RESEARCH ARTICLE

Generation of Genetically Modified Rats Using CRISPR/Cas9 Genome-Editing System to Reveal Novel Vitamin D Actions

Authors

Miyu Nishikawa[1], Kaori Yasuda[2], Hiroki Mano[2], Shinichi Ikushiro[1], and Toshiyuki Sakaki[2]

Affiliations

1. Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama, 939-0398, Japan
2. Department of Pharmaceutical Engineering, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan

Address correspondence to:

Toshiyuki Sakaki

5180 Kurokawa, Imizu, Toyama 939-0398, Japan.

Fax: +81-766-56-2498

E-mail: tsakaki@pu-toyama.ac.jp

Abstract

We previously revealed that the anti-proliferative activity of 25-hydroxyvitamin D_3 (25(OH)D_3) in human prostate PZ-HPV-7 cells depends on the direct action of 25(OH)D_3 through the vitamin D receptor (VDR). We then attempted to confirm the direct action of 25(OH)D_3 *in vivo* using *Cyp27b1* knockout (KO) mice. Daily administration of 25(OH)D3 at 250 μg/kg bw/day rescued the rachitic conditions in *Cyp27b1* KO mice. The plasma levels of calcium, phosphorus, and parathyroid hormonea as well as the bone mineral density and female sexual cycle were normalized via the administration of 25(OH)D_3. These results strongly suggest the direct action of 25(OH)D_3. However, to our surprise, normal levels of 1α,25(OH)$_2D_3$ were detected in the plasma of *Cyp27b1* KO mice, probably due to Cyp27a1, which has a weak 1α-hydroxylation activity toward 25(OH)D_3.

Next, we generated a novel *in vivo* system using genetically modified (GM) rats deficient in the *Cyp27b1* or *Vdr* gene to reveal the molecular mechanisms of vitamin D action. Human type II rickets model rats with mutant *Vdr* (R270L), which recognizes 1,25(OH)$_2D_3$ with an affinity equivalent to that of 25(OH)D_3, were also generated. *Cyp27b1*-knockout (KO), *Vdr*-KO, and *Vdr* (R270L) rats showed symptoms of rickets, including growth retardation and abnormal bone formation. Among these model animals, *Cyp27b1*-KO rats had notably low levels of calcium in the blood and the most severe growth retardation, while *Vdr*-KO rats showed abnormal skin formation and alopecia. Administration of 25(OH)D_3 restored rickets symptoms in *Cyp27b1*-KO and *Vdr* (R270L) rats. As shown in *Cyp27b1*-KO mice, 1,25(OH)$_2D_3$ was also synthesized in *Cyp27b1*-KO rats. In contrast, the effects of 25(OH)D_3 on *Vdr* (R270L) rats strongly suggest that 25(OH)D_3 exerts a direct action via VDR-genomic pathways. These results suggest that our novel *in vivo* system containing three different types of GM rats is useful for elucidating the molecular mechanism of vitamin D action.

Key words: vitamin D, vitamin D receptor, genome editing, CYP27B1, rickets, genome editing

Introduction

Vitamin D exerts various biological activities, including calcemic, osteogenic, and anti-cancer activities, along with the generation of immune responses [1]. Vitamin D_3 is an animal vitamin D form obtained from diet via intestinal absorption or through biosynthesis from 7-dehydrocholesterol by UV irradiation of the skin. Vitamin D_3 is sequentially converted to biologically active 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2D_3$) in the body. Moreover, 25-hydroxyvitamin D_3 (25(OH)D_3), which is converted from vitamin D3 via the hydroxylation of C-25 by CYP2R1 and CYP27A1 in the liver, circulates through the body by binding to vitamin D-binding protein (DBP). DBP-bound 25(OH)D_3 is taken into the proximal tubular cells in the kidney through megalin-mediated endocytosis of DBP, and then converted to 1,25(OH)$_2D_3$ via 1α-hydroxylation by CYP27B1, which is a key enzyme in the production of 1,25(OH)$_2D_3$ [2] (Fig. 1). Furthermore, 1,25(OH)$_2D_3$ is a ligand with a high affinity for the vitamin D receptor (VDR), whose binding affinity is approximately 1000-fold higher than that of 25(OH)D_3. The transcriptional activity of

$1,25(OH)_2D_3$ by binding to VDR is a classic molecular mechanism of vitamin D, which is observed in tissues highly expressing VDR, such as the intestine, kidneys, and bones. Once $1,25(OH)_2D_3$ binds to VDR, it forms a heterodimer with 9-cis retinoid X receptor (RXR) to regulate the expression of target genes by binding to the vitamin D-responsive element (VDRE) in the promoter region of target genes involved in calcium homeostasis and osteogenesis [3]. This transcriptional activity is also recognized as a VDR-dependent genomic action of $1,25(OH)_2D_3$. Dysfunction of the genomic action of $1,25(OH)_2D_3$ causes typical bone disorders, such as osteomalacia and rickets associated with hypocalcemia and hyperparathyroidism. Recently, novel molecular mechanisms of vitamin D have been reported. Hence, there may be five types of molecular actions of vitamin D: (1)VDR-dependent genomic or non-genomic effects of $1,25(OH)_2D_3$ (VDR-$1,25(OH)_2D_3$) [4, 5], (2) VDR-independent effects of $1,25(OH)_2D_3$ (non VDR-$1,25(OH)_2D_3$) [6], (3) VDR-dependent effects of $25(OH)D_3$ (VDR-$25(OH)D_3$) [7, 8], (4) VDR-independent effects of $25(OH)D_3$ (non VDR-$25(OH)D_3$) [9], and (5) ligand-independent effects of VDR (VDR-no ligand) [10, 11] (Fig. 1 and Table 1). The elucidation of these vitamin D actions and biological activities is useful for the discovery of new therapeutic targets, because the classical vitamin D action (VDR-dependent genomic action of $1,25(OH)_2D_3$) has a high potency of exhibiting calcemic effect, which often causes side effects, such as hypercalcemia.

To reveal the molecular mechanisms underlying the action of vitamin D, it is important to identify the active form of vitamin D_3. A wide variety of vitamin D metabolites are present in the body to tightly regulate the concentration of $1,25(OH)_2D_3$ in the plasma. For example, $25(OH)D_3$ and $1,25(OH)_2D_3$ are also sequentially converted to biologically inactive metabolites by CYP24A1, whose conversion starts with C-23 and C-24 hydroxylation, respectively (Fig. 1). In the current review, we introduce new molecular mechanisms of action of vitamin D, which is revealed through a detailed analysis of vitamin D metabolism using cultured cells and genetically modified (GM) rickets model animals.

1. Direct biological action of $25(OH)D_3$ via VDR in PZ-HPV-7 cells.

Recent reports have demonstrated that $25(OH)D_3$ can regulate gene expression by directly binding to the VDR [12-15]. We have demonstrated that $25(OH)D_3$ is a potential VDR ligand in immortalized human prostate PZ-HPV-7 cells [7]. Whereas the affinity of $25(OH)D_3$ toward VDR is less than 100-fold lower than that of $1,25(OH)_2D_3$ [16], the plasma concentration of $25(OH)D_3$ is several hundred-fold higher than that of $1,25(OH)_2D_3$ in the DBP-bound form. Based on its K_d value for the VDR and the plasma concentration of $25(OH)D_3$, these biological and biochemical findings suggested that $25(OH)D_3$ could be a physiologically important agonist of VDR. We revealed that transcellular localization of VDR was induced by 100 nM $25(OH)D_3$ or 1 nM $1,25(OH)_2D_3$ in PZ-HPV-7 cells, both of which are physiological conditions of vitamin D [7]. We further examined the effect of vitamin D on cell growth because previous studies have shown that $1,25(OH)_2D_3$ has cell growth-inhibitory activity mediated by VDR

[17]. In agreement with VDR trans-localization, 1 and 10 nM $1,25(OH)_2D_3$ significantly inhibited cell growth. Similarly, 10 and 100 nM $25(OH)D_3$ exerted inhibitory effects on cell growth. We measured the amount of $1,25(OH)_2D_3$ synthesized from 100nM $25(OH)D_3$ in the PZ-HPV-7 cells, and confirmed that cell-derived $1,25(OH)_2D_3$ did not trigger the trans-localization of VDR. We further confirmed that the inhibition of cell growth was not inhibited by CYP27B1 knockdown by siRNA, strongly suggesting that $25(OH)D_3$ acts as a potential VDR ligand in PZ-HPV-7 cells [7].

2. Cyp27b1-independent $1,25(OH)_2D_3$ production in *Cyp27b1*-KO mice.

To confirm the direct action of $25(OH)D_3$ *in vivo*, we examined its effect on osteogenesis in *Cyp27b1* knockout (KO) mice [18]. *Cyp27b1-KO* mice have no detectable $1,25(OH)_2D_3$ in the plasma and exhibit all the hallmarks of vitamin D-dependent rickets (VDDR) type I, such as lower bone mineral density and hypocalcemia. $25(OH)D_3$ administration at 150 $\mu g \cdot kg^{-1} \cdot day^{-1}$ normalized bone and calcium phenotypes in *Cyp27b1-KO* mice. Unexpectedly, a normal level of $1,25(OH)_2D_3$ was detected in *Cyp27b1-KO* mice administered $25(OH)D_3$ [18]. We previously reported that CYP27A1 has a weak 1α-hydroxylation activity toward $25(OH)D_3$, which is a liver mitochondrial enzyme involved in 25-hydroxylation of vitamin D, and has 40% homology in amino acid sequence compared to CYP27B1 [19]. Based on the activity of 1α-hydroxylation towards $25(OH)D_3$ in liver mitochondrial fractions prepared from *Cyp27b1-KO* mice, we assumed that Cyp27a1 compensatory converted $25(OH)D_3$ to $1,25(OH)_2D_3$ [18]. Thus, high doses of $25(OH)D_3$ promote compensatory production of $1,25(OH)_2D_3$ by liver Cyp27a1 in *Cyp27b1*-KO mice, suggesting that $25(OH)D_3$ treatment would be useful for patients with CYP27B1-disfunction, including VDDR type I rickets and chronic kidney disease. However, we failed to demonstrate the direct action of $25(OH)D_3$ in *Cyp27b1*-KO mice.

3. Elucidation of direct action of $25(OH)D_3$ via Vdr in *Vdr* (R270L) mutant rats.

We hypothesized that the multiple deletion of CYP27B1 and CYP27A1 also fails to demonstrate the direct action of $25(OH)D_3$ via VDR because CYPs form a gene superfamily and their substrate specificity often overlaps with others. Thus, we focused on mutated VDR derived from VDDR type II patients. Furthermore, we selected a rat model to generate GM animals because pharmacokinetic analysis of vitamin D metabolites using liquid chromatography/mass spectrometry (LC/MS) often requires a large volume of blood samples. To elucidate the new biological actions of vitamin D, including the direct action of $25(OH)D_3$ via VDR, we used *Cyp27b1*-KO, *Vdr*(R270L)-knocked in, and *Vdr*-KO rats generated by the clustered regularly interspaced palindromic repeat/caspase 9 (CRISPR/Cas9) genome editing system.

Rat Vdr mutation, R270L, which is an ortholog of human VDR mutation, R274L, isolated from the VDDR type II rickets patient [20-22], exerts significantly reduced affinity towards $1,25(OH)_2D_3$ as Arg270 and Arg274

in rats and human VDR play pivotal roles in hydrogen bond formation with 1α hydroxy group of $1,25(OH)_2D_3$. The affinity of these mutant VDRs toward $1,25(OH)_2D_3$ is approximately 1000-fold lower than that of the wild-type [22], which has an equivalent affinity toward $25(OH)D_3$. The patients with VDR (R274L) were hardly responsible for $1,25(OH)_2D_3$. Hence, there is no ligand with high affinity for the mutated Vdr in *Vdr*(R270L) rats, which enables us to evaluate the direct action of exogenous $25(OH)D_3$ via Vdr(R270L) because the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ is almost negligible for VDR-mediated action in *Vdr*(R270L) rats. In fact, bone disorders associated with hypocalcemia and subsequent hyperparathyroidism were observed in *Vdr*(R270L) rats, while the plasma levels of $1,25(OH)_2D_3$ were significantly elevated after weaning, indicating that $1,25(OH)_2D_3$ could not act as a high-affinity ligand of Vdr(R270L) [23].

Table1. vitamin D signals including canonical (VDR-$1,25(OH)_2D_3$) and non-canonical actions [23].

GM strain	Mode of actions				
	(1) VDR-$1,25(OH)_2D_3$	(2) non VDR-$1,25(OH)_2D_3$	(3) VDR-$25(OH)D_3$	(4) non VDR-$25(OH)D_3$	(5) VDR-no ligand
WT	Yes	Yes	Yes	Yes	Yes
Vdr(R270L)	No	Yes	Yes	Yes	Yes
Cyp27b1-KO	No	No	Yes	Yes	Yes
Vdr-KO	No	Yes	No	Yes	No

We compared the phenotypes of GM rats to determine the biological effects of different vitamin D actions [23]. As these GM strains lack different vitamin D actions, comparative analysis might reveal the biological effect of each vitamin D action (Fig. 1 and Table1). For example, as *Vdr*(R270L) rats are deficient in Vdr-dependent action of $1,25(OH)_2D_3$, abnormal phenotypes of *Vdr*(R270L) compared to wild type would be the effect of Vdr-dependent action of $1,25(OH)_2D_3$. Similarly, the effect of Vdr-independent action of $1,25(OH)_2D_3$ was revealed by comparison with *Cyp27b1*-KO and *Vdr*-KO rats. All GM rats showed rickets symptoms, including bone disorders associated with hypocalcemia. Among these GM rats, *Cyp27b1*-KO rats showed severe rickets symptoms, including notably low levels of calcium in the blood and the most severe growth retardation, suggesting that Vdr-independent $1,25(OH)_2D_3$ action is also involved in these phenotypes in addition to Vdr-dependent action of $1,25(OH)_2D_3$. In contrast, *Vdr*-KO rats showed abnormal skin formation and alopecia, whereas skin phenotypes *Vdr*(R270L) rats were almost normal, suggesting that the Vdr-dependent effect of $25(OH)D_3$ or the ligand-independent effect of Vdr might be involved in these skin phenotypes [23]. Thus, these GM rats are useful for revealing the molecular mechanisms of vitamin D, including novel and conventional signaling, which lead to novel molecular targets for vitamin D-related diseases (Table 1).

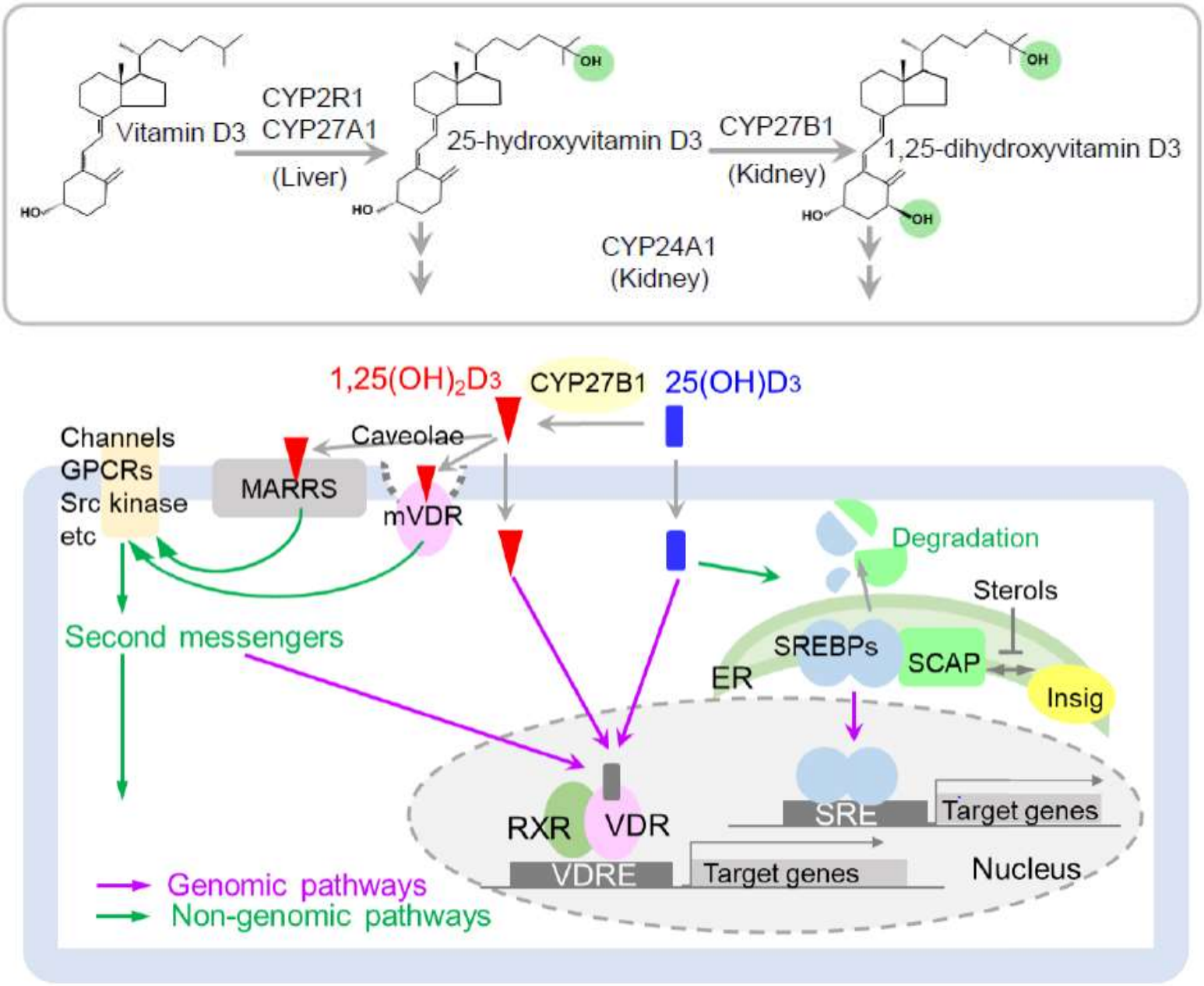

Fig.1. Metabolism and putative molecular mechanisms of vitamin D_3

Vitamin D_3 is converted into 25(OH)D_3 by CYP2R1 and CYP27A1 in the liver, and then further convertted into 1,25$(OH)_2D_3$ by CYP27B1 in the kidney. The resultant 1,25$(OH)_2D_3$ is inactivated by CYP24A1 in the kidney

Purple and green arrows indicate genomic and non-genomic pathway, respectively.

GPCRs, G protein-coupled receptor; MARRS, membrane associated, rapid response steroid-binding receptor; VDR, vitamin D receptor; mVDR, membrane-bound vitamin D receptor; RXR, retinoid X receptor; VDRE, vitamin D response element; ER, endoplasmic reticulum; SREBPs, sterol regulatory element–binding proteins; SCAP, SREBP cleavage activating protein; SRE, sterol regulatory element.

Finally, we treated *Cyp27b1*-KO (VDDR type I model) and *Vdr*(R270L) (VDDR type II model) rats with 25(OH)D_3 at 200 μg・kg^{-1}・day^{-1} after weaning to evaluate the biological activities of 25(OH)D_3 [23]. 25(OH)D_3 treatment fully normalized their rickets phenotypes, including hypocalcemia and bone disorders. As expected, plasma 1,25$(OH)_2D_3$ deficiency in *Cyp27b1*-KO rats was normalized by 25(OH)D_3 in accordance with a study using *Cyp27b1*-KO mice [23]. In contrast, 25(OH)D_3 decreased plasma 1,25$(OH)_2D_3$ to the normal level in *Vdr*(R270L) rats, whose plasma 1,25$(OH)_2D_3$ levels were significantly elevated in the absence of 25(OH)D_3 treatment. These results suggest the following: (1) In Cyp27b1 deficient conditions, high-dose treatment of 25(OH)D_3 is useful as a source for extrarenal 1,25$(OH)_2D_3$ synthesis, and hepatic Cyp27a1 might be involved in 1α–hydroxylation of 25(OH)D_3 under these conditions. (2) 25(OH)D_3 itself acts as a ligand for Vdr(R270L) at a high dose of 25(OH)D_3 [23] (Fig. 2). 1,25$(OH)_2D_3$ is clinically used for treatment of rickets, even in VDDR type II patients who are hardly responsible for 1,25$(OH)_2D_3$. Our findings demonstrated the usefulness of 25(OH)D_3 as a wide-safety-region compound for CYP27B1 deficient or VDR(R274L).

In conclusion, we demonstrated novel vitamin D actions involved in calcium homeostasis and osteogenesis by a comparative approach using GM rats deficient in different vitamin D signals. Our novel *in vivo* system is useful for elucidating the molecular mechanism of vitamin D action.

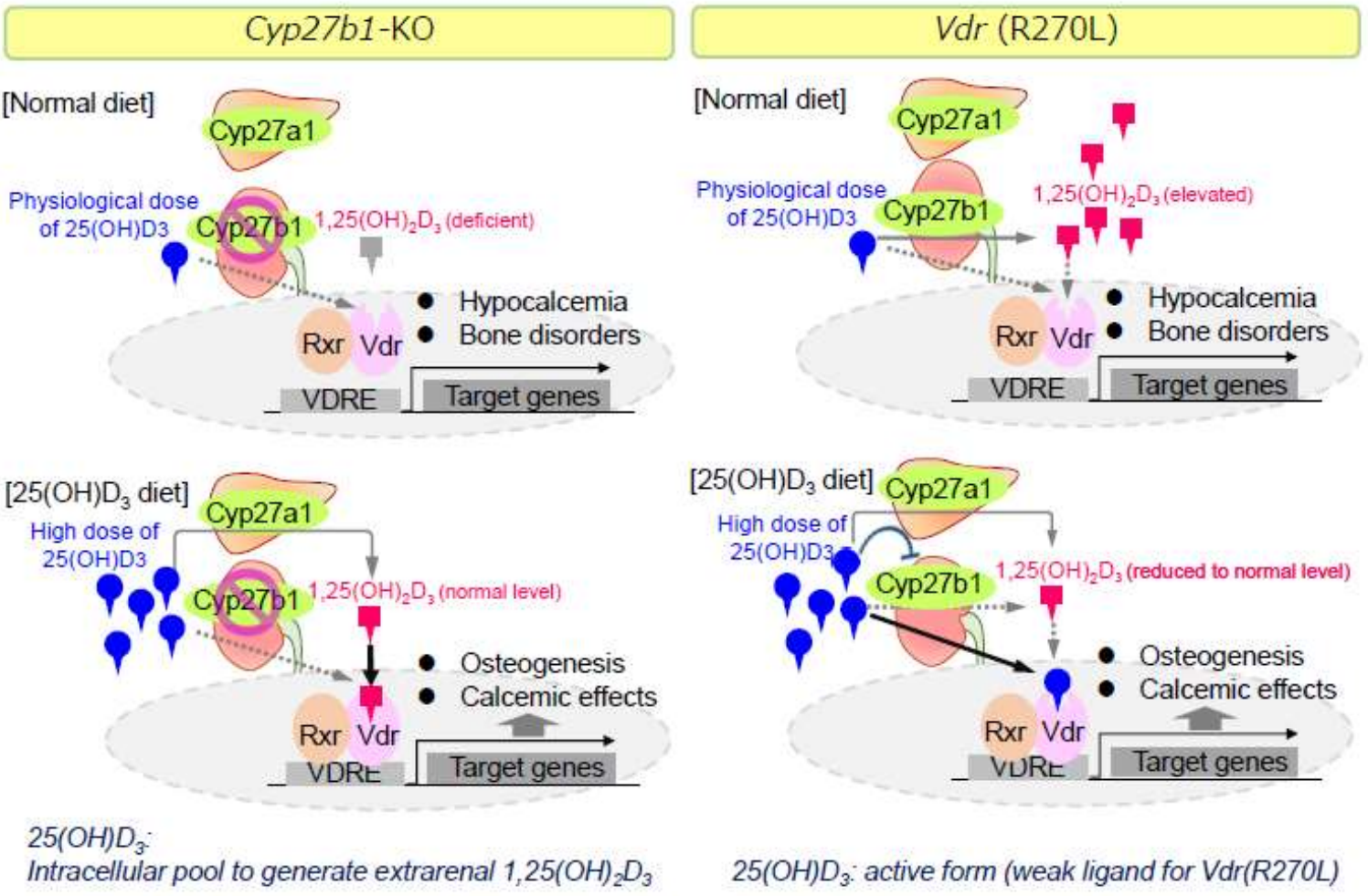

Fig.2. Biological roles of 25(OH)D$_3$ in *Cyp27b1*-KO and *Vdr*(R270L) rats [23]
In the case of *Cyp27b1*-KO rats fed a normal diet, plasma 1,25(OH)$_2$D$_3$ level was dramatically reduced to result in hypocalcemia and bone disorders.
In the case of *Cyp27b1*-KO rats fed a 25(OH)D$_3$ containing diet, plasma 1,25(OH)$_2$D$_3$ level was increased to a normal level probably by Cyp27a1 to result in normal plasma

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