



Functions of vitamin D in bone

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Abstract

Vitamin D, synthesized in the skin or absorbed from the diet, undergoes multi-step enzymatic conversion to its active form, 1,25-dihydroxy vitamin D [1,25(OH)₂D], followed by interaction with the vitamin D receptor (VDR), to modulate target gene expression. Loss-of function mutations in the genes encoding the enzymes regulating these processes, or in the VDR, result in human diseases, which have demonstrated the paramount role of 1,25(OH)₂D in mineral and skeletal homeostasis. Mouse genetics has been used to create disease phenocopies which have produced considerable insight into the mechanisms of 1,25(OH)₂D regulation of mineral and skeletal metabolism. Hypophosphatemia resulting from 1,25(OH)₂D deficiency or resistance can inhibit apoptosis in hypertrophic chondrocytes leading to abnormal development of the cartilaginous growth plate in rickets. Decreased 1,25(OH)₂D may also cause decreased vascular invasion associated with reduced chondroclast and osteoclast activity and thereby contribute to growth plate abnormalities. Reduced 1,25(OH)₂D-mediated intestinal and renal calcium transport can reduce calcium availability, increase parathyroid hormone secretion and phosphaturia, and impair mineral availability for normal matrix mineralization, resulting in reduced growth plate mineralization and osteomalacia. 1,25(OH)₂D may exert an anabolic effect in bone, apparently via the VDR in mature osteoblasts, by increasing osteoblast activity and reducing osteoclast activity. High ambient levels of exogenous 1,25(OH)₂D, or of elevated endogenous 1,25(OH)₂D in the presence of reduced calcium balance, can enhance bone resorption, and apparently prevent mineral deposition in bone. These actions demonstrate the critical role of vitamin D in regulating skeletal homeostasis both indirectly and directly via the 1,25(OH)₂D/VDR system.

Keywords Vitamin D · Mineral homeostasis · Cartilaginous growth plate · Bone formation · Bone resorption

Introduction

Vitamin D₃ (cholecalciferol) may be synthesized endogenously in the skin via ultraviolet irradiation of a precursor, 7-dehydrocholesterol, which can be photoconverted to previtamin D₃ and then to vitamin D₃, which enters the bloodstream. Alternatively, vitamin D may be absorbed from the diet, in the gut, as vitamin D₃ or as vitamin D₂ (ergocalciferol) and can then enter the bloodstream. Vitamin D (D₂ or D₃), from either endogenous or exogenous

sources, circulates in the blood bound to vitamin D binding protein (DBP) and is converted in the liver, mainly via, a cytochrome P450 mixed-function oxidase (CYP) with 25-hydroxylase activity, termed CYP2R1, to 25-hydroxyvitamin D (25OHD). 25OHD, bound to DBP, is then transported to the kidney where it can be 1 α -hydroxylated by CYP27B1, a mitochondrial CYP with 1-hydroxylase (1OHase) activity, to produce the bioactive hormonal moiety, 1,25-dihydroxyvitamin D [1,25(OH)₂D]. Alternatively, 25OHD may be 24-hydroxylated in the kidney by CYP24A1, a 24-hydroxylase (24OHase), to 24,25-dihydroxyvitamin D [24,25(OH)₂D], which is the first step in the eventual degradation of vitamin D to calcitroic acid. 1,25(OH)₂D may also be converted to 1,24,25 by the 24OHase (Bikle et al. 2014). Both the renal 1OHase and the renal 24OHase are tightly regulated by hormones and ions. Extra-renal 1OHase and 24OHase activity have also been reported in a number of tissues; however, the most important site of synthesis and

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degradation of the active form of vitamin D, $1,25(\text{OH})_2\text{D}$ is the kidney.

The biological action of $1,25(\text{OH})_2\text{D}$ is mediated by the vitamin D receptor (VDR), a member of the steroid receptor family (Pike and Christakos 2017). The VDR contains a DNA-binding domain (DBD) and a ligand-binding domain (LBD). $1,25(\text{OH})_2\text{D}$ binding to VDR induces a conformational change that facilitates interaction with the retinoid X receptor (RXR) and coregulatory complexes, and binding to specific DNA sequences [vitamin D response elements (VDREs)], thus resulting in the transcription of target genes. The actions of $1,25(\text{OH})_2\text{D}$ involve regulation of gene activity at a range of locations, often many kilobases from the transcription start site of target genes.

Loss-of-function mutations in the VDR (Malloy et al. 1999, 2006) or overexpression of a protein that binds to the VDRE and prevents interaction with the VDR (Chen et al. 2003) are both characterized by resistance to the action of $1,25(\text{OH})_2\text{D}$, and result in the recessive disorders Hereditary Vitamin D-Resistant Rickets (HVDRR) A and B [also called Vitamin D-Dependent Rickets, type 2A (VDDR2A) and VDDR2B, respectively]. A loss-of-function mutation in CYP27B1 is characterized by the inability to synthesize $1,25(\text{OH})_2\text{D}$, and results in the autosomal recessive disease Pseudodeficiency Rickets [also called Vitamin D-Dependent Rickets, type 1A (VDDR1A)] (Fu et al. 1997). Loss-of-function mutations in CYP2R1, characterized by decreased ability to synthesize 25OHD (Thacher et al. 2015), cause the recessive disorder Vitamin D-Dependent Rickets, type 1B (VDDR1B). Loss-of-function mutations of CYP24A1, characterized by impaired clearance of $1,25(\text{OH})_2\text{D}$ (Schlingmann et al. 2011; Streeten et al. 2011), result in the disorder idiopathic infantile hypercalcemia (IIH). Targeted gene deletion in mice has produced phenocopies of these disorders (Yoshizawa et al. 1997; Li et al. 1998; Panda et al. 2001; Dardenne et al. 2001; St-Arnaud et al. 2000; Zhu et al. 2013) and shed considerable insight into vitamin D biology. What is striking about all these disorders is that they are all characterized most strikingly by abnormalities in mineral and skeletal homeostasis. Thus, although vitamin D has been implicated in many pleiotropic biological effects, these genetic disorders emphasize the importance of the mineral and bone actions of vitamin D irrespective of any other biological consequences.

Vitamin D and mineral homeostasis

A primary function of vitamin D, in the postnatal state, is to maintain calcium homeostasis by increasing intestinal absorption of calcium. This is especially critical in hypocalcemic situations (Fig. 1). Although much of this action occurs in the duodenum, distal segments of the intestine, in

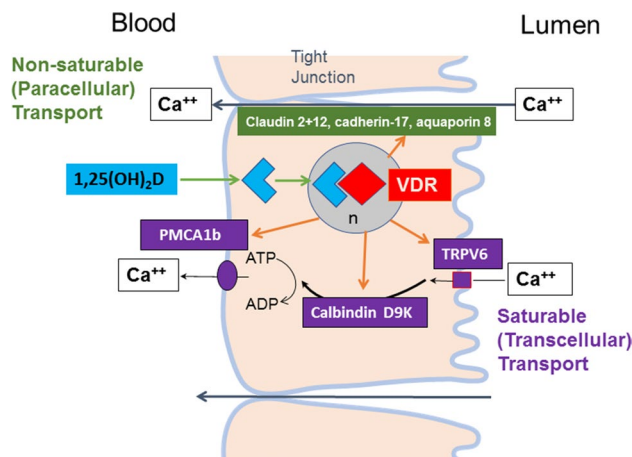


Fig. 1 $1,25(\text{OH})_2\text{D}$ /VDR-mediated calcium (Ca^{++}) transport in intestinal epithelium. $1,25(\text{OH})_2\text{D}$ can stimulate saturable (transcellular) transport in the proximal small intestine. $1,25(\text{OH})_2\text{D}$ enters the cell, binds to VDR in the nucleus (n), and increases gene transcription of the apical membrane Ca^{++} channel TRPV6, resulting in increased Ca^{++} uptake from the intestinal lumen into the cell. The $1,25(\text{OH})_2\text{D}$ /VDR system increases gene expression of calbindin 9 k, which can then shuttle Ca^{++} from the apical membrane to the basolateral membrane. The $1,25(\text{OH})_2\text{D}$ /VDR system can also upregulate the basolateral plasma membrane protein PMCA1b, facilitating Ca^{++} extrusion from the intestinal cell into the blood via an ATP-dependent process. $1,25(\text{OH})_2\text{D}$ /VDR signaling can also increase gene expression of the tight junction proteins claudin 2 and 12, cadherin-17, and aquaporin 8, and in this way facilitate passive, diffusional, and paracellular Ca^{++} fluxes across the intestine, particularly in the jejunum and ileum

addition to the duodenum, appear to be targets of vitamin D action. Thus, when dietary calcium is low, $1,25(\text{OH})_2\text{D}$ stimulates transcellular intestinal calcium transport by increasing the expression of the apical membrane calcium channel TRPV6 (transient receptor potential vanilloid 6) and calcium-binding protein calbindin-D9k. The extrusion of calcium across the basolateral membrane is mediated by PMCA1b (plasma membrane calcium ATPase 1b). Other calcium transporters may also be involved. When calcium intake is high, paracellular calcium transport predominates, but this pathway may also be regulated by $1,25(\text{OH})_2\text{D}$. Thus, the paracellular-associated proteins including claudin-2 and claudin-12, which are transmembrane components of tight junctions, cadherin-17, a cell adhesion protein, and aquaporin 8, a tight junction channel, can also be regulated by $1,25(\text{OH})_2\text{D}$ (Fig. 1).

In the kidney, distal tubular calcium re-absorption involves an active transcellular mechanism in which calcium enters the distal tubule cell through TRPV5, calcium is transferred in the cytoplasm by binding to calbindin-D9k and calbindin-D28k, and is extruded on the apical surface by NCX1 (the sodium/calcium exchanger 1) and plasma membrane calcium pump 1b. The $1,25(\text{OH})_2\text{D}$ /VDR system is known to enhance gene expression of several calcium

transporters including Trpv5, calbindin-D9k, calbindin-D28k, and likely, NCX1 (Van Abel et al. 2003; Song et al. 2003).

The increased calcium absorbed from the intestine and reabsorbed from the kidney can facilitate skeletal mineralization, but can also activate the calcium sensing receptor in the parathyroid gland and inhibit release of parathyroid hormone (PTH), which is elevated in the presence of hypocalcemia, and results in secondary hyperparathyroidism. The elevated PTH can stimulate the renal 1(OH)ase to increase 1,25(OH)₂D when hypocalcemia prevails, whereas reduction of PTH after 1,25(OH)₂D-mediated increases in extracellular calcium, subsequently decreases 1,25(OH)₂D, achieving a new steady state (Fig. 2).

It has been suggested that 1,25(OH)₂D can also stimulate active phosphate absorption in the intestine; however, the mechanism involved is uncertain. In the kidney, elevated PTH can stimulate the internalization and lysosomal degradation of the sodium–phosphate cotransporters NPT2a and NPT2c in the proximal tubules (Bacic et al. 2006), thereby reducing proximal tubular phosphate re-absorption. Increased 1,25(OH)₂D can, therefore, indirectly enhance renal phosphate re-absorption by reducing the concentrations of circulating PTH. Nevertheless 1,25(OH)₂D can also enhance release of the phosphaturic factor, fibroblast growth

factor (FGF)23 from osteocytes in bone (Fig. 2) (Nguyen-Yamamoto et al. 2017), which can decrease NPT2a and NPT2c in the proximal tubules and increase expression of α Klotho expression in the distal tubule (Forster et al. 2011), factors that facilitate renal phosphate loss, and this could negate the effects occurring via PTH.

Effects of reduced vitamin D status on the cartilaginous growth plate

Until after weaning, mice with targeted deletion of VDR, i.e., VDR^{-/-} mice (which display vitamin D resistance), and with targeted deletion of 1 α (OH)ase, i.e., 1 α (OH)ase^{-/-} mice (which manifest vitamin D deficiency), are normal in length and morphology and the mineral content of the growth plates and long bones are normal. However, after weaning, long bone growth and features of rickets such as expansion of the epiphyseal growth plate are observed, due to widening and disorganization of the hypertrophic zone with impaired apoptosis of hypertrophic chondrocytes. Low serum phosphate levels due to the vitamin D resistance or deficiency appear to decrease apoptosis of hypertrophic chondrocytes via the caspase-9-mediated mitochondrial pathway (Donohue and Demay 2002; Sabbagh et al. 2005)

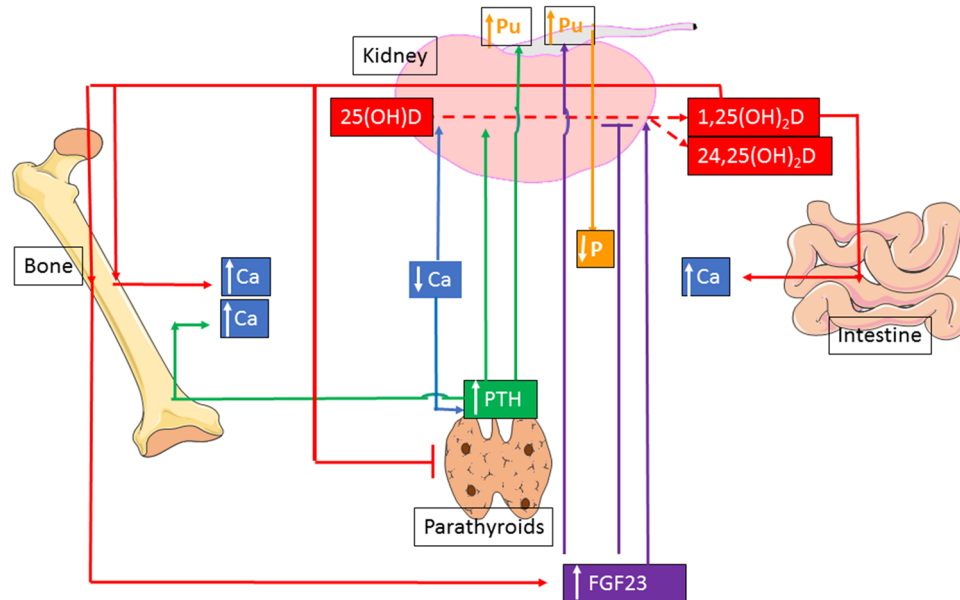


Fig. 2 Hormonal regulation of serum calcium (Ca) and phosphorus (P). A low serum calcium (↓Ca), due to negative calcium balance, can stimulate renal conversion of 25(OH)D to 1,25(OH)₂D, and can stimulate the release of PTH from the parathyroid glands. The increased PTH (↑PTH) can further stimulate 25OHD conversion to 1,25(OH)₂D and can promote increased urine phosphorus (↑Pu), which can result in decreased serum phosphate (↓P). PTH and 1,25(OH)₂D can also produce bone resorption to elevate serum calcium (↑Ca). The ele-

vated 1,25(OH)₂D can increase FGF23 release from bone, which can also enhance urine phosphorus and produce low serum phosphorus. The increased 1,25(OH)₂D can also enhance calcium absorption from intestine and elevate serum calcium. FGF23 will reduce conversion of 25OHD to 1,25(OH)₂D and increase 24,25(OH)₂D, thus further reducing the elevated 1,25(OH)₂D. The increased serum calcium will restore normocalcemia, thus reducing elevated 1,25(OH)₂D, which reduces increased FGF23, and inhibiting further PTH release

(Fig. 3), resulting in the widening and expansion of the growth plate. However, in addition, the production of chondroclasts and osteoclasts at the chondro-osseous junction may be defective due to decreased expression of receptor activator of nuclear factor κ B ligand (RANKL) and due to decreased vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 leading to decreased vascular invasion; this leads to diminished removal of hypertrophic chondrocytes (Fig. 3). The enlargement of the cartilaginous growth plate, particularly the hypertrophic zone, may, therefore, be at least in part due directly to reduced activity of the $1,25(\text{OH})_2\text{D}/\text{VDR}$ system, resulting in altered cartilage growth plate remodeling (Lin et al. 2002).

Effect of reduced vitamin D status on mineralization

Mineralization of bone is a well-regulated multiphasic process (van Driel et al. 2017) Initially, calcium is taken up, via calcium-binding acidic phospholipids and calcium-binding proteins (Anderson 1995, 2003) by nano-sized extracellular matrix vesicles (EVs) that bud from the cell membrane of osteoblasts. The phosphate concentration inside the vesicles is regulated by phosphohydrolases such as alkaline phosphatase that use inorganic pyrophosphate as substrate. When the solubility product of calcium and phosphate is exceeded, hydroxyapatite (HA) mineral deposits are formed inside the extracellular vesicles. Subsequently, the preformed HA crystals are deposited (Anderson 2003) outside these vesicles and propagate in the extracellular matrix with a resulting accumulation of mineral (Ali et al. 1970; Anderson 1995).

A large number of other proteins may also contribute to mineralization (Thouverey et al. 2011; Xiao et al. 2007; Staines et al. 2014) either by promoting or inhibiting this process. Thus, in addition to alkaline phosphatase, bone-specific phosphatase PHOSPHO1 may augment mineralization by accumulating phosphate within the extra-cellular vesicles (Roberts et al. 2007; Stewart et al. 2006; Yadav et al. 2011). Important inhibitors are pyrophosphatase phosphodiesterase 1 (NPP1) and ankylosis protein (ANK). NPP1 generates pyrophosphate (PPi) and the transmembrane protein ANK allows PPi to pass through the plasma membrane to the extracellular matrix, therefore, inhibiting HA formation in the extracellular vesicles (Kim et al. 2010; Millan 2013). Osteopontin (Opn) is a highly phosphorylated extra-cellular matrix sialoprotein that has strong mineral-binding capacities and that may also inhibit mineralization. Nevertheless, the effects of vitamin D deficiency on reduced mineralization of the cartilage in the growth plate in rickets and the reduced mineralization of the bone matrix in osteomalacia may be indirect. In $1\alpha(\text{OH})\text{ase}^{-/-}$ (Panda et al. 2004; Dardenne et al. 2003) and $\text{VDR}^{-/-}$ mice (Amling et al. 1999) maintained on a normal diet, hypocalcemia, hypophosphatemia, and secondary hyperparathyroidism are observed after weaning with typical histological features of rickets and osteomalacia, including, inadequate mineralization of cartilage, primary spongiosa, and cortical bone, and an increase in unmineralized osteoid in both trabecular and cortical bone. Treating these animals with a high calcium, high phosphate, high lactose “rescue” diet (lactose enhances intestinal absorption of these minerals) restored serum mineral and PTH levels to normal, and the growth plate mineralization was improved and osteoid was reduced. Similarly,

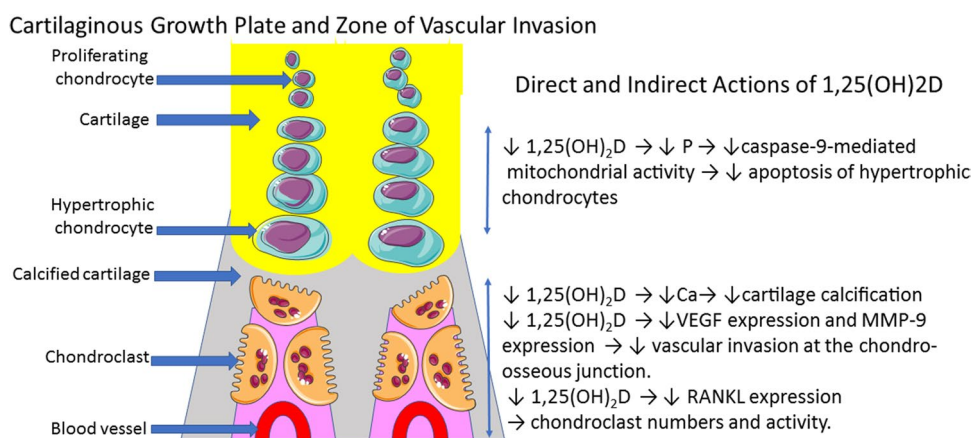


Fig. 3 Direct and indirect actions of $1,25(\text{OH})_2\text{D}$ on the growth plate. Reduced (\downarrow) $1,25(\text{OH})_2\text{D}$ can result in diminished extracellular fluid phosphorus which can decrease apoptosis of hypertrophic chondrocytes via reducing activity of the caspase-mediated mitochondrial pathway. Reduced $1,25(\text{OH})_2\text{D}$ can also reduce availability of calcium and diminish calcification of cartilage. Decreased $1,25(\text{OH})_2\text{D}$

can result in decreased VEGF and decrease vascular invasion and also decrease RANKL resulting in reduced number and activity of chondroclasts/osteoclasts. These mechanisms combine to produce the rachitic growth plate changes of decreased $1,25(\text{OH})_2\text{D}$ or resistance to $1,25(\text{OH})_2\text{D}$

in humans, improvement of rickets and osteomalacia can be obtained even in patients with HVDRR after administering calcium intravenously (al-Aqeel et al. 1993). Therefore, the rachitic and osteomalacic changes appear to be due to an indirect effect of the $1,25(\text{OH})_2\text{D}/\text{VDR}$ system, mediated by reduced extracellular mineral levels because of the absence of $1,25(\text{OH})_2\text{D}$ or VDR.

Osteomalacia has been reported in animal models where pronounced increases of $1,25(\text{OH})_2\text{D}$ have been induced, including mice with deletion of VDR from the intestine of mice which are profoundly hypocalcemic, (Lieben et al. 2012) and mice with deletion of the 24OHase enzyme which is responsible for $1,25(\text{OH})_2\text{D}$ clearance, and which are hypercalcemic (St-Arnaud et al. 2000). The very elevated $1,25(\text{OH})_2\text{D}$ levels in the former model were associated with increased expression of ectonucleotide pyrophosphatase phosphodiesterase (Enpp1 and Enpp3, which generate PPI from trinucleotides, and by increased levels of progressive ankylosis (Ank), which enhances the transport of PPI (Kim et al. 2010), thus increasing PPI levels and inhibiting mineralization (Millan 2013). $1,25(\text{OH})_2\text{D}_3$ also increased expression of osteopontin, another potent mineralization inhibitor. It was suggested that the $1,25(\text{OH})_2\text{D}_3$ -mediated inhibition of bone matrix mineralization may contribute to preserving normal serum calcium levels (Lieben et al. 2012) in situations of severe hypocalcemia.

Effect of the $1,25(\text{OH})_2\text{D}/\text{VDR}$ system on bone turnover

Bone undergoes continuous turnover or remodeling throughout life, with osteoblastic bone formation building bone (an anabolic effect) and osteoclastic bone resorption removing bone (a catabolic effect). These actions are generally coupled, although not necessarily balanced. Cells of the osteoblast lineage are derived from mesenchymal stem cells in the bone marrow and differentiate through osteoblasts through to mature osteoblasts and osteocytes. Osteoclasts are derived from hematogenous precursors and result from fusion of mononuclear cells of the monocyte/macrophage lineage.

Effect of the $1,25(\text{OH})_2\text{D}/\text{VDR}$ system on bone formation

In both $1\alpha(\text{OH})\text{ase}^{-/-}$ mice and $\text{VDR}^{-/-}$ mice that were hypocalcemic, hypophosphatemic, and exhibited secondary hyperparathyroidism, the numbers of osteoblasts, but not osteoclasts lining bone surfaces were increased and trabecular bone in the primary spongiosa was augmented, although the increased bone volume is largely due to increased unmineralized osteoid (Li et al. 1997;

Panda et al. 2004; Dardenne et al. 2001). This suggested an uncoupling of bone turnover in the presence of a defective $1,25(\text{OH})_2\text{D}/\text{VDR}$ system, even though PTH, which can augment osteoclasts, was elevated. When the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice were then treated with a rescue diet, and serum calcium, phosphorus, and PTH were normalized, the amounts of mineralized bone increased but were below the levels seen in wild-type mice. In contrast, treatment of the $1(\text{OH})\text{ase}^{-/-}$ mice with $1,25(\text{OH})_2\text{D}_3$ restored normal mineral and PTH levels and completely normalized their bones. Consequently, in these studies, normalizing calcium, phosphorus, and PTH appeared to unmask a requirement for the $1,25(\text{OH})_2\text{D}/\text{VDR}$ system for normal bone formation. In other studies using a model of $1\alpha(\text{OH})\text{ase}^{-/-}$ mice, it was also concluded that the rescue diet did not appear as effective as $1,25(\text{OH})_2\text{D}_3$ replacement therapy, since bone growth remained impaired (Dardenne et al. 2003; Panda et al. 2004); this was completely reversed by treatment with $1,25(\text{OH})_2\text{D}_3$. Furthermore, $1,25(\text{OH})_2\text{D}$ administered to young, genetically modified mice with deficiency in both $1,25(\text{OH})_2\text{D}$ and PTH, promoted endochondral bone growth independent of PTH (Xue et al. 2005). Nevertheless, a rescue diet, which normalized calcium, phosphorus, and PTH, has been reported to completely normalize bones of $\text{Vdr}^{-/-}$ mice at 10 weeks relative to wild-type littermates fed regular chow (Amling et al. 1999); the source of the discrepant results in these models is unclear.

In humans with HVDRR, administration of calcium alone has been reported to normalize bone; however, treated HVDRR patients frequently exhibit residual low normal or low calcium and high normal or elevated serum PTH even after calcium administration (Tiosano et al. 2011). The extent to which remaining increases in circulating PTH contribute to the apparent normalization of bone mass remains uncertain.

A direct anabolic effect of the $1,25(\text{OH})_2\text{D}$ system in bone is further supported by studies in a transgenic mouse model in which the VDR was expressed in late osteoblasts/early osteocytes (Gardiner et al. 2000). A major increase in cortical bone was observed associated with an increase in periosteal bone formation and a reduction in bone resorption. Trabecular bone was also increased (Triliana et al. 2016). Therefore, VDR activity in more mature osteoblasts appears to be both anabolic and anticatabolic. The VDR-induced anti-resorptive effect appears to be mediated by decreasing the ratio of the osteoclast-stimulating cytokine, receptor activator of nuclear factor kappaB ligand (RANKL) to its naturally occurring soluble decoy receptor termed osteoprotegerin (OPG). In contrast, the anabolic effect may be the result of increased expression of lipoprotein receptor-related protein (LRP)-5, a co-receptor in wingless (Wnt) signaling, a pathway known to mediate anabolic effects in osteoblasts

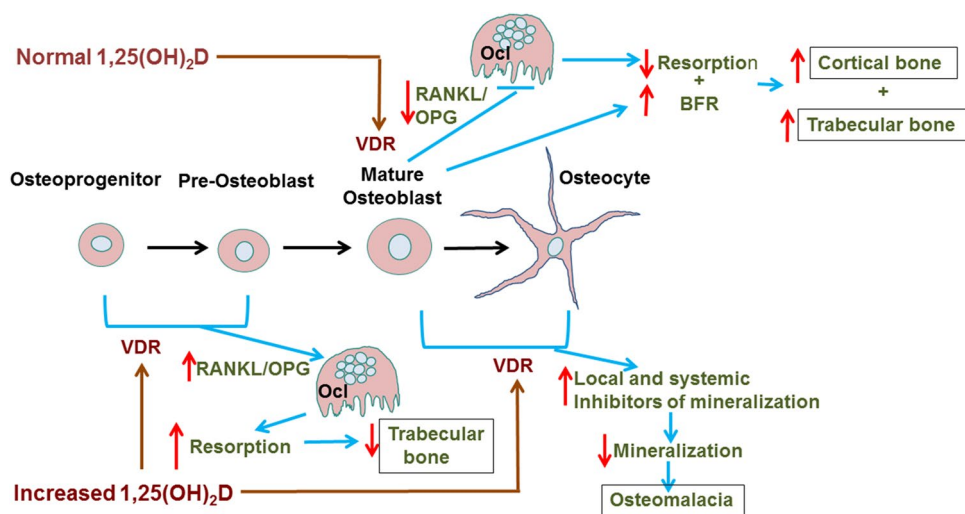


Fig. 4 Tentative scheme of direct actions of 1,25(OH)₂D/VDR on bone. Normal levels of 1,25(OH)₂D act via the VDR in mature osteoblasts to decrease the ratio of RANKL/OPG and reduce osteoclastic bone resorption. As well, 1,25(OH)₂D action via the VDR in mature osteoblasts increases the bone formation rate (BFR). The net result is increased cortical and trabecular bone. Increased levels

of 1,25(OH)₂D acting via the VDR in less mature osteoblasts may increase RANKL/OPG, stimulate osteoclastic bone resorption, and reduce trabecular bone. The action of high levels of 1,25(OH)₂D in mature osteoblasts and osteocytes can increase local and systemic inhibitors of osseous mineralization and decrease mineralization of bone leading to osteomalacia

(Fretz et al. 2006). Consequently, the 1,25(OH)₂D/VDR system may exert anabolic effects in bone via its actions in late osteoblastic and osteocytic cells (Fig. 4).

Effect of vitamin D on bone resorption

1,25(OH)₂D is a potent stimulator of osteoclastogenesis in vitro, and administration of high doses of 1,25(OH)₂D can bind the VDR in osteoblasts and increase expression and release of RANKL from osteoblasts (Kitazawa et al. 2008; Kim et al. 2006) and thus increase the ratio of RANKL to OPG. RANKL can bind to its cognate receptor RANK in osteoclasts, and increase osteoclast formation and action (Boyle et al. 2003). Increased endogenous 1,25(OH)₂D in the presence of hypocalcemia may also increase osteoclastogenesis to restore normocalcemia (Lieben et al. 2012). Studies of conditional deletion of the VDR from the osteoblast lineage suggest that early osteoblastic cells may mediate an increase in bone resorption induced by 1,25(OH)₂D (Yamamoto et al. 2013) (Fig. 4).

The effects of vitamin D on bone turnover may, therefore, depend on the state of calcium balance of the organism (Christakos et al. 2016) but may also depend on the stage of osteoblast differentiation which may determine the 1,25(OH)₂D response. Thus, there may be a differential impact of 1,25(OH)₂D on the transcriptomes of pre-osteoblastic and differentiated osteoblastic cells (St John et al. 2014) reflecting the dynamic changes occurring to the genome when cells differentiate. Furthermore, the relative

numbers of less differentiated and more mature cells of the osteoblast lineage may change during growth and aging (Kim et al. 2017; Farr et al. 2016) and alter the nature of the action of 1,25(OH)₂D at different stages of the life cycle. Nevertheless, the resorptive action of 1,25(OH)₂D appears to be the most commonly occurring mechanism influencing skeletal economy.

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