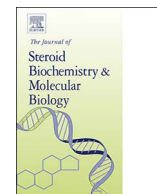




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## Review

## Identification of tumor-autonomous and indirect effects of vitamin D action that inhibit breast cancer growth and tumor progression

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## ABSTRACT

Several epidemiological studies have found that low vitamin D levels are associated with worse prognosis and poorer outcomes in patients with breast cancer (BCa), although some studies have failed to find this association. In addition, prior research has found that BCa patients with vitamin D deficiency have a more aggressive molecular phenotype and worse prognostic biomarkers. As vitamin D deficiency is common in patients diagnosed with BCa, elucidating the cause of the association between poor outcomes and vitamin D deficiency promises to have a significant impact on improving care for patients with BCa including enabling the development of novel therapeutic approaches. Here we review our recent findings in this area, including our data revealing that reduction of the expression of the vitamin D receptor (*Vdr*) within BCa cells accelerates primary tumor growth and enables the development of metastases, demonstrating a tumor autonomous effect of vitamin D signaling to suppress BCa metastases. We believe that these findings are likely relevant to humans as we discovered evidence that a mechanism of VDR regulation identified in our mouse models is conserved in human BCa. In particular, we identified a negative correlation between serum 25(OH)D concentration and the level of expression of the tumor progression factor *ID1* in primary tumors from patients with breast cancer.

## 1. Introduction

Vitamin D is the precursor to the potent steroid hormone, 1 $\alpha$ , 25-dihydroxyvitamin D<sub>3</sub> (calcitriol). Vitamin D, induced by sunlight or ingested in the diet, is readily converted in the liver to 25(OH)D, which is then converted into calcitriol by the mitochondrial enzyme 25-hydroxyvitamin D 1 $\alpha$ -hydroxylase (CYP27B1) in the kidney or extra-renal sites [1–3]. In order to maintain calcium and phosphorus homeostasis, one of the major physiological roles of calcitriol, renal calcitriol synthesis is tightly regulated by other key calcitropic hormones, parathyroid hormone and fibroblast growth factor [2,3].

Calcitriol and its analogs bind to and activate the vitamin D receptor (VDR), a member of the steroid nuclear receptor family. This activation of the VDR leads to heterodimerization with the retinoid X receptor (RXR) and occupancy of vitamin D response elements (VDREs) in the DNA. The occupancy of VDREs initiates the recruitment of other co-factors to form a regulatory complex that modulates the expression of target genes [4,5]. This mode of activation enables vitamin D to have complex and context-specific actions. Indeed, over the past decades, a

large body of work in numerous laboratories has established that vitamin D action extends far beyond its role in calcium homeostasis to participate in a variety of physiological processes from hair growth to metabolism. In addition, perturbations in vitamin D action have been connected to the pathogenesis of a number of diseases [6]. Many studies have shown that vitamin D deficiency is common in the United States, even using the less rigorous cut-point of 20 ng/mL, especially in people of color [7]. In this manuscript, we will review the connections between vitamin D signaling and cancer with a primary concentration on two studies that examined tumor-autonomous actions as well as vitamin D actions on the tumor microenvironment in breast cancer (BCa).

## 2. Vitamin D action in the breast

Although the kidney is the principal site of CYP27B1 activity regulating the generation of circulating calcitriol, this enzyme is also expressed in multiple extra-renal sites along with the VDR leading to local synthesis and paracrine actions [2,8,9]. These findings suggest that there are multiple local tissue-specific vitamin D actions. For example,

**Abbreviations:** calcitriol, 1 $\alpha$ , 25-dihydroxyvitamin D<sub>3</sub>; 25(OH)D, 25-hydroxyvitamin D; CYP27B1, 1 $\alpha$ , -hydroxylase; VDR, vitamin D receptor; RXR, retinoid X receptor; VDRE, vitamin D response element; ID1, Inhibitor of Differentiation 1; OVX, ovariectomized; BCa, breast cancer

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CYP27B1 is highly expressed in breast tissue [10–12]. Consistent with a role for vitamin D action in the breast tissue, the VDR and CYP27B1 are expressed in both normal breast and BCa tissue [11,13]. Mice that have the VDR gene knocked-out have accelerated development and increased branching of the ducts in the breast compared to wild-type controls [14], confirming an important role for vitamin D action in breast tissue physiology and development.

### 3. Vitamin D action in breast cancer

Mounting evidence supports the concept that vitamin D has anti-cancer actions that may be beneficial in the prevention and/or treatment of several types of cancers including prostate, colon and breast [15]. These studies indicate that calcitriol has effects on cell proliferation, differentiation, and apoptosis. Furthermore, increased expression of VDR in BCa tumors is associated with a reduced risk of death [16,17]. Recent studies have also shown that increased vitamin D action suppresses BCa cell migration and metastasis [15,18–22]. Together these, and multiple additional studies have raised optimism that increasing the levels of vitamin D could have beneficial effects against BCa in humans.

In addition to potential benefits of the pharmacological use of vitamin D and calcitriol, some (but not all) epidemiological studies suggest an inverse association between BCa risk and physiological vitamin D stores [20,23–25]. For example, some studies examining the association between serum 25(OH)D levels and BCa prognostic indicators report that patients with vitamin D deficiency [low serum 25(OH)D] have a more aggressive molecular phenotype of BCa and worse prognostic indicators [26–29]. If confirmed, these results indicate that vitamin D action has a physiological role in tumor suppression and would support increasing efforts to reduce the prevalence of vitamin D deficiency. Furthermore, given the widespread prevalence of vitamin D deficiency in the population in general and in cancer patients in particular [30], understanding the relationship between vitamin D deficiency and BCa progression could have broad implications and impact.

#### 3.1. Tumor-autonomous effects of vitamin D action in BCa

Breast tissue is composed of multiple cell types, as well as an intricate extracellular matrix, and all of these components are known to have important roles in both physiology as well as the pathogenesis of BCa [31–33]. Therefore, it is of both scientific and medical interest to localize various actions of vitamin D within BCa and, in particular, specify which compartments and actions are responsible for any anti-tumor vitamin D activity.

To test the effect of vitamin D deficiency on BCa tumor progression, we recently performed studies using two different mouse models of vitamin D deficiency [21]: First, we used the classic model of diet-induced vitamin D deficiency that occurs when mice are fed low vitamin D diet (25 IU vitamin D<sub>3</sub>/kg) (or controls with standard chow containing 500 IU vitamin D<sub>3</sub>/kg) for 10 weeks. Once vitamin D deficiency was established, we orthotopically implanted BCa cells into the mammary fat pad. We found that mice with diet-induced vitamin D deficiency had increased rate of BCa growth, with tumors becoming palpable ~1 week prior to mice with sufficient vitamin D levels [21].

While these results are highly intriguing, indicating that the association between vitamin D deficiency and worse BCa outcomes may result from cause-and-effect mechanisms, the studies did not resolve the question of whether this effect of vitamin D deficiency was occurring because of tumor-autonomous or non-autonomous changes. To address this important question, we established, a second model, using non-metastatic 168FARN BCa cells [34]. From this parental line, we established cells with VDR levels knocked-down (168FARN-*Vdr* KD) and subsequently rescued with a *Vdr* expression plasmid (control cells) in order to control for any off-target effects. We propose that knocking-

down the VDR decreases vitamin D signaling in a manner analogous to vitamin D deficiency, as there is reduced ligand/receptor activation, but the method enables uncoupling of vitamin D deficiency in tumor cells from the local and systemic extra-tumor environments. Therefore, we could evaluate the tumor-autonomous effects of vitamin D deficiency by transplanting these cells into mice that were systemically vitamin D sufficient to resolve the site of relevant activity. Tumors established with the 168FARN-*Vdr* KD cells grew significantly faster compared to tumors from control cells. Strikingly, we also discovered that 60% of the 168FARN-*Vdr* KD tumors were metastatic while none of the tumors established with the control cells metastasized. This finding indicates that tumor-autonomous vitamin D action in BCa is a critical and sufficient regulator of metastasis and might explain the worse prognosis in patients that have vitamin D deficiency [21].

In order to understand the molecular mechanisms that govern these effects, we performed unbiased expression profiling in the tumors harvested from the mice and identified Inhibitor of Differentiation 1 (*Id1*) as differentially expressed in 168FARN-*Vdr* KD versus control cells. Using chromatin immunoprecipitation and luciferase assays, we identified a negative VDRE in the *Id1* gene that was occupied by calcitriol/VDR, establishing *Id1* as a direct target gene.

##### 3.1.1. Vitamin D deficiency results in dysregulation of *Id1*

*Id1* belongs to a family proteins that act as dominant negative regulators of the basic helix-loop-helix family of transcription factors, regulating cell cycle and tumorigenesis [35] and promoting epithelial-to-mesenchymal transition, angiogenesis, invasion and metastasis [36,37]. *Id1* over-expression increases BCa metastasis in mouse models [38]. Importantly, an increase in *Id1* expression has been associated with more aggressive disease and poor clinical outcomes in BCa patients [39]. Therefore, we hypothesized that dysregulation of *Id1* expression in 168FARN-*Vdr* KD tumors was responsible for the acquisition of metastatic potential. Indeed, we also found that knocking-down the overexpression of *Id1* in 168FARN-*Vdr* KD cells restored their rate of migration to control levels in transwell assays [21].

In order to begin to test if this pathway is conserved in humans, we obtained data from a clinical trial to establish the relationship between serum 25(OH)D levels and *ID1* expression in breast tumor samples from patients undergoing BCa resections. Using archived de-identified BCa specimens from this clinical trial, we demonstrated an inverse correlation between serum 25(OH)D levels and *ID1* expression in breast tumor indicating that systemic vitamin D deficiency/insufficiency is associated with higher *ID1* mRNA levels with BCa tumors in humans [21].

#### 3.2. Non-tumor-autonomous effects of vitamin D in BCa

While our [15,21] and other's (i.e. [40]) studies established critical tumor-autonomous effects of vitamin D action, this does not exclude the possibility that non-tumor-autonomous actions are also relevant. For example, studies indicate that the tumor microenvironment could be an important contributor to the protective effects of vitamin D in cancer [32]. In pancreatic ductal adenocarcinoma progression, for instance, VDR in tumor stromal cells modulate tumor volumes and impacts survival in humans [41]. In a recent nested case-control study, serum 25(OH)D levels during summer negatively correlated not only with tumor risk but also elevated stromal VDR levels [42]. Similarly, in colon cancer, the level of VDR expression and induced gene signature in the fibrous stroma play a predictive role in determining the clinical outcome [43]. In the breast microenvironment, both the stromal and tumor cells produce estrogen, a known driver of BCa growth, particularly in postmenopausal women [44,45]. In an obese setting, the adipose stroma is inflamed leading to an unfavorable microenvironment, with increased production of estrogen, thereby promoting BCa progression [46]. Therefore, we examined the effects of vitamin D in mitigating the adverse effects of BCa in obesity [47]. We injected VDR-expressing

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