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Mitochondrial pyruvate carrier blockade results in decreased osteoclastogenesis and bone resorption via regulating mitochondrial energy production

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Running title: MPC blockade decreases bone resorption

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ABSTRACT

It’s widely accepted that increasing mitochondrial respiration plays a pivotal role during osteoclastogenesis. Mitochondrial pyruvate carrier (MPC) is the key transporter that links glycolysis to mitochondrial respiration but little is known about its role during osteoclastogenesis. Our goal was to determine the effects of its blockade on osteoclastogenesis and bone resorption in vivo and in vitro. To address this issue, we performed gene knockdown or pharmacologically inhibited MPC in primary bone marrow-derived monocytes (BMMs) or in an ovariectomized mouse model. We also studied the metabolic changes in RANKL-induced differentiating BMMs with MPC blockade and performed rescue experiments. We found that MPC blockade resulted in decreased osteoclastogenesis both in vivo and in vitro and inhibiting MPC significantly alleviated ovariectomy-induced trabecular bone loss. Further investigations showed that MPC blockade significantly reversed the metabolic profile related to RANK activation, including decreased intermediates involved in citric acid cycle and glutamine metabolism. Moreover, metabolic flux analysis verified that MPC blockade decreased flux into TCA cycle with no significant effect on glycolysis. Additionally, MPC blockade resulted in suppressed mitochondrial biogenesis in addition to oxidative phosphorylation. Rescue experiments revealed that targeting pyruvate dehydrogenase kinase (PDK) via sodium dichloroacetate (DCA), but not targeting glutamine metabolism, could reverse the effects of MPC blockade on osteoclastogenesis. These implied that the effects of MPC blockade were mediated by reduced pyruvate fuel into citric acid cycle in multiple aspects. Taken together, our data demonstrated the inhibitory effects of MPC blockade on osteoclastogenesis, which was mediated by decreased mitochondrial energy production.

Keywords: mitochondrial pyruvate carrier, osteoclast, energy production, mitochondrial biogenesis, osteoporosis
Abbreviations: MPC: mitochondrial pyruvate carrier; RANK: receptor activator of NF-κB; BMMs: bone marrow-derived monocytes; TRAP: tartrate-resistant acid phosphatase; OXPHOS: oxidative phosphorylation; RANKL: receptor activator of NF-κB ligand; OVX: ovariectomy; UK: UK5099; PDK: pyruvate dehydrogenase kinase; DCA: sodium dichloroacetate; α-KG: α-ketoglutarate; HCA: l-homocysteic acid; FBS: fetal bovine serum; SPF: specific pathogen-free; μCT: microcomputed tomography; Tb.Th: trabecular thickness; BV/TV: bone volume per total volume; Tb.N: trabecular number; Tb.Sp: trabecular spacing; Ct.Ar: cortical area, Ct.Th: cortical thickness, Ct.Ar/Tt.Ar: relative cortical-area-to-total-area ratio; MT: Masson’s trichrome; Oc.S/BS: osteoclast surface, Ob.S/BS: osteoblast surface; N.Oc/B.Pm: osteoclast number; PCR: polymerase chain reaction; SMPDB: small molecular pathway database; TEM: transmission electron microscopy; LPL: lipoprotein lipase; ND1: NADH Dehydrogenase subunit 1; PCA: principal component analysis; orthoPLS-DA: orthogonal partial least squares discriminant analyses; TCA cycle: citric acid cycle; CREA: creatinine; CREB: cyclic AMP response element binding; mtCN: mitochondrial DNA copy number; GSH: glutathione; PTH: parathyroid hormone; LPL: lipoprotein lipase; OC: osteoclast.
Introduction

Upon activation of receptor activator of NF-κB (RANK), bone marrow-derived monocytes (BMMs) firstly differentiate into tartrate-resistant acid phosphatase (TRAP)-positive pre-osteoclasts, which then fuse into mature multinucleated osteoclasts mediating bone resorption(1). It is widely accepted that this process demands for metabolic reprogramming which results in massive biosynthesis and bioenergy generation(2,3). Recently, glucose has been identified as the main energy source for osteoclastogenesis in vitro and osteoclast differentiation has been demonstrated to require both glycolysis and mitochondrial respiration(4). However, energy required for osteoclastogenesis derives mainly from mitochondrial oxidative phosphorylation (OXPHOS) while glycolysis provides bone degradation activities(4,5), indicating the central status of mitochondrial function during osteoclast formation. On one hand, receptor activator of NF-κB ligand (RANKL) stimulation induces mitochondrial biogenesis leading to increases in mitochondrial size and number, deficiency of master regulators such as PGC-1β in osteoclast progenitors impairs not only mitochondrial function but also osteoclast formation(6-8). On the other hand, RANKL enhances mitochondrial respiration and OXPHOS(9), disruption of mitochondrial function impairs osteoclastogenesis and bone resorption(10). Above studies have revealed the suppressive effect of mitochondrial dysfunction on osteoclastogenesis and highlighted the potential antiresorptive therapeutic role of targeting mitochondrial function of osteoclast precursors.

Pyruvate is a critical metabolite that links cytoplasmic glycolysis to mitochondrial metabolism. Generated mainly through glycolysis, pyruvate is transported into mitochondria via mitochondrial pyruvate carrier (MPC), a protein complex including two evolutionally conserved mitochondrial inner-membrane protein components MPC1 and MPC2. Although its kinetics, specificity for substrates and inhibitors have been studied since 1975(11), the molecular structure of MPC was just identified in the past decade and less is known about its expression and function in different cell types(12,13). Recent studies proved that this complex controlled
mitochondrial pyruvate uptake and was required for OXPHOS in several mammal cell types\textsuperscript{(14,15)}. However, the role of MPC in osteoclast differentiation and function has never been studied. In this work, we have investigated the effects of MPC blockade on osteoclastogenesis and bone resorption in vitro and in vivo. We have demonstrated that inhibition of MPC could significantly suppress osteoclast formation and ameliorate ovariectomy (OVX)-induced bone loss, its effects on osteoclast formation could be explained by dampened mitochondrial pyruvate uptake and reduced mitochondrial bioenergy generation.

Results

Pharmacological inhibition of MPC blocks osteoclastogenesis and formation of bone-resorptive osteoclasts

To investigate the role of MPC complex during osteoclast differentiation, we cultured BMMs isolated from young male mice and induced osteoclastogenesis in response to RANKL. Protein expressions of the two MPC components MPC1 and MPC2 were assessed after induction for different time periods (0, 1, 3, 5 days) and Western blot results showed that both expressions of MPC1 and MPC2 were upregulated upon RANKL stimulation. UK5099 is the most common used specific MPC inhibitor\textsuperscript{(11,16)}, here we demonstrated that BMM cell viability was not affected by UK5099 at different concentrations from 5\(\mu\)M to 20\(\mu\)M via CCK8 tests (Figure 1B). TRAP staining showed that UK5099 suppressed RANKL-driven osteoclast differentiation at 5\(\mu\)M to 20\(\mu\)M in a dose-dependent manner (Figure 1C). At different stages (day 3 and day 5) of the RANKL-induced differentiation course, RT-qPCR revealed that transcription levels of osteoclast-specific functional or regulatory genes \textit{Mmp9}, \textit{Ctsk}, \textit{Trap}, and \textit{Nfatc1} were significantly decreased by 20\(\mu\)M UK5099 (Figure 1D), and Western blot results confirmed that protein levels of MMP9, CTSK, TRAP, NFATc1 and the main osteoclastogenesis regulator c-fos were diminished by 20\(\mu\)M UK5099 (Figure 1E). In addition, we found that the inhibitory effect of UK5099 on protein expressions of MMP9, CTSK, TRAP, NFATc1 and c-fos was also dose-
dependent (Figure 1F). As osteoclastogenesis normally occurs on cortical or trabecular bone surface in vivo, then we sought to test whether UK5099 dampened formation of bone-resorptive osteoclasts differentiated from pre-osteoclasts on bone slices or not. We detected that addition of various doses of UK5099 effectively eliminated resorption pit formation on bone slices (Figure 1G). Because bone resorption function is tightly linked to F-actin ring formation(17), we also performed F-actin ring staining on bone slices and observed that UK5099-treated osteoclasts were more incapable of forming actin rings (Figure 1H). Taken together, these results illustrated that MPC inhibitor UK5099 could dose-dependently inhibit osteoclast differentiation and active osteoclast formation in vitro.

**MPC1 knock-down attenuates osteoclast differentiation and activation of osteoclastic bone-resorption while overexpression of MPC1 accelerates osteoclastogenesis in vitro**

MPC1 and MPC2 together form a protein heterodimer which controls mitochondrial pyruvate import(12,13). Thus, we speculated that the effects of UK5099 on osteoclast were not its off-target effects. To down MPC1 in BMMs via lentivirus transduction of shRNAs, the protein expressions of infected BMMs were evaluated by Western blot analysis (Figure 2A) and the two shRNAs (shMPC1#1 and shMPC1#2) that efficiently silenced MPC1 expression were applied to the subsequent experiments. We found that both MPC1 and MPC2 protein expressions were significantly knocked down (Figure 2B), while only transcription level of *Mpc1* but not *Mpc2* was downregulated (Figure 2C) when BMMs were infected with lentivirus expressing shMPC1#1 or shMPC1#2. Previous studies reported that the two MPC components stabilized each other and silencing either one resulted in reduced protein expression of the other(18-20), so we thought that the above-mentioned phenomenon should be due to similar mechanism. Furthermore, we found that knock-down of MPC1 with lentivirus expressing shMPC1#1 or shMPC1#2 impaired osteoclast differentiation in well plates (Figure 2D), inhibited osteoclastic bone-resorption (Figure 2F) and F-actin ring formation (Figure 2G) on bone slices.
Meanwhile BMMs infected with lentivirus expressing shMPC1#2 showed decreased protein levels of osteoclast-specific markers during osteoclast differentiation (Figure 2E), all these above implied that MPC1 played an essential role during osteoclast differentiation and activation. Moreover, lentivirus expressing shMPC1#2 acquired better knockdown of MPC1 in both transcriptional and protein levels compared to shMPC1#1 and was chosen to use in subsequent experiments.

To further explore the effect of MPC1 overexpression on osteoclastogenesis, we performed adenovirus transduction in primary BMMs and found that both transcription and protein expression level of MPC1 were extensively elevated (Figure 3A), resulting in acceleration of osteoclast differentiation and bone-resorptive osteoclast formation (Figure 3B, 3C). We further demonstrated that overexpression of MPC1 could completely reverse the effects of MPC1 knockdown on osteoclastogenesis (Figure 3D). Our in vitro experiments demonstrated the suppressive effects of MPC blockade on osteoclast differentiation, based on it, we further explored the potential effects of UK5099 on pathological bone loss caused by osteoclast overactivation using an OVX-induced osteoporosis mouse model. We collected tibias and femurs of mice after treatment with different doses of UK5099 or vehicle for 8 weeks. The doses and route of drug administration were set in consideration of the effective doses and related routes reported in earlier mouse experiments (16,21,22). We firstly analyzed the distal femur μCT data to assess trabecular bone mass: it was noticed that OVX induced significantly decreased BV/TV, decreased Tb.Th, decreased Tb.N and increased Tb.Sp; low- and high-dose UK5099 treated SHAM groups demonstrated similar BV/TV, Tb.Th, Tb.N and Tb.Sp compared to vehicle treated SHAM group while low- and high-dose UK5099 treated OVX groups demonstrated significant
increases in BV/TV (1.6-fold, 1.9-fold) and significant decreases in Tb.Sp (31% decrease, 39% decrease) compared to vehicle treated OVX group (Figure 4A, 4C). Although low-dose UK5099 treated OVX group didn’t show significantly different Tb.Th or Tb.N (p=0.061, p=0.052), high-dose UK5099 treated OVX group showed significantly increased Tb.Th and Tb.N (1.2-fold, 1.6-fold) compared to vehicle treated OVX group (Figure 4A, 4C). We next performed midshaft μCT data analysis of femur to assess cortical bone mass and found that neither low- nor high-dose UK5099 had any distinguishable effect on cortical bone parameters (Ct.Th, Ct.Ar, Tt.Ar, Ct.Ar/Tt.Ar) no matter in SHAM groups or in OVX groups (Figure 4B, 4D). Thus, inhibition of MPC via UK5099 reduces trabecular bone mass but doesn’t improve cortical bone mass in the ovariectomized mice.

To determine the in vivo effects of UK5099 on osteoclastogenesis and osteoblastogenesis, we performed histological and histomorphometric analyses on sections. Immunostaining revealed the presence of intracellular MPC1 on the trabecular bone surface, which was labelled by TRAP staining as TRAP-positive osteoclasts (Figure 5A) indicating detectable MPC expressed in osteoclasts in vivo. In view of no discernable impact on cortical bone mass in the OVX mouse experiment, histomorphometric measurements in vehicle treated SHAM group and OVX group showed that both N.Oc/B.Pm (20% decrease, 43% decrease) and Oc.S/BS (16% decrease, 38% decrease) were significantly decreased but N.Ob/BS remained nearly unchanged in low- and high-dose UK5099 treated OVX groups compared to vehicle treated OVX group (Figure 5B, 5C, 5D). These results suggested that UK5099 markedly suppressed osteoclast formation but barely impacted osteoblast formation in vivo.

**UK5099 significantly reverses the cellular metabolic alterations during osteoclast differentiation in vitro**

Previous studies reported that the main pharmacological effect of MPC blockade via UK5099 on mammal cells was defective mitochondrial pyruvate flux and decreased cell bioenergetics(14,18,20). Therefore, we were curious about how UK5099
influenced metabolic profile alterations induced by RANK activation during osteoclast formation. BMMs were cultured without RANKL or underwent DMSO or 20μM UK5099 treatment with RANKL induction and then subjected to metabolomics analysis. The principal component analysis (PCA) plot showed the distinct metabolite profiles among three groups (Figure 6A). Because most of the differentially expressed metabolites between BMMs (BMM group) and differentiated BMMs (BMM+RANKL group), and that between differentiated BMMs (BMM+RANKL group) and UK-treated differentiated BMMs (BMM+RANKL+UK group) were metabolites with small fold changes (<2.0) (Figure 6B, 6C), we plotted all these differentially expressed metabolites with p<0.05 volcano plots, which revealed that much more significantly elevated metabolites (191 out of 792, p<0.05) compared to reduced metabolites (42 out of 792, p<0.05) by UK5099 during RANKL stimulation while most differentially expressed metabolites were downregulated (30 out of 792, p<0.05). Next, we sought to determine the most important, differentially expressed metabolites that contributed to metabolic patterns in above comparisons via orthogonal partial least squares discriminant analyses (orthoPLS-DA) and found that the highest ranked metabolites related to RANKL effect belonged to various metabolic processes: different amino acid syntheses, lipid metabolism, citric acid (TCA) cycle, etc. While most of top ranked metabolites related to MPC blockade during osteoclast formation belonged to three important processes: citric acid cycle, glutamine metabolism and carnitine metabolism (Figure 6D, 6E). To further characterize the effect of our treatments on metabolic processes, we did pathway analyses and enrichments based on SMPDB and discovered that RANKL induced divergent and comprehensive metabolic pathway alternations related to bioenergetics and biosynthesis (Figure 6F, 6H). On the other hand, UK5099 treatment predominately influenced glutamine metabolism, citric acid cycle and related processes (Figure 6G, 6I), in consistent with the former orthoPLS-DA analysis (Figure 6E). Interestingly, we found that citric acid cycle was significantly affected
while glycolysis was not significantly influenced in both two situations (RANKL induction and UK5099 treatment) (Figure 6F, 6G). Moreover, we wondered if UK5099 reduced levels of most upregulated metabolites during RANKL induction. Thus, we did Venn plots and noticed that most upregulated metabolites upon RANKL stimulation (157 out of 191) could be downregulated by UK5099 during osteoclast differentiation (157 out of 343), further enrichment analysis uncovered that these 157 metabolites belonged to various metabolic processes (Figure 6J). Considering that mitochondrial pyruvate flux was the essential and direct source of citric acid cycle and tightly connected with glycolysis and energy metabolism (14,20), we analyzed the fold changes of stable intermediates of citric acid cycle and glycolysis among the groups. Results demonstrated that most intermediates were not significantly altered except 3-phosphoglyceric acid, 2-phosphoglyceric acid, pyruvic acid and lactic acid (Figure 6K), we thought that alterations of these could be explained by downstream blockade. The alteration of latter one was related to increased aerobic glycolysis during osteoclast differentiation(4). Unlike glycolysis, seven stable intermediates in TCA cycle all significantly altered among three groups: upregulated upon RANKL induction and downregulated upon UK5099 addition. Notably, three nearby intermediates (citric acid, cis-aconitic acid and isocitric acid) located upstream of MPC displayed most significant changes both upon RANKL induction (p=0.002, p<0.001 and p=0.002) and UK5099 treatment (p<0.001 for all) (Figure 6L). Finally, data also showed that UK5099 reduced ADP level and increased ATP/ADP while no significant change was detected in ATP level among groups, indicating that UK5099 dampened energy consumption during osteoclastogenesis (Figure 6M). Hence metabolomics illustrated the powerful reversion effect of MPC blockade on metabolite profile changes during RANKL induction.

Stable isotope tracing reveals that UK5099 decreases pyruvate flux into mitochondria for oxidation via TCA cycle
Based on the results that UK5099 decreased levels of all stable intermediates in TCA cycle while left most of stable intermediates in glycolysis unchanged (Figure 6), we further performed in vitro isotope tracing to determine the effects of MPC blockade on the fate of glucose-derived carbon flux in differentiated BMMs. Uniformly labeled glucose ([U-13C]-D-glucose) is utilized into glycolysis, the pentose phosphate pathway and the citric acid pathway (Figure 7A). After culture with [U-13C]-D-glucose for 6 hours, we detected extensive M+6 labelling of glucose and glucose-6-phosphate, M+3 labelling of pyruvic acid and lactic acid, and M+5 labelling of ribulose-5-phosphate (Figure 7B). Notably, we found comparable incorporation of 13C-glucose-derived carbons into glycolytic intermediates (glucose, glucose-6-phosphate, pyruvic acid) between control and UK5099-treated cells, except that UK5099 led to a very modest but significant enrichment and decreased M+0 enrichment of lactic acid compared to the control (Figure 7B). In contrast, we found significantly decreased flux into TCA cycle caused by UK5099 which was illustrated by decreased M+2 isotopologues of citric acid, succinic acid, fumaric acid, malic acid, glutamic acid and decreased M+4 and M+6 isotopologues of citric acid (p<0.05 for all) (Figure 7C). Besides, M+5 ribulose-5-phosphate, a stable intermediate involved in the pentose phosphate pathway, was significantly decreased in UK5099-treated cells (p<0.001) (Figure 7B). Together, these results revealed that MPC blockade had less effects on glucose uptake and glycolysis but significantly decreased glucose-derived carbon flux into TCA cycle and the pentose phosphate pathway.

**UK5099 and knock-down of MPC1 both lead to defects in oxidative phosphorylation (OXPHOS) and mitochondrial biogenesis**

Since metabolomics analysis revealed the severe decline in TCA cycle intermediates caused by MPC blockade, we decided to validate the impact of MPC inhibition on OXPHOS which is coupled with TCA cycle carbon flux. We measured oxygen consumption rate (OCR) which is closely related to OXPHOS at osteoclast differentiation day 3 and day 5, when mononuclear preosteoclasts and multinuclear
osteoclasts were induced to form respectively. Results confirmed that both 20μM UK5099 and MPC1 knockdown via shMPC1#2 significantly decreased OCR at day 3 and day 5 respectively (Figure 8A, 8B, 8C, 8D). The process of osteoclast differentiation is linked to mitochondrial biogenesis(23), for the reason that pharmacological inhibition and knockdown of MPC1 both led to decreased osteoclastogenesis, we wondered if the impact of MPC blockade on mitochondrial bioenergy generation could be partially due to defective mitochondrial biogenesis besides decreased TCA cycle carbon flux. To verify this, we examined mitochondrial DNA copy number (mtCN, normalized to genomic DNA content) and assayed expressions of OXPHOS protein components, which increased following RANKL induction and usually associated with mitochondrial biogenesis(7,23). We found that both pharmacological inhibition and knockdown reduced mtCN and downregulated expressions of several OXPHOS components at different stages during osteoclast differentiation (Figure 8E, 8F, 8H). One step further, we analyzed mitochondrial morphology and observed decreased cross-sectional area of mitochondria caused by UK5099 or MPC1 knock-down (Figure 8I, 8J). Previous studies suggested that transcriptional coactivator PGC1-β was the major factor linking osteoclastogenesis to mitochondrial biogenesis(23,24). In addition, PGC-1β was reported to interact and regulate reactive oxygen species (ROS) signaling and the phosphorylation of cyclic AMP response element binding (CREB) pathway to fulfill its role in mitochondrial biogenesis(24). Therefore, we tested the effect of UK5099 on PGC1-β expression and CREB phosphorylation, data showed that UK5099 significantly suppressed protein expressions of PGC1-β and phosphorylation of CREB during osteoclastogenesis (Figure 8K).

**Inhibitory effects of UK5099 on osteoclast formation can be significantly rescued by pharmacological inhibition of PDK but not by supplement of α-KG or HCA**

Besides TCA cycle, metabolomics analysis identified glutamine metabolism including glutamate metabolism and glutathione metabolism as the most critical process affected by UK5099 during osteoclastogenesis (Figure 6G, 6I). Glutamine was proved
to maintain TCA cycle function when glucose-derived mitochondrial pyruvate transport was impaired(25). Thus, we next explored the alterations of important stable intermediates involved in glutamine metabolism and tried to determine if interfering with this process could compensate for MPC blockade during RANK activation. The process of glutamine metabolism contains two approaches that interact with TCA cycle or mitochondrial oxidative stress: one supplies α-KG for TCA cycle and the other support glutathione (GSH) biosynthesis to regulate intracellular oxidative stress (Figure 9A). Metabolomics data showed that both intracellular glutamine and glutamate were significantly increased response to RANKL induction and decreased upon addition of UK5099 (Figure 9B). Oxidized glutathione was dramatically reduced by UK5099 while glutathione, γ-glutamylcysteine and L-cysteine were also reduced but the differences were not statistically significant. L-aspartate was increased by RANKL stimulation but not altered by UK5099 (Figure 9B). These results suggested that MPC blockade resulted in impaired glutamate metabolism and reduced glutathione synthesis and oxidized. Then we tried to fuel glutamate metabolism by elevating α-KG concentration in medium and enhance glutathione synthesis by adding HCA to suppress cystine/glutamate antiporter. α-KG is the ideal intermediate utilized by cells to provide glutamate(26), also activity of cystine/glutamate antiporter is strongly correlated with intracellular GSH production(27). The concentrations of α-KG and HCA used in cell culture were determined according to a previous study on primary BMMs(28). Interestingly, we observed that both different concentrations (0, 0.25, 0.5, 1μM) of α-KG and that of HCA couldn’t rescue the inhibitory effects of UK5099 on TRAP-positive osteoclast formation (Figure 9C, 9E) or on protein levels of osteoclast-specific transcriptional factors NFATc1 and c-fos (Figure 9D, 9F). Moreover, there existed one tendency for α-KG to enhance the suppressive effect of UK5099 and the other for HCA to slightly block the inhibition on osteoclastogenesis (Figure 9C, 9E).

We next wanted to uncover if increasing citric acid flux in mitochondria could rescue the inhibition of osteoclastogenesis caused by MPC blockade. Suppressing
PDK by DCA can increase the generation of citrate in TCA cycle (29). Impressively, the PDK inhibitor DCA brought significant increases in both mature osteoclasts and expressions of NFATc1 and c-fos at 2µM to 4µM (Figure 9H, 9I) which was tested to not harm BMM cell viability (Figure 9G), in a dose-dependent manner. Besides, DCA also significantly recovered formation of resorptive osteoclast and F-actin ring in a dose-dependent manner (Figure 9J, 9K). To sum up, our data revealed that inhibiting PDK significantly rescued suppressive effects of UK5099 on osteoclastogenesis.

**Inhibiting PDK via DCA also partially recovers the altered metabolite profile generated by MPC blockade during osteoclast differentiation.**

Considering the effects of DCA on impaired osteoclastogenesis caused by UK5099, we applied metabolomics analysis to investigate if this phenomenon was accompanied with extensive metabolite changes. BMMs were cultured with DMSO or 20µM UK5099 or 20µM UK5099 coupled with 4mM DCA and stimulated with RANKL for 5 days and then subjected to metabolomics analysis. The PCA plot showed the distinct metabolite profiles between DMSO (BMM+RANKL group) and UK-treated (BMM+RANKL+UK group) differentiated BMMs, but differentiated BMMs treated with UK and DCA (BMM+RANKL+UK+DCA group) presented a metabolite profile that intersected with both two other groups (Figure 10A). For the same reason as that in Figure 6, we plotted all differentially expressed metabolites (p<0.05) between DMSO (BMM+RANKL group) and UK-treated (BMM+RANKL+UK group) differentiated BMMs, and that between UK-treated (BMM+RANKL+UK group) and UK plus DCA-treated (BMM+RANKL+UK+DCA group) differentiated BMMs in the volcano plots (Figure 10B, 10C, 10D, 10E). The volcano plots showed: UK5099 caused more downregulation (185 out of 672 if p<0.05, 230 out of 672 if p<0.1) than upregulation (13 out of 672 if p<0.05, 24 out of 672 if p<0.1) in metabolites, while UK plus DCA induced more upregulation (67 out of 672 if p<0.05, 153 out of 672 if p<0.1) than downregulation (3 out of 672 if p<0.05, 5 out of 672 if p<0.1). The orthoPLS-DA revealed that the highest ranked metabolites related to the rescue effect of DCA belonged to metabolic processes that closely related to or upstream or
downstream of TCA cycle such as lipid synthesis and glucose metabolism (Figure 10F). Pathway analysis and enrichment based on SMPDB pointed that citric acid cycle and glutamine metabolism were significantly influenced, and other processes including ketone body metabolism and amino acid metabolism were mainly affected (Figure 10G, 10H). The Venn plots indicated that upregulated metabolites in BMM+RANKL+UK+DCA group intersected with downregulated metabolites in BMM+RANKL+UK group, and when we set $p$ value<0.1, it was noticeable that most downregulated metabolites (117 out of 230) caused by UK5099 could be upregulated by adding DCA (117 out of 153). And latter enrichment analysis uncovered that these 117 metabolites belonged to metabolic processes including citric acid cycle, glutamine metabolism and processes closely related to TCA cycle such as fatty acid oxidation and pentose phosphate pathway. The fact that citric acid cycle and glutamine metabolism were the highest ranked bioenergy processes affected by UK5099 (Figure 6G, 6I), we calculated the fold changes of stable intermediates in these processes among the groups and found that: all intermediates in TCA cycle were upregulated by adding DCA in varying degrees where citric acid, isocitric acid and succinic acid were most significantly elevated (Figure 10J); intermediates involved in GSH synthesis were upregulated at a significant although alterations for the latter were not significant (Figure 10K). Furthermore, we examined the effects of DCA on OCR and OXPHOS component expressions and found that the inhibitory effects of UK5099 could also be rescued by adding DCA (Figure 10L, 10M). Hence metabolomics illustrated the powerful reversion effect of MPC blockade on metabolite profile changes during RANKL induction. In a word, these data demonstrated that DCA could rescue the suppression on metabolic processes including citric acid cycle and glutamine metabolism resulted from MPC blockade and recover the mitochondrial respiration.

**Discussion**
Emerging evidence supports the theory that energy metabolism feature is quite different between osteoblastogenesis and osteoclastogenesis. In osteoblasts, lactate is the major end metabolite derived from glucose regardless of oxygen conditions, and besides, Wnt signaling activates aerobic glycolysis via mTOR while parathyroid hormone (PTH) stimulates aerobic glycolysis through IGF-1(9,30). During osteoclastogenesis, RANK activation increases mitochondrial biogenesis and oxidative phosphorylation (OXPHOS) which is thought to be the main energy source for osteoclasts(5,31). Therefore, based on this theory we considered targeting OXPHOS through controlling pyruvate flux into citric acid cycle could possibly be a good manner to treat osteoporosis caused by overactivation of osteoclasts, while bone anabolism mediated by osteoblasts might be preserved.

In the present study, we investigated the role of MPC in osteoclast activation and function. Firstly, we proved that MPC blockade either by pharmacological inhibition or gene knock-down predominantly impacted on osteoclastogenesis rather than on osteoblastogenesis through in vitro and in vivo experiments (Figure 1, 2, 4, 5). What’s more, we demonstrated that overexpression of MPC1 promoted osteoclast formation and could rescue the suppressive effects of MPC1 knockdown (Figure 3). These together explained the essential role of MPC1 during osteoclastogenesis. Secondly, we identified metabolic alterations during osteoclastogenesis and found that most of these RANKL-induced changes could be reversed by UK5099 (Figure 6). Glucose metabolism shifts to a much more oxidative status when pre-osteoclasts and osteoclasts form from precursors(5,24), and this process undergoes massive metabolic changes which directly motivate osteoclast-related gene expressions(32,33). However, on one hand few articles focus on intracellular metabolite profile changes during this course. On the other hand, although it’s known that increasing OXPHOS plays a vital role in osteoclastogenesis(4,10), the relationship between it and other metabolic changes remains unknown. In consideration of these, here we demonstrated that MPC played an essential role in osteoclast-mediated bone-resorption and that downregulated metabolites related to MPC blockade and upregulated metabolites
related to RANK activation had most features in common (Figure 6), indicating that mitochondrial pyruvate flux crosslinked with other important metabolic pathways and acted as a central driving force to promote metabolic reprogramming during osteoclastogenesis. Further, we studied the effects of MPC blockade on glucose-derived carbon flux via metabolic flux analysis, results verified the fact that MPC blockade suppressed pyruvate flux into TCA cycle and the pentose phosphate pathway but had less effects on glucose utilization into glycolysis in differentiated osteoclasts (Figure 7). Nevertheless, we think that isotope-labelled metabolic flux analysis based on a conditional MPC1-knock-out mouse model is needed in the future study to validate the exact interaction between carbon flux related to MPC and that from other important metabolic pathways during osteoclastogenesis.

It was very interesting that targeting MPC had such a dramatic influence on RANKL-induced, osteoclastogenesis-related metabolic reprogramming, thus we deduced that MPC blockade must have impacts on mitochondria, at least on its respiratory function which is coupled with pyruvate sourced from glucose-derived pyruvate to OXPHOS. Subsequent experiments revealed that MPC blockade led to not only mitochondrial respiration but also mitochondrial biogenesis. Recent pioneering researches elucidated that RANKL-induced osteoclast differentiation and mitochondrial biogenesis were controlled by independent pathways, so we thought that the effect of MPC blockade on mitochondrial biogenesis which was related to inhibition of PGC1-β expression and CREB phosphorylation (Figure 8K), might be independent from that on osteoclastogenesis. However, the defective mitochondrial biogenesis could in turn contribute to impaired pyruvate flux because of decreased consumption ability (Figure 8F, 8H). Therefore, the reduced mitochondrial biogenesis should aggravate suppressed differentiation-related metabolic reprogramming caused by MPC blockade, although the upstream regulation mechanisms for the two events might be independent.

Besides decreased metabolism of citric acid cycle (Figure 6L), glutamine
metabolism was also significantly decreased by UK5099 (Figure 6I, 9B). Despite the significant changes in glutamine metabolism upon MPC blockade, we failed to rescue UK5099-mediated suppression on osteoclastogenesis through targeting the two main approaches involved in glutamine metabolism (Figure 9C, 9D, 9E, 9F). A recent study performed by Lee et al. revealed that α-KG negatively regulated osteoclastogenesis through epigenetic regulation of glutathione generation (28), and our finding that α-KG couldn’t rescue the inhibitory effects but led to more decreased osteoclast differentiation was consistent with their conclusions. Differently, HCA was thought to decrease intracellular glutathione production thus increase reactive oxygen species (ROS) (27), and increase mature osteoclast formation. In our study, we found that HCA could not significantly rescue suppression on osteoclastogenesis by UK5099 at the same concentrations that certainly facilitated osteoclastogenesis in the former study (28). We speculated that there were two reasons: firstly, glutathione anabolism and oxidation were decreased to low levels by UK5099 so that targeting glutathione to decrease antioxidants had limited effects; secondly, UK5099 had much more negative influence on ROS than decreasing glutathione could rescue.

Finally, we succeeded in recovering impaired osteoclast formation and function resulted from MPC blockade through targeting PDK via DCA (Figure 9H, 9I, 9J, 9K), and DCA also significantly restored metabolic status of osteoclast in the presence of UK5099 (Figure 10). The major pharmacological effect of DCA is to increase carbon flux into citric acid cycle (29), we confirmed this by detecting that the first three stable intermediates significantly increased upon addition of DCA (Figure 10J). Besides, reduced OXPHOS and mitochondrial biogenesis had also been rescued (Figure 10K, 10L), implying that DCA targeted multiple vital pathways mediating the effects of UK5099. A graphic abstract of this article is presented in Figure 11.

In conclusion, our results reflected that MPC blockade suppressed mainly osteoclastogenesis rather than osteoblastogenesis and its inhibitory effects were due to decreased fuel in mitochondrial respiration. There exist some limitations in this study:
firstly, conditionally gene knock-out animals were not available in this study; secondly, metabolic influx analysis based on these animals was not included; thirdly, the exact mechanism how MPC blockade impacted mitochondrial biogenesis was not fully explained. Therefore, future investigation is needed.

**Experimental Procedures**

Reagents, primary BMM isolation, in vitro osteoclast differentiation on plastics and cell proliferation assay

Recombinant soluble murine M-CSF and RANKL used in this study were purchased from R&D Systems. MPC-specific inhibitor UK5099 (UK), and pyruvate dehydrogenase kinase (PDK)-specific antagonist sodium dichloroacetate (DCA) were both supplied by Medchem Express (UK, K1128) and L-homocysteic acid (HCA, H9633) Sigma-Aldrich. Cell permeable DCA, α-KG and HCA were titrated to pH=7.0 by sodium hydroxide before used for cell culture.

BMMs were isolated from femurs and tibias of 6-week-old C57BL6/J male mice following the previously described (33). BMMs were cultured with α-MEM (HyClone) containing 10% FBS (Gibco), 100U/ml penicillin, 100μg/ml streptomycin and 30ng/ml recombinant soluble murine macrophage colony-stimulating factor (M-CSF) (R&D Systems). BMMs were seeded on 96-well plates at 1.0×10⁴ cells/well and induced with α-MEM containing 10% FBS, 30ng/ml M-CSF and 50ng/ml RANKL for an additional 5 days to differentiate into mature osteoclasts. TRAP staining using a kit (Sigma-Aldrich, 387A-1KT) was performed and TRAP-positive cells with 3 or more nuclei was identified as osteoclasts and counted.

Cell proliferation and viability were assessed via CCK8 assay (Boster Biotechnology). BMMs were seeded on 96-well plates at 3.0×10³ cells/well and cultured with α-MEM containing different concentrations of UK5099 (or DCA), 10% FBS and 30ng/ml M-CSF for up to 5 days and then assay was performed.

**Lentivirus-mediated shRNA expressions and adenovirus-mediated...**
overexpression of MPC1 in BMMs

The lentiviral vectors expressing shRNAs against murine Mpc1, and control vector expressing scrambled shRNA were generated by Vigene Biosciences. The shRNA sequences used for MPC1 knock-down were: shRNA1, 5’-GACCAGCTCTTTGAAAGAGACATTCAAGAGATGTCTCTTTCAAGAGCTGGTCTTTTTT-3’; shRNA2, 5’-GTCAGCATGAGTAGTCTGCTTTCAAGAGAGCACGACTACTCATGCTGACTTTTTT-3’; shRNA3, 5’-GCTGCCTTATCAATGCTAAACTTCAAGAGAGTTTAGCATTGATAAGGCAGCTTTTTT-3’. The scrambled sequence used as control was: 5’-TTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTT-3’. For overexpression of MPC1, adenoviral plasmid expressing wildtype MPC1 (pAdM-CMV-MPC1) and control plasmid were constructed by Vigene Biosciences. Vector-based lentivirus packaging was completed in Shanghai GeneChem Company and plasmid-based adenovirus packaging was completed in Vigene Biosciences. Lentivirus and adenovirus particles were used to transduce primary BMM cells with polybrene.

Ovariectomy-induced mouse model and animal experiment design

Female wild type C57BL6/J mice were supplied by Beijing Huafukang Bioscience Co., Ltd (Beijing, China). All animal experiments and related procedures were ethically approved by the Institutional Animal Ethics Committee of Tongji Hospital (No. TJH-201903021), and were conducted strictly following the guidelines for care and use of laboratory animals. 11-week-old mice were housed in a specific pathogen-free (SPF) animal laboratory in Tongji Hospital with controlled conditions (18-22°C and a 12:12 h light-dark cycle). Mice were randomly divided into four groups and subjected to bilateral ovariectomy or sham operation under anesthesia via pentobarbital, and treatment was initiated one week after surgery (12 weeks of age). Treatment for each group was: sham-operated group treated with vehicle (SHAM group, sample size n=6), sham-operated group treated with low-dose UK5099
(10mg/kg body weight, n=7), sham-operated group treated with vehicle (OVX group, n=7), OVX group treated with low-dose UK5099 (10mg/kg body weight, n=7), OVX group treated with high-dose UK5099 (30mg/kg body weight, n=6). We determined different doses of UK5099 based on the previous in vivo studies (16,21,22), mice were weighted weekly and were treated with UK5099 or vehicle (200μL sterile phosphate-buffered saline with 9% DMSO and 2.5% 2-hydroxypropyl beta cyclodextrin) daily via intraperitoneal injection respectively. After specified treatment for 8 weeks, all mice were sacrificed and femurs were excised. No adverse effect was recorded during the course of treatment.

**Bone microcomputed tomography (μCT) scans, histomorphometric analyses and immunofluorescence**

Femur bones of the female C57BL6/J mice were fixed in 4% paraformaldehyde for 48h. μCT scanning was conducted using a Scanco vivaCT 40 instrument (Scanco Medical) and parameters for visualizing calcified tissue were set as we previously described (33). We reconstructed three-dimensional images at 10μm resolution via the built-in software and measured the parameters for trabecular and cortical morphometry including bone volume per total volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), cortical area (Ct.Ar), cortical thickness (Ct.Th), total area (Tt.Ar) and relative cortical-area-to-total-area ratio (Ct.Ar/Tt.Ar). Decalcified femurs were paraffin-embedded and then cut. 5-μm-thick sections were stained by tartrate-resistant acid phosphatase (TRAP) method (Sigma-Aldrich, 387A-1KT) and Masson’s trichrome (MT) method for histomorphometric analysis. Digital images were captured at 200x magnification, and we took measurements 200μm underneath growth plates and cortical bones. Osteoclasts were identified as multinucleated TRAP-positive cells which always lied on bone surface on TRAP-stained sections and osteoblasts were identified as red-stained rod-like or fish-like cells adhered to bone surface on MT-stained sections. Analysis was performed using Bioquant Osteo software (v18.2.6; Bioquant Image
Analysis Corp.) for the quantification of parameters including osteoclast surface (Oc.S/BS), osteoblast surface (Ob.S/BS) and osteoclast number (N.Oc/B.Pm).

Frozen sections and subsequent immunofluorescent staining were performed following the method described by Kusumbe et al. (34). Anti-MPC1 primary antibody was purchased from Abcam (ab74871) and FITC-labelled secondary antibody was purchased from Thermo Fisher Scientific. Fluorescent images were captured at 200× magnification and analyzed by Image J software (National Institutes of Health, USA).

**F-actin ring staining and osteoclast resorptive activity assay**

BMMs were seeded on bovine cortical bone slices placed in well plates and cultured in α-MEM containing 10% FBS, 30ng/ml M-CSF and 50ng/ml RANKL for 7 days to differentiate into mature osteoclasts. For F-actin ring staining, cells were fixed with 4% paraformaldehyde, then incubated with FITC-phalloidin at room temperature for 30min and washed 3 times. Fluorescent images were captured at 400× magnification using a confocal microscopy (FV3000, Olympus) and nuclei were visualized via DAPI staining. For osteoclast resorptive activity assay, BMMs were firstly seeded in 6-well plates and cultured with differentiation medium containing 30ng/ml M-CSF and 50ng/ml RANKL for 3 days. Then the differentiating pre-osteoclasts were digested by 0.25% trypsin and equal number of cells were seeded on bone slices cultured with medium containing M-CSF and RANKL until mature osteoclast formation confirmed by TRAP staining. Next bone slices were incubated with 2N sodium hydroxide and cells were wiped away. After that, we incubated bone slices with peroxidase-conjugated wheat germ agglutinin (Sigma, L3892) for 30min and performed DAB (Abcam, ab64238) staining for 10min, digital images were captured at 200× magnification and the area of resorption pits were measured and analyzed by Image J software (National Institutes of Health, USA).

**RNA expression analysis via real-time quantitative PCR (RT-qPCR)**

Total RNA was extracted and reverse transcribed into cDNA as we previously described (33). The primer sequences for genes of interest were listed in Table 1. RT-qPCR was carried out in the CFX Connect™ RT-qPCR detection system (Bio-Rad)
using SYBR Green qPCR Master Mix (Thermo Fisher Scientific). Relative mRNA expressions for genes of interest were calculated by comparative Ct \( (2^{-\Delta \Delta CT}) \) method and we used expression of murine β-actin for normalization.

**Western blotting analysis**

Cells were harvested and cell lysates were extracted. Western blotting experiments were performed as we previously described\(^{(33)}\). The following antibodies were used: anti-MPC1 (14462), NFATc1 (8032), c-fos (2250), and β-actin (3700) purchased from Cell Signaling Technology (CST); anti-tartrate-resistant-acid-phosphatase (TRAP, ab96372), cathepsin K (CTSK, ab19027), PGC-1β (ab72328), CREB (ab32515), p-CREB (ab32096) purchased from Abcam; anti-MPC2 (14462-1-AP) and MMP9 (10375-2-AP) purchased from Proteintech Group, Inc.; total OXPHOS rodent WB antibody cocktail (ab110412); anti-tartrate-resistant-acid-phosphatase (TRAP, ab96372), cathepsin K (CTSK, ab19027), PGC-1β (ab72328), CREB (ab32515), p-CREB (ab32096) purchased from Abcam; anti-MPC2 (14462-1-AP) and MMP9 (10375-2-AP) purchased from Proteintech Group, Inc.; total OXPHOS rodent WB antibody cocktail (ab110412).

**Metabolomics analysis via liquid chromatography-mass spectrometry/mass spectrometry**

To elucidate the effects of MPC blockade on metabolome changes of BMMs during osteoclast differentiation, BMMs isolated from different wild type (WT) C57BL6/J male donors were cultured in α-MEM containing 10% FBS and 30ng/ml M-CSF (BMM group, sample size n=4), or subjected to α-MEM containing FBS and 20μM UK5099 (BMM+RANKL+UK group, sample size n=4) or DMSO (BMM+RANKL group, sample size n=4) induced with M-CSF and RANKL for 5 days. To investigate the possible metabolome changes of simultaneous PDK inhibition on differentiating BMMs with MPC blockade, BMMs isolated from different WT donors were cultured with DMSO (BMM+RANKL group, sample size n=3), 20μM UK5099 (BMM+RANKL+UK group, sample size n=4), or 20μM UK5099 accompanied by 4mM DCA (BMM+RANKL+UK+DCA group, sample size n=3) induced with M-CSF and RANKL for 5 days. Then harvested cell samples were prepared and underwent extraction and purification procedures. Purified samples were injected into the LC-MS/MS system. LC-MS/MS analyses were performed using an ExionLC™ AD system (SCIEX) coupled with a QTRAP® 6500+ mass spectrometer (SCIEX) in
Novogene Co., Ltd. (Beijing, China). All parameters and conditions were set the same as those used in a previous study (35), and the raw data generated by the LC-MS/MS system were analyzed using the software SCIEX OS Version 1.4 to integrate and correct the chromatographic peak. All metabolomics data were analyzed by the website-based tool MetaboAnalyst (http://www.metaboanalyst.ca). Particularly, we used univariate analysis (t test) to evaluate the statistical significance (P value) and screened differentially expressed metabolites (P value<0.05 or P value<0.1), and in the differential metabolites, we did pathway analysis based on The Small Molecular Pathway Database (SMPDB) and the SMPDB entry that met the condition P value<0.05 was identified as significant enriched.

**In vitro U-13C glucose labelling and metabolic flux analysis.**

BMMs from different WT male donors were cultured with 20μM UK5099 (BMM+RANKL+UK group, sample size n=4) or DMSO (BMM+RANKL group, sample size n=4) and induced with 30ng/ml M-CSF and 50ng/ml RANKL for 5 days. On day 5 of differentiation, media was changed to a glucose free α-MEM containing M-CSF, RANKL, and [U-13C] D-glucose (Cambridge Isotopes, CLM-1396-5) and cells were cultured for another 6 hours under the stimulation of RANKL and M-CSF with or without treatment of UK5099 or DMSO. Then cell samples were harvested and underwent extraction and purification procedures. Purified samples were used for metabolic flux analysis. Chromatographic separation was performed on a Thermo Fisher Ultimate 3000 ultra-high performance liquid chromatography (UHPLC) system with a Waters BEH Amide column (2.1mm × 100 mm, 1.7 μm). The column temperature was 15°C. The mobile phases consisted of water with 0.01% formic acid and 2 mM ammonium formate (phase A) and acetonitrile (phase B). A linear gradient elution was performed with the following program: 0 min, 90%B; 4 min, 85%B; 11 min, 75%B; 14 min, 70%B, 14.5 min, 50%B and held to 17 min; 17.1 min, 90%B and held to 20 min. The eluents were analyzed on a Thermo Fisher Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometry in heated electrospray ionization negative mode. Spray voltage was set to 4000 V.
Capillary and Probe Heater Temperature were both 320 °C. Sheath gas flow rate was 35 (Arb, arbitrary unit), and Aux gas flow rate was 10 (Arb). S-Lens RF Level was 50 (Arb). The full scan was operated at a high-resolution of 70000 FWHM (m/z=200) at a range of 70-1050 m/z with AGC Target setting at 3×106. Raw data were analyzed using Xcarlibur software (Thermo Fisher Scientific, version 4.0.27.19) and corrected according to the published method(36).

**Extracellular oxygen consumption rate (OCR) assay**

Primary BMMs were either cultured with M-CSF or induced with M-CSF and RANKL for 5 days to differentiate into mature osteoclasts. Then cells were subjected to different interventions for an additional 48h. Extracellular OCR was measured using a commercially available assay kit (ab197243, Abcam, following manufacturer’s instructions. Signals were read via a SpectraMax i3x microplate reader (Molecular Devices) at default parameters.

**Transmission electron microscopy for visualizing mitochondrial morphology and measurement of cross-sectional area**

Primary BMMs were subjected to different interventions and cultured with M-CSF and RANKL on bovine cortical bone slices for 7 days to induce osteoclast differentiation. Cells were fixed for three times and harvested, followed by fixation in 2.5% glutaraldehyde at room temperature. Next sample cells were dehydrated, embedded in resin and cut into 70-nm-thick sections. These ultrathin sections were prepared for mitochondrial morphology examination using a HT7700 transmission electron microscope (120kv, Hitachi) and images were taken at 7000× magnification and 20000× magnification respectively. The mitochondrial cross-sectional area was measured on images (20000× magnification) by Image J software using the previously reported method(23).

**Quantification of mitochondrial abundance**

Total DNA was extracted using a DNA extraction kit (TIANGEN Biotechnology, Beijing). 15ng of total DNA per sample was used for the subsequent qPCR detection in the CFX Connect™ RT-qPCR detection system (Bio-Rad) using SYBR Green
qPCR Master Mix (Thermo Fisher Scientific). The relative abundance of mitochondrial marker gene ND1 (NADH Dehydrogenase subunit 1) to genomic marker gene LPL (lipoprotein lipase) was determined by qPCR. The relative mitochondrial copy number was calculated via $2^{-(C_{t \text{ND1}} - C_{t \text{LPL}})}$. Primer sequences were listed in Table 2.

**Statistical analysis**

GraphPad PRISM 8.0 software (Graphpad Software Inc., USA) was used for statistical analysis. Two-tailed unpaired Student’s t test was performed for comparison of two groups. One-way ANOVA with Sidak’s multiple comparison tests was used for comparison of more than two groups. $P$ values less than 0.05 were considered statistically significant. Every result was reproduced in three independent experiments respectively and the representative image was presented.

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**Ethics approval and consent of participate:**

The study protocol was approved by ethical committee of Tongji Hospital.

**Declaration of interests:**

The authors declare no conflicts of interest.

**Data availability statement:**

The data that support the findings of this study are available in the methods and supplementary material of this article.
CRediT author statement (authors’ contributions):
Qian Guo: Conceptualization, Methodology, Visualization, Formal analysis, Software, Writing-Original Draft. Hongjian Zhao: Data Curation, Formal analysis. Haozhe Cheng: Formal analysis. Honglei Kang: Date Curation, Methodology. Yimin Dong: Visualization. Renpeng Peng: Investigation. Meipeng Zhu: Date Curation. Zhong Fang: Funding acquisition, Supervision. Feng Li: Conceptualization, Funding acquisition, Project administration. All authors read and approved the final manuscript.
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FIGURE LEGENDS

Figure 1. UK5099 suppresses osteoclast differentiation and bone resorption in vitro. Primary BMMs were cultured with MCSF (30ng/ml) and RANKL (50ng/ml) for up to 5 days to differentiate into osteoclasts. (A) RANKL/MCSF induced BMMs for 0, 1, 3 or 5 days, the protein expressions of MPC1 and MPC2 were determined by Western blot. (B) BMMs were cultured with MCSF and treated with different concentrations of UK5099 (0, 5, 10, 20μM) for 1, 3 or 5 days. The cell proliferation and viability were examined by CCK8 tests. (C) BMMs were cultured with RANKL/MCSF for 5 days in the presence of different concentrations of UK5099 (0, 5, 10, 20μM), osteoclastogenesis was evaluated by counting multinucleated TRAP-positive cells with 3 or more nuclei. (D, E) BMMs were induced with RANKL/MCSF in the presence of 20μM UK5099 for 0, 1, 3 or 5 days, relative mRNA levels of osteoclast makers were evaluated by qPCR (D), protein levels of indicated osteoclast markers were determined by Western blot (E). (F) BMMs were cultured with various concentrations of UK5099 (0, 5, 10, 20μM) accompanied with RANKL/MCSF induction for 3 days, protein levels of indicated osteoclast markers were determined by Western blot. (G) BMMs were induced to differentiate into pre-osteoclasts, and were digested and seeded on bone slices and cultured with RANKL/MCSF and different concentrations of UK5099 (0, 5, 10, 20μM) until mature osteoclast formation was confirmed by TRAP staining in control group (0μM UK5099), then resorption pits were assayed by DAB staining. The representative images of DAB-stained resorption pits were presented, scale bar represents 200μm. 5 distinct resorption areas in each bone slice were quantified by image J to assess bone resorption area, osteoclast number was counted by image J and resorption area per osteoclast was calculated. (H) BMMs were induced with RANKL/MSCF in the presence of different concentrations of UK5099 (0, 5, 10, 20μM) for 7 days on bone slices to differentiate into osteoclasts, then F-actin rings and nuclei were visualized by fluorescent staining. Representative images were
presented, scale bar represents 50µm. 5 distinct areas in each bone slice were quantified by image J to calculate the percent of F-actin ring positive cells. For Western blot, β-actin was served as loading control. The p values were calculated by one-way ANOVA with Sidak’s multiple comparison tests.

Figure 2. Knock-down of MPC1 decreases osteoclast differentiation and bone resorption in vitro. Lentivirus expressing shRNAs against MPC1 were used to knock down MPC1 expression in BMMs. (A) Knock-down of MPC1 in BMMs via lentivirus expressing shRNAs was examined by Western blot. (B, C) BMMs were infected with control virus, lentivirus expressing shMPC1#1 or shMPC1#2 and protein levels of MPC1 and MPC2 were assayed by Western blot. (D) relative mRNA levels of MPC1 and MPC2 were assessed in BMMs infected with control virus, lentivirus expressing shMPC1#1 or shMPC1#2 were cultured with RANKL/MCSF for 5 days, multinucleated TRAP-positive cells with 3 or more nuclei were counted, scale bar represents 200µm. (E) BMMs infected with control virus or lentivirus expressing shMPC1#2 were cultured with MCSF or induced with RANKL/MCSF for 3 days, protein levels of indicated osteoclast markers were determined. (F) BMMs were induced to differentiate into pre-osteoclasts and infected with control virus, lentivirus expressing shMPC1#1 or shMPC1#2, and digested and seeded on bone slices and induced with RANKL/MCSF until mature osteoclast formation was confirmed by TRAP staining in control group, then resorption pits were assayed by DAB staining. The representative images of DAB-stained resorption pits were presented, scale bar represents 200µm. 5 distinct resorption areas in each bone slice were quantified by image J to assess bone resorption area, osteoclast number was counted by image J and resorption area per osteoclast was calculated. (G) BMMs were infected with control virus, lentivirus expressing shMPC1#1 or shMPC1#2 and induced with RANKL/MSCF for 7 days on bone slices to differentiate into osteoclasts, then F-actin rings and nuclei were visualized by fluorescent staining. Representative images were presented, scale bar
represents 50μm. 5 distinct areas in each bone slice were quantified by image J to calculate the percent of F-actin ring positive cells. For Western blot, β-actin was served as loading control. We performed semi-quantitative densitometry analysis of each Western blot for three times with BMMs from three different donors. The p values were calculated by one-way ANOVA with Sidak’s multiple comparison tests.

Figure 3. Overexpression of MPC1 promotes osteoclast formation and activation of osteoclastic bone-resorption, and completely rescues suppression of osteoclastogenesis caused by MPC1 knockdown. (A) Adenovirus expressing wildtype MPC1 was used for overexpression of MPC1 BMMs. Overexpression of MPC1 in BMMs was examined by Western blot and qPCR. BMMs infected with control virus or adenovirus expressing MPC1 were cultured with RANKL/MCSF for 5 days, multinucleated TRAP-positive cells with 3 or more nuclei were counted, scale bar represents 200μm. (B) BMMs infected with control virus or adenovirus expressing MPC1 were induced to differentiate into pre-osteoclasts and infected with control virus or adenovirus expressing shMPC1#1, and were digested and seeded on bone slices and cultured with RANKL/MCSF until mature osteoclast formation was confirmed by TRAP staining in control group, then resorption pits were assayed. Representative images of DAB-stained resorption pits were presented, scale bar represents 200μm. (C) 5 distinct resorption areas in each bone slice were quantified by image J to assess bone resorption area, osteoclast number was counted by image J and resorption area per osteoclast was calculated. (D) BMMs infected with control viruses, lentivirus expressing shMPC1#1, lentivirus expressing shMPC1#2, adenovirus expressing MPC1 combined with lentivirus expressing shMPC1#1 or adenovirus expressing MPC1 combined with lentivirus expressing shMPC1#2, were cultured with RANKL/MCSF for 5 days, multinucleated TRAP-positive cells with 3 or more nuclei were counted, scale bar represents 200μm. For Western blot, β-actin was served as loading control. The p values were calculated by two-tailed Student’s t tests except that in figure 3E the p values were calculated by one-way ANOVA with Sidak’s multiple comparison tests.
**Figure 4.** UK5099 predominately shows protective effects on OVX-induced trabecular bone loss. Distal femurs from SHAM groups and OVX groups treated with different doses of UK5099 were analyzed. (A) Representative μCT reconstruction images of the distal femurs for each group (above is the longitudinal view, below is the axial view of metaphyseal region), scale bar represents 1 mm. (B) Representative μCT reconstruction images of the midshaft femur for each group (axial view), scale bar represents 1 mm. (C) Trabecular bone morphometric parameter results: trabecular number (Tb.N), bone volume/total volume (BV/TV), trabecular thickness (Tb.Th), trabecular spacing (Tb. Sp). (D) Cortical bone morphometric parameter results: cortical thickness (Ct.Th), total area (Tt.Ar), cortical area (Ct.Ar) and relative cortical-area-to-total-area ratio (Ct.Ar/Tt.Ar). The p values were calculated by one-way ANOVA with Sidak’s multiple comparison tests.

**Figure 5.** UK5099 suppresses osteoclastogenesis while not significantly influences osteoblastogenesis in vivo. Distal femurs from SHAM groups and OVX groups treated with different doses of UK5099 were analyzed. (A) TRAP-stained section image and immunostaining of MPC1 in the same area (nuclei visualized by DAPI staining), scale bar represents 50 μm. (B, C) Representative image of metaphyseal region in the TRAP-stained section for each group (B), and in the Masson’s trichrome (MT)-stained section for each group (C), scale bar represents 200 μm. (D) Static histomorphometric parameter results: osteoblast surface/bone surface (Ob.S/BS), osteoclast number (N.Oc/B.Pm), and osteoclast surface/bone surface (Oc.S/BS). The p values were calculated by one-way ANOVA with Sidak’s multiple comparison tests.

**Figure 6.** UK5099 reverses RANKL-induced cellular metabolic changes in vitro. BMMs isolated from different wildtype murine donors were cultured with MCSF (BMM group), treated with DMSO (BMM+RANKL group) or 20 μM UK5099 (BMM+RANKL+UK group) accompanied with MCSF/RANKL induction for 5 days.
Then cells were harvested and used to determine metabolite levels, all data were analyzed and plotted by Metaboanalyst. (A) Principal component analysis (PCA) plot for all three groups. (B, C) Volcano plots for differentially expressed metabolites (p<0.05) between BMM+RANKL group and BMM group (B) and for that between BMM+RANKL+UK group and BMM+RANKL group (C). (D, E) Orthogonal partial least squares-discriminant analyses (orthoPLS-DA) for the most important, differentially expressed metabolites between BMM+RANKL group and BMM group (D) and for that between BMM+RANKL+UK group and BMM+RANKL group (E). (F, G) Pathway analyses based on SMPDB for pathways involved in metabolite profile difference between BMM+RANKL group and BMM group (F) and for that between BMM+RANKL+UK group and BMM+RANKL group (G). Size of data point represents the number of metabolites involved. (H, I) Enrichments based on SMPDB for top 25 metabolite sets involved in the comparison between BMM+RANKL group and BMM group (H) and for that between BMM+RANKL+UK group and BMM+RANKL group (I). (J) Venn plots for the intersection between downregulated metabolites in BMM+RANKL group compared to BMM group and upregulated metabolites in BMM+RANKL+UK group compared to BMM+RANKL group, and for that between upregulated metabolites in BMM+RANKL group compared to BMM group and downregulated metabolites in BMM+RANKL+UK group compared to BMM+RANKL group (lower left), pathway enrichment based on SMPDB for top 25 metabolite sets involved in the intersection between upregulated metabolites in BMM+RANKL group compared to BMM group and downregulated metabolites in BMM+RANKL+UK group compared to BMM+RANKL group (right). (K, L) The fold change of every stable intermediate in each group involved in glycolysis (K) or citric acid cycle (L) was normalized to the control (BMM group) and was plotted. (M) The fold change of ATP, ADP or ATP/ADP in each group was normalized to the control (BMM group) and was plotted. The p values in figure 6K, 6L and 6M were calculated by one-way ANOVA with
Sidak’s multiple comparison tests. Metabolomics data was shown in Supplementary Table 1.

**Figure 7. UK5099 does not affect $^{13}$C-glucose-derived glycolytic flux but decreases $^{13}$C-glucose-derived pentose phosphate pathway and TCA cycle intermediates.** BMMs from different donors were cultured with 20µM UK5099 (BMM+RANKL+UK group, sample size n=4) or DMSO (BMM+RANKL group, sample size n=4) and induced with 30ng/ml M-CSF and 50ng/ml RANKL for 5 days. On day 5 of differentiation, glucose in the media was changed to 5.5mM [U-$^{13}$C]-D-glucose (Cambridge Isotopes, CLM-1396-5) for 6 hours. Then cell samples were harvested for the determination of metabolites. (A) Atom mapping for [U-$^{13}$C]-D-glucose tracing into the glycolysis, pentose phosphate pathway and TCA cycle intermediates. White circles are $^{12}$C atoms, black circles are $^{13}$C atoms. (B) Labelling pattern of [U-$^{13}$C]-D-glucose incorporation into D-glucose, glucose-6-phosphate, pyruvic acid, lactic acid, ribulose-5-phosphate (pentose phosphate pathway intermediate) in differentiated BMMs treated with DMSO or 20µM UK5099, with CLM-1396-5 for 6 hours. (C) Labelling pattern of [U-$^{13}$C]-D-glucose incorporation into citric acid, succinic acid, fumaric acid, malic acid (TCA cycle intermediates) and glutamic acid (closely related to TCA cycle) in differentiated BMMs treated with DMSO or 20µM UK5099, with CLM-1396-5 for 6 hours. Results were expressed as fraction of pool. The p values were calculated by one-way ANOVA with Sidak’s multiple comparison tests and raw data of metabolites are shown in Supplementary Table 2.

**Figure 8. Pharmacological inhibition and knock-down of MPC1 both decrease oxidative phosphorylation and suppress mitochondrial biogenesis.** BMMs were cultured with MCSF (30ng/ml) and RANKL (50ng/ml) for 3 days to differentiate into preosteoclasts or for 5 days to differentiate into mature osteoclasts. (A, B) Extracellular oxygen consumption rate (OCR) was assayed and calculated for BMMs...
after cultured with RANKL/MCSF in the presence of 20µM UK5099 or DMSO for 3 (A) or 5 (B) days. (C, D) BMMs were infected with control virus or lentivirus expressing shMPC1#2, and extracellular OCR was assayed and calculated after RANKL/MCSF induction for 3 (C) or 5 (D) days. (E) RANKL/MCSF induced BMMs in the presence of 20µM UK5099 or DMSO for 0, 3 or 5 days and the relative mitochondrial abundance was determined by qPCR. (F) RANKL/MCSF induced BMMs in the presence of 20µM UK5099 or DMSO for 3 or 5 days and the expressions of oxidative phosphorylation (OXPHOS) components were determined by Western blot. (G) BMMs were infected with control virus or lentivirus expressing shMPC1#2, and underwent RANKL/MCSF induction for 3 or 5 days and the relative mitochondrial abundance was determined by Western blot. (H) RANKL/MCSF induced BMMs on bone slices in the presence of 20µM UK5099 or DMSO for 0, 3 or 5 days and cells were harvested and subjected to mitochondrial morphology examination by transmission electron microscopy (TEM), scale bar represents 1µm. (J) BMMs were infected with control virus or lentivirus expressing shMPC1#2 and underwent RANKL/MCSF induction on bone slice for 7 days, cells were harvested and subjected to mitochondrial morphology examination by transmission electron microscopy (TEM), scale bar represents 1µm. (K) RANKL/MCSF induced BMMs in the presence of 20µM UK5099 or DMSO for 0, 3 or 5 days and expressions for indicated proteins were assessed by Western blot. For Western blot, β-actin was served as loading control. In figure 8J, we performed semi-quantitative densitometry analysis of each Western blot for three times with BMMs from three different donors. The p values were calculated by two-tailed Student’s t tests except that in figure 8E, 8G and 8K the p values were calculated by one-way ANOVA with Sidak’s multiple comparison tests.

Figure 9. Inhibiting PDK by DCA but not supplement of α-KG or HCA can
**Rescue impaired osteoclastogenesis caused by UK5099.** (A) A diagram for intracellular glutamine metabolism. (B) The fold change of every stable intermediate in each group involved in glutamine metabolism was normalized to the control (BMM group) and was plotted. (C) RANKL/MCSF induced BMMs for 5 days in the presence of DMSO, or 20μM UK5099 accompanied by different concentrations of α-KG (0, 0.25, 0.5, 1mM), multinucleated TRAP-positive cells with 3 or more nuclei were counted as osteoclasts, scale bar represents 200μm. (D) BMMs were cultured with MCSF or induced with RANKL/MSCF for 3 days in the presence of DMSO, or 20μM UK5099 accompanied by different concentrations of α-KG (0, 0.25, 0.5, 1mM), protein levels of NFATc1 and c-fos were determined by Western blot. (E) RANKL/MCSF induced BMMs for 5 days in the presence of DMSO, or 20μM UK5099 accompanied by different concentrations of α-KG (0, 0.25, 0.5, 1mM), multinucleated TRAP-positive cells with 3 or more nuclei were counted as osteoclasts, scale bar represents 200μm. (F) BMMs were cultured with MCSF or induced with RANKL/MSCF for 3 days in the presence of DMSO, or 20μM UK5099 accompanied by different concentrations of α-KG (0, 0.25, 0.5, 1mM), protein levels of NFATc1 and c-fos were determined by Western blot. (G) RANKL/MCSF induced BMMs for 5 days in the presence of DMSO accompanied by different concentrations of DCA (0, 2, 4mM), multinucleated TRAP-positive cells with 3 or more nuclei were counted as osteoclasts, scale bar represents 200μm. (H) RANKL/MCSF induced BMMs for 5 days in the presence of DMSO accompanied by 0, 2, 4mM DCA, or 20μM UK5099 accompanied by 0, 2, 4mM DCA, multinucleated TRAP-positive cells with 3 or more nuclei were counted as osteoclasts, scale bar represents 200μm. (I) BMMs were cultured with MCSF or induced with RANKL/MSCF for 3 days in the presence of DMSO, or 20μM UK5099 accompanied by 0, 2, 4mM DCA, protein levels of NFATc1 and c-fos were determined by Western blot. (J) BMMs were induced to differentiate into pre-osteoclasts, and were digested and seeded on bone slices and cultured with RANKL/MCSF in the presence of DMSO accompanied by 0, 2, 4mM DCA, or 20μM UK5099 accompanied by 0, 2, 4mM DCA until mature osteoclast formation was
confirmed by TRAP staining in control group (DMSO + 0mM DCA), then resorption pits were assayed by DAB staining. The representative images of DAB-stained resorption pits were presented, scale bar represents 200µm. 5 distinct resorption areas in each bone slice were quantified by image J to assess bone resorption area, osteoclast number was counted by image J and resorption area per osteoclast was calculated. (K) BMMs were induced with RANKL/MSCF in the presence of DMSO accompanied by 0, 2, 4mM DCA, or 20µM UK5099 accompanied by 0, 2, 4mM DCA for 7 days on bone slices to differentiate into osteoclasts, then F-actin rings and nuclei were visualized by fluorescent staining. Representative images were presented, scale bar represents 50µm. 5 distinct areas in each bone slice were quantified by image J to calculate the percent of F-actin ring positive cells. In Western blot, β-actin was served as loading control. The p values were calculated by one-way ANOVA with Sidak’s multiple comparison tests.

Figure 10. DCA partially recovers the altered metabolite profile related with UK5099 during osteoclast differentiation. BMMs isolated from different wildtype murine donors were cultured with DMSO (BMM+RANKL group), 20µM UK5099 (BMM+RANKL+UK group) or 20µM UK5099 accompanied by 4mM DCA (BMM+RANKL+UK+DCA group) induced with MCSF/RANKL induction for 5 days. Then cells were harvested and used to determine metabolite levels, all data were analyzed and plotted by Metaboanalyst. (A) Principal component analysis (PCA) plot for all three groups. (B, C) Volcano plots for differentially expressed metabolites (p<0.05) between BMM+RANKL+UK group and BMM+RANKL group (B) and for that between BMM+RANKL+UK+DCA group and BMM+RANKL+UK group (C). (D, E) Volcano plots for differentially expressed metabolites (p<0.1) between BMM+RANKL+UK group and BMM+RANKL group (D) and for that between BMM+RANKL+UK+DCA group and BMM+RANKL+UK group (E). (F) Orthogonal partial least squares-discriminant analyses (orthoPLS-DA) for the most important, differentially expressed metabolites between BMM+RANKL+UK+DCA...
group and BMM+RANKL+UK group. (G) Pathway analyses based on SMPDB for pathways involved in metabolite profile difference between BMM+RANKL+UK+DCA group and BMM+RANKL+UK group. (H) Pathway enrichments based on SMPDB for top 25 metabolite sets involved in the comparison between BMM+RANKL+UK+DCA group and BMM+RANKL+UK group. (I) Venn plots for the intersection between downregulated metabolites in BMM+RANKL+UK group compared to BMM+RANKL group and upregulated metabolites in BMM+RANKL+UK+DCA group compared to BMM+RANKL+UK group (upper left p<0.05, upper right p<0.1), pathway enrichment based on SMPDB for top 25 metabolite sets involved in the intersection between downregulated metabolites in BMM+RANKL+UK group compared to BMM+RANKL group and upregulated metabolites in BMM+RANKL+UK+DCA group compared to BMM+RANKL+UK group (lower). (J, K) The fold change of every stable intermediate in each group involved in citric acid cycle (J) or glutamine metabolism (K) was normalized to the control (BMM+RANKL group) and was plotted. (L) Extracellular oxygen consumption rate (OCR) was assayed and calculated for BMMs after cultured with RANKL/MCSF in the presence of DMSO, 20μM UK5099 or 20μM UK5099 accompanied by 0, 2, 4mM DCA. (M) RANKL/MCSF induced BMMs in the presence of DMSO, 20μM UK5099 accompanied by 0, 2, 4mM DCA, or 20μM UK5099 accompanied by 0, 2, 4mM DCA, and the expressions of oxidative phosphorylation (OXPHOS) components were determined by Western blot. For Western blot, β-actin was served as loading control. The p values in figure 10J, 10K and 10L were calculated by one-way ANOVA with Sidak’s multiple comparison tests. Metabolomics data was shown in Supplementary Table 3.

Figure 11. A graphic abstract of this article.
### TABLES

#### Table 1. Murine qPCR primers for mRNA analysis.

| Gene | Forward primer (5’- to 3’-) | Reverse primer (5’- to 3’-) |
|------|-----------------------------|-----------------------------|
| Mpc1 | GAGGACGACTTATCAACTACGAGAT   | GCACGACTACTCATGCTGACA       |
| Mpc2 | CCGCTTTACAACCACCACGGCA      | CAGCACACACCAATCCCCATTTCA    |
| Nfatc1 | CAACGCCCTGACCACCGATAG      | GGGAAAGTCAGAAGTGGTGGGA     |
| Mmp9 | TCCAGTACCAAGACAAGCCCTA     | TTGCACACTGACCGGTTGAA       |
| Trap | TACCTGTGAGACATGACC         | TCCATAGTGAAACCGC            |
| Ctsk | TGTATAACCGCCACGGCAAA      | TTATCAGGTCACA               |
| \(\beta\)-actin | AGCTTCTTTGAGCTCCTTC    | AGCTTATTCCCACC               |

#### Table 2. Murine qPCR primers for mitochondrial abundance quantification.

| Gene | Forward primer (5’- to 3’-) | Reverse primer (5’- to 3’-) |
|------|-----------------------------|-----------------------------|
| Nd1  | CCCATTCGCGTTATCTT           | AAGTTGATCGTAACGGAAGC        |
| Lpl  | GGATGCGGTGCTCCTT            | ATCCAAGGGTAGCAGACAGGT       |
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BMM+RANKL+UK vs. BMM+RANKL

Scores Plot

BMM+RANKL+UK+DCA vs. BMM+RANKL+UK

3 Stg. Down

67 Stg. Up

BMM+RANKL+UK+DCA vs. BMM+RANKL+UK

5 Stg. Down

153 Stg. Up

BMM+RANKL+UK+DCA vs. BMM+RANKL+UK

Overview of Enriched Metabolite Sets (Top 25)

Ketone Body Metabolism
Glutamine and Glutamate metabolism
Sodium Oxidation of Very Long Chain Fatty Acids
Pyruvate Metabolism
Alpha Ketoglutarate Shunt
Arginine and Spermidine Metabolism
Nicotinate and Nicotinamide Metabolism
Glutathione Metabolism
Aminobutyric Acid Metabolism
Tryptophan metabolism
Asparagine and Aspartate Metabolism
Glycine, serine and threonine metabolism
D-glutamine metabolism
Lysine degradation
Tryptophan metabolism
Citric acid (TCA) cycle
Gluatamine metabolism
DCA (mM)

UK5099

UQCRCC2

MTCO1

SDHB

NDUFB8

β-actin

D5

D5

D5

D5

D5

D5
