Published in final edited form as:

J Steroid Biochem Mol Biol. 2018 March ; 177: 36–45. doi:10.1016/j.jsbmb.2017.06.002.

The impact of VDR expression and regulation in vivo

Seong Min Lee^a, Mark B. Meyer^a, Nancy A. Benkusky^a, Charles A. O'Brien^b, and J. Wesley Pike^{a,*}

^aDepartment of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706, United States

^bUniversity of Arkansas for Medical Sciences, Little Rock, AR 72205, United States

Abstract

The vitamin D receptor (VDR) mediates the pleiotropic biological actions of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). These actions include orchestration of mineral homeostasis which is coordinated by the kidney, intestine, bone and parathyroid gland wherein the VDR transcriptionally regulates expression of the genes involved in this complex process. Mutations in human VDR (hVDR) cause hereditary vitamin D resistant rickets, a genetic syndrome characterized by hypocalcemia, hyperparathyroidism and rickets resulting from dysregulation of mineral homeostasis. Expression of the VDR is regulated by external stimuli in a tissue-specific manner. However, the mechanisms of this tissue-specificity remain unclear. Studies also suggest that phosphorylation of hVDR at serine 208 impacts the receptor's transcriptional activity. These experiments were conducted in vitro, however, and therefore limited in their conclusions. In this report, we summarize (1) our most recently updated ChIP-seq data from mouse tissues to identify regulatory regions responsible for the tissues-specific regulation of the VDR and (2) our studies to understand the mechanism of hormonal regulation of *Vdr* expression in bone and kidney in vivo using transgenic mouse strains generated by mouse mini-genes that contain comprehensive genetic information capable of recapitulating endogenous *Vdr* gene regulation and expression. We also defined the functional human *VDR* gene locus in vivo by using a human mini-gene comparable to that in the mouse to generate a humanized VDR mouse strain in which the receptor is expressed at normal levels (normal expressor). The present report also shows that a humanized mouse model in which the VDR is expressed at levels about 10- fold lower than the normal expressor mouse rescued the VDR-null phenotype despite its reduced transcriptional activity relative to wildtype expression. We also generated an additional humanized mouse model expressing hVDR bearing a mutation converting serine 208 to alanine (hVDR-S208A). In spite of the mutation, target gene expression induced by the ligand was unchanged relative to a mouse strain expressing comparable levels of wildtype hVDR. Further characterization also showed that serum calcium and parathyroid hormone levels were normal and alopecia was not observed in this hVDR-S208A mouse strain as well. Taken together, our in vivo studies using ChIP-seq analyses and the mini-

*Corresponding author at: Department of Biochemistry, University of Wisconsin-Madison, Hector F. Deluca Laboratories, Room 543D, 433 Babcock Drive, Madison, WI, 53706, United States. pike@biochem.wisc.edu (J.W. Pike).

Conflict of interest

The authors have no conflict of interest to declare.

gene transgenic mice improve our understanding of the tissue-specific regulatory mechanisms of controlling VDR expression and the mechanisms of action of the VDR.

Keywords

Vitamin D receptor; 1,25-dihydroxyvitamin D₃; Hereditary vitamin D resistant rickets; Humanized VDR mouse model; Bacterial artificial chromosome; ChIP-seq

1. Introduction

1.1. Role of the vitamin D receptor

The vitamin D receptor (VDR) is a nuclear transcription factor that mediates the biological actions of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the active form of vitamin D [1–3]. These actions include maintenance of mineral homeostasis in higher vertebrates, which is an outcome of the ability of the VDR to regulate gene expression in target tissues [4]. Although numerous cell types and tissues have been reported to express the VDR and suggested to be 1,25(OH)₂D₃ targets, the vitamin D endocrine system primarily regulates calcium and phosphate homeostasis through its action in intestine, kidney, bone and parathyroid gland (PTG) [5].

The importance of the 1,25(OH)₂D₃/VDR system in human health is recognized through studies of hereditary 1,25-dihydroxyvitamin D resistant rickets (HVDRR) and vitamin D dependency rickets type 1 (VDDR-1). The former syndrome results from the loss of vitamin D activity due to VDR mutations [6] whereas the latter is a disease caused by mutations in the *CYP27B1* gene resulting in a failure of 1,25(OH)₂D₃ production [7]. Further studies of mouse models of these diseases that include VDR-null [8–11] and *Cyp27b1*-null mice [12–14], have shown that the mouse models share many aspects of the human diseases, such as abnormal mineral homeostasis, early onset rickets and growth retardation, confirming the importance of the vitamin D endocrine system in mineral homeostasis.

In addition to the 1,25(OH)₂D₃-mediated actions of the VDR, an alopecic phenotype is found in VDR-null mice but not in *Cyp27b1*-null mice, suggesting that alopecia is due to the receptor's actions in hair formation in the absence of ligand. This idea was later confirmed by recovery of alopecia in VDR-null mice using keratinocyte-specific expression of a mutant VDR which lacked 1,25(OH)₂D₃ binding activity [15]. It has been found that some of HVDRR patients display alopecia but VDDR-1 patients do not [6]. Mutations found in the patients with alopecia abrogated DNA binding activity of the VDR or prohibited the expression of the VDR (nonsense mutation) whereas those without alopecia abolished ligand binding activity or coactivator recruitment [6], which also supports the idea of ligand-independent action of the VDR in hair formation. Additional ligand-independent actions of the VDR as a transcription repressor/de-repressor in mouse tissues has been suggested [16,17].

1.2. Studies on the tissue-specific regulation of Vdr expression

The VDR is expressed in specific cell types in many tissues. These tissues include not only those involved in mineral homeostasis but also skin [18], pancreatic beta cells [19], reproductive tissues [20], placenta [21] and immune cells [22]. Expression of the VDR is also regulated in a tissue-specific manner at the transcriptional level by various hormones including parathyroid hormone (PTH), all trans retinoic acid (atRA), and by its ligand, $1,25(\text{OH})_2\text{D}_3$ [23–25]. Furthermore, it has been suggested that calcium as well as $1,25(\text{OH})_2\text{D}_3$ are required for renal VDR transcripts above a basal level [26]. This report also demonstrated that low dietary calcium results in hypocalcemia and high renal *Cyp27b1* expression, likely caused by high serum PTH levels. Interestingly, renal *Vdr* expression was reduced under this condition but its duodenal expression was not, providing another examples of the tissue-specific regulation of *Vdr* expression [27].

Despite these lines of evidence for tissue specific regulation, the mechanisms responsible for tissue/cell type-specific expression of the VDR remain to be clarified. To understand the molecular mechanisms responsible for the regulation of the *Vdr* expression, we have performed ChIP-chip and ChIP-seq analyses using bone cells and identified intronic as well as upstream regulatory regions that were occupied by the VDR, phosphorylated CREB (pCREB) and retinoic acid receptor (RAR), transcription factors that mediate the actions of the hormones that regulate *Vdr* expression [24,25,28]. To test if *Vdr* expression is regulated by the identified regions, we generated a mini-gene comprised of a segment of a bacterial artificial chromosome containing the *Vdr* gene locus which was defined by two boundary CCCTC-binding factor (CTCF) binding sites, an N-terminal HA-tag and a C-terminal reporter cassette [28]. We first found that this mini-gene mimics the hormonal regulation of the *Vdr* expression in cell culture [28]. We then generated mutant mini-genes in which the regulatory regions were deleted from the wildtype mini-gene and found that these regions play unique and cooperative roles in the hormonal regulation of the *Vdr* gene in vitro [25].

For further assessment of the characteristics of this mini-gene in vivo, we introduced this DNA as a transgene into mice in a VDR-null background and found that the mini-gene not only recapitulated tissue-specific expression of endogenous *Vdr* gene but also rescued the VDR-null phenotypes, suggesting that the mini-gene contains sufficient genetic information to mimic properties of the endogenous gene in vivo [29]. We then explored the roles of the enhancers in the regulation of *Vdr* expression in vivo by generating transgenic mice containing mutant transgenes and confirmed the involvement of the enhancers in the regulation of *Vdr* expression in bone [25].

1.3. Studies on the role of human VDR in vivo

In addition to understanding the regulatory mechanisms of *Vdr* expression, it is important to understand the function of the VDR in vivo. Although numerous mutations in the human VDR (hVDR) have been found to cause HVDNR and the activity of these mutant proteins assessed through in vitro assays as summarized in [6], our understanding of these mutations has emerged largely as a result of more basic molecular biological studies of VDR structure/function in cell culture. Thus, to overcome this limitation, we have used a human version of the mouse VDR mini-gene described above as a transgene to generate a humanized VDR

mouse model in the VDR-null background [29]. The hVDR derived from this mini-gene recapitulated cell type- and tissue-specific expression of the endogenous VDR and further rescued VDR-null phenotypes [29]. We have also generated mutant humanized VDR mice which produce a VDR lacking 1,25(OH)₂D₃ binding activity to examine the systemic effect of the loss of this activity in vivo [30]. Through the characterization of this mutant mouse model, we have discovered actions of the VDR that appear independent of 1,25(OH)₂D₃ in intestine, bone and PTG as well as skin [16].

Phosphorylation of the hVDR has been reported in the 1980s [31] and found to be increased by 1,25(OH)₂D₃ treatment in vitro [31,32]. Later, several kinases, such as protein kinase C, casein kinase II, protein kinase A and ataxia telangiectasia mutated, were suggested to be responsible for the phosphorylation by in vitro assays [33–36]. It has been shown that casein kinase II-mediated phosphorylation occurs at the serine 208 residue in the hVDR [34,37] and that the transcriptional activity of the VDR was enhanced by the phosphorylation [38]. Furthermore, it has been shown that phosphorylation of the hVDR at serine 208 increases interaction between the VDR and VDR-interacting protein 205 (DRIP205), a subunit of a coactivator complex that is important for transcription [39]. Finally, it has been suggested that increased phosphorylation of the hVDR by treatment of a protein phosphatase inhibitor enhances the interaction of the DRIP205 to increase ligand-mediated transcriptional activity but the phosphorylation at serine 208 is not involved in the phosphatase inhibitor-mediated enhancement of VDR-DRIP205 interaction and the transcriptional activity [40]. Despite the efforts to understand mechanisms and roles of hVDR phosphorylation, the effect of this post-translational modification of the hVDR has not been clarified in vivo, largely because of the limits imposed in studying human proteins in vivo. This has been particularly problematic since serine 208 in the hVDR is not conserved in the murine VDR.

1.4. Current knowledge of the VDR regulation

As described above, ChIP-seq analysis and transgenic mouse models generated by mouse VDR mini-genes have advanced our understanding of molecular mechanisms essential for tissue specific regulation of *Vdr* expression. In the present report, we summarize our previous ChIP-seq data together with current results from the kidney and intestine of *Cyp27b1*-null mice to identify regulatory regions responsible for tissue-specificity. We also generated a new mini-gene transgenic mouse model not only to confirm the bone-specific hormonal regulation of *Vdr* expression but also to understand its auto-regulation mechanism in kidney in vivo. Furthermore, we generated two additional humanized VDR mouse models. One model expresses reduced levels of wildtype hVDR relative to wildtype mice and the second expresses mutant hVDR in which serine 208 was replaced with alanine. Analyses of the former revealed that while low level of the VDR desensitizes tissues to 1,25(OH)₂D₃ genetic response, this reduction in gene expression has no effect on the rescue of mineral homeostasis in the VDR-null mouse. Therefore, our studies reveal surprising tissue-specific regulatory mechanisms of *Vdr* gene expression, and that very low concentrations of the VDR are sufficient to rescue the complex skeletal phenotype of the VDR-null phenotype. Finally, we show that the absence of phosphorylation at S208 in the hVDR does not alter its transcriptional activity in vivo.

2. Materials and methods

2.1. Cell Culture

MC3T3-E1 mouse osteoblastic cells were cultured in Minimum Eagle's medium alpha (MEM α) modification (Mediatech, Inc.) supplemented with 10% FBS (Hyclone Laboratories) and 1% penicillin-streptomycin (Invitrogen). Cells were treated with vehicle or 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ for 3 h and then subjected to ChIP-seq analysis [41,42].

2.2. Generation of *Cyp27b1*-null mice

Cyp27b1-null mice were produced by GenOway with a strategy similar to that previously described [43]. In brief, mouse *Cyp27b1* gene was amplified by PCR using genomic DNA from C57BL/6 embryonic stem cell (ES) cells as a template and cloned into the pCR4-TPOP vector (Invitrogen) to create a targeting vector (Fig. 1A). Two *loxP* sites were inserted in intron 6 and downstream of exon 9 and a positive selection marker, neomycin resistance gene which is flanked by FRT sites, was introduced between exon 9 and downstream *loxP* site. Diphtheria Toxin A (DTA) was also introduced into the upstream of *Cyp27b1* gene as a negative selection marker to reduce the isolation of non-homologous recombined ES clones and to enhance the chance to isolate ES cell clones containing the distal *loxP* site. The targeting vector was linearized and electroporated into ES cells. The recombined ES cell clones (Fig. 1A) identified by the selection, PCR confirmation and Southern blot analysis were then injected into C57BL/6 blastocysts to create chimeric mice, and the injected blastocysts were re-implanted into OF1 pseudo-pregnant females and allowed to develop. To remove the selection marker flanked by FRT sites and exon 7 through 9 of *Cyp27b1* gene flanked by *loxP* sites, the recombinant mice were sequentially bred with Flp-recombinase expressing deleter mice and then Cre-recombinase expressing deleter mice (Fig. 1A). For genotyping, a primer set amplifying a DNA region between the last exon and downstream of the last exon (880 bp) and a primer set amplifying a DNA region between intron 5 and downstream of the last exon (560 bp) were used for wildtype and the deleted alleles, respectively. Primers for genotyping were obtained from IDT and the sequences are available upon request. *Cyp27b1*-null phenotypes previously reported [12–14] were reproduced in the mice that we generated (data not shown).

2.3. Construction of VDR mini-genes

The wildtype mouse and human VDR mini-genes were previously generated from bacterial artificial chromosome clone RP23-136G8 [28] and RP11-89H19 [29], respectively (Fig. 1B and C). Deletion of the individual enhancer regions from the wildtype mouse VDR mini-gene was performed using the galactokinase system as previously described [28,44]. The mutation in serine 208 (Fig. 1C) was introduced by altering the triplet codon, AGT, in exon 8 to GCT coding alanine as previously described [28,44]. The deletions and mutation were confirmed by PCR analysis and DNA sequencing.

2.4. Generation of transgenic mouse and humanized VDR mouse strains

Transgenic mouse strains containing wildtype or mutant VDR mini-gene mentioned above (Fig. 1B and C) were generated as previously reported [29]. Genotypes of the transgenic

mice were identified by luciferase assay using lysates obtained from tail clips, as previously described [29]. To generate wildtype and mutant humanized VDR mice in the VDR-null background, the hVDR transgenic mice were crossed with VDR-null mice created by Demay group [9] and obtained from The Jackson Laboratory. The genotypes of the humanized mice were identified as previously reported [29]. Primers for genotyping were obtained from IDT and the sequences are available upon request.

2.5. Animal study

VDR-null and *Cyp27b1*-null mice and transgenic strains were maintained as heterozygotes through outbreeding with C57BL/6 mice (The Jackson Laboratory). All mice were fed standard rodent chow diet (5008; Harlan Teklad) after weaning except VDR-null mice to examine alopecic phenotype at 6 month of age. In this case, the mice were fed on a diet containing 20% lactose, 2% calcium and 1.25% phosphate diet (TD.96348; Harlan Teklad). For gene expression analysis, mice were treated with 1,25(OH)₂D₃ (10 ng/g body weight, SAFC) for 6 h, atRA (1 ng/g body weight, Sigma, R2625) for 4 h, fibroblast growth factor 23 (FGF23, 50 ng/g body weight, R & D Systems, 2629-FG-025) for 3 h or PTH (230 ng/g body weight, Bachem, H-1370.0100) and dibutyryl cyclic AMP (db-cAMP, 0.1 ng/g body weight, Sigma, D0260) for 1 h. For ChIP-seq analysis, mice were treated with 1,25(OH)₂D₃ (10 ng/g body weight) for 1 h. Mouse treatments were performed by intraperitoneal injection. The mice sacrificed for gene expression and ChIP-seq analyses were 8–10 weeks old of both genders. Bone mineral density (BMD) was measured using 8 weeks old male mice. Images of mouse gross appearance to check the presence of alopecia was taken at 6 month of age. Mice were exposed to a 12-h light-dark cycle. All animal studies were reviewed and approved by the Research Animal Care and Use Committee of University of Wisconsin-Madison.

2.6. Gene expression analysis

Total RNA was prepared from cells or mouse tissues using TRIzol Reagent (Life Technologies) and then subjected to reverse transcription using the High Capacity cDNA Reverse Transcription Kit following the manufacturer's protocols as previously described [29,43]. Gene expression was assessed by TaqMan-mediated quantitative PCR (qPCR) on a StepOnePlus (Applied Biosystems). Customized TaqMan primer detecting mouse VDR mini-gene-derived *Vdr* gene expression (HA- *Vdr*) generated no signal in tissues of C57BL/6 mice, suggesting that the customized primer is specific to the exogenous *Vdr* transcript (data not shown). Information on TaqMan primers is available upon request

2.7. Chromatin immunoprecipitation coupled to DNA sequencing analysis (ChIP-seq)

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) analyses in MC3T3-E1 cells was previously reported [41,42]. ChIP-seq analyses in kidney [45] and intestine [46] from wildtype mice were previously carried out. For ChIP-seq analysis of these tissues from *Cyp27b1*-null mice, the mice were treated with 1,25(OH)₂D₃ (10 ng/g body weight) for 1 h and subjected to immunoprecipitation using either a control IgG or the indicated antibodies as previously described [41,42]. ChIP antibodies to VDR (C-20, sc-1008) was purchased from Santa Cruz Biotechnology, Inc. Antibodies for Lys 4-monomethylated histone H3 (H3K4me1, ab8895) and Lys 27-acetylated histone H3 (H3K27ac, ab4729-100) were

obtained from Abcam. A genome-wide analysis of the VDR in the intestine from wildtype mice injected with vehicle or 1,25(OH)₂D₃ has been previously reported [46]. The genome-wide analyses of ChIP-seq data of mouse kidney and intestine will be published elsewhere.

2.8. Western blot analysis

Tissue lysates were obtained and subjected to western blot analysis using anti-VDR antibody (9A7; 1/1000 dilution) as previously described [47]. Anti-β-tubulin (sc-9104; Santa Cruz; 1/1000 dilution) antibody was used to provide a loading control.

2.9. Characterization of phenotypes of humanized VDR mice

Serum calcium levels were determined using QuantiChrom Calcium Assay Kit (BioAssay Systems) as previously described [29]. Serum PTH concentrations were measured in EDTA-plasma using Mouse PTH 1–84 ELISA Kit (Immutopics) according to the manufacturer [29]. To measure bone mineral densities (BMD), 8 weeks old males were scanned and analyzed as previously described [29]. The alopecic phenotype was visually assessed at 6 month of age.

2.10. Statistical analysis

Student's unpaired *t*-test was used to identify significant differences between groups (*p* < 0.05).

3. Results and discussion

3.1. Regulatory enhancer regions responsible for tissue-specific regulation of *Vdr* expression

We have shown that *Vdr* expression is differentially regulated in bone, kidney and intestine by 1,25(OH)₂D₃, atRA and db-cAMP, a PTH surrogate [25]. To understand the regulatory mechanisms of *Vdr* expression by various hormones involved in mineral homeostasis, we further assessed its expression in the tissues from mice treated with 1,25(OH)₂D₃, FGF23 and PTH. As shown in Fig. 2A, *Vdr* expression was induced by 1,25(OH)₂D₃ and PTH in calvaria and by 1,25(OH)₂D₃ and FGF23 in kidney, whereas it was not significantly changed by hormonal treatments in intestine, a result comparable to our previous findings [25]. It has been shown previously that VDR levels in kidney are decreased under hypocalcemic condition whereas the levels in intestine are not changed [27]. We therefore compared *Vdr* expression in wildtype mice with *Cyp27b1*-null mice that display hypocalcemia and secondary hyperparathyroidism due to the loss of ability to produce 1,25(OH)₂D₃. The results (Fig. 2B) revealed that expression was lower in the kidneys of *Cyp27b1*-null mice than wildtype controls at the transcriptional level as well as the protein level (data not shown), but it was not different in intestine between the strains. Therefore, these data suggest that the *Vdr* expression is differentially regulated in each tissue.

Aside from tissue-specific hormonal regulation, VDR expression is also highly tissue-specific in that it is observed in bone, kidney, intestine and PTG but almost undetectable in liver and muscle in mice under normal condition [29]. To determine the regulatory regions involved in the differential regulation of *Vdr* gene, we have performed genome-wide ChIP-

seq analyses and identified the binding of the VDR and histone marks representing enhancers and active DNA regions near the *Vdr* gene in bone cells as well as in intestine and kidneys of wildtype mice [25]. In Fig. 3A, we compared VDR binding in MC3T3-E1 cells and in kidney and intestine of wildtype mice as well as in *Cyp27b1*-null mice treated with either vehicle or 1,25(OH)₂D₃, and confirmed that VDR binding is distinctive in bone cells. VDR binding was also found not only in the third intron but also 20–40 kb upstream in the kidney of wildtype mice whereas this binding was not as pronounced in *Cyp27b1*-null mice; these results are potentially compromised by low expression of the VDR in kidney of *Cyp27b1*-null mice (Fig. 2B). In intestine, VDR binding is found similarly in both wildtype and *Cyp27b1*-null mice, although the locations of most of these sites are different from those of the kidney. To find enhancer regions at the *Vdr* gene locus, we examined the presence of the epigenetic enhancer signature H3K4me1 in the vehicle-treated samples and compared them with ChIP-seq data of kidney and intestine from C57BL/6 mice available via the ENCODE project [48]. The data showed that the enhancers marked by this histone modification were unique in bone cells and aligned with VDR binding while broad H3K4me1 enrichment was observed in the first and the third introns as well as in the upstream region of *Vdr* gene in kidney and intestine (Fig. 3B). There was no significant difference between sites of activity in our data and the ENCODE data. It was also found that all the VDR binding regions were included in the enhancer regions but that the pattern of the histone marks was different between the tissues. Since the VDR is expressed in kidney and intestine of wildtype and *Cyp27b1*-null mice under normal condition, we next assessed active chromatin regions marked by H3K27ac binding in the same samples. As shown in Fig. 3C, active regions found in wildtype and *Cyp27b1*-null mice were indistinguishable in the same tissues whereas the binding pattern of H3K27ac was quite different between the tissues. In particular, H3K27ac activity in the upstream region was more noticeable in intestine than kidney. Taken together, these data suggest that these regions may be involved in the tissue-specific hormonal regulation as well as the tissue-specific expression of the VDR. It will be necessary to dissect the roles of each of these regions to better understand the mechanisms for the tissue-specificity of the VDR expression.

3.2. Regulatory mechanisms of *Vdr* gene expression by hormones in bone and kidney

The ChIP-seq data summarized above raise many additional questions, such as the exact locations for the tissue-specific expression and the identity of the transcription factors that are involved in this regulation. To answer these questions, we first addressed the mechanism of hormonal regulation of *Vdr* expression in bone [25]. To identify the regulatory regions involved in the induction of *Vdr* gene by 1,25(OH)₂D₃, PTH and atRA in bone, we performed ChIP-chip and ChIP-seq analyses using both MC3T3-E1 [28,42] and UAMS-32PB cell lines [45]. As summarized in Fig. 4A, we discovered that the transcription factors responsible for the hormonal response, such as VDR, pCREB and RAR, bind to 2 intronic and 1 upstream regions designated S1, S3 and U1 and that these 3 regions were highly specific to bone cells (Fig. 3A). Further in vitro assays using (1) MC3T3-E1 cells stably transfected with a mini-gene which contains wildtype or enhancer-deleted mouse *Vdr* gene constructs shown in Fig. 1B, (2) UAMS-32PB cells genomically edited using the CRISPR/Cas9 system and (3) conventional reporter plasmids allowed us to define the

importance of S1, S3 and U1 enhancers in the regulation by $1,25(\text{OH})_2\text{D}_3$, atRA and PTH, respectively [25].

To examine the roles of these enhancers in vivo, we first used the wildtype VDR mini-gene to generate transgenic mice in a VDR-null background and showed that the mini-gene recapitulates the expression and regulation of the endogenous gene and rescues VDR-null phenotypes, suggesting that the mini-gene contained sufficient genetic information to mimic the endogenous *Vdr* gene [29]. We then generated transgenic mouse strains using the mini-gene in which U1 or a combination of the U1/S1 enhancers were deleted to confirm the function of the enhancers identified in the vitro assays [25]. In the present study, we generated an additional transgenic mouse strain using a dual S1/S3- enhancer deleted mini-gene and compared the hormonal responses of this transgene in bone between wildtype and the enhancer-deleted transgenic mouse strains to confirm further the roles of the enhancers in the hormonal regulation in bone. As summarized in Fig. 4B, the U1 deletion (U1 KO and U1/S1 KO) compromised the induction of mini-gene- derived *Vdr* expression by db-cAMP as previously reported [25]. In the transgenic mice containing the S1-deleted mini-genes (U1/S1 KO and S1/S3 KO), the responses to $1,25(\text{OH})_2\text{D}_3$ in calvaria was abolished while the S3-deleted (S1/S3 KO) transgenic mice lost atRA-response in the tissue. These data, therefore, confirm the specific role of each enhancer in the regulation of *Vdr* expression by the hormones in vivo, which is highly relevant to the binding of individual transcription factors (Fig. 4A).

As shown in Fig. 2A, *Vdr* expression is auto-regulated by its ligand in kidney as well as bone. It was also found that in bone, auto-regulation occurred primarily through VDR binding at the S1 enhancer region (Fig. 4). Interestingly, ChIP-seq analyses (Fig. 3A) showed that VDR binding at the S1 enhancer is also observed in kidney but not in the intestine, consistent with the absence of regulation by $1,25(\text{OH})_2\text{D}_3$ as well (Fig. 2A). To assess whether the S1 enhancer is involved in auto-regulation in kidney as in bone, the transgenic mice containing wildtype or enhancer-deleted mini-genes were treated with $1,25(\text{OH})_2\text{D}_3$ and mini-gene-derived *Vdr* expression (HA-*Vdr*) was measured in kidney. As can be seen in Fig. 4C, the induced expression of the *Vdr* gene by $1,25(\text{OH})_2\text{D}_3$ in kidney of wildtype mice (Fig. 2A) was recapitulated in the wildtype mini-gene transgenic mice (WT). Furthermore, its induction remained intact in U1-deleted transgenic mice (U1 KO) while deletion of S1 enhancer (U1/S1 KO and S1/S3 KO) abolished auto-regulation in kidney. Therefore, these data suggest that the $1,25(\text{OH})_2\text{D}_3$ -mediated increase of *Vdr* expression in kidney is attained in the same manner as the auto-regulation observed in bone.

3.3. Effect of low amount of the VDR on its response to $1,25(\text{OH})_2\text{D}_3$ and VDR-null mouse phenotypes

As mentioned above, it has been suggested that *Vdr* expression is downregulated in mouse kidney under specific conditions of low serum calcium level and $1,25(\text{OH})_2\text{D}_3$ [26,27] and that renal but not intestinal expression of the VDR is reduced in *Cyp27b1*-null mice relative to wildtype mice (Fig. 2B). However, the consequence of this low expression of the VDR is unclear. In previous studies, we generated a humanized VDR mouse in a VDR-null background using a human VDR mini-gene (Fig. 1C) in which the expression level of the

hVDR was similar to that of the endogenous mouse VDR (termed a hVDR-normal expressor) [29]. To explore the effect of low amounts of the VDR in vivo, we generated an additional humanized wildtype VDR mouse strain in which both the transcript and the protein levels of human *VDR* gene were about 10-fold less than hVDR-normal expressor (termed a hVDR-low expressor) (Fig. 5A). To test if the reduction in hVDR has an effect on response to $1,25(\text{OH})_2\text{D}_3$, hVDR-normal and -low expressor mice were injected with the ligand and target gene expression was measured. As shown in Fig. 5B, the treatment induced *Cyp24a1* expression in kidney and intestine of both strains. However, the level of induction was considerably lower in the hVDR-low expressor mouse than in the hVDR-normal expressor mouse although basal levels of *Cyp24a1* expression were not significantly different between the strains. When we measured other target genes in intestine such as *Trpv6*, *S100g* and *Atp2b1*, their induced levels were also lower in hVDR-low expressor mice (data not shown). Therefore, these data suggest that lower amount of the VDR reduces genetic response to $1,25(\text{OH})_2\text{D}_3$ in a given tissue.

To test if the reduced response to the ligand affected the ability of the mini-gene to rescue the VDR-null phenotype, we measured serum calcium and PTH levels of hVDR-low expressor mice and compared the results with those obtained from wildtype and VDR-null littermates. As can be seen in Fig. 5C, hypocalcemia and secondary hyperparathyroidism were normalized even in the hVDR-low expressor mouse. The renal basal expression of *Cyp24a1* or *Cyp27b1* has been shown to be suppressed or induced, respectively, likely due to the high serum PTH level in VDR-null mice [29,49]. Therefore, the normalization of these systemic factors is consistent with our results which show that the renal basal expression of *Cyp24a1* (Fig. 5B) and *Cyp27b1* (data not shown) are not different between hVDR-normal and -low expressor mice. While low BMD is a feature of VDR-null mice, total body BMD of male hVDR-low expressor mice was also not different from that of wildtype controls as seen in Fig. 5D. Femoral BMDs in the males showed similar results (data not shown). Alopecia which is a ligand-independent action of the VDR was also not observed in hVDR-low expressor mice (Fig. 5E). Therefore, these data suggest that the low amount of the VDR in hVDR-low expressor mice is sufficient to rescue the VDR-null phenotype under normal physiological conditions in spite of reduced transcriptional response to $1,25(\text{OH})_2\text{D}_3$. The ability of the low expressor mouse to rescue the VDR-null phenotype in the context of alternative conditions such as dietary challenge, age or pregnancy will require further examination.

Carmeliet and colleagues have generated a VDR-null mouse strain which expresses the VDR exclusively in intestine at a reduced level (about 15% of wildtype mice) [50]. Interestingly, these mice displayed sub-normal level of serum calcium even though the remaining VDR-null phenotypes relative to mineral homeostasis, such as secondary hyperparathyroidism, low serum FGF23 and high serum $1,25(\text{OH})_2\text{D}_3$, were not rescued, suggesting that the low amount of the VDR in intestine improves intestinal calcium uptake but $1,25(\text{OH})_2\text{D}_3$ -mediated VDR actions in kidney and skeleton may still be required for full recovery of the VDR-null phenotypes. Despite the incomplete rescue of the VDRnull phenotypes in this mouse strain, this study supports our results suggesting that the low expression of the VDR is still sufficient for full function in a given tissue.

We have generated a unique HVDRR mouse model without alopecia using a hVDR mini-gene in which leucine residue 233 was mutated to serine to prevent 1,25(OH)₂D₃ binding activity [30], and used as a model to explore ligand-independent actions of the VDR [16]. Three mouse strains of this mutant which were distinguished by VDR expression levels (approximately 10%, 20% and 50% of hVDR-normal expressor mice) were examined [30]. Interestingly, defects in hair formation which represent a ligand independent function of the VDR were fully rescued by the mutant VDRs regardless of expression levels whereas rachitic phenotypes were not rescued due to the loss of 1,25(OH)₂D₃ binding activity [30]. These data support the idea that expression of a concentration of the VDR lower than that seen in the normal mouse is sufficient to mediate ligand independent functions as well.

An interesting question is why VDR levels are downregulated in the kidney under conditions of low serum calcium such as seen in *Cyp27b1*-null mice (Fig. 2B) and in wildtype mice fed a low calcium diet [26,27]. The kidney expresses the *Cyp27b1* gene encoding the 25-hydroxyvitamin D₃ 1-alpha-hydroxylase as well as *Cyp24a1* gene encoding the 25-hydroxyvitamin D₃ 24-hydroxylase. Thus, this tissue is responsible for controlling serum 1,25(OH)₂D₃ levels not only through production but also through degradation. When an elevation of serum calcium level is required, the kidney enhances the production of 1,25(OH)₂D₃ following the induced *Cyp27b1* expression by PTH to assist in increased calcium uptake by the intestine and increased calcium resorption from bone [5,51,52]. At the same time, it is likely that *Cyp24a1* expression is suppressed through reciprocal regulation to reduce the degradation of the hormone in the kidney. This idea is supported by *Cyp24a1* suppression in the kidney of VDR-null mice and *Cyp27b1*-null mice where there is a high demand to increase serum calcium levels [29,49]. Since PTH has been shown to reduce *Cyp24a1* expression in AOK-B50 cells, a porcine kidney proximal tubule cell line [53] and elevated serum PTH levels are a common phenotypic feature of VDR-null mice [9–11] and *Cyp27b1*-null mice [12,13], it is possible that high serum PTH level under hypocalcemic conditions directly suppress *Cyp24a1* expression. Expression of the *Cyp24a1* gene is also highly induced by 1,25(OH)₂D₃ in the tissues where the VDR is expressed, by which local levels of the hormone are regulated through a negative feedback mechanism. The data demonstrated above (Fig. 5) indicate that low amounts of the VDR desensitize tissues to the ligand under the normal physiological conditions. Therefore, it is possible that the reduction of the renal VDR expression, observed in *Cyp27b1*-null mice (Fig. 2B) and the mice fed low calcium diet [26,27], not only maintains low basal *Cyp24a1* expression levels but also desensitizes the kidney to prevent *Cyp24a1* expression from being induced strongly in an effort to increase 1,25(OH)₂D₃ levels in the blood by reducing its turnover.

3.4. Role of phosphorylation at serine 208 in the hVDR in vivo

In vitro assays demonstrate that serine 208 is phosphorylated in the hVDR [34] and the phosphorylation of the VDR increases interaction between the VDR and DRIP205 to enhance its transcriptional activity [38,39]. Examining the role of this phosphorylation site is difficult, however, because it is specific to the hVDR and is not conserved in the mouse. However, the humanized VDR mouse strain described above provides an opportunity to examine this phosphorylation site functionally in vivo [16,29,30]. Accordingly, we created a mutation into the wildtype hVDR mini-gene to replace serine 208 with alanine (Fig. 1C) and

introduced the mutated mini-gene into the VDR-null mouse background (hVDR-S208A). Since it was shown that the amount of the VDR affects its transcriptional response to $1,25(\text{OH})_2\text{D}_3$ (Fig. 5A and B), we first assess the level of hVDR-S208A expression and compared it to levels seen in our hVDR low and normal expressor mice. As shown in Fig. 6A, expression levels of the hVDR-S208A mice were almost identical to that of the wildtype hVDR-low expressor mice in kidney, intestine and western blot analysis confirmed this similar expression level in kidney and intestine between the strains (Fig. 6B). We therefore injected both wildtype hVDR-low expressor mice and hVDR-S208A mice with vehicle or $1,25(\text{OH})_2\text{D}_3$ and compared VDR target gene expression. As can be seen in Fig. 6C, *Cyp24a1* expression was induced in kidney and intestine by the ligand treatment and the induced levels between wildtype and hVDR-S208A mice were not significantly different. Induction levels of *Trpv6*, *S100g* and *Atp2b1* observed in the intestine were also not different between the strains (data not shown). These data suggest that the loss of the phosphorylation site in the hVDR-S208A mutant did not alter the ability of the VDR to activate gene expression in vivo.

To assess whether the hVDR-S208A mutation affected the overall actions of the VDR, we measured serum calcium and PTH levels in these mice. When compared to that seen in the hVDR-low expressor mice, the mutation did not alter VDR activity (Fig. 6D), which is consistent with the absence of effect on basal levels of *Cyp24a1* (Fig. 6C) in the kidney. The absence of any change in VDR transcriptional activity and the ability of this mutant to rescue the VDR-null phenotype suggests that the S208A mutation has no effect on the ability of the VDR to bind ligand and to function normally. As shown in Fig. 6E, these animals also did not exhibit alopecia. Therefore, these data suggest that the mutation in the serine 208 residue does not have a significant effect on the activity of the VDR under the normal condition in vivo. However, it cannot be ruled out that the phosphorylated VDR may have actions that are highly specific for certain genes or that this modification affects other characteristics of the VDR protein that were not measured here. It is also possible that mechanisms involved in the phosphorylation of hVDR at serine 208 and/or to produce outcomes of the phosphorylated serine 208 are absent in mice due to the lack of conservation of the serine residue in mouse VDR.

4. Conclusions

This report offers several lines of evidence for tissue-specific regulation of VDR expression in vivo and identifies potential regulatory regions responsible for this tissue-specificity supported by genome wide in vivo ChIP-seq analyses. For a more comprehensive understanding of the mechanisms of the regulation of the VDR expression, we identified the hormonal regulatory mechanisms of VDR expression in bone and kidney in vivo using mini-genes which behave in a fashion similar to that of the endogenous gene. Using the hVDR mini-gene which is also able to recapitulate endogenous mouse VDR behavior, we further drew possible implications for the surprising ability of low concentrations of the VDR in tissues to rescue the VDR-null mouse. Our results also emphasize the necessity for in vivo studies to understand the mechanisms of VDR activity including assessment of the role of phosphorylation in VDR action. In this context, it remains formally possible that phosphorylation may impact a feature of the VDR for which we have not yet explored.

Taken together, however, success in understanding VDR regulation in the skeleton using mini-genes and ChIP-seq analyses and the studies on humanized VDR mice using various hVDR mini-genes provide information that brings us closer to understanding the regulation and function of this important nuclear receptor.

Acknowledgments

Funding

This study was supported by the National Institutes of Health grants DK-072281, DK-073995 and AR-045173 to JWP.

We thank members of the Pike laboratory for their contributions to this work. We also acknowledge Dustin Irving, Regina Berget and Douglas Jacobson for the animal husbandry associated with this study. We acknowledge ENCODE Consortium and Bing Ren's laboratory at the Ludwig Institute for Cancer Research (LICR) who generated and analyzed ChIP-seq data available for the ENCODE project.

Abbreviations

1,25(OH)₂D₃	1,25 dihydroxyvitamin D ₃
atRA	all trans retinoic acid
BMD	bone mineral density
db-cAMP	dibutyl cyclic AMP
ChIP	chromatin immunoprecipitation
ChIP-chip	ChIP followed by microarray
ChIP-seq	ChIP followed by sequencing
FGF23	fibroblast growth factor 23
H3K27ac	Lys 27-acetylated histone H3
H3K4me1	Lys 4-monomethylated histone H3
HVDRR	hereditary 1,25-dihydroxyvitamin D ₃ resistant rickets
pCREB	phosphorylated CREB
PTG	parathyroid gland
PTH	parathyroid hormone
qPCR	quantitative polymerase chain reaction
RAR	retinoic acid receptor
VDDR-1	vitamin D dependency rickets type 1
VDR	vitamin D receptor

References

1. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The nuclear receptor superfamily: the second decade. *Cell*. 1995; 83(6):835–839. [PubMed: 8521507]
2. Pike JW, Meyer MB, Bishop KA. Regulation of target gene expression by the vitamin D receptor—an update on mechanisms. *Rev Endocr Metab Disord*. 2012; 13(1):45–55. [PubMed: 21870057]
3. Bouillon R, Carmeliet G, Verlinden L, van Etten E, Verstuyf A, Luderer HF, Lieben L, Mathieu C, Demay M. Vitamin D and human health: lessons from vitamin D receptor null mice. *Endocr Rev*. 2008; 29(6):726–776. [PubMed: 18694980]
4. Pike JW, Meyer MB, Lee SM, Onal M, Benkusky NA. The vitamin D receptor: contemporary genomic approaches reveal new basic and translational insights. *J Clin Invest*. 2017; 127(4):1146–1154. [PubMed: 28240603]
5. Jones G, Strugnelli SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. *Physiol Rev*. 1998; 78(4):1193–1231. [PubMed: 9790574]
6. Feldman D, Malloy PJ. Mutations in the vitamin D receptor and hereditary vitamin D-resistant rickets. *Bonekey Rep*. 2014; 3:510. [PubMed: 24818002]
7. Feldman, D., Malloy, P.J., Miller, W.L. Genetic disorders of vitamin D synthesis and action. In: Thakker, R.V., Whyte, M.P., Eisman, J., Igarashi, T., editors. *Genetics of Bone Biology and Skeletal Disease*. Elsevier; San Diego, CA, USA: 2013. p. 537–552.
8. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, Kato S. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet*. 1997; 16(4):391–396. [PubMed: 9241280]
9. Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R, Demay MB. Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. *Proc Natl Acad Sci U S A*. 1997; 94(18):9831–9835. [PubMed: 9275211]
10. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, Stockmans I, Van Herck E, Kato S, Bindels RJ, Collen D, Carmeliet P, Bouillon R, Carmeliet G. Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc Natl Acad Sci U S A*. 2001; 98(23):13324–13329. [PubMed: 11687634]
11. Erben RG, Soegiarto DW, Weber K, Zeitz U, Lieberherr M, Gniadecki R, Möller G, Adamski J, Balling R. Deletion of deoxyribonucleic acid binding domain of the vitamin D receptor abrogates genomic and nongenomic functions of vitamin D. *Mol Endocrinol*. 2002; 16(7):1524–1537. [PubMed: 12089348]
12. Dardenne O, Prud'homme J, Arabian A, Glorieux FH, St-Arnaud R. Targeted inactivation of the 25-hydroxyvitamin D(3)-1(alpha)-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets. *Endocrinology*. 2001; 142(7):3135–3141. [PubMed: 11416036]
13. Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN, Goltzman D. Targeted ablation of the 25-hydroxyvitamin D 1alpha -hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. *Proc Natl Acad Sci U S A*. 2001; 98(13):7498–7503. [PubMed: 11416220]
14. Vanhooke JL, Pahl JM, Kimmel-Jehan C, Mendelsohn M, Danielson EW, Healy KD, DeLuca HF. CYP27B1 null mice with LacZ reporter gene display no 25-hydroxyvitamin D3-1alpha-hydroxylase promoter activity in the skin. *Proc Natl Acad Sci U S A*. 2006; 103(1):75–80. [PubMed: 16371465]
15. Skorija K, Cox M, Sisk JM, Dowd DR, MacDonald PN, Thompson CC, Demay MB. Ligand-independent actions of the vitamin D receptor maintain hair follicle homeostasis. *Mol Endocrinol*. 2005; 19(4):855–862. [PubMed: 15591533]
16. Lee SM, Pike JW. The vitamin D receptor functions as a transcription regulator in the absence of 125-dihydroxyvitamin D3. *J Steroid Biochem Mol Biol*. 2016; 164:265–270. [PubMed: 26323657]
17. Huet T, Laverny G, Ciesielski F, Molnár F, Ramamoorthy TG, Belorusova AY, Antony P, Potier N, Metzger D, Moras D, Rochel N. A vitamin D receptor selectively activated by gemini analogs

- reveals ligand dependent and independent effects. *Cell Rep.* 2015; 10(4):516–526. [PubMed: 25620699]
18. Reichrath J, Schilli M, Kerber A, Bahmer FA, Czarnetzki BM, Paus R. Hair follicle expression of 125-dihydroxyvitamin D3 receptors during the murine hair cycle. *Br J Dermatol.* 1994; 131(4): 477–482. [PubMed: 7947199]
 19. Pike J. Receptors for 1,25-dihydroxyvitamin D3 in chick pancreas: a partial physical and functional characterization. *J Steroid Biochem.* 1982; 16(3):385–395. [PubMed: 6283269]
 20. Zarnani AH, Shahbazi M, Salek-Moghaddam A, Zareie M, Tavakoli M, Ghasemi J, Rezaei S, Moravej A, Torkabadi E, Rabbani H, Jeddi-Tehrani M. Vitamin D3 receptor is expressed in the endometrium of cycling mice throughout the estrous cycle. *Fertil Steril.* 2010; 93(8):2738–2743. [PubMed: 19896660]
 21. Pike J, Gooz L, Haussler M. Biochemical evidence for 125-dihydroxyvitamin D receptor macromolecules in parathyroid, pancreatic, pituitary, and placental tissues. *Life Sci.* 1980; 26(5): 407–414. [PubMed: 7366331]
 22. Provvedini DM, Tsoukas CD, Deftos LJ, Manolagas SC. 125-dihydroxyvitamin D3 receptors in human leukocytes. *Science.* 1983; 221(4616):1181–1183. [PubMed: 6310748]
 23. Strom M, Sandgren ME, Brown TA, DeLuca HF. 125-Dihydroxyvitamin D3 upregulates the 1,25-dihydroxyvitamin D3 receptor in vivo. *Proc Natl Acad Sci U S A.* 1989; 86(24):9770–9773. [PubMed: 2481316]
 24. Zella LA, Kim S, Shevde NK, Pike JW. Enhancers located within two introns of the vitamin D receptor gene mediate transcriptional autoregulation by 125-dihydroxyvitamin D3. *Mol Endocrinol.* 2006; 20(6):1231–1247. [PubMed: 16497728]
 25. Lee SM, Meyer MB, Benkusky NA, O'Brien CA, Pike JW. Mechanisms of enhancer-mediated hormonal control of Vitamin D receptor gene expression in target cells. *J Biol Chem.* 2015; 290(51):30573–30586. [PubMed: 26504088]
 26. Healy KD, Zella JB, Pahl JM, DeLuca HF. Regulation of the murine renal vitamin D receptor by 125-dihydroxyvitamin D3 and calcium. *PNAS.* 2003; 100(17):9733–9737. [PubMed: 12900504]
 27. Healy KD, Frahm MA, DeLuca HF. 125-Dihydroxyvitamin D3 up-regulates the renal vitamin D receptor through indirect gene activation and receptor stabilization. *Arch Biochem Biophys.* 2005; 433(2):466–473. [PubMed: 15581603]
 28. Zella LA, Meyer MB, Nerenz RD, Lee SM, Martowicz ML, Pike JW. Multifunctional enhancers regulate mouse and human vitamin D receptor gene transcription. *Mol Endocrinol.* 2010; 24(1): 128–147. [PubMed: 19897601]
 29. Lee SM, Bishop KA, Goellner JJ, O'Brien CA, Pike JW. Mouse and human BAC transgenes recapitulate tissue-specific expression of the Vitamin D receptor in mice and rescue the VDR-Null phenotype. *Endocrinology.* 2014; 155(6):2064–2076. [PubMed: 24693968]
 30. Lee SM, Goellner JJ, O'Brien CA, Pike JW. A humanized mouse model of hereditary 125-dihydroxyvitamin D-resistant rickets without alopecia. *Endocrinology.* 2014; 155(11):4137–4148. [PubMed: 25147982]
 31. Pike J, Sleator N. Hormone-dependent phosphorylation of the 125-dihydroxyvitamin D3 receptor in mouse fibroblasts. *Biochem Biophys Res Commun.* 1985; 131(1):378–385. [PubMed: 2994658]
 32. Hsieh JC, Dang HT, Galligan MA, Whitfield GK, Haussler CA, Jurutka PW, Haussler MR. Phosphorylation of human vitamin D receptor serine-182 by PKA suppresses 125(OH)2D3-dependent transactivation. *Biochem Biophys Res Commun.* 2004; 324(2):801–809. [PubMed: 15474498]
 33. Hsieh JC, Jurutka PW, Galligan MA, Terpening CM, Haussler CA, Samuels DS, Shimizu Y, Shimizu N, Haussler MR. Human vitamin D receptor is selectively phosphorylated by protein kinase C on serine 51, a residue crucial to its trans-activation function. *Proc Natl Acad Sci U S A.* 1991; 88(20):9315–9319. [PubMed: 1656468]
 34. Jurutka PW, Hsieh JC, MacDonald PN, Terpening CM, Haussler CA, Haussler MR, Whitfield GK. Phosphorylation of serine 208 in the human vitamin D receptor. The predominant amino acid phosphorylated by casein kinase II, in vitro, and identification as a significant phosphorylation site in intact cells. *J Biol Chem.* 1993; 268(9):6791–6799. [PubMed: 8384219]

35. Jurutka PW, Hsieh JC, Haussler MR. Phosphorylation of the human 125-dihydroxyvitamin D3 receptor by cAMP-dependent protein kinase, in vitro, and in transfected COS-7 cells. *Biochem Biophys Res Commun.* 1993; 191(3):1089–1096. [PubMed: 8385450]
36. Ting HJ, Yasmin-Karim S, Yan SJ, Hsu JW, Lin TH, Zeng W, Messing J, Sheu TJ, Bao BY, Li WX, Messing E, Lee YF. A positive feedback signaling loop between ATM and the vitamin D receptor is critical for cancer chemoprevention by vitamin D. *Cancer Res.* 2012; 72(4):958–968. [PubMed: 22207345]
37. Hilliard GT, Cook R, Weigel N, Pike J. 1,25-dihydroxyvitamin D3 modulates phosphorylation of serine 205 in the human vitamin D receptor: site-directed mutagenesis of this residue promotes alternative phosphorylation. *Biochemistry.* 1994; 33(14):4300–4311. [PubMed: 8155647]
38. Jurutka PW, Hsieh JC, Nakajima S, Haussler CA, Whitfield GK, Haussler MR. Human vitamin D receptor phosphorylation by casein kinase II at Ser-208 potentiates transcriptional activation. *Proc Natl Acad Sci U S A.* 1996; 93(8):3519–3524. [PubMed: 8622969]
39. Arriagada G, Paredes R, Olate J, van Wijnen A, Lian JB, Stein GS, Stein JL, Onate S, Montecino M. Phosphorylation at serine 208 of the 1 α ,25-dihydroxy Vitamin D3 receptor modulates the interaction with transcriptional coactivators. *J Steroid Biochem Mol Biol.* 2007; 103(3–5):425–429. [PubMed: 17368182]
40. Barletta F, Freedman LP, Christakos S. Enhancement of VDR-mediated transcription by phosphorylation: correlation with increased interaction between the VDR and DRIP205, a subunit of the VDR-interacting protein coactivator complex. *Mol Endocrinol.* 2002; 16(2):301–314. [PubMed: 11818502]
41. Meyer MB, Benkusky NA, Pike JW. The RUNX2 cistrome in osteoblasts: characterization, down-regulation following differentiation, and relationship to gene expression. *J Biol Chem.* 2014; 289(23):16016–16031. [PubMed: 24764292]
42. Meyer MB, Benkusky NA, Lee CH, Pike JW. Genomic determinants of gene regulation by 125-dihydroxyvitamin D3 during osteoblast-lineage cell differentiation. *J Biol Chem.* 2014; 289(28):19539–19554. [PubMed: 24891508]
43. Onal M, St John HC, Danielson AL, Pike JW. Deletion of the distal tnfsf11 RL-D2 enhancer that contributes to PTH-Mediated RANKL expression in osteoblast lineage cells results in a high bone mass phenotype in mice. *J Bone Miner Res.* 2016; 31(2):416–429. [PubMed: 26332516]
44. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res.* 2005; 33(4):e36. [PubMed: 15731329]
45. Meyer MB, Benkusky NA, Pike JW. Selective distal enhancer control of the mmp13 gene identified through clustered regularly interspaced short palindromic repeat (CRISPR) genomic deletions. *J Biol Chem.* 2015; 290(17):11093–11107. [PubMed: 25773540]
46. Lee SM, Riley EM, Meyer MB, Benkusky NA, Plum LA, DeLuca HF, Pike JW. 125-Dihydroxyvitamin D3 controls a cohort of Vitamin D receptor target genes in the proximal intestine that is enriched for calcium-regulating components. *J Biol Chem.* 2015; 290(29):18199–18215. [PubMed: 26041780]
47. Lee SM, Bishop KA, Goellner JJ, O'Brien CA, Pike JW. Mouse and human BAC transgenes recapitulate tissue-specific expression of the Vitamin D receptor in mice and rescue the VDR-null phenotype. *Endocrinology.* 2014; 155(6):2064–2076. <http://dx.doi.org/10.1210/en.2014-1107>. [PubMed: 24693968]
48. Consortium EP. An integrated encyclopedia of DNA elements in the human genome. *Nature.* 2012; 489(7414):57–74. [PubMed: 22955616]
49. Panda D, Miao D, Bolivar I, Li J, Huo R, Hendy G, Goltzman D. Inactivation of the 25-hydroxyvitamin D 1 α -hydroxylase and vitamin D receptor demonstrates independent and interdependent effects of calcium and vitamin D on skeletal and mineral homeostasis. *J Biol Chem.* 2004; 279(16):16754–16766. [PubMed: 14739296]
50. Lieben L, Verlinden L, Masuyama R, Torrekens S, Moermans K, Schoonjans L, Carmeliet P, Carmeliet G. Extra-intestinal calcium handling contributes to normal serum calcium levels when intestinal calcium absorption is suboptimal. *Bone.* 2015; 81:502–512. [PubMed: 26319498]

51. Fu GK, Lin D, Zhang MY, Bikle DD, Shackleton CH, Miller WL, Portale AA. Cloning of human 25-hydroxyvitamin D-1 α -hydroxylase and mutations causing vitamin D-dependent rickets type 1. *Mol Endocrinol.* 1997; 11(13):1961–1970. [PubMed: 9415400]
52. Murayama A, Takeyama K, Kitanaka S, Kodera Y, Kawaguchi Y, Hosoya T, Kato S. Positive and negative regulations of the renal 25-hydroxyvitamin D3 1 α -hydroxylase gene by parathyroid hormone, calcitonin, and 1 α ,25(OH) 2D3 in intact animals. *Endocrinology.* 1999; 140(5): 2224–2231. [PubMed: 10218975]
53. Zierold C, Reinholz GG, Mings JA, Prahl JM, DeLuca HF. Regulation of the porcine 125-dihydroxyvitamin D3-24-hydroxylase (CYP24) by 1,25-dihydroxyvitamin D3 and parathyroid hormone in AOK-B50 cells. *Arch Biochem Biophys.* 2000; 381(2):323–327. [PubMed: 11032422]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1.

Schematic structures of the targeting vector and *Cyp27b1* gene locus for *Cyp27b*-null mice and the mini-genes for transgenic mouse models. (A) Schematic structures of the targeting vector and the *Cyp27b1* gene locus in the mice produced in the process to generate *Cyp27b1*-null mice in which exon 7 through 9 of the gene are deleted are presented (see Materials and methods). Numbered hatched boxes represent each exon. FRT sites and *loxP* sites are shown double gray triangles and a black triangle, respectively. Selectable markers, diphtheria toxin A (DTA) and neomycin (neo) resistance genes, are indicated. (B, C) Schematic structures of mouse (B) and human (C) VDR mini-genes are presented. Each mini-gene includes the entire mouse or human *VDR* gene locus and its surrounding intergenic segments. Location of each enhancer (S1, S3 and U1) of mouse VDR mini-gene is indicated by an arrow (B). The S208A mutation was introduced through nucleotide mutagenesis (AGT to GCT) as indicated (C). Exons and introns are represented by black and gray boxes, respectively. Direction of transcription is indicated by an arrow at the TSS. Insertion sites of HA-tag (HA) and a cassette containing an IRES-driven luciferase (LUC) reporter and a TK promoter (TK)-driven neomycin resistance gene (Neo^r) are shown in the translation start site and in the 3'-UTR, respectively. The sizes of upstream and downstream intergenic sequence included in the mini-genes are indicated.

Fig. 2.

Tissue specific regulation of endogenous *Vdr* expression. (A) Wildtype mice were intraperitoneally injected with vehicle (Veh), 1,25(OH)₂D₃ (1,25), FGF23 or PTH and expression of endogenous *Vdr* gene in the indicated tissues was measured by qPCR. (B) Expression of endogenous *Vdr* gene was measured in the indicated tissues obtained from *Cyp27b1*-null mice (C27KO) and wildtype littermates (WT). The expression level represents relative quantitation (RQ) normalized to *Gapdh* (CT) and data are displayed as the means for each group ± SEM (5–7 mice per group). **p* < 0.05 compared with vehicle-treated samples (A) or wildtype littermates (B).

Fig. 3.

Genetic and epigenetic features of mouse *Vdr* gene locus. (A) Representative ChIP-seq tracks of triplicate samples for the VDR at mouse *Vdr* gene locus in MC3T3-E1 cells (MC3T3), kidney and intestine obtained from *Cyp27b1*-null mice (C27KO) and wildtype littermates (WT) are presented. Binding of the VDR in vehicle- and 1,25(OH)₂D₃-treated samples is shown in yellow and blue, respectively. Overlaps in binding are shown in green. (B, C) Representative ChIP-seq tracks of triplicate samples for Lys 4-monomethylated histone H3 (B, H3K4me1) and Lys 27-acetylated histone H3 (C, H3K27ac) at mouse *Vdr* gene locus in MC3T3-E1 cells (MC3T3) and kidney and intestine from *Cyp27b1*-null mice (C27KO) and wildtype littermates (WT) treated with vehicle are presented in yellow. ChIP-seq data of the same histone marks in kidney and intestine obtained from C57BL/6 mice (ENCODE Consortium) are also displayed in gray (BL6). Exons and introns of *Vdr* and adjacent *Tmem106c* genes are shown in boxes and lines, respectively. Transcriptional direction of a gene is indicated by an arrow at TSS and genomic location and scale are provided. Maximum height of tag density for the data track is indicated on each track.

CCCTC-binding factor binding sites (ENCODE Consortium) commonly found in kidney and intestine are indicated below the tracks with arrows (C, CTCF). Potential enhancer regions in each sample are highlighted in red boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4.

Regulation of *Vdr* mini-gene expression by hormones in the transgenic mice. (A) Binding of the VDR, phosphorylated CREB (pCREB) or RAR was determined by ChIP-chip and ChIP-seq analyses using MC3T3-E1 cells (Bone) or kidney from wildtype mice (Kidney). Major and minor binding of the indicated transcription factors at S1, S3 and U1 in the samples is summarized as large and small circles, respectively. Exons and introns of the *Vdr* gene are shown in boxes and lines, respectively. Transcriptional direction of a gene is indicated by an arrow at TSS and the scale is provided at the bottom of the gene. (B, C) The transgenic mice generated by wildtype (WT) and U1- (U1KO), U1 and S1- (U1/S1 KO) and S1 and S3-deleted (S1/S3 KO) mouse VDR mini-genes were intraperitoneally injected with vehicle

(Veh), 1,25(OH)₂D₃ (1,25), db-cAMP (cAMP) or atRA and the expression of the *Vdr* gene from the mini-genes (HA- *Vdr*) in calvaria (B) and kidney (C) was measured by qPCR. The expression level was calculated as a relative quantitation (RQ) normalized to *Gapdh* (CT). Data are displayed as the means of fold induction relative to vehicle-treated samples for each group±SEM (5 mice per group). **p* < 0.05 compared with vehicle-treated sample.

Fig. 5.

Characterization of hVDR-low expressor mice. (A) Levels of hVDR transcripts and proteins were measured in the indicated tissues obtained from hVDR-normal and -low expressor mice by qPCR and western blot analysis, respectively. Level of β -tubulin was used as a loading control. * $p < 0.05$ compared with hVDR-normal expressors. (B) The hVDR-normal and -low expressor were intraperitoneally injected with vehicle (Veh) or 1,25(OH)₂D₃ (1,25) and the expression of *Cyp24a1* gene in the indicated tissues was measured by qPCR. * $p < 0.05$ compared with vehicle-treated samples, # $p < 0.05$ compared with hVDR-normal expressors received the same treatment. The expression level (A, B) is relative quantitation (RQ) normalized to *Gapdh* (CT) and data are displayed as the means for each group $\pm$ SEM (5–7 mice per group). (C, D) Serum calcium and PTH levels (C) and total body BMDs (D, males) in hVDR-low expressor mice were measured and compared with those in VDR-null mice (VDR KO) and wildtype littermates (WT). Each value is the average of 5–7 mice per strain $\pm$ SEM. * $p < 0.05$ compared with wildtype littermates, # $p < 0.05$ compared with VDR-null mice. (E) The images show gross appearance of a representative mouse of wildtype (WT), VDR-null mice (VDR KO) or hVDR-low expressor mice at 6 month of age.

Fig. 6.

Characterization of hVDR-S208A mini-gene mice. (A, B) Levels of hVDR transcripts (A) and proteins (B) were measured in the indicated tissues obtained from hVDR-low expressor and hVDR-S208A mice by qPCR (A) and western blot analysis (B), respectively. Level of β -tubulin was used as a loading control. No difference in the expression level was found in all tissues examined. (C) The hVDR-low expressor and hVDR-S208A mice were intraperitoneally injected with vehicle (Veh) or 1,25(OH) $_2$ D $_3$ (1,25) and the expression of *Cyp24a1* gene in the indicated tissues was measured by qPCR. * $p < 0.05$ compared with vehicle-treated samples. No difference was found between two mouse strains. The level of transcript (A, C) represents relative quantitation (RQ) normalized to *Gapdh* (Ct) and data are displayed as the means for each group $\pm$ SEM (5–7 mice per group). (D) Serum calcium and PTH levels in hVDR-low expressor and hVDR-S208A mice were measured. Each value is the average of 5 mice per strain $\pm$ SEM. No difference was found in the levels of the

serum factors. (E) The images show gross appearance of a representative mouse of hVDR-low expressor and hVDR-S208A mice at 6 month of age.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript