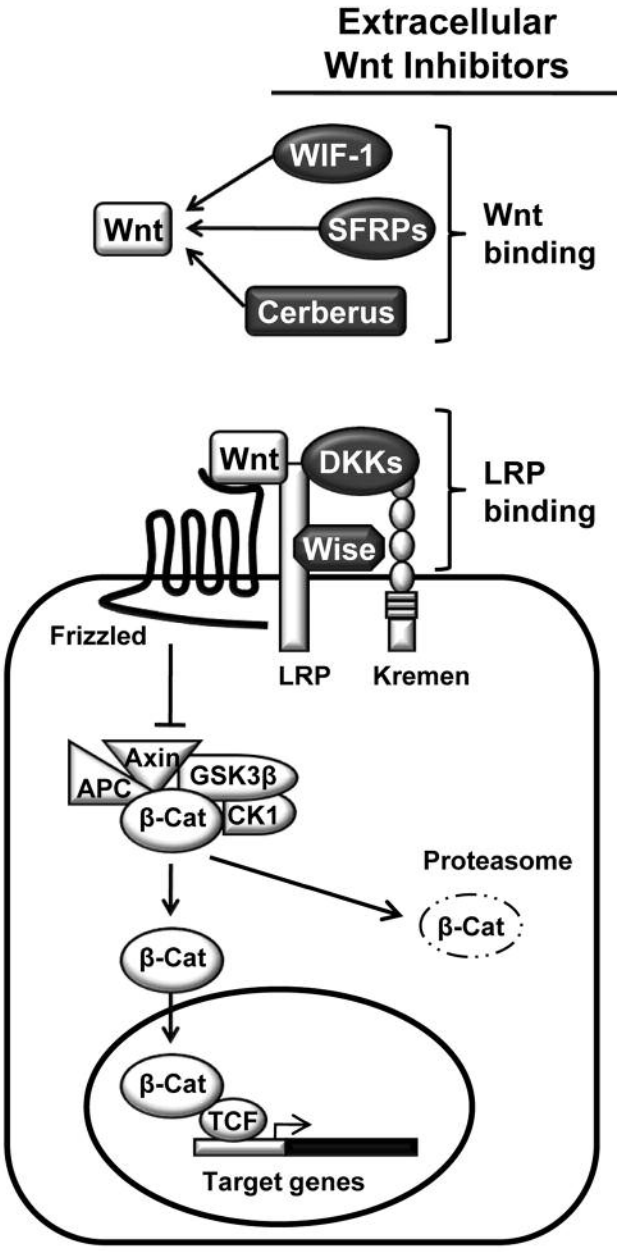

Figure 1. *Extracellular inhibitors of the Wnt/β-catenin pathway. Wnt signalling leads to stabilization of cytosolic β-catenin through the inactivation of a multiprotein complex which phosphorylates β-catenin and targets it for degradation by the proteasome. Stabilised β-catenin enters the cell nucleus and associates with LEF/TCF transcription factors, modulating the transcription of Wnt-target genes. There are two types of Wnt/β-catenin pathway extracellular inhibitors: on one hand, secreted Frizzled-related proteins (SFRPs), Wnt inhibitory factor-1 (WIF-1), and Xenopus Cerberus that bind directly to Wnt factors and block their interaction with Frizzled proteins; and on the other hand, DKK -1 and -4, and in some cases DKK-2, and Wise that bind to LRP5/6 and block Wnt signal transduction by preventing Wnt-Frizzled-LRP interaction and/or inducing LRP endocytosis in the presence of the DKK co-receptors Kremen proteins.*

ligands, free β-catenin is phosphorylated by casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β) in a destruction complex that contains the scaffolding proteins axin and APC (Figure 1). Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase β-transducin repeat containing protein (β-TrCP) and targeted for proteasomal degradation. Wnt binding to Frizzled/LRP induces the co-clustering of receptors in LRP-signalosomes, which leads to the phosphorylation of LRP by GSK3β and CK1γ (25, 26). Axin docking to the phosphorylated residues in LRP promotes the inactivation of the destruction complex and the accumulation of β-catenin. Then a population of β-catenin molecules translocates into the cell nucleus where it partners with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors to activate the transcription of target genes (Figure 1). Numerous TCF or Wnt target genes have been identified in diverse biological systems. For a comprehensive, updated overview of TCF target genes, the reader is referred to the Wnt homepage (http://www.stanford.edu/~rnusse/wntwindow.html).

Wnt reception is modulated by secreted extracellular Wnt antagonists which can be divided into two functional classes: those that bind directly to Wnts (secreted Frizzled-related proteins (SFRPs), Wnt inhibitory factor-1 (WIF-1), and *Xenopus* Cerberus), thereby altering their ability to bind to the Wnt receptors; and those that inhibit Wnt signalling by binding to LRP5/6 (Dickkopf (Dkk) proteins, and Wise) (27) (Figure 1). The *Dickkopf* family encodes secreted proteins of 255-350 aminoacids and consists of four main members in vertebrates (Dkk-1 to -4) (28). Dkk-1, the most widely studied member of this family, and Dkk-4 proteins act as pure inhibitors of Wnt/β-catenin signalling. In contrast, *Dkk-2* and Dkk-3 can activate or inhibit the pathway depending on the cellular context (28-30). The inhibitory effect of Dkks may be brought about by two mechanisms. First, Dkk binding to LRP5/6 can directly block the LRP-Wnt interaction (31). And second, Dkks can form a ternary complex with LRP5/6 and another class of high affinity Dkk receptors named Kremen (Krm1/2), which induces rapid endocytosis and removal of LRP56 from the plasma membrane, thereby presumably blocking Wnt/β-catenin signalling (32, 33).

Abnormal Wnt/β-catenin signalling is associated with many human diseases, including cancer, osteoporosis, degenerative disorders and with aging (34, 35). Mutations that strongly and constitutively activate the Wnt/β-catenin pathway are involved in the initiation and progression of several types of cancer. The best-known example of a disease involving a Wnt pathway mutation that produces tumours is familial adenomatous polyposis (FAP), an autosomal, dominantly inherited disease in which patients inherit one defective *APC* allele (36, 37) and as a consequence develop large numbers of colon adenomas, or polyps, in early adulthood. Polyps are benign, clonal outgrowth of epithelial cells in which often the second *APC* allele is later inactivated causing some of them to progress into malignant adenocarcinoma. Loss of both *APC* alleles occurs in the large majority of sporadic colorectal carcinomas (38) leading to inappropriate stabilization of β-catenin. In rare cases where *APC* is not mutated, *AXIN2* is mutant (39) or activating mutations in *CTNNB1* (β-catenin) are found (40).

Functional Interplay between $1,25(OH)_2D_3$ and the Wnt/β-catenin Pathway

Results from our group have demonstrated that $1,25(OH)_2D_3$ and several analogues can antagonize canonical Wnt signalling in human colorectal cancer cells (13). In SW480-ADH cells, $1,25(OH)_2D_3$ inhibits the transcriptional activity of β-catenin by two mechanisms. Firstly, 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 LEF/TCF target genes in the opposite way to β-catenin. This effect is independent of E-cadherin, as it takes places in LS-174T cells that lack E-cadherin expression (13). Secondly, 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, an effect that has recently been shown to be abolished *in vitro* and *in vivo* by SNAIL1 (41). The nuclear export of β-catenin is concomitant to E-cadherin protein induction. These results indicate that $1,25(OH)_2D_3$ down-regulates the Wnt/β-catenin signalling pathway, which may control the phenotype of colon epithelial cells. Upon β-catenin stabilization in colon cancer cells, due to its own mutation or that of *APC* or *AXIN*, 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, we found that nuclear β-catenin transiently potentiates VDR transcriptional activity before β-catenin moves out of the nucleus and/or VDR is extinguished (13).

Shah and colleagues have confirmed our results and showed that the effects of β-catenin on VDR activity were due to interaction between the activator function-2 (AF-2) domain of the VDR and the *C*-terminal region of β-catenin (42). Moreover, acetylation of the β-catenin *C*-terminal region differentially regulates its ability to activate LEF/TCF or VDR-regulated promoters and the mutation of a specific residue in the AF-2 domain, which renders a VDR that can bind hormone, but is transcriptionally inactive in the context of classical co-activators, still allows interaction with β-catenin and ligand-dependent activation of VDRE-containing promoters. Interestingly, VDR antagonists, which block the VDRE-directed activity of the VDR and recruitment of classical co-activators, do allow VDR to interact with β-catenin, which suggests that these and perhaps other ligands would permit those functions of the VDR that involve β-catenin interaction (42).

In the skin, the canonical Wnt pathway controls both epidermal stem cell renewal and lineage selection (43-45). Likewise, *VDR* is essential for adult epidermal homeostasis (46) and mutations in the *VDR* gene in humans result in familial $1,25(OH)_2D_3$-resistant rickets, which can be associated with alopecia (47). *In vivo*, the expression of a mutant VDR that can bind β-catenin, but not $1,25(OH)_2D_3$ rescues alopecia in Vdr null mice, demonstrating ligand-independent functions of VDR in the skin (48). Recently, two independent groups have shown that the absence of VDR impairs canonical Wnt signalling in keratinocytes and leads to the development of alopecia (49, 50). Cianferotti and colleagues found a gradual decrease in the size of the stem cell compartment in $Vdr^{-/-}$ epidermis and this correlated with a failure of β-catenin to induce proliferation (49). In contrast, Palmer *et al*. saw no evidence that VDR loss impaired the proliferative response to β-catenin (50, 51). Alternatively, they have demonstrated that β-catenin is a co-activator of VDR in epidermal keratinocytes and that a number of Wnt target genes in the skin are likely to be regulated through VDREs. For these researchers, the primary role of the VDR/β-catenin interaction in the skin is to promote the transcription of genes associated with differentiation of the hair follicle lineages. Constitutive activation of the Wnt pathway leads to ectopic hair follicle formation and, subsequently, to a type of benign tumour called trichofolliculloma. In the presence of the $1,25(OH)_2D_3$ analogue EB1089 (Seocalcitol), the differentiation of ectopic hair follicles is stimulated and trichofolliculloma development is blocked. Conversely, in the absence of VDR, differentiation of ectopic follicles is inhibited and the tumours that develop in response to β-catenin are undifferentiated basal cell carcinomas (50). Thus, vitamin D analogues may be beneficial in the treatment of tumours in which the canonical Wnt pathway is activated inappropriately. An interesting corollary to this work is that β-catenin can no longer be considered as chiefly an activator of LEF/TCF target genes. The interaction of β-catenin with other transcription factors, such as VDR, is likely to contribute to the pleiotropic effects of the Wnt pathway, which has different target genes in different cell types.

The skeleton is also a direct target of vitamin D action, which modulates the proliferation of osteoblast precursors, their differentiation into mature osteoblasts and their functional activity (52). Some of these effects of $1,25(OH)_2D_3$ are reminiscent of those orchestrated by the Wnt signalling pathway (53). Indeed, the Wnt co-receptor LRP5 is now known to play a particularly important role in bone formation such that loss of this component results in a reduction in osteoblast number, a delay in mineralization and a reduction in peak bone mineral density (54, 55). Interestingly, it has recently been reported that the *LRP5* gene is a direct transcriptional target of $1,25(OH)_2D_3$ (56) highlighting the existence of a functional interaction between the Wnt and $1,25(OH)_2D_3$ pathways in this tissue.

$1,25(OH)_2D_3$ Induces the Expression of DICKKOPF-1 Gene

Dickkopf (Dkk)-1, the founding member of the *Dkk* gene family, was originally identified as an embryonic head inducer and Wnt antagonist in *Xenopus* (57). Since then, Dkks have been identified in other vertebrates including humans as well as in invertebrates such as *Dictyostelium*, cnidarians, urochordates and ascidians (58-61), but not in *Drosophila* or *Caenorhabditis elegans*. Thus, *Dkks* are an evolutionarily ancient gene family that was already present in the last common ancestor of cnidarians and bilaterians and which was probably secondarily lost during evolution in protostomes. A distant *Dkk* family member, *soggy* (*sgy*; also called *Dickkopf-like protein 1*, *Dkkl1*), has been described in vertebrates (61) and shows unique homology to *Dkk3*.

Human *DKK-1* seems to have wide and complex effects on cell proliferation and differentiation, it induces the proliferation of human adult bone marrow stem cells (62) and inhibits osteoblastic differentiation (63) which is in line with the finding that high circulating levels of DKK-1 in patients with multiple myeloma are associated with osteolytic lesions (64). Moreover, glucocorticoids, which are associated with bone loss and osteoporosis (65), enhance *DKK-1* expression in osteoblasts (66). In contrast, *DKK-1* expression promotes preadipocyte differentiation (67), and in the mouse small intestine and colon, forced *Dkk-1* expression inhibits the proliferation of the crypt progenitor cells that is induced by the transcriptional activity of β-catenin/TCF (68, 69). Additionally, human *DKK-1* was reportedly induced by p53 (70), although in another study *DKK-1* was induced by DNA damage and sensitized to apoptosis in a p53-independent manner (71).

Thus, DKK-1 seems to have distinct effects depending on the cell type, which agrees with the different effects of Wnt/β-catenin signalling. While Wnt/β-catenin promotes proliferation and blocks differentiation in colon epithelial cells, in mesenchymal precursors it stimulates osteoblastogenesis and represses differentiation to alternative cell types, such as adipocytes (72, 73). Also, the role of Wnt signalling in melanoma cells is controversial as it probably depends upon a regulated, temporal expression pattern of Wnts. Expression at a particular time will lead a cell to tumourigenesis and invasion, while expression at other times may have the opposite effect, resulting instead in cellular apoptosis (74).

In breast cancer, the role of *DKK-1* is controversial. It has been reported that *DKK-1* is expressed in hormone-resistant breast tumours and thus it has been proposed as a new prognosis marker (75). On the other hand, Mikheev *et al*.

described tumour suppression in breast carcinoma cells mediated by *DKK-1*. Ectopic expression of DKK-1 in these cells was associated with increased phosphorylation and degradation of β-catenin and inhibition of cyclin D1 (*CCND1*) and *c-MYC* oncogenes (76). Likewise, the overexpression of *DKK-1* in hepatocellular carcinoma cell lines down-regulates *CCND1* and *c-MYC*, so inhibiting cell growth and migration during the metastatic process (77). Moreover, *DKK-1* is down-regulated by the neural (n)-*MYC* (*MYCN*) oncogene and inhibits neuroblastoma cell proliferation (78). The *DKK-1*-inducible neuroblastoma IMR32-DKK-1 cell line showed impaired proliferation upon DKK-1 expression. Surprisingly, DKK-1 expression did not inhibit the canonical Wnt/β-catenin pathway, suggesting a role of DKK-1 in an alternative route (78). Also in lung and oesophageal carcinomas, DKK-1 has been proposed as prognostic and a serological marker. Gene expression profiling of both carcinomas has revealed that *DKK-1* was highly transactivated in the majority of lung squamous cell carcinomas and serum DKK-1 levels were higher in lung and oesophageal cancer patients than in healthy controls (79). In conclusion, the antitumoural activity of DKK-1 is strictly dependent on the tissue and cancer type.

Curiously, over-expression of DKK-1 has also been associated with neuronal degeneration in the brain of Alzheimer's patients. In this case, the cascade was triggered by the β-amyloid peptide which up-regulates *TP53*. Subsequently, DKK-1 expression was enhanced and the Wnt/β-catenin pathway inhibited (80).

We and others have observed that the transcription of the *DKK-1* gene is enhanced by β-catenin/TCF acting on several sites in the promoter region (81-83). Our group reported also that *DKK-1* is down-regulated in colon cancer (82), indicating the loss of a negative feedback control of the Wnt/β-catenin pathway in this neoplasia. We also showed that *DKK-1* down-regulation occurs, at least in part, due to promoter methylation, which is specifically found in 25% of advanced, less differentiated tumours (Dukes' stages C and D) (84). Interestingly, DKK-1 seems to have antitumoural effects independently of the antagonism of β-catenin/TCF transcriptional activity in H28 and MS-1 mesothelioma, HeLa cervical, and JAR and JEG3 human placental choriocarcinoma cancer cells (23, 85, 86). Activation of the Jun N-terminal kinase (JNK) pathway is involved in some of these tumour suppressor effects (23, 85). Also in DLD-1 colon cancer cells, which bear a truncated *APC* gene and so have a constitutively active Wnt/β-catenin pathway, transfection of *DKK-1* decreases cell growth *in vitro* and tumour formation in immunodeficient mice (84). These data indicate that DKK-1 can inhibit tumourigenesis by different mechanisms. Nevertheless, further studies will be necessary to reveal whether DKK-1 may be acting in ways other than inhibiting the canonical Wnt signalling pathway or holding back the pathway in an unknown manner.

The gene expression profile associated with exposure of human SW480-ADH colon cancer cells to $1,25(OH)_2D_3$ has shown that numerous genes are modulated by this hormone, including many involved in transcription, cell adhesion, DNA synthesis, apoptosis, redox status, and intracellular signalling (23). Among them, *DKK-1* seemed to be up-regulated by $1,25(OH)_2D_3$. We have validated that $1,25(OH)_2D_3$ increases the level of *DKK-1* RNA and protein in SW480-ADH cells. This effect is slow and depends on the presence of a transcription-competent VDR (87). The regulation of *DKK-1* expression by $1,25(OH)_2D_3$ is transcriptional, but indirect. The slow kinetics of *DKK-1* RNA accumulation and the lack of VDR binding to the promoter region that is activated by the hormone, together with the absence of effect on the half-life of *DKK-1* RNA and the requirement of VDR transcriptional activity strongly suggest that $1,25(OH)_2D_3$ up-regulates the transcription of *DKK-1 via* intermediate proteins encoded by early response genes that remain uncharacterized. The induction of *DKK-1* by $1,25(OH)_2D_3$ constitutes a third mechanism by which this hormone antagonizes the Wnt/β-catenin pathway. The existence of several mechanisms of Wnt/β-catenin signalling antagonism by $1,25(OH)_2D_3$ reinforces the importance of this pathway and of its regulation for the biology of the colonic epithelium.

Another interesting finding is that *DKK-1* is up-regulated by ectopic E-cadherin in SW480-ADH cells and that a blocking antibody against E-cadherin inhibits $1,25(OH)_2D_3$-mediated *DKK-1* induction. These data strongly indicate that the regulatory effect of $1,25(OH)_2D_3$ is an indirect consequence of the induction of the epithelial adhesive phenotype (87).

The finding that *DKK-1* expression is silenced by promoter methylation in a subset of advanced, typically dedifferentiated colorectal tumours and the association of *DKK-1* with the differentiated phenotype suggest the interesting hypothesis that *DKK-1* silencing is not only concomitant with, but also plays a role in the dedifferentiation process. This may thus explain the correlation between *DKK-1* and *VDR* expression in human tumours: VDR expression has been reported to be a marker of differentiation in colon carcinoma cells (18, 88) and is lost through colon cancer progression together with that of E-cadherin in parallel to the up-regulation of *SNAIL1* (20, 21, 41).

DICKKOPF-4 Induces a Malignant Phenotype in Colon Cancer Cells and is Repressed by $1,25(OH)_2D_3$

DICKKOPF-4 (*DKK-4*) is the least studied and characterized member of the *DKK* family. This gene was first described by Krupnik and collegues in 1999 (61) but information is limited in the scientific literature. Probably,

one of the main reasons is that during adult life *DKK-4* is not expressed or its levels are very low. In fact, its pattern of expression is unclear. Although Northern blot analysis of several adult and fetal human tissues did not detect *DKK-4* RNA, a survey of a human cDNA library panel by PCR with specific primers generated products from libraries prepared from cerebellum, activated human T-lymphocytes, lung and oesophagus (61). *Dkk-4* mRNA was also detected in the pre-placodes in a murine model, being expressed at sites of presumptive epithelial-mesenchymal interactions during appendage morphogenesis including the dental lamina, mammary gland, eccrine gland, and primary and secondary hair follicles (89).

Interestingly, the expression of *DKK-4* has also recently been detected in some pathological processes such as inflammation, cancer and squizophrenia. Aung *et al*. have reported that the RNA level of *DKK-4* was increased in 11 out of 44 (25%) gastric cancer biopsies, even though they only detected DKK-4 protein in 2 out of 151 (1.3%) by immunohistochemistry (90). In another study, significantly higher expression of *DKK-1* and *DKK-4* RNA and protein was detected in the distal squamous mucosa of the oesophagus in oesophagitis patients compared to healthy controls and patients with Barrett's oesophagus. In this case, the authors suggested that those genes might play a role in the development of different injuries in response to pathological gastro-oesophageal acid reflux (91). Also, DKK-4 was the molecular marker that showed the highest expression in microarray (46.9-fold increase) and quantitative RT-PCR (138-fold increase) analyses in the endometrium of Hong Kong Chinese women with endometrial cancer (92). Moreover, *DKK-4* RNA levels were increased in patients with ulcerative colitis (93) and also with colon cancer (94, 95). Finally, Proitsi *et al*. identified single nucleotide polymorphisms (SNPs) in the *DKK-4* gene, which is located in genome regions previously linked to schizophrenia, suggesting that *DKK-4* might play a role in this disease (96).

Regarding its biological activity, DKK-4 protein has been described as an antagonist of Wnt/β-catenin signalling (33, 61) and has been shown to be transcriptionally induced by this pathway (89, 95) as is DKK-1 (81-83) (Figure 2). DKK-4 is a weaker Wnt inhibitor than DKK-1, although its effect is increased if Kremen 2 is overexpressed ((33) and our unpublished data). In apparent contradiction, DKK-4 inhibits the Wnt/β-catenin pathway and is overexpressed in several pathological diseases including some types of cancer (90-92, 94, 95). As stated above, we and others have found *DKK-4* RNA expressed in human colorectal tumours while it was undetectable in normal adjacent tissue (94, 95). This result contrasts with the common silencing of the *DKK-4* gene in colon cancer cell lines that we and others (97) have found and that may be related to cell culture conditions.

To investigate whether *DKK-4* up-regulation in human colon cancer could have functional implications for tumour progression, we expressed *DKK-4* ectopically in two human colon cell lines, SW480-ADH, which expresses low levels of the endogenous gene, and DLD-1, with undetectable expression. Exogenous *DKK-4* enhanced the migratory and invasive potential *in vitro* of both cell lines. These effects were partially inhibited by the transfection of *DKK-4* siRNA oligonucleotides. Moreover, the migration and morphogenetic capacity of primary human microvascular endothelial cells (HMVEC) were robustly increased in the presence of conditioned medium from DKK-4-expressing cells or recombinant DKK-4 protein (95). The ability to induce and sustain angiogenesis is essential for incipient neoplasias to grow, and the capability for invasion enables cancer cells to metastasise. Thus, although DKK-4 can act as a Wnt inhibitor, these findings support new roles for this protein in human colon cancer, probably inducing β-catenin-independent actions during the progression of this neoplasia.

Wnt antagonists other than *DKK-4* are also up-regulated and may contribute to tumourigenesis in different systems. For example, *SFRP4* is expressed in the stromal cells surrounding endometrial and breast carcinomas, but is barely detectable in the stroma of secretory or menstrual endometrium (98). Moreover, the expression of *SFRP1* and *SFRP2* is up-regulated in glioma-derived cell lines, and *SFRP2* promotes tumour growth in nude mice (99). Additionally, *SFRP1* induces angiogenesis in chick chorioallantoic membranes and increases migration and organization of endothelial cells into capillary-like structures (100). Strong *DKK-3* expression has been detected in tumour endothelial cells of glioma, high-grade non-Hodgkin's lymphomas, melanoma and colorectal carcinoma (101, 102), and the authors proposed that *DKK-3* might be a marker for endothelial cell activation during tumour angiogenesis. In contrast, *DKK-3* is frequently inactivated in lung cancer by promoter hypermethylation (29). In conclusion, up-regulation of *DKK-4* and several Wnt inhibitors in some cancer cell types imply their involvement in roles other than the control of this signalling pathway.

Unlike *DKK-1*, the available data indicate that *DKK-4* is a target gene induced by Wnt/β-catenin that remains up-regulated in colon tumours. The pro-tumourigenic actions of *DKK-4* in cultured cells suggest that they overcome its weak inhibitory effect on the Wnt/β-catenin pathway (Figure 2). The discrepancy between the regulation of *DKK-4* in colon and breast tumours, in which variable levels but no up-regulation was found in a first study (95), reveal tissue-specific actions and promotes interest in extending the study of DKK-4 effects to other types of carcinomas. Notably, $1,25(OH)_2D_3$ inhibits *DKK-4* expression in colorectal cancer cells and diminishes transcription from the *DKK-4* promoter

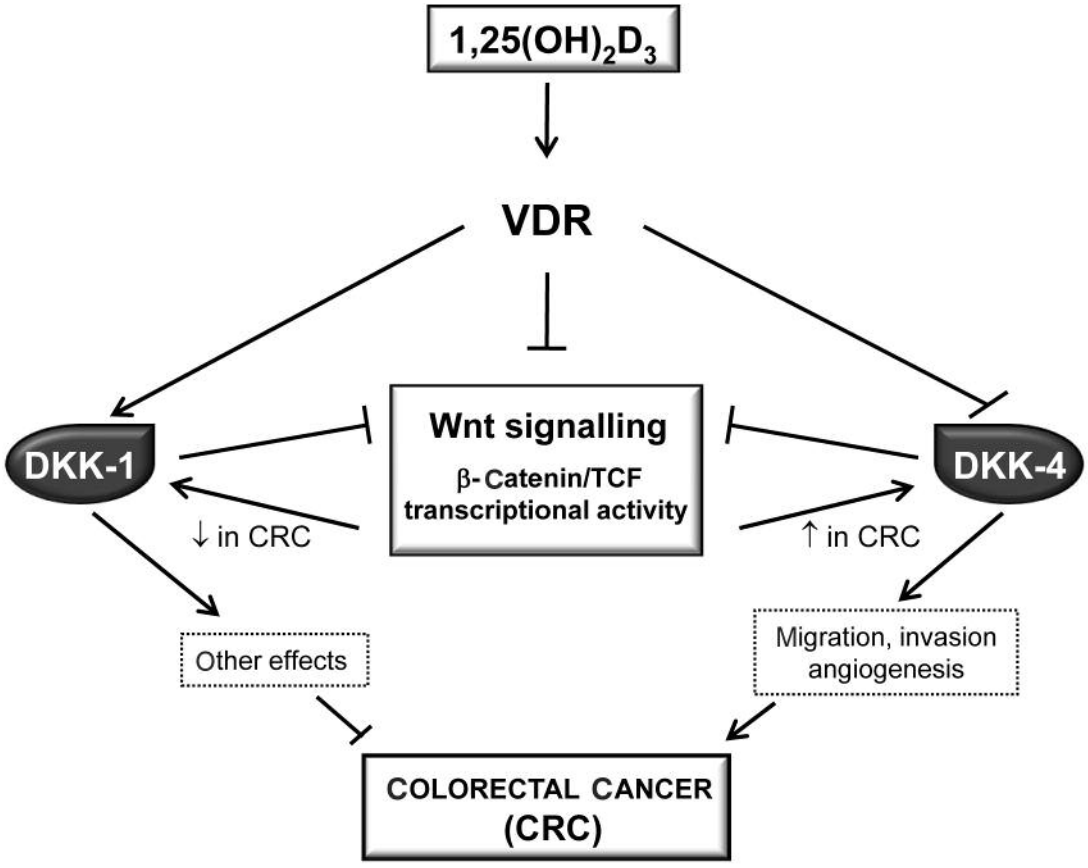

Figure 2. *Multifaceted effects of 1,25(OH)$_2$D$_3$ on the Wnt/β-catenin pathway in colorectal cancer through the induction of direct β-catenin–VDR interaction and the opposite modulation of DKK-1 and DKK-4 genes. Both DKK genes are transcriptionally induced by Wnt/β-catenin signalling, and the corresponding encoded proteins are secreted and inhibit this pathway. However, DKK-1 is down-regulated in colon cancer while DKK-4 is overexpressed. Additionally, DKK-1 has other anticancer effects still to be characterized. In contrast, DKK-4 potentiates the migratory, invasive and pro-angiogenic phenotype of tumour cells.*

in SW480-ADH and in three human breast cancer cell lines (MCF-7, MDA-MB-468, MDA-MB-453) (95). The repression appears to be direct as, again in contrast to DKK-1, 1,25(OH)$_2$D$_3$ promotes the binding of VDR and also of the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) co-repressor to a consensus sequence adjacent to the transcription initiation site and the abrogation of histone H4 acetylation. Interestingly, the inverse correlation found between *VDR* and *DKK-4* RNA levels in human colorectal tumours suggests that the regulation of DKK-4 observed in cell lines also occurs in patients.

The regulation and expression of *DKK-1* and *DKK-4* occur in opposite directions in human colorectal cancer. While the induction of *DKK-1* by 1,25(OH)$_2$D$_3$ is slow and requires intermediate proteins (87), 1,25(OH)$_2$D$_3$ represses *DKK-4* rapidly promoting direct VDR binding to the gene promoter region (95). Both, DKK-1 and DKK-4 proteins putatively have β-catenin-independent activities that, however, must differ markedly. While DKK-1 has anti-tumoural effects (84), the effects of DKK-4 on the phenotype of colon cancer cells and its up-regulation in colon cancer indicate tumour-promoting actions (95). The induction of *DKK-1* and the repression of *DKK-4* by 1,25(OH)$_2$D$_3$ agrees with and may contribute to its protective effects against this neoplasia. The elucidation of the roles of DKK-1 and DKK-4 proteins in colon cancer cells may be important for understanding the biology of colon cancer and 1,25(OH)$_2$D$_3$ action.

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