

Chapter 2

The Molecular Cancer Biology of the VDR

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Abstract The development of an understanding of the role the vitamin D receptor (VDR) endocrine system plays to regulate serum calcium levels began approximately three centuries ago with the first formal descriptions of rickets. The parallel appreciation of a role for the VDR in cancer biology began approximately 3 decades ago and subsequently a remarkable increase has occurred in the understanding of its actions in normal and malignant systems.

Principally, much of this understanding has focused on understanding the extent and mechanism by which the VDR influences expression of multiple proteins whose combined actions are to govern cell cycle progression, induce differentiation, and contribute to the regulation of programmed cell death, perhaps in response to loss of genomic integrity. Predominantly, although not exclusively, these increases in target proteins reflect the transcriptional control exerted via the VDR. Reflecting the expanding understanding of how chromatin architecture is sensed and altered by transcription factors, the actions of the VDR have been defined through the large transcriptional complexes it is found in. The diversity of these complexes is large, and presumably underpins the pleiotropic biological actions that the VDR is associated with. The VDR is neither mutated nor deleted in malignancy but instead polymorphic variation distorts its ability to function, as indeed does expression of a number of associated cofactors, thereby skewing the ability to transactivate target genes.

Exploitation of this understanding into cancer therapeutic settings may occur through several routes, but perhaps a more systems orientated approach may yield insight by identifying and modeling points where the VDR, and closely related nuclear receptors, exert the most dominant control over cellular processes such as cell cycle control.

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Abbreviations

AR	Androgen receptor
bHLH	Basis helix loop helix
9 cRA	9 <i>cis</i> retinoic acid
1 α ,25(OH) ₂ D ₃	1 α ,25DihydroxyvitaminD ₃
DREAM	Downstream regulatory element antagonist modulator
ER	Estrogen receptor
FXR	Farnesoid X-activated receptor
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HSP	Heat shock protein
LCOR	Ligand-dependent nuclear receptor corepressor
LCA	Lithocholic acid
LXR	Liver X receptor
NCOR1	Nuclear receptor corepressor 1
NCOR2/SMRT	Silencing mediator of retinoid and thyroid hormone receptors/Nuclear receptor corepressor 2
NR	Nuclear receptor
PPAR	Peroxisome proliferator activated receptor
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
SLIRP	SRA stem loop-interacting RNA-binding protein
SRC	Steroid receptor coactivator
TRIP2/DRIP205	Thyroid hormone receptor interactor 2
TRIP15/COPS2/Alien	Thyroid hormone receptor interactor 15
VDR	Vitamin D receptor

2.1 Choreography of VDR Signaling

2.1.1 General Findings for VDR Transcriptional Actions

1 α ,25(OH)₂D₃ and its precursor 25(OH)D₃, in common with most NR ligands, are highly hydrophobic and transported in the aqueous blood stream associated with a specific binding protein (DBP) [1–3]. At the cell membrane they are free to diffuse across the lipid membrane, although the identification of Megalin as an active transport protein for 25(OH)D₃ suggests that transport into the cell of vitamin D₃ metabolites may be more tightly regulated than merely by passive diffusion alone [4]. Once in the cells of the target organ, 1 α ,25(OH)₂D₃ associates with the VDR.

In the absence of ligand, the VDR may be distributed throughout the cell, although predominantly located in the nucleus. There is evidence of cytoplasmic expression and cell-membrane-associated VDR that may mediate non-genomic

signal transduction responses [5, 6]. This is a feature of several NRs, such as the ER α , where the NR is cycled through caveolae at the cell membrane to initiate signal transduction pathways [6, 7]. The contribution of these actions to the overall functions of 1 α ,25(OH) $_2$ D $_3$ remains to be clarified fully. Interestingly, there is also evidence for the VDR to be actively trafficked into the nucleus upon ligand activation, in tandem with the heterodimeric partner RXRs [8], each in association with specific importins [9].

The majority of findings to date have addressed a nuclear function for the VDR associated with transcription. Structurally, the VDR is uncommon, compared to other NRs (NRs), as it does not contain an activation domain at its amino terminus (AF1). In most other receptors, this is an important domain for activation, for example, for autonomous ligand-independent AF function domain. The VDR instead relies on a domain in the carboxy terminus (AF-2) for activation and other domains for heterodimerization with RXR [10]. The VDR ligand-binding pocket contains hydrophobic residues such as His-305 and -397 that are important in the binding of 1 α ,25(OH) $_2$ D $_3$. Ligand binding specifically requires interaction of the hydroxyl group of the A ring at carbon 1 of 1 α ,25(OH) $_2$ D $_3$, which is added by the action of the 1 α hydroxylase enzyme. The binding of ligand causes an LBD conformational change, which allows the C-terminal helix 12 of the AF2 domain to reposition into an active conformation, exposing a docking surface for transcriptional co-regulators [11–13]. This switch of conformation of the LBD in the presence of ligand is a common feature in all ligand-binding NRs, as is the capacity to undergo receptor–cofactor interactions. Thus, both the unliganded and liganded VDR associates with a large number of different proteins involved with transcriptional suppression and activation, respectively.

When located within the nucleus and in the absence of ligand, the VDR exist in an “apo” state associated with RXR and corepressors (e.g., NCOR1 and NCOR2/SMRT) [14, 15] as part of large complexes (~2.0 MDa) [14, 16] and bound to RE sequences. These complexes in turn actively recruit a range of enzymes that post-translationally modify histone tails, for example, histone deacetylases (HDACs) and methyltransferases, and thereby maintain a locally condensed chromatin structure around response element sequences [17–20]. Ligand binding induces a so-called *holo* state, facilitating the association of the VDR-RXR dimer with coactivator complexes. A large number of interacting coactivator proteins have been described, which can be divided into multiple families including the p160 family, the non-p160 members, and members of the large “bridging” TRAP/DRIP/ARC complex, which links the receptor complex to the co-integrators CBP/p300 and basal transcriptional machinery [21, 22].

The complex choreography of these events has recently emerged from the study of the VDR [17, 23–28] and other NRs [29–32], and involves cyclical rounds of promoter-specific complex assembly, gene transactivation, complex disassembly, and proteosome-mediated receptor degradation coincident with corepressor binding and silencing of transcription. This gives rise to the characteristic periodicity of NR transcriptional activation and pulsatile mRNA and protein accumulation. However, the periodicity of VDR-induced mRNA accumulation of target genes is not shared, but

rather tends toward patterns that are specific for individual target genes and suggests that promoter-specific complexes combine to determine the precise periodicity [23, 24].

2.1.2 *VDR Signal Specificity*

Historically, researchers have tended to consider transcription factor actions in a somewhat monochrome view, for example, as illustrated for MYC and AP-1. These views are currently being revised in the light of surveys of genome binding sites and dissection of biological actions in a broader context (for example, reviewed in [33, 34]). These findings suggest that the functions of a given transcription factor superfamily are distilled through interaction with multiple cellular processes such that the normal capacity represents an extremely flexible and integrated signaling module. In malignancy, however, these transcriptional choices and phenotypic outputs generally become restricted [35].

The diversity of VDR expression sites, being detected in virtually all cells of a human, and the disparate phenotypic effects, from regulating calcium transport to sensing redox potential and DNA damage, also suggests that the cell specificity of actions may be distilled in a cell-type-specific manner. Therefore, the questions emerge as to what governs the temporal regulation of VDR-dependent transcriptions, among different cell types. Recent findings suggest that a high level of specificity of the timing and choice of VDR cofactor interactions may provide a mechanistic basis for signaling specificity. Combined expression and choice of interacting cofactors yield a high degree of NR transcriptional plasticity over choice, and timing of gene regulation [32, 36, 37].

Of the principal corepressors, it remains to be established to what extent specificity and redundancy occur. The expression, localization, and isoforms of NCOR1 and NCOR2/SMRT corepressors strongly influence the spatio-temporal equilibrium between repressing and activating NR complexes and transcriptional outputs [38]. The specificity of these corepressor interactions is beginning to emerge. Ncor1 and Ncor2/Smrt knockouts are embryonically lethal, whereas stem cell components from these mice and conditional approaches are revealing tissue-specific interactions [39–41] with distinct interacting domains being used to distinguish NR recognition [42]. Equally, the list continues to grow of novel corepressor proteins that the VDR interacts with.

Compared to the relatively massive size of the corepressors NCOR1 and NCOR2/SMRT, a number of smaller molecules have emerged as showing corepressor function. TRIP15/COPS2/Alien has been demonstrated to interact with the VDR and act as a corepressor, in an AF-2 independent manner that may not require the same interactions with HDACs that NCOR1 does [43]. Intriguingly, this protein contributes to the lid sub-complex of the 26S proteasome and thereby potentially links VDR function with the regulation of protein stability [44]. Similarly, SLIRP [45] has also emerged as a repressive factor for the VDR, although to date very little is known about the specificity, in terms of tissue and target gene.

Other repressors appear to demonstrate more specific phenotypic specificity. Hairless blocks VDR-mediated differentiation of keratinocytes, whereas addition of $1\alpha,25(\text{OH})_2\text{D}_3$ displaces Hairless from the promoter of target genes and recruits coactivators to promote differentiation [46–48]. Similarly, DREAM (downstream regulatory element antagonist modulator) usually binds to direct repeat response elements in the promoters of target genes to enhance transcription in VDR and RAR target genes, in a calcium-dependent manner, and suggests that specificity arises from the interactions of VDR with further tissue-specific cofactors [49].

Finally, the Williams syndrome transcription factor (WSTF), contained within WINAC complex, identified by Kato and colleagues, directly interacts with unliganded VDR and mediates binding to promoter sequences and can then bind and recruit other co-regulatory proteins. WINAC has ATP-dependent chromatin-remodeling activity and contains both SWI/SNF components and DNA replication-related factors. WINAC mediates the recruitment of unliganded VDR to its promoter target sites, and may organize local nucleosomal positioning to allow promoters access to co-regulators. This suggests a novel mechanism in transcriptional regulation, in which VDR binds to gene promoters before ligand is present [50, 51].

A similar level of coactivator specificity is also beginning to emerge. Members of the TRAP/DRIP complex were identified independently in association with the VDR and other NRs including the GR [52, 53] and TR [54–56]. The exact specificity of many of the co-regulatory factors remains to be established fully, although there are some suggestions that certain co-activators are VDR-specific, for example, NCoA-62 [57]. Similarly, knockout of TRAP220, which has multiple NR interacting domains, has begun to reveal distinct interactions, and notably disrupts the ability of the VDR to regulate hematopoietic differentiation [58, 59]. In keeping with the skin being a critical target for VDR actions, the specificity of VDR interactions with cofactor complexes has been dissected in detail by Bikle and colleagues who have demonstrated the timing and extent of coactivator binding, and established a role for SRC3 during specific stages of keratinocyte differentiation [60, 61].

Aside from the established co-regulators, some chaperone proteins have been reported to be regulators of VDR-mediated transcription. HSP70 down-regulates VDR to repress transcription [62], whereas BAG1L, an HSP70 binding protein, has been shown to bind to the VDR, and enhances VDR-mediated transcription [63]. Similarly, p23 and HSP90 have been shown to release the VDR/coactivator complex from the promoter of target genes in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$ [64]. The association of these HSPs suggests a natural cross-talk with other NRs, such as the AR, that associate with these chaperones in the cytoplasm.

Posttranslational modifications (PTM) possibly confer further VDR specificity of function. PTMs resulting from signal transduction processes, for example, bring about phosphorylation, acetylation, and ubiquitinylation events on the AR [65]. The VDR has been less extensively studied, but crucial roles have emerged for the phosphorylation of serine and threonine residues [66]. Subsequently, several residues have been identified that appear to regulate DNA binding and cofactor recruitment. The zinc finger DNA-binding domain is located at the N terminal of the VDR and

adjacent to this domain is the Serine 51 residue. This residue appears crucial for ligand-induced and phosphorylation-dependent transcriptional activation by the VDR. When Ser51 is mutated, phosphorylation of the VDR, by PKC at least, is all but completely abolished and its transcriptional activity is markedly reduced [67]. It is intriguing that the crucial site of PKC activity is located so close to the DNA-binding domain, but whether there are allosteric or biochemical changes that alter the ability of the VDR to bind DNA remains to be elucidated.

The common NR partner RXR can also be phosphorylated and as a result alters recruitment of cofactors to its *holo*-complexes. Ser260 is located within the ligand-binding domain of the RXR and appears crucial for mediating cofactor binding and ligand-induced transcriptional responses. When phosphorylated, Ser260 allows binding between the RXR and VDR, but presumably through allosteric changes to the complex, limits the recruitment of cofactors to the complex [68].

The recruitment of cofactors to the VDR *holo*-complex also appears to be regulated further by the presence of PTMs, for example, kinase CK-II. The phosphomimic mutant VDRS208D does not increase or decrease VDR–DNA, VDR–RXR, or VDR–SRC interactions but it does increase the levels of VDR–DRIP205 complexes present. CK-II which specifically phosphorylates Ser208 enhances $1,25(\text{OH})_2\text{D}_3$ -induced transactivation of VDR targets [69, 70]. In addition, phosphatase inhibitors (okadaic acid) in combination with $1,25(\text{OH})_2\text{D}_3$ shifts the cofactor preference from NCOA2/GRIP-1 to TRIP2/DRIP205 [71]. Taken together, these data suggest that the TRIP2/DRIP205 coactivator complex enhances the transcriptional response by VDR and is recruited by CK-II dependent phosphorylation of the VDR at Ser208.

2.1.3 Vitamin D Response Elements

A further level of specificity may arise from the specificity of binding sequence contained within the REs sequences of genomic targets. Simple REs are formed by two recognition motives and their relative distance and orientation contributes to receptor-binding specificity. Thus, the first identified VDRE was the DR3 – an imperfect hexameric direct repeat sequence AGTTCA with a spacer of three nucleotides. In the DR3 configuration, RXR, the heterodimer partner is believed to occupy the upstream half-site and VDR the downstream motif with two half-sites spaced by three nucleotides. Other types of VDREs have since been identified. One such VDRE is a palindromic sequence with a nine base-pair nucleotide spacer (IR9). This sequence was identified in the human calbindin D9K gene and like most VDREs the VDR/RXR binds this sequence in a 5′-RXR-VDR-3′ polarity (reviewed in [72]). More recently, a novel everted repeat sequence with a six base-pair nucleotide spacer (ER6) has been identified in the gene for *CYP3A4* (an enzyme important in xenobiotic metabolism) in addition to the DR3 already known to be present in this gene [73]. An inverted repeat with no spacer (IR0) has also been identified in the *SULT2A1* gene [74].

Similarly, the ability of VDR to display transrepression, that is, ligand-dependent transcriptional repression has received significant interest and reflects emerging themes for other NRs, for example, PPARs [75, 76], and highlights further the hitherto unsuspected flexibility of the VDR to associate with a diverse array of protein factors to adapt function [77, 78]. For example, analysis of the avian PTH gene has revealed a ligand-dependent repression of this gene by VDR [79]. The element mediating this effect was identified as a DR3, and since it resulted in transcriptional repression, the motif was referred to as a negative nVDRE. A similar nVDRE has been identified in the human kidney in the *CYP27b1* gene [80]. Interestingly, the VDR does not bind directly to this sequence; binding has been shown to be mediated by an intermediary factor known as a bHLH-type transcription factor, VDR interacting repressor (VDIR). It has since been shown that liganded VDR binds to the VDIR and indirectly causes repression through HDAC mechanisms [77].

More recently, larger and integrated responsive regions have been identified, suggesting a yet more intricate control involving integration with other transcription factors, for example, p53 and C/EBP α as demonstrated on the promoter/enhancer regions of *CDKN1A* and *SULT2A1*, respectively [23, 81]. Thus, the combinatorial actions of the VDR with other TFs most likely go some way toward explaining the apparent diversity of VDR biological actions. Again, for other NRs (e.g., AR and ER α), more dominant transcription factors, so-called pioneer factors, appear to be highly influential in determining choice and magnitude of transcriptional actions [82]. Recently, C/EBP family members have been demonstrated to act in a similar cooperative manner with the related PPAR γ [36] and it remains to be established to what extent the VDR interacts similarly with other transcription factors. The above findings are suggestive of a similar mechanism.

Efforts to understand VDR function have at their basis the antagonism between these *apo* and *holo* receptor complexes and the ability of these complexes to sense and regulate a diverse range of histone modifications. Histone modifications at the level of meta-chromatin architecture appear to form a stable and heritable “histone code,” such as in X chromosome inactivation (reviewed in [83]). The extent to which similar processes operate to govern the activity of micro-chromatin contexts, such as gene promoter regions, is an area of debate [84, 85]. The *apo* and *holo* NR complexes initiate specific and coordinated histone modifications [86, 87] to govern transcriptional responsiveness of the promoter. There is good evidence that specific histone modifications also determine the assembly of transcription factors on the promoter, and control individual promoter transcriptional responsiveness [88–90]. It is less clear to what extent complexes containing NRs in general, and VDR specifically, recognize basal histone modifications on target gene promoters; functional studies of the SANT motif contained in the corepressor NCoR2/SMRT support this latter idea [91]. This is a complex and rapidly evolving area and the reader is referred to an excellent recent review [75].

Collectively, these findings support the concept that the VDRs transcriptional actions reflect a convergence of multiple complexes, the details of which are still emerging and reflect the cross-talk, both cooperatively and antagonistically

between different cellular-signaling systems. Furthermore, the arena for VDR actions and interplay extends beyond the nucleus and integrates levels of cytoplasmic signal transduction, genomic and epigenomic regulation. Establishing the specificity of function and selectivity of VDR interactions has to an extent been limited by technical approaches. Unbiased approaches are now required to dissect VDR interactions (in the membrane, cytoplasm, and nucleus) in either individual cells or very pure populations, thereby to generate a comprehensive understanding of the spatial temporal network of its interactions.

2.2 Integrated VDR Actions

2.2.1 *Lessons from Murine Models*

The VDR plays a well-established endocrine role in the regulation of calcium homeostasis by regulating calcium absorption in the gut and kidney, and bone mineralization. $1\alpha,25(\text{OH})_2\text{D}_3$ status is dependent upon cutaneous synthesis initiated by solar radiation and also on dietary intake – a reduction of either one or both sources leads to insufficiency, although UV-initiated cutaneous $1\alpha,25(\text{OH})_2\text{D}_3$ synthesis is the principal route 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. That is, the 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. Current recommendations for daily vitamin D_3 intake are in the range of 400–800 IU/day [92]. More recently, reassessment of the $1\alpha,25(\text{OH})_2\text{D}_3$ impact on the prevention of osteoporosis has suggested that the correct level may be as high as 2–3,000 IU/day, which may reflect more accurately “ancestral” serum levels [93].

The importance of the relationship between UV exposure and calcium homeostasis has driven the endocrine view of $1\alpha, 25(\text{OH})_2\text{D}_3$ synthesis and signaling. In parallel, local generation of $1\alpha, 25(\text{OH})_2\text{D}_3$ in target tissues has become apparent and supported a separate autocrine role to regulate cell proliferation and differentiation, and other functions including the modulation of immune responses.

Key insights into these functions have been gained in *Vdr*-deficient mice [94–96]. The *Vdr* is expressed widely during murine embryonic development in tissues involved in calcium homeostasis and bone development. *Vdr* disruption results in a profound phenotype in these models, which is principally observed post-weaning and is associated with the alteration of duodenal calcium absorption and bone mineralization, resulting in hypocalcemia, secondary hyperparathyroidism, osteomalacia, rickets, impaired bone formation, and elevated serum levels of $1\alpha,25(\text{OH})_2\text{D}_3$. In parallel, a range of more subtle effects are seen more clearly when the animals are rescued with dietary calcium supplementation

and may represent autocrine and non-calcemic actions. The animals became growth-retarded, display alopecia, uterine hypoplasia, impaired ovarian folliculogenesis, reproductive dysfunction, cardiac hypertrophy, and enhanced thrombogenicity.

2.2.2 Self-renewing Epithelial Systems

The sporadic, temporal acquisition of a cancer phenotype is compatible with models of disruption of the self-renewal of epithelial tissues. It has become increasingly clear that breast, colon, and prostate tissues, in common with other epithelial tissues and many other cell types in the adult human, are self-renewing and contain committed stem cell components [97–102]. These stem cells are slowly proliferating and are able to undergo asymmetric divisions to give rise both to other stem cells and transiently amplifying (TA) populations of progenitor cells, that in turn give rise to the differentiated cell types, which typify the functions of these tissues and are subsequently lost through programmed cell death processes and replaced by newly differentiated TA cells. The mechanisms that control the intricate balance of these processes of division, differentiation, and programmed cell death are subjects of significant investigations. These studies have revealed common roles for Wnt and hedgehog signaling and the actions of other signal transduction processes that govern cell cycle progression, with gene targets such as the cyclin-dependent kinase inhibitor *CDKN1A* (which encodes p21^(waf1/cip1)) emerging as points of criticality upon which numerous signal pathways converge.

Against this backdrop, the Vdr operates in several self-renewing tissues. The Vdr is readily detected in keratinocytes and co-treatment of calcium and $1\alpha,25(\text{OH})_2\text{D}_3$ decreases proliferation and promotes differentiation of cultured keratinocytes [103]. The Vdr is also detected in outer root sheath and hair follicle bulb, as well as in the sebaceous glands [104] and the Vdr $-/-$ mice develop hair loss and ultimately alopecia totalis, associated with large dermal cysts, that is not prevented by the high calcium rescue diet. The alopecia arises due to a complete failure to initiate anagen, which is the first postnatal hair growth phase. Subsequently, the hair follicles convert into epidermal cysts [105]. Hair follicle formation requires highly coordinated signaling between different cell types including contributions from the stem cells components and therefore the alopecia phenotype has attracted significant research interest as it may represent a role for the VDR in stem cell maintenance. Subsequent studies have demonstrated that a failure to maintain hair follicles in Vdr $-/-$ animals does not actually reflect a loss of follicle stem cells but rather an inability of the primitive progenitor cells to migrate along the follicle at the onset of anagen [106].

Interestingly, these effects appear independent of ligand binding, in that they can be rescued even when Vdr is mutated in the LBD, but not completely if the AF2 domain is interrupted, suggesting that the association with cofactors is

required [107]. Notably, the corepressor, Hairless plays a clear role in hair formation with either knockout or mutation resulting in alopecia strikingly similar to that observed in the Vdr null mice [108, 109].

Wnt signaling is one of the major processes regulating postmorphogenic hair follicle development. Interestingly, the development of dermal cysts and increase in sebaceous glands observed in the Vdr and Hairless $-/-$ mice are also similar to mice expressing a keratinocyte-specific disruption to β -catenin [110, 111]. These findings have raised the possibility that one function of the Vdr may be to co-regulate aspects of Wnt signaling, a concept that is supported further by the physical association of VDR in a complex with β -catenin and other Wnt components [112].

Another unexpected finding of the Vdr $-/-$ animals was the uterine hypoplasia and impaired ovarian function in the females that leads to dramatically reduced fertility. Similarly to the hair phenotype, this was not restored by the rescue diet of high calcium [94]. Estradiol supplementation, however, of the female mice restored uterine function and fertility and suggests the fault lies with an inability to generate estrogen. The mammary gland has also been studied extensively, in a comprehensive series of experiments by Welsh and coworkers [113, 114] and represents an intriguing tissue where endocrine (calcemic8) and autocrine (antimitotic, pro-differentiative, pro-apoptotic) effects of the VDR appear to converge.

These phenotypes underscore the integrated nature of VDR signaling. That is, the biology of hair regeneration and mammary gland function reflects the choreographed actions of VDR, with other NRs, alongside other regulatory processes including Wnt signaling. Dysfunction of multiple aspects of this is seen in many cancer phenotypes.

2.3 VDR Transcriptional Networks in Malignancy

Defining the mechanisms by which the VDR exerts desirable anticancer effects has been an area of significant investigation since the early 1980s. In 1981, $1\alpha,25(\text{OH})_2\text{D}_3$ was shown to inhibit human melanoma cell proliferation significantly *in vitro* at nanomolar concentrations [115], and was subsequently found to induce differentiation in cultured mouse and human myeloid leukemia cells [116, 117]. Following these studies, anti-proliferative effects have been demonstrated in a wide variety of cancer cell lines, including those from prostate, breast, and colon [118–125]. To identify critical target genes that mediate these actions, comprehensive genome-wide *in silico* and transcriptomic screens have analyzed the anti-proliferative VDR transcriptome and revealed broad consensus on certain targets, but has also highlighted variability [118, 126–128]. This heterogeneity may in part reflect experimental conditions, cell line differences, and genuine tissue-specific differences of cofactor expression that alter the amplitude and periodicity of VDR transcriptional actions.

2.3.1 Cell Cycle Arrest

A common anti-proliferative VDR function is associated with arrest at G_0/G_1 of the cell cycle, coupled with upregulation of a number of cell cycle inhibitors including p21^(waf1/cip1) and p27^(kip1). Promoter characterization studies have demonstrated a series of VDREs in the promoter/enhancer region of *CDKN1A* [23, 129]. By contrast, the regulation of the related CDKI p27^(kip1) is mechanistically enigmatic, reflecting both transcriptional and translational regulation such as enhanced mRNA translation, and attenuating degradative mechanisms [130–133].

The up-regulation of p21^(waf1/cip1) and p27^(kip1) principally mediate G_1 cell cycle arrest, but $1\alpha,25(OH)_2D_3$ has been shown to mediate a G_2/M cell cycle arrest in a number of cancer cell lines via direct induction of *GADD45 α* [127, 134, 135]. Again, this regulation appears to combine direct gene transcription and a range of posttranscriptional mechanisms. These studies highlight the difficulty of establishing strict transcriptional effects of the VDR, as a range of posttranscriptional effects act in concert to regulate target protein levels. Concomitant with changes in the cell cycle there is some evidence that $1\alpha,25(OH)_2D_3$ also induces differentiation, most clearly evidenced 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, the expression of CDKIs such as p21^(waf1/cip1), and the induction of cellular differentiation [136].

Historically, hematological malignancies combined an ease of interrogation with robust classification of cellular differentiation capacity which was envied by investigators of solid tumors. It is therefore no coincidence that these cell systems yielded many important insights for cancer cell biologists generally, such as chromosomal translocations and instability, and the role of committed adult stem cells.

Indeed, the capacity to readily differentiate in response to external and internal signals has fascinated leukemia researchers as they have sought to understand why leukemia cells appear to fail at certain stages of differentiation. It is within this context that in the 1980s, investigators [137, 138] considered a role for the VDR and the related retinoic acid receptor (RAR) to reactivate dormant differentiation programs in so-called differentiation therapies. Over the following 2 decades, researchers began to reveal how these receptors instill mitotic restraint and facilitate differentiation programs and how discord over the control and integration of these processes is central to leukemogenesis. Despite these efforts, clinical exploitation of these receptors has largely proved to be equivocal. The one exception to this translational failure has been the exploitation of RAR signaling in patients with acute promyelocytic leukemia. Again, understanding the basic signaling behind this application proved significant to the developing understanding of epigenetic regulation of transcription and the promise of HDAC inhibitors [139].

Against this backdrop, various groups, including that of Studzinski, have worked consistently exploring mechanisms of resistance to VDR signaling and methods of exploitation and recently demonstrated, elegantly, a role for VDR to down-regulate miR181a, which when left unchecked degrades p27^(kip1). Thus, indirectly VDR

activation elevates expression of $p27^{(kip1)}$, initiates cell cycle arrest, and commits cells toward differentiation. Transcriptional control of miRNAs and their biological effects are clearly a field of rapid expansion, and members of the NR superfamily are implicated in their regulation [140, 141]. A role for the VDR to govern the expression of this regulatory miRNA and, importantly, place its role in the well-understood map of differentiation is highly novel.

2.3.2 Sensing DNA Damage

An important and emergent area, both in terms of physiology and therapeutic exploitation, is the role the liganded VDR appears to play in maintaining genomic integrity and facilitating DNA repair. There appears to be close cooperation between VDR actions and the p53 tumor suppressor pathway. The maintenance of genomic fidelity against a backdrop of self-renewal is central to the normal development and adult function of many tissues including the mammary and prostate glands, and the colon. For example, in the mammary gland p53 family members play a role in gland development and maintenance. *P63* $-/-$ animals have an absence of mammary and other epithelial structures, associated with a failure of lineage commitment (reviewed in [142]), whereas *p53* $-/-$ animals have delayed mammary gland involution, reflecting the *Vdr* $-/-$ animals, and wider tumor susceptibility (reviewed in [143]).

The overlap between p53 and VDR appears to extend beyond cellular phenotypes. The VDR is a common transcriptional target of both p53 and p63 [144, 145] and VDR and p53 share a cohort of direct target genes associated with cell cycle arrest, signal transduction, and programmed cell death including *CDKN1A*, *GADD45A*, *RB1*, *PCNA*, *Bax*, *IGFBP3*, *TGFB1/2*, and *EGFR* [23, 128, 135, 146–150]. At the transcriptional level, both VDR heterodimers and p53 tetramers associate, for example, with chromatin remodeling factors CBP/p300 and the SWI/SNF to initiate transactivation [51, 151]. By contrast, in the gene repressive state VDR and p53 appear to associate with distinct repressor proteins, for example, p53 with SnoN [152], and VDR with NCOR1, suggesting the possibly association with distinct sets of histone deacetylases. Indeed, *CDKN1A* promoter-dissection studies revealed adjacent p53 and VDR-binding sites, suggesting composite responsive regions [23]. Together, these findings suggest that $1\alpha,25(\text{OH})_2\text{D}_3$ -replete environments enhance p53 signaling to regulate mitosis negatively.

Similarly the role of $1\alpha,25(\text{OH})_2\text{D}_3$ in the skin is also suggestive of its chemopreventive effects. UV light from sun exposure has several effects in the skin; UVA light induces DNA damage through increasing the level of reactive oxygen species (ROS), but importantly UVB light also catalyzes the conversion of 7-dehydroxysterol to $25(\text{OH})\text{-D}$ and induces the expression of VDR.

In addition, antimicrobial and anti-inflammatory genes are another subset of VDR targets that are induced by UV radiation. Suppression of the adaptive inflammatory response is thought to be protective for several reasons. Inflamed tissues

contain more ROS, which in turn can damage DNA and prevent proper function of DNA repair machinery. Also the induction of cytokines and growth factors associated with inflammation act to increase the proliferative potential of the cells. NF- κ B is a key mediator of inflammation and the VDR attenuates this process by negatively regulating NF- κ B signaling [153]. This control by VDR is underscored by studies showing *Vdr*^{-/-} mice are more sensitive to chemicals that induce inflammation than their wild-type counterparts [154]. The normally protective effect of inflammation that occurs under other conditions is lost through VDR-mediated suppression but is compensated for by the induction of a cohort of antimicrobial and antifungal genes [155–157]. The induction of antimicrobials not only prevents infection in damaged tissue but can be cytotoxic for cells with increased levels of anion phospholipids within their membranes, a common feature of transformed cells [158]. Finally, and most recently, network strategies have been used in different strains of mice with altered sensitivity toward skin cancer. Remarkably, in such unbiased screens, the VDR emerges as a key nodal control point in determining sensitivity toward skin tumors as it regulates both turnover of self-renewal and inflammatory infiltrate [159].

The key question, and central to exploiting any therapeutic potential of this receptor, is why should the VDR exert such pleiotropic actions? One possible explanation for this pleiotropism is that it represents an adaptation of the skin to UV exposure, coupling the paramount importance of initiating $1\alpha,25(\text{OH})_2\text{D}_3$ synthesis with protection of cell and tissue integrity. Thus, VDR actions are able to maximize UV-initiated synthesis of $1\alpha,25(\text{OH})_2\text{D}_3$ production, whilst controlling the extent of local inflammation that can result from sun exposure. To compensate for the potential loss of protection associated with immunosuppression, the VDR mediates a range of antimicrobial actions. Equally, local genomic protection is ensured through the upregulation of target genes which induce G_0/G_1 arrest, cooperation with p53, and the induction of cell differentiation. It remains a tantalizing possibility that the functional convergence between p53 family and VDR signaling, which arose in the dermis as an evolutionary adaptation to counterbalance the conflicting physiological requirements of vitamin D synthesis and genome protection, are sustained in epithelial systems, such as the lining of the mammary gland, to protect against genotoxic insults derived from either the environment or local inflammation.

2.3.3 Programmed Cell Death

VDR actions, notably in MCF-7 breast cancer cells, are associated with a profound and rapid induction of apoptosis, irrespective of p53 content. This may reflect the VDR role in the involution of the post-lactating mammary gland. The direct transcriptional targets which regulate these actions remain elusive, although there is evidence of an involvement of the BAX family of proteins [160, 161]. Induction of programmed cell death following $1\alpha,25(\text{OH})_2\text{D}_3$ treatment is also associated with increased ROS generation. $1\alpha,25(\text{OH})_2\text{D}_3$ treatment up-regulates *VDUP1* encoding

vitamin D up-regulated protein 1, which binds to the disulfide reducing protein thioredoxin and inhibits its ability to neutralize ROS, thereby potentiating stress-induced apoptosis [162, 163]. In other cells, the apoptotic response is delayed and not so pronounced, and probably reflecting less direct effects. Taken together, these data suggest that extent and timing of apoptotic events depend on the integration of VDR actions with other cell signaling systems. This regulation of apoptosis in human cancer cell lines reflects, of course, the absence of apoptosis in chondrocytes in the Vdr $-/-$ animals [7].

2.4 Mechanisms of Resistance Toward the VDR

A major limitation in the therapeutic exploitation of VDR in cancer therapies is the resistance of cancer cells toward $1\alpha,25(\text{OH})_2\text{D}_3$. An understanding of the molecular mechanisms of resistance has emerged.

2.4.1 *Reduced Local Availability of $1\alpha,25(\text{OH})_2\text{D}_3$*

Tumors, such as breast cancer appear to distort the VDR signaling axis locally, with reduced *CYP27b1* mRNA and protein levels, and comparative genome hybridization studies have found that *CYP24* is amplified in human breast cancer [164, 165]. Thus, cancer cells maybe associated with low circulating concentrations of $25(\text{OH})\text{D}_3$, arising as a result of reduced exposure to sunlight, altered dietary patterns, and exacerbated further by impaired local generation of $1\alpha,25(\text{OH})_2\text{D}_3$. In support of these in vitro findings, a large number of epidemiological studies have identified an association between environments of reduced serum $25(\text{OH})\text{D}$ and cancer rates.

2.4.2 *Dominant Signal Transduction Events*

In terms of distribution, evidence is emerging that the normally dynamic flux of the VDR becomes altered in more transformed and aggressive cancer cells, becoming restricted to the nucleus [166, 167]. These findings that the normal transport rates, such as importin-mediated processes, become distorted in malignancy and may result in a reduced ability for the VDR to sample the cytoplasm for $1\alpha,25(\text{OH})_2\text{D}_3$.

Reflecting the cooperative and integrated nature of the VDR to function as a transcription factor, a number of workers have identified mechanisms by which more dominant signaling process are able either to ablate or attenuate VDR

signaling. For example, Munoz and coworkers have dissected the interrelationships between the VDR, E-cadherin, and the Wnt signaling pathway in colon cancer cell lines and primary tumors. In these studies, the induction of *CDH1* (encodes E-Cadherin) was seen in subpopulations of SW480 colon cancer cells, which express the VDR and respond to $1\alpha,25(\text{OH})_2\text{D}_3$. The VDR thereby limits the transcriptional effects of β -catenin by physically and directly binding it in the nucleus, and by upregulating E-cadherin to sequester β -catenin in the cytoplasm. In malignancy, these actions are corrupted through downregulation of *VDR* mRNA, which appears to be a direct consequence of binding by the transcriptional repressor SNAIL; a key regulator of the epithelial-mesenchyme transition, which is overexpressed in colon cancer [168–170]. Equally underscoring the central importance of β -catenin, it has recently been shown to be posttranslationally modified to act as VDR coactivator and supports a model of checks and balances between these two signaling processes [168, 171].

2.4.3 Genetic Resistance

In cancer, and outside of the very limited pool of mutations reported in the VDR in type II rickets, the receptor, generally, is neither mutated nor does it appear to be the subject of cytogenetic abnormalities [172]. By contrast, polymorphic variations of the *VDR* have been widely reported. Thus polymorphisms in the 3' and 5' regions of the gene have been described and variously associated with risk of breast, prostate, and colon cancer, although the functional consequences remain to be established clearly. For example, a start codon polymorphism in exon II at the 5' end of the gene, determined using the *fok*-I restriction enzyme, results in a truncated protein. At the 3' end of the gene, three polymorphisms have been identified that do not lead to any change in either the transcribed mRNA or the translated protein. The first two sequences generate *Bsm*I and *Apa*I restriction sites and are intronic, lying between exons 8 and 9. The third polymorphism, which generates a *Taq*I restriction site, lies in exon 9 and leads to a silent codon change (from ATT to ATC) which both inserts an isoleucine residue at position 352. These three polymorphisms are linked to a further gene variation, a variable length adenosine sequence within the 3' untranslated region (3'UTR). The poly(A) sequence varies in length and can be segregated into two groups; long sequences of 18–24 adenosines or short ones [173–176]. The length of the poly(A) tail can determine mRNA stability [177–179] so the polymorphisms resulting in long poly(A) tails may increase the local levels of the VDR protein.

Multiple studies have addressed the association between *VDR* genotype and cancer risk and progression. In breast cancer, the *Apa*I polymorphism shows a significant association with breast cancer risk, as indeed have *Bsm*I and the “L” poly(A) variant. Similarly, the *Apa*I polymorphism is associated with metastases to bone [180, 181]. The functional consequences of the *Bsm*I, *Apa*I, and *Taq*I polymorphisms are unclear, but because of genetic linkage may act as a marker for the

poly(A) sequence within the 3'UTR, which in turn determines transcript stability. Interestingly, combined polymorphisms and serum 25OH-D levels have been shown to compound breast cancer risk and disease severity further [182].

Earlier studies suggested that polymorphisms in the VDR gene might also be associated with risk factor of prostate cancer. Ntais and coworkers performed a meta-analysis of 14 published studies with four common gene polymorphisms (*Taq1*, poly A repeat, *Bsm1*, and *Fok1*) in individuals of European, Asian, and African descent. They concluded that these polymorphisms are unlikely to be major determinants of susceptibility to prostate cancer on a wide population basis [183]. Equally, studies in colon cancer have yet to reveal conclusive relationships and may be dependent upon ethnicity of the population studied.

2.4.4 Epigenetic Resistance

In cancer cells, the lack of an antiproliferative response is reflected by a suppression of the transcriptional responsiveness of anti-proliferative target genes such as *CDKN1A*, *CDKN1B*, *GADD45A* and *IGFBPs*, *BRCA1* [120, 135, 184, 185]. Paradoxically, VDR transactivation of other targets is sustained or even enhanced, as measured by induction of the highly $1\alpha,25(\text{OH})_2\text{D}_3$ -inducible *CYP24* gene [186, 187]. Together these data suggest that the lack of functional VDR alone cannot explain resistance and instead the VDR transcriptome is skewed in cancer cells to disfavor anti-proliferative target genes. It has been proposed that this apparent $1\alpha,25(\text{OH})_2\text{D}_3$ -insensitivity is the result of epigenetic events that selectively suppress the ability of the VDR to transactivate target genes [188].

The epigenetic basis for such transcriptional discrepancies has been investigated intensively in prostate cancer. VDR-resistant prostate cancer cells are associated with elevated levels of NCOR2/SMRT [135, 184]; these data indicate that the ratio of VDR to corepressor may be critical to determine $1\alpha,25(\text{OH})_2\text{D}_3$ responsiveness in cancer cells. An siRNA approach toward *NCoR2/SMRT* demonstrated a role for this corepressor to regulate this response *GADD45 α* in response to $1\alpha,25(\text{OH})_2\text{D}_3$. By contrast, knockdown of NCOR1 does not restore anti-proliferative responsiveness toward $1\alpha,25(\text{OH})_2\text{D}_3$ but does reactivate transcriptional networks governed by PPARs [189]. These data support a central role for elevated NCOR2/SMRT levels to suppress the induction of key target genes, resulting in loss of sensitivity to the anti-proliferative action of $1\alpha,25(\text{OH})_2\text{D}_3$; other workers have reinforced these concepts [190, 191].

Parallel studies have demonstrated a similar spectrum of reduced $1\alpha,25(\text{OH})_2\text{D}_3$ -responsiveness between nonmalignant breast epithelial cells and breast cancer cell lines. Again, this was not determined solely by a linear relationship between the levels of $1\alpha,25(\text{OH})_2\text{D}_3$ and VDR expression. Rather, elevated corepressor mRNA levels, notably of *NCoR1*, in ER α negative breast cancer cell lines and primary cultures, were associated with $1\alpha,25(\text{OH})_2\text{D}_3$ insensitivity [192]. Elevated NCOR1

has also been demonstrated to suppress the VDR responsiveness of bladder cancer cell lines [166], notably toward the VDR ligand lithocholic acid (LCA) [193], suggesting a role for epigenetic disruption of the capacity of cells to sense and metabolize potential genotoxic insults.

The epigenetic lesion rising from elevated NCOR1 can be targeted by co-treatment of either $1\alpha,25(\text{OH})_2\text{D}_3$ or its analogs, plus the HDAC inhibitors such as trichostatin A, and can restore the $1\alpha,25(\text{OH})_2\text{D}_3$ -responses of androgen-independent PC-3 cells to levels indistinguishable from control normal prostate epithelial cells. This reversal of $1\alpha,25(\text{OH})_2\text{D}_3$ insensitivity was associated with reexpression of gene targets associated with the control of proliferation and induction of apoptosis, notably *GADD45A* [120, 135, 185]. Similarly, targeting in breast cancer cells through co-treatments of $1\alpha,25(\text{OH})_2\text{D}_3$ with HDAC inhibitors coordinately regulated VDR targets and restored anti-proliferative responsiveness [192, 194]. Similarly, other workers have used combinatorial chemistry to combine aspects of the structure of $1\alpha,25(\text{OH})_2\text{D}_3$ and HDAC inhibitors into a single molecule that demonstrates very significant potency [195].

Together, these data support the concept that altered patterns of corepressors inappropriately sustain histone deacetylation around the VDRE of specific target gene promoter/enhancer regions, and shifts the dynamic equilibrium between *apo* and *holo* receptor conformations, to favor transcriptional repression of key target genes. Furthermore, targeting this epigenetic lesion with co-treatments of vitamin D_3 compounds plus HDAC inhibitors generates a temporal window where the equilibrium point between *apo* and *holo* complexes is shifted to sustain a more transcriptionally permissive environment.

These findings compliment a number of parallel studies that have established cooperativity between $1\alpha,25(\text{OH})_2\text{D}_3$ and butyrate compounds, such as sodium butyrate (NaB) [196–201]. These compounds are short-chain fatty acids produced during fermentation by endogenous intestinal bacteria and have the capacity to act as HDAC inhibitors. Stein and coworkers have identified the effects in colon cancer cells of $1\alpha,25(\text{OH})_2\text{D}_3$ plus NaB co-treatments to include the coordinate regulation of the VDR itself. Together these studies underscore further the importance of the dietary-derived milieu to regulate epithelial proliferation and differentiation beyond sites of action in the gut.

2.5 Toward an Integrated Understanding of the VDR

A highly conserved VDR is found widely throughout metazoans, even in certain non-calcified chordates such as the lamprey (reviewed in [202]). Within prokaryotes there appears to be the capacity to undertake UV-catalyzed metabolism of cholesterol compounds and suggests that the evolution of vitamin D biochemistry is very ancient. These findings suggest that the VDR system has been adapted to regulate calcium function and retains other functions that are calcium-independent and include the capacity to sense the local environment.

Phylogenetic classification has defined seven NR subfamilies, and within these the VDR is in the group 1 subfamily, sharing homology with the LXRs and FXR, and more distantly the PPARs [203, 204]. The receptors within this subfamily preferentially form homo- or heterodimeric complexes with RXR acting as a common central partner for VDR, PPARs, LXRs, and FXR. Thus, the receptors in the group appear to be all responsive to either bile acid or xenobiotic receptors and, therefore, widely integrated with bile acid homeostasis and detoxification. In keeping with this capacity, the bile acid lithocholic acid (LCA) has recently been shown to be a potent ligand for the VDR all be it with lower millimolar affinity [193].

VDR biology participates in at least three fundamental areas of biology required for human health, and which are disrupted in human disease. It participates in the regulation of serum calcium, and by implication the maintenance of bone integrity; the control of cell proliferation and differentiation; and by implication the disruption of these actions in malignancy; and as a modifier of immune responses and by implication contributes toward auto-immune diseases [205]. The divergence of these actions may make the VDR a particularly challenging receptor to understand in terms of biology and to exploit therapeutically.

Specifically dietary-derived fatty acids and bile acids cycle rapidly in response to dietary intake and work hormonally to coordinate multiple aspects of tissue function in response to changing energetic status. Thus, it is unlikely that the VDR alone plays a key and dominant role in cell and tissue function by acting singularly, but instead is intimately linked to the actions of related NRs (e.g., PPARs, FXR, and LXR) and cofactors. In this manner, the actions of the VDR to regulate cell growth and differentiation, as part of a network of environmental and dietary sensing receptors, may be the central and common function for the VDR. The differentiated phenotype of these cells then participates in diverse biology that range from calcium transport to dermis formation and mammary gland function.

For “next generation” developments to occur it will be necessary to adopt a broader view of VDR signaling. Historically, researchers have studied the abilities of single NRs such as the VDR to regulate a discrete group of gene targets and influence cell function. This has led to substantial knowledge concerning many of these receptors, individually. Cell and organism function, however, depends on the dynamic interactions of a collection of receptors, through the networks that link them, and against the backdrop of intrinsic cellular programs, such as those governing development and differentiation.

In such a view, it is apparent that NRs act as an adaptive homeostatic network in several tissues to sense environmental dietary and xenobiotic lipophilic compounds and sustain the cell, for example, through the diurnal patterns of fast and feeding (reviewed in [204, 206]). The VDR was originally described for a central endocrine role in maintenance of serum calcium levels. Similarly, the FXR and LXRs were described for their central role in cholesterol metabolism and bile acid synthesis in the enterohepatic system. However, their expression in multiple target tissues such a broader role. Examination of the known target genes for VDR, RARs, PPARs, FXR, and LXRs reveals that they share in common the regulation of cell cycle, programmed cell death, differentiation, and xenobiotic and metabolic clearance.

The challenge is to model the spatio-temporal actions of the NR network and, in particular, the extent to which the VDR exerts critical control over transcription and translation. Such an understanding requires a clear awareness of the chromatin architecture and context of the promoter regions (e.g., histone modifications, DNA methylation), genomic organization, gene regulation hierarchies, and $1\alpha,25(\text{OH})_2\text{D}_3$ -based metabolomic cascades, all within the context of specific cell backgrounds. The ultimate research goal will be to translate this understanding to strategies that can predict the capacity of subsets of VDR actions to be regulated and targeted in distinct cell types and exploited in discrete disease settings.

The current lack of an integral view of how these interactions bring about function and dysfunction, for example, in the aging human individual, can be attributed to the limitations of previously available techniques and tools to undertake such studies. The implementation of post-genomic techniques together with bioinformatics and systems biology methodology is expected to generate an integral view, thereby revealing and quantifying the mechanisms by which cells, tissues, and organisms interact with environmental factors such as diet [207, 208].

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