The vitamin D receptor functions as a transcription regulator in the absence of 1,25-dihydroxyvitamin D_3

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Abstract

The vitamin D receptor (VDR) is a critical mediator of the biological actions of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). As a nuclear receptor, ligand activation of the VDR leads to the protein's binding to specific sites on the genome that results in the modulation of target gene expression. The VDR is also known to play a role in the hair cycle, an action that appears to be $1,25(OH)_2D_3$-independent. Indeed, in the absence of VDR as in hereditary 1,25-dihydroxyvitamin D resistant rickets (HVDRR) both skin defects and alopecia emerge. Recently, we generated a mouse model of HVDRR without alopecia wherein a mutant human VDR lacking $1,25(OH)_2D_3$-binding activity was expressed in the absence of endogenous mouse VDR. While $1,25(OH)_2D_3$ failed to induce gene expression in these mice, resulting in an extensive skeletal phenotype, the receptor was capable of restoring normal hair cycling. We also noted a level of secondary hyperparathyroidism that was much higher than that seen in the VDR null mouse and was associated with an exaggerated bone phenotype as well. This suggested that the VDR might play a role in parathyroid hormone (PTH) regulation independent of $1,25(OH)_2D_3$. To evaluate this hypothesis further, we contrasted PTH levels in the HVDRR mouse model with those seen in *Cyp27b1* null mice where the VDR was present but the hormone was absent. The data revealed that PTH was indeed higher in *Cyp27b1* null mice compared to VDR null mice. To evaluate the mechanism of action underlying such a hypothesis, we measured the expression levels of a number of VDR target genes in the duodena of wildtype mice and in transgenic mice expressing either a normal or a hormone-binding deficient mutant VDRs. We also compared expression levels of these genes between VDR null mice and *Cyp27b1* null mice. In a subset of cases, the expression of VDR target genes was lower in mice containing the VDR as opposed to mice that did not. We suggest that the VDR may function as a selective suppressor/de-repressor of gene expression in the absence of $1,25(OH)_2D_3$.

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1. Introduction

The vitamin D receptor (VDR) is a nuclear factor which mediates the biological actions of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). One of the prominent activities of this hormone is to maintain mineral homeostasis in higher vertebrates via the regulation of gene expression by the VDR in intestine, kidney, bone and parathyroid gland (PTG) [1]. Parathyroid hormone (PTH), a calciotropic regulator secreted from the PTG, directly influences $1,25(OH)_2D_3$ target tissues such as kidney and bone but not the intestine. Indeed, PTH profoundly upregulates the expression of renal *Cyp27b1* to increase the production of $1,25(OH)_2D_3$ which in turn activates the VDR [2]. $1,25(OH)_2D_3$-bound VDR modulates the expression of a network of genes to raise calcium absorption in intestine [3] while influencing bone cell differentiation in conjunction with PTH to affect bone remodeling [4]. Increased levels of $1,25(OH)_2D_3$ also exert a suppressive negative feedback loop in the PTG to reduce the production of PTH, although the molecular mechanism of such action remains unclear [5]. In kidney, ligand-activated VDR regulates target genes not only to repress *Cyp27b1* gene expression to control $1,25(OH)_2D_3$ production but also to facilitate calcium reabsorption [2].

The action of the VDR is initiated through its interaction with $1,25(OH)_2D_3$, which results in the formation of a heterodimer with retinoid X receptor (RXR), the binding of this heterodimer to vitamin D response element (VDRE) at target genes, and the recruitment of various molecular machines that are capable of altering chromatin structure [6–10]. Our recent unbiased genome-wide studies using ChIP-sequencing (ChIP-seq) analysis coupled with to RNA-sequencing (RNA-seq) analysis in bone cells [11] and mouse small intestine [3] suggest that VDR binding sites are enriched for VDREs but also contain adjacent sequences capable of interacting with additional transcription factors that may be involved. These studies have also revealed that $1,25(OH)_2D_3$/VDR target genes are regulated through multiple enhancers located predominantly within introns and intergenic regions but less frequently in regions near target gene promoters.

Since the syndrome of hereditary 1,25-dihydroxyvitamin D resistant rickets (HVDRR) was first identified [12], the underlying involvement of the VDR was suspected. Further biochemical and genetic studies of patient samples and cells not only supported but eventually confirmed this hypothesis [13], prompting the development of a mouse model in which the VDR was similarly mutated [14–17]. Further understanding of the roles of the $1,25(OH)_2D_3$/VDR system were extended through studies of the syndrome of vitamin D dependency rickets type 1 (VDDR-1), a disease caused by mutations in the *CYP27B1* gene that resulted in a failure of $1,25(OH)_2D_3$ production [18], and the development of cognate *Cyp27b1* null mouse models [19–21]. These human syndromes and mouse models in which $1,25(OH)_2D_3$-mediated transcriptional activity of the VDR was compromised share a similar biological phenotype, such as abnormal mineral homeostasis and skeletal defects that include rickets and growth retardation, indicating the importance of $1,25(OH)_2D_3$-dependent activation of the VDR. This transcriptional activity was also suggested to modulate proliferation and differentiation of epidermal keratinocytes in skin as well [22]. However, prompted by selective differences in the presence or absence of alopecia in humans as well as the observation that *Vdr* null but not *Cyp27b1* null mice are alopecic, the concept

emerged that the VDR might function in keratinocytes in the absence of $1,25(OH)_2D_3$. This concept was further supported by studies of VDR null mice in which a mutant human VDR lacking $1,25(OH)_2D_3$ binding activity due to a leucine to serine replacement at amino acid 233 (L233S) was expressed specifically in keratinocytes and led to normal hair growth [23].

We recently generated a humanized VDR mouse model using a bacterial artificial chromosome (BAC) that contained either the entire wildtype human *VDR* gene [24] or a mutant version (L233S) as just documented [25]. Although the wildtype VDR fully rescued transcription, mineral homeostasis and skin abnormalities, the mutant VDR rescued only the alopecia, supporting the concept that the control of hair follicle cycling by the VDR is $1,25(OH)_2D_3$ ligand-independent. Interestingly, we found that the presence of this mutant form of the VDR also led to an exaggerated phenotype relative to PTH secretion and lower bone mineral density (BMD) than that seen in the VDR null mouse, suggesting the possibility that $1,25(OH)_2D_3$-independent actions of the VDR might extend beyond the skin [25]. In the present report, we present data suggesting that the VDR expressed both in the mutant humanized mouse models we generated and in the *Cyp27b1* null mouse model may function to suppress/de-repress specific target genes in the absence of $1,25(OH)_2D_3$ not only in skin but in other mouse tissues as well.

2. Materials and Methods

2.1. Animal Study

VDR null mice [15] and *Cyp27b1* null mice [21] used in this study were obtained from the Jackson Laboratories. Generation of humanized VDR mice ($hVDR^{WT}/VDR^{-/-}$) and mutant humanized VDR mice ($T805/VDR^{-/-}$, $T806/VDR^{-/-}$ and $T807/VDR^{-/-}$) were previously described [24, 25]. Wildtype mice were littermates from C57BL/6 mice (Harlan). All mice were fed standard rodent chow diet (5008; Harlan Teklad) after weaning until sacrifice at 8–10 weeks of age. Mice were exposed to a 12-hour light-dark cycle and all animal studies were reviewed and approved by the Research Animal Care and Use Committee of University of Wisconsin-Madison.

2.2. Serum PTH measurement

Blood collection and measurement of serum PTH levels were previously described [24].

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Preparation of RNAs and cDNAs from tissues were previously described [24]. Quantitative polymerase chain reaction (qPCR) was performed as previously described [25]. TaqMan primers (Applied Biosystems) for qPCR are available upon request.

2.4. Statistical Analysis

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

3. Results and Discussion

3.1. Potential role of the VDR as a de-repressor of PTH production in the absence of 1,25(OH)$_2$D$_3$

Our previous studies describe the introduction of BAC clone transgenes containing the human VDR gene and its regulatory features into the VDR null background [24, 25]. In addition to several wildtype hVDR strains, three transgenic strains designated T805/VDR$^{-/-}$, T806/VDR$^{-/-}$, and T807/VDR$^{-/-}$ were also created that produced a human L233S mutant VDR protein incapable of binding 1,25(OH)$_2$D$_3$ [25]. VDR protein expression in the tissues of these mice ranged from that seen in wildtype mice (T807/VDR$^{-/-}$) to levels approximately 10-fold lower (T805/VDR$^{-/-}$) [25]. None of these mice exhibited alopecia; however, all were hypocalcemic and phosphatemic, exhibited high levels of PTH and displayed skeletal defect analogous to those seen in VDR null mice due to a failure of the VDR to direct 1,25(OH)$_2$D$_3$-dependent transcription [25]. Interestingly, while serum PTH levels as documented in Fig. 1 were normalized in VDR null mice rescued through expression of the wildtype human VDR, levels of this hormone were substantially higher in VDR null mice expressing the mutant L233S VDR form [25]. As this finding suggested a potential ligand-independent de-repressing action of the VDR in the PTG, we compared the levels of PTH in these mice with those in *Cyp27b1* null mice where wildtype mouse receptor was expressed normally in the absence of 1,25(OH)$_2$D$_3$. As can be seen in Fig. 1, PTH levels in these mice were much higher than in VDR null mice as well. Importantly, these data are consistent with an earlier report in a similar strain of hypocalcemic mice [26]. The authors at that time also concluded that the increased levels of PTH seen in *Cyp27b1* null mice correlated directly with PTG size that exceeded that seen in VDR null mice and that this effect was independent of calcemic conditions [26]. Importantly, their further studies revealed that PTG size was also greater in *Cyp27b1* null mice even in the absence of the VDR. This study also suggested an alternative possibility that PTG cell proliferation was regulated by serum calcium and 1,25(OH)$_2$D$_3$ and not by the VDR, although the idea that VDR protein might exert a direct impact on this tissue to accelerate PTH production separate from the hormone cannot be ruled out.

3.2. Role of the VDR in bone in the absence of 1,25(OH)$_2$D$_3$

L233S VDR mutant mice also display reduced skeletal BMD relative to VDR null mice [25]. This contrast with VDR null mice was also observed in a recent study in mice in which a mutation was introduced into the endogenous mouse *Vdr* gene different from ours [27]; this created a unique form of the mouse VDR that was unable to bind 1,25(OH)$_2$D$_3$ as well, but could be activated by a synthetic ligand. These data suggest that the presence of the VDR protein could exert an effect on not only the PTG but on the skeleton as well, although this phenotype could also be derived from higher levels of PTH. Interestingly, mice with the knock-in mutation exhibited a more severe bone defect than seen in VDR null mice, as manifested by shorter femur length [27]. This defect was not observed in our own studies despite similarities in both experimental age and dietary content. Although a reduced femur shaft length was not seen in earlier studies of the *Cyp27b1* mouse, this investigation was conducted on animals that were considerably older and maintained under different dietary conditions [26]. We conclude that while the skeletal defects seen in each of these three

studies appear to be inconsistent, and sensitivity of skeletal elements to high levels of PTH could account for the results observed, the idea that a ligand binding-deficient receptor could be active in the skeleton remains a viable possibility.

3.3. Role of the VDR as a transcription repressor in the absence of 1,25(OH)$_2$D$_3$

As with 1,25(OH)$_2$D$_3$-dependent activity, ligand-independent actions of the VDR are likely to occur at the level of transcription. Given the complexity associated with mechanisms related to PTH upregulation and the potential for this hormone to impact the skeleton separate from the VDR, we focused our search for additional 1,25(OH)$_2$D$_3$-independent actions at genes that were expressed in the intestine in our mouse strains and not influenced by PTH. Accordingly, we chose genes such as *Trpv6* and *S100g* (calbindin D9k) that represent direct targets of vitamin D action and that are involved in calcium absorption. As can be seen in Fig. 2, residual expression of both genes was highest in normal wildtype mice and in mice that expressed the wildtype hVDR transgene in a mouse VDR null background. In contrast, the expression of these genes in mice producing various levels of the L233S mutant VDR were significantly depressed. It is interesting that the highest level of expression of the L233S mutant in the T807/VDR$^{-/-}$ strain was capable of the greatest suppression of basal *Trpv6* and *S100g* expression and while the level of suppression in the two strains with lower level of VDR expression (T806/VDR$^{-/-}$ and T805/VDR$^{-/-}$) was less relative to the two control mouse strains, it was still highly significant. This observation is consistent with our earlier finding that while lower levels of VDR protein were also able rescue the alopecia seen in this VDR null rescue strain (T805/VDR$^{-/-}$), it was clear that the skin phenotype of the T805/VDR$^{-/-}$ strain was not entirely normal [25]. This finding directly at the level of gene expression in the intestine, a tissue unaffected by high circulating levels of PTH, suggests that in the absence of 1,25(OH)$_2$D$_3$-activation, the VDR is indeed capable of suppressing the expression of genes which are in turn induced in the presence of the hormone. Importantly, the recent study of VDR knock-in mutant mice also revealed a reduction in basal expression of *Trpv6*, *Cyp24a1* and *Slc30a10* in the intestine [27].

We have recently identified a gene network in the intestine of *Cyp27b1* null mice that is regulated by 1,25(OH)$_2$D$_3$ and plays a role in the active uptake of duodenal calcium [3]. To confirm and extend the results obtained in the VDR null mouse strain expressing the human L233S VDR mutant, we contrasted in a final study the basal expression of not only *Trpv6* and *S100*g but other newly discovered target genes as well in *Cyp27b1* null mice with that of VDR null mice maintained on a normal calcium and phosphate diet. As documented in Fig. 3, although *Trpv6*, *S100g*, *Atp2b1* and *Slc30a10* were all significantly lower in *Cyp27b1* null mice vs VDR null mice, other genes such as *Slc37a1*, *Lrrc26*, *Pgap1* and *Mctp2* were not repressed. These data combined with VDR protein-dependent suppression seen in the L233S mutant strains suggest that the VDR is indeed capable of the selective repression of target genes in the absence of ligand. This effect is consistent with the 1,25(OH)$_2$D$_3$-independent effects of the VDR in hair follicle cycling and could highlight a role for the VDR in the regulation of gene expression that is more extensive that currently appreciated. These data also suggest that the VDR may be similar to other nuclear receptors such as the

thyroid and retinoid receptors that are known to bind to DNA and to act as repressors of gene expression in the absence of their cognate ligands [28–32].

3.4. Advancing insights into 1,25(OH)$_2$D$_3$-independent actions of the VDR

Further studies to define the molecular mechanisms that enable the VDR to modulated genes in the absence of 1,25(OH)$_2$D$_3$ will be necessary. The first requirement will be to identify fully the sites of action of the VDR at genes whose expression can be suppressed by the VDR alone. Accordingly, we have recently performed genome-wide in vivo ChIP-seq analysis using intestine from vehicle- or 1,25(OH)$_2$D$_3$-treated wildtype mice [3]. This analysis has identified the presence of VDR binding sites at target genes such as *Trpv6* and *Cyp24a1* reported earlier [33–35] as well as near genes such *S100g*, *Atp2b1*, *Cldn2* and *Pdlim2*, for which sites were previously unknown. Regulatory sites were also identified for a broader arrays of genes that were regulated by 1,25(OH)$_2$D$_3$ as well. It is at these sites that the actions of the VDR will need to be examined. An important complexity of this regulation is the finding that most genes are controlled by multiple regulatory regions often located at sites that are highly remote relative to the genes' transcriptional start sites. Thus, an understanding of the interacting roles of these regulatory regions will also be necessary. Assuming that unliganded VDR functions at the level of DNA, a second requirement will be to identify sites on the genome to which the VDR is prebound prior to ligand activation. In this regard, recent genome-wide studies in both cells in culture and in mice in vivo suggest that while the majority of VDR DNA binding occurs in response to 1,25(OH)$_2$D$_3$, a smaller subset of binding sites on DNA are indeed pre-occupied by the VDR even in the absence of the hormone [9, 36–38]. The presence of the VDR at sites for which gene suppression has been noted will therefore be a likely prerequisite. Finally, we would anticipate that VDR-mediated suppression of gene expression will likely require the recruitment of coregulatory factors such as a histone deacetylase (HDAC) by the unliganded VDR that would serve to condense chromatin surrounding these gene loci thus restricting the expression of the gene of interest. Fully defining these principles will not be an easy task.

4. Conclusions

Although the VDR is a well-established critical mediator of the biological actions of 1,25(OH)$_2$D$_3$, the protein's actions in tissues in the absence of this ligand are not fully understood. Nevertheless, 1,25(OH)$_2$D$_3$-independent action of the VDR within the hair follicle to facilitate cycling is now fully accepted, although the molecular mechanisms integral to this process remain elusive. In this report, we present several distinct features of the mouse models in which the ability of VDR to regulate gene expression in response to 1,25(OH)$_2$D$_3$ is abrogated yet support a potential role of the VDR as a transcription regulator in the absence of the hormone in vivo.

Acknowledgments

Abbreviations

VDR	vitamin D receptor
1,25(OH)$_2$D$_3$	1,25-dihydroxyvitamin D$_3$
PTG	parathyroid gland
PTH	parathyroid hormone
RXR	retinoid X receptor
VDRE	vitamin D response element
ChIP-seq	ChIP-sequencing
RNA-seq	RNA-sequencing
HVDRR	hereditary 1,25-dihydroxyvitamin D resistant rickets
VDDR-1	vitamin D dependency rickets type 1
BAC	bacterial artificial chromosome
qPCR	quantitative polymerase chain reaction

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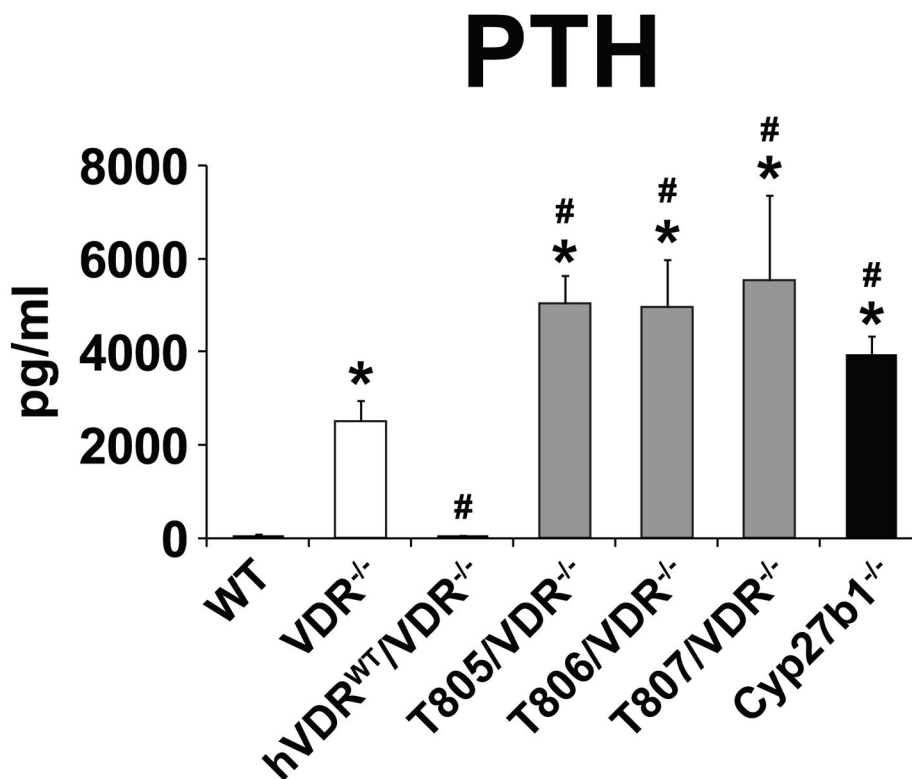
PTH

Fig. 1.
Analysis of serum PTH levels. PTH levels were assessed in EDTA-plasma obtained from wildtype (WT), VDR null mice (VDR$^{-/-}$; white bar), wildtype humanized VDR mice (hVDR^{WT}/VDR$^{-/-}$), 3 mutant humanized VDR mice (T805/VDR$^{-/-}$, T806/VDR$^{-/-}$ and T807/VDR$^{-/-}$; gray bars) and *Cyp27b1* null mice (Cyp27b1$^{-/-}$; black bar) as indicated. Data are presented as the mean ± SEM (n=5–7 per strain). *$p < 0.05$ compared with wildtype mice, #$p < 0.05$ compared with VDR null mice.
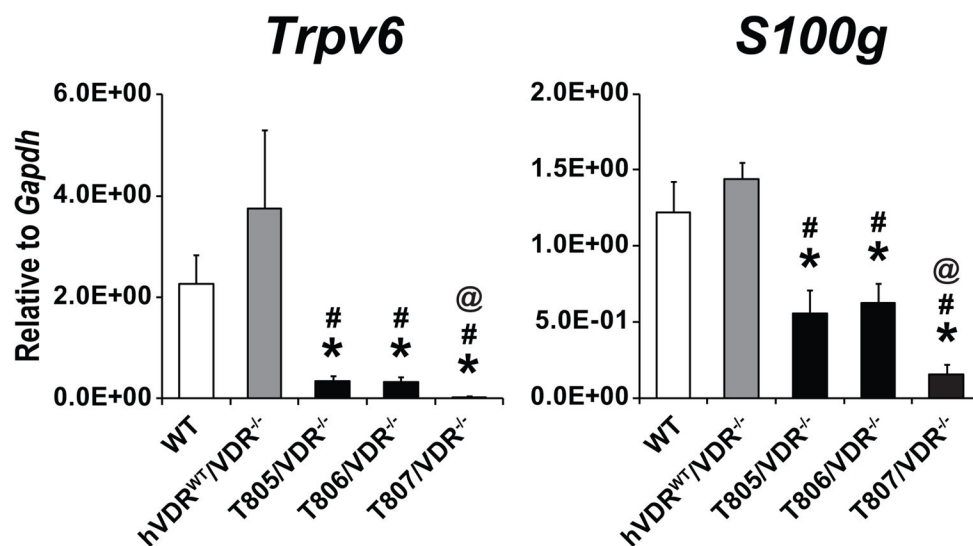
Fig. 2.
Basal expression of VDR target genes in duodenum of humanized VDR null mice.
Expression of the indicated VDR target genes was measured in duodenum of wildtype (WT;
white bars), wildtype humanized VDR mice (hVDR^{WT}/VDR$^{-/-}$; gray bars) and 3 mutant
humanized VDR mice (T805/VDR$^{-/-}$, T806/VDR$^{-/-}$ and T807/VDR$^{-/-}$; black bars) by
qPCR. Expression levels of the gene transcripts were normalized to *Gapdh* and presented as
the mean ± SEM (5–7 mice per strain). *$p < 0.05$ compared with wildtype mice, #$p < 0.05$
compared with wildtype humanized VDR mice, @$p < 0.05$ compared with T805/VDR$^{-/-}$
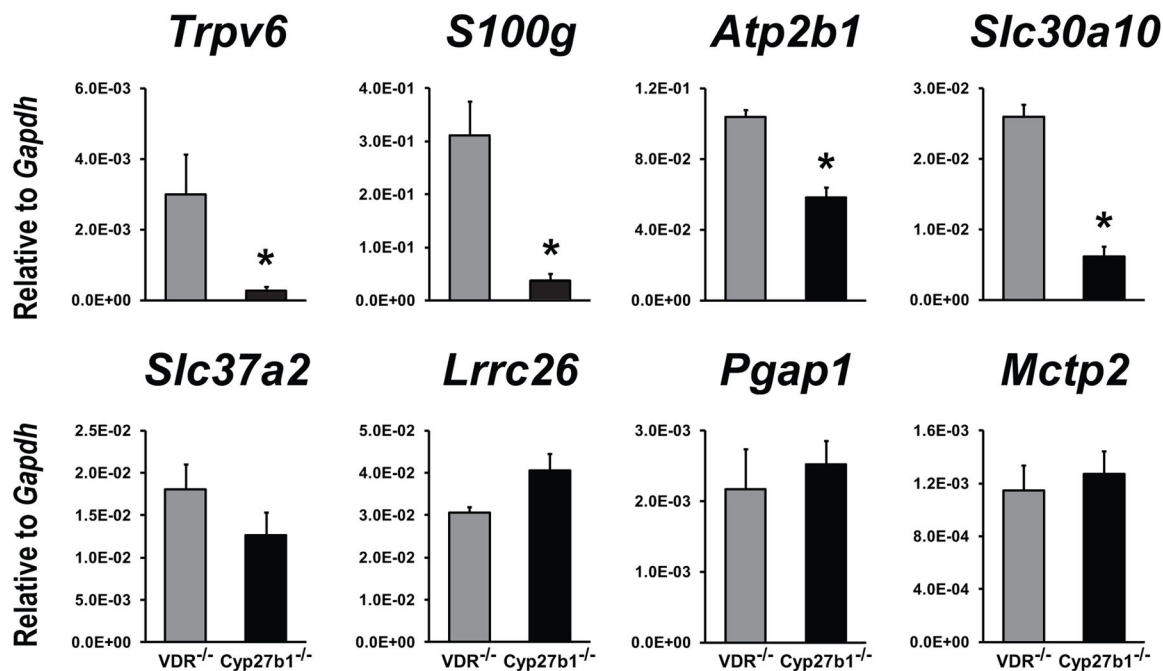and T806/VDR$^{-/-}$ mice.

Fig. 3.
Basal expression of VDR target genes in duodenum of *Cyp27b1* null mice. Expression of the indicated VDR target genes was measured in duodenum of VDR null mice (VDR$^{-/-}$; gray bars) and *Cyp27b1* null mice (Cyp27b1$^{-/-}$; black bars) by qPCR. Expression levels of the gene transcripts were normalized to *Gapdh* and presented as the mean ± SEM (5–7 mice per strain). *$p < 0.05$ compared with VDR null mice.