Best Practice & Research Clinical Endocrinology & Metabolism 25 (2011) 561–572

Contents lists available at ScienceDirect

Best Practice & Research Clinical Endocrinology & Metabolism

journal homepage: www.elsevier.com/locate/beem

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Calcemic actions of vitamin D: Effects on the intestine, kidney and bone

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Keywords:
vitamin D
intestinal calcium transport
bone homeostasis
renal calcium reabsorption

The analysis of mice that lack systemically the actions of the active form of vitamin D, $1,25(OH)_2D$, has shown that $1,25(OH)_2D$ is an essential regulator of calcium homeostasis and that its actions are aimed at maintaining serum calcium levels within narrow limits. Especially the stimulation of intestinal calcium transport by $1,25(OH)_2D$ is important for calcium and bone homeostasis. The involved transporters are however still elusive. The targeted deletion of $1,25(OH)_2D$ action in chondrocytes has provided compelling evidence for a paracrine control of bone development and endocrine regulation of phosphate homeostasis by $1,25(OH)_2D$. Targeting vitamin D receptor (VDR) function in other tissues will further enhance our understanding of the cell-type specific action of $1,25(OH)_2D$. In this review, we will discuss the current understanding and remaining questions concerning the calcemic actions of $1,25(OH)_2D$ in the intestine, kidney and bone, with special focus on the evidence obtained by the use of transgenic mouse models.

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doi:10.1016/j.beem.2011.05.008

Introduction

Normal serum Ca^{2+} levels are essential to guarantee the optimal functioning of multiple vital processes, and they depend on the coordinated handling of calcium in several tissues including the intestine, the site of net absorption; the kidney, the site of net secretion; and the skeleton, the largest repository of calcium in the body. The active form of vitamin D, $1,25(OH)_2D$, plays a crucial role in regulating multiple processes in these target tissues, and in doing so, in the maintenance of normal serum Ca^{2+} levels.

The regulation of serum Ca^{2+} and $1,25(OH)_2D$ levels are (partly) interconnected and comprise several feedback loops. Briefly, a decrease in extracellular Ca^{2+} levels results in the release of parathyroid hormone (PTH). PTH attempts to restore serum calcium levels by increasing renal calcium reabsorption as well as bone resorption and by promoting the hydroxylation of 25(OH)D to its biologically active form, $1,25(OH)_2D$, via the induction of renal *Cyp27b1* expression. $1,25(OH)_2D$ in turn, increases intestinal calcium absorption, renal calcium reabsorption and bone resorption. Overcompensation is avoided by correction of the eliciting factor (extracellular Ca^{2+}), and because $1,25(OH)_2D$ induces a negative feedback loop. Indeed, $1,25(OH)_2D$ inhibits the expression of *Pth* and *Cyp27b1*, and it increases the expression of *Cyp24a1*, the enzyme that degrades $1,25(OH)_2D$. In addition, $1,25(OH)_2D$ induces fibroblast growth factor 23 (*Ffg23*) expression in bone, which negatively affects *Cyp27b1* and *Pth* (Fig. 1).[1] A more comprehensive review on this topic will be given elsewhere in this issue.

Insight in the actions of $1,25(OH)_2D$ has been obtained by the analysis of transgenic mouse models, the most important being mice with impaired $1,25(OH)_2D$ signaling either due to inactivating mutations in the vitamin D receptor (*Vdr*)[2–5] or in *Cyp27b1*.[6,7] In accordance with the anticipated role of $1,25(OH)_2D$ in the regulation of calcium and phosphate homeostasis, *Cyp27b1* and *Vdr* null mice develop hypocalcemia, hyperparathyroidism, hypophosphatemia, and rickets. They only differ from each other in serum $1,25(OH)_2D$ levels; these are undetectable in *Cyp27b1* null mice, but very high though ineffective, in the *Vdr* null strains.

In this review, we will discuss the current understanding and remaining questions concerning the action of $1,25(OH)_2D$ in the intestine, kidney and bone, with special focus on the evidence generated by studying the consequences of genetic inactivation of enzymes and receptors involved in vitamin D metabolism in mice.

Intestinal calcium absorption

Dietary calcium absorption is crucial for calcium homeostasis, as it is the predominant source of calcium acquisition. In this section, the proposed mechanisms and recent progress in the understanding of intestinal calcium transport will be reviewed.

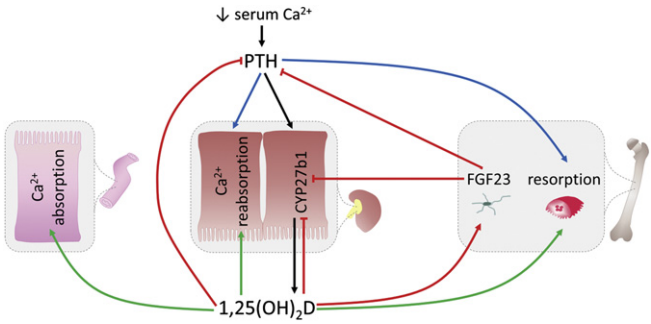

Fig. 1. Regulation of calcium and vitamin D homeostasis. A reduction in extracellular Ca^{2+} levels stimulates the release of PTH, which induces calcemic responses (blue arrow) and promotes the formation of the active form of $1,25(OH)_2D$ (black arrow). This hormone increases calcium absorption in the intestine, calcium reabsorption in the kidney and calcium release from bone (green arrows); and initiates negative feedback loops to avoid hypercalcemia (red arrow).

Passive versus active, 1,25(OH)$_2$D-mediated, calcium transport in the intestine

Calcium transport in the intestine is considered to comprise an active and a passive mechanism. The active transcellular pathway provides an efficient means of calcium absorption, occurs predominantly in the duodenum, is to a large extent regulated by 1,25(OH)$_2$D, and requires energy. The passive route involves paracellular, concentration-dependent diffusion that takes place throughout the intestine and does not require energy.[1]

The contribution of passive versus active transport to the total calcium absorption largely depends on the dietary calcium content and changes with age. Active calcium transport delivers most of the absorbed calcium during low dietary calcium intake, whereas the contribution of the active pathway declines when calcium intake is high.[4]

Moreover, large differences in the balance between paracellular and transcellular transport are reported with age. During the first weeks after birth, intestinal calcium absorption depends on passive diffusion and not on a 1,25(OH)$_2$D-mediated process,[8] a finding that is confirmed in suckling rats.[9] The lack of response of the intestine to 1,25(OH)$_2$D can be explained by the relative absence of the VDR in the intestine during early neonatal life.[10] During growth, the paracellular pathway is gradually replaced by a 1,25(OH)$_2$D-dependent saturable component, which is fully active by the time of weaning in rats. This observation may explain the coincidence of the development of hypocalcemia in *Vdr* and *Cyp27b1* null mice with the time of weaning.[3,7] Another possible explanation for the absence of hypocalcemia before weaning is that the high calcium/lactose intake during lactation prevents earlier development. In accordance herewith, the onset of the bone defects coincides with the time of weaning, even when this time point is accelerated (2 weeks) or postponed (4 weeks).[5] At a later age, the efficacy of 1,25(OH)$_2$D-mediated calcium transport decreases, and it is significantly compromised from 60 years on in humans[11] and in 12-months-old rats.[12] This defect is mainly attributed to the low 1,25(OH)$_2$D levels in serum, which are frequently noticed in the elderly. Intestinal 1,25(OH)$_2$D resistance is also suggested as a contributing factor, whereas manifest changes in intestinal VDR levels are not observed.[12]

In summary, the relative contribution of the active, 1,25(OH)$_2$D-mediated transport versus the passive, paracellular pathway to the overall intestinal calcium absorption varies, and largely depends on the diet and age.

1,25(OH)$_2$D action is crucial for adequate intestinal calcium transport

The importance of 1,25(OH)$_2$D for optimal calcium transport in the small intestine is convincingly demonstrated in mice. Indeed, intestinal calcium absorption is reduced by 40% in *Vdr* null mice as measured by oral gavage[4] and by the *in situ* ligated loop technique.[13] Conversely, 1,25(OH)$_2$D treatment increases intestinal calcium transport.[4] In addition, reintroducing the *Vdr* specifically in the intestine of *Vdr* null mice almost completely rescues the *Vdr* null phenotype, i.e. intestinal calcium absorption, serum calcium and phosphate levels, as well as growth plate morphology are normal compared to *Vdr* null littermates.[14,15] Rescue of the calcium and bone homeostasis is also achieved by feeding *Cyp27b1*[16–18] and *Vdr*[19,20] null mice a high calcium/lactose diet from weaning onwards.

These findings underscore that the major function of 1,25(OH)$_2$D is to increase intestinal calcium absorption during low/normal calcium intake, and that the changes in serum and bone parameters related to *Vdr* deficiency are secondary to the compromised 1,25(OH)$_2$D-mediated intestinal calcium absorption.

Calcium transporters involved in 1,25(OH)$_2$D-mediated intestinal calcium transport

It has been suggested that 1,25(OH)$_2$D regulates primarily the transcellular pathway of intestinal calcium transport, consisting of calcium entry through an apical calcium transporter, followed by intracellular buffering and an energy-dependent calcium extrusion by the plasma-membrane calcium ATPase (PMCA$_{1b}$). Evidence for this transcellular model is provided by the finding that *in vitro* inhibition of the basolateral calcium ATPase manifestly reduces the 1,25(OH)$_2$D-stimulated calcium transport.[21] Two members of the apical transient receptor potential vanniloid (TRPV) family, TRPV5 and especially TRPV6, and the calcium-binding protein Calbindin-D$_{9k}$, were identified as plausible candidates to mediate 1,25(OH)$_2$D-dependent active transport. This claim is mainly based on the

observation that $1,25(OH)_2D$ regulates their expression. Indeed, $1,25(OH)_2D$ treatment of mice increases the intestinal expression of *Trpv6*, *Trpv5*, and *Calbindin-D_{9k}*, whereas *$Pmca_{1b}$* is only marginally upregulated. Conversely, the decrease in intestinal calcium absorption in *Vdr* null mice is accompanied by a reduction in the expression levels of these transport proteins.[4] These gene-expression data support the hypothesis that $1,25(OH)_2D$ mediates active intestinal calcium absorption, by regulating *Trpv6*, *Trpv5* and *Calbindin-D_{9k}* expression (Fig. 2, upper panel).

Recent genetic studies, however, question the critical role of TRPV6, TRPV5 and Calbindin-D_{9k} in active, $1,25(OH)_2D$-mediated intestinal calcium absorption. Indeed, duodenal calcium absorption is not decreased in *Trpv6*,[22,23] *Calbindin-D_{9k}*,[22,24] or *Calbindin-D_{9k}/Trpv6*[22] double null mice, fed a normal calcium diet. Hence, serum calcium levels and bone homeostasis are not affected in these genetic models. When *Trpv6* and *Calbindin-D_{9k}/Trpv6* double null mice are however receiving a low calcium diet, they respond with a less pronounced increase in duodenal calcium transport compared to wild-type mice,[22] and *Trpv6* null mice develop hyperosteoidosis in these dietary conditions.[25] A second argument in support of TRPV6 and Calbindin-D_{9k} not being critical for $1,25(OH)_2D$-mediated intestinal calcium transport, is that $1,25(OH)_2D$ treatment increases calcium transport to a similar extent in *Trpv6* and *Calbindin-D_{9k}/Trpv6* double null mice as in wild-type mice.[22,27] Also TRPV5 seems not essential for intestinal calcium absorption, as *Trpv5* ablation even results in increased intestinal calcium transport. The change in calcium absorption is caused by the increased $1,25(OH)_2D$ levels and compensates for the renal calcium wasting due to *Trpv5* inactivation.[28,29] Together, these data suggest that TRPV6, TRPV5 and Calbindin-D_{9k} are redundant for [$1,25(OH)_2D$-mediated] intestinal calcium absorption when dietary calcium intake is normal, yet TRPV6 has a limited but definite role when dietary intake is low.

It is evident that $1,25(OH)_2D$ increases intestinal calcium transport, but the generation of several transgenic mouse models has shown that the suggested transporters are not critically involved, indicating that the molecular mediators remain to be identified. Possibly, other (unknown) apical transport proteins are involved. On the other hand, it has recently been proposed that $1,25(OH)_2D$ may facilitate the paracellular pathway. $1,25(OH)_2D$ treatment increases Claudin (Cldn) 2 and 12 expression, which are suggested to act as paracellular channels for cations. Their protein levels are, conversely, decreased in *Vdr* null mice. Moreover, *in vitro* overexpression and knockdown experiments have provided functional arguments that CLDN2 and -12 contribute to $1,25(OH)_2D$-mediated calcium transport.[30] Solid genetic evidence, by studying $1,25(OH)_2D$-mediated intestinal calcium transport in *Cldn2* and *Cldn12* null mice, is however still lacking. $1,25(OH)_2D$ also decreases the gene expression of several adhesion molecules in the duodenum of rats, such as *Cldn3* and *Cadherin 14*.[31] Together, these studies provide evidence for the regulation of paracellular calcium transport by $1,25(OH)_2D$, but the *in*

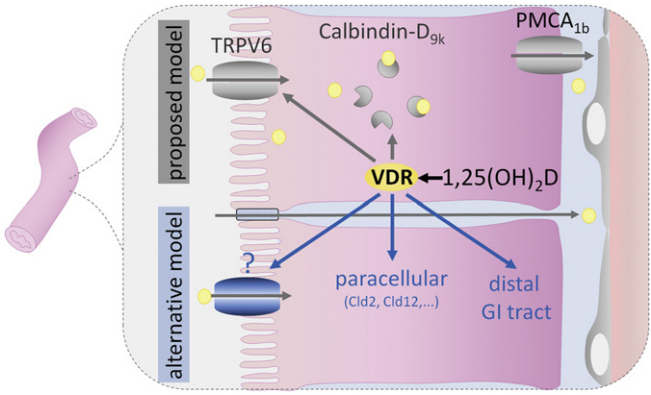

Fig. 2. $1,25(OH)_2D$-mediated intestinal calcium absorption. It is well established that $1,25(OH)_2D$ stimulates intestinal calcium transport, but the molecular mediators are still elusive. Transgenic mouse studies have shown that the suggested proteins, TRPV6 and Calbindin-D_{9k}, are redundant (proposed model). Recently, $1,25(OH)_2D$ has been shown to facilitate paracellular calcium transport by increasing the expression of *Cld2* and *Cld12*, though conclusive *in vivo* evidence is still lacking. Possibly, other (unidentified) proteins or calcium transporters in the distal part of the gastrointestinal (GI) tract are also involved (alternative model).

vivo significance remains to be determined (Fig. 2, lower panel). Uncertainty has arisen not only concerning the type of transport and the identity of the molecular mediators, but also about the part of the intestine where $1,25(OH)_2D$-mediated calcium transport occurs. The duodenum has generally been considered as the major side of $1,25(OH)_2D$-mediated calcium transport. However, it has recently been suggested that calcium transport in the more distal parts of the gastrointestinal tract may also contribute significantly to active intestinal calcium transport.[32]

In conclusion, the finding that the *Vdr* null phenotype is prevented by dietary calcium/lactose supplements[16] or by reintroducing intestinal *Vdr* expression,[14,15] underscores the importance of $1,25(OH)_2D$ action on intestinal calcium absorption when dietary calcium intake is low to normal. To answer the question whether the VDR-mediated pathway is critical during normal calcium intake, and whether compensatory mechanisms are initiated when $1,25(OH)_2D$-mediated calcium transport fails, additional studies are required. Moreover, the transporters and mechanisms involved in $1,25(OH)_2D$-mediated intestinal calcium transport remain to be characterized.

Renal calcium reabsorption

The kidney plays a critical role in calcium homeostasis. Approximately 50% of free, ionized Ca^{2+} in serum is filtered through the glomerulus. 85% of the filtered Ca^{2+} is passively reabsorbed. Reabsorption of the remaining Ca^{2+} is regulated by an active transport mechanism in the distal nephron. The process resembles intestinal calcium absorption, albeit by means of different molecular mediators, and consists of calcium entry through TRPV5, cytosolic transfer by binding to (Calbindin-D_{9k} and) Calbindin-D_{28k}, and extrusion by the Na^+/Ca^{2+} exchanger (NCX1) and $PMCA_{1b}$[1] (Fig. 3).

In contrast to the limited role of TRPV6 in intestinal calcium absorption, TRPV5 is critical and the rate-limiting step in renal calcium reabsorption. *Trpv5* inactivation in mice results in a manifest decrease in renal calcium reabsorption, leading to severe hypercalciuria.[28] Normocalcemia is maintained by a compensatory increase in $1,25(OH)_2D$, resulting in calcium hyperabsorption in the intestine.[29] Klotho, a type I membrane β-glucosidase-like protein, ensures TRPV5 activity by entrapping TRPV5 to the plasma membrane via the hydrolysis of extracellular sugar residues.[33] Accordingly, *Klotho* null mice exhibit renal calcium loss.[34] Calbindin-D_{9k}[24,26] and Calbindin-D_{28k}[35] are redundant for urinary calcium reabsorption. However, combined deletion of *Calbindin-D_{9k}* and *Calbindin-D_{28k}* and dietary calcium restriction,[36] or inactivation of *Calbindin-D_{28k}* and *Vdr* but with a normal calcium diet[37] does result in severe abnormalities in calcium homeostasis and in premature lethality. The *in vivo* role of NCX1 in renal calcium absorption could not be assessed due to embryonic lethality.[38] In conclusion, genetic evidence confirms that TRPV5 is the rate-limiting step in renal calcium reabsorption, and that Klotho is important to activate TRPV5, whereas Calbindin-D_{28k} and Calbindin-D_{9k} are largely redundant.

Renal calcium reabsorption is, among many factors, regulated by $1,25(OH)_2D$.[39] Indeed, renal calcium reabsorption is compromised in *Vdr* null mice, demonstrated by the inappropriately high urinary calcium levels. These are observed in hypocalcemic mice when they receive a normal calcium, but also in normocalcemic conditions when they are given a rescue diet.[2,40] This is associated with a reduction in *Calbindin-D_{9k}*, whereas *Trpv5* and *Calbindin-D_{28k}* are only affected in some *Vdr* null strains.[2,4,40] In *Cyp27b1* null mice, *Trpv5*, *Calbindin-D_{9k}*, *Calbindin-D_{28k}* and *Ncx1* levels are reduced, and

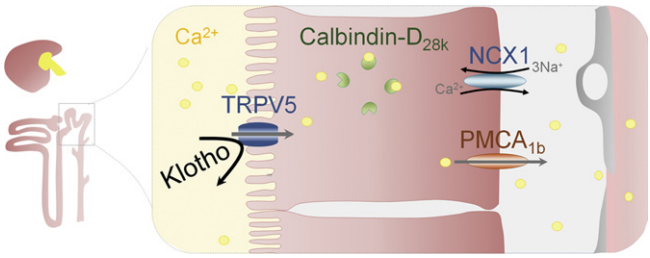

Fig. 3. Active calcium reabsorption in the kidney. Active calcium absorption occurs in the distal nephron and involves Ca^{2+} entry through TRPV5, intracellular calcium binding to Calbindin-D_{28k} and basolateral extrusion by NCX1 and $PMCA_{1b}$. TRPV5 is entrapped to the apical membrane via hydroxylation by Klotho.

normal levels are restored by $1,25(OH)_2D$ treatment.[41] It is however still elusive to what extent the $1,25(OH)_2D$-mediated renal calcium reabsorption adds to calcium homeostasis under physiological conditions, an issue that should be addressed by tissue-specific *Vdr* inactivation.

Thus, in contrast to the poor characterization of the intestinal calcium transport proteins, the mechanisms that underlie renal calcium transport are well characterized, and point to TRPV5 as a crucial mediator of calcium reabsorption.

Bone homeostasis

The lack of systemic $1,25(OH)_2D$ activity is associated with manifest bone defects including rickets and osteomalacia. These bone abnormalities are only noticed after weaning, indicating that VDR signaling is redundant during embryonic development and lactation.[5,7,42] Moreover, the observation that this phenotype is completely prevented by adapting dietary calcium content[18,19] or by reintroducing the *Vdr* in the intestine,[14,15] indicates that the most critical role of $1,25(OH)_2D$ is to enhance intestinal calcium absorption, and that $1,25(OH)_2D$ signaling in bone cells is not a prerequisite for bone development and homeostasis when intestinal calcium transport is guaranteed. Nevertheless, multiple bone cells express the VDR,[2,43] and recent studies underscore the hypothesis that $1,25(OH)_2D$ action in bone cells directly controls specific aspects of bone and mineral homeostasis. The next sections highlight the contributions of $1,25(OH)_2D$ to growth plate, bone and phosphate homeostasis (Fig. 4).

1,25(OH)$_2$D action in chondrocytes

The major skeletal hallmark of the systemic loss of $1,25(OH)_2D$ signaling is rickets, a pathology characterized by an expansion and widening of the growth plate with marked disorganization of the chondrocytes. These growth plate abnormalities most likely result from hypophosphatemia, which impairs phosphate-induced activation of Erk1/2 phosphorylation, and leads to decreased caspase-mediated apoptosis and consequently to expansion of the hypertrophic chondrocytes layer.[44–46] The hypothesis that hypophosphatemia underlies the growth plate pathology is supported by the finding that several hypophosphatemic conditions, independent of the cause, result in similar growth plate abnormalities.[45] In addition, the development of rickets is prevented in normophosphatemic *Vdr* and *Cyp27b1* null mice, i.e. during suckling and when fed a rescue diet.[3,6,18,20] Final proof was provided by chondrocyte-specific *Vdr* and *Cyp27b1* deletion mutants which do not show any signs of rickets.[47,48] Thus, hypophosphatemia, and not the lack of the VDR *per se*, causes rickets.

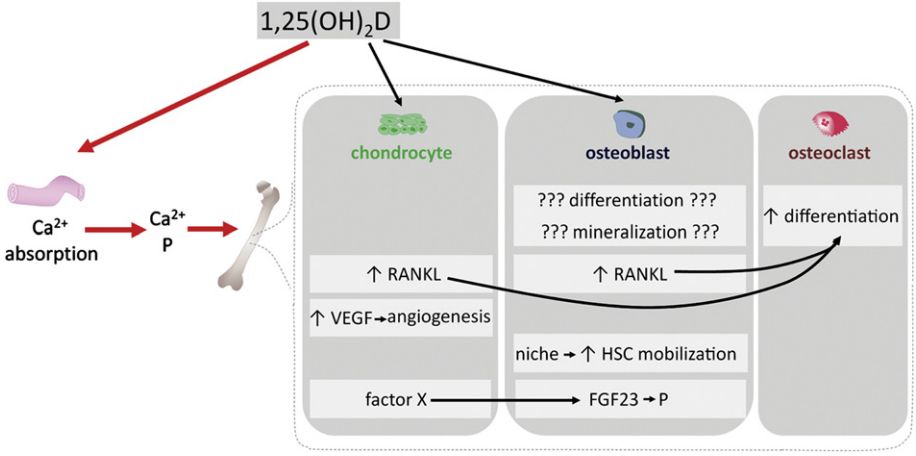

Fig. 4. Direct and indirect effects of $1,25(OH)_2D$ on bone homeostasis. The role of $1,25(OH)_2D$ in bone homeostasis is largely indirect by controlling serum calcium and phosphate levels; calcium via increased intestinal absorption and phosphate via the suppression of PTH. However, $1,25(OH)_2D$ does exert paracrine and endocrine effects on chondrocytes and regulates indirectly, via the osteoblasts, the formation of osteoclasts and the mobilization of HSC, as schematically depicted.

VDR and CYP27b1 actions in chondrocytes have thus no manifest cell autonomous role in chondrocyte differentiation,[47,48] yet their action is required to counterbalance the negative effects of hypophosphatemia on the growth plate in mutant mice lacking the sodium-phosphate cotransporter type II, *Npt2a*.[49] These mice suffer from persistent hypophosphatemia, but the increase in $1,25(OH)_2D$ serum levels results in the recovery of rickets. In conclusion, $1,25(OH)_2D$ signaling in chondrocytes seems redundant when mineral homeostasis is not manifestly disturbed, but is critical to prevent the rachitic growth plate abnormalities when hypophosphatemia is present.

Moreover, the VDR expressed in the 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 $1,25(OH)_2D$ controls vascular invasion and osteoclast formation in the primary ossification center by increasing the angiogenic factor VEGF (vascular endothelial growth factor) and the pro-osteoclastic factor RANKL [receptor activator of nuclear factor NF-$_k\beta$ (RANK) ligand].[47] Similar findings were observed when *Cyp27b1* was inactivated in chondrocytes.[48] The decrease in blood vessel density and osteoclast number in the primary spongiosa of chondrocytes-specific *Vdr* and *Cyp27b1* null mice persists during fetal and early post natal life, and results in an increase in trabecular bone volume, which is however transient and completely normalized at 8-wk of age.[47,48]

Together, these observations suggest that $1,25(OH)_2D$ signaling in chondrocytes is not essential for chondrocyte differentiation, but indicates that this pathway controls bone development in a paracrine manner. It also contributes significantly to the endocrine function of the skeleton, which will be discussed further on.

$1,25(OH)_2D$ action in osteoblasts

The bone phenotype of hypocalcemic *Vdr* null mice is not only characterized by growth plate defects, but also by unbalanced bone remodeling and osteomalacia. More specifically, a high number of osteoblasts are lining the bone surfaces but mineral apposition is impaired. As a result, total bone mass is increased, due to an excess in unmineralized bone matrix.[7,20] These changes likely result from the anabolic action of the increased PTH levels. Evidence for this hypothesis is provided by the increased levels of runt-related transcription factor 2 (Runx2), a crucial factor for osteoblast differentiation, and known to mediate the anabolic consequences of PTH.[50,51] Normalizing serum calcium and PTH levels in *Vdr* null mice reverses the increase in *Runx2* to normal, and reduces osteoblast number to normal/subnormal levels.[16,20] To date, the relationship between PTH, calcium and Runx2 has been extensively studied but remains incompletely understood, particularly regarding the transcriptional regulation.

Defects in $1,25(OH)_2D$ signaling affects osteoblast differentiation and bone formation, but predominantly via its role in the intestine.[14,15,20] Yet the VDR may also exert some direct, although subtle, effects on cells of the osteoblastic lineage. Indeed, overexpression of the VDR in differentiating osteoblasts in mice increases bone volume. This phenotype is accompanied by an increase in osteoblast activity, as demonstrated by dynamic bone histomorphometry, and by a reduction in osteoclast number.[52] *In vitro* experiments have also provided evidence for the direct regulation of osteoblast differentiation by VDR action, although contradictory results have been reported. Cultures of calvarial osteoblasts isolated from *Vdr* null mice display an increased differentiation potential,[53] yet opposite results are reported for bone marrow stromal cell cultures.[16] The treatment of osteoblastic cell lines with $1,25(OH)_2D$ has even further complicated the story, as differentiation- and species-dependent effects are observed. Indeed, $1,25(OH)_2D$ induces the differentiation of human pre-osteoblasts,[54] whereas in rat osteoblastic cells, $1,25(OH)_2D$ enhances only the differentiation of late stage osteoblasts but inhibits differentiation when given to early pre-osteoblasts.[55] The differentiation of mouse osteoblastic cells is at all times inhibited by $1,25(OH)_2D$ treatment, independent of the differentiation stage.[56] Additional studies are needed to assess the *in vivo* outcome of manipulating $1,25(OH)_2D$ signaling in osteoblasts, and will require the generation of osteoblast-specific *Vdr* null mice, with inactivation of the *Vdr* at specific stages of differentiation.

Normalizing serum calcium and phosphate levels completely rescues the osteomalacia in *Vdr* null mice, indicating that the mineralization defects in conditions lacking $1,25(OH)_2D$ signaling are caused by an insufficient supply of mineral to the bone matrix.[14,20] On the other hand, hypervitaminosis D is also often associated with bone mineralization defects, despite elevated serum calcium and phosphate levels.

Indeed, continuous administration of high doses of $1,25(OH)_2D$ causes bone loss and results in the presence of abundant unmineralized bone matrix in rats.[57] Defects in the metabolism of $1,25(OH)_2D$, like in *Cyp24* null mice, results in hypervitaminosis D, hypercalcemia and impaired bone matrix mineralization. The bone phenotype and hypercalcemia are rescued by genetic inactivation of *Vdr* signaling.[58] Hypervitaminosis D is also caused by interfering with the regulation of $1,25(OH)_2D$ synthesis. Indeed, inactivation of *Fgf23*[59] is associated with hypervitaminosis D, hypercalcemia, hyperphosphatemia, and osteoid excess. Thus, low as well as excessive $1,25(OH)_2D$ levels are associated with impaired bone matrix mineralization, the first being due to inadequate mineral supply and the latter likely being caused by direct effects of $1,25(OH)_2D$. Validation of the inhibitory action of $1,25(OH)_2D$ on bone matrix mineralization, as well as the underlying molecular mechanisms, require further investigation.

Osteoblasts are also part of specific niches in the bone microenvironment in which the hematopoietic stem cells (HSC) reside. It has recently been shown that the number of HSC in the bone marrow of *Vdr* null mice is manifestly reduced, whereas their number is increased in the spleen; changes that are largely corrected by dietary calcium treatment. This finding indicates that VDR signaling, via its effect on extracellular calcium, is involved in restricting splenic hematopoiesis and promoting retention of HSC in the bone marrow.[60] In addition, VDR signaling in osteoblasts, and not extracellular calcium, is required to suppress the osteoblastic niche and induce mobilization of HSC in the circulation, in response to adrenergic stimulation. Indeed, mobilization of the HSC is hampered in *Vdr* null mice, even on a rescue diet.[61]

Thus, although $1,25(OH)_2D$ affects the skeleton mainly via its actions on the intestine and the regulation of mineral homeostasis, directs effects are also observed, especially related to osteoblast differentiation, bone matrix mineralization, and the osteoblastic niche.

1,25(OH)$_2$D action in osteoclasts

$1,25(OH)_2D$ regulates osteoclast differentiation by acting on the osteoblasts; more precisely, by increasing in osteoblastic cells the expression of the pro-osteoclastic factor RANKL and reducing the expression of the anti-osteoclastic factor osteoprotegerin (OPG).[62] Indeed, co-culture experiments of osteoblasts and splenocytes have shown that $1,25(OH)_2D$ is only able to induce osteoclast formation *in vitro* when the VDR is present in the osteoblasts, whereas a functional VDR is not required in the osteoclast precursors. Other osteoclastogenic hormones, including PTH, induce osteoclast formation regardless of the presence of VDR.[63] This finding may explain the presence of osteoclasts in *Vdr* and *Cyp27b1* null mice; yet it remains elusive why the number was not increased, as one would expect when hyperparathyroidism is present.[16,20] More precisely, PTH levels were manifestly increased in *Vdr* null mice, the number of osteoclasts, however, was not altered[20] or even reduced.[18] This finding may be explained by a blunted skeletal response to PTH, a response which is frequently observed following prolonged hyperparathyroidism. Although the pathogenesis of this desensitization remains to be elucidated, it is most likely attributed to PTH receptor downregulation.[64] Another possible explanation is that the absence of VDR in bone cells hampers the response to PTH. The transcriptional control of *Rankl* expression by PTH and $1,25(OH)_2D$ has indeed been shown to involve a common enhancer, that contains a functional cAMP-binding domain that mediates PTH signaling, in addition to a vitamin D responsive element. Deletion of this region diminishes the PTH- and $1,25(OH)_2D$-induced *Rankl* expression and osteoclastogenesis, which suggests a cross talk between PTH and $1,25(OH)_2D$.[65]

Thus, $1,25(OH)_2D$ is not essential for osteoclast formation and bone resorption, yet the hampered response to PTH in *Vdr* null mice remains to be fully characterized.

1,25(OH)$_2$D and the skeletal control of phosphate homeostasis

FGF23, produced by differentiated osteoblasts and osteocytes in bone, is an important regulator of phosphate homeostasis.[66] FGF23, like PTH, induces renal phosphate wasting by suppressing renal NPT2a expression. FGF23 exerts its function at the transcriptional level, PTH by inducing protein internalization and degradation.[67]

Fgf23 expression in bone is positively regulated by $1,25(OH)_2D$, evidenced by increased *Fgf23* expression after $1,25(OH)_2D$ treatment,[68] and undetectable FGF23 levels in *Vdr* null mice.[69] Skeletal

VDR action is, however, not an absolute prerequisite for the induction of *Fgf23*, as *Fgf23* levels did increase in *Vdr* null mice receiving a high calcium diet.[69] $1,25(OH)_2D$ increases FGF23 levels via two pathways, i.e. by direct stimulation of *Fgf23* expression in osteoblasts and by activating the release of a soluble factor from chondrocytes, that in turn increases *Fgf23* expression in osteoblasts.[47] As a consequence, FGF23 levels were reduced in chondrocyte-specific *Vdr* and *Cyp27b1* null mice.[47,48] To date, the exact mechanism of *Fgf23* induction by $1,25(OH)_2D$ is still not fully characterized.

Vice versa to the positive regulation of FGF23 by $1,25(OH)_2D$, FGF23 suppresses $1,25(OH)_2D$ levels, as it decreases the expression of *Pth*[70] and *Cyp27b1*[59] (Fig. 1). Consequently, *Fgf23* null mice do not only display hyperphosphatemia, due to insufficient suppression of renal phosphate reabsorption, but they also exhibit hypervitaminosis D, leading to hypercalcemia.[59] A similar phenotype is observed in *Klotho* null mice,[71,72] as Klotho is required for efficient FGF23 signaling.[73] As a consequence of the hyperphosphatemia, ectopic calcifications develop. Reversing hypercalcemia and hyperphosphatemia via the elimination or reduction of $1,25(OH)_2D$ activity by dietary or genetic means, rescues the ectopic calcifications.[74,75] Additionally, inactivation of *Npt2a* in *Fgf23* and *Kotho* null mice shows that merely the normalization of phosphate levels was sufficient,[76,77] indicating that the reduction of phosphate levels in the presence of hypercalcemia prevents the ectopic calcifications.

$1,25(OH)_2D$ also regulates the expression of the other phosphaturic hormone, PTH, as part of a negative feedback loop (Fig. 1). Restricted deletion of the *Vdr* in parathyroid gland of mice results in a moderate increase in PTH levels, despite normal levels of serum calcium and phosphate. This finding confirms that VDR signaling in the parathyroid gland is indeed involved in the suppression of *Pth*.[78]

Phosphate homeostasis is thus, in part, regulated by $1,25(OH)_2D$-induced *Fgf23* expression in the skeleton.

Conclusion

The use of transgenic mouse models that lack $1,25(OH)_2D$ signaling systemically and that only exhibit VDR activity in the intestine, has greatly contributed to the current insight in the function of $1,25(OH)_2D$. These studies have shown that $1,25(OH)_2D$ especially targets intestinal calcium absorption during low/normal calcium intake, and that the $1,25(OH)_2D$-mediated pathway of intestinal calcium transport is crucial to preserve normal calcium and bone homeostasis. Moreover, the generation of chondrocyte-specific deletion mutants has demonstrated that $1,25(OH)_2D$ has no manifest cell autonomous function in the chondrocytes, but exerts rather paracrine and endocrine functions that regulate bone and phosphate homeostasis. In a next step, the generation of other mutants that target VDR function in a cell-specific manner will provide tools to understand thoroughly the *in vivo* contribution of direct $1,25(OH)_2D$ action on specific tissues.

Research agenda

- To assess the relative importance of $1,25(OH)_2D$-mediated intestinal calcium absorption to calcium homeostasis, when VDR signaling is functional in other calcemic target tissues including the kidney and bone.
- To identify the calcium transporters involved in $1,25(OH)_2D$-mediated intestinal calcium absorption.
- To determine the contribution of the VDR-mediated pathway to the overall calcium reabsorption in the kidney.
- To evaluate the direct effect of VDR signaling on osteoblast differentiation *in vivo*.
- To confirm the inhibitory action of excess $1,25(OH)_2D$ on bone matrix mineralization, as well as the characterization of the underlying molecular mechanisms.
- To characterize the reduced response to PTH in *Vdr* null.
- To identify the exact mechanism of FGF23 induction by $1,25(OH)_2D$, including the identification of the FGF23-stimulating factor secreted by the chondrocytes and the characterization of the direct transcriptional control in osteoblasts.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by a grant from the Fund for Scientific Research-Flanders (FWO: G.0508.05 and G.0587.09) and the Japan Society for the promotion of sciences (JSPS: 22592071).

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