Vitamin D and Wnt/β-Catenin Signaling

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Running title: Tissue-dependent Vitamin D-Wnt/β-Catenin Interplay
Summary

Wnt factors regulate tissue formation during development and tissue homeostasis in adult life. Upon binding to plasma membrane receptors, Wnt factors trigger Wnt/β-catenin (canonical) signaling pathway or several other β-catenin-independent (non-canonical) pathways. Activation of Wnt/β-catenin signaling leads to nuclear accumulation of β-catenin which acts as a transcriptional coactivator of T-cell factor/Lymphoid enhancer factor (TCF/LEF) target genes involved in numerous cellular processes. Aberrant activation of Wnt/β-catenin signaling occurs in several human cancers and other diseases. The most active vitamin D₃ metabolite 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and its receptor (VDR) regulate Wnt/β-catenin signaling in a tissue-specific manner. 1,25(OH)₂D₃/VDR inhibits β-catenin/TCF transcriptional activity in colon cancer cells, while upregulation of the Wnt/β-catenin pathway by either ligand-activated or unliganded VDR promotes bone formation or hair follicle differentiation, respectively. This crosstalk with Wnt/β-catenin signaling may contribute to the protective action of 1,25(OH)₂D₃ against colon cancer and to its effects on tissue homeostasis.

Key Words: Vitamin D, Vitamin D Receptor, Wnt, β-Catenin, Wnt inhibitors, Dickkopf, Colon Cancer, Bone, Skin
I. INTRODUCTION

The most active vitamin D₃ metabolite 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃, calcitriol), is a pleiotropic hormone with cell type-dependent effects on cell proliferation, survival, differentiation and function. 1,25(OH)₂D₃ exerts its actions via binding and modulation of the vitamin D receptor (VDR), a member of the nuclear receptor superfamily of transcriptional regulators. 1,25(OH)₂D₃ triggers a complex network of gene regulatory (genomic) effects. It also modulates in a transcription-independent manner the activity of several types of signaling enzymes and ion channels (non-genomic effects). In addition, 1,25(OH)₂D₃/VDR regulates cell phenotype and function through crosstalk with other signal transduction pathways. Research in the last decade has revealed that an important mechanism of 1,25(OH)₂D₃/VDR action in certain tissues is related to its interaction with the Wnt/β-catenin pathway. In this chapter, we will review the evidence and comment on the significance of this interplay, which highlights the biological relevance of 1,25(OH)₂D₃ and opens new possibilities for the therapeutic use of vitamin D and its analogues.

II. WNT SIGNALING

Wnt factors are a family of highly conserved secreted glycoproteins that regulate cell proliferation, differentiation, polarity, movement, and survival during development and in adult tissue homeostasis [1-3]. The 19 human Wnts are cystein-rich molecules with 350-400 aminoacids and highly related structures [4]. In addition to bearing an N-terminal signal peptide, Wnts undergo N-glycosylation which is required for secretion. They are also modified by the addition of two lipids, palmitic and palmitoleic acids, which may accounts for the hydrophobicity and poor solubility of Wnt proteins, and thus explain their low range distribution and predominantly autocrine-paracrine activity [5, 6]. Lipid modification is also required for the signaling activity of the secreted protein [7].
Several membrane receptors and coreceptors for Wnt proteins have been described: Frizzled family members (10 in humans), low-density lipoprotein receptor-related proteins (LRP) 5 and 6 (Arrow, in Drosophila), and RYK (Derailed, in Drosophila) and ROR2 families of single-pass transmembrane receptor tyrosine kinases. Upon binding to their receptors, Wnts trigger different signaling pathways: the so-called canonical or Wnt/β-catenin pathway and the β-catenin-independent, non-canonical pathways [3].

A. The Wnt/β-Catenin Pathway

In normal epithelial cells, β-catenin protein is bound to the intracellular domain of the transmembrane E-cadherin protein in the intercellular adhesion structures adherens junctions. β-Catenin links E-cadherin to the actin cytoskeleton through its simultaneous binding to α-catenin, which is directly and indirectly bound to actin filaments. In the absence of Wnt factors β-catenin is mostly found at adherens junctions (Figure 1A). Free β-catenin is phosphorylated by casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β) in a so-called destruction complex that also contains the scaffolding proteins Axin and APC. Phosphorylated β-catenin is ubiquitinated by the E3 ubiquitin ligase β-transducin repeat-containing protein (β-TrCP), and thus targeted for degradation by the proteasome [3] (Figure 1A). Wnt binding to Frizzled and the subsequent formation of a ternary complex with LRP5/6 coreceptor induces β-catenin protein stabilization (Figure 1B). Wnt binding to Frizzled/LRP triggers co-clustering of receptor complexes in signaling structures named LRP-signalosomes, which leads to phosphorylation of LRP by GSK3β and CK1γ located in the vicinity of the plasma membrane [8, 9]. Axin docking to the phosphorylated residues in LRP promotes the inactivation of the destruction complex and the accumulation of β-catenin (Figure 1B). Then, a population of β-catenin molecules translocate into the cell nucleus, where they join members of the T-cell factor/Lymphoid enhancer factor (TCF/LEF: TCF1,
TCF2/LEF1, TCF3 and TCF4) family of transcription factors (Figure 1B). TCF/LEFs bind to specific DNA sequences referred to as Wnt responsive elements (WRE: CCTTTGA/TA/T). In the absence of Wnt signals, TCF/LEF proteins are mostly repressors although in some cases they may activate transcription of their target genes. TCF/LEFs repress gene expression by interacting with corepressors such as Groucho/TLE1, which promotes histone deacetylation and chromatin compaction (Figure 1A). Binding of β-catenin to TCF/LEFs displace corepressors [10] and recruits a plethora of coactivators which trigger transcriptional activation of TCF/LEF-bound genes that were previously repressed (Figure 1B). β-Catenin-associated coactivators include BCL9 and Pygo, Mediator complex, p300/CBP and TRRAP/TIP60 histone acetyltransferases (HATs), MLL1/2 histone methyltransferases (HMTs), the SWI/SNF family of ATPases for chromatin remodelling, telomerase, and the PAF1 complex for transcription elongation and histone modification [11-13]. Intriguingly, recent evidence suggests that during Wnt-induced transcription β-catenin and its coactivators cycle on and off the WRE with a 60 min periodicity and are replaced by Groucho/TLE1 [3, 14, 15]. Recently also, APC has been found to antagonize β-catenin/TCF-dependent transcription by promoting the exchange of coactivators with corepressors within the nucleus in a stepwise and oscillating manner [14]. Thus, both coactivators and corepressors appear to be active during β-catenin-mediated transcription.

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Numerous β-catenin/TCF or Wnt target genes have been identified in diverse biological systems and they are mostly cell- and context-specific [16]. Products of Wnt target genes have a large variety of biochemical functions including cell cycle kinase regulation, cell adhesion, hormone signaling, and transcription regulation. The plurality and diversity of these biochemical functions reflect the variety of different biological effects of the Wnt pathway, including activation of cell cycle progression and proliferation, inhibition of
apoptosis, and regulation of embryonic development and cell differentiation, growth and migration [16]. For a comprehensive, updated overview of β-catenin/TCF target genes, we recommend Roel Nusse’s Wnt homepage (http://www.stanford.edu/~rnusse/wntwindow.html).

Deregulation or abnormal activation in adult life of the Wnt/β-catenin signaling pathway contributes to the emergence and progression of several types of human cancer. This is not surprising as the Wnt1 gene (Int1 was its first name) was discovered in mouse mammary carcinomas as a target of insertional activation by the mouse mammary tumor virus [17]. Cancer cells with mutationally activated Wnt pathway overexpress at least 20 target genes that activate proliferation including proto-oncogenes c-MYC, c-JUN, and CCND1/cyclin D1 [16].

B. Non-Canonical Wnt Pathways

Non-canonical Wnt pathways are very diverse and are still evolving into an increasing number of branches [18]. Among them, Wnt/planar cell polarity (PCP) and Wnt/calcium pathways are relatively better characterized.

The Wnt/PCP pathway was described in Drosophila melanogaster. It regulates the polarity of epithelial cells within the plane of the epithelium, e.g. orienting Drosophila wing hairs or regulating the organization of the ommatidia in the fly eye [19]. In vertebrates, a similar pathway has been described regulating convergent extension movements during gastrulation or neurulation and migration of neural crest cells [20-22]. The core element of the Wnt/PCP pathway includes the activation of Rho GTPases such as RhoA, Rac, or Cdc42 that can activate more downstream mediators like Jun N-terminal kinase (JNK) or Rho kinase (ROCK). Our understanding of PCP signaling has increased dramatically in the past years and its role in diseases such as cancer is an area of intense research (reviewed in [23]).
Some Wnts (e.g. Wnt5a or Wnt11) can induce a rapid release of intracellular calcium that depends on heterotrimeric G proteins [24] and the activation of the phosphatidylinositol cycle [25]. This increase in calcium concentration activates different intracellular calcium-sensitive enzymes such as protein kinase C [26, 27], calcium-calmodulin-dependent kinase II [28], and the phosphatase calcineurin, which subsequently activates the transcription factor NFAT [29, 30]. This pathway is involved in dorsoventral patterning of early *Xenopus laevis* and zebrafish embryos [28, 29, 31], regulation of the Wnt/β-catenin pathway [32], tumor formation [33-35], and the regulation of epithelial-mesenchymal transitions [36].

### C. Wnt Inhibitors

In line with its crucial roles in essential processes during development and adult life, Wnt action is antagonized or modulated by numerous molecules that act at different locations and by different mechanisms. Extracellular inhibitors are secreted molecules that either bind Wnt factors in solution, thus preventing their interaction with the plasma membrane receptors or inhibit Wnt signaling by binding to LRP5/6 (Figure 2). The first group comprises secreted Frizzled receptor-related proteins (SFRPs), Wnt inhibitory factor-1 (WIF-1), Crescent and *Xenopus* Cerberus [37]. Wnt inhibitors that bind LRP5/6 include the Dickkopf (Dkk) and WISE/SOST families [38-41] (Figure 2). In addition, an increasing number of intracellular inhibitors of Wnt signaling are known. Some of them function in the cytosol such as Naked or Axin2/Conductin [42-44], while others such as Chibby or ICAT block β-catenin action within the cell nucleus either by direct binding or by binding to β-catenin partners and the promotion of β-catenin nuclear export [45, 46].

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The *Dickkopf* gene family encodes secreted proteins of 255-350 aminoacids and comprises four main members in vertebrates (*Dkk-1* to *-4*) [41]. A distant *Dkk* family
member, Dkk-Lsgy (Dickkopf-like protein 1 or soggy), has been described in vertebrates [47] and shows unique homology to Dkk-3. Dkk-1 and Dkk-4 proteins act as pure inhibitors of Wnt/β-catenin signaling. In contrast, Dkk-2 and Dkk-3 can activate or inhibit the pathway depending on the cellular context [41, 48, 49]. The inhibitory effect of Dkks may be induced by two mechanisms. First, Dkk binding to LRP5/6 can directly block the Wnt-Frizzled-LRP interaction [50]. 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 LRP5/6 from the plasma membrane, thereby blocking Wnt/β-catenin signaling [51, 52]. Recent biochemical and genetic studies suggest that Dkk-1 disruption of the Wnt-induced Frizzled-LRP6 complex is a more likely mechanism [53], with Kremen playing a minor modulatory role in specific tissues [54].

III. ANTAGONISM OF WNT/β-CATENIN PATHWAY BY 1,25(OH)₂D₃ IN COLON CANCER

A. Wnt Signaling in Normal Colon and Colon Cancer

Colon is lined with a specialized simple epithelium organized into crypts (deep invaginations) and a flat surface epithelium. The bottom half of the crypts host highly proliferative progenitor cells named transit-amplifying cells which give rise to two different cell lineages: the absorptive enterocytes and the secretory cells (mucus-secreting goblet cells and hormone-secreting enteroendocrine cells). Maturation of progenitor cells coincides with upward migration. Upon reaching the surface epithelium, the differentiated cells undergo apoptosis and are shed into the lumen. The self-renewing capacity of the colon depends on the presence of stem cells at the crypt bottom [55, 56].

The Wnt/β-catenin pathway is the main driving force behind the proliferation of epithelial cells in the colonic crypts and functional studies have confirmed that this pathway
constitutes the master switch between proliferation and differentiation of the epithelial cells [57, 58]. Thus, active Wnt signaling is essential for the maintenance of crypt progenitor compartments in the intestine. This is evidenced by mice lacking the Tcf4 transcription factor [59], by the conditional depletion of β-catenin from the intestinal epithelium [60, 61], and by transgenic inhibition of extracellular Wnt signaling through Dkk-1 [62, 63]. In all cases, a dramatic reduction of proliferative activity was observed. In the converse experiment, activating the Wnt pathway through transgenic expression of the Wnt agonist R-Spondin-1 resulted in a massive hyperproliferation of intestinal crypts [64].

Although Wnt signaling is essential to the normal physiology of the intestine, it was first characterized by its association with colorectal cancer, one of the most common cancers in industrialized countries. Fearon and Vogelstein [65] have proposed that colorectal cancer results from an ordered sequence of mutations in what is called the suppressor pathway. Invariably, the initiating mutation occurs in a gene (APC, CTNNB1/β-catenin, or AXIN2) that encodes for a protein involved in the Wnt/β-catenin pathway. Loss of the tumor suppressor APC is the signature of the great majority of human intestinal tumors, both in the hereditary familial adenomatous polyposis and in sporadic colorectal cancers [66-68]. In the rare cases where APC is not inactivated, human colon tumors arise from activating mutations in CTNNB1/β-catenin itself [69, 70], or from loss-of-function mutations in AXIN2 [71]. As a common result, β-catenin accumulates in the nucleus, constitutively binds to TCF/LEF transcription factors and induces the expression of targets genes mainly involved in cell proliferation, leading to the formation of benign yet long-lived adenomas. Subsequently, other mutations follow (e.g. in K-RAS, SMAD4, or TP53…), ultimately resulting in metastasizing carcinomas.

B. 1,25(OH)_{2}D_{3} Inhibits the Transcriptional Activity of β-Catenin/TCF Complexes
Results from our group have demonstrated that 1,25(OH)$_2$D$_3$ and several analogues antagonize the Wnt/β-catenin signaling pathway in human colorectal cancer cells. 1,25(OH)$_2$D$_3$ inhibits the transcriptional activity of β-catenin by two mechanisms. First, it rapidly increases the amount of VDR bound to β-catenin, thus reducing the interaction between β-catenin and TCF4 [72] (Figure 3A). In this way, 1,25(OH)$_2$D$_3$ modulates TCF/LEF target genes in the opposite way to β-catenin: those induced by β-catenin/TCF4 such as c-MYC, TCF1, PPAR-δ or CD44 are repressed by 1,25(OH)$_2$D$_3$, while zonula occludens (ZO)-1, which is inhibited by β-catenin/TCF4 becomes activated by 1,25(OH)$_2$D$_3$ [72]. Second, 1,25(OH)$_2$D$_3$ induces β-catenin nuclear export and relocation to the plasma membrane adherens junctions. As β-catenin is no longer in the nucleus, transcription of TCF/LEF target genes is halted (Figure 3B). This happens concomitantly to an increase in E-cadherin protein expression. 1,25(OH)$_2$D$_3$ induction of E-cadherin in SW480-ADH human colon cancer cells was observed soon after treatment and peaked at 48-72 h, and correlated with a change to a more differentiated phenotype [72]. Thus, 1,25(OH)$_2$D$_3$ may cause a reduction in nuclear β-catenin concentration by promoting its sequestration at the adherens junctions bound to the newly synthesized E-cadherin (Figure 3B). Alternatively, 1,25(OH)$_2$D$_3$ might stimulate β-catenin nuclear export through yet unknown mechanisms. Although APC has been proposed as a contributor to β-catenin nuclear export [73], the relocation of β-catenin takes place in SW480-ADH cells that express a truncated APC protein.

We have shown that transient activation of the RhoA small GTPase is necessary for the induction by 1,25(OH)$_2$D$_3$ of the expression of CDH1/E-cadherin and many other genes in several cell types [74]. Interestingly, RhoA activity is also required for the promotion of β-catenin nuclear export and for the inhibition of β-catenin/TCF4 transcriptional activity and cell proliferation [74], suggesting a relationship between E-cadherin expression and nuclear
export. Moreover, it is worth noticing that although the export of β-catenin out of the nucleus contributes to the inhibition of β-catenin/TCF4-mediated transcriptional activity in some cell lines, the global antagonism of 1,25(OH)_2D₃ on this signaling pathway must be independent of E-cadherin induction, as it takes places in LS-174T colon cancer cells that lack E-cadherin expression [72].

In addition to 1,25(OH)_2D₃, the vitamin D analogues EB1089, MC903 and KH1060 [72] and the superagonistic fluorinated CD578, WU515 and WY113 compounds [75] also inhibit β-catenin/TCF transcriptional activity in a strictly VDR-dependent fashion, as inhibition does not occur in SW480-R or SW620 cells that lack VDR expression. Moreover, the transcription factors SNAIL1 and SNAIL2 (also called SLUG), which repress VDR expression [76, 77], abolish this effect in vitro and in vivo [77, 78].

These results indicate that 1,25(OH)_2D₃ downregulates the Wnt/β-catenin signaling pathway, and may thus 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 AXIN2, 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 linked to SNAIL1/SNAIL2 upregulation. 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 [72].

Shah and cols. [79] confirmed and extended the finding of VDR/β-catenin interaction. These authors have characterized the interacting domains in VDR and β-catenin: the activator function-2 (AF-2) domain of VDR and the C-terminal region of β-catenin. Moreover, they showed that acetylation of β-catenin C-terminal region differentially regulates its ability to activate TCF/LEF or VDR-regulated promoters. 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 coactivators, still allows interaction with β-catenin and ligand-dependent activation of VDRE-containing promoters. Interestingly, VDR antagonists, which block the recruitment by VDR of classical coactivators, do allow VDR to interact with β-catenin, suggesting that some ligands would permit those functions of VDR that involve β-catenin interaction [79]. In addition to human colon cancer cells, the inhibition of β-catenin/TCF transcriptional activity by 1,25(OH)2D3 or its analogues QW and BTW has been found in rat Rama 37 mammary cells [80]. In these cells different β-catenin transcriptional complexes distinctly modulate the activation of the OPN/Osteopontin gene promoter by ligand-activated VDR: β-catenin/LEF1 enhances the activation while β-catenin/TCF4 diminishes it. Recently, Egan and cols. [81] have reported that the inhibition of the transcriptional activity of β-catenin/TCF complexes by 1,25(OH)2D3 in colon cancer cells is enhanced by wild-type APC. In addition, these authors have shown that the VDR ligand lithocolic acid also inhibits β-catenin transcriptional activity but to a lesser extent than 1,25(OH)2D3 and concordantly it is also less effective than 1,25(OH)2D3 in promoting VDR binding to β-catenin.

Nuclear hormone receptors other than VDR have also been reported to regulate β-catenin transcriptional activity in several types of cells and tissues, reflecting a complex crosstalk between hormonal systems and Wnt signaling [82, 83].

Notably, a novel mechanism of Wnt/β-catenin signaling pathway inhibition by 1,25(OH)2D3 has recently been reported by Kaler and cols. [84]. These authors have shown that THP-1 macrophages activate the Wnt/β-catenin signaling pathway in HCT116 and Hke-3 colon carcinoma cells via the STAT-1-mediated production and secretion of interleukin (IL)-1β, which blocks GSK3β activity and hence increases β-catenin/TCF transcriptional activity and proliferation of carcinoma cells (Figure 3D). This mechanism, which might contribute to the tumorigenic effect of tumor-associated macrophages in vivo, is repressed by
1,25(OH)₂D₃ through the inhibition of the constitutive activation of STAT-1 and the production of IL-1β in macrophages [84] (Figure 3D).

C. 1,25(OH)₂D₃ Regulates the Wnt Inhibitor DICKKOPF-1

We have reported that 1,25(OH)₂D₃ increases the level of DKK-1 RNA and protein in SW480-ADH human colon cancer cells and this effect depends on the presence of a transcription-competent VDR [85]. 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)₂D₃ upregulates the transcription of DKK-1 via intermediate proteins encoded by early 1,25(OH)₂D₃ target genes that remain uncharacterized. In addition, DKK-1 is upregulated by ectopic E-cadherin in SW480-ADH cells, and a blocking antibody against E-cadherin inhibits 1,25(OH)₂D₃-mediated DKK-1 induction. These data indicate that the regulatory effect of 1,25(OH)₂D₃ is an indirect consequence of the induction of E-cadherin and the epithelial adhesive phenotype [85]. The induction of DKK-1 by 1,25(OH)₂D₃ constitutes yet another mechanism by which this hormone antagonizes the Wnt/β-catenin pathway (Figure 3C). The existence of several mechanisms of Wnt/β-catenin signaling antagonism by 1,25(OH)₂D₃ reinforces its importance for the biology and the maintenance of the normal status of the colonic epithelium.

Since most colorectal cancer cells have mutations in APC that render an active Wnt/β-catenin pathway, the relevance of DKK-1 induction by 1,25(OH)₂D₃ is uncertain. Interestingly, DKK-1 seems to have antitumoral 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 [86-88]. In line with this, we
have shown that 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 tumor formation in immunodeficient mice [89]. Activation of the Jun N-terminal kinase (JNK) pathway is involved in some of these tumor suppressor effects [86, 88]. Thus, DKK-1 may control signaling cascades independently of LRP5/6 and β-catenin [41, 90]. Additionally, LRP5/6 may have β-catenin-independent effects under the control of DKK-1 [91]. These data indicate that DKK-1 can inhibit tumorigenesis by different mechanisms and that its induction might be of unforeseen importance for the anticancer action of 1,25(OH)$_2$D$_3$.

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We and others have observed that, in addition to 1,25(OH)$_2$D$_3$, the transcription of the DKK-1 gene is enhanced by β-catenin/TCF itself acting on several sites in the promoter region [92-94]. Our group also reported the downregulation of DKK-1 in human colon cancer [93] indicating the loss of this negative feedback control of the Wnt/β-catenin pathway in this neoplasia. Reduced DKK-1 expression is due, at least in part, to promoter methylation, which is specifically found in 25% of advanced, less differentiated tumors (Dukes’ stages C and D) [89]. Thus, the induction of DKK-1 expression by 1,25(OH)$_2$D$_3$ may restore DKK-1 antitumoral effects in those colon tumors in which DKK-1 downregulation is not due to promoter methylation. The finding that DKK-1 expression is silenced in dedifferentiated colorectal tumors and the association of DKK-1 expression with the differentiated phenotype suggests that DKK-1 accumulation is not only concomitant with, but also plays an active role in the differentiation process. Accordingly, we have also demonstrated a significant correlation between the expression of VDR and DKK-1 in human colon cancer [85]. VDR is considered a marker of differentiation in this neoplasia [95, 96] and its expression is lost during colon cancer progression together with that of E-cadherin, and presumably in parallel.
to the upregulation of the transcription factors SNAIL1 and SNAIL2 that repress both genes [76, 77, 97].

D. 1,25(OH)2D3 Represses DICKKOPF-4 and Induces TCF4 in Colon Cancer Cells

DKK-4 protein has been described as an antagonist of Wnt/β-catenin signaling [47, 52] and, like DKK-1, it has also been shown to be transcriptionally induced by this pathway [98, 99]. DKK-4 is a weaker Wnt inhibitor than DKK-1, although its effect is increased if Kremen 2 is overexpressed ([52] and our unpublished data). In apparent contradiction, DKK-4 inhibits the Wnt/β-catenin pathway but is overexpressed in several pathological diseases including some types of cancer, inflammation and schizophrenia [99-103]. We and others have found DKK-4 RNA expression in human colorectal tumors but not in adjacent normal tissue [99, 102, 104]. Moreover, DKK-4 RNA levels are already increased in patients with inflammatory bowel disease [105, 106]. These results contrast with the common silencing of the DKK-4 gene in colon cancer cell lines that we and others [107] have found and that may be related to cell culture conditions. They also contrast with results from Baehs and cols. [108] who have reported DKK-4 downregulation in colorectal cancer.

Notably, 1,25(OH)2D3 inhibits DKK-4 expression in human colorectal (SW480-ADH, Caco-2) and breast (MCF-7, MDA-MB-468, MDA-MB-453) cancer cell lines [81, 99]. The mechanism of DKK-4 repression is unclear. While in SW480-ADH cells a direct transcriptional repression mediated by VDR binding to the promoter is found [99], in Caco-2 cells a mutant VDR deficient in DNA binding mediates similar repression of DKK-4 to wild-type VDR [81]. In SW480-ADH cells, 1,25(OH)2D3 promotes the binding of VDR and also of the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) corepressor to a consensus sequence adjacent to the transcription initiation site and the abrogation of histone H4 acetylation. Interestingly, our group showed an inverse correlation between VDR
and DKK-4 RNA levels in human colorectal tumors which suggests that the regulation of DKK-4 observed in cell lines also occurs in patients [99].

In order to characterize DKK-4 effects in colon cancer and thus the importance of its regulation by 1,25(OH)₂D₃, we ectopically expressed DKK-4 in two human colon cancer cell lines: SW480-ADH, which expresses low levels of the endogenous gene, and DLD-1, in which its expression is not detected. Exogenous DKK-4 protein enhanced the migratory and invasive potential in vitro of both cell lines. 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 [99]. Thus, DKK-4 enhances the capacity of colon cancer cells to invade and to induce sustained angiogenesis, both essential for incipient neoplasias to grow and metastasise. These findings suggest that DKK-4 inhibition by 1,25(OH)₂D₃ could explain some of the antitumoral effects of the hormone in colon cancer.

Although DKK-4 can act as a Wnt inhibitor, these data support new roles for this protein in human colon cancer, probably inducing β-catenin-independent actions during the progression of this neoplasia. In addition, they suggest that the tumorigenic actions of DKK-4 could overcome its weak inhibitory effect on the Wnt/β-catenin pathway [99]. The effects may be dose related: small amounts of DKK-4 may predominantly be inhibitory for Wnt signaling while higher levels may promote cell malignancy. Accordingly, Wnt antagonists other than DKK-4 are also upregulated and may contribute to tumorigenesis in different systems [109-111]. Thus, upregulation of DKK-4 and other Wnt inhibitors in some cancer cell types implicate them in roles other than the control of this signaling pathway.

Byers and cols. [112] have recently reported a decreased expression of Tcf4 (product of the TCF7L2 gene in humans) in the mammary gland of Vdr null mice. In addition, these authors found that 1,25(OH)₂D₃ increases TCF4 RNA and protein levels in several human
colon cancer cell lines by an indirect mechanism that requires *de novo* protein synthesis and is completely dependent on VDR. This induction is unique to TCF4, as other TCF/LEF family members are not upregulated. Although it is generally assumed that binding of β-catenin to members of the TCF/LEF family is cancer-promoting, recent studies have indicated that TCF4 functions instead as a transcriptional repressor with growth inhibitory activity. Thus, RNAi-mediated disruption of TCF4 expression facilitates β-catenin activity and cell growth in both DLD-1 cells (APC mutation) and HCT116 cells (activating *CTNNB1*/β-catenin mutation) [113]. Also, recent data show that TCF4 expression is lost in human breast cancers but abundant in the surrounding normal tissue, indicating that *TCF4* might be a tumor suppressor in this tissue [114]. Consequently, it is possible that the 1,25(OH)$_2$D$_3$/VDR-mediated increase in TCF4 may have a protective role in colon and breast cancer.

**IV. WNT AND 1,25(OH)$_2$D$_3$ IN THE BONE**

**A. Wnt Signaling and Bone Homeostasis**

The observation that Wnt signaling is critical in bone biology has been a major development in the area over the past few years. Bone marrow-derived mesenchymal stem cells (BMSCs) can potentially differentiate into adipocytes, chondrocytes, or osteoblasts. Although the precise orchestration of Wnt signaling during bone development is dependent on complex microenvironmental cues, data from several groups suggest that Wnt signaling is central to osteoblastogenesis while it represses differentiation of BMSCs to alternative cell types, such as adipocytes [115, 116]. A number of different Wnt proteins play a role in bone formation. *Wnt10b*−/− mice have decreased trabecular bone and serum osteocalcin [117], while transgenic mice that express Wnt10b in bone marrow show increased bone mass and strength [117]. The expression of Wnt10b in mesenchymal progenitors induced the expression of Runx2 and
Osterix, two transcription factors associated with osteoblast differentiation, and stimulated osteoblastogenesis [117]. Likewise, Wnt3a+/− and Wnt5a+/− mice showed a decrease in bone mass [118] which associates with a reduced number of osteoblasts. Wnt5a does not activate Wnt/β-catenin signaling but a non-canonical pathway, and has been shown to induce osteoblastogenesis by inactivation of peroxisome proliferator-activated receptor-γ (PPARγ), a key adipogenic transcription factor, and activation of Runx2 [118]. Thus both canonical and non-canonical Wnt signaling pathways play a role in osteoblast differentiation and bone formation.

The involvement of Wnt/β-catenin signaling in the control of bone biology is further supported by findings related to the LRP5 gene. Loss-of-function mutations in this gene were associated with low bone mass in the osteoporosis pseudoglioma syndrome (OPPG, [119]), whereas a single amino-acid substitution (G171V) in the same gene was associated with a high bone mass state in two kindreds [120, 121]. This mutation inhibits the ability of DKK-1 and potentially other proteins to bind to LRP5 and inhibit Wnt signaling. In line with this, mice with disruption of Lrp5 in all cells, similar to patients with OPPG, have a low bone mass phenotype which is secondary to reduced osteoblast proliferation [122].

Recently, Yadav and cols. [123] have demonstrated that Lrp5 has an important role inhibiting serotonin biosynthesis in the gut. Serotonin had previously been implicated in the regulation of bone mass [124] and gut-specific deletion of Lrp5 was shown to result in a low bone mass phenotype [123]. In contrast, osteoblast-specific deletion of Lrp5 did not cause a similar defect. Thus, bone formation appears to be controlled by Lrp5-mediated serotonin inhibition in the intestine. It is possible that LRP6, rather than LRP5, is the critical coreceptor for Wnt signaling in bone. Consistent with this, the Lrp6 loss of function bone phenotype is much more severe than the Lrp5 loss of function phenotype. Although Lrp6+/− mice die at birth [125], Lrp6 heterozygous mice display reduced bone mass [126]. In this regard, a
missense mutation in *LRP6* that resulted in impaired Wnt signaling was reported in a family with autosomal dominant early coronary artery disease, metabolic risk factors, and osteoporosis [127].

Wnt extracellular inhibitors also have a relevant role in bone biology. Dkk-1 overexpression in transgenic mice resulted in severe osteopenia with a 49% reduction in the number of osteoblasts [128]. In contrast, mice engineered to lack Dkk-1 showed increased bone formation and bone mass [129]. In humans, elevated expression of DKK-1 in myeloma patients has been associated with bone disease [130].

SFRP1 has also been identified as a regulator of osteoblast and osteocyte survival, with effects on trabecular bone mass. Expression of SFRP1 increases with advancing osteoblast differentiation and peak expression of SFRP1 occurs at the pre-osteocyte stage [131]. Mechanistically, deletion of *Sfrp1* in mice led to a decrease in osteoblast and osteocyte apoptosis with a resultant increase in osteocyte number *in vivo* [132].

The Wnt inhibitor Sclerostin, product of the *SOST* (SclerOSTeosis) gene, also has a critical role in the control of bone mass. Sclerostin is produced exclusively by osteocytes and inhibits bone formation. Inactivating mutations in *SOST* result in sclerosteosis, a sclerosing bone dysplasia [133, 134]. Targeted deletion of the *Sost* gene in mice results in increased bone formation and strength [135]; conversely transgenic mice overexpressing *Sost* have low bone mass [136].

In summary, activation of the Wnt/β-catenin pathway leads to increased bone mass while suppression results in bone loss.

**B. 1,25(OH)\(_2\)D\(_3\) Promotes Wnt/β-Catenin Signaling in Osteoblasts**

The important role of 1,25(OH)\(_2\)D\(_3\) in bone homeostasis is well known (See chapters “Development of the Skeleton”, “Osteoblasts” and “Osteoclasts”). The function of VDR in
osteoblasts seems to be modulated as a function of the differentiation stage of the cells. Indeed, calvarial osteoblasts from Vdr null mice displayed enhanced osteogenesis in vitro, suggesting that VDR activation in pre-osteoblasts suppresses bone formation [137], whereas the expression of a Vdr transgene in mature osteoblasts results in increased bone mass [138]. This differentiation-dependent effect may however be species-specific, as it is not observed in humans (Hans van Leeuwen, personal communication).

Some of the effects of 1,25(OH)\(_2\)D\(_3\) in the bone are reminiscent of those orchestrated by Wnt signaling suggesting a crosstalk between both pathways. Indeed, it has been shown that 1,25(OH)\(_2\)D\(_3\) can induce binding of the VDR to a response element within the mouse Lrp5 gene in both primary osteoblasts and osteoblastic cell lines [139]. This interaction between 1,25(OH)\(_2\)D\(_3\)-activated VDR and the Lrp5 gene led to both a modification in chromatin structure within the mouse Lrp5 locus and the induction of Lrp5 mRNA transcripts in vivo as well as in vitro [139]. Thus, through the induction of Lrp5 expression, 1,25(OH)\(_2\)D\(_3\) enhances Wnt signaling in mouse osteoblasts. Interestingly, whereas the regulatory region in the mouse Lrp5 gene is highly conserved in the human genome, the vitamin D response element is not [139], which argues against a conserved mechanism for 1,25(OH)\(_2\)D\(_3\)/Wnt signaling interaction in the bone.

Studies using BMSCs derived from Vdr-null mice showed that ablation of Vdr did not alter osteoblastic differentiation [140]. However, when cultured under adipogenic conditions, these BMSCs expressed higher mRNA levels of PPAR\(\gamma\) and other markers of adipogenic differentiation, and also of mRNA encoding the Wnt inhibitors Dkk-1 and Sfrp2 [140]. This increase was, at least in part, due to ligand-dependent actions of the VDR, since 1,25(OH)\(_2\)D\(_3\) suppressed Dkk-1 and Sfrp2 expression in wild-type cultures. Thus, it is concluded that ligand-dependent actions of the VDR in mouse BMSCs promote canonical Wnt signaling by inhibiting the expression of Dkk-1 and Sfrp2 and inducing the expression of Lrp5, leading to
a repression of adipogenic differentiation (Figure 4). 1,25(OH)\(_2\)D\(_3\) effects on Wnt signaling are, however, complex and cell- or tissue-dependent. 1,25(OH)\(_2\)D\(_3\) enhances the induction of the negative regulator of bone formation and Wnt inhibitor \(SOST\) gene by bone morphogenetic protein in human osteoblasts [141] while it represses the \(SOST\) homologue \(WISE/SOSTDC1\) gene in keratinocytes [142]. Similarly, Dkk-1 expression is inhibited by 1,25(OH)\(_2\)D\(_3\) in BMSCs while it is induced by the hormone in colon cancer cells [85, 140] (Figure 4).

V. WNT AND 1,25(OH)\(_2\)D\(_3\) IN THE SKIN

A. Wnt Signaling in the Epidermis

The Wnt/\(\beta\)-catenin signaling pathway controls stem cell differentiation in the skin [143, 144]. \(\beta\)-Catenin transcriptional activity promotes differentiation of the hair follicle lineages in embryonic and adult epidermis and, in certain circumstances, can expand the stem cell compartment [145, 146]. In contrast, \(\beta\)-catenin inhibits sebaceous gland differentiation [143, 147] and actively suppresses interfollicular epidermis differentiation in developing skin [145, 146]. Sonic hedgehog and Jagged-1, ligands of the Hedgehog and Notch signaling pathways, respectively, are \(\beta\)-catenin target genes in the epidermis and both pathways act downstream of \(\beta\)-catenin to induce stem cell expansion and follicle formation [148, 149].

Consistent with the relation between aberrant Wnt signaling and cancer, deletion of \(\beta\)-catenin renders the epidermis resistant to chemically-induced tumors [150]. Moreover, prolonged activation of \(\beta\)-catenin in transgenic mice is sufficient to induce benign hair follicular tumors (pilomatricomas and trichofolliculomas) [151], which regress when the pathway is no longer active [147]. Interestingly, human pilomatricomas have been found to harbor activating \(\beta\)-catenin mutations [152].
B. The Vitamin D Receptor Mediates Wnt/β-Catenin Signaling in the Epidermis

VDR is essential for adult epidermal homeostasis [153] and mutations in the VDR gene in humans result in familial 1,25(OH)_{2}D_{3}-resistant rickets, which can be associated with alopecia [154]. In vivo, the expression of a mutant Vdr that can bind β-catenin but not 1,25(OH)_{2}D_{3} rescues alopecia in Vdr null mice, demonstrating ligand-independent functions of VDR in the skin [155]. Vdr null mice fail to undergo the first postnatal hair cycle; instead, the hair follicles are converted to cysts of interfollicular epidermis. Two independent groups have shown that the absence of Vdr impairs Wnt/β-catenin signaling in keratinocytes and leads to alopecia [156, 157]. Cianferotti and cols. reported that Vdr ablation results in gradual depletion of the hair follicle stem cell pool which correlated with a failure of β-catenin to induce proliferation [156]. In contrast, Pálmer and cols. [158] defend that the degeneration of Vdr null follicles does not reflect a loss of follicle stem cells. Alternatively, they suggest that the failure to maintain the hair follicle may represent an inability of the stem cells to migrate along the follicle [158]. These authors also found that many genes that are upregulated by active β-catenin contain vitamin D response elements and that several of them are induced independently of TCF/LEF. They conclude that unliganded VDR is a Wnt effector and that β-catenin acts as VDR coactivator in epidermal keratinocytes [157]. 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. Although these genes are activated by Wnt signals in the absence of 1,25(OH)_{2}D_{3}, the combined treatment has a synergic effect [157] (Figure 4).

Prolonged activation of β-catenin in the absence of VDR results in the development not of benign trichofolliculomas but of undifferentiated tumors resembling basal cell carcinomas [157]. Conversely, activation of β-catenin in the presence of VDR and the vitamin D analog EB1089 prevents β-catenin-induced formation of trichofolliculomas.
Interestingly, human trichofolliculomas have cells with high levels of nuclear β-catenin and VDR, whereas infiltrative human basal cell carcinomas have high β-catenin levels and low VDR levels [157]. Thus, vitamin D analogues may be beneficial in the treatment of skin tumors in which the canonical Wnt pathway is activated inappropriately. A corollary to these results is that β-catenin can no longer be considered as chiefly an activator of TCF/LEF target genes. The interaction of β-catenin with VDR and possibly other transcription factors is likely to contribute to the pleiotropic effects of the Wnt pathway, which has different target genes in different cell types (Figure 4).

**PLEASE, INSERT FIGURE 4 HERE**

**VI. CONCLUSIONS**

1,25(OH)\(_2\)D\(_3\) and VDR regulate the Wnt/β-catenin signaling pathway depending on the cell or tissue type: while 1,25(OH)\(_2\)D\(_3\) and analogues inhibit β-catenin transcriptional activity and target genes in colon tumor cells, upregulation of the pathway by either ligand-activated or unliganded VDR occurs in osteoblasts and keratinocytes. The mechanisms of Wnt signaling control by 1,25(OH)\(_2\)D\(_3\) and VDR are diverse: direct VDR/β-catenin interaction, induction of β-catenin nuclear export, variable regulation of the expression of Wnt inhibitors such as DKK-1 and -4, WISE/SOSTDC1, SOST and Sfrp2, of the Wnt coreceptor Lrp5 (in mouse cells) or of the nuclear β-catenin partner TCF4, and repression of IL-1β production by stromal macrophages (Figure 4).
Figure Legends

Figure 1. Wnt/β-catenin signaling pathway. (A) In the absence of Wnt factors, β-catenin (β-Cat) is located at plasma membrane adherens junctions bound to E-cadherin. Free β-catenin is phosphorylated by GSK3β and CK1 in a destruction complex that includes also APC and Axin. This phosphorylation targets β-catenin for degradation by the proteasome. The transcription of Wnt/β-catenin target genes is inhibited by TCF/LEF via the recruitment of corepressors such as Groucho(Gro)/TLE1. (B) Wnt factors promote the stabilization of cytosolic β-catenin through the inactivation of the destruction complex. β-Catenin enters the cell nucleus and associates with TCF/LEF proteins activating the transcription of Wnt/β-catenin target genes. Activated Wnt pathway reduces E-cadherin expression through the induction of transcriptional repressors of CDH1/E-cadherin gene and of proteases that degrade E-cadherin protein (for a review see [159]).

Figure 2. Extracellular inhibitors of Wnt signaling. WIF-1, SFRPs and Cerberus bind directly to Wnt factors and block their interaction with Frizzled receptors. DKK and WISE/SOST families bind to LRP coreceptors and prevent Wnt-Frizzled-LRP interaction and signaling. In addition, DKKs induce LRP endocytosis in the presence of Kremen proteins.

Figure 3. 1,25(OH)_{2}D_{3} inhibits Wnt/β-catenin signaling in colon cancer cells by several mechanisms. (A) Ligand-activated VDR binds to β-catenin and inhibits the formation of transcriptionally competent β-catenin/TCF4 complexes. (B) 1,25(OH)_{2}D_{3} induces E-cadherin expression and promotes β-catenin nuclear export and relocation at plasma membrane adherens junctions bound to newly synthesised E-cadherin. (C) 1,25(OH)_{2}D_{3} induces the
expression of the DKK-1 Wnt inhibitor. (D) 1,25(OH)_{2}D_{3} inhibits IL-1β secretion by tumor-associated macrophages and thus blocks the IL-1β-dependent inhibition of GSK3β activity and subsequent β-catenin stabilization in cancer cells.

**Figure 4.** The interplay between Wnt/β-catenin pathway and 1,25(OH)_{2}D_{3}/VDR depends on the cell or tissue type. In colon cancer cells, 1,25(OH)_{2}D_{3}/VDR inhibits β-catenin transcriptional activity and target genes through the inhibition of β-catenin/TCF interaction, the induction of β-catenin nuclear export, and the regulation of DKK-1, DKK-4, TCF4 and IL-1β. In mouse osteoblasts and bone marrow stem cells, 1,25(OH)_{2}D_{3}/VDR upregulates Wnt/β-catenin signaling through the induction of Lrp5 and the repression of Dkk-1 and Sfrp2. In human osteoblasts, 1,25(OH)_{2}D_{3} potentiates SOST induction by bone morphogenetic proteins. Unliganded VDR mediates the activation of Wnt/β-catenin target genes in mouse keratinocytes promoting hair follicle differentiation and inhibiting the formation of infiltrative basal cell carcinomas. In human keratinocytes, 1,25(OH)_{2}D_{3} represses WISE/SOSTDC1.
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