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Vitamin D receptor expression in mature osteoclasts reduces bone loss due to low dietary calcium intake in male mice

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Abstract

Mature osteoclasts express the vitamin D receptor (VDR) and are able to respond to active vitamin D (1α, 25-dihydroxyvitamin D_3; $1,25(OH)_2D_3$) by regulating cell maturation and activity. However, the *in vivo* consequences of vitamin D signalling directly within functionally mature osteoclasts is only partially understood. To investigate the *in vivo* role of VDR in mature osteoclasts, conditional deletion of the VDR under control of the cathepsin K promoter ($Ctsk^{Cre}/Vdr^{-/-}$), was assessed in 6 and 12-week-old mice, either under normal dietary conditions (NormCaP) or when fed a low calcium (0.03%), low phosphorous (0.08%) diet (LowCaP). Splenocytes from $Ctsk^{Cre}/Vdr^{-/-}$ mice were co-cultured with MLO-Y4 osteocyte-like cells to assess the effect on osteoclastogenesis. Six-week-old $Ctsk^{Cre}/Vdr^{-/-}$ mice demonstrated a 10% decrease in vertebral bone volume ($p < 0.05$), which was associated with increased osteoclast size ($p < 0.05$) when compared to $Vdr^{fl/fl}$ control mice. Control mice fed a LowCaP diet exhibited extensive trabecular bone loss associated with increased osteoclast surface, number and size ($p < 0.0001$). Interestingly, $Ctsk^{Cre}/Vdr^{-/-}$ mice fed a LowCaP diet showed exacerbated loss of bone volume fraction (BV/TV%) and trabecular number (Tb.N), by a further 22% and 21%, respectively ($p < 0.05$), suggesting increased osteoclastic bone resorption activity with the loss of VDR in mature osteoclasts under these conditions. Co-culture of $Ctsk^{Cre}/Vdr^{-/-}$ splenocytes with MLO-Y4 cells increased resulting osteoclast numbers 2.5-fold, which were greater in nuclei density and exhibited increased resorption of dentine compared to osteoclasts derived from $Vdr^{fl/fl}$ splenocyte cultures. These data suggest that in addition to RANKL-mediated osteoclastogenesis, intact VDR signalling is required for the direct regulation of the differentiation and activity of osteoclasts in both *in vivo* and *ex vivo* settings.

KEYWORDS

Introduction

1α,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) is necessary for maintaining calcium homeostasis and skeletal health. Much of this activity is associated with the action of 1,25(OH)$_2$D$_3$ on the intestine, where it stimulates calcium absorption to maintain serum calcium levels, and to provide adequate calcium for mineralisation of bone [1, 2]. It is well established that high circulating 1,25(OH)$_2$D$_3$ levels stimulate osteoclast formation indirectly through osteoblastic Receptor activator of nuclear factor kappa-B ligand (RANKL), Tumour necrosis factor Superfamily, Member 11 (TNFSF11)-mediated osteoclastogenesis [3, 4]. Osteoclasts are large multinucleated cells derived from haemopoietic mononuclear precursors principal in the process of bone resorption [5, 6]. The induction of osteoclastogenesis is triggered by the combined effects of macrophage colony-stimulating factor (M-CSF) and RANKL, among others [5, 7]. RANKL-RANK signaling induces NFATc1 expression, thus triggering a cascade of osteoclast maturation and activation events. The process of bone resorption is mediated by a number of osteoclast-expressed factors such as vacuolar H$^+$ATPase (V-ATPase), carbonic anhydrase II (CA2), Cathepsin K (CTSK) and tartrate resistant acid phosphatase (TRAP/ACP5) [5, 8-10]. While the vitamin D receptor (VDR) is well known to play a role in the regulation of RANKL expression and thus osteoclastogenesis [11], the role VDR plays in osteoclasts to regulate cellular maturation and activity is only partially understood.

We have previously reported that splenocytes derived from VDR-null mice results in increased osteoclastogenesis and increased resorptive ability [12]. Furthermore, we have shown that the absence of VDR in mature osteoclasts, using Ctsk-Cre-driven deletion (Ctsk^{Cre}/Vdr$^{-/-}$

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), resulted in reduced femoral bone mineral volume in young females under normal conditions [13]. In contrast, alternative Cre models of VDR deletion in osteoclasts have resulted in no observable effect on osteoclast function, indicating that the role of VDR in osteoclasts may be to determine osteoclastic activity and bone volume only under certain circumstances [14, 15]. We now report that VDR deletion within CTSK-expressing cells leads to bone loss, which is further exacerbated when fed a low calcium diet, which is consistent with previous observations that increased osteoclastic bone resorption activity occurs with the loss of VDR in mature osteoclasts [13].

Materials and Methods

Animals and Diet

Conditional osteoclast-specific VDR knockout animals were generated using the well-defined *Cre-LoxP* system [16]. Briefly, floxed-Vdr (Vdr$^{fl/fl}$) mice [17] were mated with the Cathepsin-K-Cre (Ctsk-*Cre)* mouse line [18]. Mice inheriting the homozygous floxed-*Vdr* recombination and the Ctsk-*Cre* transgene resulted in the deletion of exon 2 of the *Vdr* gene, causing a frame-shift and early termination of *Vdr* mRNA [19].

Three-week old male Ctsk^{Cre}/Vdr$^{-/-}$ and Vdr$^{fl/fl}$ mice (n = 5-7/group) were fed a normal calcium (0.8%), normal phosphorus (0.7%) diet (NormCaP) or a low calcium (0.03%), low phosphorous (0.08%) diet (LowCaP; Specialty Feeds, WA, Australia) for 3 weeks with drinking water provided *ad libitum*.

Homozygous floxed control (Vdr$^{fl/fl}$) and conditional knockout mice (Ctsk^{Cre}/Vdr$^{-/-}$) were housed at 21-23°C with a 12-hour light/dark cycle, group-housed up to 5 animals per cage. Mice were humanely killed at 6 weeks of age, with long bones, soft tissues and serum collected. Spleens from male Ctsk^{Cre}/Vdr$^{-/-}$ and Vdr$^{fl/fl}$ littermate control mice at 8 weeks of age were

utilised for *ex vivo* splenocyte co-culture investigations. All animal procedures were performed under the Animal Welfare Code of Practice of Australia and with approval provided by the University of South Australia Animal Ethics Committee (Approval 28/12).

3D Micro-Computed Tomography, Reconstruction and Realignment of Cross-Sectional Images

After formalin fixation for 3-4 days, vertebral micro-architecture was analysed using a high-resolution micro-CT system, using an 1174 micro-CT system (Skyscan 1174, Bruker, Belgium) to obtain x-ray transmission images. Cross-sectional images were reconstructed using the nRecon software (Version 1.6.9.18) (Bruker), with an isotropic voxel size of 6.5µm. Realignment of datasets was performed using Dataviewer software (v.1.5.1, Bruker). All bone quantification from reconstructed and realigned datasets was performed using CTan software (v.1.7, Bruker). Whole L1 vertebrae were analysed to quantify trabecular bone volume parameters within this region.

Bone Histology and Histomorphometry

After micro-CT scanning, vertebra were embedded into methyl methacrylate [13] Embedded samples were cut using a motorised microtome (RM2500, Leica) and consecutive serial sections were cut (moistened with water) at 5 µm. Sections were placed on gelatin-coated glass Superfrost slides (Menzel-Glaser, Lomb Scientific Pty Ltd) as previously described [13] Sagittal sections (5 µm) were stained for TRAP or left unstained for double fluorochrome (calcein; 10mg/kg BW and xylenol orange; 90mg/kg BW) analysis. Fluorochromes were administered intraperitoneally (IP) 6 and 2 days before mice were humanely killed. The OsteoMeasure[TM] Version 3.3.0.2 (OsteoMetrics, Inc. Decatur, GA, USA) was used for TRAP-stained slides to measure osteoclast perimeter (Oc.Pm, mm), number of osteoclasts (N.Oc,

#/mm), osteoclast surface per bone surface (Oc.S/BS, %), number of osteoclasts per tissue area ($N.Oc/T.Ar$, $\#/mm^2$), number of osteoclasts per bone perimeter (N.Oc/B.Pm, #/mm) and osteoclast size (μm^2). Unstained slides allowed for the measurement of inter-label distance and to calculate the mineral apposition rate (MAR, µm/day) and bone formation rate (BFR, $\mu m^3/\mu m^2/day$).

Biochemistry

Serum calcium (Ca), phosphate (P) and alkaline phosphatase (ALP) levels were measured using the KoneLab 20XT Clinical Chemistry Analyser (ThermoScientific, MA, USA), using standard protocols and reagents (Thermo-Scientific, USA). Serum C-terminal telopeptide (cross-laps, CTX) and TRAP5b levels were measured using a RATLaps EIA or TRAP5b Kit, respectively (Immunodiagnostic Systems Limited, UK).

Molecular Biology

Tissues were harvested from $Ctsk^{Cre}/Vdr^{-/-}$ and $Vdr^{fl/fl}$ animals to assess *Vdr* deletion specificity. Briefly, tail samples were digested overnight and centrifuged, supernatant was collected and processed to extract DNA, as described previously [20]. DNA was amplified with a total reaction volume of 20 µl, containing 10x buffer (2 µl) 50 mM $MgCl_2$ (0.4 µl), forward and reverse primers. Amplified products were then run on agarose (1%) to verify *Vdr* deletion [13].

Splenocyte-derived Osteoclast Co-Culture with MLO-Y4 Cells

To establish the role of the VDR in the generation and activity of osteoclasts, Ctsk^{Cre}/Vdr$^{-/-}$ and floxed control splenocytes were co-cultured with MLO-Y4 cells, as previously described [21] with modifications. Briefly, 96-well tissue culture plates were coated with type I collagen. Spleens were excised and placed in media (αMEM; Sigma Chemical Co., St Louis, MO, USA) containing 10% charcoal stripped foetal bovine serum (FBS; HyClone, Logan, UT, USA). Spleens were dissected and the pieces gently ground then passed through a cell strainer (40 µm). Red cell lysis buffer was added, and the cells washed by centrifugation three times. Cells were pooled per genotype and counted using a haemocytometer.

For these co-cultures, sub-optimal concentrations of exogenous RANKL/M-CSF (10 ng/ml) were added to ensure sufficient osteoclast formation occurred, and to allow for indirect effects of vitamin D metabolites on the osteocytes to be discerned. Thus, cells were seeded into tissue cell culture plate wells and treated with combinations of 10 ng/ml recombinant M-CSF (Millipore, Temecula, CA, USA) and 10 ng/ml RANKL (Millipore), in the presence or absence of 1,25(OH)$_2$D$_3$ (1 or 10 nM) or 25D (100 nM).

Measurement of Osteoclastogenesis

To assess osteoclastogenesis, Vdr$^{fl/fl}$ and Ctsk^{Cre}/Vdr$^{-/-}$ splenocyte cells cultured in 96-well tissue culture plates were stained for TRAP activity [11], at day 10 of culture. Osteoclasts were defined as magenta-stained, multinucleated cells (MNC) containing three or more nuclei. Osteoclast number and nuclei number was assessed at day 10 of culture using a 4x and a 10x objective (respectively) on an Olympus CKX41 light microscope attached to a camera (Olympus DP20).

Measurement of Resorption Activity

Dentine slices were prepared and used for the measurement of osteoclastic resorption [22]. Briefly, dentine (sperm whale tooth, kindly provided by Prof. David Haynes, University of Adelaide [23]) was cut into slices (5 x 5 x 0.1 mm) and placed into 100% ethanol overnight. Slices were then soaked in αMEM overnight, air-dried and finally sterilised under UV light for 20-30 minutes in a Class II Biohazard cabinet. Dentine was then carefully placed into individual wells within the 96-well plate and seeded with splenocytes (2×10^5 cells/well) prepared as above. After 24 hours, medium was replaced with differentiation media, and the cells cultured for 18-22 days (with media changed every 3-4 days). Cells were removed from the dentine by first washing in detergent (0.1% v/v Extran), rinsing three times with RO water and then 6 M ammonium hydroxide solution for 1 hour. Slices were then washed three times in water and dehydrated in an ethanol series (50%, 70%, 90%, 100%) before being air-dried overnight. The dentine slices were then mounted on aluminium stubs and carbon-coated to allow for visualisation on a scanning electron microscope (SEM) (Quanta 450). Sites of resorption were quantified from SEM images using IMAGE J software (NIH, Bethesda, MD, USA).

Statistics

Statistically significant changes in individual parameters between Ctsk^{Cre}/Vdr$^{-/-}$ and Vdr$^{fl/fl}$ controls were determined using unpaired, two-tailed T-tests. Two-way analysis of variance (two-way ANOVA) with Tukey's multiple comparisons test was used for the dietary studies. All analysis was performed using GraphPad Prism software (Version 8.0.0; GraphPad Software Inc., CA, USA).

Results

Demonstration of VDR knockdown in Ctsk^{Cre}/Vdr$^{-/-}$ mice.

Recombination of the floxed *Vdr* allele was not observed in kidney, liver or intestine, however, deletion of exon 2 was detected in fat, heart, skin and muscle (**Supplementary Figure 1**). We have previously shown that excision of exon 2 of the *Vdr* within bone of Ctsk^{Cre}/Vdr$^{-/-}$ mice resulted in a 70% reduction in the expression of the *Vdr* gene within whole bone compared to Vdr$^{fl/fl}$ littermate controls [13].

The effects of Osteoclastic VDR deletion and Dietary Calcium and Phosphorus restriction in Ctsk^{Cre}/Vdr$^{-/-}$ Mice.

In males fed a LowCaP diet for 3 weeks, bodyweight was significantly reduced when compared to NormCaP fed mice ($p < 0.05$). However, there was no difference in bodyweight between Ctsk^{Cre}/Vdr$^{-/-}$ and Vdr$^{fl/fl}$ mice (**Table 1**). Similarly, LowCaP fed mice demonstrated reduced femur length ($p < 0.05$) and a non-significant increase in femoral growth plate height when compared to NormCaP fed mice, without differences between Ctsk^{Cre}/Vdr$^{-/-}$ or Vdr$^{fl/fl}$ controls (**Table 1**).

While serum calcium levels remained unchanged, the serum levels of phosphorous ($p < 0.01$), alkaline phosphatase ($p < 0.0001$) and serum cross-laps ($p < 0.0001$) were elevated in male LowCaP fed mice, with only ALP levels demonstrating a significant increase between Ctsk^{Cre}/Vdr$^{-/-}$ and Vdr$^{fl/fl}$ control mice fed a LowCaP diet ($p < 0.01$) (**Table 2**).

Both Ctsk^{Cre}/Vdr$^{-/-}$ and Vdr$^{fl/fl}$ control mice fed the LowCaP diet exhibited decreased vertebral bone volume when compared to those fed the NormCaP diet ($p < 0.01$) (**Fig. 1A & B**). Male Ctsk^{Cre}/Vdr$^{-/-}$ mice demonstrated further decreases in bone volume, representing a 22% decline in vertebral bone volume when compared to LowCaP fed control mice. The decline in bone volume due to diet and genotype was resultant of a decline in both Tb.Th ($p < 0.01$) and Tb.N

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($p < 0.01$) (**Fig. 1B**). Ctsk^{Cre}/Vdr$^{-/-}$ fed the LowCaP diet appeared to exhibit a greater decline in Tb.N but this did not reach statistical significance (21%, $p = 0.08$) (**Fig. 1A & B**).

The osteoclastic parameters osteoclast surface per bone surface, number of osteoclasts per bone perimeter were not significantly altered in either Ctsk^{Cre}/Vdr$^{-/-}$ or Vdr$^{fl/fl}$ mice fed a LowCaP diet when compared to the respective controls fed a NormCaP diet. However, osteoclast size was increased similarly in both genotypes fed a LowCaP diet ($p < 0.01$) (**Fig. 1C**).

Mineral apposition rate (MAR) in LowCaP fed Ctsk^{Cre}/Vdr$^{-/-}$ mice was reduced by over 50% when compared to Vdr$^{fl/fl}$ mice ($p < 0.05$) fed the same diet (**Table 3**). Ctsk^{Cre}/Vdr$^{-/-}$ mice exhibited no differences to Vdr$^{fl/fl}$ mice in terms of the bone formation parameters MS/BS and BFR/BS on either diet (**Table 3**).

Effects of VDR deletion on osteoclastogenesis in co-cultures of splenocytes with MLO-Y4 cells.
To further examine the effects of osteoclast-lineage specific deletion of *Vdr* on osteoclastogenesis, we employed an osteoclast-forming co-culture model. Splenocytes derived from Vdr$^{fl/fl}$ or Ctsk^{Cre}/Vdr$^{-/-}$ mice were seeded onto layers of MLO-Y4 osteocyte-like cells, a cell line with an intact VDR-signalling system and known to support osteoclastogenesis [21, 24]. In the absence of exogenous $1,25(OH)_2D_3$, TRAP-positive MNC numbers increased with time in both co-culture types. However, compared to Vdr$^{fl/fl}$ co-cultures, dramatically increased osteoclastogenesis was observed in Ctsk^{Cre}/Vdr$^{-/-}$/MLO-Y4 co-cultures by day 10 ($p < 0.05$) (**Fig. 2A**), suggesting an intrinsic increased propensity to form osteoclasts in the absence of VDR. In the presence of exogenous $1,25(OH)_2D_3$ (1 nM), osteoclastogenesis was enhanced in co-cultures of both genotypes, consistent with the (stromal) osteocyte supplying additional pro-osteoclastic factors in response to $1,25(OH)_2D_3$. However, osteoclast formation was further increased in Ctsk^{Cre}/Vdr$^{-/-}$/MLO-Y4 co-cultures at all time points assayed relative to Vdr$^{fl/fl}$ co-cultures (**Fig. 2A**). $1,25(OH)_2D_3$ treated Ctsk^{Cre}/Vdr$^{-/-}$ co-cultures contained more large

osteoclasts (containing greater than 10 nuclei) when compared to control cultures ($p = 0.019$, **Fig. 2B**). In the presence of 1,25(OH)$_2$D$_3$, the surface area resorbed was increased significantly only in Ctsk^{Cre}/Vdr$^{-/-}$ osteoclast cultures ($p < 0.05$) (**Fig. 2C & D**). Together, these findings are consistent with VDR expression in mature osteoclasts imparting an ameliorating effect to their resorbing activity.

Discussion

The role for VDR signalling within osteoclasts in the maintenance of bone health is a controversial topic, with previous studies suggesting little evidence of VDR expression by osteoclasts or a role for VDR in the determination of bone mass during basal conditions [15, 25]. However a strong body of evidence exists that VDR plays a role in osteoclast formation and activity [11-13]. To examine the role for VDR-mediated activity in osteoclasts *in vivo*, we generated an osteoclast-specific *Vdr* knockout mouse model using the Cre-LoxP system[13, 16, 18]. *Vdr* deletion in the Cstk^{Cre}/Vdr$^{-/-}$ mice was confirmed, both at the level of genomic DNA, demonstrating excision of exon 2 of the *Vdr* gene, and at the level of gene expression showing a 70% reduction in total *Vdr* mRNA levels in bone.

At 6 weeks of age, Ctsk^{Cre}/Vdr$^{-/-}$ mice demonstrated a 10% reduction in vertebral bone volume, which was associated with increased osteoclast size when compared to Vdr$^{fl/fl}$ controls, interestingly, no overt change to osteoclast number was observed in these animals. In order to determine whether the presence of the VDR in mature osteoclasts is essential for the maintenance of bone resorption in this model, particularly during times of physiological stress in young animals (6 weeks of age), Ctsk^{Cre}/Vdr$^{-/-}$ and Vdr$^{fl/fl}$ mice were subjected to dietary calcium and phosphorus restriction (LowCaP diet) from weaning until 6 weeks of age. All young mice fed LowCaP exhibited reduced bone volume when compared to animals fed adequate calcium and phosphorus levels. However, Ctsk^{Cre}/Vdr$^{-/-}$ mice under these conditions exhibited exacerbated vertebral bone loss (22%) when compared to that in control mice (10%). The vertebrae of Ctsk^{Cre}/Vdr$^{-/-}$ mice demonstrated the lowest bone mineral levels when compared to other groups, yet there was no change to serum levels of the bone resorption marker, cross-laps (CTX). This suggests that enhanced vertebral bone resorption gave rise to lower bone volume, however this was not reflected by changes in biochemical markers of bone resorption. While dietary restriction of calcium and phosphorus caused a significant increase

in osteoclastogenesis and activity, no changes in osteoclastic histomorphometric parameters were observed in Ctsk^{Cre}/Vdr$^{-/-}$ mice when compared to control mice. Thus, any change to osteoclastic activity in Ctsk^{Cre}/Vdr$^{-/-}$ mice to describe the lower bone volume is not easily measurable using standard histological and biochemical measures. These findings, however, suggest that the effects of VDR signalling *in vivo* in osteoclasts is to regulate their activity rather than their formation/differentiation, as previously proposed [11].

Interestingly, the bone loss observed in LowCaP fed Ctsk^{Cre}/Vdr$^{-/-}$ mice was associated with reduced osteoblastic differentiation and reduced mineral deposition, perhaps to a greater extent than changes to osteoclastic activity alone. This is potentially explained by possible effects of VDR signalling in osteoclasts being linked to the coupling of bone resorption to bone formation; osteoclast activity is intrinsically linked to the bone formation response and suppression of resorption is well known to also suppress bone formation [26]. Despite previous studies suggesting a role for VDR in inhibiting bone formation [17], in our investigation of the Ctsk^{Cre}/Vdr$^{-/-}$ mouse model there was no observed increase in bone volume that would correspond to this effect.

In order to further understand the role of the VDR in mature osteoclasts in the regulation of osteoclast generation and activity in an *ex vivo* setting, we utilised splenocytes harvested from Ctsk^{Cre}/Vdr$^{-/-}$ mice in osteoclast-forming co-culture with MLO-Y4 osteocyte-like cells, which have an intact VDR signalling mechanism. In contrast to previous findings using global *Vdr*$^{-/-}$ cells [11], splenocyte-derived osteoclasts from Ctsk^{Cre}/Vdr$^{-/-}$ mice exhibited increased osteoclastogenesis as well as bone resorbing activity. The observed increase in the number of large osteoclasts observed in Ctsk^{Cre}/Vdr$^{-/-}$ cultures may reflect either enhanced fusion of smaller osteoclasts into larger cells or enhanced survival. While there was no change in osteoclast numbers in the bone tissue of these animals, overall, our data are consistent with

osteoclasts formed in the absence of *Vdr* expression resorbing more bone than those in control Vdr$^{fl/fl}$ mice.

In a recent study, Verlinden and colleagues [14] investigated the role of Vdr signalling in osteoclasts using M lysozyme-driven Cre expression, a Cre 'knock-in' model. The use of this method resulted in the deletion of *Vdr* within a spectrum of myeloid cells, as it targets cells from monocytes through to macrophages and pre-osteoclasts. A bone phenotype in this model was not observed under basal conditions or under conditions of increased bone resorption such as calcium insufficiency, with the conclusion there was no role for VDR signalling in osteoclasts [14]. However, there are some differences between the Verlinden [14] studies and ours which could account for the disparate findings. We examined the role for VDR-mediated activity in mature osteoclasts *in vivo*, by generating an osteoclast-specific *Vdr* knockout mouse model using the Cre-LoxP system [16, 18]. The choice of the Ctsk^{Cre} founder line to delete the VDR in osteoclasts was based on its documented expression of *Cre* specifically in mature osteoclasts [18]. TheVerlinden [14] model targets immature cells as well as mature cells. We do not refute that there may not be a role for VDR signalling within early osteoclasts and suggest that if *Vdr* is deleted earlier in the osteoclast lineage that the system may be able to adapt and the resorption activity in the mature cell may be unaffected. Further, the use of a knock-in Cre model can lead to endogenous expression levels of the gene driving the Cre expression (M-Lysozyme) to be disrupted, which could have systemic effects. Besides the Cre model used for the conditional deletion of *Vdr*, Verlinden *et al.* [14] reported findings from a different gender and skeletal site, females, whereas we report here findings in males, and tibial metaphysis, whereas we report effects in vertebrae. There are also subtle but potentially important differences in diets utilised with respect to the concentrations of calcium and phosphorous. However, we clearly demonstrate that within mature, actively resorbing osteoclasts there is a significant role for VDR in ensuring appropriate bone resorption.

Conclusions

Overall, our findings are consistent with the notion that direct VDR signalling within osteoclasts may ameliorate osteoclastogenesis and resorptive activity, consistent with our previous observations in human models [11]. Therefore, in addition to adequate dietary intake, an intact 1,25D/VDR signalling mechanism is required for the physiological moderation of osteoclastic resorption activity.

Disclosures

The authors have nothing to disclose.

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Table 1: Body Weight (g), Femoral Length (mm) and Growth Plate Height (µm) in male floxed Vdr control ($Vdr^{fl/fl}$) and osteoclast-specific Vdr knockout ($Ctsk^{Cre}/Vdr^{-/-}$) mice at 6 weeks of age fed a NormCaP or LowCaP diet. Values presented as means±SEM, n=5-12. Two-way ANOVA (NormCaP vs LowCaP Floxed Controls [a]p<0.01 and NormCaP vs LowCaP $Ctsk^{Cre}/Vdr^{-/-}$ [b]p<0.01).

	NormCaP		LowCaP	
	$Vdr^{fl/fl}$	$Ctsk^{Cre}/Vdr^{-/-}$	$Vdr^{fl/fl}$	$Ctsk^{Cre}/Vdr^{-/-}$
Body Weight (g)	20.41 ± 0.60	21.06 ± 0.39	17.81 ± 0.90[a]	17.34 ± 0.42[a]
Femur Length (mm)	13.70 ± 0.23	13.92 ± 0.11	12.10 ± 0.26[a]	12.06 ± 0.41[a]
Growth Plate Height (µm)	66.35 ± 3.23	58.97 ± 2.38	72.02 ± 5.16	72.06 ± 6.68

Table 2: Serum Biochemistry for levels of Ca (mmol/L), Calcium; P (mmol/L), Phosphorous; ALP (mmol/L), Alkaline Phosphatase and CTX (ng/mL), Cross-Laps in male $Vdr^{fl/fl}$ and $Ctsk^{Cre}/Vdr^{-/-}$ mice at 6 weeks of age fed a NormCaP or LowCaP diet. Values presented as means±SEM, n=5-12. Two-way ANOVA (NormCaP vs LowCaP Floxed Controls [a]p<0.01; NormCaP vs LowCaP $Ctsk^{Cre}/Vdr^{-/-}$ [b]p<0.01; LowCaP $Vdr^{fl/fl}$ vs $Ctsk^{Cre}/Vdr^{-/-}$ [c]p<0.01).

	NormCaP		LowCaP	
	$Vdr^{fl/fl}$	$Ctsk^{Cre}/Vdr^{-/-}$	$Vdr^{fl/fl}$	$Ctsk^{Cre}/Vdr^{-/-}$
Ca (mmol/L)	2.16 ± 0.12	2.52 ± 0.12	2.49 ± 0.09	2.42 ± 0.05
P (mmol/L)	1.71 ± 0.14	2.26 ± 0.11	2.45 ± 0.15[a]	2.40 ± 0.14
ALP (mmol/L)	196.57 ± 43.20	221.21 ± 35.85	812.38 ± 22.85[a]	1176.24 ± 82.22[a,b]
CTX (ng/mL)	97.23 ± 11.53	116.04 ± 21.86	287.49 ± 31.44[a]	261.39 ± 47.73[a]

Table 3: Bone Formation Parameters. MS/BS (%), Mineralising surface per bone surface; MAR (µm/d), Mineral apposition rate and BFR/BS (µm^3/µm^2/t), Bone formation rate per bone surface in male floxed Vdr control (Vdr$^{fl/fl}$) and osteoclast-specific Vdr knockout (Ctsk^{Cre}/Vdr$^{-/-}$) mice at 6 weeks of age fed a NormCaP or LowCaP diet. Values presented as means±SEM, n=5-12. Unpaired T-Test ([*]p<0.05) vs Vdr$^{fl/fl}$ fed LowCaP.

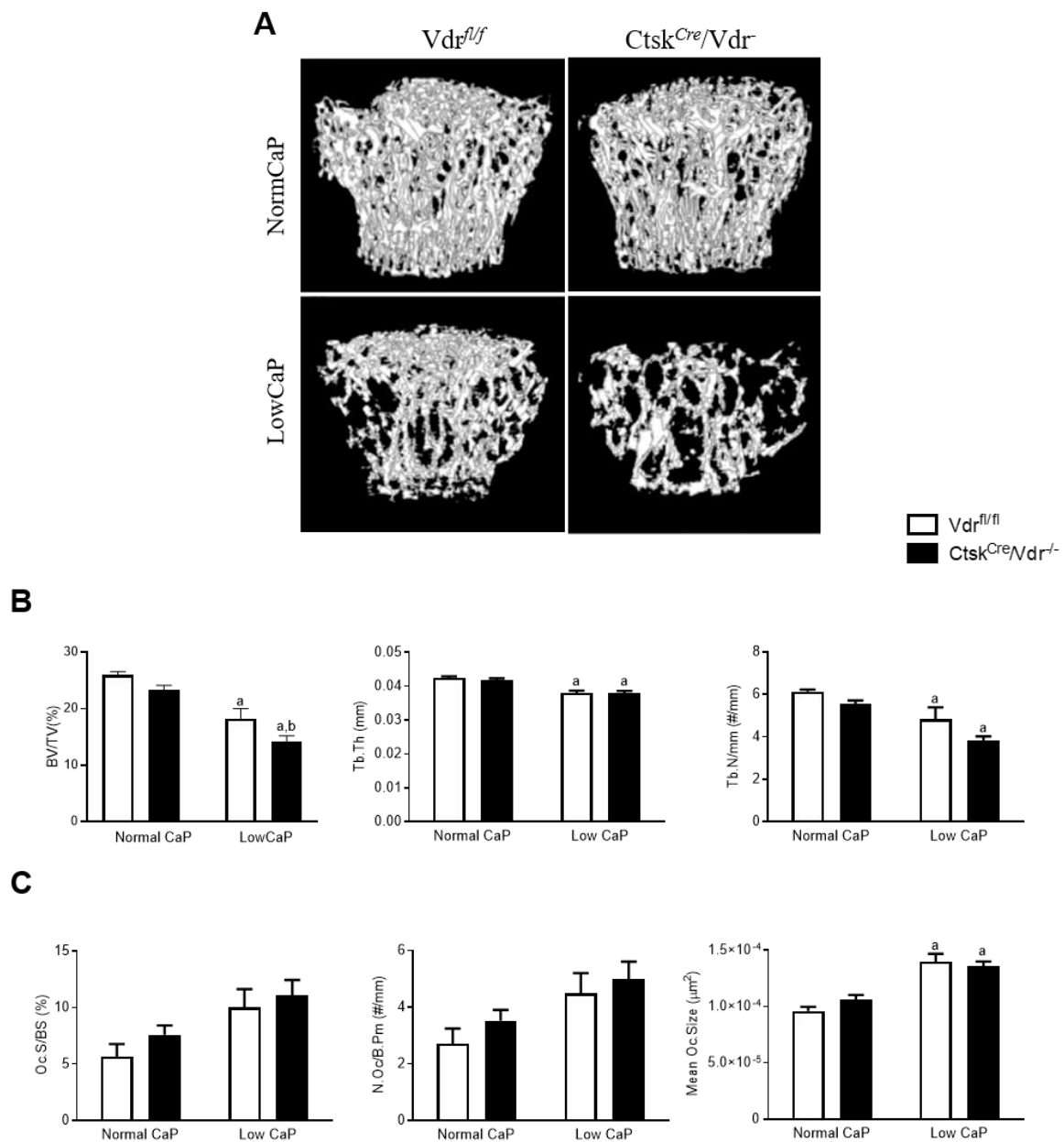|	*NormCaP*		*LowCaP*	
	Vdr$^{fl/fl}$	Ctsk^{Cre}/Vdr$^{-/-}$	Vdr$^{fl/fl}$	Ctsk^{Cre}/Vdr$^{-/-}$
MS/BS *(%)*	21.48 ± 1.64	23.61 ± 2.70	13.3 ± 1.60	17.55 ± 7.35
MAR *(µm/d)*	2.38 ± 0.37	1.77 ± 0.05	3.43 ± 0.21	1.53 ± 0.11[a]
BFR/BS *(µm^3/µm^2/t)*	0.48 ± 0.04	0.41 ± 0.04	0.45 ± 0.03	0.34 ± 0.06

Figure 1: A) Representative reconstructed 3-D models of L1 vertebrae. 6-week-old floxed control (Vdr$^{fl/fl}$) and osteoclast-specific Vdr knockout (Ctsk^{Cre}/Vdr$^{-/-}$) mice fed a NormCaP or a LowCaP diet. **B)** Vertebral Bone Volume (L1)**.** BV/TV (%), Bone volume per tissue volume; Tb.Th (mm), trabecular thickness and Tb.N (#/mm), trabecular number. **C)** Vertebral Osteoclastic Resorption. Oc.S/BS (%), osteoclast surface per bone surface; N.Oc/B.Pm (#/mm), number of osteoclasts per bone perimeter and Mean Oc.Size (μm^2). Data are presented as means ± SEM, $n = 5–12$ per group. Significant differences are shown due to [a]diet and [b]genotype ($p < 0.05$).
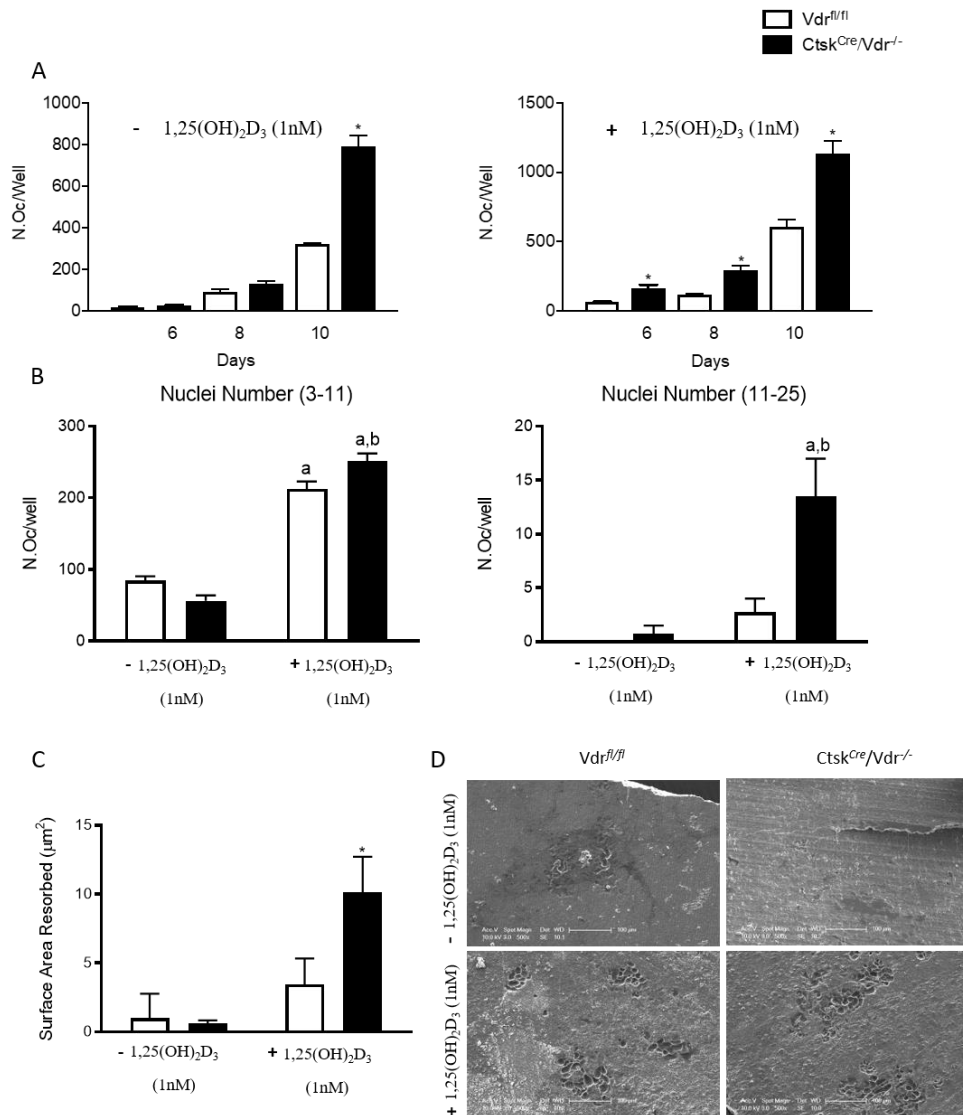
Figure 2: The effect of VDR deletion and vitamin D metabolite addition in mature osteoclasts under co-culture with MLOY-4 cell line. Co- cultures of splenocytes with MLO-Y4 cells in the presence or absence of $1,25(OH)_2D_3$ (1 nM) were performed, as described in Materials and Methods for the times indicated and assayed for TRAP$^+$, multinucleated cell formation and resorption of dentine. **A)** Total TRAP$^+$ cell numbers were combined from two independent experiments, each using splenocytes pooled from at least 4 animals/group. Data are shown as means ± SEM; significant difference is indicated by $^*p < 0.05$. **B)** TRAP+ osteoclasts were categorised as those containing 3-11 nuclei/cell and osteoclasts containing 11-25 nuclei/cell. Data shown are combined counts from three independent experiments, each using splenocytes pooled from at least 4 animals/group and shown as means ± SEM. Significant differences are shown due to [a]$1,25(OH)_2D_3$ exposure and [b]genotype ($p < 0.01$). **C)** Resorption activity on dentine. Average total surface area resorbed. Data are means ± SEM of quadruplicate wells and are representative of 2 independent experiments. Significant difference is indicated by $^*p < 0.05$. **D)** Representative scanning electron microscopy images of dentine resorption.

Supplementary Figure 1: Non-quantitative genomic DNA analysis of the deleted VDR product (visible bands) in different tissues harvested from osteoclast-specific VDR knockout mice (Ctsk^{Cre}/Vdr$^{-/-}$).