

The vitamin D receptor: contemporary genomic approaches reveal new basic and translational insights

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The vitamin D receptor (VDR) is the single known regulatory mediator of hormonal 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] in higher vertebrates. It acts in the nucleus of vitamin D target cells to regulate the expression of genes whose products control diverse, cell type-specific biological functions that include mineral homeostasis. In this Review we describe progress that has been made in defining new cellular sites of action of this receptor, the mechanisms through which this mediator controls the expression of genes, the biology that ensues, and the translational impact of this receptor on human health and disease. We conclude with a brief discussion of what comes next in understanding vitamin D biology and the mechanisms that underlie its actions.

Introduction

In the early 1970s, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] was identified as both the exclusive, metabolically active form of vitamin D and a key component of what proved to be an exquisite endocrine system that regulates numerous biologic processes in higher vertebrates (1, 2). This pioneering discovery ended a decades-long quest to understand the dynamic processes that govern the formation of vitamin D in the skin and its sequential activation to 25(OH)D₃ in the liver and then to 1,25(OH)₂D₃ in the kidney. These findings stimulated numerous subsequent mechanistic studies aimed at defining the regulation of CYP27B1, the renal enzyme responsible for the production of 1,25(OH)₂D₃ (3). These early achievements in hormone identification were followed closely by the discovery and characterization of a receptor molecule (later termed the vitamin D receptor [VDR]) that was hypothesized to mediate the actions of 1,25(OH)₂D₃ in the nucleus of target cells (4, 5). Importantly, the cloning of this receptor well over a decade later confirmed that the vitamin D hormone was indeed part of a true steroid-like hormone system whose physiologic functions were dictated through receptor-mediated activities that were mechanistically similar to that of other steroid endocrine systems (6–8). Vitamin D plays a major role in orchestrating the maintenance of mineral homeostasis through specific actions in the intestine, skeleton, and kidney. It also regulates the synthesis and production of additional calciotropic and phosphotropic hormones including parathyroid hormone (PTH) and FGF23 (9–11), the latter a skeletally produced phosphatonin hormone that controls phosphate balance via the kidney (12). Importantly, alterations in vitamin D hormone production in the kidney and/or its ubiquitous degradation in the kidney and virtually all other target tissues can lead to a wide variety of diseases of mineral dysregulation or other significant pathophysiological states (13). In this Review we discuss the most recent contemporary genomic advances in understanding the vitamin D system and the regulatory molecules that are centrally

involved (14, 15). With this background, we then comment briefly on the current translational impact of several features of VDR action and function on human health and disease.

The VDR

VDR tissue distribution. The VDR was discovered initially in tissues involved in the regulation of calcium and phosphate homeostasis, namely the intestine, bone, kidney, and parathyroid glands (16). Given that these tissues hold the highest levels of VDR expression, this finding was not surprising. However, as VDR expression emerged in other tissues (17–19), it became clear that vitamin D action in many cellular targets was unrelated to mineral regulation, suggesting the likelihood for additional vitamin D hormone functions (20). The VDR is now known to be expressed in many non-calcium-regulating cell types including dermal fibroblasts and keratinocytes of skin, immune cells, selected cardiovascular cell types, and cellular components of numerous other tissues (21). Much of the biology associated with the actions of vitamin D in these tissues has been or is in the process of being delineated (Figure 1). Although this wider distribution is not in dispute, the concept that the VDR is ubiquitously distributed has been highly overstated. Indeed, the VDR is often expressed in one or more cellular subsets that frequently represent only minor components of more complex organs. Thus, there has been difficulty in detecting the VDR in tissues such as muscle (22), liver (23), and particularly the CNS (24), where the incredible sensitivity of quantitative reverse transcriptase PCR analysis provides evidence of low levels of VDR RNA transcripts that are then difficult to confirm at the protein level using much less sensitive, antibody-based detection methods.

The identity of VDR-positive cells is important for understanding the biology of vitamin D, as it is well recognized that specific VDR-positive immune cells such as macrophages are commonly embedded in tissues and their abundance in response to inflammation and other conditions can be increased (25). While mice with tissue-specific *Vdr* deletion have been engineered (26, 27), there are many complexities inherent to this method that can complicate experimental outcomes and their interpretation. Additionally, as

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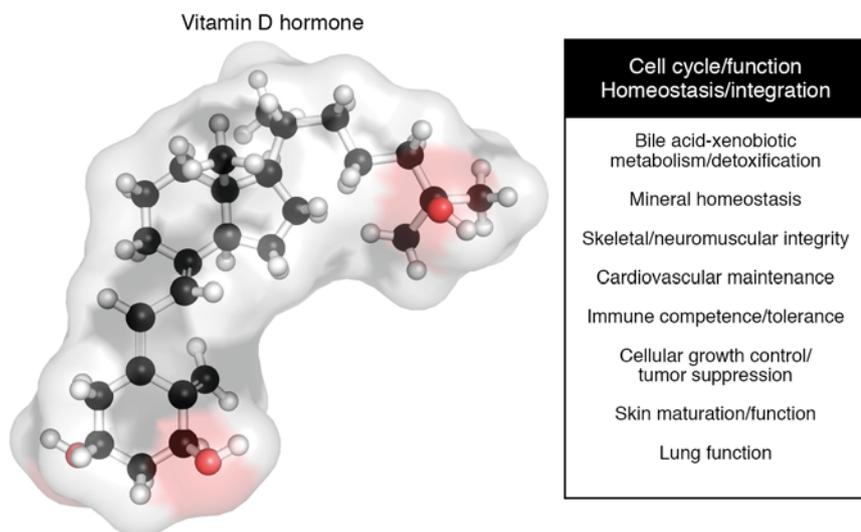


Figure 1. Biological roles of the vitamin D hormone. The three-dimensional structure of the vitamin D hormone is shown, with several of the major biological activities indicated.

many regulatory proteins are dynamically modulated during disease processes, the observation that VDR is expressed in pathological states raises the question as to whether the protein is correspondingly expressed in the normal tissue counterpart. The issue of whether a complex tissue represents a target tissue for vitamin D, and the controversy that it frequently engenders, will no doubt continue.

VDR gene regulation. The selective presence of the VDR in specific cell types supports the idea that expression from the *VDR* gene itself is uniquely regulated. Accordingly, there is evidence for *VDR* transcriptional modulation through multiple signaling pathways in different cell types (28–30), including its regulation in immune cells by TLRs (25). Nevertheless, a re-assessment of many of the earlier findings of the mechanisms associated with *VDR* gene regulation is now underway due to results from recent genome-wide studies using ChIP deep sequencing (ChIP-seq) and other analyses. These studies broadly support the idea that although genes can be regulated through promoter-proximal elements, the majority appear to be regulated through multiple control elements that can be located tens if not hundreds of kilobases distal to their transcriptional start sites (TSSs) (31–33). Indeed, due to technical limitations (discussed below), distal sites for genes were largely undetected using early molecular biological approaches, although clinical studies provided support for this principle (34). Our recent studies using these contemporary and largely unbiased methods in intestine, bone, and kidney have revealed numerous potential regulatory sites located upstream of *VDR* as well as within several of the gene's large introns in both the mouse and human genomes (35, 36). Several of these distal enhancer regions and the regulatory *cis* elements within have been explored in bone cells and shown to mediate unique cell-specific autoregulation of *VDR* by $1,25(\text{OH})_2\text{D}_3$ and direct stimulation by PTH both in vitro and in the mouse in vivo (36, 37). As both mouse and human genes are highly conserved, it was not surprising that large DNA segments spanning each of these genes and their corresponding distal regulatory regions were capable of fully rescuing the biological phenotype of the *VDR*-null mouse and, in the case of the human segments,

humanizing the mouse in turn. The success of this approach has enabled subsequent examination of the individual enhancers that regulate *VDR* gene expression in vivo and of the functional consequences of expressing mutant human VDR proteins from these transgenes (38, 39). As this gene represents a key determinant of vitamin D action, understanding the mechanisms through which VDR is expressed in individual tissues is extremely important.

VDR protein structural insights. The cloning of nuclear receptors including VDR enabled an extensive analysis of the overall domain structure of this transcription factor family (40–42). Dissection of the receptor with molecular biological manipulation combined with recombinant expression and mutagenesis was followed by crystallographic determination of its 3D structure (43, 44). Most nuclear receptors form either

homodimers or, as in the case of the VDR and other members of this subclass, heterodimers with retinoid X receptors (RXRs) (41). Since these molecules also recruit large coregulatory protein complexes, more recent techniques using cryo-electron microscopy have focused on the elucidation of larger and more complex quaternary structures at the level of DNA (45). These latter studies have revealed not only the overall organization of receptor dimer pairs bound to their cognate DNA regulatory elements, but have also identified some of the molecular interactions that permit selective dimerization. These relationships have been thoroughly explored by Moras, Rochel and colleagues (45–47), who have defined the structure of the VDR/RXR heterodimer and the spatial interrelationships that exist between the heterodimer and key coregulatory components that mediate the DNA-specifying activity of the receptor itself. Future studies are likely to establish the overall structural organization of even larger complexes that are nucleated through unique VDR binding activity at specific gene targets. Interestingly, these VDR structural insights have enabled an advanced molecular understanding of the functional consequence of *VDR* mutations that cause the human syndrome of hereditary resistance to $1,25(\text{OH})_2\text{D}_3$ (discussed below).

Mechanisms of action. Molecular studies over the past several decades have revealed that the VDR functions in the nucleus much like other receptors of its class (40). Thus, the receptor is first activated by its cognate hormone, a process that is characterized by conformational changes that prompt numerous downstream events. While the order of these events is less certain, the ligand-bound VDR localizes to regulatory sites on DNA, with accessibility determined by the overall chromatin state of the target cell and governed by both lineage and differentiation status (14, 48). Whether heterodimerization with free RXR precedes VDR binding to DNA or occurs at sites that are pre-marked by RXR remains uncertain, although numerous genome-wide studies (discussed below) suggest that many unoccupied VDR binding sites contain a prebound form of RXR (49, 50). It seems unlikely that the VDR will prove to be a pervasive repressor of gene expression in the absence

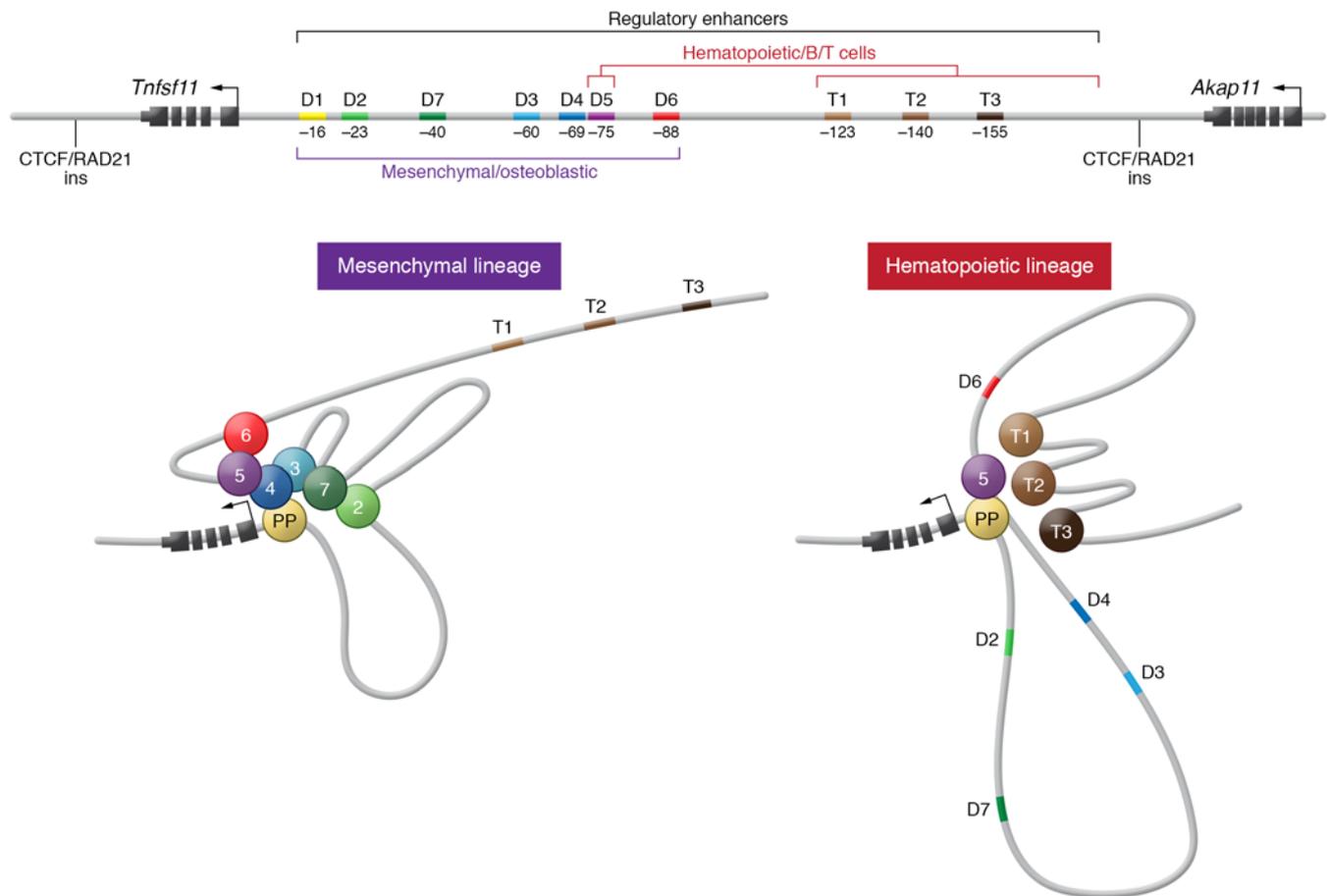


Figure 2. Schematic of the mouse *Tnfsf11* (RANKL) gene and its regulatory components. Top: Locations of the upstream *cis*-acting regulatory components that control expression of the *Tnfsf11* gene in mesenchymal and hematopoietic lineage cell types. The *Tnfsf11* and *Akap11* genes (with exons) are shown (arrows indicate direction of transcription), the locations of CTCF insulator elements are identified, and the locations of the individual *Tnfsf11* enhancers (D1–D7 and T1–T3) are shown. Bottom: Hypothetical three-dimensional DNA looping organization of the *Tnfsf11* gene and its engaged regulatory regions responsible for cell type–specific expression in mesenchymal/skeletal (left) and hematopoietic (right) cells. The spheres numbered 1–7 (left) represent the enhancer complexes and correspond to D1–D7 in the top figure. PP, proximal promoter.

of ligand, as VDR/DNA binding is heavily dependent upon VDR activation by $1,25(\text{OH})_2\text{D}_3$ (33, 49–51). Numerous coregulatory factors have also been shown to interact with the nuclear receptor family, some in a highly specific manner (52). In case of the VDR/RXR heterodimer, only a limited number of studies have demonstrated the recruitment of such factors and have been largely limited to fundamental coregulators such as CREB-binding protein/p300, the steroid receptor coactivator family, and remodeling factors such as SWI/SNF complexes, among others (35, 53–56). The functions of coregulatory proteins are highly diverse, but clearly affect the chromatin architecture in a gene-specific manner by influencing histone identity and epigenetic status and perhaps the overall organization of the gene locus itself (57). Additional complexity is certain to be discovered, as the spatial and temporal relationships through which these molecular events unfold are likely to emerge in the coming years.

Genome-wide principles of the VDR cistrome. Early studies of transcriptional regulation focused largely upon specific genes, relying heavily upon methodologies that were highly biased in favor of promoter-localized control elements. Unfortunately, these methodologies were also free of the context of the endogenous gene locus

itself, relative to the presence of not only additional regulatory features of the gene but also its natural chromatin environment. In retrospect, given the knowledge that enhancer elements interact in three dimensions, the absence of these key components of gene regulation seemed certain to affect interpretation. Most of these issues have been overcome with the emergence of genome-scale methodologies that have enabled almost unlimited annotation of natural cellular genomes with respect to transcription factor binding sites (cistrome), histone identification and post-translational status, DNA modification, chromatin structural architecture, and other features (31, 57). Indeed, it is now possible to assess the impact of the epigenome not only genome-wide, but at single sites as a function of environmental change, systemic factor stimulation, or in response to differentiation and/or disease perturbation. Such studies have provided widespread annotation of the genomes of multiple cell types in mouse and human tissues both *in vitro* and *in vivo* and have resulted in novel interpretations of genomic function (58).

Our recent studies of the genome in bone cells and other cell types highlight some of the insights that have been gained regarding the properties of VDR interactions with the genome, including identification of VDR genetic targets, the consequences of

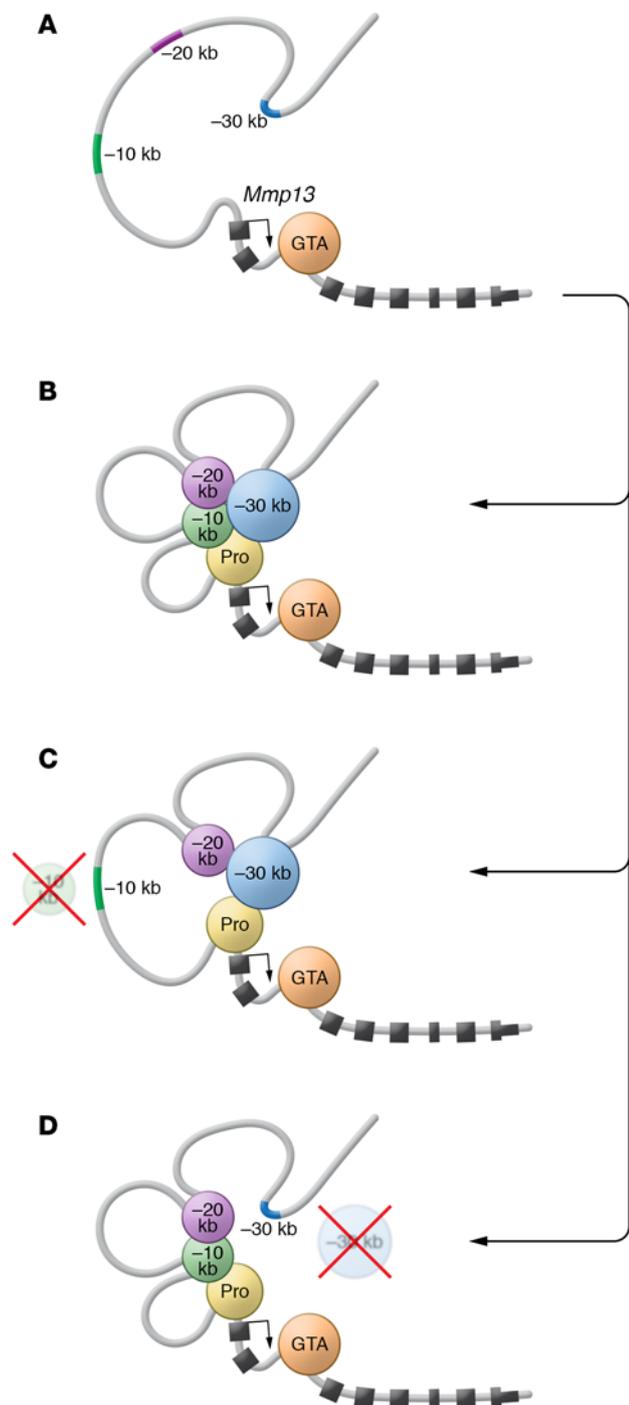


Figure 3. Organization of the mouse *Mmp13* gene and its regulatory components. (A–D) Hypothetical three-dimensional looping organization of the *Mmp13* gene in the absence (A) and fully engaged presence (B) of *trans*-acting regulatory factors, in the absence of the structural influence of the VDR-regulated –10-kb enhancer (C), and in the absence of the RUNX2-regulated –30-kb enhancer (D). GTA, general transcriptional apparatus; Pro, promoter region.

VDR binding on gene output in response to $1,25(\text{OH})_2\text{D}_3$, and the effects of differentiation on these features of VDR action (refs. 49, 50, 59–61 and reviewed in ref. 15). Genome-wide analyses of osteoblast lineage cells beginning with mesenchymal stem cell (MSC) precursors and extending to osteoblasts and terminally

differentiated osteocytes revealed VDR cistromes that are heavily dependent upon $1,25(\text{OH})_2\text{D}_3$ activation for broad DNA binding at sites of transcriptional regulation across the genome. While ligand-independent DNA binding occurs at limited locations in all of these osteoblast lineage cells, the number of sites that contain prebound RXR increases 8- to 10-fold upon pretreatment with $1,25(\text{OH})_2\text{D}_3$. De novo analysis of the DNA sequences most overrepresented at these sites reveals the presence of one or more classic vitamin D response elements (VDREs), each comprising two directly repeated imperfect hexameric half-sites separated by three base pairs. These results confirm earlier traditional studies of single genes such as osteocalcin (62), osteopontin (63), and *Cyp24a1* (64), the latter considered a quintessential and highly induced target of vitamin D action. As mentioned earlier, the vast majority of these VDR binding sites across osteoblast lineage cells were found within introns or intergenic regions either upstream or downstream many kilobases distal to transcriptional targets, sites that could not be identified by traditional methods. Analogous to the above discussion of VDR, we confirmed the functionality of many of these distal sites at known vitamin D target genes using large, genomically integrated segments of DNA (36, 37, 60, 65) and via genomic deletion analyses (66–69) both in vitro and in vivo. A classic example of a highly complex enhancer profile and of the factors that are associated with these regulatory sites is illustrated by *Tnfrsf11* (the RANKL gene) in Figure 2, together with speculative looping profiles (reviewed in ref. 70) for the two distinct cell types in which it is expressed (71). Importantly, while the presence of some of the enhancers previously identified near gene promoters using traditional means were confirmed by these unbiased methods, others were not, suggesting that traditional analyses in the absence of endogenous chromatin context can frequently result in false positives. Many genes that rely upon promoter proximal elements were also regulated by more distal elements, as we showed for the *Cyp24a1* gene (65). Although cistromes for each of the cell types examined were highly cell specific overall, their general characteristics were common. Finally, most VDR-bound enhancers are modular and contain adjacent binding sites for additional transcription factors (49, 50, 61). Indeed, we discovered in bone cells that the osteoblast master regulator runt-related transcription factor 2 (RUNX2) and the remodeling factor C/EBP β were present at over 40% of the VDR binding sites and displayed a distinctive organizational pattern. Importantly, treatment with $1,25(\text{OH})_2\text{D}_3$ altered the expression and genome-binding activity of these two factors following differentiation (discussed below), thereby genetically influencing the gene-specific activity of vitamin D in a given cellular state. This rather frequent functional regulatory unit unique to bone cells was termed a consolidated osteoblast enhancer complex (49). In summary, these and other genome-wide analyses have not only confirmed many of the fundamental principles of vitamin D action identified earlier, but also provided new and unexpected insights.

VDR binding sites and transcriptional regulation. Although the presence of VDR binding sites in osteoblast lineage cells correlates with many genes that are regulated by $1,25(\text{OH})_2\text{D}_3$, the VDR cistrome is far more complicated relative to transcriptional regulation. This is partly due to the distal nature of many VDR binding sites, making it difficult to link these regions directly to the genes

they regulate based upon spatial proximity alone. Thus, while many genes located near VDR binding sites are regulated by $1,25(\text{OH})_2\text{D}_3$, others similarly located are not (49, 61). Surprisingly, many genes regulated by $1,25(\text{OH})_2\text{D}_3$ do not contain VDR binding sites within or near their surrounding loci. There are multiple explanations for these seemingly paradoxical results. It is possible that the VDR is capable of binding to sites of historical significance (earlier cellular states of differentiation). Binding sites may be located many hundreds of kilobases from the regulated gene at locations not readily identified. Alternatively, many genes may require the regulatory influence of additional signaling inputs for activation (72). It is also possible that certain functional consequences of VDR binding cannot be recognized or that some sites may be nonfunctional. Regardless of the explanation, these findings highlight the unexpected complexity that characterizes gene regulation when examined on a genome-wide scale in known target tissues and cells.

The impact of differentiation on the biological response to $1,25(\text{OH})_2\text{D}_3$

The regulatory sites that mediate the actions of vitamin D as well as other regulatory hormones are highly dynamic. A comparison of the VDR cisomes in osteoblast lineage cells prior to and following differentiation into mature mineralizing osteoblasts in vitro (49) or in osteoblasts prior to and after their transition into terminally differentiated osteocytes (60) revealed striking changes to the genome after these cellular transitions. These changes demonstrate that the cisomes for VDR and for other transcription factors such as RUNX2 and C/EBP β (61) can undergo significant changes both in expression and in genomic distribution. Importantly, these changes correlate with similar changes in gene expression. In the case of the VDR cisome, much of the restriction in VDR binding may be due to suppressed expression of the *VDR* gene itself, which results in a striking downregulation of VDR protein in more differentiated osteoblastic lineage cells (49). This downregulation, which may result from the trophic effects of $1,25(\text{OH})_2\text{D}_3$ on VDR autoregulation in bone cells, also occurs in vivo. This finding highlights the not unexpected fundamental importance of transcription factor expression to changes in transcriptomic output that can occur as a function of cellular differentiation; however, changes in cellular response to external cues are far more complex than just alterations in transcription factor expression. Interestingly, while many genes no longer responded to $1,25(\text{OH})_2\text{D}_3$ following osteoblast differentiation, a moderate cohort of genes exhibited unique patterns of response or acquired unusual sensitivity to $1,25(\text{OH})_2\text{D}_3$ (49). This alteration in gene response in spite of the suppression in receptor expression signals an increased complexity in vitamin D hormone response that likely involves additional cellular components that are genetic and/or epigenetic in nature, and is certain to extend the range and diversity of response to $1,25(\text{OH})_2\text{D}_3$ both qualitatively and quantitatively in metabolically complex tissues such as bone.

Differentiation and changes in genetic factor expression. A classic example of a change in response to $1,25(\text{OH})_2\text{D}_3$ and the co-regulatory role of RUNX2 and C/EBP β was found in the *Mmp13* gene, which is expressed in a wide variety of cell types (including chondrocytes and osteoblasts), responds to multiple environmen-

tal cues, and directs the synthesis of collagenase-3 (73–75). *Mmp13* is relatively insensitive to induction by the vitamin D hormone in osteoblast lineage cells at early stages of development, but is strongly upregulated by the hormone following differentiation into mature mineralizing osteoblasts, particularly in chondrocytes (50, 69). ChIP-seq studies revealed that *Mmp13* was also regulated by RUNX2, C/EBP β , and VDR, although each of these factors was bound to distinct upstream regions, which we collectively termed dispersed enhancers (Figure 3). Changes in RUNX2 and C/EBP β binding to this gene influence its upregulation following differentiation. Interestingly, each of these regions influenced basal *Mmp13* expression. Furthermore, while vitamin D response was uniquely mediated by a VDR-binding enhancer located 10 kb upstream, a RUNX2-bound enhancer located even further upstream at –30 kb exerted a hierarchical influence on the other enhancers and on the overall expression of *Mmp13* (50). Accordingly, loss of the RUNX2-regulated enhancer through CRISPR/Cas9 nuclease-directed deletion experiments resulted in almost complete loss of *Mmp13* expression as well as regulation by $1,25(\text{OH})_2\text{D}_3$, likely due in part to an apparent failure of each of the regulatory components in this distal region to coalesce around the *Mmp13* promoter. While the activated reorganization of these dispersed enhancers is reminiscent of the more common consolidated versions, these results highlight the complexity inherent in the regulation of genes for which expression levels are controlled by more than one modulatory region. This complexity may be particularly important for *Mmp13*, a gene whose enzymatic product plays multiple structural and functional roles in a myriad of different tissues not limited to bone.

Differentiation and changes in epigenetic modification. That additional components such as RUNX2 and C/EBP β and their respective regulation contribute to the activation of genes by vitamin D represents only a partial explanation for changes in sensitivity to $1,25(\text{OH})_2\text{D}_3$. Differentiation may have the most profound impact on the epigenetic landscape across the genome and in particular at genetic loci that contain functional VDREs. On a genome-wide basis, we found that the histone H3 lysine 4 methylation, a histone modification that marks the general locations of regulatory enhancers (57), was frequently either enriched or depleted as a function of differentiation; in some cases, this histone mark was newly commissioned at specific sites upon differentiation, suggesting that the initial lack of responsiveness to $1,25(\text{OH})_2\text{D}_3$ in pre-osteoblasts is due to restricted DNA access that is reversed as the cells undergo differentiation (50). The dynamic nature of epigenetic modifications across the genome suggests that transcriptional regulation and therefore cellular phenotype may be much more plastic than previously thought. In that vein, our recent studies of the epigenetic landscape of bone marrow-derived MSCs suggest an inherent epigenetic predisposition for osteoblastogenesis (61). Thus, while MSCs can be prompted to undergo adipogenesis in response to an appropriate inducer cocktail in vitro, displaying an epigenetic profile that is distinct relative to osteoblasts, these adipocytes can be readily redirected into osteoblast-like cells following subsequent exposure to an osteogenic cocktail, perhaps via a trans-differentiation process. Thus, differentiation influences the ability of cells to respond to signals via regulated modulators such as the VDR.

Biological functions of the vitamin D hormone

The above mechanisms likely underlie the diverse biological effects of vitamin D that are exerted in a tissue- and cell type-specific fashion in all higher vertebrates. These include not only the role of vitamin D in mineral homeostasis through orchestrated actions on intestine, kidney, and bone, but also in immune cells, skin, and tissues of the cardiovascular system, muscle, liver, and brain. Advances in our understanding of the multiple roles of vitamin D have been covered extensively in numerous reviews (13, 76–81) and will not be considered in any depth here; however, it is noteworthy that the vast majority of the biological activities of the vitamin D hormone have emerged through either clinical discovery or through studies of the numerous genetically modified mouse models that have been created wherein either *VDR* or *Cyp27b1* have been removed in tissues either globally or selectively (27, 82–84). Clinical observation of humans with mutant VDRs that underlie hereditary $1,25(\text{OH})_2\text{D}_3$ -resistant rickets (HVDRR) (85–87) presaged the creation of virtually all mutant *VDR* mouse models, many of which have been extensively described in recent reviews (76, 88). Another example is the *Cyp27b1*-null mouse, which also recapitulates human *CYP27B1* dysfunction (89). It seems likely that the novelty and selectivity of these mouse models of human disease will only increase as a consequence of the democratization of gene-editing methods such as those exemplified by the CRISPR/Cas9 approach (90). Indeed, the efficiency with which genetically modified mice can be made suggests that not only can they represent unique models for phenotypic analysis, but they may also serve as novel biological reagents designed to further our understanding of important mechanisms of transcriptional regulation.

Translational impact of the VDR

Hereditary disease. As the principle mediator of vitamin D hormone activities, VDR can represent both the cause of human disease as well as a therapeutic target. Early studies suggested that certain forms of rickets might be due to inherited mechanistic defects (91). This theory was confirmed when molecular examination of *VDR* sequences from patients with HVDRR revealed mutations within the DNA binding domain that compromised the protein's ability to regulate transcription (85, 87). These mutations resulted in a failure to absorb calcium that led to hypocalcemia, highly disrupted mineral homeostasis, and striking skeletal and hard-tissue deformities. Most patients respond to calcium supplementation but not to treatment with the hormone itself (92). To date, over 100 patients and 45 mutations have been identified largely through the significant efforts of Malloy and Feldman and their colleagues (93). These mutations map to virtually every known functional domain within the VDR protein and cause unique underlying VDR defects linked to compromised expression, DNA binding, heterodimer formation with RXR, binding to $1,25(\text{OH})_2\text{D}_3$, affinity for the hormone, and coregulator recruitment; most of these defects result in generally similar early-stage clinical outcomes (94). Interestingly, a subset of patients exhibit features of partial or total alopecia, a phenotype that was eventually correlated to VDR mutations that result from either the absence of the VDR or the absence of critical domains but not from the ability of the mutant protein to bind $1,25(\text{OH})_2\text{D}_3$. Further studies revealed that hair follicle cycling requires VDR but is not dependent on ligand

binding and is the single biological process known to date to be a ligand-independent function of the VDR (95).

Given the dramatic defects associated with loss of VDR function, it is not surprising that the literature is replete with studies that attempt to link disease with changes in VDR expression in tissues. While it seems intuitive that a reduction in VDR might result in a reduced ability to regulate gene expression, the abundance of the receptor in most tissues, together with the fact that the majority of these receptors in a given tissue are not bound by $1,25(\text{OH})_2\text{D}_3$ under normal physiologic situations, suggests a significant gap in our understanding of VDR biology. It is also clear not only that additional components are required for VDR-mediated transcriptional regulation but also that they can alter the receptor's activity on specific genes, as discussed above (14, 49). No studies illustrate the effort to relate receptor expression to disease phenotype more thoroughly than those that have attempted to correlate specific RFLPs or single nucleotide variants (SNVs) within or near the *VDR* locus to different human clinical manifestations (96). Unfortunately, these studies have yielded relatively modest insight into the relationship between VDR and clinical phenotypes. The reason for the limited nature of these correlations is unclear, although most of the genetic variations that have been explored extensively within the VDR locus do not reside in regions that are currently known to modulate receptor expression. Moreover, the linkage between minor changes in VDR expression and biological function in tissues have not been clearly established. Two SNVs represent notable exceptions: a polymorphism (termed Fok1 RFLP) located near the *VDR* translational start site has been suggested to affect activity (97–99), and a second SNV located in a potential caudal type homeobox-2 (CDX2) non-coding region upstream of the VDR TSS (termed the CDX2 SNP) may affect intestinal VDR expression (100, 101); however, further studies will be required to verify these effects. Nevertheless, it is possible that these or perhaps other identified SNVs could affect VDR expression either globally or in a tissue-specific manner and therefore influence biological outcomes.

Therapeutic intervention via the VDR. The VDR represents a natural regulatory target for vitamin D analogs and mimetics that could reduce or cure human disease. The number of VDR-associated diseases is extremely large, prompting considerable effort in this area (see multiple recent reviews: refs. 13 and 102–105). Only a few of the many thousands of analogs and mimetics that have been synthesized have become drugs, with most displaying relatively limited efficacy at their indicated disease targets. Separation of the natural calcemic activities of most vitamin D analogs from their potential beneficial effects on most clinical indications remains an enigma relative to therapeutic efficacy. Mechanistic insight that might provide the structural basis for addressing this confounding calcemic “side effect” of the receptor has not yet emerged. Despite the incredible wealth of new structural and functional information that has accrued over the past several decades for nuclear receptor activities in general, few compounds have been identified that have been able to take advantage of these advances and have emerged as drugs. Given the uniformly broad biological effects of not only the VDR but of other receptors in this class, it is likely that efforts will continue in the attempt to take advantage of nuclear receptors for therapeutic purposes.

Deficits and future directions

Many questions remain to be answered about vitamin D and its actions. While many unique activities have emerged, biological actions in specific cell types in receptor-poor tissues such as muscle, liver, and the CNS will require further probing. At present, age-associated vitamin D deficiency can result in muscle weakness, although it is unclear whether this effect is direct (106, 107). Recent studies suggest the presence of the VDR in activated hepatic stellate cells (23, 108). It is unknown whether VDR is expressed in inactive stellate cells *in vivo*, although the receptor is not immunologically detectable in the liver. Additionally, while vitamin D appears to act on CNS neurons, the specific activities of vitamin D in this tissue are not fully defined (24, 109, 110). The mechanisms of VDR action relative to coregulatory factors requires further exploration, including how coregulatory factors are recruited and how their properties are exploited to modulate transcriptional output. Some evidence has emerged that the VDR may function in a ligand-independent manner (i.e., hair follicle cycling) (39, 95, 111–113); however, since VDR/DNA binding is largely ligand dependent both *in vitro* and *in vivo*, only a small subset of genes are likely to represent direct targets of this activity (49). It is also unclear how the numerous epigenetic modifications that have been identified across the genome and that are influenced by transcription factors such as the VDR influence genetic output (114). How these modifications are interpreted by histone reader complexes is a particular focus. While VDR primarily acts at the level of DNA, considerable evidence suggests that the receptor may modulate the activity of other DNA-bound transcription factors through tethering mechanisms (115), although again additional evidence for this mechanism is still

needed. The same can be said for the proposed non-genomic actions of $1,25(\text{OH})_2\text{D}_3$, which have not been discussed in this Review but have been recently summarized (116). Mechanistic underpinnings have been well established now for several of the steroid hormones but remain largely obscure for vitamin D. Finally, from a much broader perspective, molecular mechanisms that govern the expression of the VDR targets *Cyp27b1* and *Cyp24a1* in the kidney, whose products modulate $1,25(\text{OH})_2\text{D}_3$ levels in the blood, remain unknown (3). Delineation of these mechanisms is important because while *Cyp27b1* is also expressed at low levels in numerous non-renal cell types, the contribution of these local sources of $1,25(\text{OH})_2\text{D}_3$ to the overall activity of circulating renal $1,25(\text{OH})_2\text{D}_3$ remains to be determined (25, 117). These and other distinctions have led to the prevailing clinical question of the relevance of maintaining high circulating $25(\text{OH})\text{D}_3$ levels for activities separate from those that are favorable for skeletal integrity, and of the importance of vitamin D supplementation (118). It is possible that this broader distribution of $1,25(\text{OH})_2\text{D}_3$ -producing cells may provide unique opportunities for the development of highly targeted, disease-specific therapeutics.

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