Curr Osteoporos Rep (2017) 15:443–449
DOI 10.1007/s11914-017-0394-8

SKELETAL BIOLOGY AND REGULATION (M FORWOOD AND A ROBLING, SECTION EDITORS)

Vitamin D Activity and Metabolism in Bone

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Published online: 14 August 2017

Abstract
Purpose of Review In addition to the actions of the endocrine hormone, 1alpha,25-dihydroxyvitamin D ($1,25(OH)_2D$) in stimulating intestinal calcium absorption, the regulation of bone mineral metabolism by $1,25(OH)_2D$ is also considered an important contributor to calcium homeostasis. However, recent evidence suggest that $1,25(OH)_2D$ acting either via endocrine or autocrine pathways plays varied roles in bone, which suggests that vitamin D contributes to the maintenance of bone mineral in addition to its catabolic roles. This review highlights the contrasting evidence for the direct action for vitamin D metabolism and activity in bone.
Recent Findings Numerous cells within bone express vitamin D receptor (VDR), synthesise and catabolise $1,25(OH)_2D$ via 25-hydroxyvitamin D 1alpha-hydroxylase (CYP27B1), and 25-hydroxyvitamin D 24-hydroxylase (CYP24A1) enzymes, respectively. Recent evidence suggests that all three genes are required to regulate processes of bone resorption, mineralization and fracture repair.
Summary The actions of vitamin D in bone appear to negatively or positively regulate bone mineral depending on the physiological and pathological circumstances, suggesting that vitamin D plays pleiotropic roles in bone.

Keywords Vitamin D · Vitamin D receptor · CYP27B1 · CYP24A1 · Mineralisation · Osteoblasts

This article is part of the Topical Collection on *Skeletal Biology and Regulation*

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Introduction

Vitamin D and calcium are important for bone health, aiding in the prevention of osteoporosis and associated fractures. Inadequate calcium and vitamin D nutrition and vitamin D deficiency are common in the elderly [1] which is associated with increased risk of fracture [2, 3]. While vitamin D supplementation is commonly recommended to prevent osteoporosis and reduce fracture risk, current controversies regarding vitamin D supplementation have been difficult to resolve. In particular, the levels of optimal circulating vitamin D (25(OH)D) for preventing fracture remains controversial. This is due, in part, to an incomplete understanding of the physiology by which vitamin D promotes bone structure and strength.

Vitamin D is well known to exert a wide variety of biological effects which extend beyond the traditional role for vitamin D in maintaining calcium homeostasis. This is due to the large number and wide variety of genes that are directly regulated by active vitamin D, $1,25(OH)_2D_3$ [4]. Probably the most commonly known gene that is induced by vitamin D is the enzyme 25-hydroxyvitamin D 24-hydroxylase or CYP24A1. In a classical endocrine negative feedback loop, this vitamin D receptor (VDR)-mediated transcriptional upregulation of CYP24A1 serves to attenuate the activity of $1,25(OH)_2D_3$ in target tissues. The range of target tissues, as determined by the expression of VDR, is wide. However, the number of tissues where the synthesis of active vitamin D occurs, as determined by the mitochondrondrial enzyme 25-hydroxyvitamin D 1alpha-hydroxylase or CYP27B1, is similarly broad as that for VDR [5, 6]. This suggests that many tissues may not only respond to $1,25(OH)_2D$ but may also have a complete autocrine system of vitamin D which involves its synthesis in addition to activity and catabolism. While examples of the importance of vitamin D autocrinology are limited, the bone is one such tissue where advancements in

the understanding of VDR, CYP27B1 and CYP24A1 activities have been made as shown in Fig. 1. These advancements have challenged the view that the only role for $1,25(OH)_2D$ is to promote osteoclastic bone resorption.

Vitamin D Receptor

Much of what has been determined regarding the role of vitamin D in various systems has been garnered from using gene deletion mouse models. While the global deletion of VDR, CYP27B1 or CYP24A1 in mouse models each demonstrate an impact on skeletal growth and homeostasis, this has been thought to largely do the indirect effects on active intestinal calcium absorption. In weanling *Vdr*- and *Cyp27b1*-null mice, a lack of vitamin D activity in the intestine rapidly results in hypocalcaemia, hyperparathyroidism and a rickets-like bone structure [7, 8]. However, although a diet containing high levels of calcium and phosphorus [7, 9–11] allows for sufficient passive calcium absorption to allow for normal bone development in young mice, by 17 weeks of age osteopenia develops despite the correction in serum calcium and phosphorus levels [12]. The decline in bone mineral volume in these adult null mice was associated with a deficit in osteoblastic activity and decline in mineral apposition, with no apparent change to bone resorption [12, 13]. A deficit in osteoblast activity has also been shown in osteoblast-specific VDR knockout mice. Using 2.3 kb type 1-collagen (T1-Col) promoter-Cre transgenic mice to delete VDR in osteoblasts, trabecular bone volume increased due to impaired RANKL-mediated osteoclastogenesis [14]. Unlike in the adult global *Vdr*-null mice, however, no apparent deficit in bone formation was observed at 16 weeks of age, suggesting that the dominant role for VDR in osteoblasts is to regulate bone resorption, at least in growing mice under normal conditions. Curiously, however, two other models of VDR deletion in the osteoblast lineage have yielded contrasting observations. When Osterix-Cre transgenic mice were recently used to delete VDR in osteoblasts, an apparent decrease in femoral trabecular bone volume was observed without an increase in bone resorption [15]. In contrast, when Dentin matrix protein 1 (DMP-1) promoter-Cre transgenic mice were used to delete VDR in osteocytes, no change in trabecular bone was observed [16••]. The lack of an effect of VDR deletion on osteocytic RANKL activity is consistent with other studies showing that RANKL was induced by $1,25(OH)_2D$ only in immature osteoblastic cells but not in mature cells [17]. Thus, while vitamin D-responsive RANKL is expressed at most stages of osteoblastic maturation, the data published to date suggest that the role for VDR in regulating RANKL-mediated osteoclastogenesis has varying levels of importance depending on the stage of osteoblastic maturation and on physiological and pathological circumstances.

Vitamin D promotes anabolic activities of osteoblasts in addition to regulating bone resorption [18, 19••, 20–24]. Yet, few animal studies to date have identified whether $1,25(OH)_2D$ can elicit positive actions on bone mineralization. Indeed, deleting or overexpressing VDR in late osteoblasts and osteocytes has produced contrasting observations on the role of vitamin D on mineralisation. For example, the absence of VDR in osteocytes in the aforementioned DMP-1-Cre/VDRKO mice prevent the effects of sustained high $1,25(OH)_2D$ administration on bone which would otherwise cause hyperosteoidosis [16••]. This suggests that chronically high levels of $1,25(OH)_2D$ in osteocytes inhibits bone mineralization, which could be achieved by the marked induction of genes such as *Enpp1* relative to alkaline phosphatase activity [16••], resulting in increased ratio of pyrophosphate (PPi) to inorganic phosphate (Pi), which results in the inhibition of mineralisation [25]. In contrast, transgenic overexpression of osteoblastic VDR in a mouse model exhibit substantially increased cortical and trabecular bone mineral volume and does

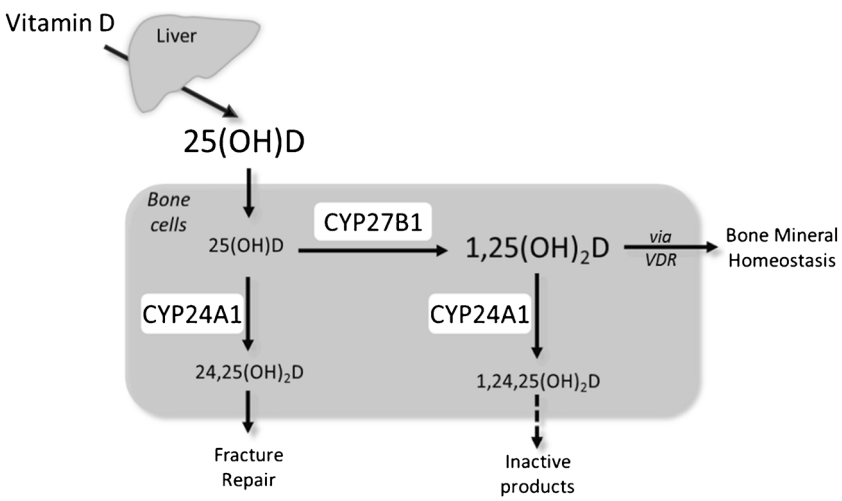

Fig. 1 Autocrine activities of vitamin D metabolism within bone cells. Serum 25(OH)D is utilised by bone cells such as osteoblasts, osteoclasts, osteocytes and chondrocytes by CYP27B1-mediated conversion to active $1,25(OH)_2D$ which regulates processes of bone mineral homeostasis. CYP24A1 inactivates $1,25(OH)_2D$ producing inactive metabolites. $24,25(OH)_2D$, produced by CYP24A1-stimulated hydroxylation of 25(OH)D, regulates processes of fracture repair

not exhibit inhibition of mineralisation at least under normal circumstances [26, 27]. Curiously, increased bone in osteoblast-specific VDR-transgenic mice has been clearly demonstrated to be due to enhanced mineral apposition on trabecular and periosteal surfaces and unexpected reduction in RANKL-mediated activity [26, 27]. These mice also are partially resistant to bone loss due to the effects of vitamin D depletion [28]. While these data suggest VDR-mediated activities may lead to anabolism, at least one study demonstrates that the osteoblast VDR-transgenic mice can exhibit lower circulating PTH levels [28], suggesting altered feedback may also contribute to lower bone resorption. However, the anabolic activities of the vitamin D analogue, eldecalcitol, increase bone volume via reducing bone resorption. The actions of eldecalcitol were ameliorated in the Osterix-Cre/VDRKO mouse model, indicating that actions of vitamin D in osteoblasts can result in reduced bone resorption at least under certain circumstances [15]. Whether these varied responses in bone can be explained by the dose and the duration of exposure of vitamin D is not yet clear. In addition, whether these varied observations can be explained by the differences in physiological circumstances in which these models are tested, (i.e. pathological verses optimal circumstances) requires further investigation.

CYP27B1

Adding to the complexity of the roles for vitamin D activity in bone is the evidence that vitamin D can be converted to its active form within osteoblasts, osteoclasts and chondrocytes [29]. With regards to osteoblasts, when 25(OH)D is converted to $1,25(OH)_2D$, in human or mouse osteoblasts, cell proliferation was inhibited and the degree of matrix mineralisation increased [19••, 22, 30]. In addition, exposure of primary osteoblasts to 25(OH)D increased the expression of mRNA species of genes associated with the mature osteoblast or osteocyte phenotype and stimulated a mature osteocyte morphology and increased mineralisation, but only in the presence of active CYP27B1 [22, 31, 32]. 25(OH)D also promotes the expression of Enpp1 and yet substantially increases the mineralisation of the osteoblastic cell-line MLO-A5 [32]. This is contrast to the effects of exogenous $1,25(OH)_2D$ on bone [16••], possibly suggesting that the expression of Enpp1 can act to promote bone mineralisation with local synthesis of $1,25(OH)_2D$. We have generated preliminary data demonstrating that the overexpression of CYP27B1 in mature osteoblasts results in increased bone volume in both male and female due to increased bone formation without a change to bone resorption, resulting in thicker trabeculae [33]. These anabolic effects occurred without changes to circulating $1,25(OH)_2D$, suggesting that local synthesis of $1,25(OH)_2D$ is more critical for bone mineral increases at least under normal growing conditions. These data are consistent the observations that *Cyp27b1*-null mice developed osteopenia with age due to reduced mineral apposition even though serum calcium and PTH levels were normalised through dietary calcium and phosphate supplementation [34]. Consistent with the notion of local $1,25(OH)_2D$ synthesis in bone, serum 25(OH)D levels are a major positive determinant of bone mineral volume in a long-term trial of varying levels of dietary vitamin D in rats [35–38]. Peak bone mineral volume was achieved with serum 25(OH)D levels between 60 and 80 nmol/L, by both reducing bone resorption and increased bone formation period (i.e. length of time osteoblasts mineralise bone) [35]. It is noteworthy that these levels of 25(OH)D exceeded the minimum level required to synthesise adequate renal $1,25(OH)_2D$ by approximately seven-fold [39]. Thus, these data collectively suggest that 25(OH)D, an inactive metabolite, is important in ensuring the local synthesis and activity of $1,25(OH)_2D$ in bone in order to regulate osteoblastic bone formation and overall bone remodelling. How these observations relate to humans and the development of osteoporosis remains somewhat unclear. Few studies to date have investigated relationship between 25(OH)D status and local metabolism of vitamin D within bone with respect to bone health. However, one such study has shown that elderly women with subcapital fracture of the femur exhibited five-times lower levels of bone-derived $1,25(OH)_2D$ levels when compared with aged-matched women without fracture, despite comparable circulating levels of $1,25(OH)_2D$ [40]. Whether the changes in local levels of $1,25(OH)_2D$ were due to changes in vitamin D metabolism in fracture patients is unknown. However, these data provides a clinical picture that warrants further investigations.

CYP24A1

Catabolism of 25(OH)D and $1,25(OH)_2D$ occurs exclusively through CYP24A1 activity [41]. The importance of CYP24A1 in regulating vitamin D activity has been demonstrated in numerous reports of CYP24A1 mutations resulting in excessive $1,25(OH)_2D$ activities, resulting in hypercalcaemia, hypercalciuria, nephrocalcinosis and nephrolithiasis [42]. In contrast, CYP24A1 over-activity has also been suggested to be associated with various diseases such as in hyper-proliferative disorders [43, 44] and in chronic kidney disease [45]. While much of the focus of altered CYP24A1 activity in disease is focused on the renal catabolism for vitamin D, there is increasing attention being placed on the putative role that CYP24A1 activity may play in non-renal tissues in the etiology of the disease. Although experimental evidence has expanded on the roles for vitamin D within the bone micro-environment, the question of whether CYP24A1 plays a critical role in directly determining bone mineral homeostasis has, however, largely remained unquestioned.

Cyp24a1 is expressed in numerous cells within the bone. Growth plate chondrocytes, osteoblasts, osteocytes, osteoclasts and precursor cells have been shown through cell culture systems to respond to $1,25(OH)_2D$ by way of rapidly inducing the expression of *Cyp24a1* [22, 32, 46–49]. The characterisation of global *Cyp24a1*-null mice has provided important experimental evidence for the impact that CYP24A1 has on bone [50]. Half of all *Cyp24a1*-null mice die before 3 weeks of age due to hypervitaminosis D and hypercalcaemia [50]. While surviving *Cyp24a1-null* mice compensate by limiting the renal synthesis of $1,25(OH)_2D$ [51], chronic elevation of $1,25(OH)_2D$ in *Cyp24a1-null* mice results in marked undermineralisation of bone, which is consistent with the effects of excessive exogenous 1,25(OH)2D3 actions on bone, as discussed earlier. The undermineralised bone in *Cyp24a1*-null mice can be rescued by crossing these mice with *Vdr*-null mice [50], which confirms that high levels of $1,25(OH)_2D$, acting through VDR, is responsible for the undermineralised bone. Interestingly, the overexpression of CYP24A1 in a transgenic rat model (Cyp24a1-Tg) also develops a rickets-like undermineralised bone phenotype [52, 53]. However, unlike in the *Cyp24a1*-null mice, the bone phenotype in Cyp24a1-Tg rats occurred without change to serum calcium, phosphate and $1,25(OH)_2D$, as renal CYP27B1 activity increased in compensation due to elevated PTH. However, serum 25(OH)D levels were strikingly low in the Cyp24a1-Tg rats, consistent with elevated CYP24A1 catabolism of 25(OH)D. Interestingly, when Cyp24a1-Tg rats were infused with 25(OH)D, the undermineralised bone phenotype was normalised. While the Cyp24a1 activity in bone was not tested directly, these data could theoretically be explained by high Cyp24a1 activity in osteoblasts abolishing the effects of 25(OH)D to $1,25(OH)_2D$ conversion. If so, the infusion of 25(OH)D may have served to improve local $1,25(OH)_2D$ synthesis.

A recent study in a unique mouse model of rickets suggests that targeting CYP24A1 activity may be of key importance to healing the bone disorder. In the rare genetic disorder of X-linked hypophosphatemia (XLH), affected children exhibit growth retardation associated with rickets and osteomalacia [54]. Its genetic basis is a mutation of the PHEX endopeptidase, leading to increased expression of the phosphaturic hormone FGF23, which in turn causes phosphate wasting, and enhancing vitamin D catabolism via CYP24A1 induction [55, 56]. The development of rickets in the mouse homolog model for XLH (*HYP*) and in other hypophosphataemic disorders is attributable to impaired chondrocyte apoptosis and to disordered function of osteoblasts and osteocytes [57, 58]. XLH was previously known as vitamin D-resistant rickets due to renal impairment of $1,25(OH)_2D$ production. The case for resistance to vitamin D activity at the tissue levels has been less clear until recently when *HYP* mice were crossed with global *Cyp24a1*-null mice [59••]. This cross was done with the intention to show the absence of vitamin D catabolism would increase serum 1,25(OH)2D3 levels, improve phosphate absorption and assist in the healing of *HYP* bone. While the compound *HYP* x *Cyp24a1*-null mice demonstrated healing of rickets, the amelioration of the animals' skeletal defects occurred in the absence of correcting low circulating $1,25(OH)_2D$ levels and severe hypophosphatemia. Furthermore, FGF23 levels were further increased in the compound *HYP* x *Cyp24a1*-null mice compared with levels detected in the *HYP* mice, perhaps reflecting the further stimulation of Fgf23 transcription by the prolonged local effects of $1,25(OH)_2D$ in osteocytes. These paradoxical findings are important as they demonstrate that the hypophosphatemic rickets that occurs in this FGF23 excess disorder is not principally a disorder of phosphate inadequacy or low circulating 1,25(OH)2D3 levels, but rather an intrinsic disorder of mineralisation itself, perhaps characterised by the active prevention of mineral deposition due to CYP24A1 activity. While it is not possible to determine whether the CYP24A1 activity in bone is responsible for the undermineralisation phenotype, this would be consistent with the undermineralisation that occurs in CYP24A1-Tg rats, as discussed earlier. Further studies are required to study whether inactivation of CYP24A1 heals the skeleton in *HYP* mice by prolonging the local half-life of $1,25(OH)_2D$ in bone, resulting in the positive effects of $1,25(OH)_2D$ on mineralisation [59••].

In addition to its catabolic effects, CYP24A1 produces the intermediate metabolite of $24,25(OH)_2D$, which has been proposed to exert biological effects (as reviewed in (48)). In particular, the less differentiated cells of the resting zone of the growth plate, respond to $24,25(OH)_2D$ by decreasing cell proliferation and stimulating differentiation and maturation [60]. $24,25(OH)_2D$–mediated stimulation of resting zone cells produces phospholipase D and promotes the conversion of phosphatidylcholine ultimately to lysophosphatidic acid (LPA) production. LPA in turn stimulates increases in alkaline phosphatase activity and protects resting zone cells from apoptosis [61]. These observations support the hypothesis that $24,25(OH)_2D$ plays a role in cartilage development. It has also been proposed that $24,25(OH)_2D$ plays a role in fracture repair. Circulating levels of $24,25(OH)_2D$ increase during fracture repair in chickens due to an increase in CYP24A1 activity [62] and administration of $24,25(OH)_2D$, in combination with $1,25(OH)_2D$, improved bone healing [63]. Furthermore, Cyp24a1-null mice exhibit a delayed bone fracture healing, supporting a role for $24,25(OH)_2D$ in mammalian fracture repair [48]. However, *Cyp27b1*-null mice did not have impaired direct intramembranous bone regeneration during distraction osteogenesis [64], a process of healing that is distinct from indirect fracture repair which consists of both intramembranous and endochondral bone formation. These data suggest that CYP24A1

and 24,25$(OH)_2$D plays a greater role in callus formation during endochondral fracture repair, rather than intramembranous fracture repair.

Conclusions

The development of novel strategies to treat or prevent bone diseases which involve altered vitamin D activity are currently hampered by an incomplete understanding of the pleiotropic roles that vitamin D plays in bone. A greater understanding of how the local synthesis and activity of vitamin D within the bone micro-environment will contribute to the rationale for vitamin D and calcium supplementation. A clearer understanding of how CYP24A1 activity in bone, as well as in other extra-renal tissues, contributes to the development of rickets in the disorder of XLH, could lead to using inhibitors to therapeutically target CYP24A1 activity. Similarly, examination of the role of CYP24A1 in the process of fracture healing may result in changes to clinical practice in orthopedics. While vitamin D activities in bone provide evidence of actions regulating important aspects of bone mineral metabolism, integration of the evidence from the endocrine and autocrine activities of vitamin D are required to establish these strategies in the future.

Compliance with Ethical Standards

Conflict of Interest Paul Anderson declares no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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