

# Chapter 4

## Vitamin D Hormone Action in the Endocrine Tissue: Implications for Prostate and Breast Carcinoma



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### Introduction

Calcitriol, the hormonally active metabolite of cholecalciferol (vitamin D<sub>3</sub>), regulates diverse physiologies in addition to its classic endocrine role in the regulation of calcium and phosphate homeostasis, and thus bone mineralization. Calcitriol action in target cells is mediated by the cognate vitamin D receptor (VDR), which is a ligand-inducible transcription factor and a nuclear receptor. In extraskeletal tissues, calcitriol-activated VDR signaling inhibits cell proliferation and angiogenesis while promoting differentiation and apoptosis. The repertoire

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of calcitriol action also includes anti-inflammatory response and induction of the drug metabolism machinery [1–3].

The normal and malignant tissues of prostate, breast, and other endocrine organs have an active vitamin D/VDR regulatory axis. Inhibition of prostate cancer and breast cancer in response to an activated VDR pathway has been extensively documented in cell culture, in xenograft settings, and in animal models of cancers arising from genetic changes or carcinogen-induced mutagenesis. Mechanisms attributed to the anticancer effects of vitamin D signaling include cell cycle inhibition at the G1/S checkpoint, reduced DNA damage, altered microRNA expression, pro-differentiation response, and apoptosis induction (reviewed in [4]). Relevance of prostate health to VDR/vitamin D signaling is highlighted by the significant association of low serum vitamin D status with aggressive prostate cancer [5–7]. Evaluation of prostate biopsies from prospectively enrolled men with abnormality in serum PSA levels and/or digital rectal examination revealed that African Americans with low (<20 ng/mL) serum 25-hydroxyvitamin D<sub>3</sub> have increased odds of prostate cancer diagnosis. This reinforces earlier results that prevalence of vitamin D deficiency among African Americans may partly account for higher incidences of aggressive prostate cancer and prostate cancer-related mortality in this race group. The same study on prostate biopsy outcomes further revealed that an increased race-independent probability for aggressive prostate cancer diagnosis, reflected in higher Gleason grades and advanced tumor stage, associated with severely low (<12 ng/mL) serum 25(OH) D<sub>3</sub> [5]. An intermediate range of 25(OH) D<sub>3</sub> (30–80 ng/mL) is considered a normal vitamin D status and may be optimal [5], since high serum vitamin D is thought to increase risks for advanced prostate cancer [8]. Inverse association has also been observed between serum vitamin D levels and breast cancer risks in postmenopausal women as well as breast cancer-related morbidity and mortality in premenopausal women [9, 10].

Prostate cancer is a common cancer in males worldwide and a leading cause of cancer deaths in US men, behind lung and colorectal cancer. Androgens, the male-prevalent sex steroids, and androgen receptor (AR), the transcription factor and nuclear receptor that mediates androgen action, regulate prostate cancer development, recurrence, and progression. Second-line inhibition of advanced prostate cancer by new-generation anti-AR/antiandrogen drugs and other intervention modalities is initially effective but non-durable [11]. A negative interplay of AR with VDR in prostate cancer cells, identified through our study and discussed in this review, leads to reduction of CYP24A1 (a cytochrome-P450 with 24-hydroxylase activity) and AR expression. Thus, a vitamin D/androgen combination may enhance inhibitory response of clinical prostate cancer to calcitriol, especially in the case of low-grade prostate cancer for which disease progression may be retarded by chemoprevention with vitamin D<sub>3</sub> supplementation.

In this article, we discuss (1) the enzyme machinery that drives calcitriol biosynthesis and metabolism to an inactive metabolite in the kidneys and local synthesis of calcitriol in prostate; (2) ligands, DNA response element, inter-domain allostery, and coregulators as determinants of VDR's transcriptional activity; and (3) inhibitory interaction of AR with VDR in prostate cancer cells. Finally, we describe

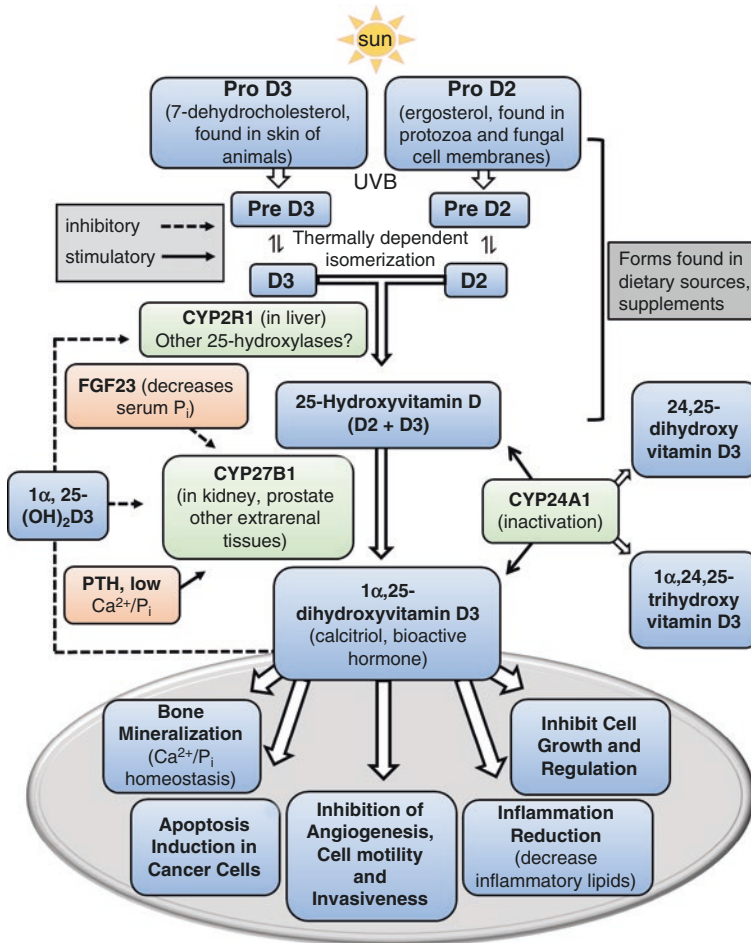
findings of preclinical studies and clinical trial outcomes for prostate cancer inhibition by calcitriol and analogs and comment on the prospects of uncovering novel interventions against prostate cancer by targeting downstream effectors of the VDR pathway in prostate. While this review primarily focuses on the current state of our understanding of the inhibitory vitamin D action on prostate cancer, a section briefly describing negative impacts of vitamin D signaling on breast cancer is also included.

## Vitamin D Metabolism

### *Synthesis, Degradation, and Roles and Regulation of Key Enzymes*

Calcitriol and its precursors are secosteroids with steroid-like structures. Figure 4.1 presents a schema of the biochemical pathway that leads to the cutaneous synthesis of cholecalciferol and its enzyme-catalyzed two-step hydroxylation, which ultimately produces 1,25-dihydroxy D<sub>3</sub> (calcitriol), the active metabolite [12]. Briefly, pro-D<sub>2</sub> (ergosterol) produced at the cell membranes of fungi and protozoa and pro-D<sub>3</sub> (7-dehydrocholesterol) produced in animal skin are secosteroid precursors of calcitriol, pro-D<sub>2</sub> being a weaker prohormone than pro-D<sub>3</sub>. Upon exposure to sunlight-derived UVB radiation, the prohormones are converted first to pre-D<sub>2</sub> and pre-D<sub>3</sub>, and then to D<sub>2</sub> (ergocalciferol) and D<sub>3</sub> (cholecalciferol), respectively—the latter conversion step requiring reversible heat-dependent isomerization. CYP2R1 is a 25-hydroxylase and the principal cytochrome P450 that converts D<sub>3</sub> or D<sub>2</sub> to the 25-hydroxy metabolite in the liver [13]. Additional 25-hydroxylase(s) may exist, since CYP 2R1 knockout caused 50% reduction of serum 25(OH)D<sub>3</sub> levels in mice [14]. A role for CYP 27A1 in the hydroxylation of vitamin D<sub>3</sub> at the carbon-25 position is ruled out, since CYP 2R1/CYP 27A1 double knockout mice did not show any further reduction of serum 25(OH)D<sub>3</sub> compared to CYP 2R1 single knockout mice. 25(OH) D<sub>3</sub> is the major circulating form of vitamin D in humans. In the event that endogenous synthesis of 25(OH) D<sub>3</sub> is not optimal, intake of cholecalciferol (vitamin D<sub>3</sub>) from dietary and supplemental sources can maintain normal vitamin D status. Exposure to endocrine-disrupting chemicals (EDCs) such as bisphenol A (BPA) and phthalates may reduce blood levels of 25(OH) D<sub>3</sub>, since urinary levels of these EDCs correlated inversely with serum vitamin D [15]. Intake of estrogen-containing oral contraceptives, in contrast, elevated serum 25(OH) D<sub>3</sub> [16]. It is of interest to know whether the effects of EDCs and estrogen are consequential to altered expression of 25 $\alpha$ -hydroxylase or to altered calcium or phosphate homeostasis.

Bioactivation of circulating 25(OH) D<sub>3</sub> to the active hormone 1 $\alpha$ ,25(OH)<sub>2</sub> D<sub>3</sub> (calcitriol) occurs primarily in the kidneys and is mediated by 1 $\alpha$ -hydroxylase (CYP27B1), which catalyzes additional hydroxylation at the carbon-1 position. Extrarenal tissues including prostate are also the sites of 25(OH)D<sub>3</sub> bioactivation to calcitriol [1, 12]. Homeostasis in cellular signaling by vitamin D is ensured by cal-



**Fig. 4.1** The pathway to calcitriol ( $1\alpha, 25(\text{OH})_2 \text{D}_3$ ) biosynthesis and metabolic inactivation. *UVB* ultraviolet B radiation, *CYP2R1* cytochrome P450 2R1, *FGF23* fibroblast growth factor 23, *PTH* parathyroid hormone, *CYP27B1* cytochrome P450 27B1, *CYP24A1* cytochrome P450 24A1,  $P_i$ , phosphate. Dotted arrows, inhibition; solid arrows, stimulation

citriol inactivation to  $1\alpha, 24, 25$  trihydroxy  $\text{D}_3$  (calcitroic acid) through a five-step oxidation process catalyzed by *CYP24A1*, which is a cytochrome P450 with 24-hydroxylase activity. *CYP24A1* also converts  $25(\text{OH}) \text{D}_3$  to the inactive  $24, 25$ -dihydroxy  $\text{D}_3$  (Fig. 4.1).

Calcitriol production is autoregulated as a result of the suppression of *CYP27B1* and *CYP2R1* gene transcription by calcitriol-activated VDR. Further, *CYP24A1*, the catabolic enzyme, is induced by a high serum concentration of calcitriol. Calcitriol homeostasis, which is vital for bone mineralization and bone health, is also regulated by calcium and phosphate levels. When phosphate levels are elevated, the bone-derived circulating peptide *FGF23* (fibroblast growth factor-23), which

regulates secretion of the parathyroid hormone (PTH), interacts with a FGF receptor-Klotho complex, to generate intracellular signaling that leads to inhibition of *CYP27B1* gene transcription. This in turn prevents calcitriol production and phosphate reabsorption in the kidneys [17, 18]. Conversely, at low phosphate and calcium levels, *CYP27B1* gene is transcriptionally stimulated by PTH-induced intracellular signaling, leading to increased renal production of calcitriol and re-adsorption of  $\text{Ca}^{2+}$  and phosphate by the kidneys.

Clinical manifestations of vitamin D deficiency are linked to mutations of the enzymes that drive synthesis and degradation of calcitriol, as observed for the loss-of-function germ line mutation of the *CYP2R1* [13, 19]. *CYP27B1* mutations, which lead to vitamin D-dependent rickets type 1, are associated with hypocalcemia and hypophosphatemia and the resultant development of fracture-prone soft, weak bone and bowed legs [20]. The biochemical hallmark of *CYP24A1* inactivation is the persistent escalation of calcitriol levels. *CYP24A1* mutations cause a range of clinical abnormalities due to hypercalcemia and hypercalciuria. These abnormalities include disrupted digestive homeostasis, aberrant renal functions, kidney stone, bone and muscle weakness, and abnormal brain functions. *CYP24A1* mutations leading to increased vitamin D sensitivity in patients with idiopathic infantile hypercalcemia have been reported [21].

### ***Vitamin D Metabolism in the Prostate***

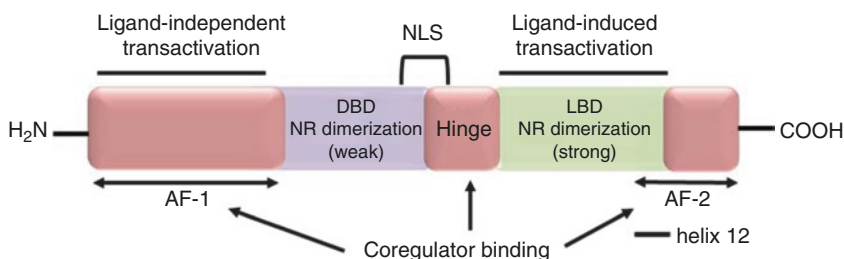
Calcitriol is synthesized in the prostate from the precursor 25(OH)  $\text{D}_3$  in the bloodstream. In a randomized clinical trial, a dose-dependent increase in calcitriol levels was detected in prostatectomy specimens from prostate cancer patients who received oral administration of cholecalciferol, and proliferation marker Ki67 levels correlated inversely with prostate calcitriol levels [22]. Ki-67 protein expression is strictly linked to cell proliferation. Calcitriol production in extrarenal tissues is essential for its intracrine, autocrine, or paracrine action, which is consistent with a ubiquitous expression pattern of *CYP27B1* and vitamin D receptor [23]. Exogenously added 25(OH)  $\text{D}_3$  converted to calcitriol in PC-3 and DU-145 (androgen receptor negative) prostate cancer cells and in cells isolated from normal and BPH prostate samples, and clotrimazole, a nonselective cytochrome P450 inhibitor, prevented this metabolic conversion [24]. 25-hydroxy  $\text{D}_3$  did not metabolize to calcitriol in LNCaP prostate cancer cells, which lack *CYP27B1* expression. It was reported that dietary supplementation of cholecalciferol and intraperitoneal injection of calcitriol reduced tumor burden from PC-3 prostate cancer xenografts at similar efficiency [25]. *CYP27B1* expression was elevated in the xenograft tumor tissue in parallel to increased circulating calcitriol when cholecalciferol was added to mouse diet. Since the *CYP27B1* level did not change in the kidneys under this condition, it was concluded that the elevated calcitriol level in circulation was due to conversion of diet-administered vitamin  $\text{D}_3$  to calcitriol in the tumor tissue [25].

## Transcription Regulation by VDR: Domain Structure and Roles for Ligands, DNA Response Elements, and Coregulators

### *Functional Domains of VDR and Inter-domain Allostery*

VDR is a ligand-inducible transcription factor and part of the 48-member human nuclear receptor (NR) family. Similar to other NRs, VDR has a functionally segmented structural organization, having a 24-amino-acid-long unstructured N-terminal domain followed by a DNA-binding domain (DBD) with two zinc finger motifs, an unstructured hinge domain, and a ligand-binding domain (LBD). Of the two zinc fingers within DBD, one associates with the retinoid X receptor (RXR), while the other aids in the formation of VDR homodimer. The unstructured hinge region grants flexibility to VDR's LBD, permitting its association with the RXR LBD. Upon DNA binding, interaction at the interface of VDR and RXR DBDs as well as interaction of the two LBDs stabilize the dimer. This stabilization circumvents the otherwise low DNA-binding affinity of a VDR monomer [26–28]. Figure 4.2 presents the basic aspects of the modular structure of VDR and other NRs. A ligand-binding pocket (LBP) is located in the interior of LBD, and a variable region (known as the F domain) follows LBD for some NR members. A coactivator- or corepressor-binding surface is generated at the agonist-/antagonist-bound LBP as a consequence of conformational realignment of 12 alpha-helices present within LBD. Conformational details for each functional domain and for the ligand-bound, DNA-associated full-length receptor came into light from structural studies of VDR and other NRs utilizing X-ray crystallography and cryo-electron microscopy and from NR's solution structures, which were analyzed by small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), and hydrogen-deuterium exchange [29–32].

Allosteric interactions among various VDR domains are evident from structural and biochemical analyses [30–33]. Hydrogen-deuterium exchange profiling of the VDR/RXR heterodimer detected increased solvent exchange at the DBD



**Fig. 4.2** Functional domains of a nuclear receptor (NR): a general scheme. *DBD* DNA-binding domain, *LBD* ligand-binding domain, *AF* activation function, *NLS* nuclear localization signal, *NH<sub>2</sub>* amino end, *COOH* carboxyl end, *Helix12* the alpha helix-12

upon ligand binding to VDR, indicating that the conformational change of LBD due to ligand occupancy impacted DBD conformation and, thus, the affinity of DBD for the cognate DNA element. This conformational change would potentially alter gene expression. Signal relay from DBD to LBD was evidenced by the result that interaction of the SRC1 coactivator with VDR and the VDR/RXR complex was altered when VDR is bound to a DR3 DNA element. NTD-DBD interplay is revealed from our result that a VDR polymorphic site (*FokI-FF*), which deletes three amino acids at the VDR NTD, abolished *CYP24A1* repression by unliganded VDR [34].

## *Naturally Occurring VDR Ligands*

### **Calcitriol, Calcidiol, and Secondary Bile Acids**

Calcitriol is the primary stimulus for VDR action, although 25(OH)D<sub>3</sub> (calcidiol) at high concentrations can bind and activate VDR and induce VDR-mediated gene transcription in vitro and in vivo [35, 36]. For example, calcidiol at high nanomolar range (400–1000 nM) induced *Cyp24a1* mRNAs in primary mouse cells isolated from the prostate and kidneys of *Cyp27b1* *-/-* mice, and in the case of LNCaP human prostate cancer cells, treatment with calcidiol at 500 nM inhibited cell proliferation with or without the presence of the 1 $\alpha$ -hydroxylase inhibitor SDZ, indicating that the inhibition was independent of calcitriol [36]. A synergistic effect of calcidiol and calcitriol in *Cyp27b1*-null cells was also observed.

Target genes for VDR include enzymes of the drug- and steroid-metabolizing machinery. VDR-mediated transactivation of genes encoding phase I oxidative enzyme, phase II conjugative enzyme, and phase III transporters has been widely reported [3, 37, 38]. Variable serum calcidiol and calcitriol levels arising from season-dependent differential sunlight exposures correlated with seasonal differences in the intestinal expression of *CYP3A4*, which is a VDR-regulated phase I gene [39].

The secondary bile acids LCA (lithocholic acid) and 3-keto LCA bind VDR with micromolar affinity as a result of relatively large ligand-binding pocket (LBP) of VDR. LCA-activated VDR facilitates metabolism of the toxic secondary bile acids by transactivating phase I and phase II genes in hepatic and enteric cells, and clearance of the LCA metabolites by inducing phase III transporter genes. Crystal structures of the VDR's LBD bound to LCA and 3keto-LCA have been solved [40]. For LCA to function as a VDR agonist, two LCA molecules must bind to the LBD [41]. The crystal structure of zebrafish LBD bound to LCA showed binding of one LCA molecule to the ligand-binding pocket (LBP) and the second LCA molecule residing on the LBD surface outside of LBP. The low-affinity second site stabilizes the active receptor conformation. The second binding site outside of LBP is a promising candidate target site for drug design and other computational studies.

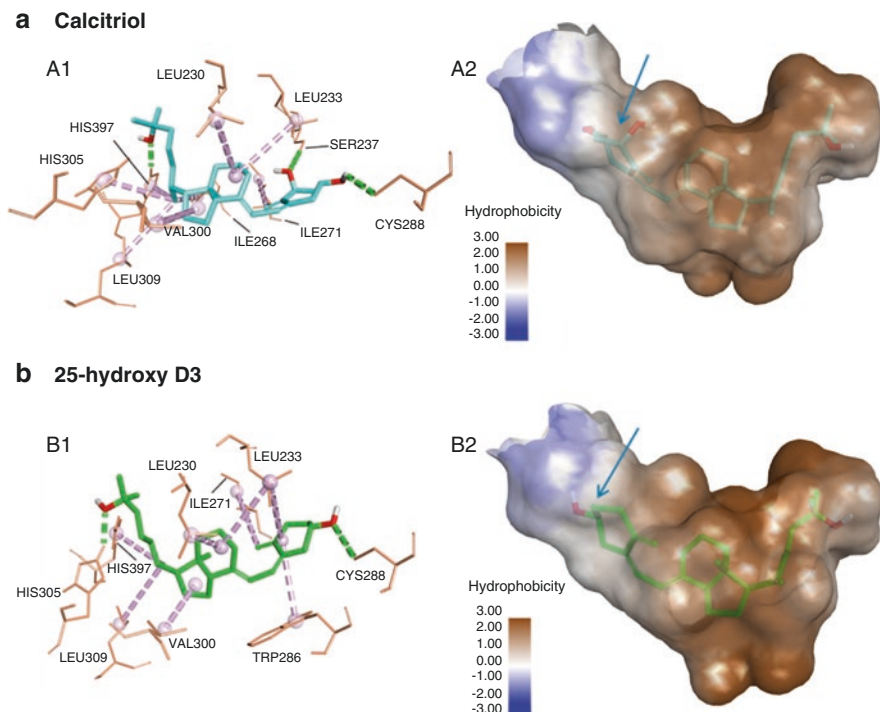
## Agonist Actions of Calcitriol and Calcidiol on VDR: Analysis by Molecular Docking

The X-ray crystallographic structure for calcitriol has been solved [42]. Molecular docking, which is a computational analysis of receptor interactions with synthetic ligands and chemically modified derivatives of natural ligands, can complement crystallography results. This approach is routinely utilized for rational drug design and development of more potent and less toxic analogs. On the basis of published information on a crystal structure of VDR in complex with calcitriol (*PDB ID: 1DB1*), we modeled interactions of the LBP of VDR with calcitriol (Fig. 4.3A1, A2) and with calcidiol (Fig. 4.3B1, B2) using the program AutoDock Vina™ [43]. The goal for this modeling exercise was to: (a) explore if calcitriol is able to bind to VDR more strongly at a site outside of LBP than a site inside of LBP—the former site potentially would lead to an allosteric influence on VDR activity; and (b) compare calcitriol vs. calcidiol binding to VDR's LBD.

We used AutoDock Vina™ in conjunction with PyRX® (version 0.8, [44]) interface to dock calcitriol and calcidiol to the LBD of VDR. The top-scoring binding pose (orientation) for calcitriol shared a pose that closely resembled the published crystal structure. Out of ten binding poses determined from our analysis, none detected ligand residency outside of LBP. Post-docking analysis using geometry optimization in Discovery Studio Visualizer® [45] revealed two polar hydrogen bonds between the hydroxyl groups (oxygen shown in red) on the carbon-1 and carbon-3 of calcitriol and polar amino acid residues SER-237 and CYS-288, respectively, of VDR (Fig. 4.3A1). An additional hydrogen bond appeared from interactions between carbon-25 of calcitriol and HIS-397 of LBP (Fig. 4.3A1). Upon calcidiol docking to VDR's LBD using similar conditions (as for calcitriol), the top-scoring binding pose of calcidiol with LBP is similar to what was reported in a molecular dynamics study [46]. The hydroxyl group at carbon-3 of calcidiol forms a hydrogen bond with CYS288 (Fig. 4.3B1) similar to the carbon-3 hydroxyl group of calcitriol. The hydroxyl group at carbon-25 of calcidiol forms hydrogen bond with HIS-305, not HIS-397 as seen for calcitriol (Fig. 4.3B1). Molecular surface rendition shows hydrophobicity of different regions of LBP residues (Fig. 4.3A2 and B2). Molecular surfaces show that hydrophobic interactions are formed mainly between the hydrophobic portions of calcitriol and calcidiol and hydrophobic residues of LBP, whereas hydrogen bonding occurs at the less hydrophobic regions of LBP. The scale for hydrophobicity goes from brown (more hydrophobic) to blue (more hydrophilic) (Fig. 4.3A2, B2).

From the above results, we infer that the additional hydrogen bond in the case of calcitriol is likely to account for the drastically increased potency of calcitriol over calcidiol observed from *in vitro* and *in vivo* experiments. Nonetheless, the favorable binding of calcidiol within the LBP, as reported earlier [46] and revealed here from our docking analysis (Fig. 4.3), supports the experimental findings that calcidiol can serve as a VDR agonist *in vitro* and *in vivo*, albeit at two orders of magnitude higher concentrations than calcitriol [35, 36]. Our modeling study is the further evidence that computational programs are useful tools in describing the agonist action of a molecule of interest. A docking approach can be insightful in the development of synthetic agonists and antagonists of VDR and other nuclear receptors.





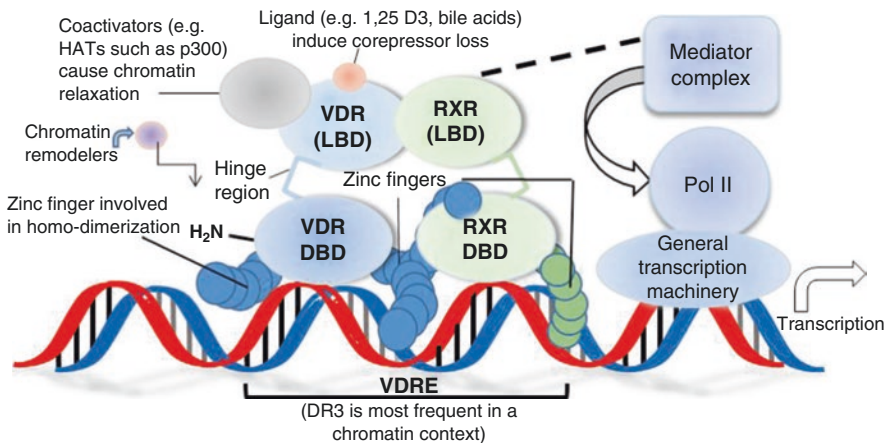
**Fig. 4.3** Docking of calcitriol (**a**) and 25-hydroxyvitamin D<sub>3</sub> (**b**) to the ligand-binding domain (LBD) of VDR and post-docking analysis. Docking was done via AutoDock Vina™. A1: *Calcitriol* (blue) docked to LBD. The top-scoring binding pose of calcitriol at LBD occurred within the ligand-binding pocket (LBP). This pose (orientation) was optimized using a Dreiding-like force field in Discovery Studio Visualizer® to maximize strengthening interactions and minimize repulsive interactions. Purple and pink dotted lines indicate hydrophobic interactions (eight total); green dotted lines indicate hydrogen bonds (three total). A2: *Molecular surface rendition of hydrophobicity of calcitriol-bound LBP*. The hydrophobicity scale: brown, most hydrophobic; blue, most hydrophilic. The blue section at left depicts the region (blue arrow) where polar residues CYS288 and SER237 form hydrogen bonds with carbon-3 and carbon-1 hydroxyl groups (red atoms) of calcitriol, respectively. B1: *25(OH)D<sub>3</sub> (calcidiol, shown in green) docked to LBD*. Top-scoring binding pose at LBD occurred within LBP. This pose was optimized as in A1 to maximize strengthening interactions and minimize repulsive interactions. Purple and pink dotted lines signify hydrophobic interactions (eight total); green dotted lines signify hydrogen bonds (two total). B2: *Molecular surface rendition of hydrophobicity of calcidiol-bound LBP*. Hydrophobicity scale: brown, most hydrophobic; blue, hydrophilic. A small blue section at left depicts the region (blue arrow) where the polar CYS288 forms a hydrogen bond with the carbon-3 hydroxyl group (red atom) of calcidiol

### ***Gene Regulation by VDR: Roles for Coregulators and DNA Response Elements***

VDR regulates transcription of more than 1000 genes in skeletal and extraskelatal tissues and thus can impact diverse physiologies. In prostate cancer cells, by

comparing transcriptome profiling of calcitriol-treated vs. untreated prostate cancer cells, we found key genes in the androgen biosynthesis and metabolism pathways are regulated by VDR. Calcitriol robustly induced CYP24A1 mRNAs in prostate cancer cells (Fig. 4.6) and in primary prostate cells [36]. Regulatory elements driving CYP24A1 induction by ligand-activated VDR have been characterized [47]. VDR-targeted genes regulating calcium and phosphate homeostasis (such as osteocalcin, osteopontin, D9K, D28K, FGF2, PTH, SLC34A2) are most abundantly expressed in the intestine, kidneys, and bone where absorption and storage of calcium and phosphate is crucial. RANKL, another VDR target, regulates osteoclast survival and differentiation. A general scheme for VDR-mediated target gene induction by VDR and roles of several genetic and epigenetic factors in this process are schematically shown (Fig. 4.4).

Ligand-free nuclear VDR remains attached to the chromatin in a repressed state through association of its LBD with corepressors such as NCoR1 (nuclear corepressor1), SMRT/NCoR2, which in turn recruit a histone deacetylase (HDAC) complex, leading to a VDR-containing compact chromatin region and gene repression similar to what is observed for the thyroid hormone receptor and retinoic acid receptor [48]. Chromatin-bound non-liganded VDR stays associated with RXR, its heterodimer partner (the  $\alpha$  isoform RXR- $\alpha$  in majority of cases). This overall picture is consistent with our result, which showed that VDR silencing elevated the basal expression of



**Fig. 4.4** Gene transcription by VDR: roles of upstream regulators. VDR/RXR heterodimer binds a VDRE (frequently DR3 type, especially in vivo). Zinc fingers maintain VDR binding to VDRE and its association with RXR. Agonist activation of VDR leads to release of VDR-associated corepressors including HDACs, which would otherwise maintain chromatin in a condensed form due to histone deacetylation. Corepressor to coactivator exchange at VDRE causes chromatin relaxation (from acetylation), recruitment of chromatin remodelers, as well as a mediator complex—the latter relaying signal (open arrow) to general transcription machinery. Dotted line reflects functional interaction. VDR vitamin D receptor, RXR retinoid X receptor, LBD ligand-binding domain, DBD DNA-binding domain,  $H_2N$  amino terminus, HATs histone acetyltransferases, Pol II RNA polymerase II, VDRE vitamin D response element, DR3 direct repeat with a 3-nucleotide spacer

CYP24A1 in breast cancer cells [34]. As a nonpermissive heterodimer, the VDR/RXR complex is activated by calcitriol but not by the RXR ligand 9-cis retinoic acid [49]. Upon agonist binding, a conformational change involving the 12 helices within the VDR's LBD leads to repositioning of the helix-12 to create an interaction surface that favors recruitment of the p160 coactivators (SRC-1, SRC-2, SRC-3) and departure of the NCoR/SMRT corepressors. A pioneer factor is involved in the initial relaxation of the chromatin [47, 50]. The p160 coactivator makes physical contact with VDR through an LXXLL (leucine xx leucine leucine) motif and recruits histone acetyltransferases (HATs) such as CBP/p300 and p/CAF. The HATs mediate acetylation of lysine and arginine residues of histones H3 and H4 locally and also at other regions where promoters are present, thus initiating transcription throughout the gene locus [51]. Chromatin relaxation by acetylation leads to the assembly of additional coregulatory complexes including histone modifiers (methyltransferase/demethylase, ubiquitin ligase/deubiquitinase, kinase/phosphatase) and chromatin remodelers (such as SWI/SNF-containing WINAC complex) [48]. A 26-subunit mediator complex integrates the final transcriptional regulation by serving as the conduit for relaying the regulatory signal from DNA-associated VDR to the basal transcription machinery and RNA polymerase II at the transcription start site [52] (Fig. 4.4).

In the chromatin, the preferred genome-wide vitamin D-responsive binding element (VDRE) for the VDR/RXR heterodimer is a direct repeat of the consensus half-site sequence AG(G/T)TCA with three intervening nucleotides (DR3) [53]. Genes with clusters of DR3 type VDREs preferably respond to the transcriptional regulation by ligand-activated VDR. VDR contacts the 6-base 5' half-site within the major groove of DNA via the first zinc finger module of DBD. The second zinc finger of VDR associates with the RXR DBD. A DR3 element mediates robust VDR-mediated transactivation of *CYP24A1* by 1,25-D<sub>3</sub> [29]. Other DR configurations (most frequently DR4, DR5) are also involved in vitamin D-mediated induction of target genes. We reported the involvement of a DR7-type VDRE in the VDR-mediated induction of *SULT2B1* sulfotransferase in transfected prostate cancer cells. *SULT2B1b* (in short *SULT2B*) is a cholesterol and DHEA sulfotransferase that is present at relative abundance in the normal human prostate. *SULT2B* levels are reduced in primary prostate cancer [54], and its expression is non-detectable in >90% cases of distant metastasis (B. Chatterjee, unpublished).

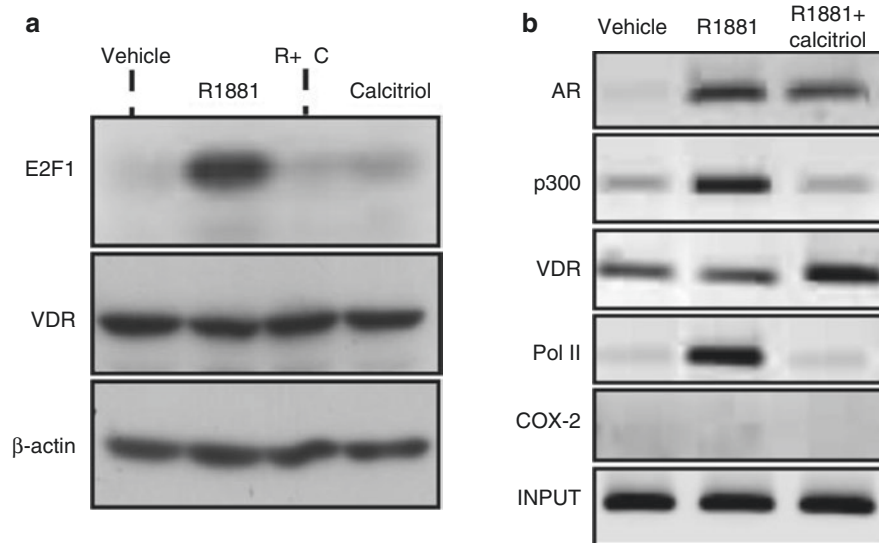
Gene repression by VDR can occur through a direct mechanism, known as transrepression [48]. Reduced *CYP27B1* gene transcription by calcitriol in the kidneys entails association of the ligand-bound VDR with several negative vitamin D response elements (nVDRE) [55]. The nVDREs in the *CYP27B1* upstream promoter are of two types—one having sequence organization resembling a positive VDRE with specific affinity for the VDR/RXR dimer in a ligand-dependent manner and the other with binding specificity for a specific transcription factor such as the VDR interacting repressor (VDIR), which is inhibited due to interference from the tethered VDR or by VDR bound to a nVDRE that bears no similarity to a classical VDRE. A DR4-type nVDRE mediates transrepression of the *CCNC* gene, which codes for cyclin C [29].

## **Interplay of Androgen and Vitamin D in Prostate: Impacts on Cell Proliferation, Vitamin D Metabolism, and the AR Pathway**

### ***Effects on Cell Cycle Regulation and E2F1, a Key Cell Cycle Regulator***

Calcitriol-mediated inhibition of normal and malignant prostate cell proliferation has been widely documented in various experimental models such as primary cells isolated from normal and malignant prostate tissue, immortalized normal epithelial cell lines, tumor-derived prostate cancer cell lines, and xenograft tumors of prostate cancer cells. The inhibition involves diverse mechanisms including the G1/S arrest of cell cycle, apoptosis, pro-differentiation changes, microRNA regulation, and diminished angiogenesis (reviewed in [4] and references therein). E2F1 transcription factor activity drives expression of DNA synthesis genes, and loss of E2F1 function, upon its sequestration by accumulated hypo-phosphorylated retinoblastoma protein (Rb), leads to cell cycle block at the G1 → S checkpoint. Hypo-phosphorylated Rb accumulates in calcitriol-treated cells due to CDK2 inactivation by elevated p21/Waf1 (a CDK2 inhibitor). Also, calcitriol triggered CDK2 exit from the nucleus, thereby preventing CDK2-cyclin interaction and, hence, CDK2 activation. Calcitriol also caused nuclear accumulation of the CDK2 inhibitor p27/Kip1 [56]. CDK2 (cyclin-dependent kinase 2) is a serine/threonine kinase with a key role in cell cycle progression, especially during the G1 to S phase transition. Partner proteins such as cyclin E or cyclin A and the cell cycle inhibitor p21/Cip1 (CDKN1A) or p27/Kip1 (CDKN1B) regulate CDK2 activity.

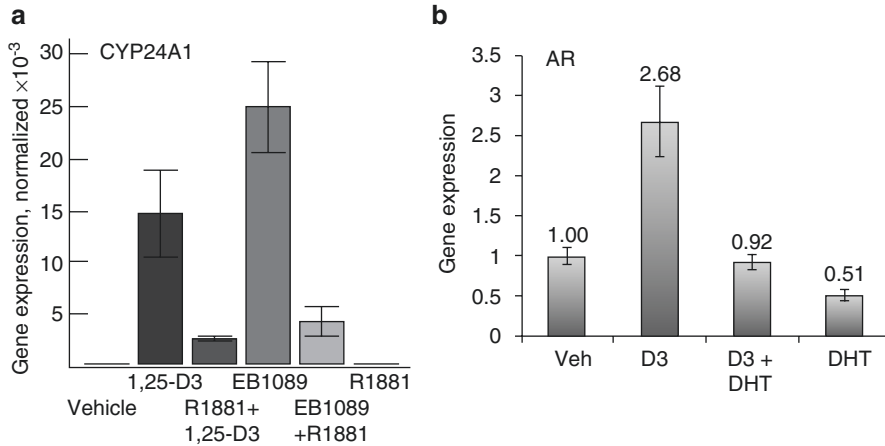
The mitogenic impact of androgen on prostate cancer cells can be blocked by calcitriol, since increase in the proliferation of androgen-stimulated LNCaP prostate cancer cells was prevented by concurrent treatment of the cells with calcitriol. E2F1 induction by androgen was also blocked by calcitriol (Fig. 4.5a), and a parallel reduction of the androgen-stimulated E2F1 promoter activity in the presence of calcitriol could be demonstrated in transfected LNCaP cells in promoter-reporter assay. Chromatin immunoprecipitation (ChIP) demonstrated engagement of the androgen receptor (AR), the coactivator p300 (a histone acetyltransferase) and the transcriptionally competent RNA polymerase II (pol II, the serine5-phosphorylated form) at an androgen-responsive E2F1 upstream promoter region in androgen-treated LNCaP cells (Fig. 4.5b). VDR occupied the same region in the E2F1 promoter independent of calcitriol treatment, which is expected since nuclear VDR remains chromatin-bound in the ligand-free state as well. AR occupancy persisted at the androgen-responsive region during E2F1 de-induction by calcitriol, indicating that AR and VDR are part of the same corepressor complex (Fig. 4.5b). Characterization of individual components of the corepressor complex may identify druggable upstream regulators involved in E2F1 gene repression by calcitriol in androgen-stimulated cells.



**Fig. 4.5** Loss of androgen-mediated induction of E2F1 in prostate cancer cells in the presence of calcitriol. **(a)** Western blot assay of E2F1 levels in LNCaP cell lysates after various treatments. R1881: synthetic androgen, non-metabolizable; C: calcitriol. **(b)** Chromatin immunoprecipitation (ChIP) from LNCaP cells for analysis of the recruitment of AR, the coactivator p300 (a histone acetyltransferase), VDR, RNA polymerase II (Pol II) to the androgen-responsive region in the E2F1 promoter after cells were treated with vehicle, R1881 and R1881 plus calcitriol. The androgen-responsive sequence in the E2F1 promoter is located at around  $-400$  upstream region. ChIP with anti-COX2 antibody was a negative control (COX-2, cyclooxygenase-2). An equal protein amount of the supernatant of sonicated cells from each treatment was used for ChIP of individual proteins, shown by similar input signals

### *Effects on Vitamin D Metabolism and on the Intratumoral AR Axis*

In certain contexts, androgen facilitates the antiproliferative activity of calcitriol. For example, calcitriol reduced the prostate size of testis intact but not castrated Sprague-Dawley rats [57], and androgen-dependent, AR-positive LNCaP prostate cancer cells were inhibited by calcitriol more robustly than androgen-independent, highly aggressive PC-3 and moderately aggressive DU-145 prostate cancer cells [58]. PC-3 and DU-145 cells are mostly considered AR-negative, although very weak AR expression in these cells were noted in some reports. Androgen nearly completely blocked marked upregulation of CYP24A1 mRNAs by calcitriol in AR-expressing C4-2B castration-resistant prostate cancer cells, while androgen alone had no detectable effect on CYP24A1 (Fig. 4.6a). A similar result on the androgen-regulated inhibition of CYP24A1 induction by calcitriol in LNCaP cells was previously reported [59]. Since CYP24A1 converts



**Fig. 4.6** Androgen-regulated loss of induction of CYP24A1 and androgen receptor (AR) mRNA in calcitriol-treated cells. Castration-resistant C4-2B prostate cancer cells were analyzed for the mRNAs using quantitative RT-PCR. The mRNA levels (indicated as gene expression) were normalized to  $\beta$ -actin mRNAs. *EB1089* a calcitriol analog, *D3* abbreviation for 1,25(OH)<sub>2</sub>D<sub>3</sub>, *DHT* 5 $\alpha$ -dihydrotestosterone

calcitriol to an inactive metabolite, its induction is normally beneficial for ensuring vitamin D homeostasis. By contrast, in the context of the vitamin D's role as an antiprostate cancer hormone, CYP24A1 induction assumes an adversarial role due to degradation of bioactive hormonal vitamin D. Indeed, pharmacologic inhibition of CYP24A1 by RC2204, a specific CYP24A1 inhibitor, or by ketoconazole, a general inhibitor of the cytochrome P450 family of enzymes, augmented calcitriol's anticancer effects on PC-3 prostate cancer cells, evidenced by heightened suppression of the clonogenic survival of the cells in culture, greater reduction of xenograft tumor burden, and more robust activation of caspase-independent apoptosis [60]. In the same study, pharmacokinetics showed that serum calcitriol levels were higher when normal mice (nontumor bearing) received calcitriol plus a CYP24A1 inhibitor compared to mice receiving calcitriol alone.

Androgen prevented calcitriol-mediated induction of AR (Fig. 4.6b). Thus inhibitory interplay between androgen and vitamin D signaling can interfere with an AR-driven growth pathway. We also observed that androgen prevented calcitriol-mediated induction of 3 $\beta$ -hydroxysteroid dehydrogenase-1 (HSD-3 $\beta$ 1), which is a major driver of testosterone and DHT biosynthesis. HSD-3 $\beta$ 1 catalyzes conversion of dehydroepiandrosterone (DHEA) to androstenedione. Association of advanced prostate cancer with HSD-3 $\beta$ 1 mutations, which enhanced stability and activity of this enzyme, has been reported [61]. Results in Fig. 4.6 suggest that a vitamin D/androgen combination therapy may be beneficial due to higher calcitriol availability and reduced androgen-AR signaling.

## Prostate Tumor Suppression by Calcitriol and Analogs: Outcomes of Preclinical Studies and Clinical Trials

### *Preclinical Studies*

Calcitriol is consistently effective in reducing tumor burden and metastases for castration-sensitive and castration-resistant prostate cancer in xenograft and allograft tumors and in prostate tumors developed in genetically altered mice. Calcitriol can also act synergistically to enhance the efficacy of chemotherapy drugs against prostate cancer [1, 62]. Dietary supplementation of vitamin D<sub>3</sub> and intraperitoneally injected calcitriol reduced tumor growth of PC-3 prostate cancer cell xenografts with similar efficacy. These treatments caused intratumoral induction of mRNAs for CYP24A1, 15-PGDH (15-prostaglandin dehydrogenase), IGFBP-3 (insulin-like growth factor-binding protein-3), p21/Cip1 (inhibitor of cyclin-dependent kinases), and repression of cyclooxygenase-2 and EP4 prostaglandin receptor mRNAs [25]. Consistent with these changes, a combination of calcitriol and a nonsteroidal anti-inflammatory drug (NSAID) caused synergistic inhibition of prostate cancer xenografts [25].

A chemopreventive role of calcitriol was revealed from its ability to prevent progression of PIN (prostate intraepithelial neoplasia) lesions in *Nkx3.1*; *Pten* mutant mice to dysplastic and neoplastic lesions [63]. Early intervention of TRAMP mice with calcitriol raised levels of E-cadherin (a pro-differentiation marker) in the tumor tissue and reduced early growth of primary prostate tumor under androgen stimulation [64]. TRAMP mice develop prostate cancer as a consequence of SV40 (simian virus 40) T antigen expression in the prostate. Continued calcitriol administration, however, enhanced distant organ metastases in these mice, indicating that long-term calcitriol therapy is potentially harmful. This result is somewhat reminiscent of the clinical finding that high serum levels of 25(OH)D<sub>3</sub> elevate risks for advanced prostate cancer [8].

A number of calcitriol analogs have been synthesized by introducing chemical modification either at the A ring, at the central CD ring, or at the side chain of the natural hormone in order to develop more potent and less calcemic vitamin D analogs [65, 66]. Many synthetic calcitriol analogs showed higher anticancer potency than the natural hormone [67, 68]. For instance, inecalcitol (19-nor-14-epi-23-yne-1,25(OH)<sub>2</sub>D<sub>3</sub>), a highly potent calcitriol analog, is several hundredfolds less hypercalcemic than calcitriol. In one study, the maximum tolerated dose of inecalcitol injected intraperitoneally three times per week was found to be 30 µg per injection per mouse, whereas the tolerated dose for calcitriol under the same condition was 0.0625 µg per injection per mouse [67]. Distinct conformations of the inecalcitol-bound versus calcitriol-bound VDR/RXR complex account for differential affinities of these complexes for VDR response elements, which results in an agonist-selective unique transcriptome profile. The markedly reduced hypercalcemia in inecalcitol-administered mice is thought to be the result of a unique profile of gene expression driven by inecalcitol-bound VDR [69].

## *Clinical Trial Outcomes*

Several large-scale trials led to the conclusion that as monotherapy, calcitriol or its synthetic analogs did not provide any clinical benefit against prostate cancer. The hypercalcemic effect of hormonal vitamin D at clinical doses elevated risks for cardiovascular disease and lethal prostate cancer [1, 67, 70]. In randomized controlled trials, patients with metastatic prostate cancer receiving a combination of  $\alpha$ -calcidol and docetaxel had no benefit for overall survival or PSA response [71]. An ongoing randomized Vitamin D and Omega-3 Trial (VITAL) on ~26,000 US participants with no prior history of major illnesses is assessing whether daily supplements of cholecalciferol (vitamin D<sub>3</sub>) at the physiological dose of 2000 international unit with or without daily omega-3 fatty acid (fish oil, 1 g) supplements will reduce risks for various cancers including prostate cancer and lower risks for stroke and cardiovascular diseases [72]. Inecalcitol combined with docetaxel is being investigated for safety and pharmacodynamics in a phase I study on metastatic castration-resistant prostate cancer patients [62].

A more promising clinical benefit may arise from the chemoprevention activity of vitamin D, especially for low-risk prostate cancer. A pilot study evaluated the effect of enhancing serum vitamin D status by vitamin D<sub>3</sub> supplements (4000 IU per day) on prostate cancer progression for 44 patients who are on active surveillance due to diagnosis of low-risk prostate cancer. When these patients took a daily physiological dose of vitamin D<sub>3</sub> supplement for 1 year, positive cores decreased in number at repeat biopsy, and Gleason scores dropped for more than 50% of the subjects. Serum PSA levels were unchanged [73]. The yearlong vitamin D<sub>3</sub> intake had no adverse health impact. In a second pilot study with 37 patients awaiting elective prostatectomy, inflammatory lesions decreased dramatically in resected prostate cancer specimens from men who took a daily physiological dose (4000 IU) of vitamin D<sub>3</sub> for 2 months prior to prostatectomy [74]. For 60% of the patients in the vitamin D<sub>3</sub> group, tumors in resected tissues either shrank or disappeared, and inflammation was drastically reduced, revealed from lower levels of inflammation-related lipids and proteins. The growth factor differentiation factor-15 (GDF-15, also known as macrophage-inhibiting anti-inflammatory cytokine-1, MIC1), which is an anti-inflammatory cytokine, was elevated in the vitamin D<sub>3</sub> arm, whereas specimens from the placebo arm had biochemical evidence for severe inflammation and showed marked reduction of GDF-15. These results are consistent with an anti-inflammatory activity of the hormonal vitamin D<sub>3</sub> [2] and add credence to the concept that inflammation is closely linked to the etiology and progression of various cancers including prostate cancer [75]. Preclinical studies showed that 1,25 D<sub>3</sub> reduced the prostaglandin level in prostate tumors by causing diminished expression of COX-2 and the prostaglandin receptor and elevated expression of 15-prostaglandin dehydrogenase. Therefore, as proposed earlier [76], impacts of combined use of vitamin D<sub>3</sub> supplements with a nonsteroidal anti-inflammatory drug (NSAID) on prostate cancer should be thoroughly assessed.



Insensitivity to VDR/vitamin D signaling can contribute to a lack of response to vitamin D-based therapy. Altered VDR expression and transcriptional activity due to single nucleotide polymorphism of the *VDR* gene can occur. For VDR *FokI* polymorphism, the longer, less active VDR variant arises from the *f* allele, and a more active shorter VDR is produced by the *F* allele due to mRNA translation from a downstream ATG initiation codon. An *f* allele *VDR* combined with a low serum vitamin D status associated with ~twofold greater prostate cancer risk compared to an *FF* or *Ff* genotype and high serum 25(OH)D<sub>3</sub> [77]. The VDR *TaqI* polymorphism giving rise to synonymous mutation at exon-9 reduces mRNA stability for VDR, thereby lowering VDR abundance and its transcriptional activity. Vitamin D homeostasis is expected to be impacted by reduced VDR activity since genes involved in vitamin D biosynthesis and metabolism (e.g., *CYP2R1*, *CYP27B1*, *CYP24A1*) are transcriptionally regulated by VDR. In meta-analysis, VDR *TaqI* polymorphism significantly associated with prostate cancer risk in Asian and African-American men [78].

Epigenetic mechanisms may also contribute to vitamin D insensitivity. Critical genes influencing vitamin D signaling and vitamin D homeostasis, such as *VDR*, *CYP2R1*, *CYP27B1*, and *CYP24A1*, are potential targets for epigenetic silencing from DNA hypermethylation since long CpG islands localize in the promoter regions of these genes. Furthermore, certain histone demethylases such as the lysine-specific demethylases and Jumonji C domain-containing histone demethylases are direct targets of VDR-mediated transcription regulation [79]. Trichostatin A (TSA), which is a pan-HDAC (histone deacetylase) inhibitor, restored antiproliferative activity of calcitriol in PC-3 cell cells [80]. In another example, calcitriol did not inhibit proliferation of prostate cancer cells that overexpressed the corepressors NCoR1 and NCoR2/SMRT, whereas corepressor silencing rescued castration-resistant prostate cancer cells from the loss of response to calcitriol [80, 81]. Elevated expression of NCoR1 and SMRT was detected in prostate cancer cell lines and in the primary culture of malignant cells isolated from prostate tumor xenografts [81, 82]. NCoR1 and SMRT physically interact with VDR, and they are part of the repressive complex that associates with ligand-free, transcriptionally inactive VDR. They are also components of the corepressor complex that regulates VDR-mediated transrepression [83].

## Breast Cancer Suppression by Calcitriol

An inverse association of serum vitamin D levels with breast cancer risks in women is indicated from epidemiology and meta-analyses, although confounders such as disease heterogeneity and variable estrogen status of the study cohort have precluded any definitive conclusions in this regard. Recent results from a prospective cohort study of 1666 breast cancer survivors showed that patients with advanced breast cancer had lower serum levels of 25-hydroxyvitamin D<sub>3</sub>, with lowest levels

in premenopausal women who present with triple negative breast cancer [10]. The triple negative phenotype signifies a lack of the estrogen receptor (ER), progesterone receptor (PR), and growth factor receptor HER2/ErbB2, thus making triple negative breast cancer nonresponsive to available targeted therapies. Furthermore, serum vitamin D levels inversely correlated with hazards of breast cancer progression and death, and women at the highest tertile of serum vitamin D showed improved overall survival compared to women at the lowest tertile [10]. However, interpretation of these results did not take into account confounders such as smoking and alcohol consumption, which may reduce serum vitamin D status while increasing risks for cancer in general [84]. Clinical trials for assessing benefits of vitamin D supplementation for breast cancer chemoprevention are in progress [85].

In experimental models, calcitriol inhibited breast cancer, revealed from studies in cell culture, in mammospheres, and in animal models of mammary tumors [1, 86–88]. Calcitriol downregulated the expression of ER- $\alpha$  as well as aromatase (CYP19A1), the latter regulating estrogen biosynthesis by catalyzing aromatization of testosterone. The calcitriol effect on aromatase expression was tissue selective, since in MCF7 breast cancer xenografts, calcitriol reduced aromatase expression in both the tumor tissue and in the surrounding mammary adipose tissue while elevating aromatase in bone marrow cells and having no effect on aromatase levels in the ovary and uterus [87]. Blocking of the estrogen signal by an aromatase inhibitor (such as letrozole) is a standard-of-care for managing ER-positive mammary tumor—a phenotype carried by ~70% of breast cancer patients.

The vitamin D/VDR signaling axis suppressed breast cancer in a tumor autonomous manner. A recently reported study demonstrated that xenografts from VDR-ablated breast cancer cells grew more rapidly and showed enhanced metastasis to distant organs, whereas ectopic expression of VDR in VDR-silenced breast cancer cells reduced xenograft growth and metastasis [89]. Enhanced cancer cell metastasis in VDR-knocked down xenografts was in part due to derepression of the ID1 (inhibitor of DNA binding 1), which is known to aid in tumor progression. For breast cancer patients, serum 25-hydroxy vitamin D<sub>3</sub> levels at presurgery inversely correlated with the abundance of ID1 in the tumor tissues of resected specimens. A negative VDRE in the *ID1* gene mediates the repressive effect of ligand-activated VDR [89]. Other examples of VDR-suppressed genes that are involved in breast cancer invasion and metastasis include those encoding uPA (urokinase-type plasminogen activator), periostin (a secreted heparin-binding protein), HbEGF (a heparin-binding EGF-like growth factor), and hyaluronan synthase [90]. VDR-mediated repression of these genes was revealed from a comparative analysis of the gene expression profiles of breast tumor cells from carcinogen-exposed wild-type vs. Vdr-null mice [86]. Periostin promotes epithelial to mesenchymal transition (EMT), while uPA is functionally linked to stromal invasion and migration of cancer cells owing to its role in the cleavage of plasminogen that leads to the activation of metalloproteases. Periostin and uPA expressions are elevated in breast cancer. Binding of the cell surface receptor CD44 to hyaluronic acid (HA), the extracellular proteoglycan which is synthesized by the catalytic action of hyaluronan synthase, triggers a sequence of events that lead to the activation of intracellular signaling induced by specific

ligand-activated growth factor receptors, thereby promoting cancer cell survival [86]. CD44-mediated cell survival signaling in triple negative breast cancer cells was suppressed by calcitriol [91]. Furthermore, repression of the breast cancer stem cell-like population by calcitriol was also demonstrated [88]. Insights into selected downstream effectors of the VDR pathway may be leveraged in pursuit of novel interventions against breast cancer.

## Conclusion

Most extraskeletal human tissues express vitamin D receptor (VDR) and thus are subject to transcription regulations by vitamin D/VDR signaling, which targets more than 1000 genes [92]. Insufficient serum 25-hydroxyvitamin D<sub>3</sub> and an errant VDR pathway elevate risks for a number of pathologies, most notably those arising from malignant cell growth, insulin resistance, atherosclerosis, and illnesses from immune system dysfunctions. Biosynthesis of calcitriol (1 $\alpha$ ,25(OH)<sub>2</sub> D<sub>3</sub>), the natural ligand for VDR, occurs primarily in the kidneys. Local synthesis of calcitriol from circulating 25(OH)D<sub>3</sub> also occurs in prostate, breast, and other VDR-responsive extraskeletal tissues. CYP27B1, the 1 $\alpha$ -hydroxylase that converts 25(OH) D<sub>3</sub> (calcidiol) to calcitriol, is expressed in both normal and cancerous cells of endocrine tissues such as the prostate and breast.

In a randomized dosing study, orally administered vitamin D<sub>3</sub> (cholecalciferol) caused a dose-dependent increase of calcitriol in the surgical specimens of prostate cancer patients who were treated for 2 months prior to prostatectomy. Serum PSA and parathyroid hormone levels also decreased, albeit modestly [22]. Reduced expression of the proliferation marker Ki-67 in the resected samples correlated with intra-prostate elevation of calcitriol. This is consistent with growth inhibition of prostate cancer cells by calcitriol—a finding that has been validated extensively in cell culture and animal experiments. Calcitriol analogs with markedly lower calcemic toxicity and robust agonist action on VDR at sub-nanomolar concentrations have demonstrated antiprostata cancer activity. Inecalcitol, a potent VDR agonist, was tolerated in mice at a ~400-fold higher dose than calcitriol [67]. However, neither calcitriol nor analog monotherapies showed a clear benefit against clinical prostate cancer. A treatment protocol that combines inecalcitol with docetaxel (a standard chemotherapy drug) is currently under evaluation for the treatment of metastatic castration-resistant prostate cancer. The combination protocol was designed following the preclinical finding that VDR agonists can sensitize prostate cancer to chemotherapeutic interventions. In a small-scale study, vitamin D<sub>3</sub> supplementation at a physiological dose prevented disease progression for low-grade, organ-localized clinical prostate cancer. A final verdict on the chemoprevention activity of vitamin D<sub>3</sub> awaits outcomes of large-scale clinical trials.

We observed that androgen prevented vitamin D-mediated induction of androgen receptor and CYP24A1 in castration-sensitive and castration-resistant prostate cancer cells. This data raises the intriguing possibility that androgen in conjunction

with calcitriol (or analog) may stem prostate cancer progression in a therapeutic setting. In this regard, we note that a high testosterone dose promoted ablation of castration-resistant prostate cancer cells in preclinical studies [93]. Further, in a pilot clinical study, bipolar androgen therapy, which entails rapid cycling from supraphysiologic to near-castrate serum testosterone levels over a 4-week cycle, lowered serum PSA and reduced radiographic progression in asymptomatic patients with castration-resistant prostate cancer and limited metastatic burden [94]. Whether daily vitamin D<sub>3</sub> supplementation can further enhance antiprostata cancer response to bipolar androgen therapy merits clinical evaluation.

Novel therapeutics against prostate and breast cancer may be identified by leveraging insights from upstream regulators and downstream effectors of the vitamin D/VDR pathway. Distinct mechanisms such as G1/S cell cycle block, apoptosis, cellular differentiation and inhibition of angiogenesis account for cell growth arrest by vitamin D action. The anti-inflammatory response elicited by vitamin D signaling may also interfere with malignant cell growth, given the close link of inflammation to cancer initiation and progression. Indeed, when patients with low-grade prostate cancer received daily vitamin D<sub>3</sub> supplementation for 2 months prior to radical prostatectomy, induction of the anti-inflammatory cytokine MIC-1 (known also as GDF-15) and reduction of inflammation in resected specimens were observed [74].

Insights into the epigenetics that play a role in the regulation of genes encoding VDR, CYP2R1, CYP27B1, CYP24A1, as well as the epigenetic mechanisms that direct VDR-mediated transcription regulation of genes encoding certain histone demethylases (such as lysine-specific demethylases and Jumonji C domain-containing demethylases) may uncover a new path to the inhibition of prostate and breast cancer. VDR-mediated microRNA regulation may also be leveraged in search of a new intervention strategy. As an example, microRNA-98, which inhibited clonogenic growth of LNCaP prostate cancer cells, was induced in calcitriol-injected mice leading to its increased level in the blood. The potential of microRNA-98 as a candidate therapeutic as well as a biomarker for vitamin D action in vivo is being explored [95]. Above examples highlight the possibility that upstream or downstream effectors of the vitamin D/VDR pathway may provide opportunities for novel targeting of breast and prostate cancer.

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## References

1. Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ. The role of vitamin D in reducing cancer risk and progression. *Nat Rev Cancer*. 2014;14:342–57.
2. Krishnan AV, Feldman D. Molecular pathways mediating the anti-inflammatory effects of calcitriol: implications for prostate cancer chemoprevention and treatment. *Endocr Relat Cancer*. 2010;17:R19–38.

3. Prakash CP, Zuniga B, Song CS, Jiang S, et al. Nuclear receptors in drug metabolism, drug response and drug interactions. *Nucl Receptor Res.* 2015;2:1–20. Article ID 101178.
4. Ahn J, Park S, Zuniga B, Bera A, Song CS, Chatterjee B. Vitamin D in prostate cancer. In: Litwack G, editor. *Vitamins & hormones*, vol. 100. New York: Elsevier/Academic; 2016. p. 321–55.
5. Murphy AB, Nyame YA, Martin IK, Catalona WJ, Hollowell CMP, et al. Vitamin D deficiency predicts prostate biopsy outcomes. *Clin Cancer Res.* 2014;20:2289–99.
6. Nelson SM, Batai K, Ahaghotu C, Agurs-Collins C, Kittles RA. Association between serum 25-hydroxy-vitamin D and aggressive prostate cancer in African American men. *Nutrients.* 2017;9:12. <https://doi.org/10.3390/nu9010012>.
7. Nyame YA, Murphy AB, Bowen DK, Jordan G, Batai K, et al. Association between serum vitamin D and adverse pathology in men undergoing radical prostatectomy. *J Clin Oncol.* 2016;34:1345–9.
8. Albanes D, Mondul AM, Yu K, Parisi D, Horst RL, Virtamo J, Weinstein SJ. Serum 25-hydroxyvitamin D and prostate cancer risk in a large nested case-control study. *Cancer Epidemiol Biomarkers Prev.* 2011;20(9):1850–60.
9. Bauer SR, Hankinson S, Bertone-Johnson ER. Plasma vitamin D levels, menopause and risk of breast cancer: dose-response meta-analysis of prospective studies. *Medicine.* 2013;92:123–31.
10. Yao S, Kwan ML, Ergas IJ, Roh JM, Cheng TYD, et al. Association of serum level of vitamin D at diagnosis with breast cancer survival: a case cohort analysis in the pathways study. *JAMA Oncol.* 2017;3:351–7.
11. Wadosky KM, Koochekpour S. Molecular mechanisms underlying resistance to androgen deprivation therapy in prostate cancer. *Oncotarget.* 2016;7:64447–70.
12. DeLuca HF. History of the discovery of vitamin D and its active metabolites. *Bonekey Rep.* 2014;3:1–8. <https://doi.org/10.1038/bonekey.2013.213>. Article number: 479.
13. Thacher TD, Fischer PR, Singh RJ, Roizen J, Levine MA. CYP2R1 mutations impair generation of 25-hydroxyvitamin D and cause an atypical form of vitamin D deficiency. *J Clin Endocrinol Metab.* 2015;100:E1005–13.
14. Zhu JG, Ochalek JT, Kaufmann M, Jones G, DeLuca HF. CYP2R1 is a major, not exclusive, contributor to 25-hydroxyvitamin D production in vivo. *Proc Natl Acad Sci U S A.* 2013;110:15650–5.
15. Johns LE, Ferguson KK, Meeker JD. Relationships between urinary phthalate metabolite and bisphenol A concentrations and vitamin D levels in U.S. adults: National Health and Nutrition Examination Survey (NHANES), 2005-2010. *J Clin Endocrinol Metab.* 2016;101:4062–9.
16. Harmon QE, Umbach DM, Baird DD. Use of estrogen-containing contraception is associated with increased concentrations of 25-hydroxy vitamin D. *J Clin Endocrinol Metab.* 2016;101:3370–7.
17. Chanakul A, Zhang MYH, Louw A, Armbrecht HJ, Miller WL, Portale AA, Perwad F. FGF-23 Regulates CYP27B1 Transcription in the kidney and in extra-renal tissues. *PLoS One.* 2013;8(9):e72816.
18. Martin A, David V, Quarles LD. Regulation and function of the FGF23/Klotho endocrine pathways. *Physiol Rev.* 2012;92:131–55.
19. Al Mutair AN, Nasrat GH, Russell DW. Mutation of the *CYP2R1* vitamin D 25-hydroxylase in a Saudi Arabian family with severe vitamin D deficiency. *J Clin Endocrinol Metab.* 2012;97:E2022–5.
20. Babiker AM, Al Gadi I, Al-Jurayyan NA, Al Nemri AM, Al Haboob AA, et al. A novel pathogenic mutation of the *CYP27B1* gene in a patient with vitamin D-dependent rickets type 1: a case report. *BMC Res Notes.* 2014;7:783.
21. Schlingmann KP, Kaufmann M, Weber S, Irwin A, Goos C, et al. Mutations in *CYP24A1* and idiopathic infantile hypercalcemia. *N Engl J Med.* 2011;365:410–21.
22. Wagner D, Trudel D, Van der Kwast T, Nonn L, Giangreco AA, et al. Randomized clinical trial of vitamin D3 doses on prostatic vitamin D metabolite levels and Ki67 labeling in prostate cancer patients. *J Clin Endocrinol Metab.* 2013;98:1498–507.

23. Morris HA, Anderson PH. Autocrine and paracrine actions of vitamin D. *Clin Biochem Rev.* 2010;31:129–38.
24. Schwartz GG, Whitlatch LW, Chen TC, Lokeshwar BL, Holick MF. Human prostate cells synthesize 1,25-dihydroxyvitamin D<sub>3</sub> from 25-hydroxyvitamin D<sub>3</sub>. *Cancer Epidemiol Biomarkers Prev.* 1998;7:391–5.
25. Swami S, Krishnan AV, Wang JY, Jensen K, Horst R, Albertelli A, Feldman D. Dietary vitamin D<sub>3</sub> and 1,25-dihydroxyvitamin D<sub>3</sub> (calcitriol) exhibit equivalent anticancer activity in mouse xenograft models of breast and prostate cancer. *Endocrinology.* 2012;153:2576–87.
26. Helsen C, Claessens F. Looking at nuclear receptors from a new angle. *Mol Cell Endocrinol.* 2013;382:97–106.
27. Orlov I, Rochel N, Moras D, Klaholz BP. Structure of the full human RXR/VDR nuclear receptor heterodimer complex with its DR3 target DNA. *EMBO J.* 2012;31:291–300.
28. Wan LY, Zhang YQ, Chen MD, Liu CB, Wu JF. Relationship of structure and function of DNA-binding domain in vitamin D receptor. *Molecules.* 2015;20:12389–99.
29. Carlberg C, Campbell MJ. Vitamin D receptor signaling mechanisms: integrated actions of a well-defined transcription factor. *Steroids.* 2013;78:127–36.
30. Huang P, Chandra V, Rastinejad F. Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. *Annu Rev Physiol.* 2010;72:247–72.
31. Watson LC, Kuchenbecker KM, Schiller BJ, Gross JD, Miles A, et al. Glucocorticoid receptor dimer interface allosterically transmits sequence-specific DNA signals. *Nat Struct Mol Biol.* 2013;20:876–83.
32. Zhang J, Chalmers MJ, Stayrook KR, Burris LL, Wang Y, et al. DNA binding alters coactivator interaction surfaces of intact VDR-RXR complex. *Nat Struct Mol Biol.* 2011;18:556–63.
33. Meijnsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR. DNA binding site sequence directs glucocorticoid structure and activity. *Science.* 2009;324:407–10.
34. Alimirah F, Vaishnav A, McCormick M, Echchgadda I, Chatterjee B, Mehta RG, Peng X. Functionality of unliganded VDR in breast cancer cells: repressive action on CYP24 Basal transcription. *Mol Cell Biochem.* 2010;342:143–50.
35. DeLuca HF, Prah J, Plum LA. 1,25-Dihydroxyvitamin D is not responsible for toxicity caused by vitamin D or 25-hydroxyvitamin D. *Arch Biochem Biophys.* 2011;505:226–30.
36. Luo YR, Molnar F, Perakyla M, Qiao S, Kalueff AV, et al. 25-Hydroxyvitamin D<sub>3</sub> is an agonistic vitamin D receptor ligand. *Steroid Biochem Mol Biol.* 2010;118:162–70.
37. Echchgadda I, Song CS, Roy AK, Chatterjee B. DHEA-sulfotransferase is a target for transcriptional induction by the vitamin D receptor. *Mol Pharmacol.* 2004;65:720–9.
38. Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, et al. Vitamin D receptor as an intestinal bile acid sensor. *Science.* 2002;296:1313–6.
39. Thirumaran RK, Lamba JK, Kim RB, Urquhart BL, Gregor JC, et al. Intestinal CYP3A4 and midazolam disposition in vivo associate with VDR polymorphisms and show seasonal variation. *Biochem Pharmacol.* 2012;84:104–12.
40. Masuno H, Ikura T, Morizono D, Orita I, Yamada S, Shimizu M, Ito N. Crystal structures of complexes of vitamin D receptor ligand-binding domain with lithocholic acid derivatives. *J Lipid Res.* 2013;54:2206–13.
41. Belorusova AY, Eberhardt J, Potier N, Stote RH, Dejaegere A, Rochel N. Structural insights into the molecular mechanism of vitamin D receptor activation by lithocholic acid involving a new mode of ligand recognition. *J Med Chem.* 2014;57:4710–9.
42. Rochel N, Wurtz JM, Mitschler A, Klaholz B, Moras D. The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Mol Cell.* 2000;5:173–9.
43. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. *J Comput Chem.* 2010;31:455–61.
44. Dallakyan S, Olson AJ. Small-molecule library screening by docking with PyRx. *Methods Mol Biol.* 2015;1263:243–50.
45. Dassault Systèmes. BIOVIA, discovery studio modeling environment, release 2017. San Diego: Dassault Systèmes; 2016.

46. Molnar F. Structural considerations of vitamin D signaling. *Front Physiol.* 2014;5:191. <https://doi.org/10.3389/fphys.2014.00191>.
47. Carlberg C. Genome-wide (overview) on the actions of vitamin D. *Front Physiol.* 2014;5:167. <https://doi.org/10.3389/fphys.2014.00167>.
48. Rosenfeld MG, Lunyak VV, Glass CK. Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev.* 2006;20:1405–28.
49. Evans RM, Mangelsdorf DJ. Nuclear receptors, RXR and the big bang. *Cell.* 2014;157:255–66.
50. Vaëisaënen S, Peraëkylaë M, Kaërkkaëinen JI, Steinmeyer A, Carlberg C. Critical role of helix 12 of the vitamin D3 receptor for the partial agonism of carboxylic Ester antagonists. *J Mol Biol.* 2002;315:229–38.
51. Pike JW, Lee SM, Meyer MB. Regulation of gene expression by 1,25-dihydroxyvitaminD3 in bone cells: exploiting new approaches and defining new mechanisms. *Bonekey Rep.* 2014;3:482. <https://doi.org/10.1038/bonekey.2013.216>.
52. Allen BL, Taatjes BJ. The mediator complex: a central integrator of transcription. *Nat Rev Mol Cell Biol.* 2015;16:155–66.
53. Tuoesmäki P, Väisänen S, Neme A, Heikkinen S, Carlberg C. Patterns of genome-wide VDR locations. *PLoS One.* 2014;9(4):e96105.
54. Seo YK, Mirkheshti N, Song CS, Kim S, Dodds S, et al. SULT2B1b sulfotransferase: induction by vitamin D receptor and reduced expression in prostate cancer. *Mol Endocrinol.* 2013;27:925–39.
55. Turunen MM, Dunlop TW, Carlberg C, Väisänen S. Selective use of multiple vitamin D response elements underlies the 1 $\alpha$ , 25-dihydroxyvitamin D3-mediated negative regulation of the human *CYP27B1* gene. *Nucleic Acids Res.* 2007;35:2734–47.
56. Yang ES, Burnstein KL. Vitamin D inhibits G1 to S progression in LNCaP prostate cancer cells through p27Kip1 stabilization and Cdk2 mislocalization to the cytoplasm. *J Biol Chem.* 2003;278:46862–8.
57. Leman ES, Arlotti JA, Dhir R, Getzenberg RH. Vitamin D and androgen regulation of prostatic growth. *J Cell Biochem.* 2003;90:138–47.
58. Bao BY, Yeh SD, Lee YF. 1 $\alpha$ , 25 dihydroxy vitamin D3 inhibits prostate cancer cell invasion via modulation of selective proteases. *Carcinogenesis.* 2006;27:32–42.
59. Luo YR, Tuohimaa P. Androgen enhances antiproliferative activity of vitamin D<sub>3</sub> by suppressing 24-hydroxylase expression in LNCaP cells. *J Steroid Biochem Mol Biol.* 2006;99:44–9.
60. Muindi JR, Yu WD, Ma Y, Engler KL, Kong RX, Trump DL, Johnson CS. CYP24A1 inhibition enhances the antitumor activity of calcitriol. *Endocrinology.* 2010;151:4301–12.
61. Chang KH, Li R, Kuri B, Lotan Y, Roehrborn CG, et al. A gain of function mutation in DHT synthesis in castration-resistant prostate cancer. *Cell.* 2013;154:1074–84.
62. Medioni J, Deplanque G, Ferrero J-M, Maurina T, Rodier J-M, et al. Phase I safety and pharmacodynamic of inecalcitol, a novel VDR agonist with docetaxel in metastatic castration-resistant prostate cancer patients. *Clin Cancer Res.* 2014;20:4471–7.
63. Banach-Petrosky W, Jessen WJ, Ouyang X, Gao H, Rao J, Quinn J, Aronow BJ, Abate-Shen C. Prolonged exposure to reduced levels of androgen accelerates prostate cancer progression in *Nkx3.1; Pten* mutant mice. *Cancer Res.* 2007;67:9089–96.
64. Ajibade AA, Kirk JS, Karasik E, Gillard B, Moser MT, Johnson CS, Trump DL, Foster BA. Early growth inhibition is followed by increased metastatic disease with vitamin D (calcitriol) treatment in the TRAMP model of prostate cancer. *PLoS One.* 2014;9:e89555.
65. Duffy MJ, Murray A, Synnott NC, O'Donovan N, Vitamin D. Analogues: potential use in cancer treatment. *Crit Rev Oncol Hematol.* 2017;112:190–7.
66. Leysens C, Verlinden L, Verstuyf A. The future of vitamin D analogs. *Front Physiol.* 2014;5:122. <https://doi.org/10.3389/fphys.2014.00122>.
67. Okamoto R, Delansorne R, Wakimoo N, Doan NB, Akagi T, Shen M, Ho QH, Saida JW, Koeffler HP. Inecalcitol, an analog of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, induces growth arrest of androgen-dependent prostate cancer cells. *Int J Cancer.* 2012;130:2464–73.

68. Perez-Stable CM, Schwartz GG, Farinas A, Finegold M, Binderup L, Howard GA, Roos BA. The Gy/T-15 transgenic mouse model of androgen-independent prostate cancer: target cells of carcinogenesis and the effect of the vitamin D analogue EB1089. *Cancer Epidemiol Biomarkers Prev.* 2002;11:555–63.
69. Verlinden L, Verstuyf A, Van Camp M, Marcelis S, Sabbe K, et al. Two novel 14-epi-analogues of 1,25-dihydroxyvitamin D-3 inhibit the growth of human breast cancer cells in vitro and in vivo. *Cancer Res.* 2000;60:2673–9.
70. Datta M, Schwartz GG. Calcium and vitamin D supplementation during androgen deprivation therapy for prostate cancer: a critical review. *Oncologist.* 2012;17(9):1171–9.
71. Attia S, Elckhoff J, Wilding G, McNeel D, Blank J, et al. Randomized double blinded phase II evaluation of docetaxel with or without doxercalciferol in patients with metastatic androgen-independent prostate cancer. *Clin Cancer Res.* 2008;14:2437–43.
72. Manson JE, Bassuk S, et al. The *VIT*amin D and *Om*ega-3 *TriaL* (VITAL): rationale and design of a large randomized controlled trial of vitamin D and marine omega-3 fatty acid supplements for the primary prevention of cancer and cardiovascular disease. *Contemp Clin Trials.* 2012;33:159–71.
73. Marshall D, Savage SJ, Garrett-Mayer E, Keane TE, Hollis BW, Horst RL. Vitamin D3 supplementation at 4000 international units per day for one year results in a decrease of positive cores at repeat biopsy in subjects with low-risk prostate cancer under active surveillance. *J Clin Endocrinol Metab.* 2012;97:2315–24.
74. Hollis BW. Vitamin D in the prevention and treatment of cancer. Talk presented at American Chemical Society 249th National Meeting and Exposition; March 22–36, Denver, CO; 2015.
75. DeMarzo AM, Platz EA, Sutcliffe S, Xu J, Grönberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG. Inflammation in prostate carcinogenesis. *Nat Rev Cancer.* 2007;7:256–69.
76. Moreno J, Krishnan AV, Peehl DM, Feldman D. Mechanisms of vitamin D-mediated growth inhibition in prostate cancer cells: inhibition of the prostaglandin pathway. *Anticancer Res.* 2006;26:2525–30.
77. Li H, Stampfer MJ, Hollis JB, Mucci LA, Gaziano JM, Hunter D, Giovannucci EL, Ma J. A prospective study of plasma vitamin D metabolites, vitamin D receptor polymorphisms and prostate Cancer. *PLoS Med.* 2007;4(3):e103.
78. Liu S, Cai H, Cheng W, Zhang H, Pan Z, Wang D. Association of VDR polymorphisms (Taq I and Bsm I) with prostate cancer: a new meta-analysis. *J Int Med Res.* 2017;45:3–10.
79. Fetahu IS, HÖbaus J, Kállay E. Vitamin D and the epigenome. *Front Physiol.* 2014;5:1–12. Article 164.
80. Abedin SA, Banwell CM, Colston KW, Carlberg C, Campbell MJ. Epigenetic corruption of VDR signaling in malignancy. *Anticancer Res.* 2006;26:2557–66.
81. Ting HJ, Bao BY, Reeder JE, Messing EM, Lee YF. Increased expression of corepressors in aggressive androgen-independent prostate cancer cells results in loss of 1 $\alpha$ ,25-dihydroxyvitamin D3 responsiveness. *Mol Cancer Res.* 2007;5:967–80.
82. Godoy AS, Chung I, Montecinos VP, Buttyan R, Johnson CS, Smith GJ. Role of androgen and vitamin D receptors in endothelial cells from benign and malignant human prostate. *Am J Physiol Endocrinol Metab.* 2013;304:E1131–9.
83. Perissi V, Jepsen K, Glass CK, Rosenfeld MG. Deconstructing repression: evolving models of corepressor action. *Nat Rev Genet.* 2010;11:109–23.
84. Braillon A. Vitamin D and breast cancer survival: the good and the bad. *JAMA Oncol.* 2017;3(8):1138–9. <https://doi.org/10.1001/jamaoncol.2016.6792>.
85. Apoe O, Jung SH, Liu H, Seisler DK, Charlamb J, et al. Effect of vitamin D supplementation on breast cancer biomarkers: CALGB 70806 (Alliance) study design and baseline data. *Am J Hematol Oncol.* 2016;12:5–9.
86. LaPorta E, Welsh J. Modeling vitamin D actions in triple negative/basal-like breast cancer. *J Steroid Biochem Mol Biol.* 2014;144 Pt A:65–73. <https://doi.org/10.1016/j.jsbmb.2013.10.022>.



87. Swami S, Krishnan AV, Wang JY, Jensen K, Peng L, Albertelli MA, Feldman D. Inhibitory effects of calcitriol on the growth of MCF-7 breast cancer xenografts in nude mice: selective modulation of aromatase expression in vivo. *Horm Cancer*. 2011;2:190–202.
88. Wahler J, So JY, Cheng LC, Maehr H, Uskokovic M, Suh N. Vitamin D compounds reduce mammo-sphere formation and decrease expression of putative stem cell markers in breast cancer. *J Steroid Biochem Mol Biol*. 2015;148:148–55.
89. Williams JD, Aggarwal A, Swami S, Krishnan AV, Ji L, Albertelli MA, Feldman BJ. Tumor autonomous effects of vitamin D deficiency promote breast cancer metastasis. *Endocrinology*. 2016;157:1341–7.
90. Narvaez CJ, Matthews D, LaPorta E, Simmons KM, Beaudin S, Welsh J. The impact of vitamin D in breast cancer: genomics, pathways, metabolism. *Front Physiol*. 2014;5:213. <https://doi.org/10.3389/fphys.2014.00213>.
91. So JY, Smolarek AK, Salerno DM, Maehr H, Uskokovic M, et al. Targeting CD44-STAT3 signaling by Gemini vitamin D analog leads to inhibition of invasion in basal-like breast cancer. *PLoS One*. 2013;8:e54020.
92. Maestro MA, Molnar F, Mourino A, Carlberg C. Vitamin D receptor 2016: novel ligands and structural insights. *Expert Opin Ther Pat*. 2016;26(11):1291–306.
93. Denmeade SR, Isaacs JT. Bipolar androgen therapy: the rationale for rapid cycling of supraphysiologic androgen/ablation in men with castration-resistant prostate cancer. *Prostate*. 2010;70:1600–7.
94. Schweizer MT, Antonarakis ES, Wang H, Ajiboye AS, Spitz A, et al. Effect of bipolar androgen therapy for asymptomatic men with castration-resistant prostate cancer: results from a pilot clinical study. *Sci Transl Med*. 2015;7:269ra2.
95. Ting HJ, Messing J, Yasmin-Karim S, Lee YF. Identification of microRNA-98 as a therapeutic target inhibiting prostate cancer growth and a biomarker induced by vitamin D. *J Biol Chem*. 2013;288:1–9.