

Vitamin D Receptor Signaling and Cancer



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KEYWORDS

• Vitamin D • Chemoprevention • Chemotherapy • TCGA • Genomics

KEY POINTS

- Preclinical and epidemiologic data justify the concept that vitamin D compounds could be exploited as a differentiation therapy for a wide range of malignancies.
- Clinical evaluation of vitamin D compounds has been more equivocal and, although biological responses can be measured in vivo, clinical responses have not justified further evaluation.
- Dissecting mechanisms of cellular resistance is one route to defining patient groups with greater precision who may respond more fully to clinical targeting.
- Large genomic and population datasets are available that can be mined to define patient responses more completely and identify which tumor types may be most effectively targeted.

A PRIMER ON VITAMIN D BIOLOGY AND MEDICINE

The primary biological action of the secosteroid hormone vitamin D ($1,25(\text{OH})_2\text{D}_3$) is to bind to the vitamin D receptor (NR1H1/VDR) and to regulate serum calcium levels. As a downstream consequence, the actions of the receptor control bone formation and maintenance. The first clinical manifestation of insufficient VDR endocrine signaling, rickets, was discovered by Daniel Whistler in the Netherlands in the 17th century; 300 years later, the VDR gene was cloned by Bert O'Malley and coworkers.¹ Between these dates, research into vitamin D was at the forefront of areas of public health, chemistry and biochemistry including the light catalyzed synthesis of vitamin D_3 by Adolf Windaus. For this work, he received the Nobel Prize in Chemistry (1928). Work in the 1960s and 1970s led to analyses of vitamin D endocrine metabolism and led to remarkable strides describing biochemical synthesis of $1,25(\text{OH})_2\text{D}_3$ and the diverse biology in which VDR participates.

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The precursor of $1,25(\text{OH})_2\text{D}_3$, cholecalciferol or vitamin D_3 , is produced in the skin and converted in the liver to 25-hydroxyvitamin D_3 , $(25(\text{OH})\text{D}_3)$; circulating levels of $25(\text{OH})\text{D}_3$ serve as a useful index of vitamin D total body stores. A further hydroxylation occurs in the principally in the kidney at the carbon 1 position by 25-hydroxyvitamin D -1 α -hydroxylase (encoded by *CYP27B1*) to produce the biologically active hormone, $1,25(\text{OH})_2\text{D}_3$. A second mitochondrial cytochrome P450 enzyme, the 24-hydroxylase (encoded by *CYP24A1*), can use both $25(\text{OH})\text{D}$ and $1,25(\text{OH})_2\text{D}_3$ as substrates, and is the first step in the inactivation pathway for these metabolites. Because of the direct role $1,25(\text{OH})_2\text{D}_3$ plays in control of serum calcium levels, elevated levels of $1,25(\text{OH})_2\text{D}_3$ block its synthesis and induce inactivation and accelerate catabolism² via induction of *CYP24A1*, in a classical negative feedback loop.

In parallel to these VDR-centered discoveries a greater awareness emerged of the 48 members of the nuclear hormone receptor (NR) superfamily, of which the VDR is a member. As a result, the VDR and other NRs represent some of the most well-studied human transcription factors and have yielded significant insight into the mechanisms of transcriptional control.

It is worth stressing the fundamental importance of the precise monitoring and regulation of serum calcium levels to human health; hence, the endocrine role of the VDR in the regulation of calcium homeostasis is critical. The levels of vitamin D depend on cutaneous synthesis initiated by solar radiation and on dietary intake; a decrease of either one or both sources leads to insufficiency. The contribution from the ultraviolet light (UV)-initiated cutaneous conversion of 7-dehydrocholesterol to vitamin D_3 is the greater, contributing more than 90% toward final $1,25(\text{OH})_2\text{D}_3$ synthesis in a vitamin D-sufficient individual.

The importance of the relationships between solar exposure and the ability to capture UV-mediated energy is underscored by the inverse correlation between human skin pigmentation and latitude and associated $25(\text{OH})\text{D}$ levels. That is, skin pigmentation was lost as humans migrated out of Africa to adjust to life with reduced solar UV exposure. As a result, individual capacity to generate vitamin D_3 in response to solar UV exposure is intimately associated with forebear environmental adaptation. The correct and sufficient level of solar exposure and serum vitamin D_3 are matters of considerable debate, and an Institute of Medicine report³ in 2010 recommended daily vitamin D_3 intake at the levels of 600 IU/d for most groups in the population (800 IU/d for those >70 years of age). However, this recommendation is not without controversy; parallel reassessment of the vitamin D impact on the prevention of osteoporosis has suggested that the correct level may be as high as 2 to 3000 IU/d, which may reflect more accurately ancestral serum levels.⁴ Another challenge is determining how a given intake relates to serum levels among individuals^{5,6} and what are the appropriate biochemical readouts for measuring systemic response.

However, given that there has been a concerted research focus on VDR signaling, there now exists a fairly sophisticated appreciation of this process, and it has been extensively reviewed.^{7–13}

WHY CONSIDER TREATING CANCER WITH VITAMIN D COMPOUNDS?

The first report that VDR actions could control cancer cell growth were discovered partly through serendipity, and partly through logical extension of other studies. Reports in the 1970s identified purified cell fractions that bound $1,25(\text{OH})_2\text{D}_3$ with high affinity,¹⁴ and encouraged investigators to begin to consider what were the molecular actions of the VDR in the classic tissues involved in calcium homeostasis, for example, skin, bone, intestine, and kidney.¹⁵ In 1981, Kay Colston and coworkers¹⁶ were first to

demonstrate that $1\alpha,25(\text{OH})_2\text{D}_3$ at nanomolar concentrations inhibited human melanoma cell proliferation in vitro. That the workers used a cancer cell model was serendipitous; cancer cell models are more readily available to study in cell culture experiments than nonmalignant counterparts, and in this case¹⁶ the cells chosen were available in an adjacent laboratory.

In parallel, it was also known that retinoids, which are also small lipophilic molecules that target NRs, could drive cell differentiation, for example, in HL60 leukemia cells lines cells.¹⁷ In turn these studies led to the pharmacologic exploitation of all-*trans* retinoic acid in acute promyelocytic leukemia (APL). The molecular cause of APL are translocations of the RAR γ receptor forming chimeric proteins such as PML-RAR γ . These chimeric proteins disrupt the control of differentiation and give rise to APL.^{18–20} Pharmacologic doses of all-*trans* retinoic acid are able to trigger differentiation and, therefore, this therapy in APL is a dramatic example of targeted cancer therapies; in addition, these findings contributed significantly to the rise of the concept of differentiation therapy.^{21–29} All-*trans* retinoic acid remains the mainstay of therapy for APL^{30,31} and this is a major catalyst for studying RARs across cancers.^{29,32,33} As a result, workers began to consider exploiting the antiproliferative actions of $1,25(\text{OH})_2\text{D}_3$ as a differentiation therapy in cancers. In the first instance, the ability of $1,25(\text{OH})_2\text{D}_3$ to induce differentiation in cultured mouse and human myeloid leukemia cells was examined.^{34,35} From the early 1980s onwards the antiproliferative effects of $1,25(\text{OH})_2\text{D}_3$ have been explored in a wide variety of cancer cell lines, which include all major solid tumors and leukemia.^{36–43}

WHAT HAS BEEN LEARNED FROM PRECLINICAL STUDIES?

One of the most highly cited papers in the last 20 years of cancer research is the *Hallmarks of Cancer* paper by Douglas Hanahan and Robert Weinberg.⁴⁴ This landmark paper defined 6 stages necessary for cancer to develop and be sustained. Although this work has been expanded to include additional steps, this original thesis provides a highly significant backdrop against which to examine anticancer VDR functions.

Insensitivity to Antigrowth Signals and Evasion of Apoptosis

Cancer cells sustain their own proliferative signals and silence cues for programmed cell death. Signaling via $1,25(\text{OH})_2\text{D}_3$ drives antiproliferative events, and counters the insensitivity to antigrowth signals and the evasion of apoptosis in cancer cells. Multiple investigators have examined the mechanistic basis for cell sensitivity to VDR antiproliferative responses. For example, early studies focused on understanding antiproliferative pathways, be they mediating cell cycle arrest^{37,45–47} or programmed cell death.^{48–50} However, other studies supported a role for $1,25(\text{OH})_2\text{D}_3$ to block or impede programmed cell death.^{51,52} Historically, hematologic malignancies combined an ease of interrogation with robust classification of cellular differentiation capacity that were envied by investigators of solid tumors. It is, therefore, no coincidence that these cell systems led to the identification of VDR control of genes that control cell cycle progression, including p21^(waf1/cip1) and p27^(kip1), as well as the direct binding sites on the gene *CDKN1A* (encodes p21^(waf1/cip1)).^{53,54} The regulation of p27^(kip1) seems to be mechanistically enigmatic and exemplifies the broad effects of VDR signaling in that both transcriptional and translational regulation, such as enhanced mRNA translation, and attenuating degradative mechanisms are described?^{55–58}

The upregulation of p21^(waf1/cip1) and p27^(kip1) principally mediate G₁ cell cycle arrest, but $1\alpha,25(\text{OH})_2\text{D}_3$ has been shown to mediate a G₂/M cell cycle arrest in a number of cancer cell lines via direct induction of GADD45 α .^{59–61} Concomitant with these

events is a downregulation of cyclins such as cyclin A, a decrease in kinase activities associated with activated complexes, and ultimately the dephosphorylation of the retinoblastoma protein and sequestration of E2F family members in a repressive complex.⁶² Concomitant with changes in the cell cycle, $1,25(\text{OH})_2\text{D}_3$ induces differentiation, most clearly evident in myeloid cell lines, but also supported by other cell types and most likely reflects the intimate links that exist between the regulation of the G_1 transition and the induction of cellular differentiation.^{63–72}

Programmed cell death has been reported in breast cancer models and leukemia models,^{73–76} with evidence that the levels of BCL-2 family of proteins are tightly regulated.⁷⁷ Treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ upregulates vitamin D upregulated protein 1, which binds to the disulfide reducing protein thioredoxin and inhibits its ability to neutralize reactive oxygen species, which in turn can lead to stress-induced apoptosis.^{78–80}

Tissue Invasion and Metastasis

VDR signaling enhances adhesion and suppresses the invasive capacity of cells; many of these effects are associated with a more differentiated phenotype. In an elegant series of studies, Munoz and coworkers have dissected the relationships between VDR signaling and invasion in colon cancer cell lines and primary tumors.^{81–86} These workers established the delicate interplay between VDR, E-cadherin, and the Wnt signaling pathway in cell lines and clinical samples. Others have examined adhesion protein expression in other cancer models, suggesting that these mechanisms may be generalizable beyond colon cancer cells.^{38,87–89}

Limitless Replicative Potential

An essential component of cancer is the ability to replicate without limits that often requires silencing of mechanisms of genomic surveillance. The VDR seems to play roles in maintaining genomic integrity and facilitating DNA repair. There is close cooperation between VDR actions and the p53 tumor suppressor pathway. Correlative data suggest that, generally, cells that respond to $1,25(\text{OH})_2\text{D}_3$ most profoundly have wild-type p53, and at the molecular level several target genes are shared by both signaling pathways, such as *CDKN1A* and *GADD45A*.^{53,54,59,90–95} Notably in the skin, VDR signaling is combined with surveillance of genomic damage to regulate mitosis negatively.^{96,97} In other epithelial tissues, close cooperation between VDR regulates *BRCA1* mRNA and protein via transcriptional activation, again supporting a role in genomic surveillance.^{98–100}

IDENTIFYING VITAMIN D RECEPTOR-MEDIATED TRANSCRIPTOMES

To identify critical target genes that mediate these actions, comprehensive genome-wide transcriptomic screens have revealed broad consensus on certain targets, but have also highlighted variability.^{36,60,101,102} There is a significant history of VDR-centric transcriptomic studies that support the cell phenotypes observed.^{36,60,61,101–111} For example, the study of isogenic cell pairs with differing sensitivities to $1,25(\text{OH})_2\text{D}_3$ signaling has identified networks that mediate antiproliferative sensitivity. In this manner, a significant role of cross-talk between VDR and transforming growth factor (TGF)- β signaling has been revealed.^{112,113} In addition, similar studies have shown that VDR transcriptional targets can distinguish leukemia aggressiveness.¹¹⁴ The list of gene targets that is common across cell models seems to be short; the most clearly shared target is *CYP24A1*. Beyond that, commonly enriched gene networks often focus on cell cycle control and signal transduction. However, substantial variations in

experimental design (eg, dose, exposure time, and use of $1,25(\text{OH})_2\text{D}_3$ or an analog) limit strict comparisons. Thus, although a formal metaanalysis to reveal common themes has not been applied,^{115,116} it seems clear that there is little overlap between the transcriptomic studies. It is also noteworthy that datasets have been developed that are aimed at noncoding RNA species.^{117,118} Therefore, the diversity of the VDR regulated transcriptome is likely to increase.

More recently, these transcriptomic studies have been complemented by VDR ChIP-Seq studies in which the VDR genomic binding patterns have been captured. VDR ChIP-seq studies have been performed in several human cell types,^{119–123} in the presence and/or absence of ligand, and revealed the impact of ligand binding on VDR genomic targeting. Arguably, VDR ChIP-seq studies are more important than transcriptomic studies because they reveal direct VDR genomic interactions, whereas transcriptomic analyses inevitably include direct and indirect VDR-mediated effects. Each VDR ChIP-Seq analysis revealed approximately 2000 to 6000 genomic loci normally distributed around transcription start sites, reflecting the binomial distributions found for other transcription factors,^{123,124} but many loci are found at considerable distance from the transcription start sites. Another important finding from these studies is that the dual hexameric DNA motif spaced by 3 bp, a so-called DR3 motif,^{125,126} is found in the majority but not all of the most prominent genomic VDR binding sites. Other binding motifs have also been suggested, for example, an inverted palindrome spaced by 9 bp, a so-called IP-9.^{127,128} The application of ChIP-Seq approaches to NRs in general has revealed greater binding site diversity than previously expected; in addition the importance of flanking regions for cofactors to be biologically important to determine function.¹²⁹ These aspects of transcriptional regulation are described in greater depth in J. Wesley Pike and Sylvia Christakos' article, "[Biology and Mechanisms of Action of the Vitamin D Hormone](#)," in this issue.

The precise frequency of DR3 type elements in part remains ambiguous, because it depends on a number of variables that include the depth of the sequence read, the precise discovery motif algorithm applied, and the statistical thresholds used. Regardless of the actual percentage of VDR binding sites that contain DR3 motifs, it is clear that the VDR binds in significant levels to genomic regions that do not contain a canonical DR3. This may be explained by the VDR interacting with the genome in both direct (VDR–DNA) and indirect (VDR–protein–DNA) modes (reviewed in reference⁸).

There is a compelling case to be made for the reanalysis of the VDR ChIP-Seq data, from genomic alignment to differential peak calling. The rationale for reanalyses is twofold. Analyses of ChIP-Seq is not trivial in terms of statistical assumptions, and the existing studies have all been analyzed in a different manner. Therefore, there is the possibility that thresholds and cutoffs differ between studies. Second, the methodologies for ChIP-Seq processing are an area of active development and advancement, and the most recent approaches display a number of benefits over earlier analytical workflows.¹³⁰

IN VIVO VITAMIN D RECEPTOR ANTICANCER ACTIONS

Given this wealth of understanding of the broad anticancer actions of the VDR, and the aim to exploit this understanding in cancer settings, the use of rodent models is a major intermediary step before clinical exploitation of VDR signaling in either chemoprevention or chemotherapy settings.

A clear difficulty in investigating the efficacy of targeting VDR with either $1,25(\text{OH})_2\text{D}_3$ or analogues that have more attractive pharmacologic properties^{33,126,131–140} is that

mice are not humans. Their spectra of age-associated malignancies are different from humans and other key metabolic differences exist. Recapitulating these lifetime effects are further compounded by the need to establish the window in which chemoprevention effects may play a role in either tumor initiation or progression.

Notwithstanding these caveats, the *Vdr*^{-/-} animals are extremely useful tools to elucidate more clearly the role for the VDR to act in a cancer preventive manner.^{2,141,142} A series of animals have been generated in which the VDR-ablated background has been crossed into animals with tumor disposition phenotypes. In the first instance, there is evidence that deleting or reducing VDR levels alters the morphology in the colon^{143,144} and breast.¹⁴⁵ Furthermore, crossing the *Vdr*-deficient and heterozygote mice with mouse mammary tumor virus-*neu* transgenic mice has generated animals that show a degree of *Vdr* haplosufficiency.¹⁴⁵ The mammary tumor burden in the crossed mice is reduced with the presence of one wild-type *Vdr* allele and further with 2 wild-type *Vdr* alleles. Alternatively, the *Vdr*^{-/-} animals demonstrate greater susceptibility to carcinogen challenge. For example, challenging *Vdr*^{-/-} mice with DMBA induced more preneoplastic lesions in the mammary glands than in wild-type mice.¹⁴⁶

Previously, other workers have established that deletion of the Adenomatous polyposis coli (*Apc*) gene in a mouse can faithfully recapitulate human colon cancer. In turn, these mice have been exploited to examine the impact of *Vdr* deletion on the progression of colon cancer¹⁴⁷; similar studies support an antitumorogenic role for the VDR in the skin.¹⁴⁸ Numerous studies have examined the ability of dietary or pharmacologic addition of vitamin D compounds to either prevent tumor formation or inhibit the growth of xenograft tumors.^{82,149–159}

One area of investigation is the impact of experimental dietary variations and their impact on tumor predisposition. Long-term studies of mice fed with a Western-style diet (eg, high fat and phosphate and low vitamin D and calcium content) have been exploited to examine the impact of vitamin D on colon cancer proliferation.¹⁶⁰ Similarly, vitamin D and calcium dietary interventions can modulate colon crypt hyperplasia¹⁶¹ and provide a rationale for how diet, inflammation, and premalignant cells could all interact and modulate cancer progression.^{143,162–165}

HUMAN EPIDEMIOLOGIC FINDINGS AND CLINICAL TRIALS

Epidemiologic studies by Cedric Garland and coworkers were the first to investigate relationships between intensity of sunlight exposure and cancer incidence and revealed an inverse correlation with risk of colon cancer, and subsequently extended these findings to implicate a relationship with other cancers.^{166–170} For example, levels of 25OH-D, the major circulating metabolite of vitamin D, are significantly lower in breast cancer patients than in age-matched controls.^{171–173} However, these relationships are clearly complex and reflect lifetime exposures, and indeed controlling for lifestyle factors can significantly impact the strength of the relationships.¹⁷⁴ Although these are all association studies, and therefore function cannot be readily inferred, there are some suggestive findings that low serum levels of 25OH-D are an unfavorable prognostic indicator^{175–178} or may trigger worse chemotherapy responses.¹⁷⁹ In other cancers, prostate for example, the relationships are more equivocal, with some positive findings,^{180,181} although more generally the results are not able to support a cancer-preventative impact of vitamin D levels.^{182–185} To address these ambiguities, investigators are now in the first stages of randomized supplementation trials,¹⁸⁶ one of which, VITAL (ViTamin D and omega-3 TriAL), has now accrued 25,000 people and is examining the impact of supplementing vitamin D and omega

3 fatty acid on a range of pathologies, principally cancer and heart disease incidence¹⁸⁷

Collectively, these preclinical studies and aspects of the epidemiologic findings encouraged academic and pharmaceutical partnerships in the design of vitamin D analogues that may have an optimal balance of in vivo properties to be used as a chemoprevention or chemotherapy agent. Optimizing vitamin D compounds for in vivo anticancer efficacy is aimed at ensuring a favorable balance between calcium mobilizing actions, which result in hypercalcemia, and enhancing the anticancer actions of targeting the VDR. Several medicinal chemistry groups undertook this goal, led in many ways by the group of Milan Uskokovic at Hoffman la Roche,^{64,188–200} alongside Lisa Binderup at Leo Pharmaceuticals,^{201–204} as well as other groups in academic settings, including Gary Posner.^{205–208} Together, these and other investigators have synthesized a blizzard of vitamin D analogues that have many promising properties, being resistant to metabolism and yet have tolerable impact on serum calcium levels.

Several of these analogues have served as the lead compounds in the search for disease settings where the anticancer actions of vitamin D compounds can be exploited. For example, phase I trials have been undertaken in a range of advanced cancers^{209,210} and led to more targeted phase II trials in pancreatic,²¹¹ liver,²¹² prostate,^{213–216} and breast cancers.^{186,217} In all cases, the regimens were well-tolerated but clinical responses were at times modest. However, this in part may reflect that the doses chosen were too conservative and the correct endpoints for these trials would be measuring cellular differentiation (or reduced proliferation or enhanced apoptosis), and this is not readily undertaken in the context of clinical trials.

These challenges are illustrated by considering prostate cancer in more detail. A number of investigators have considered the option of treating men with localized disease before surgery and then studying the prostate tumor after surgery for characterization of known VDR target genes. In a trial of nearly 40 patients with localized prostate cancer, Beer and colleagues²¹⁸ administered either 1,25(OH)₂D₃ or placebo for 4 weeks before radical prostatectomy. Expression changes in the VDR or known candidate VDR target genes or markers of cell proliferation were examined. Interestingly VDR was downregulated in the treatment group, whereas the other genes chosen (eg, *TGFBR2*) were unchanged. Others replicated this approach but with doxercalciferol and revealed significant modulation of *TGFBR2*. Interestingly, microarray studies of 1,25(OH)₂D₃ sensitivity in isogenic breast cancer cell lines established that *TGFBR2* was a critical mediator and marker of sensitivity toward 1,25(OH)₂D₃.¹¹² Other investigators have examined the question of efficacy by escalating dose to assess how well higher levels of 1,25(OH)₂D₃ can be tolerated.²¹⁹ Together these studies suggest that 1,25(OH)₂D₃ can be given to prostate cancer patients at quite high doses and changes in expression of VDR-dependent genes can be observed.

This finding has also led others to consider how chemotherapy with 1,25(OH)₂D₃ could be potentiated by combinations with other cytotoxic agents for added clinical benefit. Such combination studies are intrinsically challenging; in the vitamin D arena, Novoclea undertook such an approach in their development of DN-101 (a new formulation of 1,25(OH)₂D₃) as a cancer therapy in combination with docetaxel for men with advanced prostate cancer that had failed hormonal therapy, so-called castration resistant prostate cancer. Based on numerous preclinical studies and a single institution clinical study, Novoclea conducted a randomized phase III study (ASCENT I [AIPC Study of Calcitriol ENhancing Taxotere]) to determine whether the prostate-specific antigen response rate (defined as a >50% decline in prostate-specific antigen for >1 month) was different for the standard therapy for castration resistant prostate

cancer at the time (docetaxel 36 mg/m² weekly intravenously for 4 weeks every 6 weeks) compared with the same dose and schedule of docetaxel plus DN-101, 45 µg weekly.²²⁰ Although this study did not meet the prostate-specific antigen response criteria, it did alter the overall survival and therefore justified a large randomized trial to assess survival. This new trial (ASCENT II) was halted before full recruitment because survival in the DN-101 arm was reduced compared with standard of care. However, the ASCENT II trial design was seriously flawed: the chemotherapy in each arm was not equal in efficacy. The design of the trial was docetaxel A + DN-101 versus docetaxel B + placebo. Substantial data existed at the time that the trial was initiated that docetaxel A was clearly inferior to docetaxel B in terms of survival in men with castration resistant prostate cancer. Therefore, the trial was actually designed to ask the question, can DN-101 overcome the inferiority of docetaxel regimen A.

A more fundamental flaw of both trials was that the dose of 1,25(OH)₂D₃ chosen was neither the biologically optimal nor the maximum tolerated dose. Other studies have clearly shown that a 2 to 3 times higher doses of calcitriol can be given safely to such patients. However, the result of ASCENT II has been interpreted as “calcitriol does not potentiate docetaxel (and hence any chemotherapy) in a large clinical trial.” This is a conclusion based on no adequate data. As a result, the application of vitamin D formulations have probably been left in a challenging development point.²²¹

Given these tantalizing preclinical and epidemiologic findings, the question then is why have the clinical trials not been successful? It is clear that clinical exploitation of any drug is hard, and there is a very high attrition rate of drugs passing from preclinical development to clinical implementation.²²² There are many therapies that struggle to balance preclinical promise with clinical realities, and the clinical development pipeline is often challenged by ensuring optimal clinical trial design, as illustrated by PARP inhibitors and antiangiogenesis therapies^{223–227} that, although approved by the US Food and Drug Administration, have required further reanalyses to define optimal efficacy.^{228,229} Therefore, it is possible that vitamin D-centered chemotherapies will fall to a similar fate. It may well be that, to date, an incomplete understanding of what are the desirable anticancer actions and inappropriate clinical trial design have impeded clinical success with vitamin D compounds.

CELLULAR MECHANISMS OF RESISTANCE

A major focus emerged on dissecting how cancer cells vary in their response to 1,25(OH)₂D₃. One initial focus was on genetic variation in the 3' and 5' regions of the VDR gene itself.^{230–233} For example, a start codon polymorphism in exon II at the 5' end of the gene, determined using the *fok-I* restriction enzyme, result in a truncated protein.²³⁴ These findings were initially suggestive of a functional relationship between VDR gene genetic variation and cancer risk, but in larger studies these associations seem to be equivocal, or more nuanced.^{235–242} Indeed, this is also reflected by the fact that the National Human Genome Research Institute genome-wide association studies (GWAS) catalog does not list any genome-wide significant genetic variation that is annotated to the VDR and related to cancer phenotypes; rather the genetic variation of the VDR seems to associate with immune, diabetic, and reproduction phenotypes.^{243–245}

Also at the genetic level, various investigators have considered how cell responsiveness to 1,25(OH)₂D₃ may be determined by the expression of the activating (CYP27B1) and metabolizing (CYP24A1) enzymes. For example, comparative genome hybridization studies found that *CYP24A1* is amplified in human breast

cancer in relation to paired normal tissue.^{246,247} Others have revealed reduced *CYP27B1* mRNA and protein levels in a wide variety of cancer cell lines and primary tumors.^{248–254} Together these findings suggest that cancer cell sensitivity toward $1,25(\text{OH})_2\text{D}_3$ may primarily depend on autocrine metabolism in target cells rather than the endocrine synthesis and uptake in target cells. This raises the possibility that local control of these enzymes could be exploited in targeted VDR-centric therapies.

Finally, others have considered how epigenetic mechanisms may disrupt VDR signaling. Evidence for this approach arises from the observation that $1,25(\text{OH})_2\text{D}_3$ -reclacitrant cells still often respond transcriptionally, but lack transcriptional responsiveness to antiproliferative target genes such as *CDKN1A*, but sustain or even enhance induction of *CYP24A1* gene.^{61,100,112,118,255} These data suggest that the VDR transcriptome is skewed in cancer cells to disfavor antiproliferative target genes, and that lack of functional VDR alone cannot explain resistance. The interactions of transcriptional corepressors such as NCOR1 and NCOR2/SMRT have been examined to investigate this possibility.^{61,256–261} In turn, altered VDR–corepressor interactions may form a molecular lesion that could be targeted by cotreatment of $1,25(\text{OH})_2\text{D}_3$ plus the HDAC inhibitors.^{262–267}

LESSONS FOR BIG DATA TO OPTIMIZE VITAMIN D RECEPTOR-CENTERED THERAPIES

Biology is very clearly in the genomic era, in which the sum total of genes, transcripts, proteins and metabolites in cells are captured and analyzed. Arguably, the achievements of the Human Genome Project²⁶⁸ served as a major catalyst for this approach, and other research consortia have applied similar technologies and approaches to tackle other fundamental challenges in biology. Powerful examples are illustrated by Encyclopedia of DNA coding elements (ENCODE),^{269,270} RoadMap Epigenome,²⁷¹ Functional and Taxonomic Analysis of Metagenomes,²⁷² International Human Epigenome Consortium,^{273,274} the Cancer Genome Atlas (TCGA),²⁷⁵ and the Genotype-Tissue Expression (GTEx) project.²⁷⁶ The volume of data generated by these projects is unprecedented and truly transformative in terms of the questions that can be addressed, the manner in which they are tackled, and the how the findings are interpreted and widely translated.

Bioinformatic analyses are central to both the generation of these complex datasets and their investigation. Unbiased bioinformatics analyses can reveal organizational insight that is neither obvious nor intuitive. Unbiased and agnostic analyses are achieved by applying algorithmic approaches that depend on discrete mathematics and information theory, combined with graph theory, data mining, and computer science generally, with a central role for the statistical sciences. In this manner, bioinformatic approaches offer the promise to reveal underlying mechanisms of biology in health and disease.

For example, -omic technologies can be applied to capture genomic structural variants and mutations, gene and protein expression patterns, protein posttranslational modifications, and metabolites across cell states. Bioinformatics analyses is applied to all steps from data capture, to data processing (eg, filtering and normalization), to establishing reproducible changes between states A and B, and to more complex integrative analyses from combining different -omic datasets. The statistical sciences are central to all these steps. The ultimate goal from these workflows is to identify network changes between states, and finally to identify nodes that exert control. Such nodes would then form attractive targets for interventionist wet laboratory-based experiments.

Several points are worth stressing from this theoretic workflow. First, study design and phenotype definition are critical. Second, all analyses include a denominator (eg, the genome, the detectable transcriptome, etc) so that any change is considered against the appropriate backdrop of all events occurring in the cell. Third, all data processing includes normalization across samples, including replicates and states, and subsequent filtering to remove the large component of the signal that is unchanging to, therefore, control the penalties of false discovery. Finally, the integrative steps have very high potential for creativity and novelty. That is, as the volume of publicly available data grows, the statistical approaches and types of data integration that can occur are varied and represent where many of the key biological questions of the future will be framed.

The mechanics of VDR signaling and disruption in cancer can, therefore, be analyzed in the paradigm of mining and analyzing large biological datasets. Therefore, there are several bioinformatics approaches that are applicable to the VDR. In the first instance, applying the genome as the denominator allows the question to be addressed of where VDR biology is significant. For example, in what cancer does a significant role for the VDR emerge when considering all genetic variations or gene expression?

At the simplest, GTEx project²⁷⁷ and the TCGA data can be investigated to identify in which normal tissue is the VDR most highly expressed, or in what cancer is it most commonly altered. The GTEx data reveal that the VDR is most abundant in tissues of the colon and small intestine, and least abundant in basal ganglia and brain tissues. The TCGA data reveal that the VDR is most commonly altered by deletion in 2 cohorts of adenoid cystic carcinoma of the breast.^{278,279} Interestingly, the GTEx data clearly reflect the focus at the preclinical and epidemiologic level of investigated VDR in colon cancer. However, no studies to date have examined VDR in the context of adenoid cystic carcinoma of breast cancer.

The TCGA^{280,281} data are derived from more than 33 different cancer types that were collected from approximately 11,000 patients. The analyses of these data have been the subject of more than 350 papers to date and it is striking that none of these papers identified a genome-wide significant role for disruption or association of the VDR with tumor phenotypes. By comparison, more than 100 TCGA papers report a significant relationship with TP53.

Often, biological signals are extremely contextual. Analyses of myeloid²⁸² and megakaryocyte²⁸³ cells illustrate that there is a significant role for the VDR to act in distinct transcriptional units that control specific points of cell differentiation. These findings reveal the intricate mechanistic basis to some of the earliest cancer studies on VDR signaling in leukemia,^{284,285} which revealed that exogenous vitamin D compounds can trigger cell differentiation. Therefore, reflecting on the role VDR seems to be playing in myeloid systems, it is worth stressing the apparent importance of VDR in immune phenotypes. That is, GWAS identify significant roles for VDR genetic variation in immune phenotypes.^{244,286,287}

Perhaps reflecting the reproduction-related functions of the VDR in murine systems,²⁸⁸ the *Vdr*^{-/-} mice display a mammary gland phenotype, and this genotype can modulate cancer incidence in murine cancer models.^{145,289,290} However, transcriptional and epigenomic control of breast epithelial systems in human cells does not reveal a genome-wide significant role for the VDR,²⁹¹ and the major breast cancer papers from TCGA have not identified a genome-wide significant role for the VDR to act as a cancer driver.²⁹²⁻²⁹⁵

Putting these findings together from leukemia and common cancers suggests that the VDR itself does not act as a direct cancer driver, either through loss or gain of

function. This finding may limit the likelihood of therapeutic exploitation in the cancer context.

Other approaches can be applied to leverage public data by changing the denominator. It is possible to address questions centered around the VDR and related genes, and thereby limit the penalties of false discovery. For example, previously we analyzed 13 transcription factor families implicated in cancer, including the NR superfamily, across 3000 tumors from 6 different tumor types.^{296–300} Bootstrapping approaches³⁰¹ established that, across cancers, only the NR family was significantly downregulated, but was neither significantly mutated nor altered by copy number variation.³⁰² Within the NRs, we found that several NRs were uniquely suppressed in only one tumor site, including *VDR* in the colon cancer (COAD) cohort; this finding may reflect the strong expression of *VDR* in the normal colon. *VDR* downregulation was not found to be driven by copy number variation or mutation and thus epigenetic mechanisms may be primarily responsible for altered expression.^{301,302} There is a very well-established literature supporting links between corrupted *VDR* signaling and colon cancer.^{85,147,303–308} Our pan-cancer analyses add to these findings, suggesting that loss of *VDR*-induced growth restraint may be more apparent in colon cancer than in other cancers where alterations are not apparent.

The *VDR* ChIP-seq data also lend themselves to be combined with other types of publicly available data to ask further questions concerning *VDR* function. For example, an attractive integration approach is to examine how significant genetic variation in transcription factor binding site can relate to phenotypes and disease susceptibility. Testing the possibility that genetic variation impacts transcription factor function underpins trait differences and disease phenotypes is analytically challenging, given the size of the datasets and the potential for false discovery. Various groups have addressed this challenge; notably, both the ENCODE and Roadmap Epigenome consortia leveraged the remarkable volume of ChIP-seq data they generated and merged the binding sites with GWAS data to reveal and rank sites where single nucleotide polymorphisms (SNPs) seem to have a significant impact on the activity of multiple transcription factors.^{124,271,309}

However, given that *VDR* has not been considered in any of these consortia, we have recently integrated *VDR* ChIP-Seq^{119–123} with National Human Genome Research Institute GWAS SNPs, and SNPs in linkage disequilibrium, to provide novel insight into the interaction between disease- and phenotype-associated SNPs and *VDR* binding. From these analyses, we applied transcription factor motif searching and exploited other ChIP-Seq data to identify significant interactions between the *VDR* and other transcription factors and disease traits. In this manner, we identified genetic variation that was significant at the genome-wide level enriched in *VDR* binding sites that were shared with nuclear factor- κ B binding regions related to immune phenotypes, including self-reported allergy.³¹⁰ However, none of the GWAS SNPs identified in *VDR* binding sites were neither in a DR3 type motif, again underscoring the diversity of *VDR* binding sites, nor related to cancer phenotypes.

However, there does seem to be a significant relationship between *VDR* and colon cancer, given that the *VDR* is highly expressed in the normal colon, associated with the control of local immunity,^{82,308,311–315} and that, of all the NRs, the *VDR* is commonly and significantly downregulated in colon cancer. To test this possibility, we leveraged *VDR* ChIP-Seq data derived in LS180 colon cancer cells¹²¹ with the expression of *VDR* target genes in the TCGA–COAD cohort.²⁹⁸ Clustering the tumors by expression patterns then allowed testing the relationships between expression of *VDR* target genes and clinical outcome. Expression of *VDR* target genes were either significantly repressed or activated in the COAD cohort, suggesting that *VDR* functions in both

activating and repressing complexes at the basal (or physiologically activated) state.³¹⁶ For instance, *LGALS4* is a VDR target gene that is specific to colonic cells and is downregulated in colon cancer, acting as a tumor suppressor,^{303,317,318} and *LGALS4* quartile expression patterns significantly associated with disease-free survival in specific patient subgroups.

A further opportunity available for meaningful data integration of ChIP-Seq studies is in the judicious choice of the cell line of study. For example, there are 3 tier 1 cell lines in the ENCODE project including K562 cells, which has approximately 600 publicly available genome-wide datasets. Therefore, there is an exciting opportunity once VDR ChIP-Seq is undertaken in one of these models in terms of integrative analyses³¹⁹ that could leverage ENCODE or RoadMap Epigenome data.

FUTURE CONSIDERATIONS AND SUMMARY

Enthusiasm remains for exploiting vitamin D signaling in cancer systems. This partly reflects that the biology is now very well-understood, that the toxicities associated with vitamin D compounds are easily monitored and managed and that in an era of high dimensional biological data it is possible to measure and dissect the actions of VDR signaling in very great detail. It seems likely that efforts will continue to exploit vitamin D compounds in the clinical setting, and it may well be that by exploiting tools to very accurately measure tumor type and burden will allow vitamin D-centered therapies to be applied with great precision. It seems likely that among the actions of VDR, the immunomodulatory capacity may ultimately be the ones that are most advantageous in cancer therapies.

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