Journal Pre-proof

Vdr expression in osteoclast precursors is not critical in bone homeostasis

Lieve Verlinden, Iris Janssens, Stefanie Doms, Justine Vanhevel, Geert Carmeliet, Annemieke Verstuyf

PII: S0960-0760(19)30453-4

DOI: <https://doi.org/10.1016/j.jsbmb.2019.105478>

Reference: SBMB 105478

To appear in: *Journal of Steroid Biochemistry and Molecular Biology*

Received Date: 29 July 2019

Revised Date: 28 August 2019

Accepted Date: 18 September 2019

Please cite this article as: Verlinden L, Janssens I, Doms S, Vanhevel J, Carmeliet G, Verstuyf A, Vdr expression in osteoclast precursors is not critical in bone homeostasis, *Journal of Steroid Biochemistry and Molecular Biology* (2019), doi: <https://doi.org/10.1016/j.jsbmb.2019.105478>

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2019 Published by Elsevier.

***Vdr* expression in osteoclast precursors is not critical in bone homeostasis**

Lieve Verlinden^{1,*}, Iris Janssens¹, Stefanie Doms¹, Justine Vanhevel¹, Geert Carmeliet¹,
Annemieke Verstuyf¹

¹Clinical and Experimental Endocrinology, Department of Chronic Diseases, Metabolism, and Ageing, KU Leuven, Herestraat 49, box 902, 3000 Leuven, Belgium

* corresponding author (Lieve.Verlinden@kuleuven.be)

Highlights

- M lysozyme-driven Cre efficiently deletes *Vdr* expression in osteoclasts
- Reduced osteoclastic *Vdr* expression does not impact bone homeostasis
- Lower bone mass after dietary calcium restriction is not regulated by osteoclastic *Vdr* expression
- *In vitro* osteoclastogenesis is not influenced by the absence of *Vdr* in hematopoietic cells

Abstract

The long-recognized role of the vitamin D endocrine system is to maintain stable serum calcium concentrations, which are ensured by a complex interplay between parathyroid gland, kidney, intestine, and bone. However, although VDR is expressed in osteoclastogenic cells, the contribution of VDR-mediated signaling to osteoclast differentiation and activity remains undefined. We therefore deleted *Vdr* expression efficiently and specifically in myeloid cells by use of M lysozyme-driven Cre expression, which targets granulocytes, monocytes, macrophages and osteoclasts (*Vdr^{myel-}* mice). Bone and calcium homeostasis were investigated under basal conditions and in conditions of increased bone remodeling, by feeding *Vdr^{myel-}* and *Vdr^{myel+}*

(wildtype) mice either a normal (1%) or a low (0.02%) calcium diet from weaning onwards. *Vdr^{myel-}* mice developed normally and were normocalcemic at the age of 8 weeks, both at the normal and the low calcium diet. No differences in trabecular or cortical bone mass were observed between *Vdr^{myel-}* mice and their wildtype littermates. Dietary calcium restriction resulted in a comparable reduction of trabecular bone mass (40%) and cortical thickness (48%) in *Vdr^{myel-}* and *Vdr^{myel+}* mice, pointing to a massive transfer of calcium from the bone to the serum. In agreement with these results, osteoclastic differentiation of hematopoietic cells of *Vdr^{myel-}* mice, either induced by M-CSF and RANKL, or cocultured with osteoblasts, occurred as efficiently as osteoclastogenesis from *Vdr^{myel+}* mice. In conclusion, our data do not support a role for osteoclastic *Vdr* signaling in the control of bone homeostasis.

Key words: vitamin D receptor, osteoclasts, bone homeostasis

1 Introduction

Calcium and phosphate are vital minerals implicated in numerous biological systems, including bone formation, energy metabolism, metabolic pathways, and intracellular signaling. A complex interplay between parathyroid hormone (PTH), 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], and fibroblast growth factor 23 (FGF23) regulates mineral homeostasis by pursuing a balance between intestinal absorption, renal reabsorption, and bone influx and efflux.¹ Within bone, the vitamin D receptor (VDR) is predominantly expressed in osteoblasts and osteocytes, which are the major target cells of 1,25(OH)₂D₃ action in bone metabolism.^{2,3} However, although 1,25(OH)₂D₃ directly controls *in vitro* differentiation of osteoblast-lineage cells and bone mineralization, the vitamin D system does not appear to be a major regulator of bone homeostasis. Indeed, multiple transgenic mouse lines in which *Vdr* expression is deleted from either osteoblast precursors³ (under control of *Osterix* promoter), mature osteoblasts⁴ (under control of *Colla1* promoter), or from osteocytes⁵ (under control of *Dmp1* promoter) displayed normal bone formation. Next to osteoblasts and osteocytes, *Vdr* is also expressed in chondrocytes. Chondrocytic *Vdr* expression is involved in osteoclast formation during bone development and affects osteoblastic FGF23 expression, which subsequently leads to changes in renal expression of *Cyp27b1* and of the sodium phosphate cotransporter IIa.⁶

However, some controversy remains about the expression of *Vdr* in osteoclasts and about the role of osteoclastic *Vdr* expression in bone homeostasis. An elaborate immunohistochemical analysis by Wang *et al.* failed to demonstrate VDR staining in the osteoclasts and concluded a minor, if any, role for osteoclastic VDR expression.² However, Nakamichi *et al.* illustrated faint immunostaining of VDR in mature osteoclasts and showed that osteoclasts are responsive to treatment with 1,25(OH)₂D₃.³ Furthermore, 1,25(OH)₂D₃ is hypothesized to facilitate adhesion of

osteoclast precursors to stromal osteoblasts^{7,8} and to induce osteoblastic expression of *Rankl*⁹, favoring the formation of osteoclasts. In contrast, 25(OH)D₃ as well as 1,25(OH)₂D₃ are shown to inhibit the resorptive activity of mature osteoclasts.¹⁰ To explore the importance of osteoclastic *Vdr* expression in bone homeostasis, previous studies ablated *Vdr* expression from mature osteoclasts under the control of the *Cathepsin K (Ctsk)* promoter.^{3,11} Starczak *et al.* demonstrated that osteoclastic *Vdr* expression is a positive determinant of bone mass in young animals and contributes to preservation of bone mass after ovariectomy.¹¹ On the other hand, results from Nakamichi *et al.* argue against a role for osteoclastic *Vdr* in determining bone mass under basal conditions and indicate that osteoclastic *Vdr* expression is not involved in mediating the resorptive activity of the bone anabolic agent Eldecalcitol.³

To further delineate the role of *Vdr* expression in osteoclasts, we deleted *Vdr* expression from the myeloid lineage and investigated the bone phenotype of these mice on a normal diet as well as on a low calcium diet, to provoke increased bone remodeling with bone resorption outpacing bone formation.

2 Materials and methods

2.1 Transgenic mouse model

To obtain mice in which *Vdr* expression was deleted from the myeloid cell lineage, *Vdr*^{fl/fl} mice were crossed with M lysozyme (*LysM*)-*Cre* mice (*LysM-Cre* breeding couples provided by Prof. R. Brandes, Vascular Research Center, Frankfurt, Germany).¹² Phenotypic analysis was performed on 8 week old *Vdr*^{fl/fl;cre+} (*Vdr*^{myel-}) and their wildtype *Vdr*^{fl/fl;cre-} littermates (*Vdr*^{myel+}). Genotyping was performed by polymerase chain reaction (PCR) with Go Taq G2 Flexi DNA polymerase (Promega) on genomic DNA from toe cuts. Wildtype *Vdr* alleles were identified as 320 bp

fragments after PCR with the forward (FW) primer 5' TTT GGC CTT TCT GCT TGC CTC TTC 3' and the reverse (RV) primer 5' GGA CCA GGA AAA AAG GGG AGG TG 3'. Floxed alleles were determined as 250 bp fragments after PCR with the same FW primer as described above and the RV primer 5' AGC GAC ACT CTT GGT CTG GTT CC 3'. *Cre*⁺ mice were identified by a 534 bp fragment after amplification of genomic DNA with FW primer 5' CCC GCA GAA CCT GAA GAT G 3' and the RV primer 5' GAC CCG GCA AAA CAG GTA G 3'. Breeding pairs were maintained on a mouse breeding diet (V1124-000), whereas experimental mice were either weaned on standard chow [1% calcium, 0.7% phosphate and 1100 IU vitamin D/kg (V1535-000)] or on a low calcium diet (0.02% calcium, 0.7% phosphate and 1100 IU vitamin D/kg). All diets were obtained from Ssniff (Soest, Germany).

All mice were housed in an animal facility with 12h dark/light cycles and constant temperature. Food and water were supplied ad libitum. All animal experiments were approved by the ethical committee of the KU Leuven (P228/2013).

2.2 Serum and urine biochemistry

At the age of 8 weeks, mice were transferred to individual metabolic cages (Tecniplast, Buguggiate, Italy) to obtain 24h urine collections. Following euthanasia, blood was obtained via cardiac puncture. Serum and urinary calcium (OSR60117), phosphate (OSR6122), and creatinine (OSR6178) concentrations were measured on an AU640 chemistry analyzer (Beckman Coulter) and fractional clearance of calcium and phosphate was calculated. Serum PTH levels (Quidel, San Diego, USA) and serum C-telopeptide fragments of collagen type I (CTX-I, Ratlaps, IDS, Frankfurt, Germany) were measured with an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations.

2.3 Primary osteoclast cultures

Osteoclast differentiation was assessed by *in vitro* culture of hematopoietic cells either derived from bone marrow or from spleen in the presence of exogenous M-CSF (R&D Systems, Abingdon, UK) and receptor activator of nuclear factor κ B (RANKL, Peprotech, Rocky Hill, NJ, USA). Bone marrow cells, isolated from the long bones of hind limbs of 8 week old mice, or splenocytes were centrifuged on Ficoll-Paque gradient (Stem Cell Technologies, Grenoble, France) to isolate mononuclear cells. Cells isolated from bone marrow were plated overnight in α -MEM containing 10 ng/ml M-CSF whereas mononuclear splenocytes were incubated in α -MEM containing 25 ng/ml M-CSF (R&D systems), 10% FBS and 100 U/ml penicillin/streptomycin. The next day, non-adherent cells were harvested and plated at a density of 125,000 cells/ml (bone marrow-derived hematopoietic cells) or 500,000 cells/ml (spleen-derived hematopoietic cells) in α -MEM containing M-CSF (20 ng/ml), RANKL (100 ng/ml), 10% FBS and 100 U/ml penicillin/streptomycin in either 12-well dishes for RNA analysis (1 ml/well) or in 48-well dishes for tartrate-resistant acid phosphatase (TRAP) staining (3 ml/well). Bone marrow-derived osteoclasts were re-stimulated with M-CSF and RANKL at day 3 and analyzed at day 4 of culture, whereas spleen-derived osteoclasts were re-stimulated with M-CSF and RANKL at day 3, 5 and 7 and analyzed at day 8 of culture. To investigate the effect of $1,25(\text{OH})_2\text{D}_3$ on these osteoclast cultures, cells were either stimulated with vehicle (EtOH) or $1,25(\text{OH})_2\text{D}_3$ (10^{-7} M) during the last 24h of culture. TRAP staining was performed after a 10 min fixation of cells with 4% paraformaldehyde and permeabilization for 1 min with 0.1% Triton X-100 (Sigma-Aldrich). Cells were subsequently incubated at room temperature in 0.1 M sodium acetate containing naphthol AS-MX phosphate and Fast Red violet LB salt (Sigma-Aldrich), in the presence of 1%

dimethylformamide (Merck, Darmstadt, Germany). Positively stained cells, containing 3 or more nuclei were considered osteoclasts and were counted on digital images.

2.4 Isolation of mouse peritoneal macrophages

Peritoneal macrophages were isolated from the peritoneal cavity of 4 to 5 week old *Vdr^{myel-}* mice and their wildtype littermates. Isolated cells were resuspended in complete medium [RPMI with HEPES, 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 1.25 mg fungizone, 100 U/ml penicillin/streptomycin, 0.55 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1x non-essential amino acids (all from Invitrogen)] and left to adhere for 2 h at 37°C. Afterwards, non-adherent cells were washed away and RNA was isolated from the adherent macrophages (InnuPREP RNA isolation kit, Westburg).

2.5 Coculture of calvarial osteoblasts and hematopoietic precursor cells

Osteoblasts were isolated from calvariae of neonatal wildtype mice. Calvariae were sequentially digested with 0.1% collagenase A and 0.2% dispase (Sigma-Aldrich) as described previously.¹³ Cells released during digestion 2-6 were pooled and used in experiments. Bone marrow cells were collected from the femur of 8 week old *Vdr^{myel-}* and *Vdr^{myel+}* wildtype littermates. TRAP-positive multinucleated osteoclasts were generated by coculturing osteoblasts (20,000 cells/well in a 48-well dish or 80,000 cells/well in a 12-well dish) and bone marrow cells (200,000 cells/well in a 48-well dish or 800,000 cells/well in a 12-well dish) in α -MEM medium supplemented with 10% FBS and in the presence of 10^{-6} M prostaglandin E2 (PGE2) and 10^{-9} M 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], both from Sigma-Aldrich. At day 7 of the culture, TRAP-positive cells were

counted in triplicate wells (48-well dish) and RNA was isolated from duplicate wells (12-well dish).

2.6 Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNA for quantitative reverse polymerase chain reaction (qRT-PCR) analysis was isolated with the InnuPREP RNA isolation kit (Westburg, Leusden, The Netherlands) following the manufacturer's instructions. cDNA was synthesized from equal amounts of RNA with reverse transcriptase SuperScript II RT (Invitrogen). Gene expression of *Vdr*, *Cytochrome P450 family 24 subfamily A member 1 (Cyp24a1)*, *Calcitonin receptor (Calcr)*, *Cathepsin K (Ctsk)*, *tumor necrosis factor (ligand) superfamily member 11 (Tnfsf11 or Rankl)*, and *tumor necrosis factor receptor superfamily member 11b (Tnfrsf11b or Opg)* was assessed by real-time reverse transcriptase polymerase chain reaction (qRT-PCR) using TaqMan Fast Universal PCR master mix (Applied Biosystems, Foster City, CA, USA) in a 7500 Fast sequence detector system (Applied Biosystems). Relative gene expression was calculated with the $2^{-\Delta\Delta C_t}$ method. Expression levels of the gene of interest were normalized to the expression of β -actin and/or *hypoxanthine-guanine phosphoribosyltransferase (Hprt)*. All qRT-PCR reactions were performed in triplicate. Sequences of primers and probes are summarized in Supplemental Table 1.

2.7 Micro-computed tomography (μ CT)

Ex vivo μ CT images were taken on the high resolution Skyscan 1172 system (Bruker, Kontich, Belgium). The scanner source was set at 50 kV and 200 μ A and a 0.5 mm aluminum filter was applied. Scans were taken with a pixel size of 5 μ m with an angular increment of 0.4 degrees and a frame averaging of 2. Serial tomographs were reconstructed from raw data with the filtered Feldkamp cone-beam method (NRecon software, Bruker) and analysis was performed using the

CT Analyzer software (CTAn, Bruker) with global thresholding. The global threshold was visually determined to optimally separate the bimodal histogram into bone and soft tissue. 3D morphometric analysis was performed on the tibia in manually selected trabecular and cortical volumes of interest (between 1 to 2.5 mm distal from the growth plate to assess trabecular parameters and between 2.5 and 3 mm to calculate cortical parameters). Analysis was performed according to the guidelines of the American Society for Bone and Mineral Research (Bouxsein, JBMR, 2010). 3D models were constructed with CTvox software (Bruker).

2.8 Histological analysis and bone histomorphometry

Dissected bones were fixed overnight in 2% freshly prepared paraformaldehyde. Subsequently, bones were decalcified in 0.5 M ethylenediamine tetraacetic acid (EDTA) in phosphate buffered saline (PBS, pH 7.4) for 2 weeks at 4° C, dehydrated in graded ethanol concentrations and embedded in paraffin. Paraffin sections (4 µm) were cut, dewaxed in xylene and rehydrated before staining. For general morphological analysis, paraffin sections were stained in hematoxylin (Prosan, Merelbeke, Belgium) and eosin (H&E; 0.6% eosin Y, 1% phloxine B, 2% orange G, Sigma Aldrich), for 30 sec and 2 min, respectively. Osteoclasts were visualized on paraffin sections stained for TRAP activity and counterstained with Light Green SF Yellowish (Merck) as described previously.¹⁴ Osteoclasts covering the trabecular surfaces were measured on two TRAP-stained sections and expressed relative to the total bone surface. For trabecular analysis, three consecutive fields (0.8 mm width and 0.4 mm height) along the vertical axis of the central metaphysis were measured in each section, starting 0.2 mm from the distal end of the growth plate. For cortical analysis, osteoclast surface was quantified in two regions encompassing the bone cortex along the vertical axis of the diaphysis starting 0.4 mm from the distal end of the growth

plate (0.4 mm width and 1.2 mm height). Histomorphometric analysis was performed in the areas as indicated, and expressed according to the American Society for Bone and Mineral Research standardized histomorphometry nomenclature.¹⁵

2.9 Bone calcium content

Femurs were dried overnight at 100° C and their dry weight was recorded. Thereafter, bones were ashed for 5h at 600 °C. Ash weight was recorded and ashes were dissolved in 1 M HCl and diluted 1:100 in distilled water. Calcium concentration was measured on an AU640 chemistry analyzer.

2.10 Statistical analysis

All statistical analyses were performed with Prism 7.03 (Graphpad Software, La Jolla, CA, USA). All *in vivo* data were expressed as mean $\pm$ standard deviation (SD). Cell culture data were expressed as mean $\pm$ standard error of the mean (SEM). Differences between two groups were analyzed using the two-tailed Student's *t*-test. One- or two-way ANOVA followed by Tukey's multiple comparisons test was used to compare more groups. Differences were considered significant at $p < 0.05$.

3 Results

3.1 Decrease in bone mass induced by dietary calcium restriction is not influenced by osteoclastic *Vdr* expression

To investigate whether osteoclastic *Vdr* expression contributes to bone homeostasis, we aimed to specifically delete *Vdr* expression in myeloid cells under the control of the M lysozyme promoter, which is expressed in granulocytes, monocytes, macrophages and osteoclasts. *Vdr* expression was significantly reduced in primary osteoclast cultures initiated from bone marrow stromal cells

(Fig.1A, left panel, 67% reduction) or from splenocytes (Fig 1B, left panel, 75% reduction) of *Vdr^{myel-}* mice in comparison with cultures initiated with myeloid cells derived from their wildtype littermates. Expression of *Cyp24a1* was induced after treatment of these cultures with 1,25(OH)₂D₃, although the effect was much more pronounced in wildtype than in *Vdr^{myel-}* osteoclast cultures (Fig. 1A, B, right panels). In addition, *Vdr* expression was also significantly down-regulated in peritoneal macrophages isolated from *Vdr^{myel-}* mice (Fig. 1C, 81% reduction compared to *Vdr* expression in macrophages from *Vdr^{myel+}* mice). However, expression of *Vdr* was unaltered in other *Vdr*-target tissues such as duodenum and kidney (Fig. 1D). Interestingly, also *Vdr* expression in whole bone homogenates was similar between *Vdr^{myel-}* and *Vdr^{myel+}* mice (Fig. 1D).

Vdr^{myel-} mice developed normally and calcium homeostasis was normal in these mice as evidenced by adequate calcium levels in serum, urine, and bone in mice on standard chow (Table 1, data for 1% calcium in diet). In addition, trabecular and cortical bone mass, assessed by μ CT analysis, was identical in *Vdr^{myel-}* and *Vdr^{myel+}* mice on a normal calcium diet (Fig. 2A; Fig. 2B and Fig. 2C, black bars). To investigate the importance of osteoclastic *Vdr* expression in conditions where osteoclastic bone resorption is triggered, weanling mice were given a low calcium diet (0.02%). As expected, serum PTH levels were significantly elevated to ensure stable serum calcium levels. Indeed, serum calcium levels and fractional excretion of calcium were not significantly affected by the low calcium content of the food and were similar in *Vdr^{myel-}* and *Vdr^{myel+}* mice (Table 1). Dietary calcium restriction resulted in a substantially lower trabecular bone mass (40% reduction), cortical thinning (48% reduction) and elevated cortical porosity compared to the normal diet, but no differences were observed between *Vdr^{myel-}* and *Vdr^{myel+}* mice (Fig. 2A; Fig. 2B and Fig. 2C, white bars). To investigate microarchitectural changes in trabecular bone, bone volume was

measured within partitions of trabecular thicknesses ranging from 0.005 to 0.083 mm. In Vdr^{myel-} and Vdr^{myel+} mice on the low calcium diet, a trend was observed towards a reduced bone volume in trabeculae greater than 0.044 mm (Fig. 2D). For thinner trabeculae, bone volume was comparable between mice on the normal and on the low calcium diet. No differences were observed between Vdr^{myel-} and Vdr^{myel+} mice. Bone calcium content was reduced with approximately 50% in mice on a low calcium diet, but to a similar extent in Vdr^{myel-} and in Vdr^{myel+} mice (Table 1).

These data illustrate that *Vdr* expression was efficiently and selectively suppressed in myeloid lineage cells from Vdr^{myel-} mice. This selective deletion did, however, not affect bone phenotype under basal conditions or in circumstances of increased bone remodeling.

3.2 Osteoclastic bone resorption is enhanced by dietary calcium restriction regardless of osteoclastic *Vdr* expression

We next investigated whether the reduction in trabecular and cortical bone mass in mice on the low calcium diet was indeed accompanied by an elevated osteoclast response. Surprisingly, TRAP staining in the trabecular region of tibia sections was not elevated in mice on the low calcium diet (Fig. 3A). However, a strong induction of osteoclast number was present at the bone cortices (Fig. 3B). This elevated osteoclast number was also reflected by an elevated expression of *Ctsk* in bone (Fig. 3C) and by significantly higher serum CTx levels in mice on the low calcium diet (Fig. 3D). Furthermore, transcript levels of *Vdr* and the ratio *Rankl/Opg* were elevated in mice with dietary calcium restriction (Fig. 3E). Conclusively, all osteoclastic measurements were similarly elevated suggesting that *in vivo* osteoclast numbers and activity are identical in Vdr^{myel-} and Vdr^{myel+} mice.

3.3 *In vitro* osteoclastogenesis does not depend on the abundance of *Vdr* in hematopoietic cells

To investigate whether *in vitro* osteoclastogenesis was influenced by the abundance of *Vdr* in hematopoietic precursors, osteoclast cultures were initiated starting from either bone marrow hematopoietic cells (Fig. 4A) or from splenocytes (Fig. 4B). Osteoclastic differentiation was induced by addition of M-CSF and RANKL to the culture medium. As illustrated in Figure 4, osteoclast differentiation was equally efficient when starting from hematopoietic cells derived from *Vdr^{myel-}* mice as from *Vdr^{myel+}* mice, as illustrated by comparable osteoclast counts and by similar expression of the osteoclast markers *Calcr* and *Ctsk*. Expression of these osteoclastic markers was not altered after a short term treatment with 1,25(OH)₂D₃ (data not shown). Finally, we evaluated osteoclast differentiation in a coculture setting where calvarial osteoblasts were derived from wildtype mice. Calvarial osteoblasts were cocultured with hematopoietic cells and stimulated with PGE2 and a low dose of 1,25(OH)₂D₃ (10⁻⁹ M) to induce osteoclast formation. There was a trend towards a reduced number of osteoclasts in conditions where hematopoietic precursors had reduced *Vdr* expression (Fig. 4C). However, both inter- and intra-assay variability for osteoclast numbers was very high and no significant differences were observed. qPCR analysis of osteoclast markers did not completely match osteoclast counts and no differences were observed between cultures of either *Vdr^{myel-}* or *Vdr^{myel+}* origin (Fig. 4C). Collectively, these data suggest that suppression of *Vdr* expression in osteoclast progenitors does not influence osteoclast formation.

4 Discussion

1,25(OH)₂D₃ plays an important role in the formation of osteoclasts through its actions on osteoblasts.¹⁶ Indeed, treatment of osteoblastic cells with 1,25(OH)₂D₃ results in induction of

RANKL^{9,17}, which stimulates osteoclast formation by interacting with RANK on the surface of osteoclast precursors.⁹ On the other hand, expression of OPG, a decoy receptor for RANKL, is down-regulated after treatment with 1,25(OH)₂D₃.¹⁸ As a result, the RANKL/OPG ratio, one of the most important determinants for osteoclastogenic differentiation, is increased by 1,25(OH)₂D₃. In addition, the vitamin D system is suggested to be involved directly in osteoclast formation and function. Indeed, osteoclasts express *Cyp27b1*, the rate limiting enzyme for the activation of 1,25(OH)₂D₃¹⁰, as well as low levels of *Vdr*, required for 1,25(OH)₂D₃-mediated signal transduction. Therefore, it is plausible that 1,25(OH)₂D₃ produced in osteoclasts or osteoclast precursors is involved in the regulation of bone resorption in an autocrine or paracrine manner. As summarized by Takahashi *et al.*¹⁹, several studies reported that 1,25(OH)₂D₃ affects osteoclastic differentiation of osteoclast precursors by interfering with RANKL-induced signaling, by suppression of c-Fos (an essential transcription factor for osteoclastogenesis), by induction of IL-1 β (a suppressor of osteoclast differentiation), or by interfering with sphingosine-1-phosphatase (S1P, suggested to couple bone formation to bone resorption). To investigate the role of osteoclastic *Vdr* expression in bone homeostasis, previous studies ablated *Vdr* expression from mature osteoclasts *in vivo* by crossing floxed *Vdr* mice with *Ctsk-Cre* mice. The bone phenotype of these mice was investigated under basal conditions, as well as under conditions of accelerated bone loss or after treatment with the bone anabolic agent Eldecacitol.^{3,11} Starczak *et al.* suggested that the absence of *Vdr* in mature osteoclasts resulted in a more pronounced bone loss in 6 week old mice and after ovariectomy.¹¹ Nakamichi *et al.* on the other hand indicated that *Vdr* ablation from mature osteoclasts does not affect bone phenotype in 3 months old animals. In addition, their study suggested that not osteoclastic but osteoblastic *Vdr* expression is involved in the gain of bone mass after treatment with Eldecacitol, a suppressor of osteoclastic bone resorption.³

In our study, *Vdr* expression was deleted in the myeloid cell lineage and *Vdr^{myel-}* mice displayed an efficient and specific deletion of *Vdr* transcript levels in primary cultures of osteoclasts and peritoneal macrophages. However, *Vdr* expression was not completely deleted in the osteoclast cultures initiated from *Vdr^{myel-}* cells and these cultures remained sensitive to treatment with 1,25(OH)₂D₃, as illustrated by the transcriptional induction of *Cyp24a1*. However, induction of *Cyp24a1* by 1,25(OH)₂D₃ was much more pronounced in wildtype osteoclast cultures. This remaining *Vdr* expression and *Cyp24a1* induction may be due to an incomplete knockdown of *Vdr* expression, or may be attributed to the heterogeneous nature of these osteoclast cultures that consisted of a mixture of hematopoietic cells and stromal cells, in which *Vdr* expression was not targeted. In contrast to the results of Starczak *et al.*, deletion of *Vdr* in osteoclasts did not translate to reduced *Vdr* transcript levels in whole bones of *Vdr^{myel-}* mice. Given that osteoclasts make out only 1-2% of all cells in bone²⁰ and in view of the low *Vdr* expression in osteoclasts, it may not be surprising that osteoclastic deletion of *Vdr* expression did not result in reduced *Vdr* transcript levels in whole bone homogenates.

In analogy with the study of Nakamichi *et al.*, the basal bone phenotype of mice with reduced expression of *Vdr* in osteoclasts was similar to that of wildtype littermates, arguing that osteoclastic *Vdr* expression is not a regulator of basal bone homeostasis in (young) adult mice.³ It may be that osteoclastic VDR expression has a transient role in determining bone mass, as Starczak *et al.* did observe a slightly decreased bone mass in 6 week old animals.¹¹ As expected, low dietary calcium conditions led to a marked elevation of serum PTH levels and a major increase in osteoclast numbers, resulting in a massive transfer of calcium from bone to serum. Yet, no differences were observed between *Vdr^{myel-}* and *Vdr^{myel+}* mice, suggesting that osteoclastic *Vdr* abundance did not influence the bone phenotype in conditions of enhanced osteoclast activity. Starczak *et al.*

demonstrated that the accelerated bone loss after ovariectomy, observed when *Vdr* expression is absent in mature osteoclasts, is not associated with elevated osteoclast formation but rather with enhanced osteoclast activity as suggested by microarchitectural changes indicative of enhanced focal resorption.¹¹ However, we did not observe any differences in trabecular bone microarchitectural characteristics between *Vdr^{myel-}* and *Vdr^{myel+}* mice. In both genotypes, *Vdr* transcript levels were elevated to the same extent in whole bone homogenates of mice on the low calcium diet, suggesting that osteoblastic *Vdr* levels were induced in response to the increased calcium demand and elevated PTH levels. Accordingly, the ratio *Rankl/Opg*, a major regulator of *in vivo* osteoclastogenesis, was increased in mice on the low calcium diet, suggesting that osteoblastic, and not osteoclastic, *Vdr* expression was involved in the regulation of osteoclastic bone loss. The results of the *in vitro* osteoclastogenesis assays recapitulated the *in vivo* findings and did not reveal a role for osteoclastic *Vdr* expression in the formation of osteoclasts or for the expression of osteoclastic markers.

In conclusion, our data do not support an important role for osteoclastic *Vdr* expression in bone homeostasis under basal conditions or in conditions of accelerated bone loss and we hypothesize that the bone loss observed after dietary calcium restriction is mediated by enhanced production of RANKL by osteoblasts.

Acknowledgments

We thank Ralf Brandes for providing us with *Vdr^{myel}* breeding couples. This work was supported by University of Leuven Grant C16/18/006 and Research Foundation–Flanders Grants FWO G0D4217N-FB.

References

- 1 Christakos, S., Dhawan, P., Verstuyf, A., Verlinden, L. & Carmeliet, G. Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. *Physiol Rev* **96**, 365-408, doi:10.1152/physrev.00014.2015 (2016).
- 2 Wang, Y., Zhu, J. & DeLuca, H. F. Identification of the vitamin D receptor in osteoblasts and chondrocytes but not osteoclasts in mouse bone. *J Bone Miner Res* **29**, 685-692, doi:10.1002/jbmr.2081 (2014).
- 3 Nakamichi, Y. *et al.* VDR in Osteoblast-Lineage Cells Primarily Mediates Vitamin D Treatment-Induced Increase in Bone Mass by Suppressing Bone Resorption. *J Bone Miner Res* **32**, 1297-1308, doi:10.1002/jbmr.3096 (2017).
- 4 Yamamoto, Y. *et al.* Vitamin D receptor in osteoblasts is a negative regulator of bone mass control. *Endocrinology* **154**, 1008-1020, doi:10.1210/en.2012-1542 (2013).
- 5 Lieben, L. *et al.* Normocalcemia is maintained in mice under conditions of calcium malabsorption by vitamin D-induced inhibition of bone mineralization. *J Clin Invest* **122**, 1803-1815, doi:10.1172/JCI45890 (2012).
- 6 Masuyama, R. *et al.* Vitamin D receptor in chondrocytes promotes osteoclastogenesis and regulates FGF23 production in osteoblasts. *J Clin Invest* **116**, 3150-3159, doi:10.1172/JCI29463 (2006).
- 7 Okada, Y. *et al.* Cell-to-Cell adhesion via intercellular adhesion molecule-1 and leukocyte function-associated antigen-1 pathway is involved in 1 α ,25(OH) $_2$ D $_3$, PTH and IL-1 α -induced osteoclast differentiation and bone resorption. *Endocr J* **49**, 483-495 (2002).
- 8 Medhara, M. M. *et al.* 1 α ,25-dihydroxyvitamin D $_3$ up-regulates expression of the osteoclast integrin α v β 3. *J Biol Chem* **268**, 1456-1461 (1993).
- 9 Yasuda, H. *et al.* Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A* **95**, 3597-3602, doi:10.1073/pnas.95.7.3597 (1998).
- 10 Kogawa, M. *et al.* Osteoclastic metabolism of 25(OH)-vitamin D $_3$: a potential mechanism for optimization of bone resorption. *Endocrinology* **151**, 4613-4625, doi:10.1210/en.2010-0334 (2010).
- 11 Starczak, Y. *et al.* Absence of vitamin D receptor in mature osteoclasts results in altered osteoclastic activity and bone loss. *J Steroid Biochem Mol Biol* **177**, 77-82, doi:10.1016/j.jsbmb.2017.10.022 (2018).
- 12 Leyssens, C. *et al.* Impact on Experimental Colitis of Vitamin D Receptor Deletion in Intestinal Epithelial or Myeloid Cells. *Endocrinology* **158**, 2354-2366, doi:10.1210/en.2017-00139 (2017).
- 13 Daci, E., Verstuyf, A., Moermans, K., Bouillon, R. & Carmeliet, G. Mice lacking the plasminogen activator inhibitor 1 are protected from trabecular bone loss induced by estrogen deficiency. *J Bone Miner Res* **15**, 1510-1516, doi:10.1359/jbmr.2000.15.8.1510 (2000).
- 14 Verlinden, L. *et al.* Nrp2 deficiency leads to trabecular bone loss and is accompanied by enhanced osteoclast and reduced osteoblast numbers. *Bone* **55**, 465-475, doi:10.1016/j.bone.2013.03.023 (2013).

- 15 Dempster, D. W. *et al.* Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* **28**, 2-17, doi:10.1002/jbmr.1805 (2013).
- 16 Takeda, S. *et al.* Stimulation of osteoclast formation by 1,25-dihydroxyvitamin D requires its binding to vitamin D receptor (VDR) in osteoblastic cells: studies using VDR knockout mice. *Endocrinology* **140**, 1005-1008, doi:10.1210/endo.140.2.6673 (1999).
- 17 Pike, J. W., Lee, S. M. & Meyer, M. B. Regulation of gene expression by 1,25-dihydroxyvitamin D3 in bone cells: exploiting new approaches and defining new mechanisms. *Bonekey Rep* **3**, 482, doi:10.1038/bonekey.2013.216 (2014).
- 18 Yasuda, H. *et al.* Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. *Endocrinology* **139**, 1329-1337, doi:10.1210/endo.139.3.5837 (1998).
- 19 Takahashi, N., Udagawa, N. & Suda, T. Vitamin D endocrine system and osteoclasts. *Bonekey Rep* **3**, 495, doi:10.1038/bonekey.2013.229 (2014).
- 20 Florencio-Silva, R., Sasso, G. R., Sasso-Cerri, E., Simões, M. J. & Cerri, P. S. Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. *Biomed Res Int* **2015**, 421746, doi:10.1155/2015/421746 (2015).

Figure legends

Figure 1. *Vdr* expression is specifically deleted from myeloid cells. qRT-PCR analysis of *Vdr* transcript levels in primary osteoclasts generated from bone marrow-derived hematopoietic cells (**A, left panel**) or from splenocytes (**B, left panel**) which were isolated from *Vdr*^{myel-} mice or their wildtype littermates (n=3-4). *Cyp24a1* induction was expressed as a ratio of *Cyp24a1* transcript levels in 1,25(OH)₂D₃-treated cultures over vehicle-treated cultures (**A, B; right panels**). (**C**) Suppression of *Vdr* expression in peritoneal macrophages from *Vdr*^{myel-} mice analyzed by qRT-PCR (n=4-7). Results are expressed as means and SEM. (**D**) *Vdr* transcript levels in different vitamin D target tissues of *Vdr*^{myel-} and *Vdr*^{myel+} mice as measured by qRT-PCR (n=4-7). Results are expressed as means and SD. *p < 0.05, **p < 0.01, *** p < 0.001.

Figure 1

Figure 2. Bone mass is significantly reduced in response to a low calcium diet but is not dependent on osteoclastic *Vdr* expression. (A) μ CT images of the tibia of 8 week old female *Vdr^{myel}* mice and their wildtype littermates weaned on either a normal (1%) or on a low (0.02%) calcium diet: 3D reconstructed longitudinal views of the metaphyseal area in the upper panels and transverse views of the proximal diaphysis in the lower panels. (B) μ CT-based quantification of the ratio of bone volume over tissue volume (BV/TV) and of trabecular thickness in the proximal metaphyseal area (n=10). (C) μ CT-based analysis of cortical thickness and cortical porosity (n=10). (D) Distribution of trabecular bone volume by thickness (n=10). Results are expressed as means and SD. **p < 0.01, ****p < 0.0001.

Figure 2

Figure 3. Osteoclastic bone resorption is enhanced by dietary calcium restriction regardless of osteoclastic *Vdr* expression. (A) Representative images of TRAP-stained sections of 8 week old female *Vdr^{myel-}* mice and their wildtype littermates weaned on either a normal (1%) or a low (0.02%) calcium diet. Scale bar: 500 μ m. Black squares represent the areas of which a higher magnification image is shown (scale bar: 200 μ m). (B) Osteoclast surface relative to bone surface (OcS/BS), quantified on TRAP-stained tibias in the trabecular (left panel) or cortical (right panel) region of the proximal metaphysis of the tibia (n=4-5). (C) qRT-PCR analysis of *Ctsk* expression in the femur (n=6-10). (D) Serum CTx concentrations (n=5). (E) Transcript levels of *Vdr* (left

panel) and *Rankl/Opg* (right panel) in the femur (n=6-10). Results are expressed as means and SD.

* $p < 0.05$, ** $p < 0.01$.

Figure 3

Figure 4. Osteoclast numbers in primary cultures of bone marrow- or spleen-derived hematopoietic cells do not depend on osteoclastic *Vdr* expression. *In vitro* formed osteoclasts starting from hematopoietic cells isolated from bone marrow (A) or from spleen (B) were quantified by counting TRAP-positive cells containing 3 or more nuclei (n=3-4). (C) Osteoclasts, formed in cocultures of wildtype calvarial osteoblasts with hematopoietic cells from *Vdr^{myel+}* or *Vdr^{myel-}* mice, were quantified by counting TRAP-positive cells containing 3 or more nuclei (n=3-

4). qRT-PCR analysis was used to assess the expression of the osteoclastic markers *Calc* and *Ctsk* in these primary cultures (n=3-4). Results are expressed as means and SEM.

Figure 4

Table 1. Calcium homeostasis in *Vdr^{myel-}* mice.

Dietary calcium content	<i>Vdr^{myel+}</i>		<i>Vdr^{myel-}</i>	
	1% calcium	0.02% calcium	1% calcium	0.02% calcium
Serum				
Calcium (mg/dl)	10.6 ± 0.3	10.3 ± 0.4	10.4 ± 0.2	10.3 ± 0.5
Pi (mg/dl)	11.1 ± 0.9	10.5 ± 0.8	11.1 ± 1.1	10.6 ± 1.5
PTH (pg/ml)	26.4 ± 17.9	465.7 ± 220.6**	34.0 ± 24	407.1 ± 83.1**
Urine				
	0.51 ±		0.37 ±	
FE _{Ca} (%)	0.16	0.34 ± 0.09*	0.12	0.42 ± 0.11
				63.9 ±
FE _{Pi} (%)	18.1 ± 2.9	58.4 ± 13.3****	18.0 ± 4.2	18.3****
Bone				
Calcium (mg)	7.6 ± 1.2	3.5 ± 0.4****	7.3 ± 1.0	4.0 ± 1.1****

Values are expressed as mean and SD.

* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ (versus respective 1% calcium group)