Vitamin D and Bone

Sylvia Christakos, Shanshan Li, Jessica DeLa Cruz, Lieve Verlinden, and Geert Carmeliet

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Abstract

Vitamin D is a principal factor required for mineral and skeletal homeostasis. Vitamin D deficiency during development causes rickets and in adults can result in osteomalacia and increased risk of fracture. 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the hormonally active form of vitamin D, is responsible for the biological actions of vitamin D which are mediated by the vitamin D receptor (VDR). Mutations in the VDR result in early-onset rickets and low calcium and phosphate, indicating the essential role of $1,25(OH)_2D_3$/VDR signaling in the regulation of mineral homeostasis and skeletal health. This chapter summarizes our current understanding of the production of the vitamin D endocrine hormone, $1,25(OH)_2D_3$, and the actions of $1,25(OH)_2D_3$ which result in the maintenance of skeletal homeostasis. The primary role of $1,25(OH)_2D_3$ is to increase calcium absorption from the intestine and thus to increase the availability of calcium for

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bone mineralization. Specific actions of $1,25(OH)_2D_3$ on the intestine, kidney, and bone needed to maintain calcium homeostasis are summarized, and the impact of vitamin D status on bone health is discussed.

Keywords
1,25-Dihydroxyvitamin D3 · Bone · Calcium homeostasis · Intestine · Kidney · Vitamin D metabolism

1 The Vitamin D Endocrine System: Metabolism and Molecular Mechanism of Action

Vitamin D is a principal factor required for the development and maintenance of bone as well as for maintaining normal calcium and phosphorus homeostasis. Vitamin D deficiency during bone development causes rickets, and in adults, vitamin D deficiency, which is common in the elderly, causes secondary hyperparathyroidism that can result in osteomalacia and increased risk of fracture (Weaver et al. 2016; Bouillon and Carmeliet 2018). For vitamin D to affect mineral metabolism, it must first be metabolized to its active form. Vitamin D which is synthesized in the skin from 7-dehydrocholesterol in a reaction catalyzed by ultraviolet irradiation or taken in the diet (few foods, which include fish oils and fortified dairy products, contain appreciable amounts of vitamin D) is first hydroxylated in the liver to 25-hydroxyvitamin D_3 [$25(OH)D_3$], the major circulating form of vitamin D and the most reliable index of vitamin D status (Christakos et al. 2019). CYP2R1, a 25-hydroxylase, is likely the key vitamin D based in part on genetic evidence that patients with a mutation in CYP2R1 are deficient in $25(OH)D_3$ and develop vitamin D-dependent rickets (Cheng et al. 2003, 2004; Thacher et al. 2015). Since in *Cyp2r1* null mice levels of $25(OH)D_3$ are reduced but not abolished, it has been suggested that other hydroxylases are also involved in the conversion in the liver of vitamin D to 25(OH)D (Zhu et al. 2013). The second hydroxylation occurs in the proximal renal tubule through the action of mitochondrial 25(OH)D 1 α hydroxylase (CYP27B1) resulting in the conversion of 25(OH)D to the principal hormonal form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), which is responsible for the biological actions of vitamin D (Christakos et al. 2016). Vitamin D and its metabolites are transported in the blood by vitamin D binding protein (DBP) (Christakos et al. 2016, 2019). Mutation in the CYP27B1 gene results in vitamin D dependency rickets type 1 (VDDR-1), characterized by decreased mineralization, hypocalcemia, and low circulating $1,25(OH)_2D_3$ (Kitanaka et al. 1998). The activity of renal CYP27B1 is under stringent control. Parathyroid hormone (PTH) induced in response to hypocalcemia stimulates CYP27B1 (Jones et al. 2014). FGF23, which promotes renal phosphate excretion and requires klotho, a transmembrane protein, suppresses the expression of CYP27B1 (Shimada et al. 2004; Hu et al. 2013).

$1,25(OH)_2D_3$, as a feedback mechanism, regulates its own production by inhibiting CYP27B1, downregulating PTH synthesis by the parathyroid gland and upregulating FGF23 production in bone (Jones et al. 2014; Christakos et al. 2016). Recent mouse genomic studies by Meyer et al. (2017, 2019a) identified a kidney specific enhancer module that mediates basal and PTH-induced expression of *Cyp27b1* and FGF23 and $1,25(OH)_2D_3$-mediated repression. Studies using human kidney suggest that a kidney-specific module similar to that observed in the mouse exists in humans (Meyer et al. 2019a). These findings represent an important advance in the vitamin D field since they provide insight for the first time at the genomic level on the mechanisms that control *Cyp27b1* expression. Additional factors including sex hormones and prolactin have been reported to stimulate CYP27B1 (Tanaka et al. 1978; Ajibade et al. 2010). In addition to the kidney, CYP27B1 is also expressed in the placenta (Zehnder et al. 2002) and in small amounts in a number of different tissues, including bone (Bikle 2010). However, the role of CYP27B1 under normal physiological conditions in tissues other than kidney and placenta remains to be determined.

As an additional autoregulatory mechanism, $1,25(OH)_2D_3$ induces CYP24A1 (25-hydroxyvitamin D_3 24-hydroxylase) which accelerates the catabolism of $1,25(OH)_2D_3$ by catalyzing the conversion of $1,25(OH)_2D_3$ into 24-hydroxylated products targeted for excretion (Jones et al. 2014; Christakos et al. 2016). Mutation in the *CYP24A1* gene results in hypercalcemia, hypercalciuria, decreased PTH, and normal to high $1,25(OH)_2D_3$ levels (Schlingmann et al. 2011). The levels of 25(OH) D_3 have also been reported to be regulated through the catabolic activity of renal CYP24A1 (Jones et al. 2014; Christakos et al. 2016; Meyer et al. 2019b). CYP24A1 is present not only in kidney but also in all cells that contain VDR (Jones et al. 2014; Christakos et al. 2016). Thus, CYP24A1 not only regulates circulating $1,25(OH)_2D_3$ protecting against hypercalcemia but also may modulate the amount of $1,25(OH)_2D_3$ in target cells and control the cellular response. In the kidney CYP24A1 is reciprocally regulated when compared to CYP27B1 (suppressed by low calcium and PTH and induced by FGF23 and $1,25(OH)_2D_3$). This reciprocal regulation occurs only in the kidney. In nonrenal target cells, CYP24A1 is solely regulated by $1,25(OH)_2D_3$ (Meyer et al. 2019b). Recent studies have identified genomic mechanisms resulting in differential regulation of *Cyp24a1* in the kidney and nonrenal target tissues (e.g., intestine and bone) (Meyer et al. 2019b).

The biological activities of $1,25(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), a nuclear receptor which is a member of the steroid receptor family. $1,25(OH)_2D_3$-occupied VDR heterodimerizes with the retinoid X receptor (RXR) and together with chromatin active co-regulatory proteins interacts with specific DNA sequences (vitamin D response elements) in and around target genes resulting in activation or repression of transcription (Christakos et al. 2016; Pike and Christakos 2017). VDR-binding sites are located at proximal promoters and also many kilobases upstream and downstream and in intronic and exonic sites. Mutations in VDR result in early-onset rickets, low calcium and phosphate, and high PTH, indicating the essential role of VDR in mediating $1,25(OH)_2D_3$ regulation of mineral homeostasis and skeletal health (Malloy et al. 2014).

2 Effect of Vitamin D on Calcium Homeostasis

2.1 Intestine

The principal action of vitamin D in maintenance of calcium homeostasis is increased intestinal calcium absorption and thus increased availability of calcium for mineralization of bone. This conclusion is based in part on studies in *Vdr* null mice. Feeding *Vdr* null mice a diet which includes high calcium prevents rickets and osteomalacia and results in the normalization of serum calcium and PTH (Amling et al. 1999; Masuyama et al. 2003). In addition, in humans with a mutation in VDR characterized by resistance to $1,25(OH)_2D_3$ (hereditary vitamin D-resistant rickets; HVDRR), administration of calcium alone has been reported to normalize bone and result in normal mineralization (al-Aqeel et al. 1993). Active intestinal calcium absorption occurs when there is an increased need for calcium (during growth, pregnancy, lactation, and under low dietary calcium conditions). Active calcium absorption is mediated at least in part by a transcellular process involving calcium entry via the apical epithelial calcium channel TRPV6, calcium binding by the intracellular calcium-binding protein calbindin-D_{9k}, and calcium extrusion via the basolateral membrane calcium ATPase PMCA1b (Christakos et al. 2014). TRPV6, calbindin, and PMCA1b are regulated by $1,25(OH)_2D_3$/VDR (Van Cromphaut et al. 2001; Song et al. 2003; Lee et al. 2015). Abnormalities in the *Vdr* null mice develop only after weaning, consistent with studies showing that intestinal VDR, calbindin, and TRPV6 are induced at weaning (Yoshizawa et al. 1997; Song et al. 2003). Overexpression of TRPV6 in the intestine results in hypercalcemia and soft tissue calcification indicating a significant role for TRPV6 in intestinal calcium absorption and suggesting that the inability to transport calcium into the enterocyte may be a primary defect in VDR-dependent rickets (Cui et al. 2012). Direct evidence for a critical role of $1,25(OH)_2D_3$/VDR-mediated intestinal calcium absorption in bone homeostasis was shown in studies in which VDR expression specifically in the intestine of *Vdr* null mice prevented rickets and normalized serum calcium (Xue and Fleet 2009). These findings indicate that intestinal VDR is essential for controlling bone formation. In addition, when VDR is deleted specifically from mouse intestine, there is calcium malabsorption, bone resorption, increased bone fractures, and normal serum calcium (Lieben et al. 2012). Thus when calcium homeostasis cannot be maintained by $1,25(OH)_2D_3$/VDR-mediated intestinal calcium absorption, serum calcium will be maintained at the expense of skeletal integrity.

2.2 Kidney

When serum calcium cannot be maintained by intestinal calcium absorption, in addition to stimulation of osteoclastogenesis by PTH and $1,25(OH)_2D_3$ (see Sect. 2.3), calcium reabsorption occurs in the distal convoluted tubule and collecting tubule and is regulated by $1,25(OH)_2D_3$ and PTH (Christakos et al. 2016). Similar to the intestine, calcium is reabsorbed in the distal tubule by a transcellular process

involving calcium entry through calcium channel TRPV5 (75% sequence homology with TRPV6), calcium binding to the calcium-binding protein calbindin [calbindin-D_{9k} (9,000 Mr; only in mouse kidney) and calbindin-D_{28k} (28,000 Mr; in mouse, rat, and human kidney)], and calcium extrusion via the calcium ATPase PMCA1b and the Na^+/Ca^{2+} exchanger (NCX1 or SLC8A1). $1,25(OH)_2D_3$ induces the expression in the kidney of TRPV5 and the calbindins (Song et al. 2003). PTH has been reported to activate TRPV5 via protein kinase A phosphorylation (de Groot et al. 2009). Studies in *Trpv5* null mice show that inactivation of *Trpv5* results in diminished calcium reabsorption in the distal tubule, severe hypercalciuria, and significant changes in bone structure (Hoenderop et al. 2003). It has been suggested that calcium uptake by TRPV5 is a rate-limiting step in renal calcium reabsorption and thus in the maintenance of calcium and bone homeostasis (Hoenderop et al. 2003). The kidney is also a major site of production of $1,25(OH)_2D_3$ and its regulation (Christakos et al. 2010, 2016; Pike and Christakos 2017).

2.3 Bone

As outlined above, loss-of-function mutations in the human VDR lead to resistance toward the actions of $1,25(OH)_2D_3$ and result in the development of hereditary vitamin D-resistant rickets type II (Malloy et al. 2014). This condition is mimicked in systemic *Vdr* null mice that develop hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, and rickets (Li et al. 1997; Yoshizawa et al. 1997; Van Cromphaut et al. 2001). The rickets phenotype is characterized by progressive widening of the epiphyseal growth plates, due to a significant enlargement and disorganization of the zone of hypertrophic chondrocytes. In addition, systemic *Vdr* null mice develop hyperosteoidosis and osteomalacia, due to a delay in bone mineralization that develops when *Vdr* null mice become hypocalcemic after weaning (Li et al. 1997; Yoshizawa et al. 1997; Van Cromphaut et al. 2001). This increase in unmineralized osteoid is accompanied by an increased number of osteoblasts, whereas osteoclast numbers are not altered (Amling et al. 1999). Importantly, the bone and mineral phenotype of systemic *Vdr* null mice is fully corrected by supplementation with a high-calcium/high-lactose diet, underscoring the importance of VDR-mediated intestinal calcium absorption (Li et al. 1998; Amling et al. 1999; Van Cromphaut et al. 2001). Yet, multiple cell types within bone express the VDR, and the highest levels of VDR are present in osteoblasts and osteocytes, which are thus considered as the main mediators of $1,25(OH)_2D_3$ action in bone homeostasis. VDR expression is also present in chondrocytes and in osteoclasts, although much less abundant. As bone homeostasis is regulated by timely controlled interactions between osteoblasts and osteoclasts (Wang et al. 2014; Nakamichi et al. 2017) and between chondrocytes and osteoclasts (Masuyama et al. 2006), VDR expression in all these cell types enables $1,25(OH)_2D_3$ to affect bone development and remodeling. Here, we aim to delineate the role of $1,25(OH)_2D_3$ in bone based on the knowledge obtained from different transgenic mouse models in which *Vdr* expression is either deleted or overexpressed in one of the different bone cell

types. First, we will discuss the bone effects of $1,25(OH)_2D_3$ in conditions of a positive calcium balance, where the amount of (re)absorbed calcium equals or exceeds fecal and renal calcium losses. In this condition, serum calcium levels remain normal and allow calcium deposition in bone. Thereafter, we will focus on the effects of $1,25(OH)_2D_3$ in bone when the calcium balance is negative, as caused, for example, by insufficient intestinal calcium absorption.

2.3.1 $1,25(OH)_2D_3$ and Bone Metabolism During a Positive Calcium Balance

When dietary calcium intake is normal, $1,25(OH)_2D_3$ indirectly regulates bone homeostasis and mineralization by guaranteeing adequate calcium supply through stimulation of intestinal and renal calcium (re)absorption (Christakos et al. 2016; Goltzman 2018). In addition, VDR expression in different osteogenic cells enables $1,25(OH)_2D_3$ to directly and locally impact on bone metabolism in a paracrine or autocrine manner. Yet, these latter effects are still not fully elucidated and are likely dependent on the differentiation stage of the osteogenic cells.

Stage-Dependent Inactivation of *Vdr* Expression in Osteoblast-Lineage Cells Points to a Minor Role of Osteoblastic *Vdr* Expression in Bone Homeostasis

Osteoblasts together with chondrocytes differentiate from a common skeletal progenitor cell. Differentiation along the osteoblast lineage is governed by multiple transcription factors including Runx2 and Osterix (Osx) (Huang et al. 2007). When osteoprogenitor cells differentiate to immature osteoblasts, they start to express genes that encode for proteins of the extracellular matrix (ECM) such as type 1 collagen α1 (Col1a1), whereas mature osteoblasts are typified by the production of osteocalcin, a secreted protein with numerous endocrine functions including the regulation of glucose and energy metabolism (Tangseefa et al. 2018; Dirckx et al. 2019). Once osteoblasts become embedded in the bone matrix, they differentiate toward osteocytes, which fulfill a function in coordinating bone formation and resorption, and these cells are characterized by a high expression of dentin matrix protein 1 (Dmp-1) and the Wnt inhibitor sclerostin (Atkins and Findlay 2012).

During the last decades, several transgenic mouse models have been established in which *Vdr* expression was either deleted or overexpressed at a specific stage of osteoblast differentiation (Gardiner et al. 2000; Lieben et al. 2012; Yamamoto et al. 2013; Triliana et al. 2016; Nakamichi et al. 2017) (Fig. 1). All studies were performed in mice that received adequate supply of dietary calcium (ranging from 0.8 to 1.18% dietary calcium). Deletion of *Vdr* expression in osteoblast precursors, by *Osx*-driven Cre recombination (*Osx-Vdr*-cKO mice), does not affect calcium or bone homeostasis (Nakamichi et al. 2017) (Fig. 2). Indeed, serum concentrations of calcium, phosphate, PTH, and $1,25(OH)_2D_3$ are normal in 14-week-old *Osx-Vdr*-cKO mice, while serum FGF23 levels are slightly reduced. Trabecular bone mass in *Osx-Vdr*-cKO mice is similar to control littermates with no overt changes in bone resorption or in bone mineralization. When *Vdr* expression is deleted in immature osteoblasts, under the control of the *Col1a1* promoter (*Col1a1-Vdr*-cKO mice), no differences in bone mass are observed in 4- and 9-week-old animals (Yamamoto

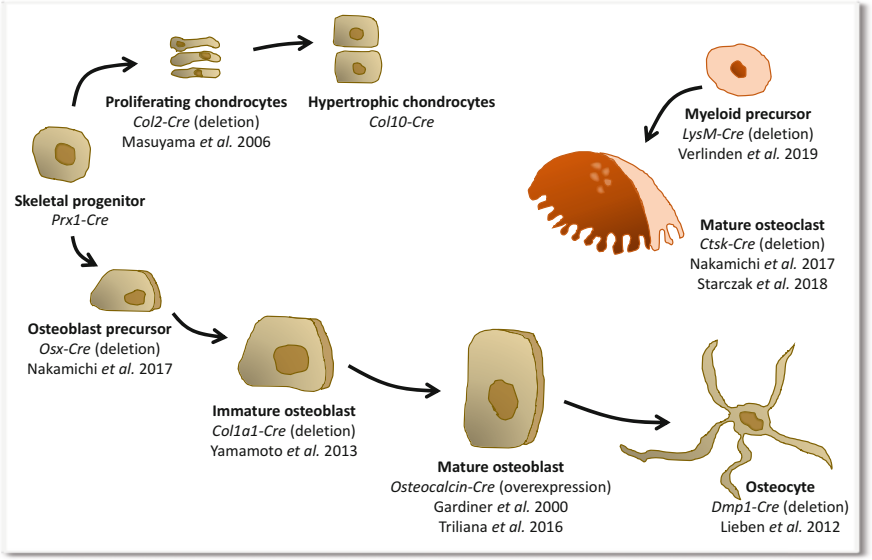

Fig. 1 Overview of different bone cell types in which *Vdr* expression was targeted

et al. 2013). However, in 16-week-old animals, a small – but significant – increase in trabecular bone mass is detected, while no significant changes in cortical bone are present. This increased bone mass is attributed to decreased bone resorption rather than to increased bone formation and is accompanied by reduced expression of the receptor activator of nuclear factor κβ ligand (RANKL), a major stimulator of osteoclastic differentiation. This bone phenotype fits with the in vitro observations showing that $1,25(OH)_2D_3$ signaling increases RANKL expression in osteoblasts (Yasuda et al. 1998), although it is not clear why the increased bone mass is only observed in older mice. Serum levels of calcium and phosphate as well as those of PTH and $1,25(OH)_2D_3$ are normal in *Col1a1-Vdr*-cKO mice.

Deletion of *Vdr* expression in mature osteoblasts and osteocytes under control of the *Dmp-1* promoter (*Dmp1-Vdr*-cKO mice) showed that VDR signaling in mature osteoblasts and osteocytes is redundant for bone homeostasis (Lieben et al. 2012). Trabecular and cortical bone mass are indistinguishable between 8-week-old *Dmp1-Vdr*-cKO mice and their wild-type littermates, as are bone resorption and mineralization. Together, these three different conditional knockout models, in which *Vdr* expression is deleted at different stages of osteoblastic differentiation, suggest that the vitamin D system does not have major direct effects on bone homeostasis in conditions of a positive calcium balance.

On the other hand, studies in mice in which *Vdr* is overexpressed in mature osteoblasts, under the control of the *osteocalcin* promoter (*osteocalcin-Vdr*-cOE mice), point toward a positive effect of VDR signaling on bone mass (Gardiner et al. 2000). Indeed, 4- and 9-month-old *osteocalcin-Vdr*-cOE mice have increased cortical and trabecular bone mass, which is accompanied by enhanced bone

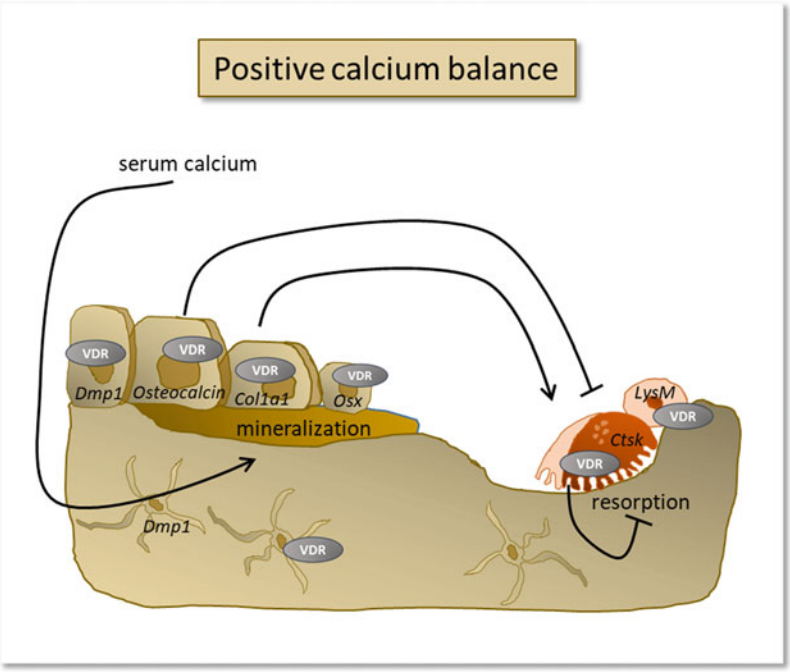

Fig. 2 Cell- and stage-specific effects of *Vdr* expression in osteogenic cells on bone homeostasis during a positive calcium balance. During a positive serum balance, normal serum calcium levels allow calcium deposition in bone and proper mineralization. *Vdr* expression in osteoprogenitors (characterized by *Osx* expression (Nakamichi et al. 2017)) or in mature osteoblasts and osteocytes (*Dmp1* (Lieben et al. 2012)) does not affect bone homeostasis. However, *Vdr* expression in immature osteoblasts (*Col1a1* (Yamamoto et al. 2013)) and in mature osteoblasts (*osteocalcin* (Gardiner et al. 2000; Triliana et al. 2016)) is reported to induce or inhibit bone resorption, respectively. *Vdr* expression in osteoclast progenitors (*LysM* (Verlinden et al. 2019)) does not influence bone resorption, whereas *Vdr* signaling in mature osteoclasts (*Ctsk*) is shown to have no (Nakamichi et al. 2017) or inhibitory effects (Starczak et al. 2018) on osteoclast activity

formation and reduced bone resorption. Mechanistically, the decrease in bone resorption in *osteocalcin-Vdr*-cOE mice is attributed to reduced RANKL and increased OPG, a decoy receptor for RANKL (Misof et al. 2003; Baldock et al. 2006). This decrease in RANKL expression is rather surprising and not fully understood, as VDR signaling has been described to stimulate RANKL expression in osteogenic cells (Yasuda et al. 1998). To investigate whether the observed bone phenotype was dependent on the FVB/N background, these *osteocalcin-Vdr*-cOE mice were backcrossed and studied in a C57Bl6 background (Triliana et al. 2016). In parallel to the previous study, an increase in cortical and trabecular bone mass is observed in 9-week-old male and female mice and is associated with increased bone formation and reduced bone resorption. However, whereas this phenotype is recapitulated in 20-week-old male mice, no differences are present between 20-week-old female *osteocalcin-Vdr*-cOE mice and their wild-type littermates.

Taken together, changes in *Vdr* signaling in osteogenic cells do not manifestly affect bone homeostasis, although some mutant *Vdr* mice develop a bone phenotype. However, both inactivation and overexpression of *Vdr* result in an elevated bone mass (Gardiner et al. 2000; Yamamoto et al. 2013; Triliana et al. 2016), and it is at present not clear how to reconcile these apparently contradictory findings. A possible explanation is that the divergent experimental settings, such as diet, age, genetic background of the animals, and gene dosage effects (overexpression versus deletion), intervene with the effect of VDR on bone homeostasis. Alternatively, the effect of $1,25(OH)_2D_3$ on bone metabolism might depend on the osteoblastic differentiation stage, as is suggested by the differential response of in vitro cultured osteoblasts and osteocytes to $1,25(OH)_2D_3$ (St John et al. 2014). Therefore, a direct comparison under controlled and identical circumstances (diet, age, background, analysis method) of different transgenic models with *Vdr* inactivation at specific stages of osteoblast differentiation is required to enhance our understanding of the direct VDR effects in osteoblasts under a positive calcium balance.

Vdr Signaling in Growth Plate Chondrocytes Transiently Regulates Bone and Phosphate Homeostasis

As outlined above, chondrocytes differentiate from the same skeletal progenitor cells as osteoblasts and express low levels of *Vdr*. Systemic *Vdr* null mice have expanded epiphyseal growth plates with a widened zone of hypertrophic chondrocytes, due to a reduced apoptosis rate. This impaired induction of caspase-mediated apoptosis of hypertrophic chondrocytes is caused by the low serum phosphate levels in the systemic *Vdr* null mice (Donohue and Demay 2002; Sabbagh et al. 2005). Correction of the hypophosphatemia by a high-calcium/high-lactose diet leads to normalization of the growth plate phenotype (Li et al. 1998; Amling et al. 1999). Accordingly, inactivation of *Vdr* expression by *Col2*-Cre-driven excision does not affect chondrocyte development (*Col2-Vdr*-cKO mice) (Masuyama et al. 2006). However, early in life (postnatal days 3 and 15), *Col2-Vdr*-cKO mice have transiently reduced trabecular bone mass, which is associated with decreased vascular invasion and reduced osteoclast number at the growth plate. Mechanistically, chondrocytic VDR signaling can stimulate osteoclast formation directly by induction of RANKL expression as evidenced in chondrocyte/splenocyte cocultures. Surprisingly, serum phosphate and $1,25(OH)_2D_3$ levels are increased in young *Col2-Vdr*-cKO mice, whereas calcium and PTH levels are normal. These changes can be explained by the lower serum levels of FGF23 in mutant mice, and in vitro cultures confirmed that *Vdr* inactivation in chondrocytes results in reduced osteoblastic expression of *Fgf23*. These decreased circulating FGF23 levels in *Col2-Vdr*-cKO mice lead to elevated renal expression of *Cyp27b1* and of the sodium phosphate cotransporter type IIa, which then cause increased serum levels of $1,25(OH)_2D_3$ and phosphate. Together these data demonstrate that VDR signaling in chondrocytes has endocrine actions and is able to affect bone mass.

Stage-Specific Deletion of Osteoclastic *Vdr* Expression Does Not Manifestly Affect Bone Homeostasis

It is well established that $1,25(OH)_2D_3$ can enhance osteoclast formation in vitro in cocultures of osteoblasts and hematopoietic cells by inducing osteoblastic RANKL production (Nakamichi et al. 2018). However, as osteoclasts express *Cyp27b1* as well as low levels of *Vdr*, it is plausible that osteoclastic VDR expression affects bone resorption in an autocrine or paracrine manner. Recent studies addressed this question by deleting *Vdr* expression either in myeloid cells by use of M lysozyme-driven Cre expression (*LysM-Vdr*-cKO mice) (Verlinden et al. 2019) or in mature osteoclasts by *Cathepsin K*-driven Cre recombination (*Ctsk-Vdr*-cKO mice) (Fig. 2) (Nakamichi et al. 2017; Starczak et al. 2018). VDR inactivation in myeloid cells results in reduced osteoclastic *Vdr* expression and lower induction of *Cyp24a1* in response to $1,25(OH)_2D_3$. However, calcium and bone metabolism are normal in 8-week-old *LysM-Vdr*-cKO mice, and no changes in bone resorption parameters are observed. Correspondingly, in vitro osteoclast formation with hematopoietic cells from *LysM-Vdr*-cKO mice occurs normally, suggesting that osteoclastic *Vdr* expression does not affect osteoclast formation and function (Verlinden et al. 2019). These findings are in agreement with observations in mature osteoclasts. Indeed, Nakamichi et al. reported that 14-week-old *Ctsk-Vdr*-cKO mice have a normal trabecular bone mass and that osteoclast surface is not different from control littermates (Nakamichi et al. 2017). In addition, osteoblast surface and dynamic bone parameters are unaltered arguing against a paracrine role of osteoclastic VDR expression on osteoblast function. However, Starczak et al. described that trabecular bone mass is slightly, but significantly, reduced in 6-week-old *Ctsk-Vdr*-cKO mice (Starczak et al. 2018). Yet, no differences are observed in osteoclast surface, number, or size, and dynamic bone parameters are also similar between *Ctsk-Vdr*-cKO mice and control littermates. After ovariectomy, *Ctsk-Vdr*-cKO mice experience an exacerbated bone loss, which is associated with enhanced osteoclastic activity but not with increased osteoclast formation. Collectively, their data suggest that osteoclastic VDR expression is a positive determinant of bone mass. This apparent discrepancy between the latter studies may point toward a transient role of osteoclastic VDR expression as Starczak et al. studied the bone phenotype of 6-week-old mice, whereas Nakamichi et al. used 14-week-old animals. Alternatively, the observed difference may be gender-related as Starczak et al. used female mice versus male mice in the study of Nakamichi et al. In analogy with the osteoblast-specific *Vdr* knockout models, controlled side-by-side analysis of transgenic models with osteoclastic *Vdr* inactivation will be required to definitively exclude a role for osteoclastic VDR expression in bone homeostasis.

2.3.2 $1,25(OH)_2D_3$ and Bone Metabolism During a Negative Calcium Balance

A negative calcium balance occurs when intestinal calcium absorption does not meet the daily calcium demand to maintain stable serum calcium concentrations and to ensure proper calcium deposition within bone. An interesting model to mimic such a negative calcium balance is the intestine-specific *Vdr* null model where intestinal

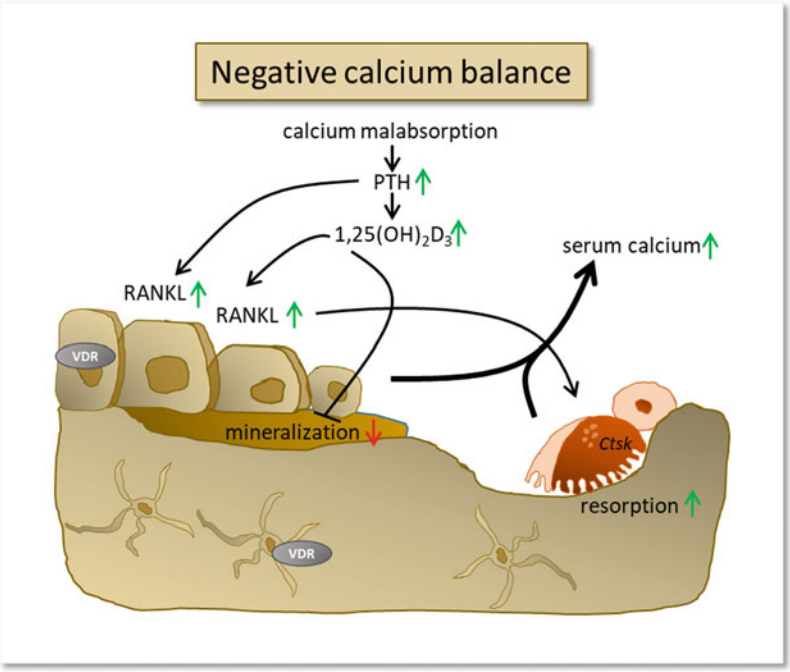

Fig. 3 Effects of a negative calcium balance on bone and the contribution of *Vdr* expression in osteoblasts. In case of intestinal calcium malabsorption, serum levels of PTH and $1,25(OH)_2D_3$ increase and stimulate the osteoblastic production of RANKL, which on its turn enhances osteoclastic bone resorption. In addition, high circulating levels of $1,25(OH)_2D_3$ decrease bone mineralization by transcriptional induction of mineralization inhibitors through *Vdr*-mediated signaling in late osteoblasts and osteocytes. Elevated bone resorption and decreased mineralization contribute both to the transfer of calcium from the bone to the blood and ensure stable serum calcium concentrations

Vdr expression is inactivated by villin-driven Cre expression (*Villin-Vdr*-cKO mice) (Lieben et al. 2012). Intestinal calcium absorption is decreased in *Villin-Vdr*-cKO mice, and in response serum levels of PTH and $1,25(OH)_2D_3$ increase, but calcium and phosphate levels remain within the normal range (Fig. 3). The normal serum calcium levels are in contrast with the hypocalcemia that develops in systemic *Vdr* null mice, suggesting that in *Villin-Vdr*-cKO mice, compensation mechanisms are installed in the kidney and bone to ensure normal serum calcium levels. Indeed, urinary calcium loss is decreased in *Villin-Vdr*-cKO mice due to elevated renal calcium reabsorption. More importantly, *Villin-Vdr*-cKO mice are characterized by a major reduction in bone mass, by enhanced cortical thinning and porosity, by a manifest increase in the amount of unmineralized matrix (hyperosteoidosis) and, finally, by a reduced mineral content of the mineralized bone (hypomineralization). Together these findings indicate a mass transfer of calcium from bone to serum. Increased bone resorption, in response to the high circulating levels of PTH and

$1,25(OH)_2D_3$, contributes to the trabecular and cortical bone loss and to the preservation of normal serum calcium levels. Indeed, suppression of osteoclastic bone resorption by administration of bisphosphonates to *Villin-Vdr*-cKO mice leads to better preservation of bone mass but concurrently reduces serum calcium levels.

In addition to the enhanced bone resorption, also impaired bone mineralization in response to the high serum levels of $1,25(OH)_2D_3$ contributes to this calcium transfer. Osteoblastic bone matrix mineralization is a multistep process, characterized by an initial formation of hydroxyapatite crystals in small extracellular matrix vesicles and subsequent deposition of hydroxyapatite minerals outside the vesicles and accumulation of minerals in the extracellular matrix (Van Driel and Van Leeuwen 2017; Goltzman 2018). This mineralization process is inhibited by high pyrophosphate levels and matrix proteins such as osteopontin (Opn). Pyrophosphate levels are regulated by pyrophosphatase phosphodiesterase enzymes (*Enpp1*, *Enpp3*), which generate pyrophosphate, and by the transmembrane ankylosis protein (encoded by *Ank*), which mediates pyrophosphate transport from intracellular to the extracellular matrix (Goltzman 2018). Interestingly, femoral transcript levels of *Ennp3* and *Ank* are significantly elevated in *Villin-Vdr*-cKO mice (Lieben et al. 2012). In addition, chromatin immunoprecipitation reactions revealed that *Enpp1*, *Enpp3*, and *Ank* are direct transcriptional target genes of $1,25(OH)_2D_3$ signaling. Together these data indicate that $1,25(OH)_2D_3$ inhibits bone mineralization by elevating pyrophosphate levels. In accordance with these findings, lowering the $1,25(OH)_2D_3$-induced pyrophosphate levels by cotreatment with tissue-nonspecific alkaline phosphatase, which reduces pyrophosphate levels, restores mineralization in the presence of $1,25(OH)_2D_3$. In addition, *Opn* gene expression is increased in *Villin-Vdr*-cKO mice, and it is known that $1,25(OH)_2D_3$ induces *Opn* gene expression via VDR-mediated transactivation of the *Opn* gene (Staal et al. 1996). Further study in *Dmp1-Vdr*-cKO mice, with *Vdr* inactivation in mature osteoblasts and osteocytes, revealed that osteocytic *Vdr* expression is involved in the inhibitory effect of $1,25(OH)_2D_3$ on matrix mineralization. Indeed, *Dmp1-Vdr*-cKO mice treated with high doses of $1,25(OH)_2D_3$ do not develop hyperosteoidosis, and the hypercalcemia is less pronounced in comparison with wild-type littermates. In conclusion, these findings revealed that during a negative calcium balance, normocalcemia is maintained at the expense of skeletal integrity. Transfer of bone calcium to serum is ensured by enhanced bone resorption as well as by reduced bone mineralization. Elevated bone resorption in response to PTH and $1,25(OH)_2D_3$ is likely due to enhanced osteoblastic expression of RANKL and not to VDR-mediated signaling in osteoclasts. Indeed, bone loss in *LysM-Vdr*-cKO mice during a negative calcium balance is not different from bone loss in control littermates and suggests that osteoclastic *Vdr* expression does not play an important role in bone homeostasis (Verlinden et al. 2019).

3 Vitamin D and Bone Health

Vitamin D deficiency, as defined by low serum 25(OH)D levels, is common in the elderly and causes secondary hyperparathyroidism which can result in decreased bone density, accelerated bone loss, osteoporosis, and increased risk of fracture (Carmeliet et al. 2015). Risk factors for vitamin D deficiency include inadequate exposure to sunshine, obesity, dark skin tone, and older age (Holick et al. 2011). With older age there is a decline in the ability of the intestine to absorb calcium, a decline in the ability of the kidney to synthesize $1,25(OH)_2D_3$, and an increase in the catabolism of $1,25(OH)_2D_3$ by CYP24A1 which may contribute to the age-related bone loss (Veldurthy et al. 2016). Although controversy exists between the association of vitamin D supplementation and protection against fracture, it is generally recognized that there is a positive relationship between vitamin D sufficiency and a reduction in the risk of fracture (Bouillon and Carmeliet 2018). Several meta-analyses of randomized controlled clinical trials have reported that vitamin D supplementation in conjunction with sufficient calcium intake has clear benefit in protection against fracture, particularly in the elderly who are vitamin D deficient (Chapuy et al. 2002; Larsen et al. 2004; Gallagher et al. 2012; Weaver et al. 2016; Bouillon and Carmeliet 2018; Macdonald et al. 2018). Vitamin D deficiency is defined by the National Institute of Medicine [now known as the National Academy of Medicine (NAM)] as serum 25(OH)D levels below 50 nM (20 ng/mL) (Ross et al. 2011). However, the Endocrine Society guidelines suggest that a threshold of 75 nM (30 ng/mL) is necessary to maintain bone health (Gallagher et al. 2012). It is not clear, however, that optimal vitamin D levels are the same for Caucasians, Black Africans, and Asians. One problem has been that assays for measuring 25(OH)D vary. Efforts are currently being made to standardize results from different laboratories and more laboratories are implementing liquid chromatography/mass spectrometry (LC/MS) to measure vitamin D metabolites (Christakos et al. 2019). Thus, in the future there may be more of a consensus for optimal 25(OH)D levels for different groups. For vitamin D supplementation, the NAM recommends 600 IU/day for ages 1–70 and 800 IU/day for those over 70 as well as 700–1,300 mg calcium/day for adults (Ross et al. 2011). A combination of vitamin D and calcium together with pharmacological intervention [bisphosphonates and RANKL inhibitor (antiresorptive compounds) and PTH (anabolic drug, teriparatide)] has been recommended for the treatment of osteoporotic patients (Rizzoli 2018). Thus correcting vitamin D deficiency together with sufficient calcium intake may optimize pharmacological treatment of osteoporosis.

4 Conclusion

Vitamin D-regulated intestinal calcium absorption is essential in order to maintain calcium homeostasis and skeletal integrity. When calcium cannot be maintained by intestinal calcium absorption (e.g., under conditions of inadequate calcium intake, vitamin D deficiency, or diminished absorption), $1,25(OH)_2D_3$ together with PTH

can stimulate osteoclastogenesis and mobilize calcium from bone and can also enhance calcium reabsorption from the renal distal tubule in order to maintain circulating calcium levels and bone homeostasis. These findings emphasize the need to use a combination of calcium and vitamin D in order to prevent or treat osteoporosis, since vitamin D alone may negatively affect bone during a negative calcium balance. During a positive calcium balance, although the mechanisms are not clearly defined, $1,25(OH)_2D_3$ can modulate bone formation by acting on certain stages of osteoblasts. Thus, through direct and indirect effects, $1,25(OH)_2D_3$ is a key factor in bone mineralization. Although the mechanisms by which vitamin D deficiency contributes to osteoporosis remain to be defined, it is generally recognized that vitamin D supplementation together with sufficient calcium intake has clear benefit in protection against fracture, particularly in the elderly who are vitamin D deficient. Future studies related to mechanisms by which inadequate vitamin D contributes to osteoporosis and the identification of novel targets of $1,25(OH)_2D_3$ action in intestine, kidney and bone involved in calcium homeostasis and changes that occur with aging will provide an increased understanding of age-related dysregulation of calcium homeostasis and may suggest candidates for targeted therapies to sustain calcium balance in the elderly.

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