CXCR4 is a response gene for parathyroid hormone which affects osteoblast and osteoclast function in vitro

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Aims

To investigate the role of CXCR4 in response to teriparatide (TPTD) treatment in osteoblasts and osteoclasts.

Methods

Primary murine and human osteoblasts and osteoclasts, MC3T3 cell lines, and hMSC-TERT4 cell lines were treated with TPTD and/or AMD3100, a pharmacological inhibitor of CXCR4. Changes in gene expression, osteoblast viability, mobility, mineralization capacity, and alkaline phosphatase activity were investigated. Osteoclastogenesis and cell fusion were also assessed in response to both treatments.

Results

TPTD increased messenger RNA levels of *CXCR4* in all stages of both murine and human osteoblast differentiation. Mineralization analysis showed that CXCR4 was involved in bone matrix formation in response to TPTD. Alkaline phosphatase activity was also impaired by CXCR4 inhibition at early stages of osteoblast differentiation, while it was promoted at late stages, suggesting that CXCR4 could produce a delay in osteoblast maturation. Moreover, we also found a direct activation of osteoclastogenesis after TPTD treatment in murine and human osteoclasts. This process seems to involve CXCR4 activity, since AMD3100-induced CXCR4 inhibition led to a reduction in both murine and human osteoclastogenesis. This process, however, could not be prevented by TPTD treatment.

Conclusion

Our results suggest that *CXCR4* is a responsive gene to TPTD treatment, involved in the regulation of osteoblast and osteoclast generation and function. Further in vivo studies are required to confirm this role, and to determine whether pharmacological strategies targeting CXCR4 could potentially improve the treatment outcome for osteoporotic patients.

Article focus

- Teriparatide (TPTD) is an effective anabolic treatment for osteoporosis, but the response is highly variable in patients.
- Genetic background could be associated with differential response to TPTD in patients with osteoporosis.
- *CXCR4* could be a response gene to TPTD treatment in both osteoblasts and osteoclasts.

Key messages

- TPTD treatment highly increases *CXCR4* expression at the RNA level in osteoblasts.
- CXCR4 seems to play a role in osteoblast viability, migration, mineralization capacity, and alkaline phosphatase activity, which could not be restored by TPTD treatment.
- TPTD increases early and transient osteoclastogenesis, a process that might occur via CXCR4 activation.

Strengths and limitations

- This is a comprehensive analysis of the role of CXCR4 in both osteoblasts and osteoclasts, using not only murine but also human in vitro models.
- The in vitro analysis might not fully mimic the physiological response to TPTD.
- The use of a pharmacological inhibitor for CXCR4 activity could also cause side effects in unreported pathways that could also be associated with TPTD response.

Introduction

Osteoporosis is a common metabolic bone disease that affects one in three women and one in five men over 50 years old worldwide; currently, more than 200 million women worldwide suffer from this disease.[1–4] It is characterized by low bone mass and alterations in the bone microstructure which lead to an increased risk of fragility fractures. More than nine million osteoporotic fractures are reported yearly, and they are associated with a major socioeconomic burden.[5] Bisphosphonates are the first line of treatment for patients with osteoporosis; they inhibit bone resorption and prevent bone loss as well as increase mineralization of existing bone.[6,7] However, they are not able to reverse the excessive bone loss in patients with severe disease at high risk of fracture, i.e. patients over 65 years old with T-score below -3.5, elderly patients with prior fragility fractures, or those who continue to fracture or lose bone mineral density (BMD) after bisphosphonate therapy.[8] They require the use of an anabolic agent such as teriparatide (TPTD), a recombinant human parathyroid hormone (1-34).[9] TPTD is more effective than bisphosphonates at increasing BMD and preventing vertebral fractures, as seen in randomized controlled trials on postmenopausal women and glucocorticoid-induced osteoporosis.[10–12] Observational studies have also reported better results than standard care, especially at the vertebral site.[13] Although TPTD is superior to antiresorptives in treating osteoporosis, the response to treatment is highly variable, with an average 13% increase in lumbar spine BMD over a period of 18 to 24 months. Individual responses range from no change to an increase of 53.4% in BMD. One out of five patients treated with TPTD also presented an increase in BMD similar to patients treated with bisphosphonates, with an average of 5% or less increase after the treatment.[13,14]

TPTD binds the parathyroid hormone 1 receptor (PTH1R) on the surface of osteoblasts and prevents their apoptosis.[15] It also plays a role in bone marrow stromal cells, promoting their commitment into osteoblast differentiation.[16] Activation of PTH1R in osteocytes increases RANKL levels,[17] leading to activation of osteoclastogenesis. To date, the molecular mechanisms underlying TPTD activity in osteoblasts are not fully understood.

Recently, a two-stage genome-wide association study on the response to TPTD identified a locus in chromosome 2 significantly associated with increased BMD in the spine after the treatment.[18] The lead single nucleotide polymorphism in this signal (rs6430612) was located near the *CXCR4* gene, and was found to be an expression quantitative trait locus (eQTL) for this gene in blood.[18] *CXCR4* encodes a chemokine receptor for stromal cell-derived factor 1 (SDF1/CXCL12). This receptor belongs to the G-protein coupled receptor superfamily, and the transduced signal leads to an increase in intracellular calcium flux via inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), as well as activation of the mitogen-activated protein kinase (MAPK)1/MAPK3 and nuclear factor kappa B (NF-kB) pathways.[19] It is involved in essential physiological processes during embryogenesis, like the development of the central nervous, cardiovascular, and haematopoietic systems.[20,21] In bone, *CXCR4* is involved in haematopoietic and mesenchymal stem cell migration,[22] and bone morphogenetic protein (BMP)2-induced osteogenic differentiation of mesenchymal progenitors.[23,24] In vivo evidence shows that *CXCR4* is required for BMP-driven osteoblast development.[25] Moreover, Wnt signalling, a master regulator of osteoblasts, modulates *SDF1* gene expression at transcriptional level in mesenchymal stem cells.[26] CXCR4 signalling in mature osteoblasts can also feedback to increase osteoclast precursor pool size, and it is involved in bone formation and resorption.[27–29] SDF1/CXCR4 signalling pathway also plays a direct role in osteoclasts, promoting chemotactic migration of cell precursors, their differentiation into bone-resorbing osteoclasts, and survival, even in the absence of macrophage colony-stimulating factor (MCSF) and receptor activator of nuclear factor-κB ligand (RANKL).[29–31]

Although *CXCR4* is involved in bone homeostasis, its role in response to anabolic treatments like TPTD has not yet been elucidated. Here, we investigate whether *CXCR4* acts as a responsive gene to TPTD treatment at both the osteoblast and osteoclast level.

Methods

A detailed description of the methodology is provided in the Supplementary Material. In summary, we investigated the role of CXCR4 in response to TPTD in both osteoblasts and osteoclasts as shown below. All cell cultures were treated with either 50 nM TPTD and/or 100 μM AMD3100, a pharmacological CXCR4 inhibitor.

Osteoblast analyses were performed using the MC3T3-E1 subclone 14 murine osteoblastic cell line, human mesenchymal stem cells (hMSC-TERT4 cell),[32] and primary human osteoblast lineage cells from patients undergoing hip arthroplasty surgery.[33] The effect of TPTD on *CXCR4* expression was examined at baseline in non-differentiated cells, as well as in bone-forming osteoblasts in both murine and human cells.[34] The role of CXCR4 in osteoblasts in response to TPTD was

assessed by measuring the intracellular calcium levels, *CXCR4* expression at RNA level, cell viability and mobility, mineralization capacity, and alkaline phosphatase activity.

MCSF-derived macrophages were obtained from long bones of lower limbs from young (two- to four-month-old) and aged (18-month-old) C57Bl/6 wild-type mice, and differentiated into osteoclasts using human RANKL. Primary human macrophages were differentiated from human $CD14^+$ monocytes isolated from buffy coats obtained from anonymous blood donations as previously described.[35–37] The role of CXCR4 in osteoclasts in response to TPTD was assessed by counting the number of differentiation osteoclasts and fusion assays.[38]

Statistical analysis

Statistical analyses were performed in SPSS v27 (IBM, USA) using both parametric and non-parametric tests, depending on the data distribution. Overall differences between controls and treated cells were assessed by Kruskal-Wallis non-parametric test or two-way analysis of variance (ANOVA) parametric test in SPSS v27. Two-group comparisons were performed by paired *t*-test for experiments with five or more biological replicates, by Mann-Whitney U test for experiments with three biological replicates, or by one-sample Wilcoxon signed-rank test/independent-samples *t*-test for comparisons relative to baseline. Comparison between TPTD and TPTD + AMD3100 was performed using Dunn's multiple comparisons test post Kruskal-Wallis analysis. Analysis of the role of CXCR4 and TPTD in human osteoclasts was performed by paired *t*-test. P-values below 0.05 were considered statistically significant. At least three independent replicates per experiment were performed. Graphs were generated using GraphPad Prism v9.2 (GraphPad Software, USA).

Results

TPTD increases CXCR4 expression in osteoblasts

The MC3T3-E1 mouse cell line was differentiated into bone-forming osteoblasts for three weeks using osteogenic media, and stimulated with 50 nM TPTD (Supplementary Figure a) for 30 hours at undifferentiated stage and after seven days in osteogenic media to generate differentiated osteoblasts. During undifferentiated stages, TPTD delayed the expression of *CXCR4* messenger RNA (mRNA), which was lower than the control at two hours and four hours (median fold change = 0.6 (IQR 0.5 to 0.8) and then increased at 24 hours (median fold change = 1.2 (IQR 1.1 to 1.9)) compared with control cells (p = 0.009, Kruskal-Wallis test) (Figure 1a). Once the cells were differentiated into osteoblasts, there was a trend of delayed *CXCR4* expression in response to TPTD at two hours of treatment, but the difference with the control was not statistically significant. After four hours of stimulation, TPTD increased the expression of *CXCR4* ($p = 9.453 \times 10^{-5}$) and it was sustained for up to 30 hours (median fold change = 7.4 (IQR 5.3 to 9.8)) (Figure 1b). This increase in expression was maintained during the whole differentiation period of three weeks on osteogenic media, with the highest difference at two weeks of differentiation (median fold change = 32 (IQR 13.4 to 35.7)) (Figure 1c). Control cells showed a short and early increase in *CXCR4* expression in differentiated osteoblasts (median fold change range = 1.5 to 4.7; p = 0.001, Kruskal-Wallis test) (Supplementary Figure b). The expression of CXCR4 in response to TPTD was also evaluated at a protein level in MC3T3 differentiated osteoblasts treated with the drug for 24 hours; results showed a trend to increased *CXCR4* levels (median = 2 (IQR 1.4 to 2.5)) (Supplementary Figure c).

Increase of *CXCR4* expression at RNA level after TPTD treatment was also investigated in human mesenchymal stem cells during their differentiation into osteoblasts (Figure 1d). Although greater variability was detected in human cells compared with MC3T3, *CXCR4* expression increased at early stages of differentiation (p = 0.031, one-sample Wilcoxon signed-rank test) when treated with TPTD, while its expression was downregulated in control osteoblasts after seven days of differentiation (p = 0.031, one-sample Wilcoxon signed-rank test) (Supplementary Figure d).

CXCR4 regulates osteoblast viability

The role of CXCR4 in osteoblast viability was evaluated in MC3T3-E1 mouse osteoblast-like cells. During their differentiation process onto bone-forming osteoblasts, cells were intermittently treated with 50 nM TPTD and 100 µM AMD3100 compound to inhibit CXCR4 activity, which was confirmed by the inhibition of intracellular calcium in osteoblasts (Supplementary Figure e). TPTD treatment showed variable effects in osteoblast viability during differentiation (with individual increases up to 56%), but these changes were not statistically significant (p = 0.250, one-sample Wilcoxon signed-rank test) (Supplementary Figure f). However, CXCR4 inhibition showed a reduction in cell viability (p = 0.008, Kruskal-Wallis test), especially after two weeks of osteoblast differentiation (median percentage of control = 40.5% (IQR 25.4 to 40.5)). The reduction in osteoblast viability due to AMD3100 could not be reverted by TPTD, and cells on combined AMD3100 and TPTD treatment showed a 56.8% median difference in viability on the second week of differentiation compared to TPTD alone (p = 0.089, Kruskal-Wallis with Dunn's multiple comparisons test) (Figure 2). This effect was sustained in time, and similar inhibition was detected after three weeks of osteoblast differentiation.

CXCR4 is involved in osteoblast migration

Treatment with TPTD and/or AMD3100 affected osteoblast migration capacity ($p = 6.494 \times 10^{-5}$, Kruskal-Wallis test; Figure 3). TPTD alone increased cell motility by a median of 18% (IQR 11% to 34%), although this change was not statistically significant (p = 0.308, Kruskal-Wallis with Dunn's multiple comparisons test) (Figure 3), while AMD3100 inhibition of CXCR4 showed a reduction in cell migration (median percentage of control = 51% (IQR 50.6 to 51.9); p = 0.041, Kruskal-Wallis with Dunn's multiple comparisons test). Combined treatment of TPTD and AMD3100 was not able to revert the inhibition on the migration capacity of osteoblasts induced by AMD3100 (median percentage migration of 54% (IQR 54.3 to 56.3) compared with TPTD alone; p = 0.041, Kruskal-Wallis with Dunn's multiple comparisons test) (Figure 3).

CXCR4 is involved in osteoblast mineralization in response to TPTD

The role of CXCR4 in the osteoblast mineralization capacity in response to TPTD was assessed in the MC3T3-E1 cell line cultured with osteogenic media to promote bone matrix

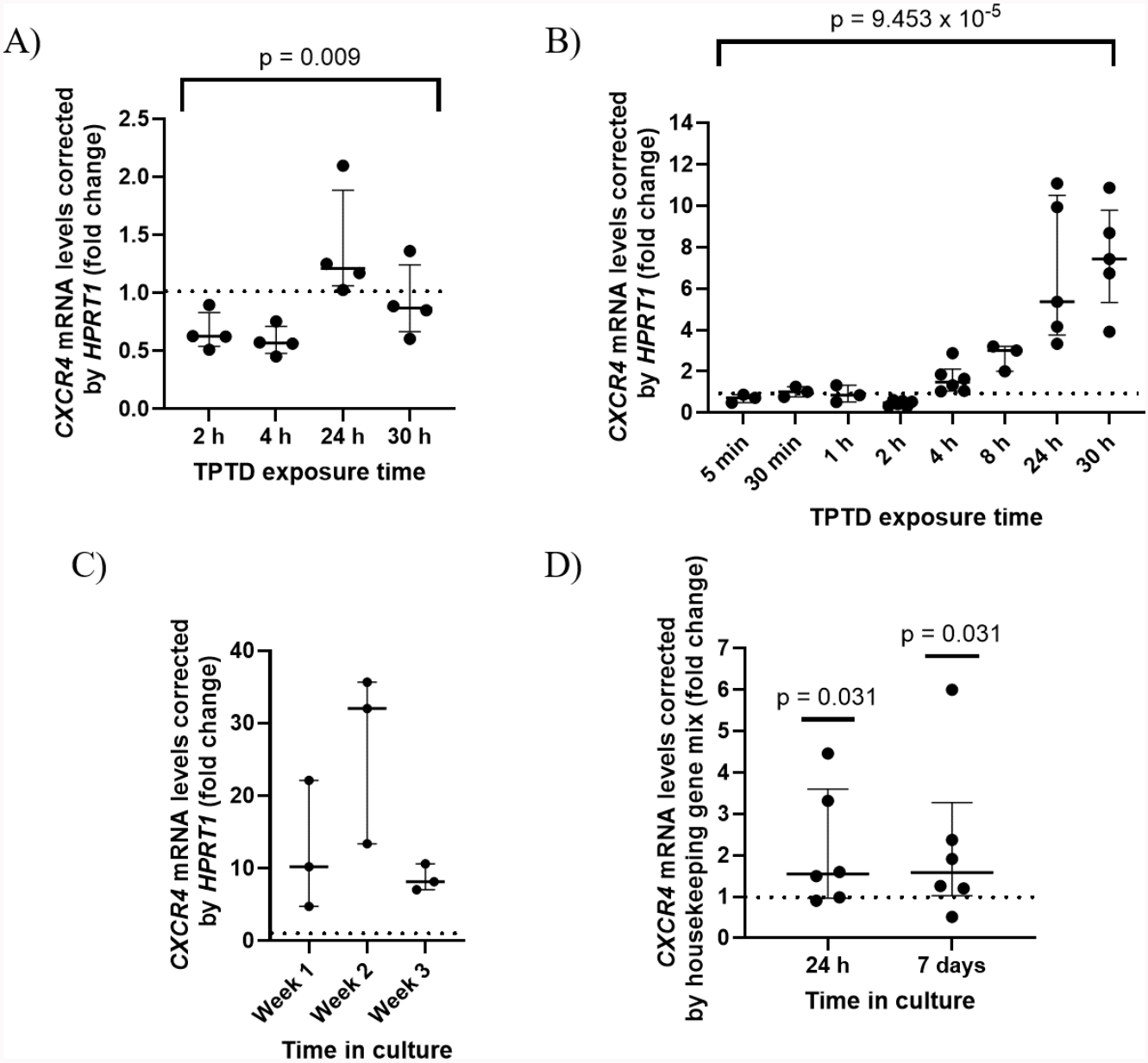

Fig. 1

CXCR4 expression at RNA level in response to teriparatide (TPTD) treatment in: a) MC3T3 undifferentiated pre-osteoblasts (biological replicates = 4; Kruskal-Wallis test); b) seven days of osteoblast differentiation (n = 3 to 6; Kruskal-Wallis test); and c) three weeks of osteoblast differentiation (biological replicates = 3). Levels are relative to control *CXCR4* expression at each timepoint. d) *CXCR4* expression at RNA level in response to TPTD treatment in human mesenchymal stem cells during the differentiation process. Housekeeping gene mix refers to the average mean of Ct values of ubiquitin C (*UBC*), TATA-box binding protein (*TBP*), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1)* genes. Values are shown as median (IQR). Biological replicates = 6. One-sample Wilcoxon signed-rank test was used to compare each timepoint to the control levels. Dotted line shows the control fold change. mRNA, messenger RNA.

mineralization. Cells were intermittently treated with 50 nM TPTD, while CXCR4 activity was continuously inhibited with 100 μM AMD3100. Both treatments played a role in osteoblast mineralization (1 week p = 0.007, 2 weeks p = 6.494 × 10^{-5}, 3 weeks p = 0.003, Kruskal-Wallis with Dunn's multiple comparisons test; Figure 4). TPTD treatment showed a trend to low mineralization rate at late stages of differentiation compared with controls (p = 0.257, Kruskal-Wallis with Dunn's multiple comparisons test; Figure 4). Impaired CXCR4 activity by AMD3100 reduced the mineralization rate by 25% at early stages and by 63% at late stages. TPTD was not able to revert this effect (1 week p = 0.025, 2 weeks p = 0.007, 3 weeks p = 0.025, Kruskal-Wallis with Dunn's multiple comparisons test; Figure 4).

Pharmacological inhibition of CXCR4 stimulates ALP activity in MC3T3 cells

MC3T3-E1 osteoblast-like cell line was differentiated towards mature osteoblasts after three weeks of culturing in osteogenic media. Alkaline phosphatase (ALP) activity was measured at different timepoints during the process, and results showed that both treatments affect ALP activity in osteoblasts, especially at late stages of differentiation (p = 0.004, Kruskal-Wallis non-parametric test; Figure 5). AMD3100 increased ALP activity (p = 0.017, Kruskal-Wallis with Dunn's

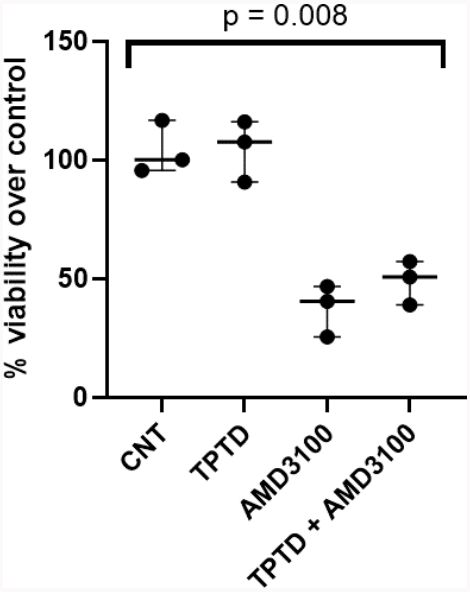

Fig. 2
Median (IQR) changes in cell viability during murine MC3T3-E1 osteoblast differentiation for two weeks in response to AMD3100 and teriparatide (TPTD) (biological replicates = 3). Kruskal-Wallis non-parametric test was used to calculate the p-value. CNT, control.

multiple comparisons test) compared to control (median fold change 3.7 (IQR 3.1 to 7.3) difference at week 3). TPTD treatment administered in combination with AMD3100 still showed higher ALP expression than the control (median fold change 2.6 (IQR 2.2 to 3.4) difference) (Figure 5).

Human osteoblasts were cultured for approximately 35 days in osteogenic media and treated with 50 nM TPTD and 100 µM AMD3100 for 72 hours. ALP activity was measured, and results suggest that there is a trend of low levels of ALP after pharmacological inhibition of CXCR4 (p = 0.402 for donor 1 and p = 0.226 for donor 2, one-way analysis of variance (ANOVA)). Treatment with TPTD was not able to revert this expression to control levels (Figure 6).

Pharmacological inhibition of CXCR4 signalling inhibits osteoclast formation

Primary osteoclasts were generated from the bone marrow of long bones of four-month-old wild-type C57Bl/6 female mice (n = 3). MCSF-driven macrophages were stimulated with 100 ng/ml RANKL until osteoclasts were formed and treated with 50 nM TPTD (intermittently) and 100 µM AMD3100 (continuously) throughout the process. Both TPTD and AMD3100 treatments affected osteoclastogenesis (Figure 7), especially in the early stages (p = 0.042, Kruskal-Wallis with Dunn's multiple comparisons test) and medium stages (p = 0.015, Kruskal-Wallis with Dunn's multiple comparisons test) of differentiation. After two days' stimulation with RANKL and MCSF, TPTD treatment increased the number of osteoclasts by 2.5 median fold change compared to controls, but their lifespan was shorter, and by day 5 the number of osteoclasts was reduced (74% reduction; p = 0.053, Kruskal-Wallis with Dunn's multiple comparisons test; Figure 7a) compared to control. Treatment with CXCR4 inhibitor AMD3100 reduced overall osteoclastogenesis (20% to 72% decrease). Combined TPTD and AMD3100 treatments failed to prevent the reduction in osteoclastogenesis caused by AMD3100 at all stages of the osteoclastogenesis process (2 days p = 0.027 and 4 days p = 0.013; Kruskal-Wallis with Dunn's multiple comparisons test) (Figures 7a to 7c).

Bone marrow was also obtained from mice aged 18 months (n = 3), and osteoclasts were generated after stimulation with MCSF and RANKL for seven days, similar to the abovementioned experiments on young adult mice. Cultures were also treated with 50 nM TPTD and/or 100 µM AMD3100 during osteoclast development. Both treatments affected osteoclastogenesis (p = 0.033 at day 3 and p = 0.008 at day 5, Kruskal-Wallis with Dunn's multiple comparisons test; Figures 7d and 7e). TPTD stimulation increased the number of osteoclasts, especially at early stages (median fold change = 1.36) compared to controls. However, inhibition of CXCR4 by AMD3100 reduced the number of osteoclasts from day 3 (22% decrease) to day 5 of differentiation (34% decrease) compared to control. During late stages of differentiation, there was also a trend of lower osteoclast number than the control, but the difference was not statistically significant due to a greater variability between replicates. The effect of TPTD in osteoclastogenesis was blunted by pharmacological inhibition of CXCR4, and no differences were found with the cultures treated with only AMD3100 (Figures 7d and 7e).

Pharmacological inhibition of CXCR4 inhibits formation of primary human osteoclasts

TPTD administration to human osteoclasts derived from $CD14^+$ blood monocytes showed a variable effect in osteoclastogenesis at early stages (Figure 8a), from a trend to decrease the number of cells after treatment in one subject (donor 3) compared with the control culture (technical replicates = 8) (p = 0.062, paired *t*-test) to an increase in osteoclast formation in two individuals (donor 1 fold change = 1.6, p = 0.001; donor 5 fold change = 1.5; p = 0.051, paired *t*-test) compared with the control culture (technical replicates per individual = 8) (Supplementary Figures g to k). Overall results showed no significant differences in the number of osteoclasts in response to TPTD compared with control (Figure 8). Pharmacological inhibition of CXCR4 by AMD3100 showed conflicting results at three days of osteoclast differentiation, increasing the number of cells in three individuals while decreasing the number of osteoclasts in the other two volunteers (Supplementary Figures g to k). Overall results showed no differences in the number of osteoclasts compared with controls at this stage (Figure 8a). However, at late stages of osteoclastogenesis (7 days on RANKL stimulation), a reduced number of osteoclasts was found in response to AMD3100 in all the individuals tested (mean fold change decrease = 2.8; p = 0.013, paired *t*-test) (Figure 9b and Supplementary Figures g to k). The number of nuclei per osteoclast was also reduced by 1.5-fold at late stages of differentiation (p = 0.004, paired *t*-test) (Figure 9 and Supplementary Figures g to k). Treatment with TPTD did not prevent the reduction in osteoclastogenesis or number of nuclei per osteoclast caused by AMD3100 (Figures 8 and 9, Supplementary Figure g).

Discussion

TPTD is an effective treatment for established osteoporosis; however, the individual response to treatment is highly variable and about 15% of patients do not respond well to

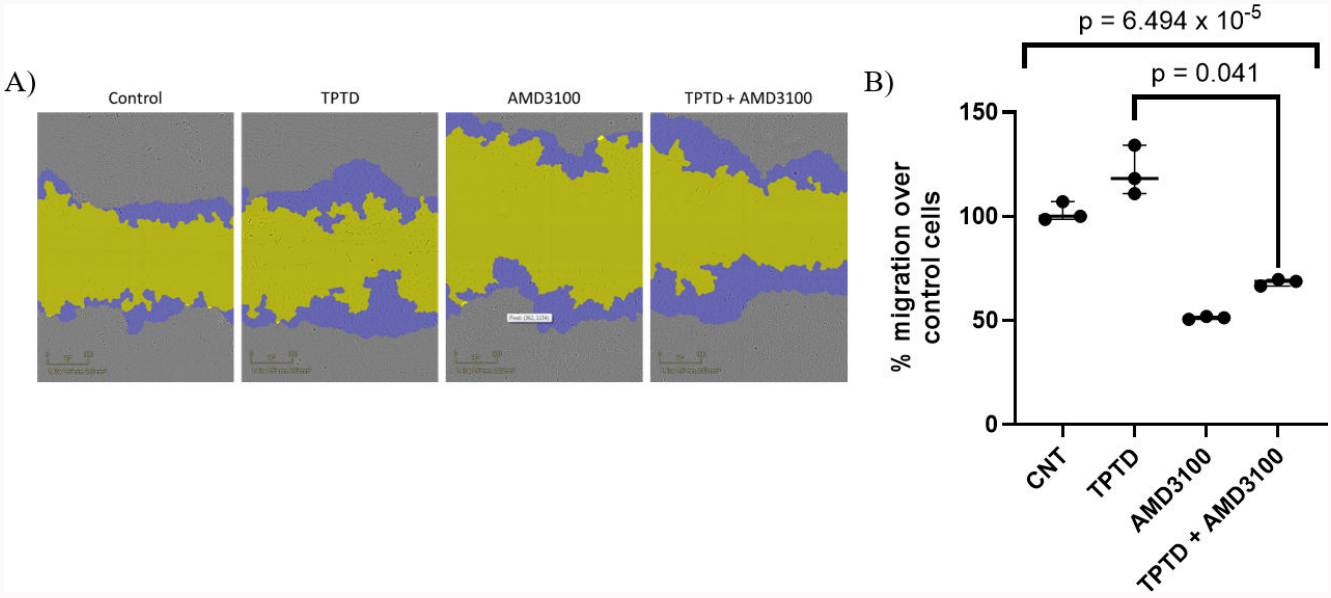

Fig. 3
Migration analysis of MC3T3-E1 differentiated osteoblasts in response to teriparatide (TPTD) or AMD3100. a) Visual representation of the migration (remaining wound area represented by yellow, mobility area represented by purple). b) Quantification of the mobility in the different treatments (median % migration (IQR), n = 3, each biological replicate representing the mean of four technical replicates). Statistical test: Kruskal-Wallis with Dunn's multiple comparisons test (TPTD vs TPTD + AM3100). CNT, control.

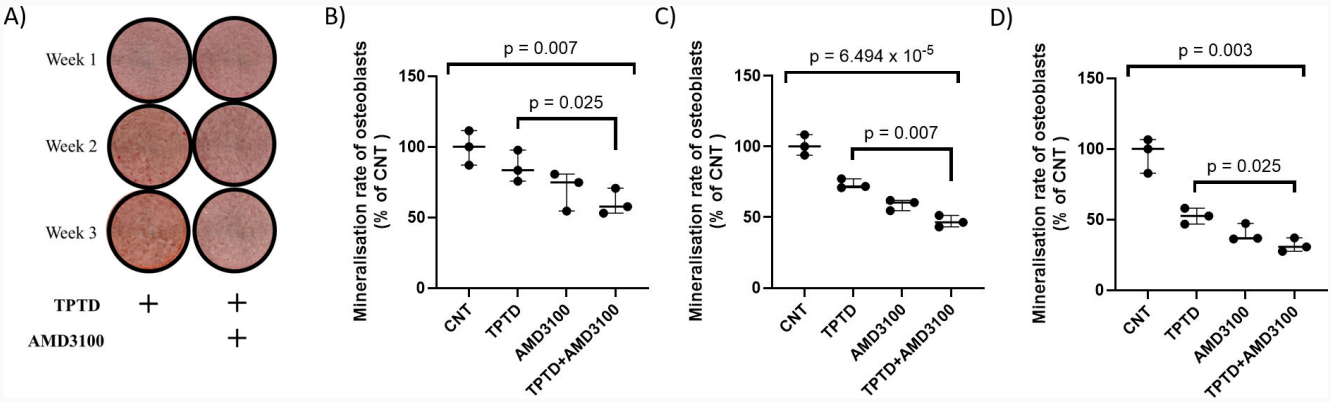

Fig. 4
Mineralization rate of MC3T3-E1 osteoblasts at different stages of differentiation in response to teriparatide (TPTD) and AMD3100. a) Alizarin red staining; quantification of mineralizing nodules after treatment b) one week, c) two weeks, and d) three weeks of osteoblast differentiation (median (IQR), biological replicates = 3). P-values calculated with Kruskal-Wallis with Dunn's multiple comparisons test (TPTD vs TPTD + AM3100). CNT, control.

the drug.[14] Recently, rs6430612 SNP,[18] near the *CXCR4* gene and regulating its expression in blood,[39] was found to be associated with TPTD response in patients with osteoporosis. *CXCR4* encodes a chemokine receptor that has demonstrated a role in bone metabolism,[40] by increasing BMP-derived bone formation.[25] CXCR4 can also regulate osteoclastogenesis, although the results are controversial.[27,41] However, to date there is no information about the role of *CXCR4* in the anabolic response to TPTD.

In this study, we confirmed the key role of CXCR4 in bone metabolism, at both an osteoblast and osteoclast level. We also showed that *CXCR4* is a response gene for TPTD.

In order to investigate the role of CXCR4 in bone metabolism, we used a pharmacological inhibitor (AMD3100), which competes with the natural ligand SDF1 to bind CXCR4 and blocks downstream activation of the receptor. This compound is highly specific for CXCR4 and has a long-lasting inhibitory effect.[42] Our results confirmed that AMD3100 blocks the CXCR4 pathway for a long period of time, by inhibiting the calcium flux in osteoblasts.

Osteoblasts are the main target cell for the anabolic effect of TPTD, via PTH1R, a G-protein coupled receptor that activates the signalling pathways upon binding of TPTD ligand. We found that TPTD treatment increased the levels of *CXCR4* mRNA at all stages of osteoblast differentiation in both murine and human osteoblasts. To our knowledge, this is the first evidence of a link between the CXCR4 pathway and TPTD response. The lower increase in *CXCR4* expression in response to TPTD found in human osteoblasts compared to murine cells could be related to the presence of a regulatory SNP that conditions the response to the treatment, in line with the GWAS findings.

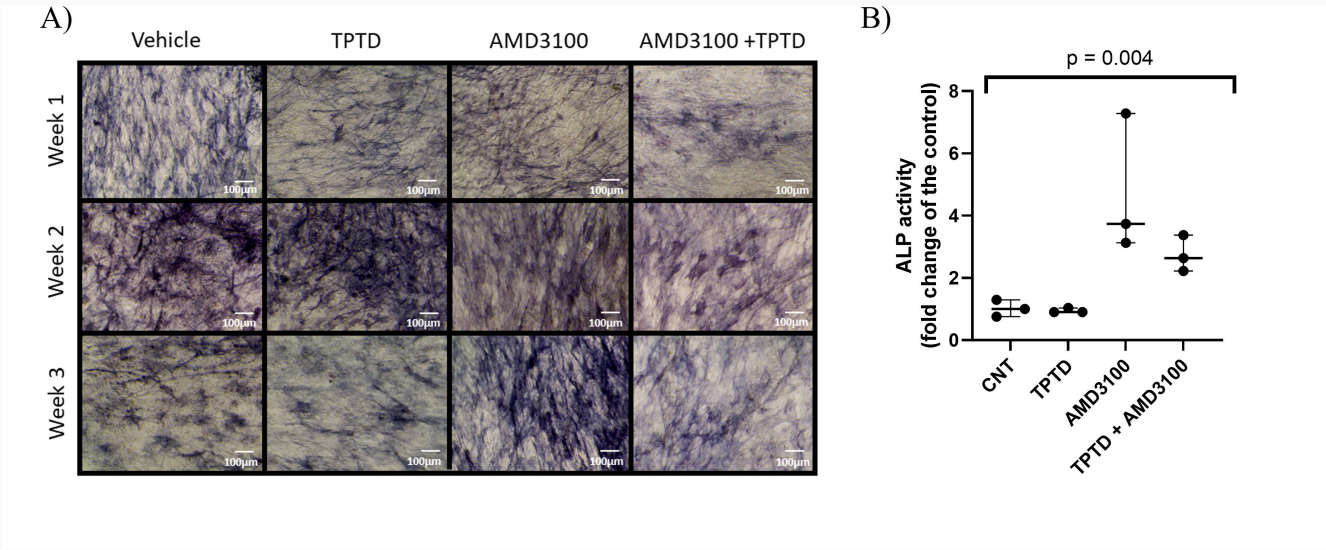

Fig. 5
Alkaline phosphatase (ALP) activity during osteoblast differentiation in response to teriparatide (TPTD) and AMD3100. a) Representative pictures. b) Quantification of ALP activity after three weeks of osteoblast differentiation (median (IQR), biological replicates = 3). The p-value was calculated with Kruskal-Wallis non-parametric test. CNT, control.

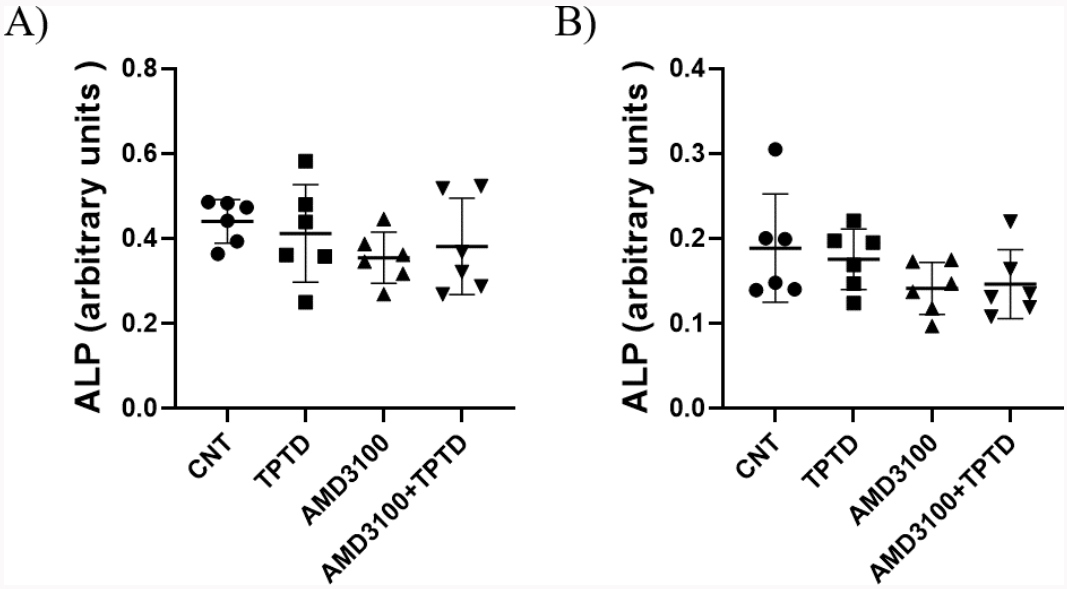

Fig. 6
Effect of teriparatide (TPTD) and AMD3100 on alkaline phosphatase (ALP) activity in primary human osteoblasts from two donors (mean (SD), biological replicates = 6). a) Donor 1 results. b) Donor 2 results. CNT, control.

Early studies suggested that the TPTD anabolic action in bone led to an increase in cell proliferation,[43] although it is now accepted that the increase in the number of osteoblasts is mainly due to a reduction in apoptosis after TPTD treatment, rather than increasing cell proliferation.[15] Our results show variable effects on increased cell viability, although not significant, after TPTD treatment through the osteoblast differentiation process. However, inhibition of CXCR4 by AMD3100 largely reduced cell viability during all stages of osteoblast differentiation. These results are in line with previous reports on CXCR4-mediated viability in mesenchymal stem cells.[44] Combined treatment with TPTD did not prevent the reduction in osteoblast viability caused by AMD3100.

TPTD treatment has been reported to promote mobilization of mesenchymal stem cells from the bone marrow.[45] In keeping with this, we also found that TPTD showed a trend to increase mobility of differentiated osteoblasts, which could probably help them to reach bone areas where new matrix is required. CXCR4 has been proven to be a key player in cell mobility by promoting cytoskeleton rearrangements and activation of integrin proteins.[46,47] This process is especially relevant to regulate homing of mesenchymal stem cells to the bone marrow environment, for tissue repair and bone healing.[48] Pharmacological inhibition

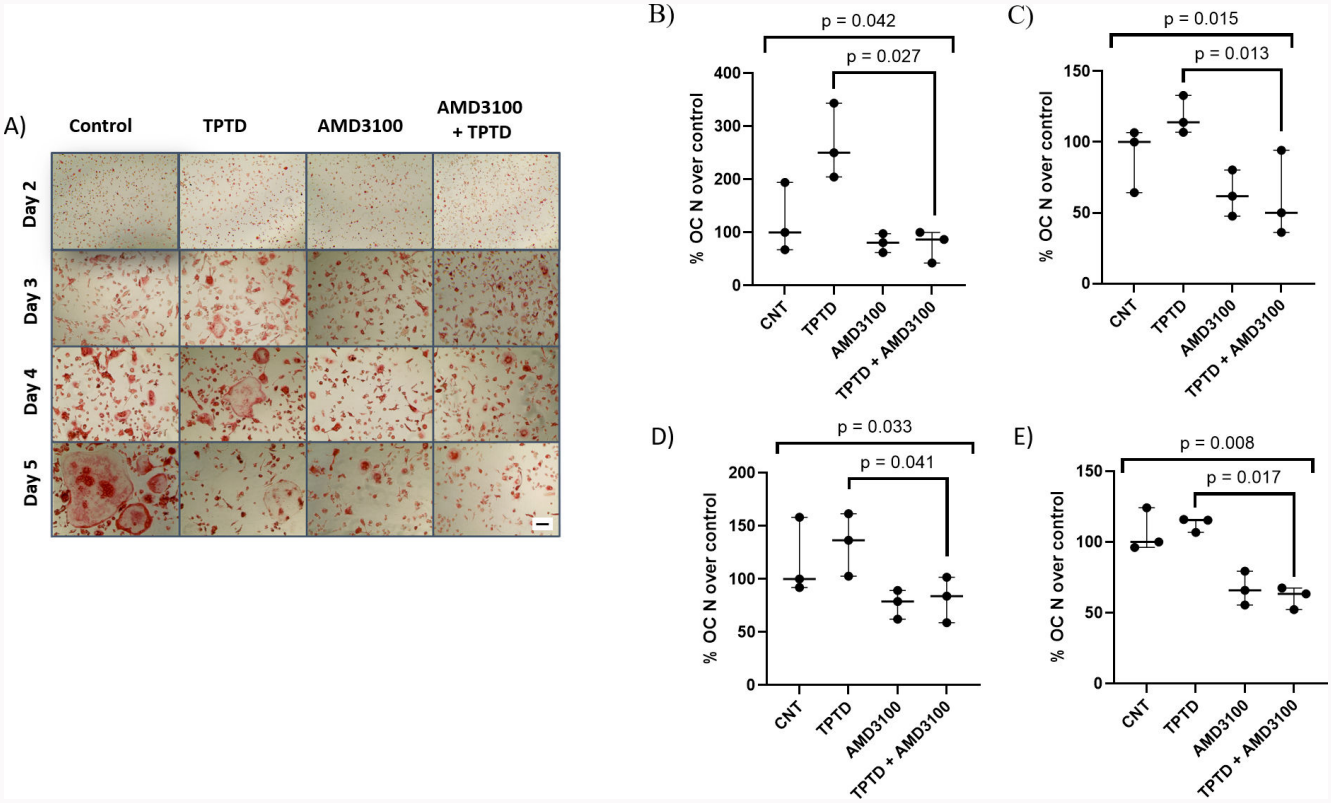

Fig. 7

Murine osteoclastogenesis in response to teriparatide (TPTD) and AMD3100. a) Tartrate-resistant acid phosphatase (TRAcP) staining for osteoclasts (OC) from young mice (scale bar: 100 µm). Median numbers of osteoclasts from: b) young mice at day 2 of differentiation; c) young mice at day 4 of differentiation; d) aged mice at day 3 of differentiation; and e) aged mice at day 5 of differentiation (error bars represent IQRs, biological replicates = 3; each biological replicate represents the mean of eight technical replicates). P-values were calculated using Kruskal-Wallis with Dunn's multiple comparisons test (TPTD vs TPTD + AM3100). CNT, control.

of CXCR4 in our osteoblast model confirmed a reduction in cell migration, a process that could not be reverted by TPTD.

Reduced CXCR4 activity also affected bone mineralization. Previous work on calcium deposition of mesenchymal stem cells stimulated with BMP2 showed a large decrease in mineralization after blocking CXCR4 by AMD3100.[23] TPTD treatment alone did not increase mineralization when compared to control osteoblasts, in line with previous in vivo reports, where TPTD increased bone strength and formation but reduced bone mineralization.[49,50] However, when combined with AMD3100, the reduction in mineralization is even higher than TPTD alone, suggesting that CXCR4 could accentuate the reduction of calcium deposition in the bone matrix driven by TPTD.

CXCR4 inhibition via AMD3100 caused a delay in the ALP activity that was only promoted at late stages of osteoblast differentiation in MC3T3 cells. ALP is a well-known early osteogenic marker for bone formation, which is secreted by pre-osteoblasts to promote phosphate concentration at the osteoblast surface and bone mineralization.[51] AMD3100-induced expression of ALP at late stages of osteoblast differentiation suggests that CXCR4 inhibition might affect osteoblast maturation, and cells might be maintained in an undifferentiated status for longer than required. These findings are also supported by previous murine studies in a *CXCR4* conditional knockout for osteoblasts. In this model, cells were arrested at early stages of development, and did not progress correctly towards maturation and bone formation, contributing in part to a reduced bone formation detected in these mice.[25] Analysis of human osteoblasts treated with AMD3100 showed a trend to low levels of ALP activity, in line with previous findings where inhibition of CXCR4 reduced ALP activity induced by BMP2 in mesenchymal stem cells[23] and C2C12 cells.[28] TPTD treatment increased ALP activity at early stages of differentiation, and in combination with AMD3100, it showed a trend to reducing the high levels of ALP activity at late stages caused by CXCR4 inhibition, although it was not able to fully revert them to control levels. The delay in osteoblast maturation by AMD3100 could also be associated with the reduction in bone mineralization we detected after inhibiting CXCR4. Although TPTD could promote osteoblast differentiation, it was not able to fully mitigate the effect of AMD3100.

Considering the regulatory crosslink between osteoblasts and osteoclasts and the requirement of both cell types for maintaining bone homeostasis,[52] we also investigated whether CXCR4 could play a role in the response to TPTD at the osteoclast level. To date, there is debate regarding how TPTD is involved in osteoclastogenesis and function. Most studies suggest that TPTD could only indirectly activate osteoclast formation, by increasing osteoblast-produced RANKL and MCSF, since these cells do not express PTH1R.[53,54] However, other groups have detected PTH1R expression in osteoclasts,[55,56] raising the possibility of direct

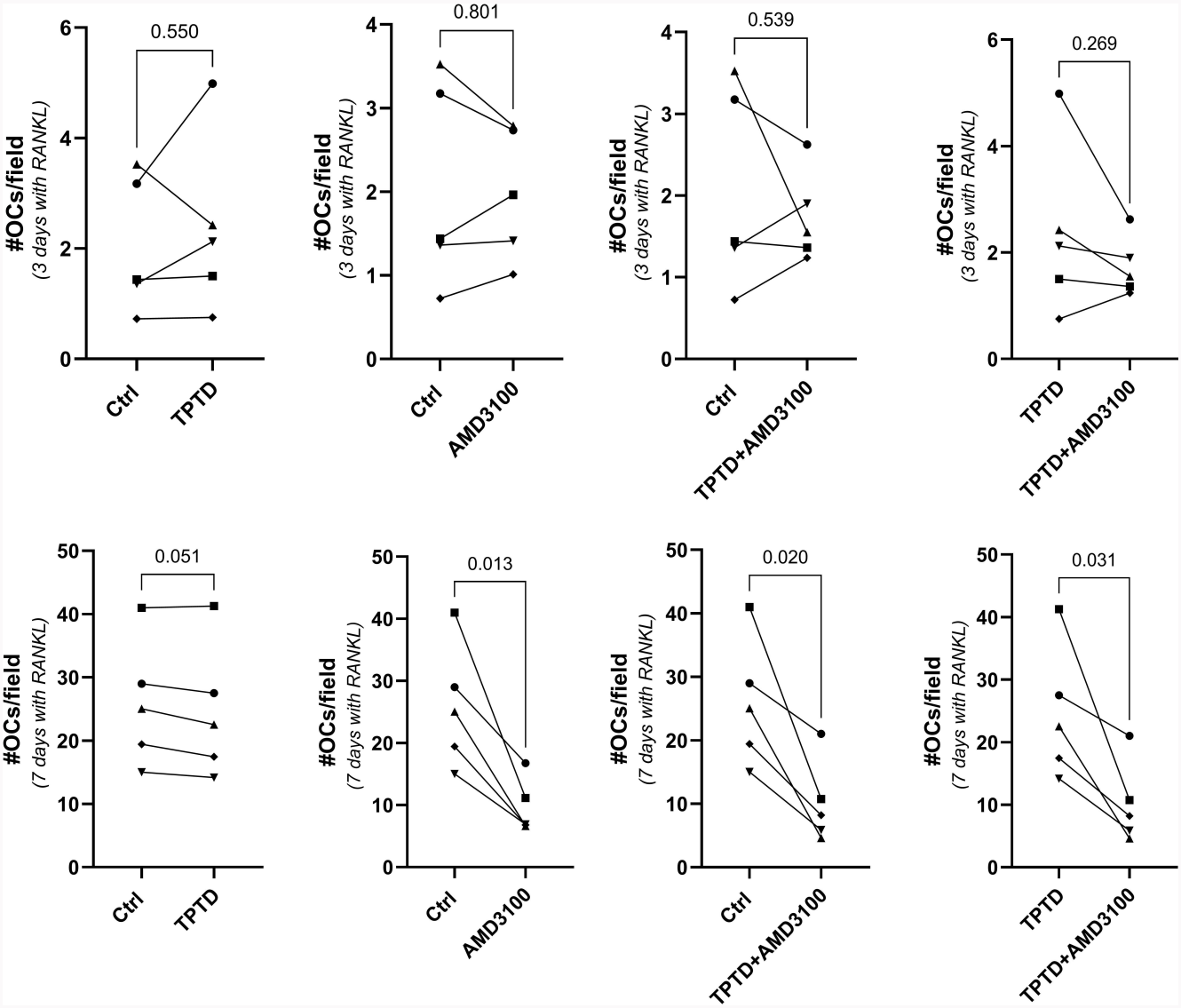

Fig. 8

Number of osteoclasts (OCs) in five human subjects after the different treatments at a) three days and b) seven days of differentiation. Each point represents the mean of eight technical replicates. All p-values calculated with paired *t*-test. Ctrl, control; RANKL, receptor activator of nuclear factor-κB ligand; TPTD, teriparatide.

regulation by TPTD. We have confirmed the expression of PTH1R in a mRNAseq analysis of murine primary osteoclasts from bone marrow,[57] but not in human osteoclasts.[58]

We also found a direct effect of TPTD treatment in murine bone marrow-derived osteoclasts from young mice, leading to an early and transient increase in osteoclastogenesis, followed by a trend of lower number of cells at late stages of differentiation compared to controls. This stimulatory effect was sustained for longer in aged mice. This short activation of osteoclastogenesis at early stages would support previous findings where initial osteoclast resorption was required for an appropriate anabolic action of TPTD.[59] Inhibition of CXCR4 by AMD3100 showed a large decrease in the number and size of osteoclasts throughout the whole differentiation process, similar to previous findings,[29] which suggested that the CXCR4 role in cell migration is crucial to attract macrophage precursors prior to generating multinucleated osteoclasts. Downregulation of CXCR4 activity would thus impair this process and prevent proper osteoclastogenesis. Combined treatment with TPTD and AMD3100 failed to show any improvement in this process.

In order to confirm these findings in humans, we generated osteoclasts from blood samples of five independent donors. The effect of TPTD in murine osteoclastogenesis could not be fully replicated, but we identified an increased number of osteoclasts at early stages of differentiation in two out of the five donors. This suggests that the effect of TPTD is not as homogeneous as in murine osteoclasts, probably due to genetic variability that could interfere with the expression or activity of PTH1R. Interindividual variability in osteoclastogenesis and osteoclast function in vitro, as well as in their response to drugs, has already been described.[37,60,61] Different factors could play a role in such variation in osteoclast development and function including clinical characteristics such as age or menopausal status, and genetic variability such as changes in DNA methylation. This could also be in line with the conflicting results reported in literature regarding the expression of the TPTD receptor.[53–56] Moreover, alternative

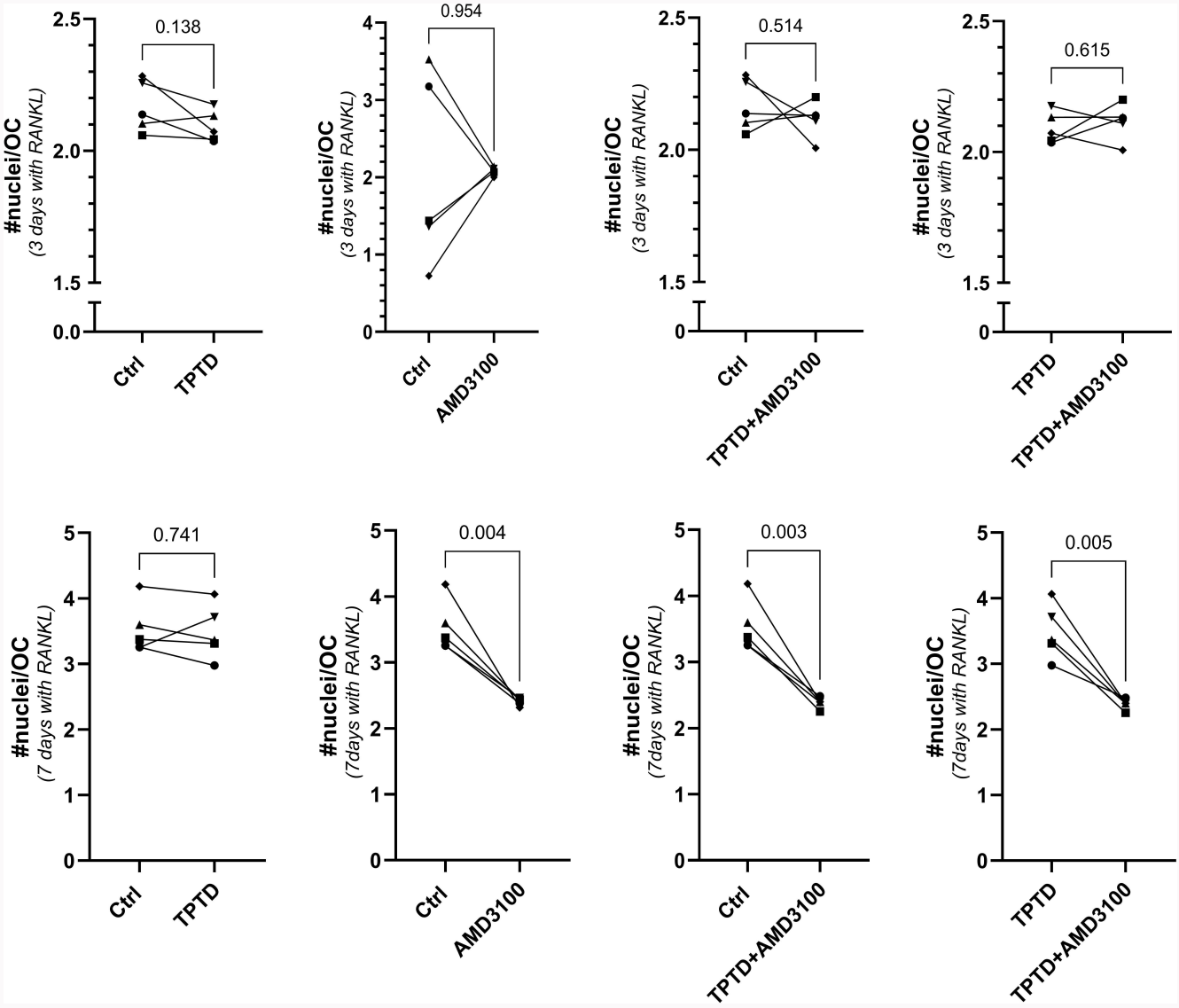

Fig. 9

Number of nuclei per osteoclast (OC) in five human subjects after the different treatments at a) three days and b) seven days of differentiation. Each point represents the mean of eight technical replicates. All p-values calculated with paired *t*-test. Ctrl, control; RANKL, receptor activator of nuclear factor-κB ligand; TPTD, teriparatide.

non-canonical signalling could also be involved in the TPTD effect in osteoclasts, and further investigation is required to identify TPTD-related pathways.

Inhibition of CXCR4 by AMD3100 in human osteoclasts consistently showed a lower number of cells and reduced number of nuclei per cell, confirming the results from murine bone marrow osteoclasts. These results were in line with previous findings, highlighting the role of CXCR4/SDF1 in chemotactic recruitment, development, and survival of both human and murine osteoclasts.[29,62] CXCR4 was also found to play a role in increasing osteoclastogenesis and bone resorption associated with cancer, such as in multiple myeloma (reviewed in Ullah[63] and in metastatic osteolysis),[64,65] although conflicting results showing an increase in osteoclastogenesis after CXCR4 disruption in a bone cancer model have also been reported.[41] Moreover, we found that combined treatment of TPTD and AMD3100 in human osteoblasts also failed to recover the number or size of osteoclasts compared with control samples, confirming the results we obtained in murine osteoclasts.

This study has strengths and limitations to be considered. We have performed an extensive analysis of the generation and function of osteoblasts and osteoclasts using both murine and human models of different ages, including primary cells and cell lines. Although this approach provides a wide assessment of the role of CXCR4 in the bone response to TPTD, it could also introduce large variability in the results and reduce the effect of the murine findings when tested in humans. Moreover, this in vitro analysis has the limitation of not reproducing in full the physiological response to TPTD. Despite the well-established use of MC3T3 cell line as a model for osteoblasts, its osteogenic behaviour varies depending on the subclone, the seeding concentration, or the duration of TPTD treatment.[66–68] Moreover, the limited response of osteoblasts to TPTD shown here could be related to the differentiation stage of the cells. Further research using mesenchymal stem cells or undifferentiated osteoblasts

may show a higher response to the drug. The present study focuses on the role of CXCR4 in differentiated osteoblasts, where the TPTD effects may be less pronounced. Our results confirm that CXCR4 was involved in osteoblast viability and function and that TPTD failed to restore them to even control levels, suggesting that its use might not be of benefit to maintain osteoblast homeostasis when CXCR4 is not fully functional. The use of a pharmacological inhibitor could also cause side effects, not only in the regulation of CXCR4, but other unreported signalling pathways that could also play a role in the response to TPTD treatment. However, our preliminary results suggest that *CXCR4* could be a responsive gene for TPTD, and support further investigation into the role of physiological levels of CXCR4 in improving the anabolic activity of TPTD using more complex in vivo models.

In summary, previous genome-wide association study results found an association of a locus on chromosome 2 and the response to TPTD. Our study suggests that *CXCR4* could be the candidate gene within this locus involved in the anabolic role of TPTD. We found that both osteoblasts and osteoclasts are directly responsive to TPTD treatment, and that *CXCR4* plays a role in osteoblast viability, migration, and mineralization, as well as in osteoclastogenesis in response to TPTD. Further in vivo studies are now warranted to validate the role of *CXCR4* in TPTD treatment, which could make it a candidate pharmacological target to improve bone formation in patients with osteoporosis.

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Supplementary material

Detailed information of the methodology and further supporting graphs on the role of CXCR4 in teriparatide response in both osteoblasts and osteoclasts.

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N. Alonso: Conceptualization, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

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Data sharing

The data that support the findings for this study are available to other researchers from the corresponding author upon reasonable request.

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Ethical review statement

Generation of primary human osteoblasts

Clinical investigations were conducted in line with principles expressed in the Declaration of Helsinki, informed consent was obtained from all donors, and have been approved by the Committee on Health Research Ethics (S-20110114).

Generation of murine osteoclasts

Ethical approval by the Ethics Committee at the University of Edinburgh (UK) was obtained to perform these experiments, which were conducted under the regulation of the UK (Project License Number 70/7964).

Generation of human osteoclasts

Anonymized buffy coats were used in accordance with Danish legislation, and all donors provided written informed consent for the use of surplus material from the donation. This procedure of using an anonymous donor with consent has been approved by

the Danish Ministry of Health and the Regional Ethics Committee of Southern Denmark.

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