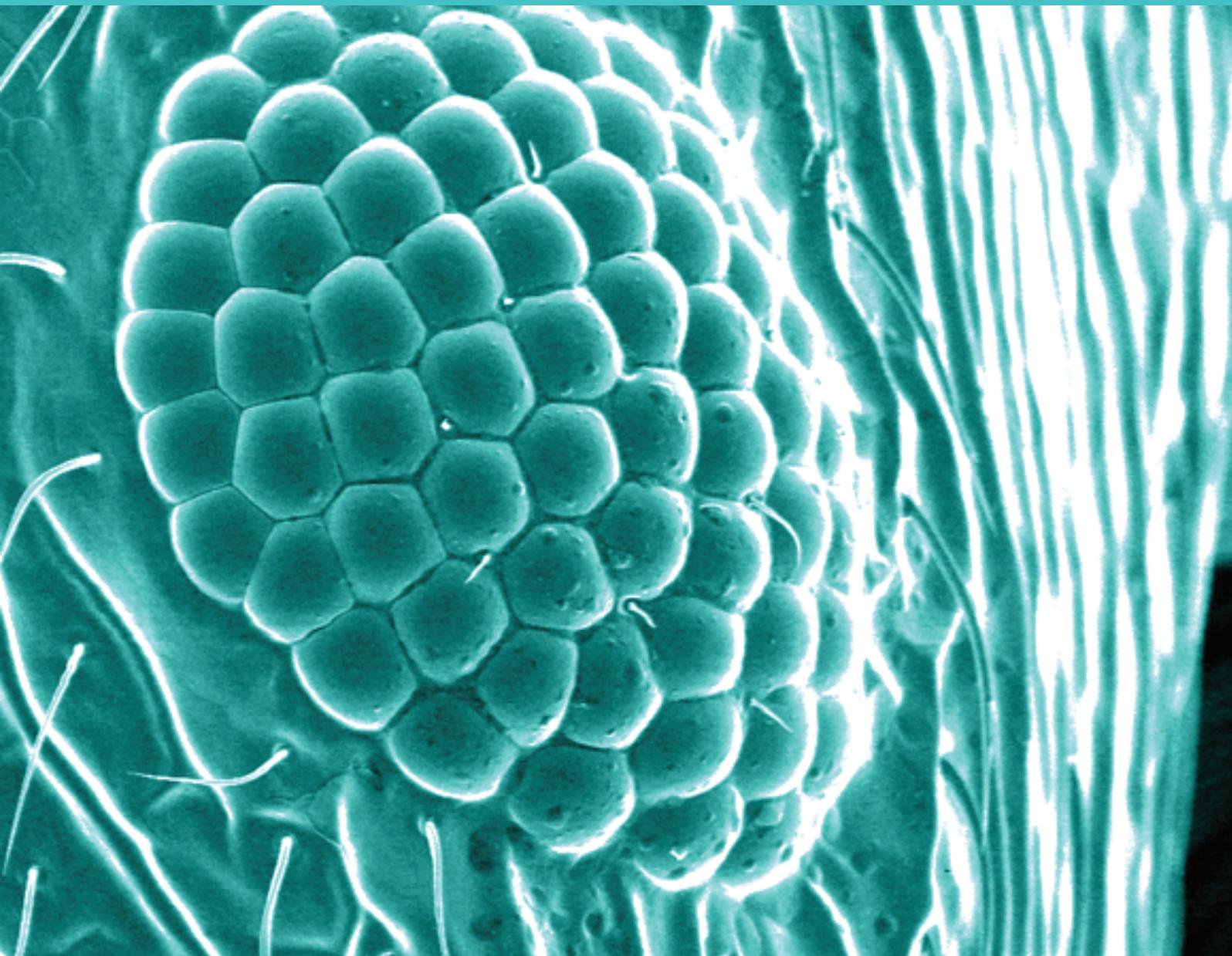


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## VITAMIN D-VDR SIGNALING IN BONE CELLS

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### ABSTRACT

Vitamin D plays a key role in mineral homeostasis, in which its main biological effect is to maintain adequate serum calcium levels. The systemic deficiency of either 1,25D or its receptor (VDR) is associated with bone alterations such as rickets and osteomalacia. This review summarizes the evidence supporting a direct effect of vit D-VDR on bone cells. The presence of vit D-hydroxylases as well as VDR in several cell types, supports an autocrine / paracrine role for vitamin D. Bone-derived cells also express VDR, and thus it is currently hypothesized that 1,25(OH)<sub>2</sub> vitamin D (1,25D) directly controls specific aspects of bone and mineral homeostasis. Several forms of vitamin D have been shown to induce specific and direct effects on different cells from bone and cartilage, such as chondrocytes, osteoblasts, osteocytes, osteoclasts and bone marrow stromal cells. Both catabolic and anabolic effects of vitamin D have been demonstrated in bone, mediated by different signal transduction mechanisms. In addition to the classic VDR mediated actions, non-classic and rapid effects of vitamin D have also been demonstrated in bone cells.

**Keywords:** vitamin D; vitamin D receptor; hydroxylases; bone homeostasis; bone marrow stromal cells

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## Introduction

Vitamin D (vit D) plays a key role in mineral homeostasis and its main biological effect is to maintain serum calcium (Ca) levels within the normal range [1]. It regulates intestinal calcium and phosphate (P) transport, maintains circulating calcium and phosphate levels that can support bone formation, exerts potent effects on cell growth and differentiation, and can modulate the immune response. Classically, vit D interacts in an endocrine manner with parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), thus regulating Ca and P levels. The presence of several vit D-hydroxylases and the receptor (VDR) in several cell types supports an autocrine/paracrine role for vit D.

Several clinical situations are associated with vit D deficiency, such as ageing and chronic kidney disease. They are characterized by impaired Ca absorption, secondary hyperparathyroidism, and bone resorption and bone loss. These observations suggest an important role for vit D in bone metabolism and Ca/P homeostasis.

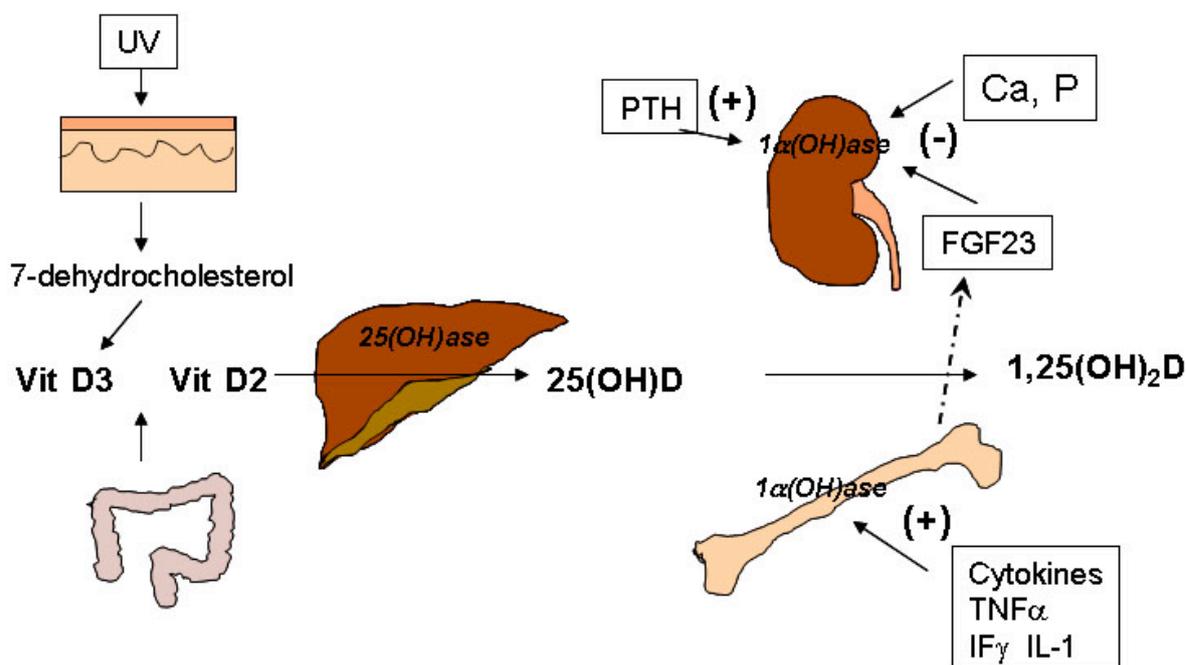
This review summarizes the existing evidence supporting a direct effect of vit D-VDR on bone cells.

## Synthesis and metabolism of vitamin D

Vit D is obtained in the skin by ultraviolet (UV) irradiation of 7-dehydrocholesterol, which thus opens its B ring to form pre-vitamin D (Figure 1) [2]. Pre-vit D can then isomerize to vit D (cholecalciferol or D<sub>3</sub>), or with continued UV irradiation can form tachysterol and lumisterol. Vit D is also found in small quantities in the diet in the form of ergocalciferol (D<sub>2</sub>) or cholecalciferol (D<sub>3</sub>), which differ in their side chains and thus show differences both in their affinity for vit D-binding protein (DBP) and in their subsequent metabolism. The liver and other organs hydroxylate vit D (either synthesized by the skin or of dietary origin), to 25-OH vit D (25D), the principal and major circulating form of vit D [2,3]. The enzyme responsible for this modification is the cytochrome P450 (CYP)-dependent 25-hydroxylase (CYP2R1) [4].

25D is then metabolized to 1,25(OH)<sub>2</sub> vit D (1,25D) principally in the renal proximal tubule by an enzyme identified as 1 $\alpha$ -hydroxylase (CYP27B1). 1,25D is the main hormonal form of vitamin D, and is responsible for most of its biological actions. Production of 1,25D by the kidney is strictly dependent on renal 1 $\alpha$ -hydroxylase activity. This enzyme is regulated by a negative feedback of 1,25D, by PTH as a signal of Ca status (PTH is secreted to stimulate CYP27B1 when serum Ca levels are low) and by FGF23 as a signal of P homeostasis (FGF23 is produced in the bone in response to elevated P levels and inhibits CYP27B1 synthesis) [4]. 1,25D is a hormone that plays a critical role in maintaining Ca homeostasis through its recognition by nuclear VDR.

## Metabolism and Regulation of Vitamin D



**Figure 1.** Vitamin D metabolism and regulation. Cholecalciferol (vit D3) is synthesized from 7-dehydrocholesterol (vit D3) in the skin by UV exposure. Vit D can also be obtained from diet in the form of ergocalciferol (vit D2) and vit D3. Both are transformed to calcidiol (25(OH)D or 25D) by cytochrome enzymes CYP2R1 and CYP27A1. Then, 25D is hydroxylated in the kidney and in other organs such as bone, by the CYP27B1/1α-hydroxylase. The renal enzyme is regulated by PTH, Ca and P levels, FGF23 and 1,25D. The skeletal 1α-hydroxylase is positively regulated by cytokines.

Other cells such as keratinocytes, parathyroid principal cells, enterocytes, macrophages, various bone cells and chondrocytes also express 1α-hydroxylase. This extra-renal production of 1,25D is regulated by different mechanisms: it can be stimulated by cytokines such as tumor necrosis factor-α, interferon-γ, and interleukin (IL)-1β [5].

25D and 1,25D can be further hydroxylated by induction of a 24-hydroxylase (CYP24A1) to form 24,25(OH)<sub>2</sub> vit D (24,25D) and 1,24,25(OH)<sub>3</sub> vit D (1,24,25D), respectively. This is generally considered the first step in the catabolism of the active metabolites. However, 24,25D and 1,24,25D may have their own biological actions; in particular, 24,25D could be the principal metabolite regulating chondrocyte function in the resting zone [2].

CYP24A1 is induced by 1,25D, and thus constitutes an important feedback mechanism to prevent vit D toxicity. Vit D metabolites are transported in blood bound to DBP and albumin, with very little vit D circulating in a free form. Individuals with liver, intestinal,

or renal diseases resulting in low levels of these transport proteins, may have low total levels of vit D metabolites but they are not vit D deficient; in fact, free vit D concentrations may actually be normal [6].

### **Vitamin D metabolites and their effects on different bone cells**

The systemic deficiency of 1,25D activity or of its receptor (VDR) is associated with bone defects such as rickets and osteomalacia. The observation that these bone abnormalities can be rescued by a high-Ca diet or by reintroducing VDR in the small intestine indicates that a critical role of 1,25D is to enhance intestinal Ca absorption. Although 1,25D signaling in bone cells is not a prerequisite for bone development and homeostasis when intestinal Ca transport is guaranteed, multiple bone cells express the VDR and it is hypothesized that through its action on these cells 1,25D directly controls specific aspects of bone and mineral homeostasis [1].

#### *Vitamin D action on chondrocytes*

The skeletal system contains mainly cartilage and bone, formed by chondrocytes and osteoblasts, respectively. Both cells are derived from mesenchymal progenitor cells. Bone tissue is constantly being renewed by a precise coupling of resorption, mediated by osteoclasts, and formation, in a process that has been termed remodeling. The quality of the bone is maintained by the action of osteocytes, a network of sensory cells mediating the effects of mechanical loading.

The major skeletal manifestation of a systemic loss of 1,25D signaling is rickets, characterized by an expansion and widening of the growth plate with marked disorganization of its associated chondrocytes. These growth plate abnormalities most likely result from hypophosphatemia, since several hypophosphatemic conditions result in similar growth plate abnormalities and normal phosphorus levels are required for growth plate maturation [7].

1,25D signaling in chondrocytes appears to be redundant when mineral homeostasis is not manifestly disturbed, but is critical to prevent the growth plate abnormalities of rickets when hypophosphatemia is present [1]. VDR expressed in chondrocytes has an important paracrine function that is directed at fine-tuning fetal and early post natal bone development. Indeed, chondrocyte-specific VDR inactivation in mice shows that 1,25D controls vascular invasion and osteoclast formation in the primary ossification center by increasing the angiogenic cytokine VEGF (vascular endothelial growth factor) and the pro-osteoclastic protein RANKL [receptor activator of nuclear factor-KB (RANK) ligand] [8]. The 1,25D and 24,25D are required for optimal endochondral bone formation [2]. Growth cartilage chondrocytes respond primarily to 1,25D, whereas resting zone cells respond primarily to 24,25D [9]. It seems that certain actions of 1,25D and 24,25D in chondrocytes do not need the VDR and are thus nongenomic [2].

### *Vitamin D effects on osteoblasts*

The bone phenotype of hypocalcemic VDR-null mice is characterized by growth plate defects, as well as by unbalanced bone remodeling and osteomalacia. Increased numbers of osteoblasts line the bone surfaces but mineral apposition is impaired. As a result, although total bone mass is increased, this is due to an excess in unmineralized bone matrix [1].

Osteocytes and osteoblasts respond to 1,25D via VDR, inducing the expression and release of FGF23 to control phosphatemia. 1,25D also repress CYP27B1 and induce CYP24A1 in these cells, in order to reduce 1,25D levels by feedback mechanisms [3].

Although defects in 1,25D signaling can affect osteoblastic differentiation and bone formation, this is predominantly due to its role in the intestine. However, *in vitro* experiments have also provided evidence for the direct regulation of osteoblast differentiation by VDR action, in a species-dependent manner. In human pre-osteoblasts, 1,25D induces differentiation [10]. These effects are biphasic in rat osteoblastic cells: 1,25D enhances differentiation at late osteoblastic stages and inhibits differentiation in early pre-osteoblasts. The differentiation of mouse osteoblastic cells is inhibited at all times by 1,25D treatment, independently of the differentiation stage [1].

Normalizing serum Ca and P levels completely rescues the bone phenotype of VDR-null mice, indicating that their mineralization defects are due to an insufficient supply of these ions to the bone matrix, caused in turn by 1,25D deficiency. However, hypervitaminosis D in rats is also often associated with bone mineralization defects such as bone loss and abundant unmineralized bone matrix [1].

Osteoblasts are functionally associated with specific niches of the bone microenvironment in which hematopoietic stem cells (HSC) reside. This fact could indicate that VDR signaling, via its effect on extracellular Ca, may be involved in promoting retention of HSC (thus favoring hematopoiesis) in the bone marrow. Other reports demonstrate that VDR signaling in osteoblasts, and not extracellular Ca, is required to suppress the osteoblastic niche and induce mobilization of HSC in the circulation, in response to adrenergic stimulation [11]. The HSC mobilization is hampered in VDR-null mice, even on a rescue diet.

In summary, although it is accepted that 1,25D affects the skeleton mainly via its actions on the intestine and the regulation of mineral homeostasis, direct effects of 1,25D are also observed in osteoblast differentiation, bone matrix mineralization and the osteoblastic niche.

### *Vitamin D action in osteoclasts*

Using an *in vitro* organ culture system, both 1,25D and 25D have been shown to increase the release of Ca from prelabeled bone into the culture medium. In these experiments, 1,25D was found to be 80 times more potent than 25D, suggesting that 1,25D is the active metabolite stimulating bone mineral mobilization [5]. An efficient mouse co-culture system to generate osteoclasts has been established with calvaria-derived osteoblasts and

spleen cells. In this co-culture system, 1,25D induces the formation of multinucleated osteoclasts. Cell-cell contact between spleen cells and osteoblastic cells, which express VDR, appears to be important for both the formation and activation of osteoclasts. 1,25D regulates osteoclast differentiation indirectly; by increasing the osteoblastic expression of the osteoclastogenic factor RANKL while reducing the expression of the anti-osteoclastic protein osteoprotegerin (OPG) [12].

VDR-deficient osteoblasts result in increased bone mass. This was reported by Yamamoto et al [13], who noted that even heterozygote ablation of VDR induced an increase of bone mass, due more to a down-regulation of osteoclast activity rather than an increase in bone formation. Further analysis indicated that VDR in osteoblasts could act as a negative regulator of bone mass through stimulation of RANKL-induced osteoclastogenesis [14].

### **Vitamin D mechanism of action on bone cells**

The effects and mechanisms of action of vit D on bone cells will depend on several factors: (a) ability to metabolize and synthesise 1,25D, (b) expression of  $1\alpha$ -hydroxylase, (c) VDR expression, and (d) presence of co-activator/repressor proteins that regulate gene transcription.

Vit D action requires binding of the active form of vit D, 1,25D, to its specific receptor VDR, thus initiating biological responses [15]. Although 25D can also activate VDR, its affinity for this receptor is 50-fold less than that of 1,25D [16]. The vit D-VDR complex then interacts with a co-receptor, the retinoid X receptor (RXR), and this heterodimer in turn will regulate the transcription of different genes. This mechanism is mediated by different vit D response elements (VDREs) present in diverse gene sequences. In addition, the regulation (activation or repression) of transcription is affected in part by the interaction of the ligand-VDR-RXR complex with co-activator or co-repressor proteins that will regulate the chromatin structure of target genes.

Transcriptional regulation has generally been believed to occur near the transcriptional start site of each gene. However, the use of new tools such as chromatin immunoprecipitation techniques (ChIP-seq) and gene scanning methodologies have provided new insights into the mechanism of action of vit D [17]. It has been suggested that VDR-transcription factor interaction depends on the specific target cells. In the case of the pre-osteoblastic MC3T3E1 cell line, distal transcriptional control has been shown to regulate most of vit D-mediated gene transcription [18]. Molecular studies have also suggested that the regulation of gene transcription depends on a variety of different transcription factors that regulate multiple signalling pathways. For instance, vit D can increase the LRP5 co-receptor gene of the Wnt pathway and also directly suppress the activity of  $\beta$ -catenin signaling molecule [19]. Interestingly, activation of the Wnt/ $\beta$ -catenin pathway is associated with increased bone formation.

In addition, evidence has been presented of a fast non-transcriptional mechanism for vit D action in skeletal muscle and osteoblastic cells [20]. These actions involve an increase in intracellular Ca and stimulation of different kinases related to signal transduction pathways. Both voltage-dependent and capacitive Ca entry channels mediate this increase

in intracellular Ca. It has been suggested that the presence of different plasma membrane receptors, or even VDR, could mediate these rapid, nongenomic actions of vit D [21].

Various bone cells and their progenitors have been found to possess the molecular machinery necessary to respond to -and metabolize- vit D [12]. They express  $1\alpha$ -hydroxylase, and regulate its expression depending on the levels of 1,25D. Osteoblasts also coordinately regulate the expression of VDR mRNA levels.

The consequences of 1,25D-VDR signalling on bone cells largely depend on Ca balance [22]. Thus, direct or indirect effects of 1,25D on bone will be compromised if necessary, in order to maintain normal serum Ca levels. Under a positive Ca balance, 1,25D will stimulate intestinal Ca absorption, indirectly affecting bone homeostasis by ensuring supply of Ca to bones. However, in the presence of a negative Ca balance (such as in dietary calcium restriction or if there is defective intestinal VDR activity), 1,25D-VDR signalling can directly enhance bone resorption and impair osteoid mineralization.

In osteoblastic cell cultures, 1,25D has been shown to play either catabolic or anabolic roles on differentiation and mineralization. The specific effect induced by vit D depends on the degree of maturation of the osteoblastic cells.

In human bone marrow stromal cells (hMSCs), 1,25D promotes their osteoblastic differentiation [23]. Osteoblastogenesis is also stimulated by 25D, an effect that depends on its conversion to 1,25D by the action of  $1\alpha$ -hydroxylase expressed by these cells. Other bone cells such as osteoblasts and osteoclasts also express this hydroxylase, supporting an autocrine/paracrine action of vit D in the bone microenvironment. 1,25D and 25D show VDR-mediated anti-proliferative and pro-differentiation effects in hMSCs, which are associated with a stimulation of alkaline phosphatase (ALP) gene expression and activity. *In vitro* treatment with 25D up-regulates CYP27B1/ $1\alpha$ -hydroxylase and IGF-I in hMSCs. IGF-I also up-regulates CYP27B1 expression and stimulates osteoblast differentiation (Table 1).

Human primary osteoblasts also respond to 1,25D and 25D, inhibiting their proliferation and stimulating their differentiation [24]. These cells express CYP27B1/ $1\alpha$ -hydroxylase and CYP24/24-hydroxylase, thus possessing the capacity to produce 1,25D and 24,25D respectively. In the presence of 24,25D, they enhance mRNA levels of differentiation genes, suggesting that other forms of vit D besides 1,25D may affect osteoblastic functions. However, the physiological relevance of these findings needs further research to be established.

Other VDR signals are involved in the anabolic effects of vit D. For instance in human trabecular bone cells, 1,25D increases the gene expression of bone matrix proteins such as osteocalcin and bone sialoprotein-1, thus enhancing osteoblastic differentiation and mineralization [15]. In addition, the 1,25D-VDR axis increases the expression of low-density lipoprotein receptor-related protein (LRP5) and upregulates the Wnt pathway in osteoblasts, where it promotes bone formation [25].

**Table 1.** Anabolic effects induced by vitamin D on bone cells

Signal	Anabolic Effects
↑ IGF-I	Osteoblastogenesis
↑ ALP	Osteoblastic differentiation
↑ Osteopontin	Osteoid mineralization
↑ LRP5	Bone formation

Under a negative Ca balance or high vit D levels, 1,25D promotes bone resorption, (Table 2). This effect, that has been demonstrated in animals as well as *in vitro* in cell culture systems [15], is mediated by an increase in RANKL and a decrease of the soluble RANKL decoy receptor OPG by osteoblasts and osteocytes [26]. In addition, 1,25D can indirectly inhibit bone formation by stimulation of FGF23 release from osteocytes. Synthesis of 1,25D in the kidney is later repressed by a feedback loop that involves FGF23. In osteoblastic cell lines, 1,25D has also been reported to inhibit the master osteoblastic transcription factor Runx2, thus potentially reducing their bone forming capacity.

**Table 2.** Catabolic effects induced by vitamin D on bone cells

Signal	Catabolic Effects
↑ RANKL	Osteoclastogenesis - ↑ Bone resorption
↓ Osteoprotegerin	Osteoblastic differentiation
↑ FGF23	↓ Mineralization
↓ Runx2	↓ Osteoblastic differentiation

## **Conclusion**

The primary role of vit D is to stimulate intestinal Ca and P absorption, ions that are required for bone formation and mineralization. In the context of normal circulating Ca and P levels, these effects on bone can largely be achieved without any direct effects of vit D metabolites on bone cells. But there is evidence that the Vit D–VDR system can induce direct effects on mature osteoblasts, chondrocytes, osteoclasts and bone marrow stromal cells. The Vit D–VDR system can have different functional roles on these cells, depending on the stage of differentiation/maturity of the osteoblastic lineage cells, as well as on the sufficiency of circulating Ca and/or P levels. Vitamin D metabolites can be catabolic, inducing bone resorption and decreasing mineral deposition so as to increase circulating Ca levels if there is a deficiency of this ion, as well as to facilitate bone balance when mineral supply is sufficient.

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