Published in final edited form as:
Arch Biochem Biophys. 2012 July 1; 523(1): 107–114. doi:10.1016/j.abb.2011.10.019.

Cellular and Molecular effects of Vitamin D on Carcinogenesis

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Abstract

Epidemiologic data suggest that the incidence and severity of many types of cancer inversely correlates with indices of vitamin D status. The vitamin D receptor (VDR) is highly expressed in epithelial cells at risk for carcinogenesis including those resident in skin, breast, prostate and colon, providing a direct molecular link by which vitamin D status impacts on carcinogenesis. Consistent with this concept, activation of VDR by its ligand 1,25-dihydroxyvitamin D (1,25D) triggers comprehensive genomic changes in epithelial cells that contribute to maintenance of the differentiated phenotype, resistance to cellular stresses and protection of the genome. Many epithelial cells also express the vitamin D metabolizing enzyme CYP27B1 which enables autocrine generation of 1,25D from the circulating vitamin D metabolite 25-hydroxyvitamin D (25D), critically linking overall vitamin D status with cellular anti-tumor actions. Furthermore, pre-clinical studies in animal models has demonstrated that dietary supplementation with vitamin D or chronic treatment with VDR agonists decreases tumor development in skin, colon, prostate and breast. Conversely, deletion of the VDR gene in mice alters the balance between proliferation and apoptosis, increases oxidative DNA damage, and enhances susceptibility to carcinogenesis in these tissues. Because VDR expression is retained in many human tumors, vitamin D status may be an important modulator of cancer progression in persons living with cancer. Collectively, these observations have reinforced the need to further define the molecular actions of the VDR and the human requirement for vitamin D in relation to cancer development and progression.

Keywords

vitamin D receptor; cancer; vitamin D; prevention; diet

I. Introduction to vitamin D and cancer

Human cancers most commonly arise from epithelial cells that accumulate genetic and epigenetic alterations that drive proliferation, survival and acquisition of invasive and metastatic capabilities. Epidemiologic and laboratory research indicates that the alterations associated with cancer development result from complex interactions between an individual's genetic makeup and their exposure to environmental risk factors. Based on data suggesting that environmental factors contribute substantially to overall cancer risk, attention has focused on exploiting specific lifestyle and dietary factors in cancer prevention strategies. Among dietary factors, vitamin D has increasingly been linked to cancer prevention in epidemiological, laboratory, animal and clinical studies. The epidemiologic

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evidence for cancer prevention by vitamin D is strongest for colon, rectal and breast cancer, but cancers of the bladder, brain, endometrial lining, esophagus, gallbladder, kidney, lungs, ovaries, pancreas, prostate, and stomach also may be vitamin D sensitive. Mechanistically, 1α,25 dihydroxyvitamin D3 (1,25D), the biologically active form of vitamin D3, is a global regulator of gene expression and signal transduction in virtually every tissue. In epithelial cells, the vitamin D receptor (VDR) and its ligand 1,25D contribute to maintenance of the quiescent, differentiated phenotype and promote pathways that defend cells against endogenous and exogenous stresses–actions that translate to reduced risk for carcinogenic conversion.

The presence of functional VDR in human tumors and cancer cell lines further indicate that this receptor might represent a target for cancer therapy–ie, that enhancing vitamin D status could improve survival or response to therapy in patients living with cancer. Indeed, multiple studies have confirmed that 1,25D induces growth arrest, triggers cell death and/or promotes differentiation of cancer cells *in vitro* and established tumors *in vivo*. Thus, it is not surprising that higher blood levels of 25D correlate with better survival of patients with cancer. These data are not entirely consistent, however, and some studies indicate that vitamin D metabolism and VDR expression become abrogated in advanced cancers, such that endogenous activity of the vitamin D pathway is no longer sufficient to trigger anti-tumor effects. In these advanced cases, more potent vitamin D based drugs may have therapeutic value. Dozens of synthetic vitamin D analogs have shown efficacy in animal models of cancer, individually and in combination with standard therapies such as chemotherapeutic drugs and radiation. However, issues with dosing, toxicity and efficacy, particularly against specific tumor types, remain to be addressed with these analogs. Approaches to monitor and overcome vitamin D resistance during tumor progression may provide alternative strategies for restoring vitamin D signaling in patients with advanced cancers.

Despite the cumulative evidence linking vitamin D biology to cancer development and progression, large randomized vitamin D supplementation studies to identify the circulating 25-hydroxyvitamin D (25D) levels associated with cancer protection have yet to be completed. However, epidemiological studies indicate that maintenance of blood 25D levels above 40 ng/mL (100 nmol/L) correlates with reduced risk of breast, colon, and rectal cancer. Depending on an individual's sun exposure, age, sex, body weight and baseline vitamin D status, supplements in the range of 2000–4000 international units daily are necessary to maintain serum 25D above 100nmol/L. Due to considerable individual variability, monitoring vitamin D status by serum 25D analysis is the most accurate way to determine the appropriate level and route of supplementation.

II. Scope of this review

As the number of literature citations linking vitamin D and cancer in the last year alone is close to 500, it is not possible to thoroughly cover all aspects of this research area in a short review. Several recent comprehensive reviews focusing on the epidemiological and clinical aspects of vitamin D and cancer, the prevalence of vitamin D deficiency and vitamin D requirements provide insight into these issues [1–3] [4, 5]. Here we will focus on newer findings related to the biology of vitamin D in relation to the common age-related cancers (skin, colon, breast, prostate) with an emphasis on cellular and molecular studies and animal models.

III. Cellular and molecular effects of vitamin D in cancer cells

General concepts

In response to the initial identification of VDR in cancer cells, numerous studies have examined the effects of its ligand 1,25D on cell phenotype. Furthermore, a large number of structural analogs of vitamin D developed by pharmaceutical companies and academic researchers have been used to probe the mechanism of action of vitamin D in cancer cells. In general, the effects of VDR agonists on multiple types of cancer cells are similar: modulation of key cell cycle regulators to arrest the cycle at either G0/G1 or G2/M, induction of differentiation markers, and/or activation of cell death (via apoptosis or autophagy). Mechanisms and pathways have recently been reviewed in detail [6, 7], and thus are briefly discussed here.

Of note, studies with cells derived from VDR null mice have definitely established that the VDR is required for the anti-proliferative effects of 1,25D *in vitro* [8, 9]. Thus, the expression and function of VDR in cancer cells is the major determinant of 1,25D sensitivity. Most normal epithelial tissues exhibit abundant VDR, however there is evidence that its expression and/or function becomes corrupted during carcinogenesis. Transcriptional repressors associated with the epithelial-mesenchymal transition such as Snail1 and Snail2 (Slug) directly bind and repress the VDR gene promoter, leading to loss of expression in colon and breast cancer cells [10, 11]. VDR activity is also disabled in cells expressing SV40 large T antigen, oncogenic ras, altered transcriptional cofactors, or HDAC3 [12–21], leading to altered target gene regulation and loss of the anti-proliferative effects of 1,25D. In many cases, inhibition of the oncogenic pathways that deregulate VDR (for example, with HDAC or kinase inhibitors) restores cellular sensitivity to 1,25D [15, 17, 20]. In addition to VDR function, other aberrations associated with carcinogenesis that may contribute to vitamin D resistance include overexpression of the vitamin D 24-hydroxylase (CYP24A1, the enzyme that catabolizes 25D and 1,25D) and reduction in the vitamin D 1α-hydroxylase (CYP27B1, the enzyme that generates 1,25D) [14, 22–25]. Furthermore, deregulation of pathways downstream of VDR (such as apoptosis) can render cells resistant to 1,25D mediated growth effects. For example, stable expression of the anti-apoptotic protein bcl-2 abolishes the induction of apoptosis by 1,25D [26–28]. Sub-clones of the MCF-7 breast cancer cell line selected for resistance to 1,25D in vitro have been independently developed and characterized. These cell lines retain low level expression of transcriptionally active VDR but exhibit changes in protein expression that alter redox status, favor autonomous growth signaling, and down-regulate the apoptotic pathway [29–33]. Notably, despite deregulation of multiple signaling pathways, the MCF-7 cells selected for 1,25D resistance are cross-resistant to structurally related vitamin D analogs but retain sensitivity to other growth inhibitory agents, including retinoids and anti-estrogens. Uncovering the molecular basis for selective vitamin D resistance will be critical in design and implementation of new vitamin D analogs for clinical use. The following sections will highlight a few of the best-characterized pathways regulated by vitamin D in specific cell types with special relevance to cancer prevention.

Skin cancer cells: epidermal differentiation and UV protection

The two most relevant effects of vitamin D for skin cancer prevention are its ability to maintain the ordered proliferation and differentiation of the stratified squamous epithelium, and its ability to prevent UV induced DNA damage. 1,25D inhibits proliferation, increases the expression of differentiation markers (involucrin, transglutaminase, loricrin, filaggrin) and enhances cornified envelope formation in keratinocytes. The sequential action of 1,25D on keratinocyte differentiation has been mechanistically linked to differential interactions of the DRIP and SRC coactivator complexes with VDR which regulate distinct gene targets as

differentiation proceeds [34]. The anti-proliferative actions of 1,25D in basal keratinocytes involve inhibition of β-catenin and hedgehog (Hh) signaling through VDR. Some evidence suggests that the parent vitamin D_3 compound (cholecalciferol), which is produced in UV-exposed skin in large quantities, directly binds components of the Hh signaling complex to repress its activity [35, 36]. Importantly, these direct effects of cholecalciferol on the Hh pathway are both 1,25D and VDR-independent. Since (as discussed in Section IV) mice lacking either VDR or CYP27B1 exhibit altered proliferation and differentiation in the skin, there is no doubt that the 1,25D/VDR complex is a major physiological regulator of epidermal biology. Thus, determining the relative contributions of VDR and non-VDR signaling and the specific roles of various vitamin D related compounds in the control of epidermal proliferation is clearly a challenge for the future.

In addition to maintenance of the differentiated epithelium, vitamin D contributes to skin cancer prevention though protection of keratinocytes from ultraviolet (UV) mediated damage [37]. *In vitro*, 1,25D enhances cell survival and reduces DNA damage of keratinocytes exposed to UV radiation through mechanisms that involve up-regulation of p53, inhibition of stress-activated kinases and suppression of nitric oxide production. These actions coordinately reduce the accumulation of UV-photoproducts and consequently limit the formation of DNA adducts that can lead to mutations that drive skin carcinogenesis. Interestingly, the photoprotective actions of 1,25D are mi-micked by vitamin D analogs that bind VDR but do not stimulate its transcription activity, indicating that they are mediated (at least in part) through non-genomic VDR signaling [38]. Since UV radiation stimulates the synthesis of cholecalciferol in the skin, it is tempting to speculate that unique vitamin D compounds might be generated in skin exposed to UV radiation that act through non-genomic mechanisms to confer protection against its damaging effects. Such compounds would presumably lack the calcemic effects associated with genomic vitamin D compounds, and thus may be useful as topical agents for prevention of UV-induced skin cancers.

In summary, although several important pathways underlying the protective effects of vitamin D/VDR in skin have been identified, there are clearly many new functions of vitamin D being discovered in the epidermis. Clarification of these novel actions and their mechanisms will likely provide insight into new strategies for prevention of human skin cancer, which some data suggests is inversely related to vitamin D status [39].

Colon cancer cells: wnt pathway and inflammation

The ability of 1,25D to induce differentiation in colon cancer cells was recognized more than 20 years ago [40], and recent molecular studies have identified specific VDR targets with an emphasis on the wnt signaling pathway, a known contributor to colorectal cancer progression [41]. Activation of the VDR represses signaling through β-catenin (a downstream mediator of the wnt pathway) via direct interactions between the activator function-2 (AF-2) domain of the VDR and the C-terminus of β-catenin [42]. n Caco-2 colorectal cancer cells [43], 1,25D suppresses β-catenin transcriptional activity and endogenous expression of the β-catenin target gene DKK-4 independent of VDR DNA-binding activity. Furthermore, the inhibition of β-catenin activity by 1,25D-VDR is significantly enhanced in the presence of the tumor suppressor APC, a known repressor of wnt signaling. Studies in this system also suggest that polymorphic variations in VDR that have been linked to colon cancer risk affect its ability to repress wnt signaling Another newly identified direct VDR target gene in colon cancer cells is cystatin D, which encodes an inhibitor of several cysteine proteases of the cathepsin family and appears to be essential for the induction of differentiation and inhibition of wnt signaling by 1,25D [44]. In addition to effects on β-catenin signaling, 1,25D increases cytosolic Ca2+ and transiently activates the RhoA-ROCK-p38MAPK-MSK pathway in colon cancer cells [45]. Inhibition of this pathway in colon cancer cells prevents the formation of epithelioid islands and abrogates the

induction of CYP24A1, cystatin D, E-cadherin, and vinculin as well as the repression of cyclin D1 by 1,25D. Thus, both genomic and non-genomic pathways contribute to the pro-differentiating effects of 1,25D in colon cancer cells.

Animal studies have linked the anti-cancer effects of vitamin D to its ability to regulate inflammation and immune responses in the gut (discussed in detail in Section IV). During colon cancer progression, tumor-associated macrophages release soluble factors (ie, IL-1β) that activate wnt signaling in epithelial cells. Through activation of VDR in macrophages, 1,25D blocks the production of IL-1β and inhibits the ability of macrophages to activate Wnt signaling in colon carcinoma cells [46]. These studies demonstrate a unique mechanism whereby 1,25D exerts chemopreventive activity by interrupting crosstalk between tumor epithelial cells and macrophages in the tumor microenvironment.

Factors that disrupt the integrity of 1,25D/VDR signaling at the level of the gut mucosa would be anticipated to increase risk for gut inflammation and development or progression of colorectal cancer. Clinical data suggests that VDR is expressed in early stages of colon cancer but reduced in aggressive disease [11]. The reduction in VDR expression during colon cancer progression has been linked to the up-regulation of transcriptional repressors such as SNAIL, which directly bind and repress the VDR promoter [47]. In addition, changes in the vitamin D metabolizing enzymes CYP27B1 and CYP24A1 in advanced colon cancer favor catabolism of both 25D and 1,25D, limiting their effectiveness in growth control. In particular, aberrant expression of CYP24A1 and the occurrence of splice variants correlate with high proliferative rate in advanced colon cancers [48, 49].

Breast cancer cells: differentiation and estrogen signaling

The majority of established breast cancer cell lines express transcriptionally active VDR and undergo growth inhibition in response to 1,25D [50]. In general, VDR expression and sensitivity to 1,25D mediated growth arrest is higher in the less aggressive, estrogen receptor (ER) positive breast cancer cell lines such as MCF-7 than in ER negative cell lines. Tumor cells derived from VDR null mice were used to conclusively demonstrate that 1,25D mediates effects in breast cancer cells via the nuclear VDR [8]. Screening for molecular changes induced by 1,25D or vitamin D analogs in various breast cancer cells has identified scores of VDR regulated genes and proteins in diverse pathways, indicating a broad range of downstream targets [32, 51, 52] involved in cell cycle (cyclins, cyclin-dependent kinases and their inhibitors), apoptosis/autophagy (bcl-2 family, caspases, cathepsins) and inflammation (NFκB, prostaglandins,cox-2). The net effect of these changes is to block mitogenic signaling, including that of estrogen, EGF, IGF-1 and KGF, and to enhance the effects of negative growth factors such as TGFβ. In many breast cancer cell lines, 1,25D mediated growth arrest is associated with the induction of differentiation markers such as casein, lipid droplets, and adhesion proteins [53, 54]. Notably, 1,25D exerts additive or synergistic effects in combination with other triggers of apoptosis, such as ionizing radiation and chemotherapeutic agents [55, 56]. Collectively, these studies indicate that a wide variety of signaling pathways, cell cycle and apoptotic regulatory proteins and proteases contribute to the anti-proliferative, pro-differentiating and apoptotic effects of 1,25D depending on the specific breast cancer cell line and/or context.

In primary cultures of normal human mammary epithelial (HME) cells, vitamin D signaling also mediates growth arrest and induction of differentiation markers such as E-cadherin, but apoptosis has not been observed [57]. In contrast to breast cancer cells, non-transformed mammary cells retain expression of CYP27B1 and generate 1,25D when incubated with physiological concentrations of 25D. Many breast cells also express the megalin-cubilin complex which mediates internalization of 25D bound to the vitamin D binding protein [58]. Autocrine metabolism of 25D triggers chemopreventive effects in breast epithelial cells

including growth inhibition, differentiation and protection from various cellular stresses [22, 57, 59]. In the intact mammary gland, the epithelium is surrounded by stromal fibroblasts and adipocytes, which provide critical growth factor signals for development and also impact on carcinogenesis. Recent evidence suggests that breast adipocytes express CYP27B1 and generate 25D which signals via adipocyte VDR to release inhibitory factors that regulate mammary epithelial cell growth [60]. Since vitamin D metabolites are stored in fat tissue, the contribution of adipocyte signaling to the tumor suppressive actions of vitamin D in mammary gland are likely of physiological importance and require further study.

As in colon cancer, acquisition of the transformed phenotype in breast cells is associated with deregulation of the vitamin D pathway [22, 61]. In HME cells, introduction of SV40 large T antigen and/or oncogenic ras induces transformation and reduces responsiveness to 25D in association with down-regulation of VDR and CYP27B1 [14]. Oncogenes and tumor suppressor genes that impact on VDR expression in breast cells include ras, p53 and slug, which act via diverse mechanisms including transcriptional regulation and mRNA instability [10, 12, 16]. The mechanisms by which transformation abrogates CYP27B1 expression in breast cells have yet to be elucidated.

Prostate cancer cells: androgen signaling and cell fate

Primary cultures derived from normal human prostate and many established prostate cancer cell lines express VDR and undergo growth inhibition in response to 1,25D. Depending on the specific cell line/context, actions triggered by 1,25D in prostate cancer cells include cell cycle arrest in G_1, apoptosis, differentiation, modulation of growth factor signaling, anti-inflammatory effects, anti-angiogenesis and inhibition of invasion and metastasis [62]. Specific 1,25D target genes identified in prostate cancer cells include those involved in growth factor signaling (IGFBP3, TGFβ-2 and TGFβ-3), prostaglandin metabolism (COX-2, 15-PDGH), inflammation (NF-κB, IL-6, IL-8) and tumor progression (VEGF, MMP-9). Recent genomic profiling of the temporal changes induced by 1,25D in immortalized but non-tumorigenic prostate epithelial cells revealed rapid suppression of wnt, Notch, NF-κB, and IGF1 signaling, early induction of genes that suppress angiogenesis and oxidative stress and sustained reductions in pro-inflammatory mediators [63]. This study identified over 250 transcripts that were regulated similarly at all time points and many of the promoters for these transcripts were found to contain putative vitamin D response elements.

Emerging studies also indicate that vitamin D signaling interacts significantly with androgen signaling in both normal and cancerous prostate cells. Genomic profiling of LNCaP prostate cancer cells treated with 1,25D ± testosterone identified over 250 genes that are not significantly regulated by either hormone alone but are synergistically modulated by the combination of the two steroids including PSA, TMPRSS2, CACNG4, KCNMB4, ITPR1, CDC20 and CCNB2. A subset of these genes lack obvious AR or VDR binding sites in their promoter regions and appear to be regulated by 1,25D and testosterone indirectly through changes in miRNA abundance [64]. miRNAs altered by 1,25D and testosterone include miR-22, miR-29ab, miR-134, miR-1207-5p and miR-371-5p (up regulated) and miR-17 and miR-20a (down regulated), the targets of these miRNAs include genes involved in regulation of cell cycle progression, lipid synthesis and calcium homeostasis.

Attention to the role of stem cells in carcinogenesis prompted Maund et al [9] to study the effects of 1,25D on progenitor/stem cells (PrP/SC) isolated from murine prostate. PrP/SC undergo cell-cycle arrest and senescence when treated with either 1,25D or 25D. Microarray analyses found that 1,25D triggers genomic changes in PrP/SC consistent with differentiation to an androgen receptor-positive luminal epithelial cell fate. Furthermore,

1,25D induces the pro-inflammatory cytokine IL-1α, which is essential for the anti-proliferative effects of both 25D and 1,25D in these cells.

Collectively, these recent genomic studies in prostate-derived cell lines have demonstrated the global nature of vitamin D signaling, identified additional mechanisms of VDR action and provided numerous additional targets that may mediate vitamin D actions or represent biomarkers of its activity.

IV. Tissue specific effects of vitamin D and VDR in animal models of cancer

General overview

The ability of vitamin D to prevent cancer has been examined in a variety of mouse models of cancer. Although mice rarely develop spontaneous cancers, specific protocols have been developed for induction of tumors in the skin, breast and colon with chemical and other carcinogens. In addition, genetically engineered mice bearing mutations that recapitulate changes in human tumors predictably develop cancers and are thus amenable to prevention studies. The effects of vitamin D supplementation and VDR ablation have been extensively studied in relation to spontaneous and induced cancers of the skin, breast, prostate and colon. As detailed below for specific tumor types, it is clear that dietary supplementation with vitamin D, or administration of synthetic VDR agonists, reduces tumor burden and/or tumor growth rates [65–67]. Furthermore, VDR ablation in mice influences tissue proliferation and apoptosis [68–70], enhances oxidative DNA damage and is associated with chronic inflammation [71, 72]. *Vdr* null mice also demonstrate enhanced sensitivity to carcinogenesis triggered by chemical carcinogens and activation of oncogenes/loss of tumor suppressor genes. As detailed below, the effects of VDR deficiency are tissue specific, yet some common mechanistic links have emerged from these studies.

Skin Carcinogenesis

A recent unbiased systems biology approach to map genetic loci that underlie susceptibility to skin cancer linked the VDR to coordinated control of epidermal barrier function, inflammation and tumor susceptibility [73]. Consistent with these findings, skin of *Vdr* null mice exhibits abnormal barrier function, altered lipid composition, enhanced proliferation and decreased differentiation and is highly sensitive to tumorigenesis triggered by chemical carcinogens or chronic UV radiation [74–76]. In *Vdr* null animals, hyperplastic epidermis and carcinogen induced tumors exhibit overexpression of components of the hedgehog pathway including sonic hedgehog (Shh) and the Gli transcription factors, which play a critical role in epidermal stem cell fate determination [77]. Of special interest, the enhanced sensitivity of skin to chemically induced tumorigenesis in *Vdr* null mice is not recapitulated in *Cyp27b1* null mice, indicating that protection against skin cancer may be mediated by unoccupied VDR, or through VDR binding to ligands other than 1,25D. However, C*yp27b1* null mice do exhibit impaired epidermal differentiation similar to *Vdr* null mice [78].

Several additional mechanisms contribute to the protective effects of vitamin D against UV-induced carcinogenesis. In *Skh:hr1* mice exposed to UV, administration of 1,25D reduced the accumulation of mutagenic cyclobutane pyrimidine dimers (CPD) which are strongly associated with tumorigenesis, suggesting a role for vitamin D in optimizing DNA repair [79]. Chronic administration of 1,25D also inhibits the development of papillomas and squamous cell carcinomas in this model. Complementary studies have shown significantly lower rates of CPD repair in the epidermis of UV-exposed *Vdr* null mice compared to their wild type counterparts [75]. The effects of vitamin D on UV-induced skin cancer may be mediated by unique mechanisms, since a non-genomic vitamin D analog that binds VDR but does not stimulate its transcriptional activity mimics the effects of 1,25D on DNA repair and tumor prevention [79].

In addition to a role for VDR in regulation of keratinocyte differentiation and DNA repair, both VDR and 1,25D modulate inflammatory responses triggered by chronic UV exposure [75, 79]. Upon UV exposure, 1,25D treatment reduces epidermal expression of the pro-inflammatory cytokine IL-6 and increases the anti-inflammatory cytokine IL-10. Although the 1,25D/VDR complex regulates IL-6 in keratinocytes, transplantation experiments have demonstrated that the regulation of IL-10 is mediated via 1,25D activation of VDR in mast cells [80]. Furthermore, VDR in mast cells is required for 1,25D abrogation of epidermal hyperplasia and ulceration following UV exposure. Collectively, these data point to a complex role for VDR in regulation of epidermal proliferation, inflammation and tumorigenesis that involves several cell types and multiple mechanisms, which may involve as-yet unidentified ligands and/or non-genomic signaling.

Colon Carcinogenesis

The effect of vitamin D on colon carcinogenesis in mice has been studied in spontaneous, chemically induced and genetic models [41, 81]. As early as 1992, it was reported that administration of 1,25D to mice prior to challenge with the colon carcinogen 1,2-dimethylhydrazine dihydrochloride (DMH) reduces the development of colon adenocarcinomas by 50% [82]. This effect is associated with abrogation by 1,25D of DMH-induction of ornithine decarboxylase activity. Consistent with an anti-tumor effect of dietary vitamin D, chronic feeding of a vitamin D deficient diet containing adequate calcium significantly enhances the growth of MC-26 murine colon cancer xenografts [83].

In a more clinically relevant model, mice chronically fed a western-style diet (containing low levels of both calcium and vitamin D) spontaneously develop benign and malignant colonic tumors which are inhibited by supplementation with dietary calcium and vitamin D [84]. Genomic profiling indicated that the western-style diet alters Paneth cell markers (a lineage normally confined to the bottom of small intestinal crypts), elevates the Wnt receptor Fzd5 and EphB2 (genes necessary for Paneth cell differentiation) and increases Wnt signaling in villus cells. Elevating dietary vitamin D and calcium, which prevents tumor development, abrogates these genomic changes.

Chronic inflammation in the gut promotes tumorigenesis in mouse models and is a risk factor for colon cancer in humans. Since vitamin D regulates immune responses in many tissues, protection against intestinal tumorigenesis by vitamin D may involve anti-inflammatory mechanisms. In support of this concept, *Vdr* null mice develop severe chronic gut inflammation when crossed to *Il-10* null mice, a model for inflammatory bowel disease [85]. *Vdr* null mice are also highly susceptible to intestinal inflammation induced by chemical irritants such as dextran sulfate sodium (DSS) [86, 87]. While control mice are mostly resistant to low doses of DSS, *Vdr* null mice demonstrate extensive ulceration, impaired wound healing and disrupted epithelial junctions leading to severe diarrhea, rectal bleeding, and marked body weight loss. The inflammation triggered by DSS also enhances tumorigenesis in mice exposed to the chemical carcinogen azoxymethane (AOM). In the AOM/DSS model, vitamin D analogs inhibit proliferation, colitis and the development of aberrant crypt foci (ACF), a pre-neoplastic lesion that predisposes to colon tumorigenesis [88, 89]. Mechanistically, vitamin D analogs such as Ro26-2198 inhibit the AOM/DSS stimulated increases in c-myc, cox-2 and pERK. More recently, the effect of dietary supplementation with vitamin D_3, 25D and 1,25D was studied in mice treated with AOM/DSS [90]. Both vitamin D_3 and 25D reduce the incidence of colon tumors by approximately 50% without adverse effects such as weight loss. Collectively, these data suggest a model whereby vitamin D and the VDR influence risk for carcinogen-induced colon cancer via effects on the development of inflammation in the gut, however, the involvement and responses of specific cell types remains to be determined. As discussed in section III, one *in vitro* study demonstrated that 1,25D acts on tumor-associated macrophages to block the

release of IL-1β which drives wnt signaling in colon cancer cells [46], however this cross-talk has not been explored *in vivo*.

The most commonly studied genetic model of colon tumorigenesis is the $Apc^{+/-}$ mouse, which spontaneously develops intestinal tumors driven by loss of *Apc* function and subsequent activation of the wnt pathway. Interestingly, western-style diets low in calcium and vitamin D (discussed above) increase and accelerate the tumor phenotype of $Apc^{+/-}$ mice, indicating that dietary and genetic modulation of intestinal tumorigenesis involve at least partially distinct and interactive effects [91]. The first study to specifically examine the efficacy of vitamin D in this model found that chronic administration of 1,25D to mice fed a standard rodent diet containing vitamin D reduces total tumor load by approximately 50% but has no effect on the number of tumors [92]. A similar study using mice fed a vitamin D deficient diet found that 1,25D administration significantly reduces both size and number of ACF and polyps [93]. Consistent with the efficacy of VDR agonists against wnt-induced colon tumorigene-sis, $Apc^{+/-}$ mice bred onto the *Vdr* null background exhibit increased numbers of ACF and larger tumors than $Apc^{+/-}$ mice on the wild-type background [94, 95]. Furthermore, tumors of $Apc^{+/-}$ mice lacking VDR exhibit increased nuclear β-catenin and higher expression of β-catenin/TCF target genes than those that develop in $Apc^{+/-}$ mice that express VDR. Although these data provide proof of principle that vitamin D signaling through VDR inhibits colon tumorigenesis driven by aberrant wnt signaling, the intervention studies utilized 1,25D administered via injection. No studies thus far have tested the impact of manipulating dietary vitamin D *per se* on tumorigenesis in $Apc^{+/-}$ mice.

Breast Cancer

VDR agonists inhibit growth and induce regression of established human breast cancer xenografts in animal models [33, 96]. In estrogen receptor (ER) positive tumors, the effects of vitamin D analogs are comparable to that of standard anti-estrogen therapies such as tamoxifen, and additive effects are observed in combination studies with tamoxifen and ionizing radiation [55, 97]. Vitamin D analog therapy is effective in both ER positive and ER negative xenografts, and the anti-tumor actions of ER and VDR ligands can be dissociated [33, 98, 99]. Of particular interest, studies on xenografts derived from WT and VDR null cells indicate that expression of functional VDR in tumor epithelial cells (rather than in accessory cells such as fibroblasts or endothelial cells) is necessary for the anti-tumor effects of the vitamin D analog EB1089 and UV generated vitamin D *in vivo* [100].

Late stage breast cancer often forms osteolytic bone lesions, and several studies have addressed the possibility that vitamin D signaling alters skeletal metastases. Although few murine models recapitulate the process of bone metastasis from primary breast tumors, several invasive human breast cancer cell lines will grow in bone after intra-cardiac or intra-tibial injection. In one study, the effect of the vitamin D analog EB1089 on growth of MDA-MB-231cells in bone was monitored after intra-cardiac injection [101]. In this model, continuous administration of EB1089 reduces the number and extent of bone metastases, prevents paralysis and markedly increases survival compared to vehicle treated animals. In another series of studies [102, 103], dietary vitamin D deficiency of sufficient magnitude to induce secondary hyperparathyroidism and accelerate bone turnover was shown to significantly enhance the growth of both ER positive and ER negative human breast cancer cells in bone. Blocking bone resorption only partially abrogates the effects of vitamin D deficiency on metastatic growth, indicating that in addition to effects on the bone microenvironment, the vitamin D deficient diet likely exerts direct effects on the tumor cells that inhibit their metastatic potential. These studies indicate that vitamin D status might be a relevant modulator of progression in women living with breast cancer, a concept which is supported by some clinical data [104–106].

Animal studies also support the concept that vitamin D signaling reduces initial development of breast cancer. Rodents fed western-style diets low in vitamin D and calcium exhibit hyperproliferation in the mammary gland and develop significantly more mammary tumors when treated with 7,12-dimethylbenzanthracence (DMBA) compared to rats fed adequate calcium and vitamin D [107]. In mouse mammary gland organ culture, 1,25D, 25D and synthetic VDR agonists reduce the incidence of pre-neoplastic lesions in response to DMBA during both the initiation and the promotion stages, demonstrating that vitamin D compounds exert direct anti-neoplastic effects on mammary gland at multiple steps [108, 109]. Prevention of N-methyl-N-nitrosourea-induced mammary tumors with vitamin D analogs provides further support that the vitamin D pathway protects against breast cancer *in vivo* [108, 110].

Vdr null mice demonstrate excess proliferation and branching as well as impaired apoptosis in the mammary gland during puberty, pregnancy and involution compared to control littermates [111, 112]. Furthermore, 1,25D blocks the growth stimulatory effects of estrogen and progesterone in organ culture of glands from wild-type mice but not in glands from *Vdr* null mice, indicating that the VDR acts in a ligand dependent manner to mediate negative growth signaling directly in mammary tissue. In response to the carcinogen DMBA, the development of mammary hyperplasia and ER negative tumors is higher in *Vdr* null mice than in their control counterparts [69]. Similarly, when crossed onto the MMTV-neu transgenic background (a model of her2 positive human breast cancer), *Vdr* heterozygote mice develop more mammary tumors than control mice [113].

Prostate Cancer

Similar to breast cancer, syngeneic and immunodeficient rodent models have demonstrated that vitamin D analogs inhibit growth of established prostate tumors [114–116]. Both androgen dependent and androgen independent prostate cells are inhibited by VDR agonists, including metastatic variants [117]. Using a model similar to that described above for breast cancer, dietary vitamin D deficiency was shown to enhance the growth of PC3 prostate cancer cells as osteolytic and osteosclerotic lesions in bone [118]. The preventive effects of 1,25D have been studied in Nkx3.1;Pten mice, a transgenic model which recapitulate stages of prostate carcinogenesis from prostate intraepithelial neoplasia (PIN) to adenocarcinoma. Chronic administration of 1,25D to Nkx3.1; Pten mice significantly reduces the formation of PIN, especially when delivered before, rather than subsequent to, the initial occurrence of PIN. Complementary studies using a more aggressive transgenic model of prostate cancer, the LPB-Tag model, assessed the impact of VDR deletion on prostate tumorigenesis. Results indicated that LPB-Tag tumors progress more rapidly in *Vdr* null mice than in control animals, and tumors lacking VDR have higher levels of cell proliferation than those expressing VDR. Interestingly, supplementation of LPB-Tag mice with testosterone abrogates these differences in tumor progression and proliferation, indicating cross-talk between the androgen and the vitamin D signaling pathways [119]. This cross talk has been further studied in the TgAPT121 mouse model of PIN in which the impact of dietary vitamin D, *Vdr* deletion and castration were evaluated [70]. In intact TgAPT121 mice, low dietary vitamin D increases prostate epithelial cell proliferation, suppresses apoptosis and enhances the severity of PIN lesions. Mice with prostate epithelial cell specific deletion of VDR (PEC VDRKO) were generated to study the direct effects of VDR on epithelial cell turnover during castration and in response to testosterone repletion. PEC VDRKO mice exhibit lower rates of apoptosis in response to castration, and higher rates of proliferation in response to testosterone administration, than control mice. These data show that low vitamin D status and VDR deletion alter cell turnover and hormonal responsiveness in normal prostate tissue - changes that likely contribute to an increased susceptibility of VDR null mice to PIN and tumorigenesis.

V. Integration of data from cellular studies and animal models

In summary, the vitamin D endocrine system has consistently been shown to exert anti-tumor effects against the common age-related human cancers: skin, colon, breast and prostate. Both VDR and CYP27B1 are highly expressed in the normal epithelial cells of these tissues. Using distinct animal models, vitamin D signaling has been shown to impact both development and progression of spontaneous, induced and genetically engineered forms of these common cancers. For several cancers, predictable changes in cancer incidence are induced during states of vitamin D deficiency and excess as well as with VDR deletion. Even under normal conditions, vitamin D signaling alters tissue homeostasis via effects on conserved pathways that regulate cell proliferation, differentiation and/or survival. In prostate and breast, vitamin D modulates tissue responsiveness to hormones (estrogen and testosterone) which are known to drive cancer in these tissues. In the colon, vitamin D regulates wnt signaling which is crucial to maintenance of appropriate stem cell differentiation along the crypt-villus axis. In the skin, vitamin D optimizes DNA repair which protects against UV induced mutations, the most common cause of skin cancer in humans. In addition to effects on epithelial cells, it is clear that vitamin D modulates inflammation, a known cancer risk factor, and also targets accessory cells within the tumor microenvironment. Importantly, the effects of vitamin D on tissue homeostasis are VDR dependent, and VDR agonists mimic the effects of natural vitamin D metabolites. A caveat to these findings is that sensitivity to vitamin D often becomes reduced as cancer progresses due to abrogated expression or activity of VDR and CYP27B1. Therefore it appears that optimization of vitamin D status will most often be beneficial prior to cancer development or during the earliest disease stages, rather than in advanced disease.

VI. Directions for Future Research

This review highlights data from cellular, molecular and animal studies to support the concept that vitamin D signaling exerts tumor suppressive actions. Clearly, the 1,25D/VDR complex triggers global changes in gene expression via classical transcriptional mechanisms that contribute to induction of quiescence and maintenance of the differentiated phenotype in epithelial cells. In addition, novel mechanisms of vitamin D signaling have been identified, including regulation of miRNAs, rapid signaling through kinase pathways and protein-protein interactions. The demonstration that vitamin D metabolites and analogs that do not activate VDR-mediated transcription can mimic some of the anti-tumor actions of 1,25D indicates that additional mechanisms of action remain to be discovered.

With respect to translational relevance, clarification of the mechanisms by VDR and CYP27B1 become deregulated in aggressive cancer cells is needed, as such information may lead to clinical strategies to restore sensitivity to vitamin D in advanced disease. Although determining the optimal vitamin D status for human cancer prevention will require large intervention trials, additional pre-clinical animal studies can provide further insight into the relationship between dietary vitamin D and serum 25D for extrapolation to human studies. In addition, animal studies can identify the life periods when optimal vitamin D status is most critical, and can be used to study interactions of vitamin D with other cancer risk factors, including dietary components, hormones and environmental modulators.

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