

PLA/nHA nanocomposites and metformin rescue aged bone repair by restoring PCK2-regulated mitochondrial metabolism

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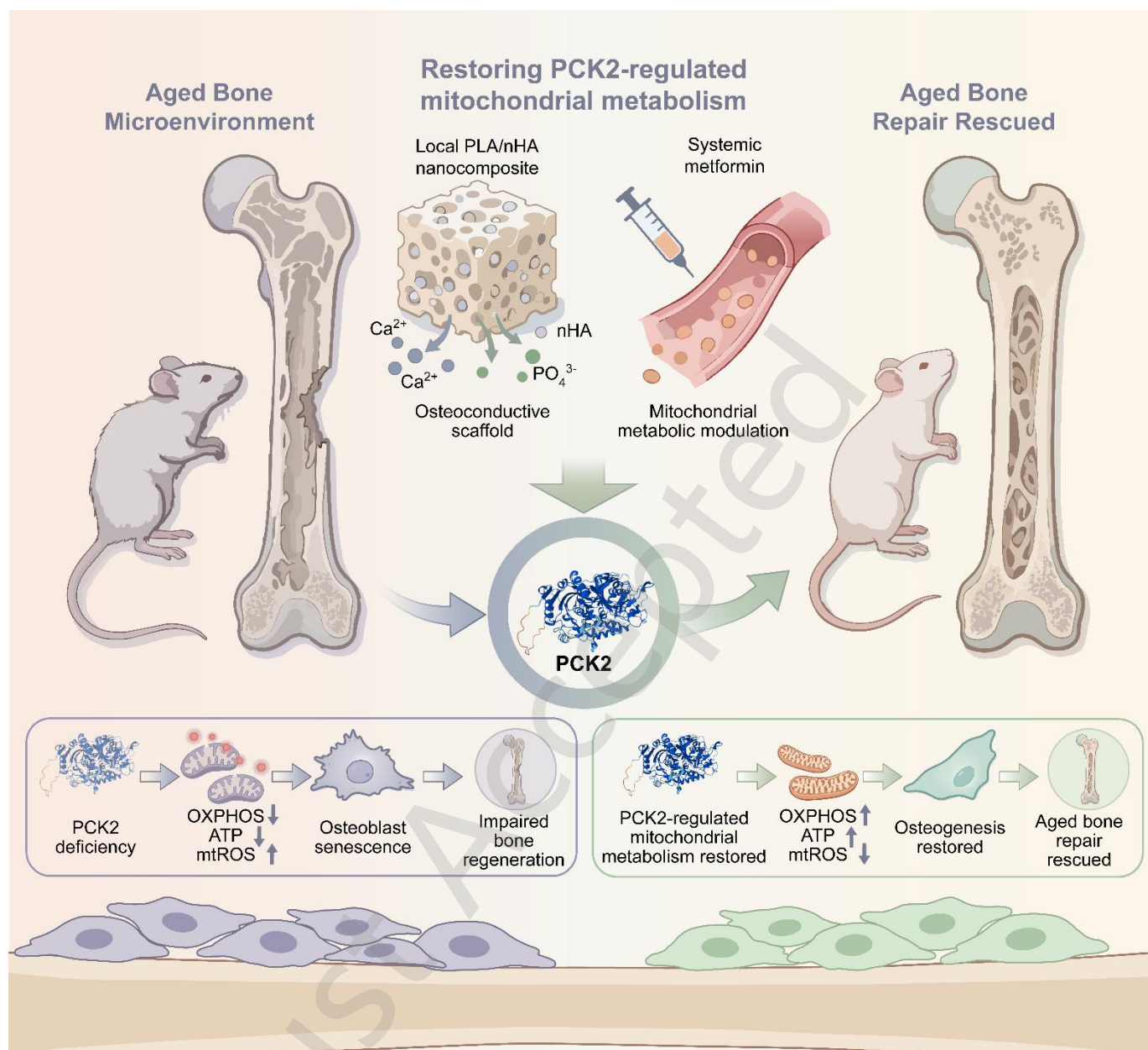
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PLA/nHA nanocomposites combined with metformin rescue aged bone repair by restoring PCK2-dependent mitochondrial metabolism and reducing osteoblast senescence.

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ABSTRACT: Bone regeneration declines with age in part because osteoblasts lose mitochondrial metabolic fitness, a defect that conventional osteoconductive scaffolds do not address. The metabolic dysfunction that connects osteoblast senescence to failed repair, however, remains poorly defined. Here, we identify mitochondrial phosphoenolpyruvate carboxykinase 2 (PCK2) as a regulator of aged bone regeneration and use this insight to design a local poly(lactic acid)/nano-hydroxyapatite (PLA/nHA) scaffold plus systemic metformin strategy. In senescent hBMSCs and osteoblast-specific Pck2 conditional knockout mice, PCK2 loss reduced oxidative phosphorylation and ATP production, increased mitochondrial superoxide, activated p53/p21-associated senescence, and impaired osteogenic differentiation. PLA/nHA provided a degradable osteoconductive matrix and sustained calcium/phosphate release, whereas metformin supported mitochondrial metabolic recovery. In combination, the treatment restored mitochondrial respiration and ultrastructure, reduced ROS accumulation, normalized senescence markers, and recovered osteogenesis without altering cell viability. In aged femoral defects, PLA/nHA/metformin reduced local senescence and accelerated new bone formation, with the clearest effect in Pck2-deficient mice. These findings place the PCK2-mitochondria axis upstream of osteoblast senescence in aged bone repair and show that scaffold-based regeneration can be strengthened by correcting the metabolic state of the repair niche.

KEYWORDS: phosphoenolpyruvate carboxykinase 2; PLA/nHA nanocomposite; metformin; mitochondrial homeostasis; osteoblast senescence; bone regeneration

1. Introduction

Bone regeneration becomes progressively less reliable with age, contributing to delayed fracture healing, bone defect nonunion, and poorer outcomes after regenerative procedures [1-3]. Although aged bone still contains cells capable of repair, these cells operate in a niche marked by chronic low-grade inflammation, oxidative stress, and altered nutrient metabolism [4, 5]. Most bone substitutes are designed to provide space maintenance and osteoconduction, but they do not correct cell-intrinsic metabolic failure in senescent osteoblast-lineage cells [6]. This mismatch helps explain why structurally adequate scaffolds can underperform in aged tissue and argues for regenerative strategies that treat the repair niche as a metabolic environment, not only a structural defect [7, 8].

Mitochondrial dysfunction is one of the most consistent features of osteoblast senescence. Reduced oxidative phosphorylation, ATP depletion, and excessive reactive

oxygen species (ROS) weaken matrix production, mineralization, and stress tolerance [9, 10]. Yet the upstream metabolic regulators that convert mitochondrial stress into poor bone repair remain incompletely defined [11, 12]. Among these regulators, mitochondrial phosphoenolpyruvate carboxykinase 2 (PCK2) is of particular relevance because its mitochondrial localization places it at a metabolic junction linking tricarboxylic acid cycle intermediates with cellular energy metabolism and redox regulation [13, 14]. In skeletal cells, PCK2 has been linked to osteogenic differentiation and skeletal homeostasis [15-18], but whether PCK2 loss is a cause of failed repair in aged bone, rather than an associated metabolic change, has not been established.

PLA/nHA composites offer a useful local framework for this question because they combine a degradable polymer matrix with hydroxyapatite-derived mineral cues [19-21]. During degradation, PLA can expose nHA and support the release of calcium and phosphate ions, which are important

for matrix mineralization and cellular signaling. However, material-derived mineral cues alone are unlikely to resolve mitochondrial stress in senescent osteoblasts. Metformin has a long clinical record and can modulate mitochondrial metabolism and AMPK-related stress responses [22–24], but systemic metabolic treatment lacks a local osteoconductive structure at the defect site. Recent studies on lactide-based nHA composites have shown that these materials can promote bone regeneration in inflammatory environments by regulating macrophage immunometabolism and polarization [25, 26]. Whether material-based regulation can be aligned with a defined osteoblast metabolic defect in skeletal aging remains unknown.

Here, we test the idea that PCK2-dependent mitochondrial failure is a tractable barrier to aged bone regeneration. We combined local PLA/nHA implantation with systemic metformin and evaluated this strategy in PCK2-deficient senescent hBMSCs and osteoblast-specific Pck2 conditional knockout mice. The results show that PCK2 loss suppresses mitochondrial respiration, increases mitochondrial superoxide, activates senescence programs, and impairs osteogenesis. The combined intervention restores mitochondrial homeostasis, reduces local senescence, and accelerates bone repair in aged defects. By linking a genetically defined metabolic dysfunction to a clinically familiar scaffold-drug strategy, this study provides a practical route for treating aged bone repair as a metabolic-regenerative problem.

2 Experimental

2.1 PLA/nHA synthesis

PLA (Sigma, GF45989881) was dissolved in chloroform at a concentration of 10% (w/v) under magnetic stirring until a clear solution was obtained. nHA (Sigma, 677418; particle size <200 nm) was dispersed in a small volume of chloroform and added to the PLA solution at a concentration of 20 wt%. The mixture was sonicated for 30 minutes to prevent nanoparticle agglomeration and ensure uniform dispersion. The resulting homogeneous suspension was cast into a glass mold and air-dried under a ventilated hood for 24 hours, followed by vacuum drying for 48 hours to remove residual solvent. The final product was a solid PLA/nHA composite block.

2.2 SEM-EDS

The surface morphology and elemental composition of untreated PLA/nHA and PLA/nHA samples soaked in simulated body fluid (SBF) for 1, 2, 3, and 4 weeks were observed under a scanning electron microscope (SEM) SU8000 (HITACHI, Japan) and analyzed using energy-dispersive X-ray spectroscopy (EDS) QUANTAX (Bruker, Germany). The materials were first dried to remove surface moisture, followed by gold sputter coating. SEM images were taken, and elemental mapping was performed to assess the distribution of calcium and phosphate ions on the PLA surface.

2.3 ICP-OES

PLA/nHA samples were soaked in SBF at a concentration of 0.2 g/mL at 37°C. The extract solution was collected at the 1st, 2nd, 3rd, and 4th week of immersion, filtered through a 0.22 µm membrane to remove suspended particles. SBF

solution served as the blank control group. The concentrations of calcium and phosphate ions were detected using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Agilent 5110, USA). The dynamic changes in ion release were monitored to evaluate the material's role in regulating the mineralization microenvironment.

2.4 pH Change of the extract liquids

Extract liquids were collected at the 1st, 2nd, 3rd, and 4th week of the experiment, filtered through a 0.22 µm membrane to remove suspended particles. The pH of the samples was measured using a pH meter. Each sample was measured three times, and the average value was used for analysis. The changes in pH were analyzed to assess the influence of the biomaterials on the microenvironment.

2.5 Cell culture

Human bone marrow mesenchymal stem cells (hBMSCs) were obtained from Sciencell Research Laboratories and cultured in α-MEM medium (Gibco, USA) containing 10% fetal bovine serum (FBS, ExcellBio, China) and 1% penicillin-streptomycin (Gibco, USA) under standard conditions of 37°C and 5% CO₂. Osteogenic induction was performed in α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin, 10 mM β-glycerophosphate (β-GP), 0.2 mM L-ascorbic acid, and 10 nM dexamethasone (Dex). For co-treatment, PLA/nHA at a concentration of 0.2 g/mL was directly added to the culture medium. Metformin (Met, Sigma, USA) was used at a concentration of 0.5 mM. Mito-TEMPO (MedChemExpress, USA) was added to the culture medium at a final concentration of 1 µM.

2.6 Animal Experiments

Pck2^{+/+} and Pck2^{+/+}; OC-Cre mice were purchased from Suzhou Cyagen Biosciences. All animal experiments were performed in Specific Pathogen Free (SPF) conditions. Pck2^{+/+} and Pck2^{+/+}; OC-Cre mice aged 6 weeks and 18 months were randomly divided into three groups: control group, PLA/nHA, and PLA/nHA/Met groups. Pck2^{+/+} mice lacking OC-Cre retained intact Pck2 expression and served as floxed littermate controls for Pck2^{+/+}; OC-Cre mice. Mice were anesthetized via intraperitoneal injection of tribromoethanol. After sterilizing the surgical site with iodine, a 2-mm incision was made on the lateral side of the femur. The muscles were bluntly dissected, and the femoral bone surface was fully exposed. A 1-mm diameter drill was used to create a bone defect 2 mm from the lateral condyle of the femur, establishing a femur defect model. For the PLA/nHA and PLA/nHA/Met groups, PLA/nHA was implanted into the defect site, and the wound was sutured. Mice in the PLA/nHA/Met group were administered Met intraperitoneally once every other day to the end of the study. Mice in the other groups received intraperitoneal injections of an equal volume of normal saline on the same schedule. Femoral and visceral tissue samples were collected 7 and 14 days post-surgery and fixed in 4% paraformaldehyde. All animal experiments were approved by the Peking University Institutional Animal Care and Use Committee (PUIRB-LA2323387) and complied with the ARRIVE guidelines.

2.7 Statistical Analysis

All results are expressed as the mean ± standard deviation (mean ± SD). GraphPad Prism Software 8.0.2 was used for

data processing, statistical analysis, and graph plotting. Before parametric analyses, data normality was assessed using the Shapiro–Wilk test. For datasets involving two independent variables, two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons tests was performed. For datasets involving a single variable, one-way ANOVA followed by Tukey's post hoc test was used. A p-value of <0.05 was considered statistically significant. The significance levels are denoted as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Additional experimental procedures are provided in the Electronic Supplementary Material (ESM).

3. Results

3.1 Degradation-coupled mineral release from PLA/nHA defines the local osteogenic niche

We first characterized the physicochemical behavior of the PLA/nHA nanocomposite to define the local mineral environment created by the scaffold. During immersion in simulated body fluid (SBF), PLA degradation gradually acidified the eluate from pH 7.4 to approximately pH 5.0 by week 4, whereas SBF alone remained near neutral (Fig. 1a). ICP-OES analysis showed a two-phase release profile for calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions (Fig. 1b, c):

release was relatively steady during the first 3 weeks and then increased once the pH dropped below 5.8, consistent with acid-accelerated nHA dissolution. Thus, scaffold degradation was coupled to sustained mineral ion delivery.

SEM confirmed that HA particles were uniformly distributed within the PLA matrix before immersion (Fig. 1d). EDS mapping further showed homogeneous calcium and phosphate distribution on the scaffold surface (Fig. 1e, f). After 1–4 weeks in SBF, the surface showed progressive erosion and pore formation, exposing internal HA while maintaining a broadly uniform mineral distribution. These data indicate that PLA/nHA provides both a stable osteoconductive framework and a degradation-responsive mineral source for modulating the defect microenvironment.

To further characterize the structural and mechanical properties of the scaffold, compression testing was performed in PBS at 37 °C. The stress-strain curves showed the compressive response of the PLA/nHA scaffolds under physiological-temperature conditions (Fig. S1). The scaffolds exhibited a compressive modulus of 4.817 ± 0.795 MPa and a porosity of $14.42 \pm 3.68\%$ (Table S1, mean $\pm$ SD, $n = 3$), suggesting a balance between shape retention and deformability that may facilitate surgical handling and placement within bone defects.

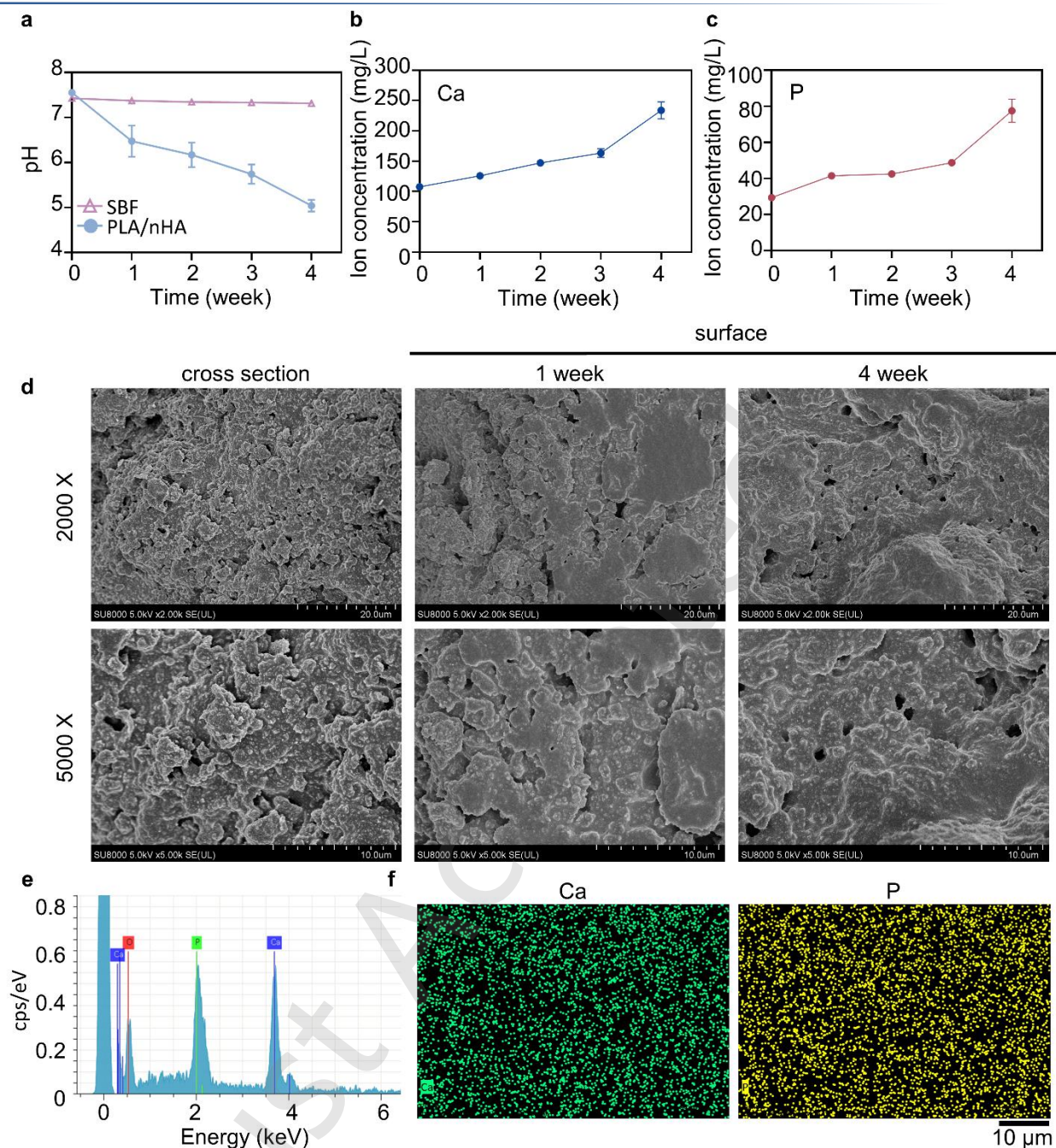


Figure 1 Physicochemical characterization and degradation-coupled ion release of PLA/nHA. (a) pH variation of PLA/nHA extracts in SBF. (b, c) ICP-OES analysis of calcium (b) and phosphate (c) release from PLA/nHA in SBF ($n = 3$). (d) SEM images showing HA distribution in the PLA/nHA cross-section and surface morphology after different degradation periods. (e, f) EDS analysis of surface elemental composition (e) and calcium/phosphorus distribution (f). Scale bar: 10 μm .

3.2 Mineral-metabolic co-intervention restores osteogenesis in PCK2-deficient senescent hBMSCs

To test whether the material-drug strategy could rescue osteogenesis under PCK2-impaired senescence, we generated PCK2-deficient senescent hBMSCs by lentiviral knockdown. Two independent shRNA sequences were used to reduce off-target concerns. Western blotting confirmed efficient PCK2 knockdown in both sh-PCK2-1 and sh-PCK2-2 cells compared with vector controls (Fig. S2), and these cells were used for subsequent functional assays.

As outlined in Fig. 2a, senescent hBMSCs (P18-20) from the NC, sh-PCK2-1, and sh-PCK2-2 groups were cultured under osteogenic conditions and treated with control

medium, PLA/nHA, or PLA/nHA plus metformin. PCK2 knockdown markedly reduced ALP staining and mineralized nodule formation, indicating impaired osteogenic differentiation (Fig. 2b, c). PLA/nHA partially restored this phenotype, whereas PLA/nHA/metformin produced a stronger rescue, as confirmed by ALP activity and ARS quantification (Fig. 2d, e). qRT-PCR analysis showed the same pattern for *ALP* and *OCN* expression (Fig. 2f, g). The reproducible response across two knockdown sequences supports that the combined intervention restores osteogenic differentiation in senescent hBMSCs with reduced PCK2 levels.

To distinguish the individual contributions of PLA/nHA and metformin, NC and PCK2-knockdown senescent hBMSCs

were treated with either component alone or in combination. Both PLA/nHA and metformin enhanced ALP activity at day 7 and matrix mineralization at day 14, while the combined treatment produced the greatest improvement, particularly in PCK2-knockdown cells (Fig. S3).

We next examined whether the osteogenic rescue reflected a true differentiation effect rather than altered survival. CCK-8 assays showed that PCK2 knockdown did not

significantly change hBMSC proliferation over 7 days (Fig. S4a). Live/Dead staining also showed comparable viability among NC, sh-PCK2-1, and sh-PCK2-2 cells under PM, PM + PLA/nHA, and PM + PLA/nHA/Met conditions (Fig. S4b). Thus, the PLA/nHA-metformin intervention rescued PCK2 deficiency-associated osteogenic impairment without measurably changing cell viability or cell death.

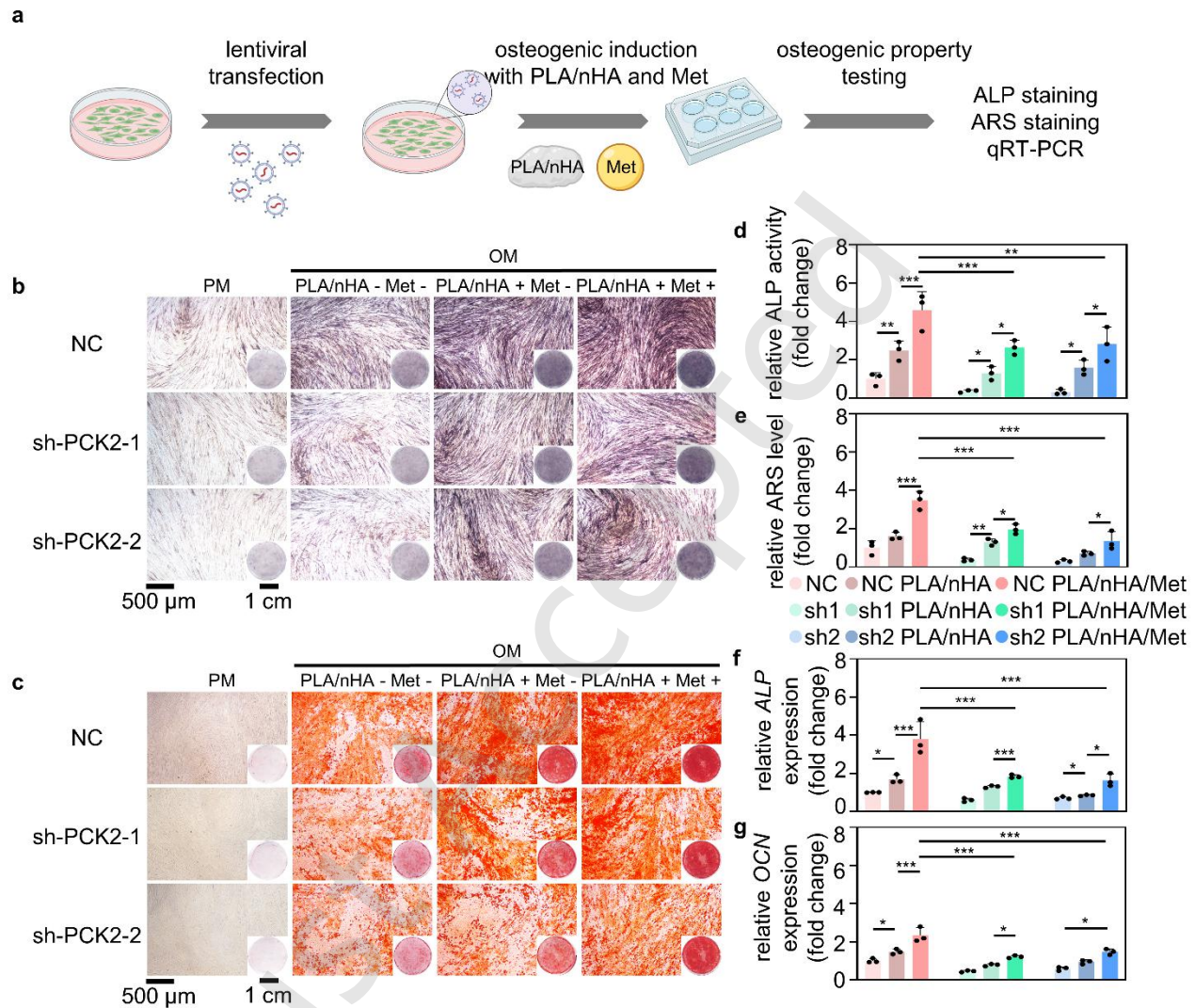


Figure 2 PLA/nHA combined with metformin restores osteogenic differentiation in PCK2-deficient senescent hBMSCs. (a) Experimental design for hBMSCs treated with PLA/nHA and metformin. (b, d) ALP staining and quantification after 7 days of osteogenic induction. Scale bar: 500 μm/1 cm. (c, e) ARS staining and quantification after 14 days of osteogenic induction. Scale bar: 500 μm/1 cm. (f, g) qRT-PCR analysis of *ALP* (f) and *OCN* (g) expression. Data are presented as mean ± SD (n = 3). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple-comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001.

3.3 Metabolic co-intervention suppresses PCK2-linked senescence and oxidative stress

Because metabolic deterioration is tightly linked to cellular aging, we next asked whether the combined intervention could suppress PCK2 deficiency-driven senescence and oxidative stress. SA-β-gal staining showed that PCK2 knockdown increased the proportion of senescent hBMSCs (Fig. 3a, c). PLA/nHA reduced this increase, and PLA/nHA/metformin produced the strongest anti-senescence effect, especially in PCK2-deficient cells. DHE staining further showed elevated intracellular ROS after PCK2 knockdown (Fig. 3b, d). PLA/nHA lowered ROS

accumulation, whereas the combined treatment produced a more pronounced reduction, indicating that metabolic rescue was accompanied by oxidative stress relief.

Consistently, immunofluorescence staining showed nuclear accumulation of the senescence-associated protein p21 (CDKN1A) in PCK2-knockdown hBMSCs (Fig. 3e). PLA/nHA and metformin reduced p21 expression. qRT-PCR further showed that PCK2 knockdown increased *p16*, *p53*, and the SASP markers *IL-1* and *IL-6*, whereas PLA/nHA/metformin normalized these senescence-associated transcripts (Fig. 3f, g). These results show that the combined strategy counteracts PCK2 deficiency-induced senescence and oxidative stress, supporting targeted cellular

rejuvenation rather than a simple viability effect.

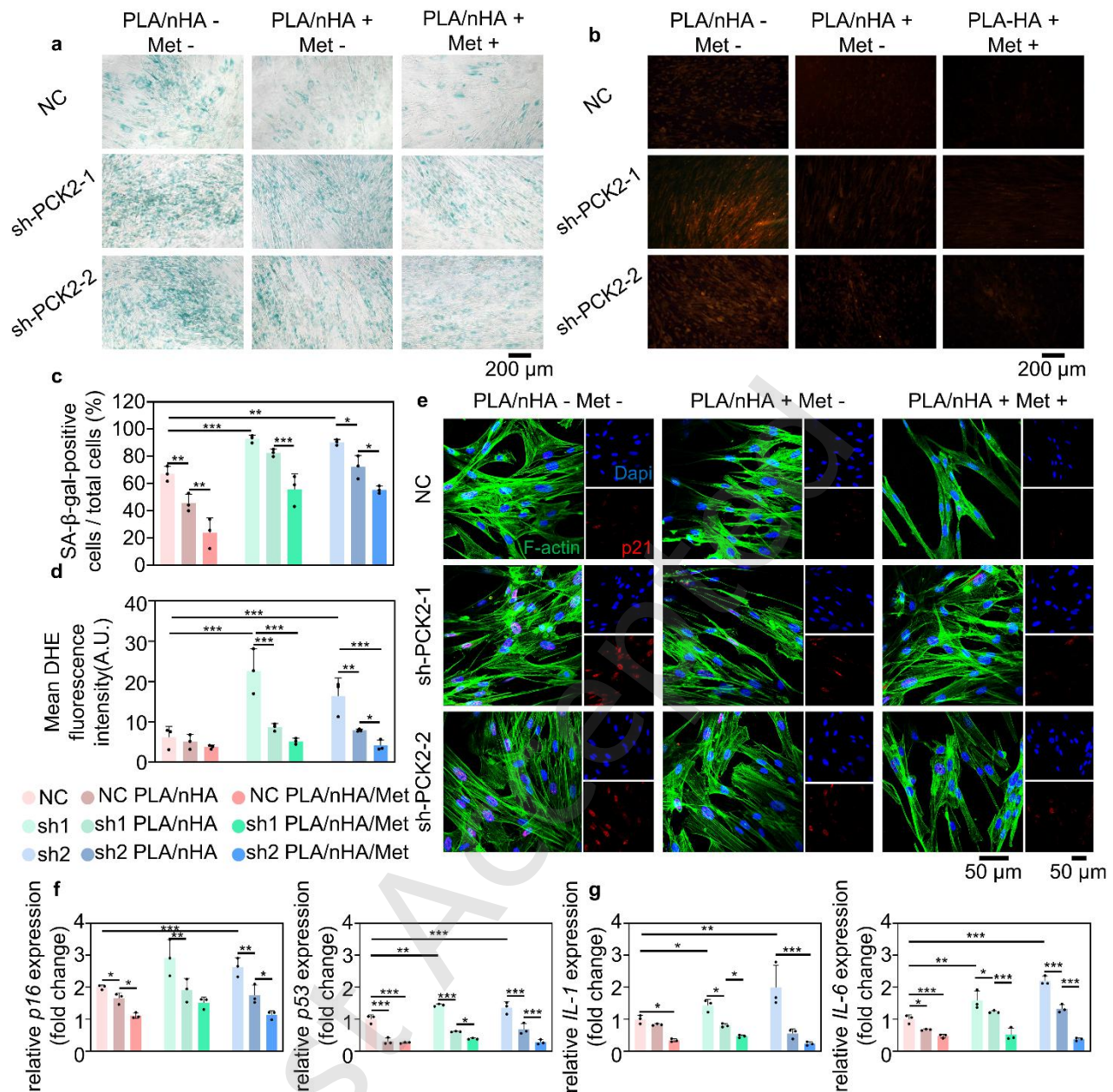


Figure 3 PLA/nHA combined with metformin attenuates PCK2 deficiency-associated senescence and oxidative stress in hBMSCs. (a) SA-β-gal staining of senescent hBMSCs after PLA/nHA and metformin treatment. Scale bar: 200 μm. (b) DHE staining of intracellular ROS (red). Scale bar: 200 μm. (c) Quantification of SA-β-gal-positive cells. (d) Quantification of mean DHE fluorescence intensity. (e) Immunofluorescence staining for p21 (red), F-actin (green), and nuclei (DAPI, blue). Scale bar: 50 μm. (f) qRT-PCR analysis of *p16* and *p53*. (g) qRT-PCR analysis of SASP markers *IL-1* and *IL-6*. Data are presented as mean ± SD (n = 3). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple-comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001.

3.4 Mechanism-based metabolic intervention accelerates aged bone repair in osteoblast-specific knockout mice

We then evaluated whether this in vitro rejuvenation translated into bone repair in vivo using osteoblast-specific *Pck2* conditional knockout mice. *Pck2*^{fl/fl}; OC-Cre mice were generated, with *Pck2*^{fl/fl} littermates serving as controls. BMSCs isolated from femurs and tibias were osteogenically induced ex vivo, and Western blotting confirmed reduced PCK2 protein in *Pck2*^{fl/fl}; OC-Cre cells compared with control cells (Fig. S5), supporting efficient osteoblast-lineage deletion.

Young (6-week-old) and aged (18-month-old) mice were used to test the intervention in metabolic dysfunction-associated bone aging. femoral defect was created, PLA/nHA was implanted locally, and metformin was administered every other day after surgery (Fig. 4a). Behavioral tests were performed before surgery and on postoperative days 3, 7, and 14 (Fig. 4b, c). By day 14, aged *Pck2*-knockout mice receiving PLA/nHA/metformin showed improved rotarod performance and increased spontaneous activity in the open-field test, suggesting better functional recovery after defect repair.

Micro-CT and 3D reconstruction were used to quantify bone regeneration on postoperative days 7 and 14 (Fig. 4d).

$Pck2^{fl/fl}$; OC-Cre mice formed less new bone than $Pck2^{fl/fl}$ controls, and aged mice regenerated less effectively than young mice, confirming both PCK2-related and age-related repair deficits (Fig. 4e, f). PLA/nHA implantation promoted new bone formation around the defect site. Metformin co-treatment further enhanced bone repair in both aged $Pck2^{fl/fl}$

controls and aged $Pck2^{fl/fl}$; OC-Cre mice, with a more pronounced improvement in the latter group. By day 14, partial scaffold degradation was accompanied by replacement with newly formed bone tissue.

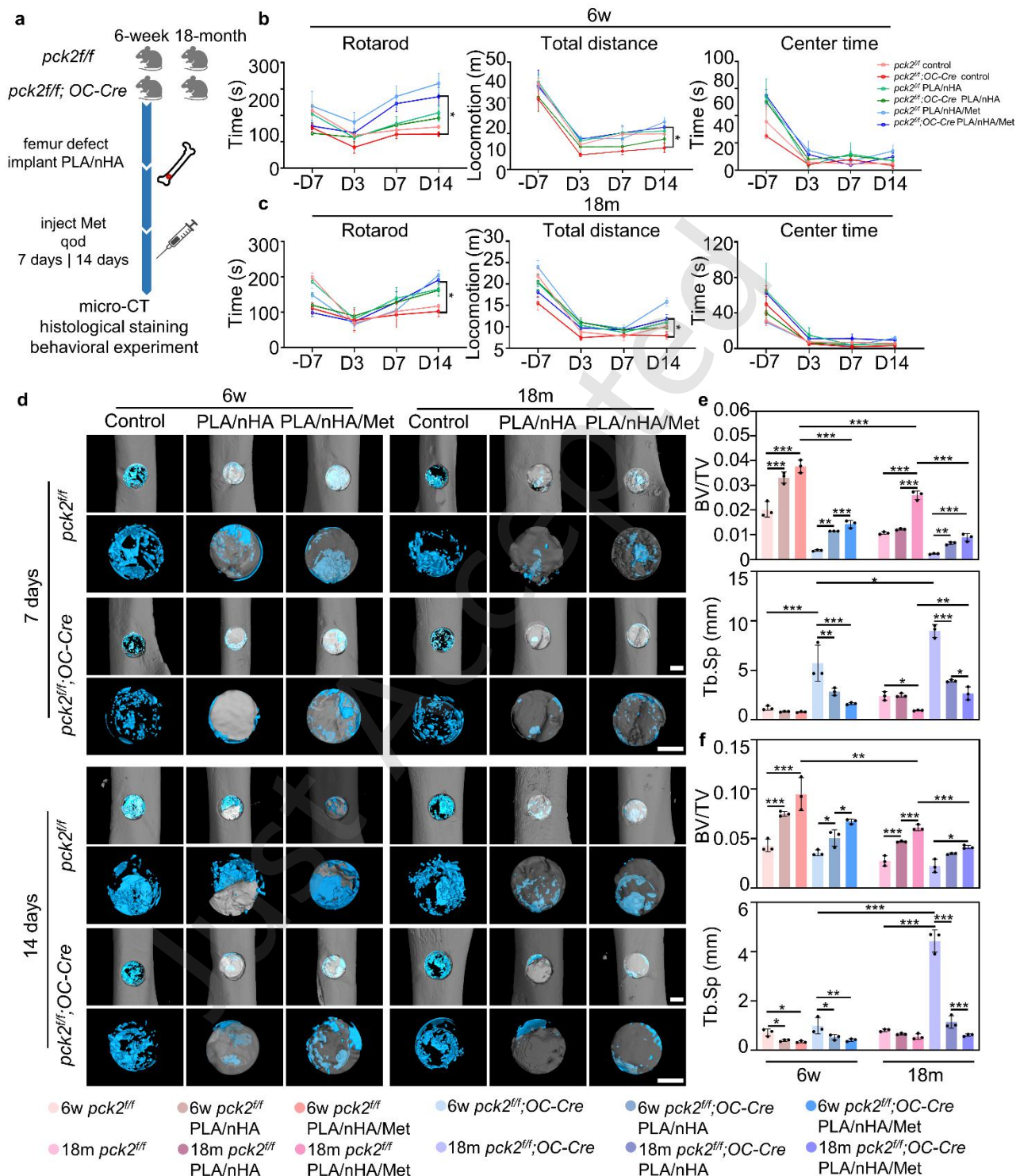


Figure 4 PLA/nHA combined with metformin enhances bone regeneration in aged mice. (a) Schematic representation of the femoral defect model in mice treated with PLA/nHA and metformin. (b, c) Behavioral assessments of preoperative and postoperative motor function in young (6-week-old) (b) and aged (18-month-old) (c) mice at each time point. (d) Micro-CT 3D reconstruction of newly formed bone and PLA/nHA scaffolds at 7 and 14 days post-surgery, assessing the therapeutic effects of PLA/nHA and metformin. Scale bar: 500 μ m. (e, f) Quantification of BV/TV and Tb.Sp of newly formed bone at 7 days (e) and 14 days (f) post-surgery. Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc multiple-comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Histological analysis further supported the regenerative

effect. H&E staining showed accelerated bone-like tissue formation in the PLA/nHA/metformin group, most

prominently in aged $Pck2^{fl/fl}$; OC-Cre mice (Fig. 5a, S6). Immunofluorescence staining at postoperative day 14 showed increased p21 and reduced OCN in $Pck2^{fl/fl}$; OC-Cre mice and in aged mice, consistent with local senescence and impaired osteogenesis (Fig. 5b, c). PLA/nHA/metformin reduced the proportion of p21-positive cells and increased

OCN expression, indicating that the intervention restored a more regenerative peri-defect microenvironment. Together, the in vivo data demonstrate that the combined strategy accelerates senescent bone defect repair by reducing local senescence and promoting osteogenesis.

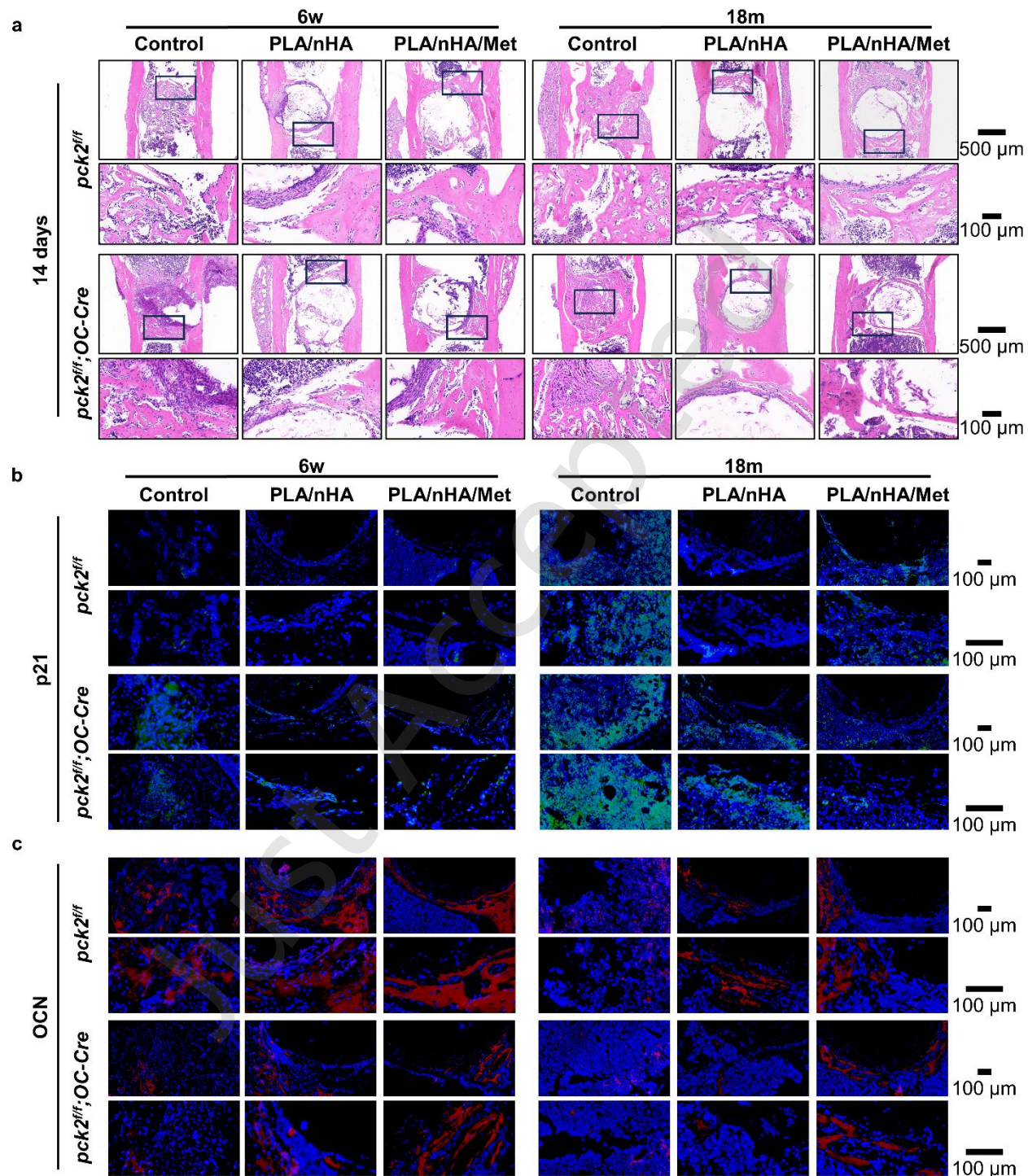


Figure 5 Histological evidence that PLA/nHA/metformin reduces local senescence and enhances osteogenesis during aged bone repair. (a) H&E staining of newly formed bone-like tissue in femoral defects at 14 days post-surgery. Scale bar: 500 μm/100 μm. (b) Immunofluorescence staining of p21 (green) in peri-defect regions and magnified local areas. Scale bar: 100 μm. (c) Immunofluorescence staining of OCN (red) in peri-defect regions and magnified local areas. Scale bar: 100 μm. Nuclei were counterstained with DAPI (blue).

3.5 Restored mitochondrial homeostasis links PCK2 metabolic rescue to senescence reversal

Because PCK2 is a mitochondrial metabolic enzyme

involved in coordinating carbon flux and bioenergetic adaptation, we next investigated how PCK2 loss reshapes the mitochondrial state of senescent hBMSCs [14]. Previous work connected PCK2 downregulation to age-related bone

loss [16], but the mitochondrial mechanism underlying this phenotype remained incompletely resolved. We therefore focused on whether PLA/nHA/metformin restores the PCK2-regulated metabolism-mitochondria axis.

Seahorse XF96 analysis showed that PCK2 knockdown reduced basal respiration, ATP production, and maximal respiration compared with NC cells (Fig. 6a-d). The blunted FCCP response indicated reduced spare respiratory capacity and impaired mitochondrial reserve. PLA/nHA partially restored these bioenergetic parameters, whereas PLA/nHA/metformin produced a stronger recovery, demonstrating that the combined intervention improves PCK2 deficiency-associated mitochondrial respiratory dysfunction.

Western blotting supported these functional findings (Fig. 6e, f, S7). PCK2 and COX I levels decreased after PCK2 knockdown, indicating impaired respiratory-chain capacity. PLA/nHA partially restored PCK2 and COX I expression, and PLA/nHA/metformin further enhanced this recovery. The p-AMPK/AMPK ratio showed a similar pattern, suggesting that the intervention reactivated AMPK-related metabolic signaling while improving mitochondrial respiratory function.

MitoTracker staining revealed marked mitochondrial network disruption in PCK2-knockdown hBMSCs, with clumped and fragmented structures replacing the rod-shaped network seen in NC cells (Fig. 6g). PLA/nHA partially improved this morphology, whereas PLA/nHA/metformin largely restored the mitochondrial network. TEM provided

ultrastructural confirmation: PCK2-deficient cells showed swollen mitochondria, disrupted cristae, and vacuolization, all of which were alleviated by PLA/nHA and more strongly corrected by the combined treatment (Fig. 6h). These observations indicate that the intervention preserves mitochondrial architecture as well as respiratory function.

MitoSOX staining showed elevated mitochondrial superoxide in PCK2-knockdown hBMSCs (Fig. 6i, j). PLA/nHA and metformin reduced both fluorescence intensity and signal distribution, indicating lower mitochondrial ROS accumulation. Mito-TEMPO similarly reduced the elevated MitoSOX signal in PCK2-deficient cells, supporting mitochondrial superoxide as a key downstream stress signal. To determine whether mitochondrial ROS functionally contributes to the impaired osteogenesis caused by PCK2 loss, senescent hBMSCs were treated with Mito-TEMPO during osteogenic induction. PCK2 knockdown reduced ALP staining and activity at day 7 and mineralized nodule formation at day 14, whereas Mito-TEMPO partially restored these osteogenic outcomes (Fig. S8a-d). Consistently, Mito-TEMPO increased ALP and RUNX2 expression at day 7 and OCN and OPN expression at day 14 compared with untreated PCK2-knockdown cells (Fig. S8e, f). These results support mitochondrial ROS as a functional mediator of the osteogenic impairment associated with PCK2 deficiency. Together, these data establish that PLA/nHA/metformin reprograms cellular metabolism, restores mitochondrial homeostasis, and counteracts PCK2 deficiency-induced senescence.

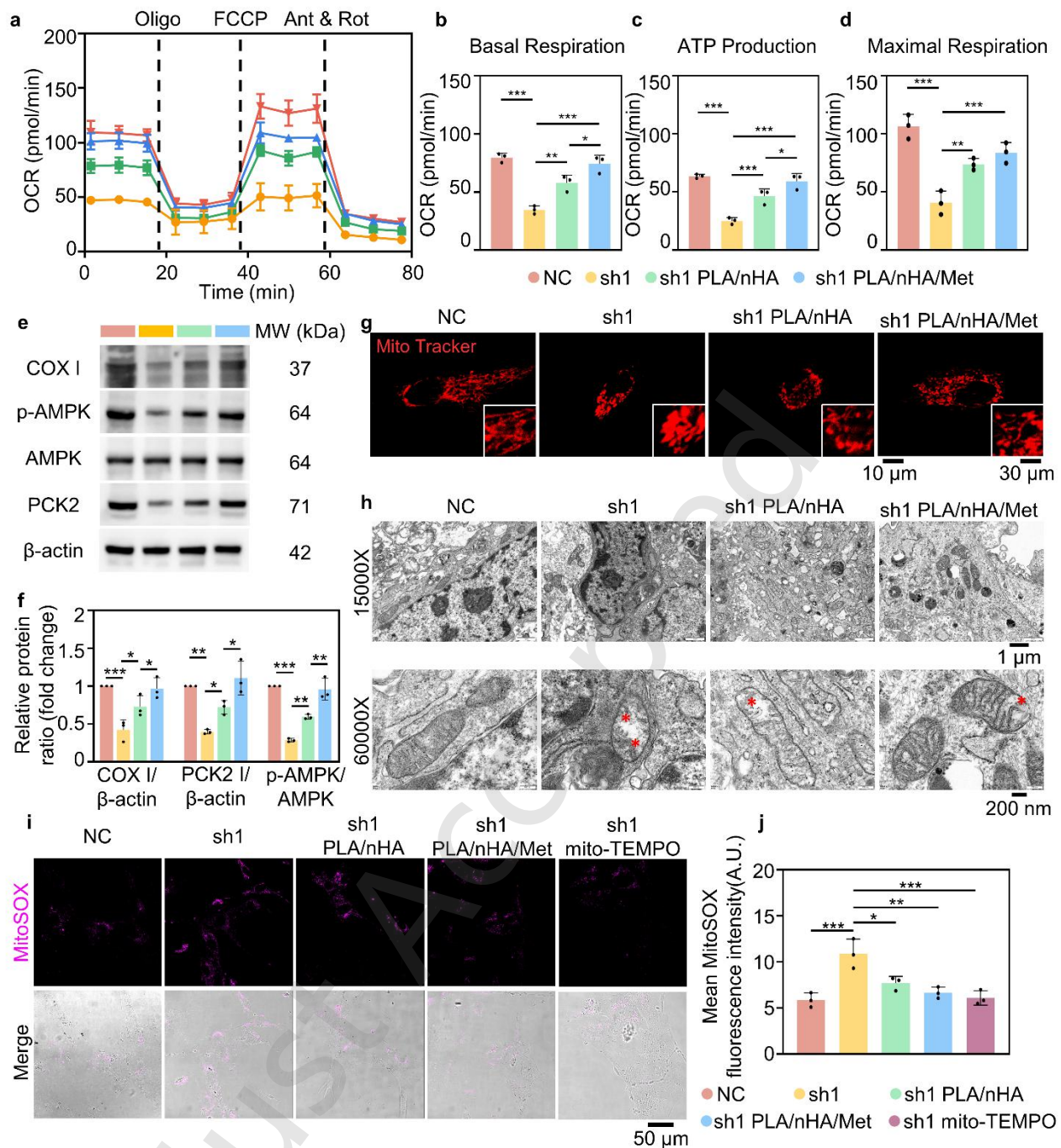


Figure 6 PLA/nHA/metformin restores the PCK2-centered metabolism-mitochondria-senescence axis. (a) OCR profiles from the Seahorse XF Mito Stress Test with sequential additions of oligomycin, FCCP, and antimycin A/rotenone ($n = 3$). (b-d) Quantification of basal respiration (b), ATP production (c), and maximal respiration (d). (e) Western blot analysis of PCK2, COX I, p-AMPK, AMPK, and β -actin. (f) Quantification of PCK2/ β -actin, COX I/ β -actin, and p-AMPK/AMPK ratios. (g) MitoTracker staining of mitochondrial morphology (red). Scale bar: 10 μ m/30 μ m. (h) TEM images of mitochondrial ultrastructure. Asterisks (*) indicate representative mitochondrial ultrastructural abnormalities, including mitochondrial swelling and disrupted cristae. Scale bar: 1 μ m/200 nm. (i) MitoSOX staining of mitochondrial superoxide accumulation, with bright-field images for cell localization. Scale bar: 50 μ m. (j) Quantification of mean MitoSOX fluorescence intensity ($n = 3$). Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4. Discussion

Age-related loss of bone regenerative capacity is closely linked to metabolic deterioration in the senescent microenvironment. Conventional biomaterials provide structural support but often fail to meet the biological demands of aged bone. This study identifies PCK2 as a pivotal osteoblast metabolic regulator. PCK2 deficiency was associated with mitochondrial dysfunction, oxidative stress,

cellular senescence, and impaired osteogenesis. We further combined local PLA/nHA implantation with systemic metformin as a mechanism-guided intervention. This combined treatment improved mitochondrial homeostasis and bone repair in the aged defect model.

Mechanistically, PCK2 connects glycolysis-derived carbon flow with mitochondrial metabolism and the TCA cycle [14]. Our genetic and in vitro evidence shows that PCK2 deficiency suppresses OXPHOS and ATP production while

increasing mitochondrial ROS and structural damage [27-31]. This stress state activates senescence-associated pathways, including the p53-p21 axis [32-34]. The PLA/nHA-metformin intervention reversed these metabolic, oxidative, and ultrastructural defects. TEM and Mito-TEMPO validation further support mitochondrial superoxide as a key mediator linking PCK2 loss to cellular senescence, providing a mechanism-based rationale for rejuvenating aged osteoblasts [35, 36].

This work also reframes PLA/nHA from a passive osteoconductive scaffold into a local component of a metabolic-regenerative strategy. Conventional bone tissue engineering often relies on structural osteoconduction or exogenous growth factors, both of which can underperform in metabolically compromised aged tissue [37-40]. In our system, PLA/nHA supplies a degradable framework and sustained calcium/phosphate release [41-43], while metformin supports mitochondrial and AMPK-related metabolic correction [24, 26, 44-48]. The combined treatment was designed to integrate local mineral cues with systemic metabolic modulation. PLA/nHA provides a local osteoconductive environment, whereas metformin may help improve the metabolic status of cells within the repair niche. This complementary design may be beneficial in the context of senescent bone repair.

The translational appeal of this approach lies in its reliance on clinically familiar components and a genetically defined disease mechanism. In aged osteoblast-specific Pck2 conditional knockout mice, PLA/nHA/metformin reduced local senescence and accelerated defect repair. Metformin has an established clinical safety record [49, 50], meanwhile PLA/nHA belongs to a widely studied family of bone-regenerative biomaterials [51, 52]. This strategy may be easier to translate than platforms requiring newly synthesized drugs or unstable biological agents. It offers a practical route for treating age-related skeletal fragility by correcting the metabolic state of the regenerative niche.

Several questions remain. First, although our findings support a role for mitochondrial homeostasis in PCK2-dependent bone repair, the links among PCK2 deficiency, mitochondrial dysfunction, and cellular senescence require further clarification. Single-cell and spatial approaches may help define cell-specific responses in aged bone [53, 54]. Second, the present intervention uses systemic metformin; future programmable or stimuli-responsive scaffolds could improve local control of metabolic modulators and reduce off-target exposure [55]. Also, although aged Pck2^{fl/fl} mice provided PCK2-intact controls, validation in genetically unmodified aged wild-type mice would further strengthen the generalizability of our findings. Finally, larger animal models and longer follow-up will be needed to evaluate mechanical recovery, scaffold degradation, and long-term safety.

5. Conclusion

In summary, this study identifies PCK2 as a causal metabolic regulator of skeletal aging and develops a mechanism-based PLA/nHA-metformin strategy for senescent bone regeneration. Genetic and in vitro evidence shows that PCK2 deficiency drives mitochondrial respiratory

failure, ROS accumulation, cellular senescence, and osteogenic impairment. The combined intervention restores mitochondrial homeostasis, reduces local senescence, and accelerates bone defect repair in aged mice, especially under osteoblast-specific Pck2 deficiency. These findings define the PCK2-mitochondria axis as a therapeutic target and provide a clinically grounded nanobiotechnology paradigm for age-related bone repair.

Electronic Supplementary Material: Additional materials and methods, supplementary figures, and supporting experimental data are available in the online version of this article at <https://doi.org/10.26599/NR.2026.94909191>.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

Muxin Yue: Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. Huiping Ma: Visualization, Methodology.

Yongsheng Zhou: Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Zheng Li: Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

All the authors have approved the final manuscript.

Informed consent

Not applicable.

Ethics statement

All animal experiments were approved by the Peking University Institutional Animal Care and Use Committee (PUIRB-LA2323387) and complied with the ARRIVE guidelines.

Use of AI statement

None.

References

- [1] Cui, J.; Shibata, Y.; Zhu, T.; Zhou, J.; Zhang, J. Osteocytes in bone aging: Advances, challenges, and future perspectives. *Ageing Res. Rev.* 2022, 77, 101608.
- [2] Farr, J. N.; Fraser, D. G.; Wang, H.; Jaehn, K.; Ogradnik, M. B.; Weivoda, M. M.; Drake, M. T.; Tchkonja, T.; LeBrasseur, N. K.; Kirkland, J. L.; Bonewald, L. F.; Pignolo, R. J.; Monroe, D. G.; Khosla, S. Identification of Senescent Cells in the Bone Microenvironment. *J. Bone Miner. Res.* 2016, 31, 1920-1929.
- [3] Farr, J. N.; Xu, M.; Weivoda, M. M.; Monroe, D. G.; Fraser, D. G.; Onken, J. L.; Negley, B. A.; Sfeir, J. G.; Ogradnik, M. B.; Hachfeld, C. M.; LeBrasseur, N. K.; Drake, M. T.; Pignolo, R. J.; Pirtskhalava, T.; Tchkonja, T.; Oursler, M. J.; Kirkland, J. L.; Khosla, S. Targeting cellular senescence prevents age-related bone loss in mice. *Nat. Med.* 2017, 23, 1072-1079.
- [4] Melis, S.; Trompet, D.; Chagin, A. S.; Maes, C. Skeletal stem and progenitor cells in bone physiology, ageing and disease. *Nat. Rev. Endocrinol.* 2025, 21, 135-153.
- [5] Ambrosi, T. H.; Marecic, O.; McArdle, A.; Sinha, R.; Gulati, G. S.; Tong, X.; Wang, Y.; Steininger, H. M.; Hoover, M. Y.; Koepke, L. S.; Murphy, M. P.; Sokol, J.; Seo, E. Y.; Tevlin, R.; Lopez, M.; Brewer, R. E.; Mascharak, S.; Lu, L.; Ajanaku, O.; Conley, S. D.; Seita, J.; Morri, M.; Neff, N. F.; Sahoo, D.; Yang, F.; Weissman, I. L.; Longaker, M. T.; Chan, C. K. F. Aged skeletal stem cells generate an inflammatory degenerative niche. *Nature* 2021, 597, 256-262.
- [6] Mi, B.; Xiong, Y.; Knoedler, S.; Alfertshofer, M.; Panayi, A. C.; Wang, H.; Lin, S.; Li, G.; Liu, G. Ageing-related bone and immunity changes: insights into the complex interplay between the skeleton and the immune system. *Bone Res.* 2024, 12, 42.
- [7] Stegen, S.; Carmeliet, G. Metabolic regulation of skeletal cell fate and function. *Nat. Rev. Endocrinol.* 2024, 20, 399-413.
- [8] Lecka-Czernik, B.; Rosen, C. J.; Napoli, N. The role of bone in whole-body energy metabolism. *Nat. Rev. Endocrinol.* 2025, 21, 743-756.
- [9] Salmonowicz, H.; Szczepanowska, K. The fate of mitochondrial respiratory complexes in aging. *Trends Cell Biol.* 2025, 35, 955-970.
- [10] Qian, K.; Gao, S.; Jiang, Z.; Ding, Q.; Cheng, Z. Recent advances in mitochondria-targeting theranostic agents. *Exploration* 2024, 4, 20230063.
- [11] Zhang, Y.; Liu, X.; Xiang, Z.; Yang, Y.; Xing, L.; Chen, Y.; Zhang, S.; Zhao, S.; Hong, Y.; Qiao, Y.; Bai, J. Mitochondrial homeostasis in bone aging: Unraveling dysfunctional pathways and developing precision therapies. *Fundamental Research* 2025.
- [12] Mendelsohn, D. H.; Walter, N.; Cheung, W.-H.; Wong, R. M. Y.; Schönmehl, R.; Winter, L.; El Khassawna, T.; Heiss, C.; Brochhausen, C.; Rupp, M. Targeting mitochondria in bone and cartilage diseases: A narrative review. *Redox Biol.* 2025, 83, 103667.
- [13] Vincent, E. E.; Sergushichev, A.; Griss, T.; Gingras, M. C.; Samborska, B.; Ntimbane, T.; Coelho, P. P.; Blagih, J.; Raissi, T. C.; Choinière, L. Mitochondrial phosphoenolpyruvate carboxykinase regulates metabolic adaptation and enables glucose-independent tumor growth. *Mol. Cell* 2015, 60, 195-207.
- [14] Yu, S.; Meng, S.; Xiang, M.; Ma, H. Phosphoenolpyruvate carboxykinase in cell metabolism: Roles and mechanisms beyond gluconeogenesis. *Mol. Metab.* 2021, 53, 101257.
- [15] Tang, M.; Sun, J.; Cai, Z. PCK2 inhibits lung adenocarcinoma tumor cell immune escape through oxidative stress-induced senescence as a potential therapeutic target. *Journal of Thoracic Disease* 2023, 15, 2601-2615.
- [16] Li, Z.; Yue, M.; Heng, B. C.; Liu, Y.; Zhang, P.; Zhou, Y. Metformin can mitigate skeletal dysplasia caused by Pck2 deficiency. *Int. J. Oral Sci.* 2022, 14, 54.
- [17] Li, Z.; Liu, X.; Zhu, Y.; Du, Y.; Liu, X.; Lv, L.; Zhang, X.; Liu, Y.; Zhang, P.; Zhou, Y. Mitochondrial Phosphoenolpyruvate Carboxykinase Regulates Osteogenic Differentiation by Modulating AMPK/ULK1-Dependent Autophagy. *Stem Cells* 2019, 37, 1542-1555.
- [18] de Zeeuw, P.; Treps, L.; García-Caballero, M.; Harjes, U.; Kalucka, J.; De Legher, C.; Brepoels, K.; Peeters, K.; Vinckier, S.; Souffreau, J.; Bouché, A.; Taverna, F.; Dehairs, J.; Talebi, A.; Ghesquière, B.; Swinnen, J.; Schoonjans, L.; Eelen, G.; Dewerchin, M.; Carmeliet, P. The gluconeogenesis enzyme PCK2 has a non-enzymatic role in proteostasis in endothelial cells. *Commun. Biol.* 2024, 7, 618.
- [19] Xue, N.; Ding, X.; Huang, R.; Jiang, R.; Huang, H.; Pan, X.; Min, W.; Chen, J.; Duan, J.-A.; Liu, P.; Wang, Y. Bone Tissue Engineering in the Treatment of Bone Defects. *Pharmaceuticals* 2022, 15, 879.
- [20] Zhu, G.; Zhang, T.; Chen, M.; Yao, K.; Huang, X.; Zhang, B.; Li, Y.; Liu, J.; Wang, Y.; Zhao, Z. Bone physiological microenvironment and healing mechanism: Basis for future bone-tissue engineering scaffolds. *Bioact. Mater.* 2021, 6, 4110-4140.
- [21] Dai, H.; Hosseinpour, S.; Hua, S.; Xu, C. Advances in porous inorganic nanomaterials for bone regeneration. *Nano TransMed* 2022, 1, e9130005.
- [22] Kulkarni, A. S.; Gubbi, S.; Barzilay, N. Benefits of Metformin in Attenuating the Hallmarks of Aging. *Cell Metab.* 2020, 32, 15-30.
- [23] Yang, Y.; Lu, X.; Liu, N.; Ma, S.; Zhang, H.; Zhang, Z.; Yang, K.; Jiang, M.; Zheng, Z.; Qiao, Y.; Hu, Q.; Huang, Y.; Zhang, Y.; Xiong, M.; Liu, L.; Jiang, X.; Reddy, P.; Dong, X.; Xu, F.; Wang, Q.; Zhao, Q.; Lei, J.; Sun, S.; Jing, Y.; Li, J.; Cai, Y.; Fan, Y.; Yan, K.; Jing, Y.; Haghani, A.; Xing, M.; Zhang, X.; Zhu, G.; Song, W.; Horvath, S.; Rodriguez Esteban, C.; Song, M.; Wang, S.; Zhao, G.; Li, W.; Izpisua Belmonte, J. C.; Qu, J.; Zhang, W.; Liu, G.-H. Metformin decelerates aging clock in male monkeys. *Cell* 2024, 187, 6358-6378.e6329.
- [24] Bharath, L. P.; Agrawal, M.; McCambridge, G.; Nicholas, D. A.; Hasturk, H.; Liu, J.; Jiang, K.; Liu, R.; Guo, Z.; Deeney, J. Metformin enhances autophagy and normalizes mitochondrial function to alleviate aging-associated inflammation. *Cell Metab.* 2020, 32, 44-55. e46.
- [25] Cao, P.; Li, N.; Liu, Y. Q.; Zhang, Y.; Zou, P. H.; Wu, J.; Cai, Y.; Yu, C. H.; Chen, L.; Luan, Q. X.; Liu, J. Poly(L-lactic-co-caprolactone)-nano hydroxyapatite promotes periodontal bone regeneration via mitochondrial metabolism-driven macrophage reprogramming. *Nano Research* 2026, 19, 94908577.
- [26] Wang, D.; Wei, L.; Hao, J.; Tang, W.; Zhou, Y.; Zhang, C.; Wang, J. PCLLA-nanoHA bone substitute promotes M2 macrophage polarization and improves alveolar bone repair in diabetic environments. *Journal of Functional Biomaterials* 2023, 14, 536.
- [27] Xu, X.; Pang, Y.; Fan, X. Mitochondria in oxidative stress, inflammation and aging: from mechanisms to therapeutic advances. *Signal Transduct. Target. Ther.* 2025, 10, 190.
- [28] Li, X.; Jiang, O.; Wang, S. Molecular mechanisms of cellular metabolic homeostasis in stem cells. *Int. J. Oral Sci.* 2023, 15, 52.
- [29] Lima, T.; Li, T. Y.; Mottis, A.; Auwerx, J. Pleiotropic effects of mitochondria in aging. *Nature aging* 2022, 2, 199-213.
- [30] Miwa, S.; Kashyap, S.; Chini, E.; von Zglinicki, T. Mitochondrial dysfunction in cell senescence and aging. *J. Clin. Invest.* 2022, 132, e158447.
- [31] Kuznetsov, A. V.; Margreiter, R.; Ausserlechner, M. J.; Hagenbuchner, J. The complex interplay between mitochondria, ROS and entire cellular metabolism. *Antioxidants* 2022, 11, 1995.
- [32] Stojanovic, B.; Jovanovic, I.; Dimitrijevic Stojanovic, M.; Stojanovic, B. S.; Kovacevic, V.; Radosavljevic, I.; Jovanovic, D.; Miletic Kovacevic, M.; Zornic, N.; Arsic, A. A. Oxidative Stress-Driven Cellular Senescence: Mechanistic Crosstalk and Therapeutic Horizons. *Antioxidants* 2025, 14, 987.
- [33] Iqbal, M. J.; Kabeer, A.; Abbas, Z.; Siddiqui, H. A.; Calina, D.; Sharifi-Rad, J.; Cho, W. C. Interplay of oxidative stress, cellular communication and signaling pathways in cancer. *Cell Commun. Signal.* 2024, 22, 7.
- [34] van Soest, D. M.; Polderman, P. E.; den Toom, W. T.; Keijer, J. P.; van Roosmalen, M. J.; Leyten, T. M.; Lehmann, J.; Zwakenberg, S.; De Henau, S.; van Bortel, R. Mitochondrial H2O2 release does not directly cause damage to chromosomal DNA. *Nat. Commun.* 2024, 15, 2725.

- [35] Lin, W.; Chen, S.; Wang, Y.; Wang, M.; Lee, W. Y.-W.; Jiang, X.; Li, G. Dynamic regulation of mitochondrial-endoplasmic reticulum crosstalk during stem cell homeostasis and aging. *Cell Death Dis.* 2021, 12, 794.
- [36] Jiang, X.; Li, W.; Ge, L.; Lu, M. Mesenchymal stem cell senescence during aging: from mechanisms to rejuvenation strategies. *Aging Dis.* 2023, 14, 1651.
- [37] Choi, I. A.; Umemoto, A.; Mizuno, M.; Park-Min, K.-H. Bone metabolism – an underappreciated player. *npj Metab Health Dis* 2024, 2, 12.
- [38] Bertels, J. C.; He, G.; Long, F. Metabolic reprogramming in skeletal cell differentiation. *Bone Res.* 2024, 12, 57.
- [39] van Gastel, N.; Carmeliet, G. Metabolic regulation of skeletal cell fate and function in physiology and disease. *Nat. Metab.* 2021, 3, 11-20.
- [40] Li, Y.; Ji, L.; Yu, J.; Zhu, F.; Xiang, Y.; Wang, X.; Wang, J.; Liu, C. Bioactive Materials-Mediated Regulation of Bone Marrow Microenvironment: Mechanistic Insights and Therapeutic Potentials. *Adv Mater* 2026, 38, e11497.
- [41] Kim, C.-D.; Koo, K.-M.; Kim, H.-J.; Kim, T.-H. Recent Advances in Nanomaterials for Modulation of Stem Cell Differentiation and Its Therapeutic Applications. *Biosensors (Basel)* 2024, 14, 407.
- [42] Bernardo, M. P.; da Silva, B. C. R.; Hamouda, A. E. I.; de Toledo, M. A. S.; Schalla, C.; Rütten, S.; Goetzke, R.; Mattoso, L. H. C.; Zenke, M.; Sechi, A. PLA/Hydroxyapatite scaffolds exhibit in vitro immunological inertness and promote robust osteogenic differentiation of human mesenchymal stem cells without osteogenic stimuli. *Sci Rep* 2022, 12, 2333.
- [43] Grigora, M.-E.; Terzopoulou, Z.; Baci, D.; Steriotis, T.; Charalambopoulou, G.; Gounari, E.; Bikiaris, D. N.; Tzetzis, D. 3D printed poly (lactic acid)-based nanocomposite scaffolds with bioactive coatings for tissue engineering applications. *J. Mater. Sci.* 2023, 58, 2740-2763.
- [44] Lee, S. H.; Duron, H. E.; Chaudhuri, D. Beyond the TCA cycle: new insights into mitochondrial calcium regulation of oxidative phosphorylation. *Biochem. Soc. Trans.* 2023, 51, 1661-1673.
- [45] Seegren, P. V.; Harper, L. R.; Downs, T. K.; Zhao, X.-Y.; Viswanathan, S. B.; Stremiska, M. E.; Olson, R. J.; Kennedy, J.; Ewald, S. E.; Kumar, P.; Desai, B. N. Reduced mitochondrial calcium uptake in macrophages is a major driver of inflammaging. *Nature Aging* 2023, 3, 796-812.
- [46] Feng, Y.; Wang, J.; Cao, J.; Cao, F.; Chen, X. Manipulating calcium homeostasis with nanoplatforms for enhanced cancer therapy. *Exploration* 2024, 4, 20230019.
- [47] de Marañon, A. M.; Díaz-Pozo, P.; Canet, F.; Díaz-Morales, N.; Abad-Jiménez, Z.; Lopez-Domenech, S.; Vezza, T.; Apostolova, N.; Morillas, C.; Rocha, M. Metformin modulates mitochondrial function and mitophagy in peripheral blood mononuclear cells from type 2 diabetic patients. *Redox Biol.* 2022, 53, 102342.
- [48] Bharath, L. P.; Nikolajczyk, B. S. The intersection of metformin and inflammation. *Am. J. Physiol.-Cell Physiol.* 2021, 320, C873-C879.
- [49] Dutta, S.; Shah, R. B.; Singhal, S.; Dutta, S. B.; Bansal, S.; Sinha, S.; Haque, M. Metformin: a review of potential mechanism and therapeutic utility beyond diabetes. *Drug Des Devel Ther.* 2023, 17, 1907-1932.
- [50] Foretz, M.; Guigas, B.; Viollet, B. Metformin: update on mechanisms of action and repurposing potential. *Nat. Rev. Endocrinol.* 2023, 19, 460-476.
- [51] Han, X.; Saiding, Q.; Cai, X.; Xiao, Y.; Wang, P.; Cai, Z.; Gong, X.; Gong, W.; Zhang, X.; Cui, W. Intelligent Vascularized 3D/4D/5D/6D-Printed Tissue Scaffolds. *Nano-Micro Letters* 2023, 15, 239.
- [52] Wan, B.; Ruan, Y.; Shen, C.; Xu, G.; Forouzanfar, T.; Lin, H.; Wu, G. Biomimetically precipitated nanocrystalline hydroxyapatite. *Nano TransMed* 2022, 1, e9130008.
- [53] Moiseeva, V.; Cisneros, A.; Sica, V.; Deryagin, O.; Lai, Y.; Jung, S.; Andrés, E.; An, J.; Segalés, J.; Ortet, L.; Lukesova, V.; Volpe, G.; Benguria, A.; Dopazo, A.; Benitah, S. A.; Urano, Y.; del Sol, A.; Esteban, M. A.; Ohkawa, Y.; Serrano, A. L.; Perdiguero, E.; Muñoz-Cánoves, P. Senescence atlas reveals an aged-like inflamed niche that blunts muscle regeneration. *Nature* 2023, 613, 169-178.
- [54] Bandyopadhyay, S.; Duffy, M. P.; Ahn, K. J.; Sussman, J. H.; Pang, M.; Smith, D.; Duncan, G.; Zhang, I.; Huang, J.; Lin, Y.; Xiong, B.; Imtiaz, T.; Chen, C.-H.; Thadi, A.; Chen, C.; Xu, J.; Reichart, M.; Martinez, Z.; Diorio, C.; Chen, C.; Pillai, V.; Snaith, O.; Oldridge, D.; Bhattacharyya, S.; Maillard, I.; Carroll, M.; Nelson, C.; Qin, L.; Tan, K. Mapping the cellular biogeography of human bone marrow niches using single-cell transcriptomics and proteomic imaging. *Cell* 2024, 187, 3120-3140.e3129.
- [55] Wang, X.; Zeng, J.; Gan, D.; Ling, K.; He, M.; Li, J.; Lu, Y. Recent strategies and advances in hydrogel-based delivery platforms for bone regeneration. *Nano-Micro Letters* 2025, 17, 73.

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Electronic Supplementary Material

PLA/nHA nanocomposites and metformin rescue aged bone repair by restoring PCK2-regulated mitochondrial metabolism

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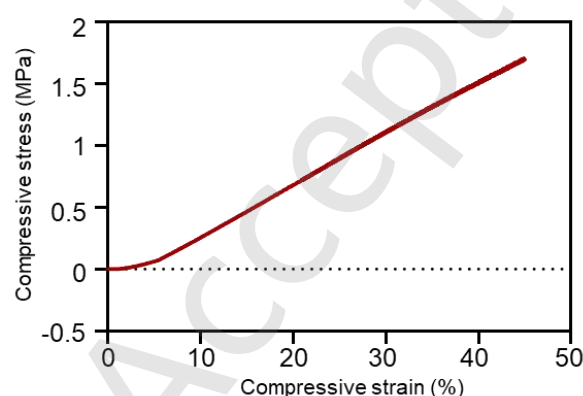


Fig. S1 Representative compressive stress-strain curves of the PLA/nHA scaffolds. Cylindrical specimens (10 mm in diameter and 20 mm in height) were compressed in PBS at 37°C at a crosshead speed of 6 mm/min.

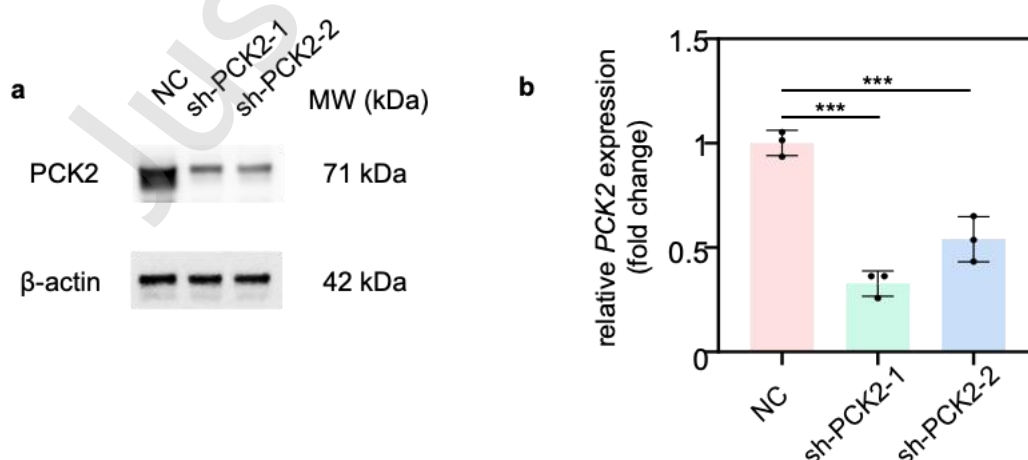


Figure S2 Validation of PCK2 knockdown efficiency in lentivirus-transduced hBMSCs by Western blot (a) and qRT-PCR (b). Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

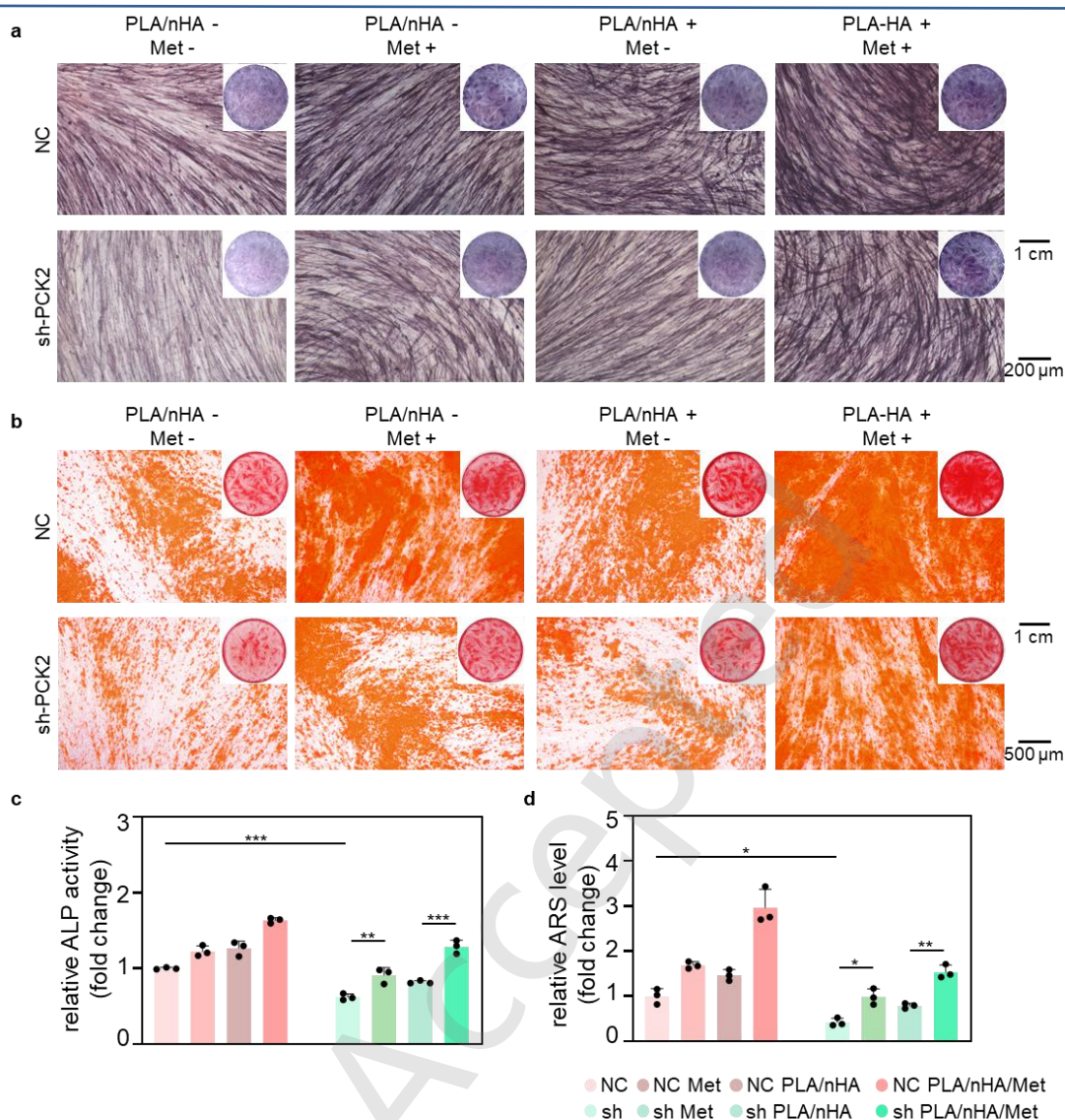


Figure S3 Individual and combined effects of PLA/nHA and metformin on the osteogenic differentiation of senescent hBMSCs. (a) ALP staining after 7 days of osteogenic induction. (b) ARS staining after 14 days of osteogenic induction. Scale bars: 200 μm and 1 cm for the whole-well images. (b, d) ARS staining and quantitative analysis after 14 days of osteogenic induction. Scale bars: 500 μm and 1 cm for the whole-well images. (c, d) Quantitative analysis of ALP activity in NC and PCK2-knockdown cells in different treatment groups. (e, f) Quantitative analysis of ARS staining in NC and PCK2-knockdown cells, respectively. Data are presented as mean ± SD (n = 3). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple-comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001.

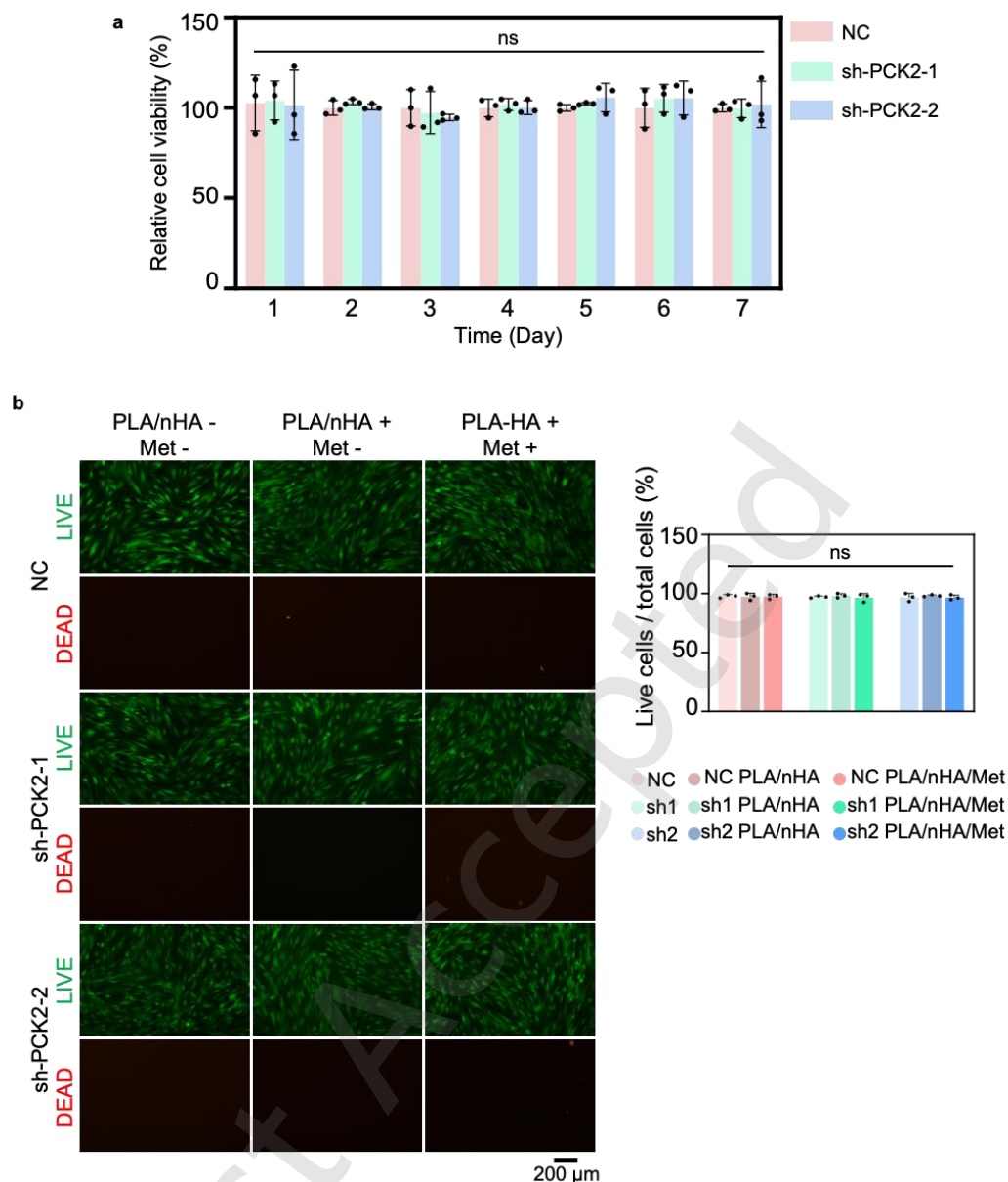


Figure S4 CCK-8 assay and Live/Dead staining of hBMSCs. (a) CCK-8 assay of NC, sh-PCK2-1, and sh-PCK2-2 hBMSCs over a 7-day culture period. (b) Representative Live/Dead staining images of NC, sh-PCK2-1, and sh-PCK2-2 hBMSCs treated with PM, PM + PLA/nHA, or PM + PLA/nHA/Met for 3 days. Live cells (green), dead cells (red). Scale bar: 200 μ m. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc multiple-comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

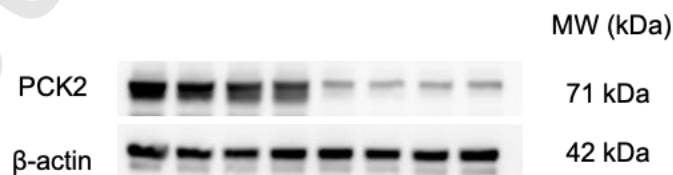


Figure S5 Validation of reduced PCK2 protein expression in osteogenically induced BMSCs from *Pck2^{fl/fl}*; OC-Cre mice by Western blot.

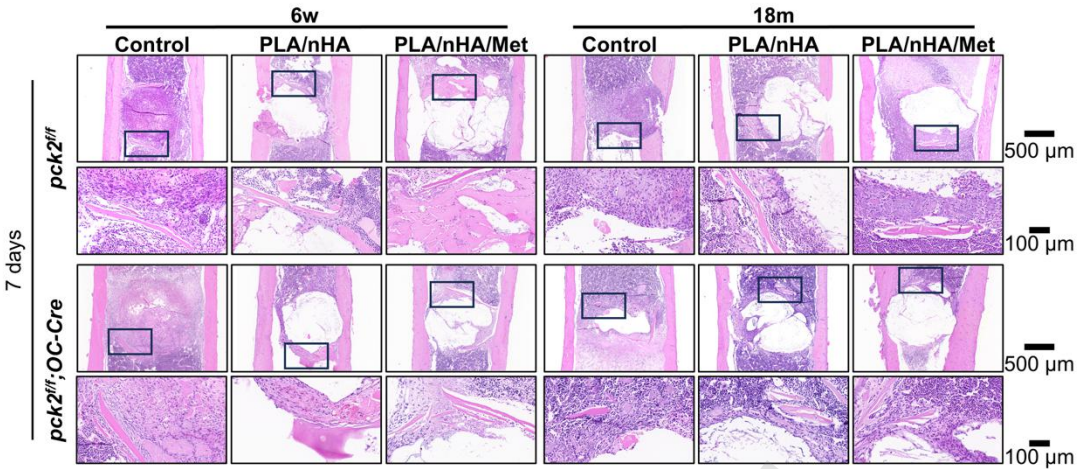


Figure S6 H&E staining of newly formed bone-like tissue in the femoral defect area at 7 days post-surgery. Scale bar: 500 μm/100 μm.

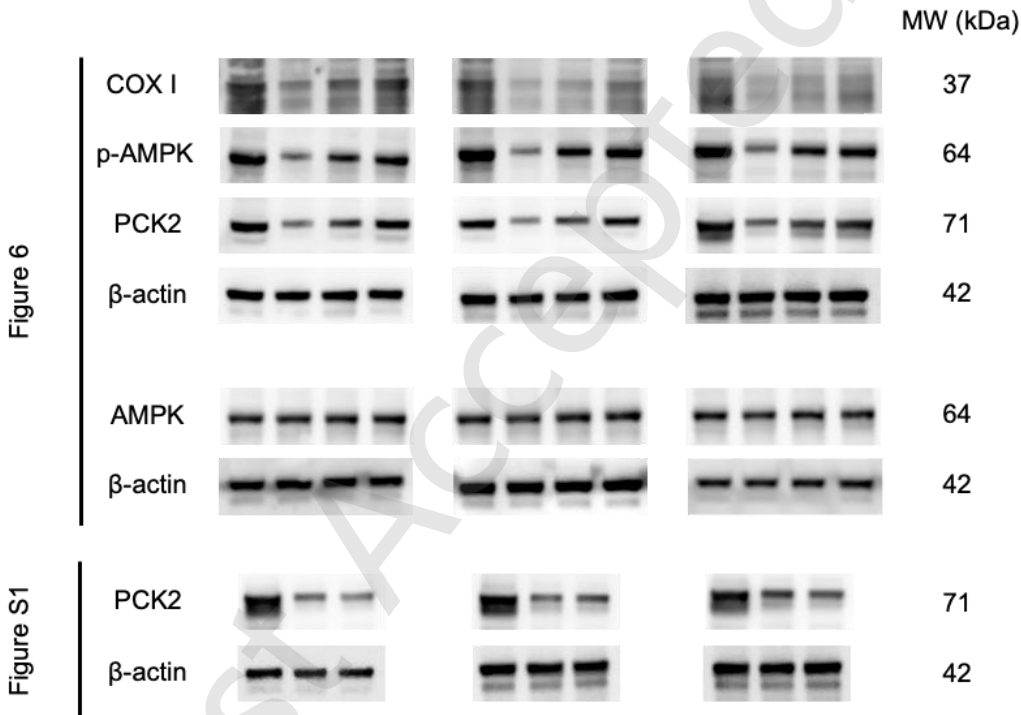


Figure S7 Western blot images of all biological replicates.

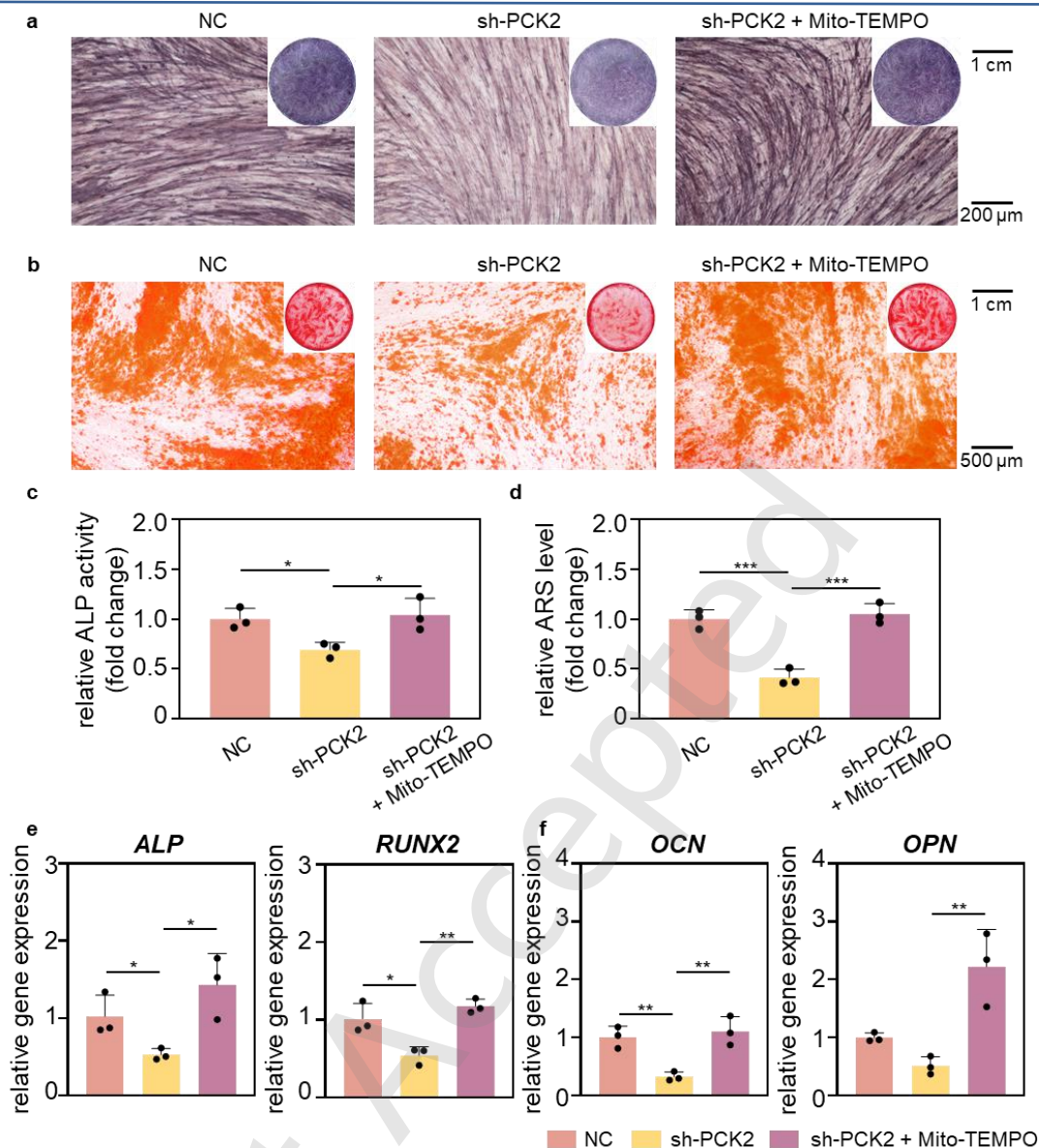


Figure S8 Mito-TEMPO partially restores osteogenic differentiation in PCK2-knockdown senescent hBMSCs. (a, c) ALP staining and quantitative analysis of ALP activity after 7 days of osteogenic induction. Scale bars: 200 μ m and 1 cm for the whole-well images. (b, d) ARS staining and quantitative analysis after 14 days of osteogenic induction. Scale bars: 500 μ m and 1 cm for the whole-well images. (e) qRT-PCR analysis of *ALP* and *RUNX2* expression at day 7. (f) qRT-PCR analysis of *OCN* and *OPN* expression at day 14. Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table S1 Porosity and compressive properties of the PLA/nHA scaffold

Scaffold	Porosity (%)	Compressive modulus (MPa)
PLA-nHA	14.42 $\pm$ 3.68	4.817 $\pm$ 0.795

Table S2 Gene Stability Values

Genes	Stability Values (M-values)	Rank
ACTB	0.216	1
RPLP0	0.216	1
18S rRNA	0.272	3
HPRT1	0.294	4
GAPDH	0.314	5
TBP	0.329	6

Table S3 Primers used in qRT-PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>GAPDH</i>	GGTCACCAGGGCTGCTTTT	GGATCTCGCTCCTGGAAGATG

<i>ACTB</i>	CACCATTGGCAATGAGCGGTTTC	AGGTCTTTGCGGATGTCCACGT
<i>18SrRNA</i>	ACCCGTTGAACCCCATTCGTGA	GCCTCACTAAACCATCCAATCGG
<i>TBP</i>	GAGAGTTCTGGGATTGTACCG	ATCCTCATGATTACCGCAGC
<i>RPLP0</i>	AGCCCAGAACACTGGTCTC	ACTCAGGATTTCAATGGTGCC
<i>HPRT1</i>	CCTGGCGTCGTGATTAGTGAT	AGACGTTCACTCTGTCCATAA
<i>PCK2</i>	GAGTGTTCCTCCCAAGG	GCGTGCCTTTGGTGATTGAG
<i>ALP</i>	GACCTCCTCGGAAGACACTC	TGAAGGGCTTCTGTCTGTG
<i>OCN</i>	GGCGCTACCTGTATCAATGG	GTGGTCAGCCAACCTCGTCA
<i>p16</i>	CTGCCCAACGCACCGAATAG	TCATGACCTGGATCGGCCTC
<i>p53</i>	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC
<i>IL-1</i>	TGACCTGAGCACCTTCTTTCC	TGTTGCTCCATATCCTGTCCC
<i>IL-6</i>	AGCCACCGGGAACGAAAGA	ACCGAAGGCGCTTGTGGAGA

Materials and methods

6.1 Porosity measurement

Porosity measurement The porosity of the PLA/nHA scaffolds was determined using the ethanol displacement method. Briefly, each dried scaffold was weighed to obtain its initial mass m_1 and then immersed in a known volume of ethanol V_1 at room temperature. The samples were sonicated for 1 min to facilitate complete penetration of ethanol into the pores, after which the total volume of the scaffold-ethanol system was recorded as V_2 . The scaffold was then removed, and its ethanol-saturated mass was recorded as m_2 . The porosity was calculated using the equation: Porosity (%) = $(m_2 - m_1) / \rho_{\text{ethanol}} (V_2 - V_1) \times 100\%$

6.2 Compressive mechanical testing

The compressive properties of the PLA/nHA scaffolds were evaluated using an Instron universal testing machine. Cylindrical specimens with a diameter of 10 mm and a height of 20 mm were used for testing. The specimens were immersed in PBS maintained at 37°C and positioned between two parallel compression platens. Uniaxial compression was applied at a crosshead speed of 6 mm/min. The recorded force-displacement data were converted into stress-strain curves based on the initial dimensions of each specimen, and the compressive modulus was calculated from the slope of the linear region of the stress-strain curve.

6.3 Lentiviral transfection

PCK2 knockdown (sh-PCK2-1, sh-PCK2-2) and control (vector) lentiviruses were transduced into P2 hBMSCs. The lentiviral titer used was approximately 1×10^9 TU/mL, and 100 μ L of lentivirus was added to 1×10^6 cells. Fresh culture medium was replaced 24 hours after transfection, and puromycin (1 μ L/mL) was added 72 hours post-transfection for selection, to improve the transfection efficiency. The sequence of lentiviruses is GGTGGCAAGCATGCGTATTAT (sh-PCK2-1), and GGGATGATATTGCTTGGATGA (sh-PCK2-2).

6.4 ALP staining and quantification

To assess osteogenic differentiation, senescent hBMSCs (P10–12) were seeded in 12-well plates. The vector, sh-PCK2-1, and sh-PCK2-2 groups were treated with osteogenic medium (OM), OM + PLA/nHA, and OM + PLA/nHA/Met. After 7 days of osteogenic induction, the cells were washed with PBS, fixed with 95% ethanol at room temperature for 30 minutes, and then stained with a 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitro blue tetrazolium (NBT) alkaline phosphatase (ALP) Color Development Kit (Beyotime, China) following the manufacturer's instructions. After staining, the cells were washed with PBS, scanned, and imaged under a light microscope.

For quantitative analysis of ALP activity, cells were lysed in 1% Triton X-100 at 4°C for 30 minutes, and the supernatant was collected after centrifugation. The protein concentration in the supernatant was determined using the BCA assay. ALP activity was measured using the ALP activity assay kit (Beyotime, China) by microplate reader at 520 nm (Biotek, USA), and the relative ALP activity was calculated.

6.5 ARS staining and quantification

After 14 days of osteogenic induction, the culture medium was removed, and the cells were washed with PBS. They were then fixed with 95% ethanol for 30 minutes and stained with Alizarin Red S (ARS). The stained cells were scanned, and images were recorded using a microscope. To quantify the mineralization, the ARS staining was dissolved with 1% hexadecylpyridinium chloride solution, and 100 μ L of the solution from each well was transferred to a 96-well plate. The absorbance was measured by microplate reader at 490 nm (Biotek, USA) to calculate the relative mineralization.

6.6 CCK-8 assay

To evaluate cell proliferation, hBMSCs from the vector, sh-PCK2-1, and sh-PCK2-2 groups were seeded into 96-well plates and cultured with proliferation medium (PM). After 1–7 days, 10 μ L of Cell Counting Kit-8 reagent (CCK-8, DOJINDO, Japan) was added to each well containing 100 μ L of culture medium, followed by incubation at 37°C for 1 h in the dark. The absorbance was measured at 450 nm using a microplate reader (Biotek, USA). Blank wells containing medium and CCK-8 reagent but no cells were used for background subtraction. The relative cell viability was calculated based on the absorbance values.

Relative cell viability (%) = $(\text{OD}_{\text{experimental}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) \times 100\%$

6.7 Live/Dead staining

To assess cell viability, hBMSCs from the vector, sh-PCK2-1, and sh-PCK2-2 groups were seeded in 24-well plates and treated with PM, PM + PLA/nHA, or PM + PLA/nHA/Met. After 3 days, cell viability was evaluated using a Live/Dead Cell Staining Kit (KeyGEN BioTECH, China) according to the manufacturer's instructions. Briefly, the cells were washed with PBS and incubated with the working solution containing calcein-AM and propidium iodide (PI) at 37°C for 20 min in the dark. After incubation, fluorescence images were captured using a fluorescence microscope. Live cells were stained green, whereas dead cells were stained red. The proportion of live and dead cells was quantified from randomly selected fields using ImageJ software.

6.8 β -galactosidase staining

To assess cellular senescence, hBMSCs from the vector, sh-PCK2-1, and sh-PCK2-2 groups were seeded in 12-well plates.

Upon reaching 90% confluence, the cells were treated with PM, PM + PLA/nHA, or PM + PLA/nHA/Met. β -Galactosidase staining was performed using a β -galactosidase staining kit (Beyotime, China). After fixation, the cells were incubated with the staining solution at 37°C for 12 hours in the absence of CO₂. The blue color indicative of β -galactosidase activity was observed under a light microscope and recorded.

6.9 Cellular ROS staining

To measure reactive oxygen species (ROS) levels, hBMSCs from the vector, sh-PCK2-1, and sh-PCK2-2 groups were seeded in 24-well plates. After reaching 90% confluence, the cells were treated with PM, PM + PLA/nHA, or PM + PLA/nHA/Met. ROS levels were assessed using the Reactive Oxygen Species Assay Kit (Solarbio, China, CA1420). Dihydroethidium (DHE) dye penetrates the cell membrane and, upon oxidation by ROS, forms ethidium, which intercalates with DNA and generates red fluorescence. After incubating with 10 μ M DHE for 30 minutes at 37°C in the dark, the cells were washed with PBS and observed under a fluorescence microscope. The average fluorescence intensity of the images was quantified using ImageJ software.

6.10 qRT-PCR

After washing the hBMSCs from each group with PBS, total RNA was extracted using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The RNA concentration was determined using a UV spectrophotometer NanoDrop 8000 (Thermo Fisher Scientific, USA). Subsequently, cDNA was synthesized using a reverse transcription kit (Accurate Biology, China) following the provided protocol. The qRT-PCR reaction mixture was prepared to a total volume of 10 μ L, consisting of 5 μ L 2 $\times$ SYBR Green PCR Master Mix (Accurate Biology, China), 1 μ L cDNA template, 0.5 μ L of each forward and reverse primer (final concentration: 1 μ M), and 3 μ L of DEPC-treated water. The reaction was performed in a 96-well PCR plate using a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, USA). The stability of multiple candidate reference genes (ACTB, GAPDH, 18S rRNA, RPLP0, HPRT1, and TBP) was first validated using the GeNorm, as shown in Table S2. Based on this analysis, ACTB, RPLP0, and 18S rRNA were selected as the most stable reference genes for normalization. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta C_t}$ method. The primer sequences used in this study are listed in Table S3.

6.11 Immunofluorescence staining

hBMSCs from each group were seeded onto coverslips and cultured until cell adhesion was achieved. Each group of cells was treated with PM, PM+PLA/nHA, and PM+PLA/nHA/Met. When cells reached 70%-80% confluence, the culture medium was discarded, and cells were gently washed with PBS. The cells were fixed at 4°C for 15 minutes with 4% paraformaldehyde, followed by three PBS washes. Cells were then permeabilized with 0.1% Triton X-100 at room temperature for 10 minutes and washed three times with PBS to remove residual detergent. To minimize nonspecific binding, cells were blocked with immunofluorescence blocking solution at room temperature for 1 hour. Afterward, the cells were incubated overnight at 4°C with primary antibody solution. The antibody used in the study was p21 Rabbit pAb (251324, ZEN-BIOSCIENCE, China). The next day, the primary antibody solution was discarded, and the cells were washed three times with washing buffer. The cells were then incubated with a fluorescence-labeled secondary antibody for 1 hour at 4°C, followed by staining with diluted Alexa Fluor 594 conjugated secondary antibody for 30 minutes at room temperature. After washing three times with washing buffer, cells were mounted using DAPI-containing antifade mounting medium (Beyotime, China). Imaging was performed under a laser scanning confocal microscope (Leica STED, Germany).

6.12 MitoTracker Deep Red staining

Cells were seeded on coverslips and treated with the indicated compounds for 48 hours. The culture medium was discarded, and the cells were stained with 100 nM Mito-Tracker Deep Red FM (Beyotime, China) at 37°C for 30 minutes. After the staining solution was removed, cells were washed with fresh culture medium preheated to 37°C. The coverslips were then removed and imaged under a laser scanning confocal microscope (Leica STED, Germany).

6.13 MitoSOX Staining

Cells treated with the blank control, PLA/nHA, or PLA/nHA/Met were washed with PBS after treatment, followed by staining with MitoSOX mitochondrial superoxide indicator (Thermo Fisher Scientific, USA). The cells were incubated at 37°C for 30 minutes. After washing three times with PBS to remove unbound probe, fluorescence images were taken using a laser scanning confocal microscope (Leica STED, Germany). The excitation wavelength was 396 nm, and the emission wavelength was 610 nm.

6.14 Mitochondria Respiration and Stress Test Assay

Cells from the NC, PCK2 knockdown (sh1), sh1 + PLA-nHA, and sh1 + PLA-nHA/Met groups were digested, counted, and seeded at a density of approximately 1×10^4 cells per well into Seahorse XF96 cell culture microplates. After a 24-hour incubation to ensure proper attachment, mitochondrial respiration was assessed using the Seahorse XF96 Analyzer. The assay was conducted according to the manufacturer's instructions, utilizing the Seahorse XF Cell Mito Stress Test Kit. This involved sequential injections of oligomycin (1.5 μ M), FCCP (1.0 μ M), and a mixture of rotenone and antimycin A (0.5 μ M each) to determine key mitochondrial parameters including basal respiration, ATP production, and maximal respiration. The injection timing was set according to the standard protocol recommended by the manufacturer, and data normalization was performed using the cell count to adjust for variations in cell number.

6.15 Western Blot

Cells from each group were washed three times with PBS, and then collected by scraping into EP tubes. RIPA lysis buffer containing phosphatase and protease inhibitors was added, and the samples were lysed on ice for 30 minutes. The lysates were centrifuged at 14,000 rpm for 20 minutes at 4°C, and the supernatants were collected on ice. Total protein concentration was measured using the BCA method. The protein samples were prepared by adding loading buffer and heating at 99°C for 5 minutes. After cooling, the samples were loaded onto SDS-PAGE gels for electrophoresis (200V, 30min). Proteins were transferred to PVDF membranes (400mA, 30min) and blocked with 10% non-fat milk in TBST for 1 hour at room temperature. After blocking, the membranes were washed three times with TBST, incubated overnight at 4°C with primary antibodies. The primary antibodies used were β -actin Mouse mAb (1:10000, 66009-1-Ig, Proteintech, China), PCK2 Rabbit mAb (1:1000,

R25292, ZEN-BIOSCIENCE, China), Phospho-AMPK α 1-T183/AMPK α 2-T172 Rabbit pAb (1:500, AP0432, ABclonal, China), AMPK Alpha Rabbit pAb (1:5000, 10929-2-AP, Proteintech, China), and MTCO1 Rabbit mAb (1:1000, A24805, ABclonal, China).

The next day, the membranes were washed with TBST and incubated for 1 hour at room temperature with species-specific secondary antibodies. The secondary antibodies used were HRP-Goat Anti-Rabbit Recombinant Secondary Antibody (1:10000, ZB2301, ZSGB-BIO, China) and HRP-Goat Anti-Mouse Recombinant Secondary Antibody (1:10000, ZB2305, ZSGB-BIO, China). After washing with TBST, the membranes were exposed using a chemiluminescent detection system (ECL, APPLIED GEN, China), and the results were recorded using a Chemidoc imaging system (VILBER BIO IMAGING, France). The images were quantified using ImageJ software to measure the relative intensities of the protein bands. The target protein levels were normalized to the β -actin band as a loading control.

6.16 Animal Behavioral Tests

The motor coordination and balance ability of mice were evaluated using the rotarod test. Mice were first trained at a low speed (5 rpm) for 1 day to familiarize them with the apparatus. In the formal test, the rotational speed was gradually increased from 5 rpm to 40 rpm over 3 minutes. Each mouse was placed on the rod, and the time it remained on the rotating rod without falling was recorded. Each mouse was tested three times, with a 10-minute interval between trials, and the average time was used for analysis.

Spontaneous activity was assessed using the open field test. Mice were individually placed in the center of an open field and allowed to explore freely for 5 minutes. The total distance traveled and the time spent in the center area were recorded for statistical analysis.

6.17 Micro-CT

After fixation, femur samples were scanned using a micro-CT scanner (Inveon instrument, Siemens, Germany). Three-dimensional reconstruction and analysis were performed using Inveon Research Workplace software (Siemens, Germany). The bone volume fraction (BV/TV) and trabecular spacing (Tb.sp) of the newly formed bone were quantitatively analyzed.

6.18 Paraffin Section Staining

Fixed femoral tissue samples were decalcified, dehydrated, paraffin-embedded, and sectioned. The tissue sections were stained with H&E and immunofluorescence staining. For H&E staining, tissue sections were first deparaffinized, rehydrated through a graded alcohol series, and then stained with hematoxylin, followed by eosin. After staining, sections were dehydrated, cleared in xylene, and mounted with a resinous mounting medium. For immunofluorescence, paraffin-embedded tissue sections were deparaffinized and rehydrated through a graded alcohol series. Antigen retrieval was performed using EDTA buffer. Sections were blocked with 3% BSA to prevent non-specific binding. Primary antibodies were incubated overnight at 4°C, followed by incubation with fluorescent secondary antibodies (1:5000 dilution) for 1 hour at room temperature. Nuclei were counterstained with DAPI (1:1000). Fluorescence images were acquired using a fluorescence microscope, and data were analyzed using ImageJ software. The antibodies used in the study were CDKN1A Rabbit mAb (A19094, abclonal, China) and OCN Polyclonal antibody (23418-1-AP, Proteintech, China).