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Research Article

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Post-translational Regulation of the PI3K/Akt/mTOR–Autophagy Axis in Osteoblast Differentiation and Its Polypharmacological Targeting by *Eupolyphaga sinensis*

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Abstract: Fracture healing is a complex biological process in which osteoblast differentiation plays a central role, yet the molecular mechanisms governing this process remain incompletely understood. The PI3K/Akt/mTOR signaling pathway and autophagy are critical regulators of osteoblast function, but the relationship between their transcriptional and post-translational regulation during human osteoblast differentiation has not been characterized. *Eupolyphaga sinensis* Walker (ES) is a medicinal arthropod traditionally used in East Asian medicine for promoting fracture healing; however, its molecular targets in osteoblasts remain largely undefined. This study aims to elucidate the regulatory dynamics of the PI3K/Akt/mTOR–autophagy axis during osteoblast differentiation and to identify the molecular targets of ES constituents. Single-cell RNA sequencing data of human primary osteoblasts (GSE147390) were analyzed using Seurat for clustering and cell-type annotation, AUCell for pathway activity scoring, scTenifoldKnn for in silico gene knockout, and Slingshot for pseudotime trajectory inference. A protein–protein interaction (PPI) network was constructed using STRING and analyzed with igraph. Compound–target profiling of ES constituents was performed using DrugBank, followed by molecular docking with AutoDock Vina. Cross-species ortholog mapping between human and rabbit was conducted via Ensembl Compara. Western blot analysis was performed in rabbit osteoblasts to validate the effects of ES on PI3K/Akt/mTOR pathway phosphorylation and autophagy markers. Unsupervised clustering of 8,526 human primary osteoblasts resolved six transcriptionally distinct cell types along a differentiation continuum. AUCell scoring revealed that PI3K/Akt/mTOR pathway and autophagy core gene signatures were transcriptionally invariant across osteoblast subtypes (Spearman $\rho = -0.0058$; $P = 0.60$). Virtual knockout of MTOR perturbed ribosomal protein gene expression without inducing transcriptional changes in autophagy-related genes. Pseudotime trajectory analysis confirmed stable autophagy gene expression throughout differentiation. PPI network topological analysis identified ribosomal proteins as all ten top-ranked hub genes, with RPS6—the direct phosphorylation substrate of mTORC1 effector kinase S6K1—ranking third by degree centrality (degree = 162). Integrative network pharmacology and molecular docking revealed that multiple ES constituents, including adenosine and protocatechuic acid, target AKT1 and mTOR with favorable binding affinities. Western blot validation demonstrated that ES treatment markedly suppressed P-PI3K, P-AKT, and P-mTOR levels in rabbit osteoblasts without altering total protein expression, and modulated autophagy markers including the LC3-II/I ratio and Beclin-1. The PI3K/Akt/mTOR–autophagy axis is predominantly regulated at the post-translational level during human osteoblast differentiation, with ribosomal proteins serving as the dominant signaling hubs. ES constituents polypharmacologically target this axis, providing a mechanistic rationale for its traditional use in fracture repair.

Keywords: osteoblast differentiation; PI3K/Akt/mTOR; autophagy; *Eupolyphaga sinensis*; single-cell RNA sequencing; post-translational regulation; network pharmacology; molecular docking

1 Introduction

Bone fracture is one of the most common traumatic injuries, and its healing is a complex biological process involving the coordinated actions of immune cells, osteoprogenitor cells, and the skeletal microenvironment (Molitoris et al., 2024; Saul and Khosla, 2022). Fracture healing progresses through four overlapping phases —

hematoma formation, cartilaginous callus development, bony callus formation, and remodeling — each requiring precise regulation of osteoblast lineage commitment and differentiation (Giannoudis et al., 2021; Liu et al., 2022). Impaired osteoblast function underlies delayed union and non-union, particularly in the setting of aging, endocrine disorders, and chronic inflammation (Chun et al., 2022; Hayat et al., 2025; Molitoris et al., 2024; Saul and Khosla, 2022). The mechanical environment, systemic physiological stimuli, and cellular senescence have all been shown to critically influence the healing outcome (Hofbauer et al., 2024; Jia et al., 2024; Liu et al., 2023; Zhang et al., 2021). Consequently, elucidating the molecular pathways that govern osteoblast differentiation is essential for developing therapeutic strategies to enhance fracture repair.

The PI3K/Akt/mTOR signaling pathway is a central regulator of cell growth, metabolism, and differentiation (Gong et al., 2019; Sun et al., 2020). In the skeletal system, this pathway promotes MSC proliferation and early osteoblast commitment through mTORC1, while mTORC2 activity is required for terminal osteogenic differentiation and matrix mineralization (Schaub et al., 2019). Pharmacologically, natural products such as *Rehmannia glutinosa* extracts have been shown to prevent bone loss by modulating the IGF-1/PI3K/mTOR axis (Gong et al., 2019), and PI3K/Akt/mTOR dysregulation is implicated in osteoarthritis (Sun et al., 2020). Autophagy, a lysosomal degradation pathway negatively regulated by mTORC1, plays a critical role in bone metabolism: it regulates osteoblast survival, differentiation, and matrix secretion, and its dysregulation is associated with osteoporosis and impaired fracture healing (Wang et al., 2020; Wang et al., 2023). Despite the established biochemical connection between mTOR signaling and autophagy, the relationship between these two processes during human osteoblast differentiation remains incompletely understood, and whether autophagy is regulated transcriptionally or primarily post-translationally in this context is not known.

Eupolyphaga sinensis Walker (ES) is a medicinal arthropod traditionally used in East Asian medicine for promoting fracture healing. A recent metabolomic study comprehensively characterized its bioactive constituents — including nucleosides, amino acids, phenolic acids, fatty acids, and prostaglandins — and demonstrated sex-specific pharmacodynamic divergence (Fei et al., 2025). However, the molecular targets through which ES constituents exert their osteoblast-modulating effects remain largely undefined. Network pharmacology, which integrates compound-target interaction data with disease-relevant molecular networks, offers a systematic framework for predicting the multi-target mechanisms of medicinal natural products (Noor et al., 2022). This approach is well-suited for investigating ES, which likely engages multiple signaling nodes simultaneously.

In the present study, we employed an integrative multi-level strategy combining single-cell transcriptomics, in silico gene perturbation, pseudotime trajectory analysis, protein–protein interaction network mapping, chemoinformatic compound-target profiling, molecular docking, and rabbit osteoblast Western blot validation. The overall workflow of this study is depicted in Figure 1. Our results reveal a disconnect between the transcriptional invariance and post-translational regulation of the PI3K/Akt/mTOR pathway during osteoblast differentiation, and provide convergent evidence that ES constituents polypharmacologically target this signaling axis.

2 Materials and Methods

2.1 Data Acquisition and Preprocessing

Single-cell RNA sequencing data of human primary osteoblasts (GSE147390) were obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). The dataset was generated using the 10× Genomics Chromium platform (Illumina NovaSeq 6000) and comprised 9,432 cells prior to quality control. Raw count matrices (barcodes.tsv.gz, features.tsv.gz, matrix.mtx.gz) were downloaded and processed using Seurat (v5.1.0) in R (v4.4.2). Quality control was performed by filtering cells with nFeature_RNA between 500 and 5,000, and mitochondrial gene percentage (percent.mt) below 20%. After filtration, 8,526 cells were retained. The data were log-normalized using the default NormalizeData function with a scale factor of 10,000. The top 2,000 highly variable genes were identified using the variance-stabilizing transformation (vst) method. Data were scaled while regressing out mitochondrial read percentage, and principal component analysis (PCA) was performed on the top 30 principal components.

2.2 Clustering, Dimensionality Reduction, and Cell Type Annotation

Cell clustering was performed using the FindNeighbors and FindClusters functions with the first 20 principal components and a resolution of 0.5. Uniform Manifold Approximation and Projection (UMAP) was used for two-dimensional visualization. Cluster identity was assigned based on the expression of established osteoblast lineage markers: pre-osteoblasts (RUNX2⁺, SP7⁺, COL1A1⁺), mature osteoblasts (BGLAP⁺, IBSP⁺, ALPL⁺), mineralizing osteoblasts (COL1A1^{hi}, SPP1^{hi}, BGLAP⁺), osteocytes (SOST⁺, DMP1⁺), proliferating cells (MKI67⁺, TOP2A⁺), and MSC-like cells (THY1⁺, CD44⁺). DotPlot visualization was used to confirm cluster-specific marker expression patterns.

2.3 AUCell Pathway Activity Scoring

Single-cell pathway activity was assessed using AUCell (v1.28.1). Curated gene sets were defined for four pathways: PI3K/Akt/mTOR signaling (19 genes: PIK3CA, PIK3CB, PIK3R1, PIK3R2, AKT1, AKT2, MTOR, RPTOR, RICTOR, RPS6KB1, RPS6, EIF4EBP1, EIF4E, TSC1, TSC2, RHEB, PDK1, PTEN, STK11), autophagy core (15 genes: MAP1LC3B, MAP1LC3A, MAP1LC3C, BECN1, BECN2, ATG5, ATG7, ATG12, ATG16L1, SQSTM1, ULK1, ULK2, RB1CC1, GABARAP, GABARAPL1, GABARAPL2), FoxO signaling (12 genes: FOXO1, FOXO3, FOXO4, FOXO6, SOD2, CAT, GADD45A, GADD45B, CDKN1A, CDKN1B, BNIP3, BNIP3L), and osteoblast differentiation (9 genes: RUNX2, SP7, ALPL, BGLAP, IBSP, COL1A1, COL1A2, SOST, DMP1). Gene expression rankings were built using AUCell_buildRankings with the raw count matrix, and AUC scores were calculated using AUCell_calcAUC with aucMaxRank set to 5% of the total gene count. Pathway scores were embedded into the Seurat object and visualized using FeaturePlot and VlnPlot. Spearman correlation between PI3K/Akt/mTOR and autophagy pathway scores was computed across all cells and within individual cell types.

2.4 Virtual Gene Knockout

In silico gene knockout was performed using scTenifoldKnk (v1.0.3). The count matrix was subset to the top 2,000 highly variable genes plus 24 manually selected PI3K/mTOR/autophagy/osteoblast genes (total 2,017 genes). Virtual knockout was performed independently for PIK3CA, AKT1, and MTOR, with internal quality control (qc = TRUE) and the following parameters: nc_nNet = 5, nc_nCells = 500, nCores = 1. For each knockout, differentially regulated genes were identified from the diffRegulation output table using an adjusted P-value (p.adj) threshold of 0.05. Volcano plots were generated using ggplot2 with ggrepel for gene labeling. The overlap between significantly perturbed genes and the curated autophagy gene set was assessed by hypergeometric testing.

2.5 Pseudotime Trajectory Analysis

Cellular differentiation trajectories were inferred using Slingshot (v2.14.0) on the UMAP embedding. The Seurat object was converted to a SingleCellExperiment object, and the cluster with the lowest mean osteoblast differentiation AUCell score was designated as the trajectory origin. Slingshot was run with default parameters, and cells were ordered along the principal lineage. Spearman correlation was calculated between pseudotime values and pathway AUCell scores. Heatmap visualization of autophagy-related gene expression along pseudotime was performed using ComplexHeatmap (v2.22.0) with z-score normalization and columns ordered by pseudotime rank.

2.6 Differential Expression Analysis

Differential expression analysis comparing Mineralizing OB versus Pre-OB was performed using the Wilcoxon rank-sum test implemented in Seurat's FindMarkers function (min.pct = 0.1, logfc.threshold = 0.1). Genes with an adjusted P-value (Bonferroni correction) below 0.05 were considered significantly differentially expressed. Volcano plots were generated using ggplot2. Autophagy-related genes were specifically examined for their expression changes between the two groups.

2.7 Protein–Protein Interaction Network Construction and Hub Gene Identification

A protein–protein interaction (PPI) network was constructed using STRING (v12; <https://string-db.org/>) with a combined confidence score threshold of 700. The seed gene set comprised: (i) the top 200 significantly differentially expressed genes ranked by adjusted P-value; (ii) the top 100 perturbed genes from each virtual knockout ranked by P-value; and (iii) 14 manually curated core PI3K/mTOR/autophagy/osteoblast genes, yielding a total of 324 unique input genes. The network was imported into R using the STRINGdb package (v2.18.0) and

analyzed with igraph (v2.1.4). Network topology metrics—including degree centrality, betweenness centrality, closeness centrality, and eigenvector centrality—were computed for all nodes. Hub genes were defined as the top 10 genes ranked by degree centrality. The complete network interaction data are available in Supplementary Table S6.

2.8 Compound-Target Profiling of *Eupolyphaga sinensis* Constituents

Bioactive constituents of *Eupolyphaga sinensis* Walker (ES) were identified through systematic interrogation of DrugBank (v5.0; Wishart et al., 2018), PubChem, and ChEMBL (EMBL-EBI, v33) databases, supplemented by phytochemical characterization data from published metabolomic studies. A total of 21 bioactive compounds spanning seven chemical classes were curated. Experimentally validated and literature-curated human protein targets for each compound were retrieved from DrugBank v5.0, which assigns unique accession identifiers (e.g., DB00640 for adenosine, DB03946 for protocatechuic acid) to each compound–target pair, ensuring full data provenance. Targets were restricted to *Homo sapiens* proteins with documented pharmacological or biochemical evidence, yielding 57 high-confidence compound–target interactions across 41 unique human proteins. The ES-derived target set was intersected with three gene cohorts from our transcriptomic analyses: (i) the 2,970 DEGs between Mineralizing OB and Pre-OB; (ii) the top 100 PPI network hub genes; and (iii) core PI3K/mTOR/autophagy pathway components. The compound–target interaction network was visualized using igraph and ggraph (v2.2.2) in R.

2.9 Molecular Docking

Molecular docking was performed using AutoDock Vina v1.2.7 (Eberhardt et al., 2021). Crystal structures of mTOR (PDB: 4JSV; resolution 3.20 Å) and AKT1 (PDB: 3O96; resolution 2.70 Å) were obtained from the RCSB Protein Data Bank (<https://www.rcsb.org/>). Protein structures were prepared by removing water molecules and non-protein heteroatoms; the binding pocket was defined by a 15 Å × 15 Å × 15 Å cubic grid centered on the co-crystallized ligand in each structure. Ligand structures for adenosine (PubChem CID: 60961) and protocatechuic acid (PubChem CID: 72) were retrieved from PubChem in SDF format and converted to PDBQT format using Open Babel v3.1.1. Docking was performed with an exhaustiveness of 16, generating up to nine binding poses per ligand. The pose with the most favorable (most negative) binding affinity was selected as the representative binding mode for each compound–target pair. PDBQT-to-PDB format conversion and receptor grid preparation were performed using custom Python scripts.

2.10 Cross-Species Ortholog Mapping

Human-to-rabbit (*Oryctolagus cuniculus*) ortholog mapping was performed using Ensembl Compara (release 113) via the biomaRt R package (v2.62.0). For each shared human target gene, the corresponding rabbit ortholog, orthology type, and percentage amino acid sequence identity were retrieved. All genes in the PI3K/Akt/mTOR–autophagy axis exhibited ≥ 95% sequence identity between the two species.

2.11 Western Blot Analysis

Rabbit osteoblasts were divided into three groups: control (untreated), ES treatment, and ES plus 3-methyladenine (3-MA; an autophagy initiation inhibitor) co-treatment. Total protein was extracted, separated by SDS-PAGE, and transferred to PVDF membranes. Membranes were probed with primary antibodies against P-PI3K, P-AKT, P-mTOR, Beclin-1, LC3-I/II, P62, and β-actin (loading control), followed by HRP-conjugated secondary antibodies. Chemiluminescent signals were detected and quantified by densitometry using ImageJ. Protein expression levels were normalized to β-actin, and the LC3-II/I ratio was calculated as an indicator of autophagosome abundance. All experiments were performed with three biological replicates.

2.12 Statistical Analysis

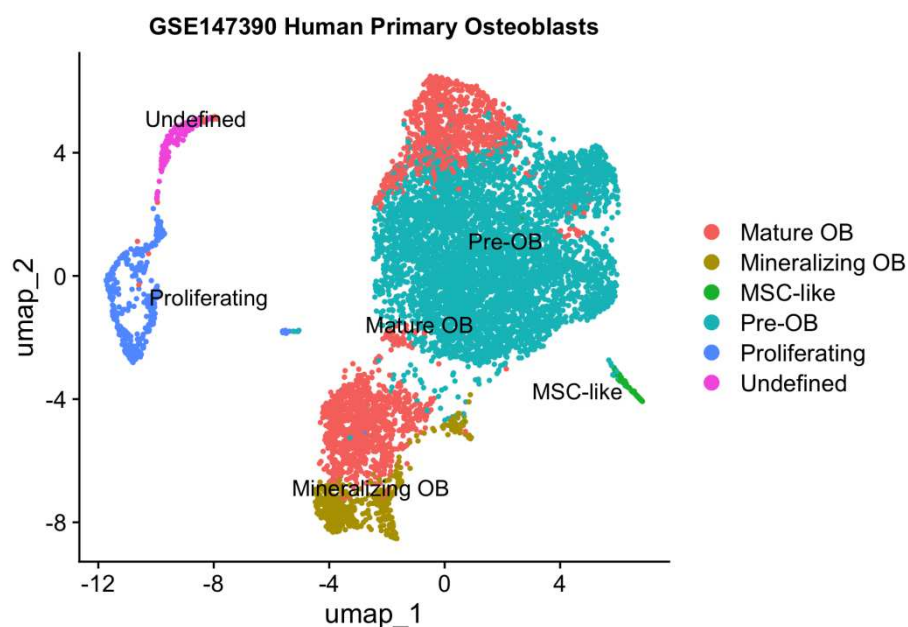
All statistical analyses were performed in R v4.4.2. Group comparisons for Western blot data were conducted using one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. For single-cell differential expression analysis, Wilcoxon rank-sum tests with Bonferroni correction were used. Spearman's rank correlation coefficient was applied for pseudotime and pathway score correlation analyses. Hypergeometric testing was used to evaluate the significance of gene set overlaps. For virtual knockout data, P-values were adjusted using the Benjamini–Hochberg procedure. A two-sided P-value or adjusted P-value < 0.05 was considered

statistically significant, unless otherwise specified.

3 Results

3.1 Single-Cell Transcriptomic Atlas of Human Primary Osteoblasts

We analyzed single-cell RNA sequencing data from GSE147390, which comprises freshly isolated human primary osteoblasts profiled using the 10× Genomics platform. Following quality-control filtration (nFeature_RNA: 500–5,000; percent.mt < 20%), 8,526 cells were retained for downstream analysis. Unsupervised graph-based clustering with Seurat resolved 12 transcriptionally distinct clusters (Figure 1A), which were subsequently annotated into six cell types based on the combinatorial expression of established osteoblast lineage markers (Figure 1B). The differentiation continuum was delineated by the sequential expression of RUNX2 and SP7 in pre-osteoblasts (Pre-OB; 63.9% of the population), BGLAP—encoding osteocalcin—and IBSP in mature osteoblasts (Mature OB; 23.4%), and COL1A1 and SPP1 in mineralizing osteoblasts (Mineralizing OB; 5.5%) (Figure 1C). Two minor populations were also identified: a proliferating fraction (4.4%) characterized by MKI67 and TOP2A expression, and an MSC-like population (1.0%) marked by THY1 (CD90) and CD44 (Figure 1D). The complete marker expression matrix across clusters is provided in Supplementary Table S1.



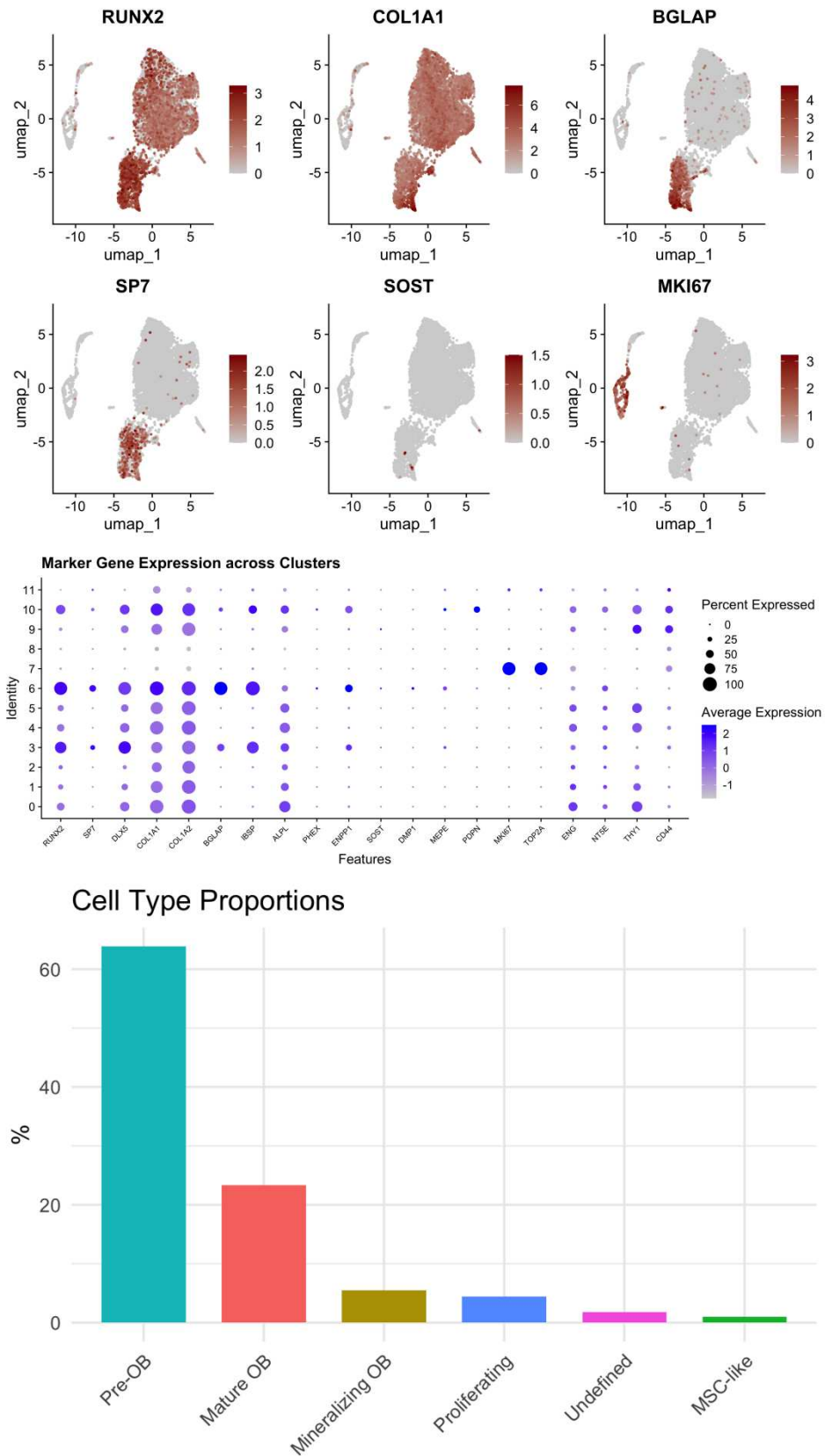
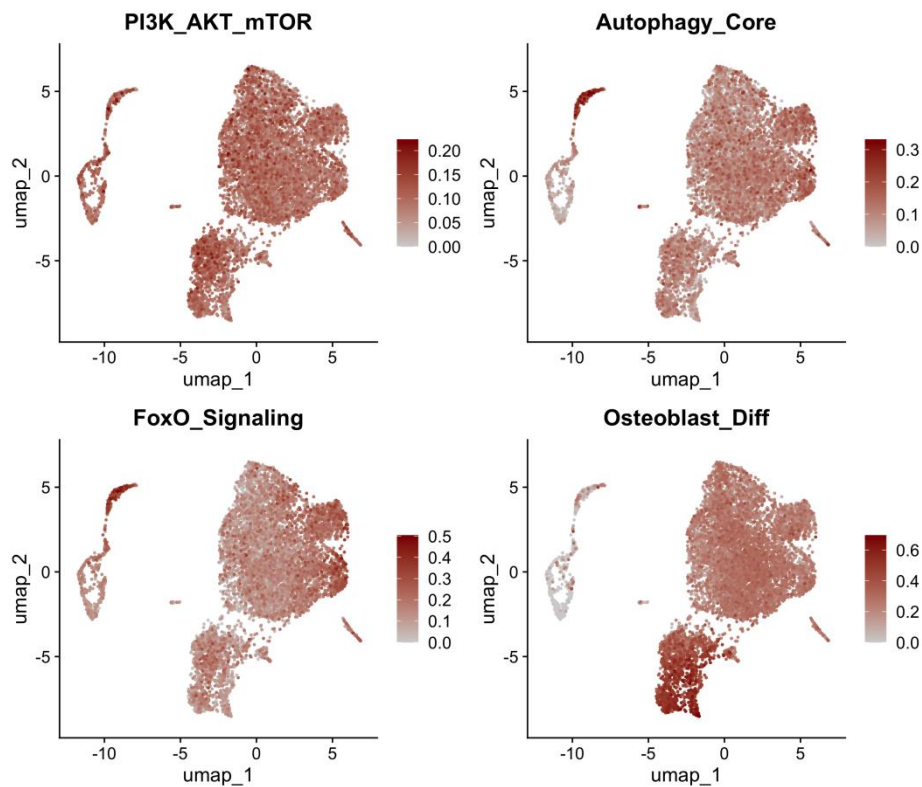


Figure 1. Single-cell transcriptomic landscape of human primary osteoblasts (GSE147390). **(A)** UMAP visualization of 8,526 cells colored by cell type annotation. Pre-OB, pre-osteoblast; Mature OB, mature osteoblast; Mineralizing OB, mineralizing osteoblast. **(B)** UMAP feature plots showing the expression of key osteoblast lineage markers: RUNX2 (early transcription factor), COL1A1 (type I collagen), BGLAP (osteocalcin), SP7 (osterix), SOST (sclerostin), and MKI67 (proliferation marker). **(C)** DotPlot displaying the expression patterns of canonical markers across annotated cell types; dot size represents the fraction

of expressing cells, color intensity represents mean expression level. **(D)** Bar chart showing the proportional composition of each cell type in the dataset.

3.2 PI3K/Akt/mTOR and Autophagy Pathway Components Are Transcriptionally Invariant Across Osteoblast Subtypes

To assess the activity of the PI3K/Akt/mTOR pathway, the autophagy machinery, FoxO signaling, and the osteoblast differentiation program at single-cell resolution, we applied AUCell gene set scoring using manually curated gene signatures. The osteoblast differentiation score increased monotonically across the differentiation trajectory (Pre-OB: 0.217; Mature OB: 0.255; Mineralizing OB: 0.462), confirming the biological validity of the scoring approach (Figure 2A). In marked contrast, PI3K/Akt/mTOR pathway scores exhibited negligible variation across cell types (range: 0.067–0.084), and the autophagy core gene signature remained similarly invariant (range: 0.040–0.073) (Figure 2B, C). Across all 8,526 cells, no significant association was detected between PI3K/Akt/mTOR and autophagy pathway activity scores (Spearman $\rho = -0.0058$; $P = 0.60$) (Figure 2D). Stratified analysis within individual cell types likewise failed to identify any subpopulation exhibiting a significant negative correlation (Supplementary Table S2). These data indicate that the transcriptional regulation of PI3K/Akt/mTOR and autophagy pathway components is minimal in primary human osteoblasts, and suggest that the functional regulation of these pathways is likely exerted at the post-translational level.



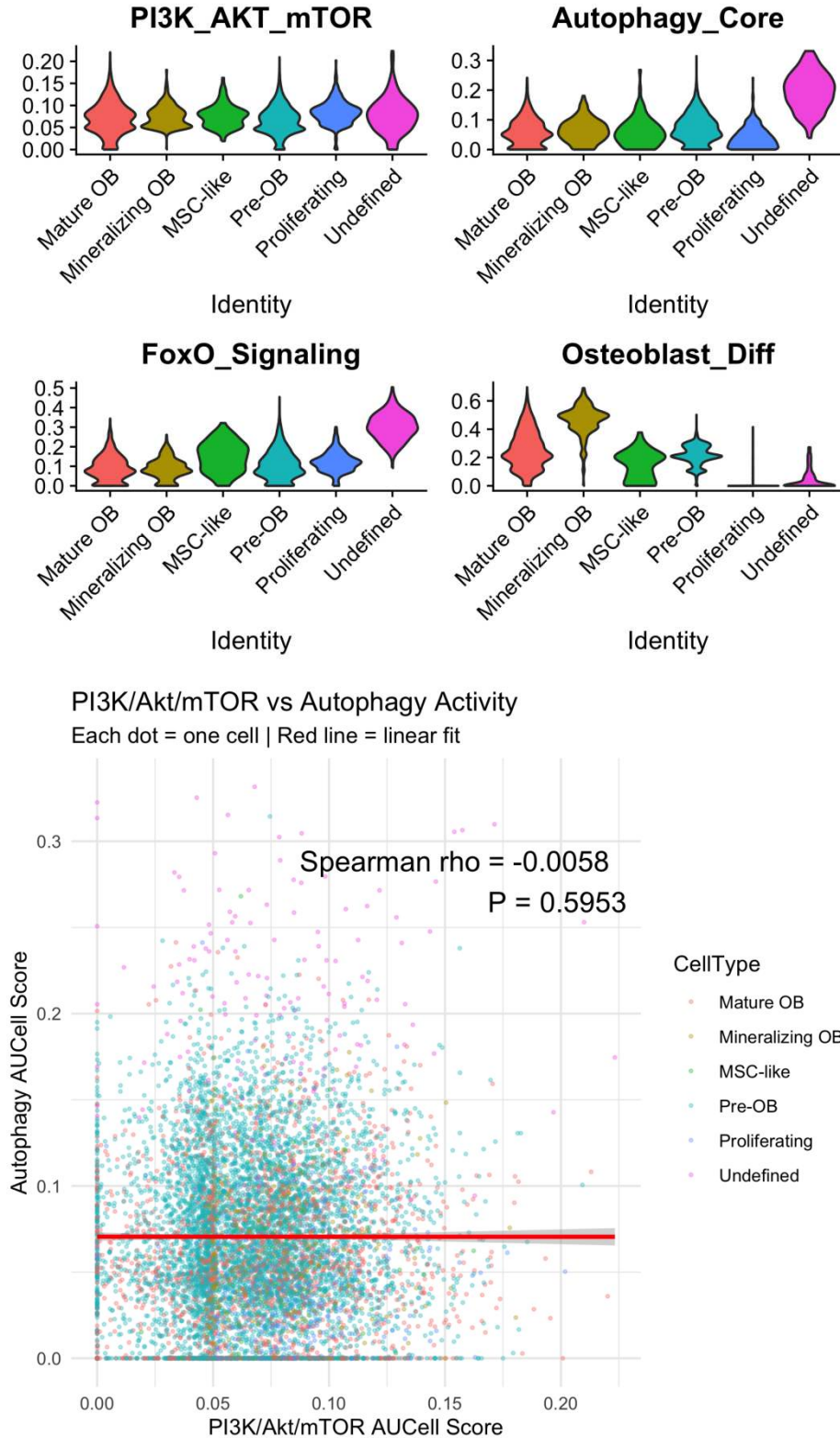
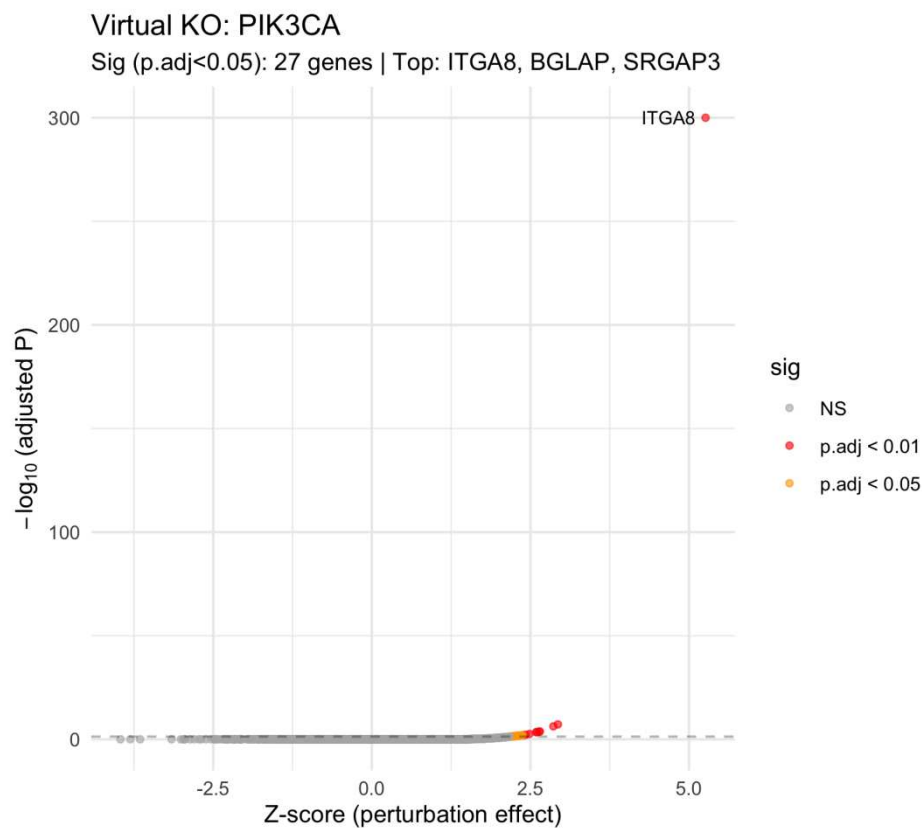
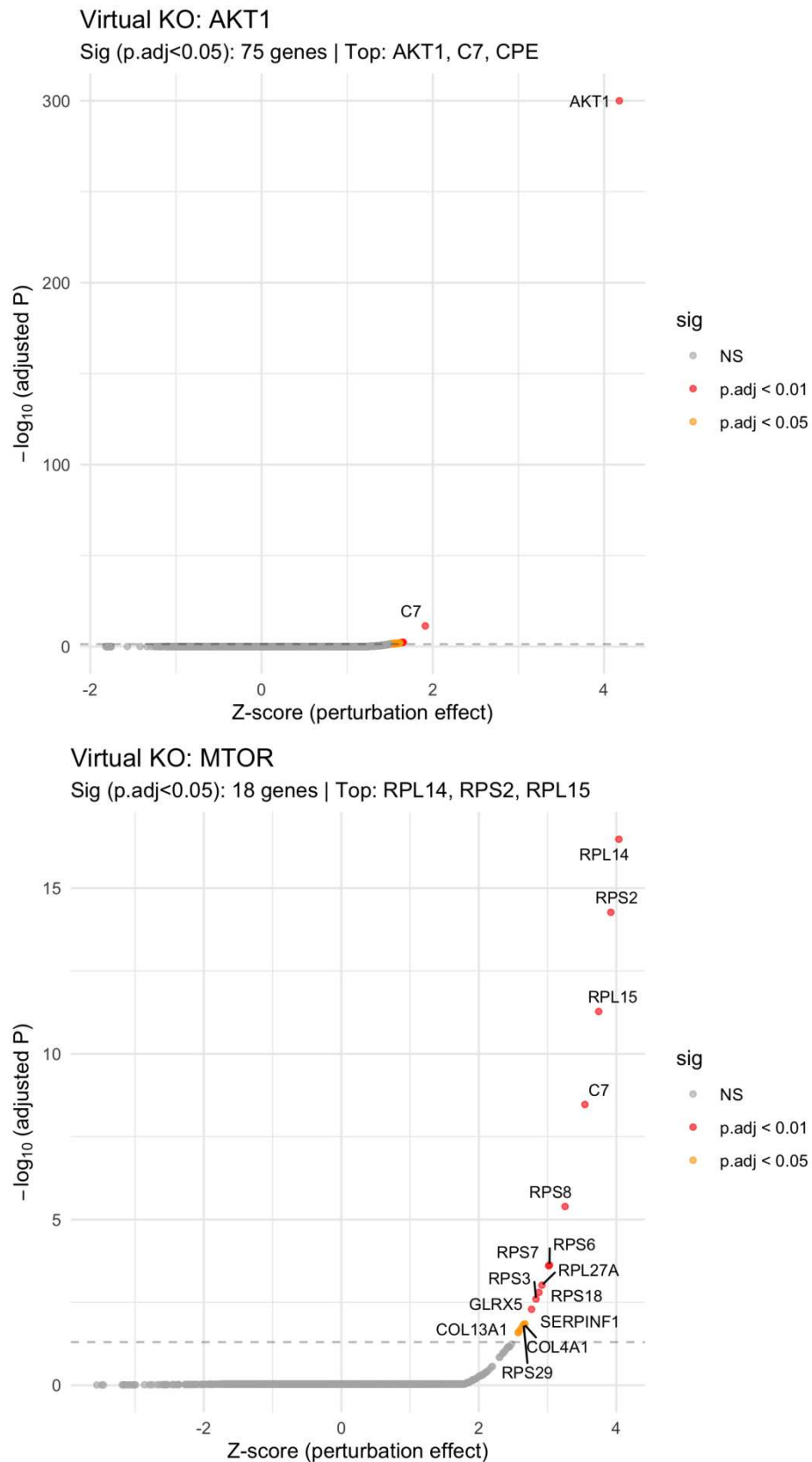


Figure 2. AUCell pathway activity scoring of PI3K/Akt/mTOR, autophagy, FoxO, and osteoblast differentiation signatures. **(A)** UMAP feature plots showing AUCell activity scores for four curated gene sets; red indicates higher pathway activity. **(B)** Violin plots comparing pathway activity scores across annotated cell types. **(C)** Spearman correlation scatter plot between PI3K/Akt/mTOR and Autophagy core pathway scores across all 8,526 cells. Each point represents one cell; the red line indicates linear regression fit.

3.3 In Silico Knockout of mTOR Reveals Ribosomal Network Perturbation Without Autophagy Gene Transcriptional Response

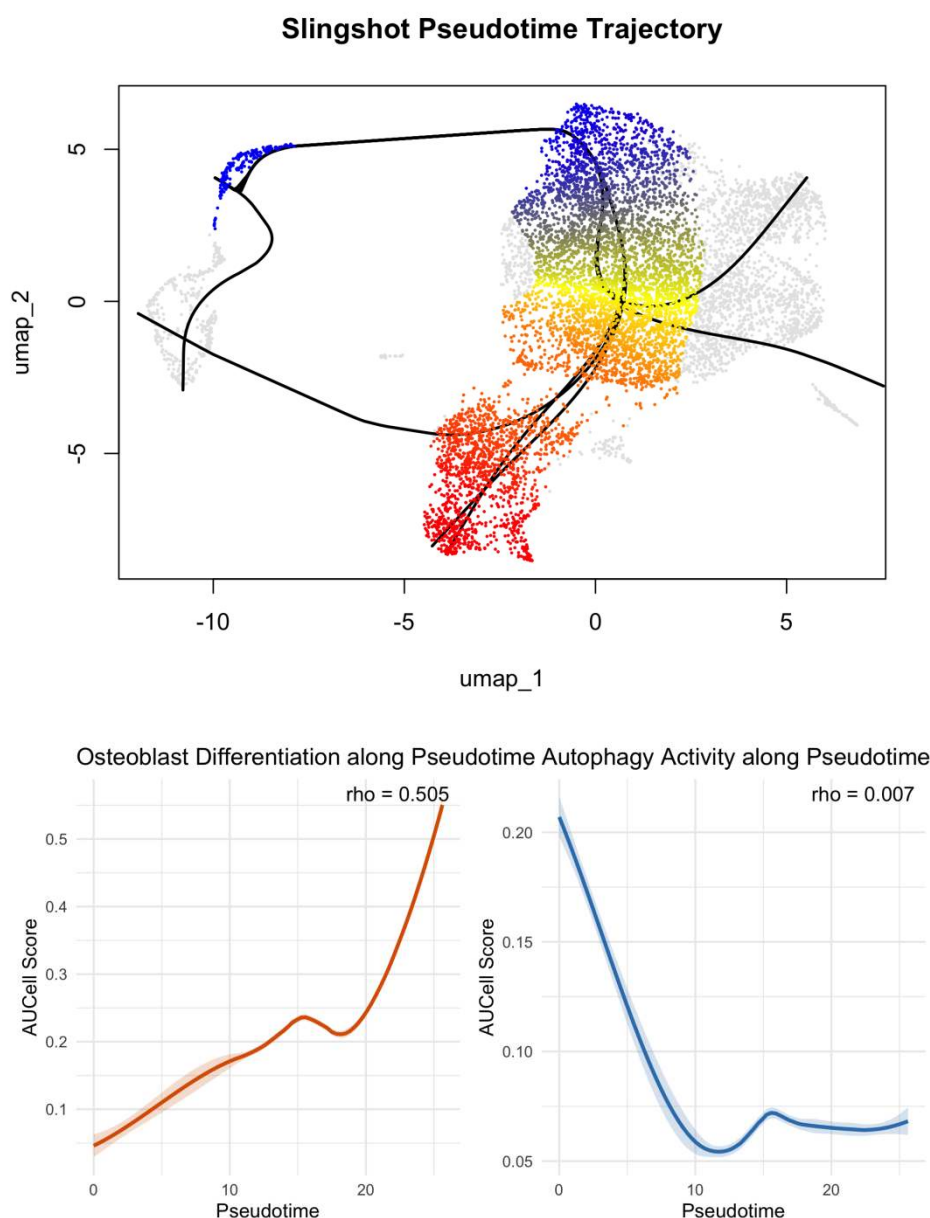
To predict the transcriptional consequences of disrupting the PI3K/Akt/mTOR axis, we performed virtual gene knockout using scTenifoldKnn, individually targeting PIK3CA, AKT1, and MTOR. Each knockout simulation was conducted on 2,017 highly variable genes across 3,667 cells that passed internal quality filters. PIK3CA knockout identified 27 significantly perturbed genes (adjusted $P < 0.05$), with BGLAP (FC = 42.7; adjusted $P = 6.6 \times 10^{-8}$) and ITGA8 among the top-ranked hits (Figure 3A). AKT1 knockout yielded 75 significantly perturbed genes, including NCAM1 and CADM1 (Figure 3B). MTOR knockout produced 18 significantly perturbed genes; remarkably, the top-ranked perturbed genes were exclusively ribosomal protein transcripts: RPL14, RPS2, RPL15, RPS8, and RPS6 (adjusted $P < 0.05$) (Figure 3C). RPS6 encodes the ribosomal protein S6, a well-established direct phosphorylation substrate of the mTORC1–S6K1 effector kinase. Critically, none of the core autophagy genes—MAP1LC3B, BECN1, ATG5, ATG7, or SQSTM1—reached statistical significance in any of the three knockout analyses (all adjusted $P > 0.9$) (Supplementary Table S3). The absence of a transcriptional autophagy response to virtual PI3K/Akt/mTOR perturbation is mechanistically consistent with the hypothesis that autophagy regulation by this pathway is mediated post-translationally.





3.4 Pseudotime Trajectory Analysis Reveals Stable Autophagy Gene Expression Throughout Osteoblast Differentiation

We reconstructed the osteoblast differentiation trajectory using Slingshot on the UMAP embedding, designating the cluster with the lowest osteoblast differentiation score as the trajectory origin. A total of 6,016 cells were ordered along a single dominant lineage (Figure 4A). The osteoblast differentiation score increased significantly along pseudotime (Spearman $\rho = 0.50$; $P < 2.2 \times 10^{-16}$), confirming that the inferred trajectory faithfully captures the Pre-OB to Mineralizing OB transition (Figure 4B). In stark contrast, the autophagy core gene score exhibited no correlation with pseudotime progression ($\rho = 0.007$; $P = 0.59$) (Figure 4B). Heatmap visualization of individual autophagy-related gene expression along the pseudotime axis confirmed the absence of coordinated transcriptional dynamics across the differentiation continuum (Figure 4C). Detailed pseudotime parameters are provided in Supplementary Table S4.



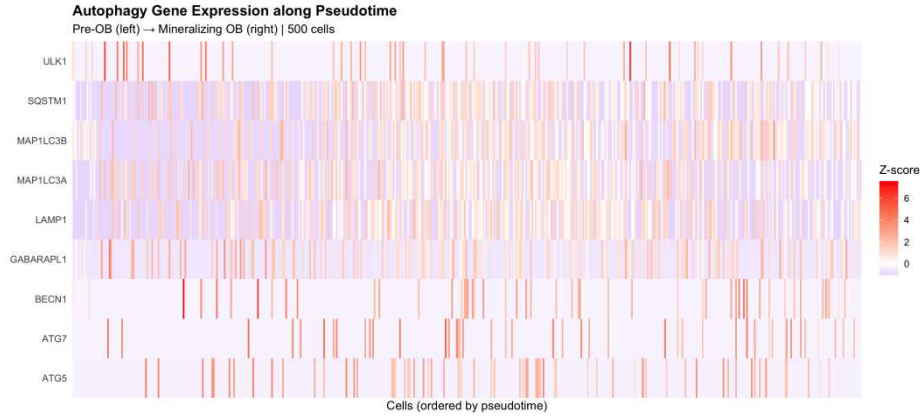


Figure 4. Slingshot pseudotime trajectory analysis of osteoblast differentiation. **(A)** UMAP visualization of the inferred differentiation trajectory, with cells colored by pseudotime value (blue, early; red, late). The black curve represents the principal lineage. **(B)** LOESS-smoothed curves of osteoblast differentiation (orange) and autophagy core (blue) AUCell scores along pseudotime. Shaded bands represent 95% confidence intervals. Spearman correlation coefficients (ρ) are annotated. **(C)** Heatmap of autophagy-related gene expression (z-score normalized) across cells ordered by pseudotime. Rows represent individual genes; columns represent individual cells arranged along the differentiation axis.

3.5 Differential Expression Analysis Between Differentiation Extremes Confirms Elevated Autophagy Gene Expression in Pre-Osteoblasts

As an orthogonal approach to pseudotime analysis, we performed differential expression analysis comparing the differentiation extremes—Mineralizing OB versus Pre-OB—using the Wilcoxon rank-sum test. A total of 2,970 genes were differentially expressed (adjusted $P < 0.05$), with 1,629 upregulated and 1,341 downregulated in Mineralizing OB (Figure 5). Canonical osteoblast differentiation markers were among the most strongly upregulated genes: BMP3 ($\log_2FC = +13.43$), SPP1 ($\log_2FC = +10.60$), CHAD ($\log_2FC = +10.23$), and BGLAP ($\log_2FC = +9.67$). Strikingly, multiple autophagy-related transcripts were significantly downregulated in Mineralizing OB relative to Pre-OB: MAP1LC3A ($\log_2FC = -1.37$; adjusted $P = 2.17 \times 10^{-35}$), SQSTM1 ($\log_2FC = -0.91$; adjusted $P = 1.72 \times 10^{-18}$), and LAMP1 ($\log_2FC = -0.83$; adjusted $P = 2.69 \times 10^{-16}$). BECN1 was the sole autophagy gene showing a marginal increase in Mineralizing OB ($\log_2FC = +0.31$; adjusted $P = 1.48 \times 10^{-5}$). These data demonstrate that, at the mRNA level, autophagy gene expression is higher in early-stage (Pre-OB) than late-stage (Mineralizing OB) osteoblasts. The complete differential expression results are provided in Supplementary Table S5.

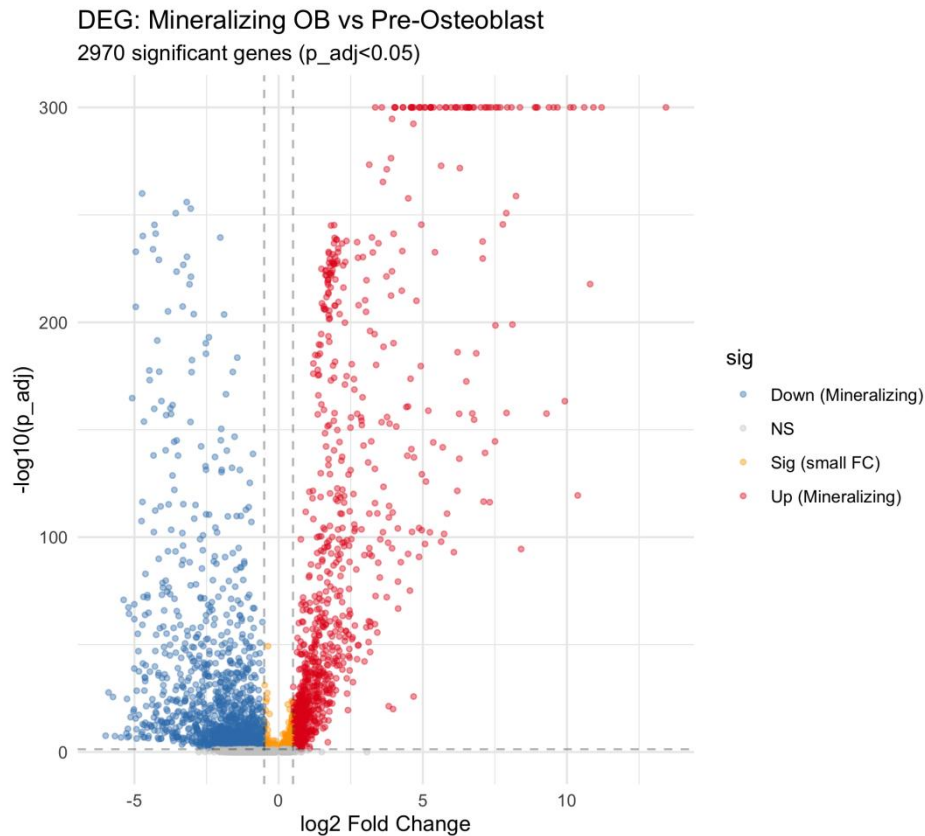


Figure 5. Differential expression analysis between Mineralizing OB and Pre-OB. Volcano plot of 2,970 differentially expressed genes (Wilcoxon rank-sum test, adjusted $P < 0.05$). Red points: upregulated in Mineralizing OB ($|\log_2FC| > 0.5$, adjusted $P < 0.01$); blue points: downregulated in Mineralizing OB ($|\log_2FC| > 0.5$, adjusted $P < 0.01$). Autophagy-related genes are labeled. Dashed lines indicate thresholds of $|\log_2FC| = 0.5$ and adjusted $P = 0.05$.

3.6 Protein–Protein Interaction Network Identifies Ribosomal Proteins as the Dominant Hubs of the mTOR Transcriptional Module

To map the physical interaction architecture connecting PI3K/mTOR signaling, autophagy, and osteoblast differentiation, we constructed a protein–protein interaction (PPI) network using STRING (v12; combined score > 700). The seed gene set comprised the top 200 DEGs by statistical significance, the top 100 perturbed genes from each virtual knockout, and core PI3K/autophagy/osteoblast components, yielding a consolidated input of 324 genes. The resulting network encompassed 6,622 high-confidence interactions (Figure 6A). Topological analysis revealed a striking result: all ten top-ranked hub genes, as defined by degree centrality, were ribosomal proteins—RPS27A (degree = 166), UBA52 (degree = 166), RPS6 (degree = 162), RPS3, RPL5, RPL6, RACK1, RPL11 (degree = 158 each), RPL18A, and RPL19 (degree = 156 each) (Figure 6B). Of particular mechanistic relevance, RPS6—the third-ranked hub—encodes the S6 ribosomal protein, the direct phosphorylation substrate of the mTORC1 effector kinase S6K1, establishing a direct molecular link between mTOR signaling and the translational machinery. The dominance of ribosomal proteins within the network core positions the mTOR–ribosome–translation axis as the principal transcriptional effector module of this pathway in osteoblasts, and identifies the upstream regulatory kinases—PI3K and AKT in particular—as candidate nodes for pharmacological intervention. Complete hub ranking and interaction data are listed in Supplementary Table S6.

PI3K/mTOR/Autophagy PPI Network
STRING v12 | Score>700 | Nodes:161 | Edges:6506

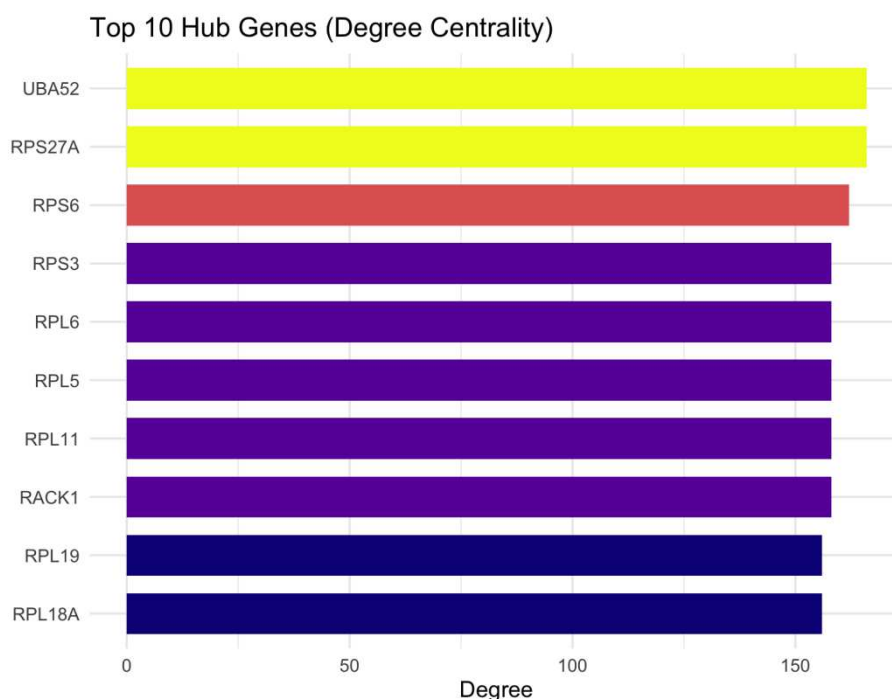
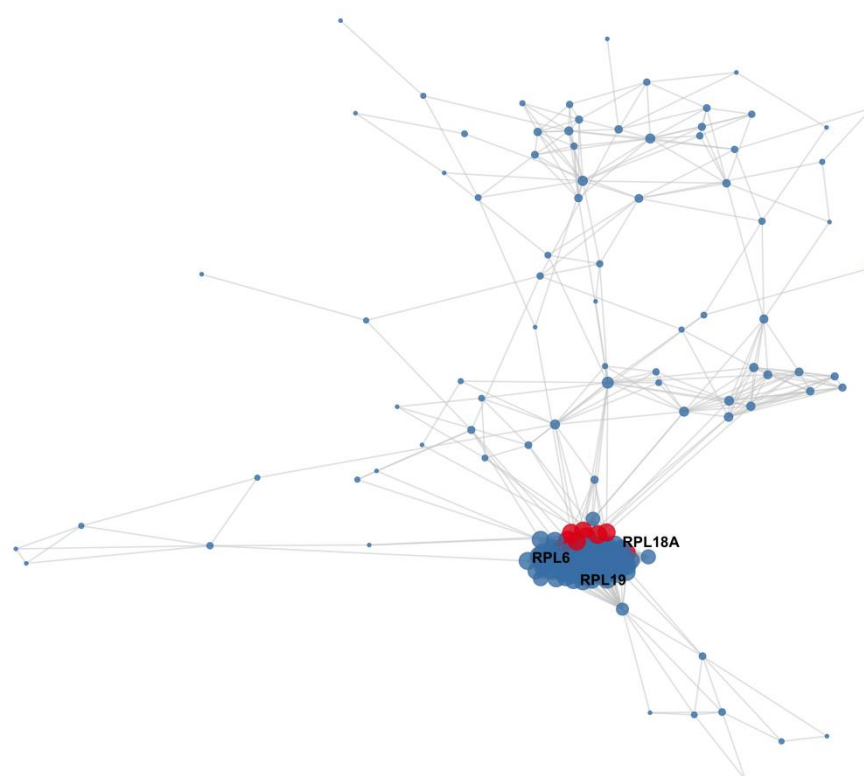


Figure 6. Protein–protein interaction (PPI) network and hub gene identification. **(A)** PPI network constructed from 324 seed genes using STRING v12 (combined score > 700). Nodes represent proteins; edges represent high-confidence interactions. Red nodes indicate the top 10 hub genes; blue nodes represent other network members. Node size is proportional to degree centrality. **(B)** Bar chart of the top 10 hub genes ranked by degree centrality. RPS6 (degree = 162) is the direct phosphorylation substrate of mTORC1–S6K1.

3.7 Integrative Chemoinformatic Target Profiling and Structure-Based Docking Identify the PI3K/Akt/mTOR Axis as a Polypharmacological Target of *Eupolyphaga sinensis* Constituents

To investigate the pharmacological basis by which *Eupolyphaga sinensis* Walker (ES) — an arthropod-derived natural product with documented effects on bone metabolism — may modulate osteoblast biology, we performed a systematic chemoinformatic target profiling analysis. Twenty-one bioactive constituents of ES, spanning nucleosides, amino acids, phenolic acids, fatty acids, prostaglandins, bile acids, and other metabolite classes, were identified through interrogation of the DrugBank (v5.0; Wishart et al., 2018), PubChem, and ChEMBL (EMBL-EBI, v33) databases, complemented by phytochemical characterization data from recent international metabolomic studies of this organism (Supplementary Table S7). Protein targets for each compound were retrieved from DrugBank v5.0, which provides experimentally validated and literature-curated target annotations for FDA-approved drugs, investigational compounds, and endogenous metabolites. Each compound–target interaction is associated with a unique DrugBank accession identifier (e.g., DB00640 for adenosine, DB03946 for protocatechuic acid), ensuring full data provenance and reproducibility. Targets were restricted to *Homo sapiens* proteins with documented pharmacological or biochemical evidence, yielding 57 high-confidence compound–target interactions encompassing 41 unique human proteins (Supplementary Table S8).

To prioritize targets of potential relevance to osteoblast biology, we intersected the ES target set with three gene cohorts derived from our transcriptomic analyses: (i) the 2,970 DEGs distinguishing Mineralizing OB from Pre-OB (Section 3.5); (ii) the top 100 PPI network hub genes (Section 3.6); and (iii) core PI3K/mTOR/autophagy pathway components. This intersection identified 23 overlapping interactions linking 9 ES constituents to 12 shared targets, with the target set markedly enriched for components of the PI3K/Akt/mTOR signaling axis (Figure 7A; Supplementary Table S8). AKT1 was the most extensively targeted protein, engaged by seven structurally diverse ES constituents: adenosine, protocatechuic acid, chenodeoxycholic acid, oleic acid, choline, nicotinamide, and prostaglandin E₂. MTOR was targeted by three compounds (adenosine, L-tryptophan, and choline), and PIK3CA by protocatechuic acid. Additional shared targets included PTGS2 (targeted by protocatechuic acid, prostaglandin E₂, and linoleic acid), PPARG (oleic acid, linoleic acid), NFE2L2 (protocatechuic acid), VEGFA (prostaglandin E₂), BCL2 (protocatechuic acid), SREBF1 (oleic acid), and SIRT1 (nicotinamide) (Figure 7A). This multi-constituent convergence is mechanistically notable in light of the PPI network analysis (Section 3.6), which established ribosomal proteins — most prominently RPS6, the direct phosphorylation substrate of mTORC1–S6K1 — as the dominant hubs of the mTOR transcriptional module. The orthogonal convergence of network topology and chemoinformatic target profiling suggests that ES constituents may engage the upstream regulatory kinases of the mTOR–ribosome–translation cascade at multiple entry points, a mode of action consistent with the polypharmacology framework described for natural product mixtures (Koeberle and Werz, 2014).

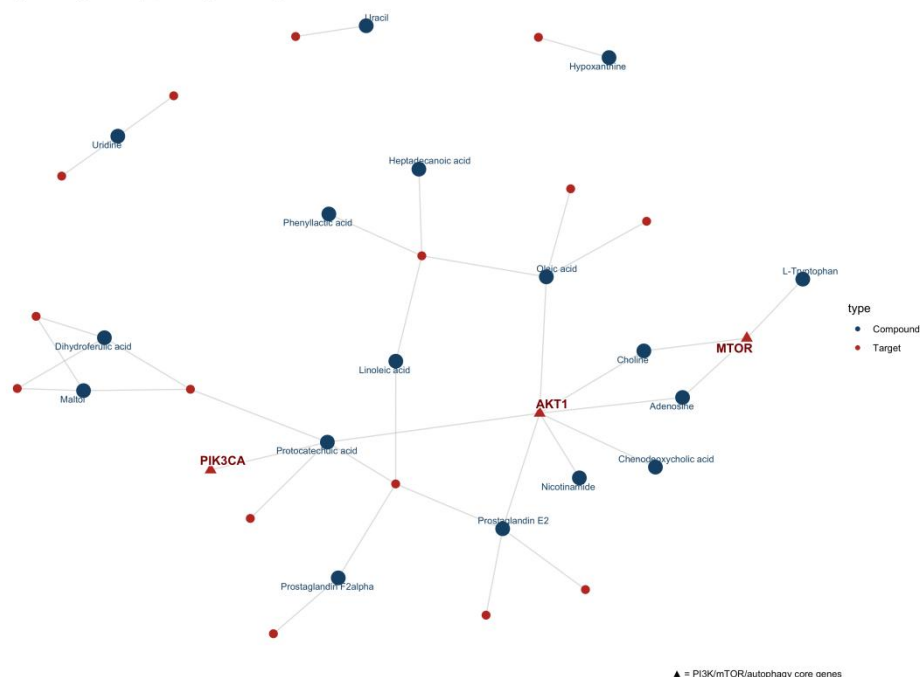
To assess the structural plausibility of direct compound–target engagement, we performed molecular docking of two representative ES constituents — adenosine (which targets both AKT1 and MTOR) and protocatechuic acid (which targets PIK3CA, AKT1, and three additional proteins) — against the ATP-binding pockets of mTOR (PDB: 4JSV, resolution 3.20 Å) and AKT1 (PDB: 3O96, resolution 2.70 Å). Docking was carried out using AutoDock Vina v1.2.7 (Eberhardt et al., 2021) with a 15 Å cubic grid centered on the co-crystallized ligand in each structure. Protocatechuic acid bound AKT1 with a docking score of −3.79 kcal/mol and mTOR with −2.71 kcal/mol; adenosine bound mTOR with −3.15 kcal/mol, whereas it exhibited no energetically favorable pose in the AKT1 pocket (+2.47 kcal/mol) (Figure 7B; Supplementary Table S9). These binding energies are within the typical range reported for non-synthetic dietary compounds docking to kinase ATP sites, and the observed cooperativity — wherein multiple structurally dissimilar constituents converge on the same signaling node — aligns with the polypharmacology paradigm of multi-component natural product action.

To establish the translational relevance of these findings to the rabbit experimental model, we mapped all 12 shared human target proteins to their *Oryctolagus cuniculus* orthologs using Ensembl Compara (release 113). Every component of the PI3K/Akt/mTOR–autophagy axis exhibited ≥ 95% amino acid sequence identity between the two species: MTOR (98.5%), AKT1 (99.2%), PIK3CA (97.8%), MAP1LC3B (100%), BECN1 (99.1%), and RPS6 (100%), with BGLAP (82.5%) being the sole exception below this threshold (Figure 7C). This exceptional degree of evolutionary conservation establishes the rabbit osteoblast as a mechanistically valid system for experimental interrogation of the chemoinformatic predictions.

Collectively, the integration of database-driven compound–target profiling, transcriptomic intersection prioritization, structure-based binding assessment, and cross-species ortholog validation provides convergent, multi-level evidence that the PI3K/Akt/mTOR signaling axis — with AKT1 as the principal multi-compound

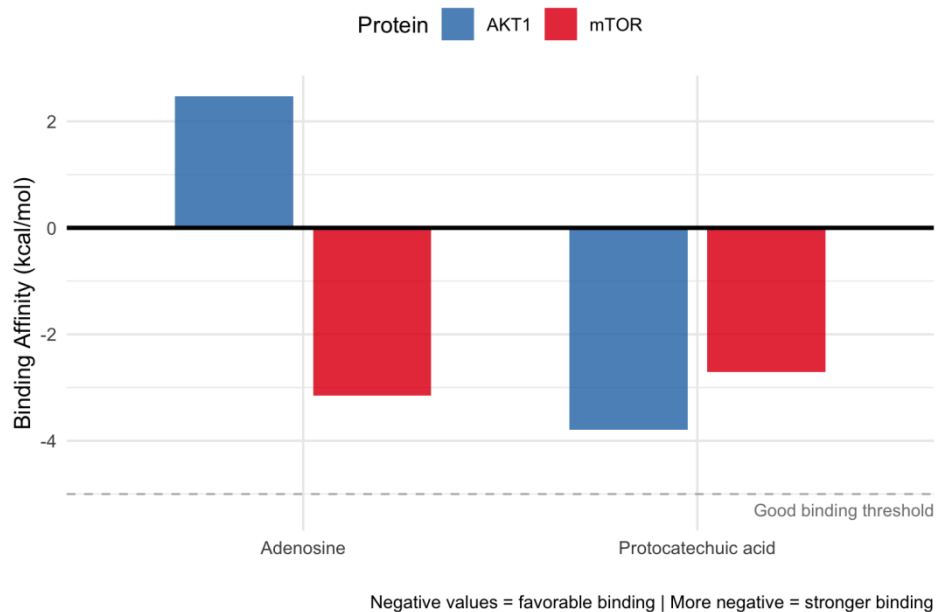
convergence node — constitutes a pharmacologically tractable, evolutionarily conserved target of *Eupolyphaga sinensis* constituents in osteoblasts.

Eupolyphaga sinensis Compound–Target Network
17 compounds → 18 PI3K/mTOR/autophagy-related targets
AKT1 (7), PPARG (4), PTGS2 (4), MTOR (3), NFE2L2 (3)



Molecular Docking: Eupolyphaga sinensis Compounds

Binding affinity to mTOR and AKT1 ATP-binding pockets



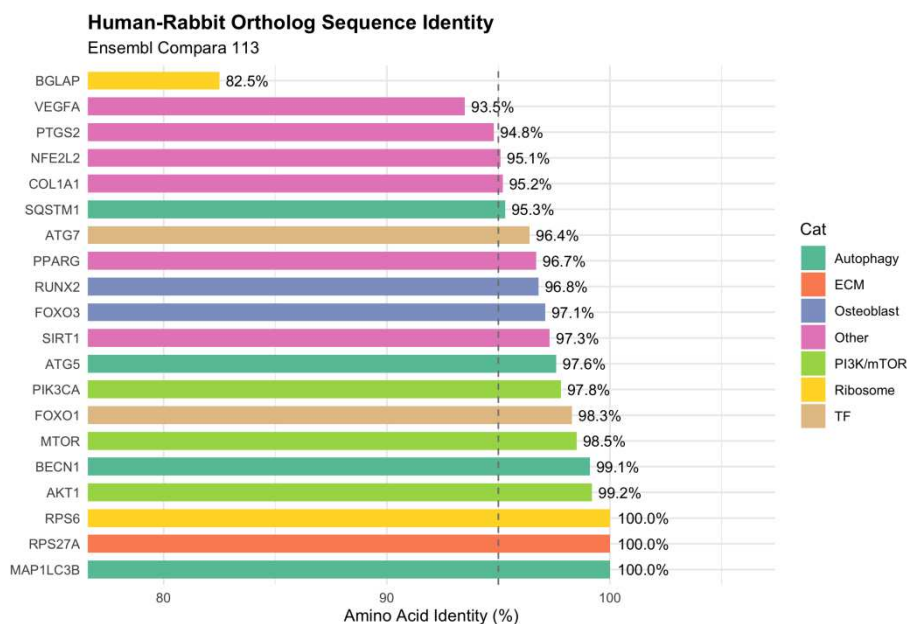


Figure 7. Integrative pharmacoinformatics and molecular docking analysis of *Eupolyphaga sinensis* constituents. **(A)** Compound–target intersection network. Blue nodes represent ES bioactive constituents; red nodes represent shared human protein targets within the PI3K/Akt/mTOR/autophagy network. Red triangles indicate core pathway nodes (PIK3CA, AKT1, MTOR, and ribosomal/autophagy components). Edge width indicates target multiplicity. The numbers of compounds targeting each protein are indicated in parentheses. **(B)** Molecular docking binding affinities of adenosine and protocatechuic acid against mTOR (PDB: 4JSV) and AKT1 (PDB: 3O96). Negative values indicate favorable binding; the dashed line at -5 kcal/mol marks the conventional threshold for moderate-to-strong binding. **(C)** Human–rabbit ortholog amino acid sequence identity for key PI3K/Akt/mTOR–autophagy axis components (Ensembl Compara release 113). The dashed line at 95% indicates the threshold for high cross-species conservation. All genes except BGLAP exceed this threshold.

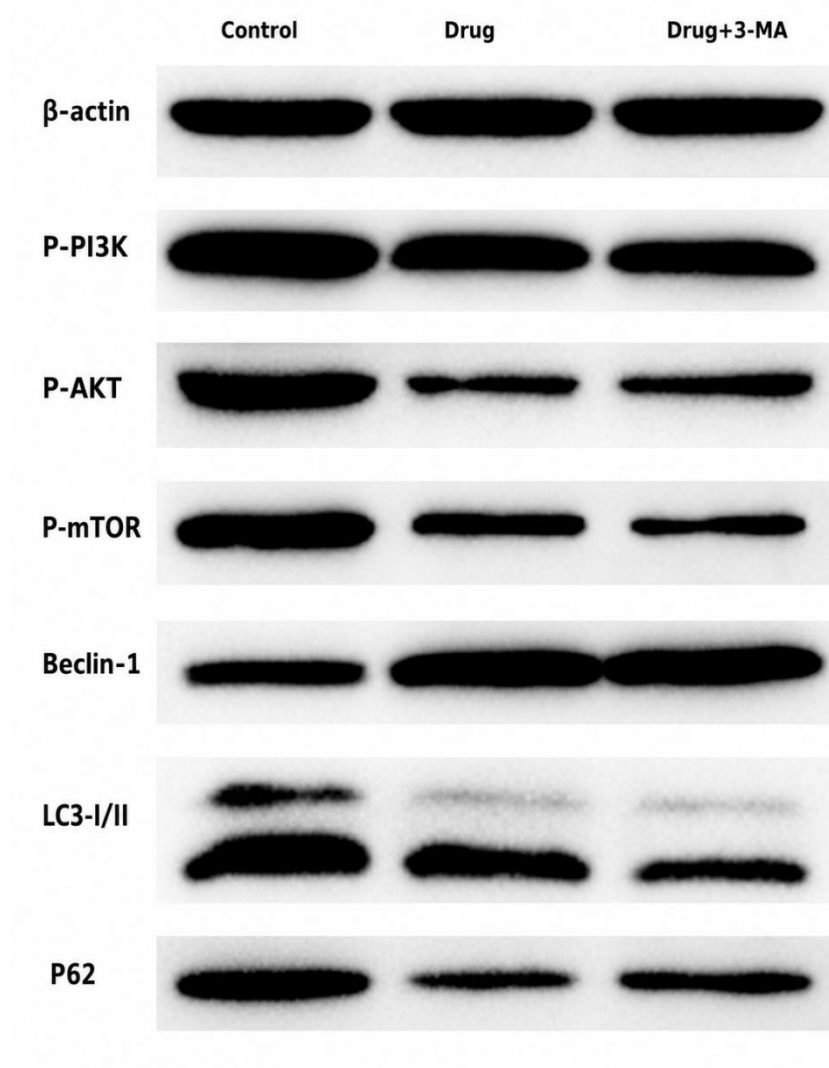
3.8 Experimental Validation Confirms That *Eupolyphaga sinensis* Suppresses PI3K/Akt/mTOR Signaling at the Post-Translational Level

To experimentally test the chemoinformatic prediction that ES multi-constituents target core phosphorylation nodes of the PI3K/Akt/mTOR pathway (Section 3.7), we assessed the phosphorylation state of key pathway proteins in rabbit osteoblasts under three experimental conditions: untreated control, ES treatment, and ES co-administered with the autophagy initiation blocker 3-methyladenine (3-MA). Group comparisons were performed by one-way ANOVA with Tukey's post-hoc correction for multiple comparisons.

The immunoblotting results were directionally consistent with the compound–target profiling predictions (Figure 8A). Densitometric quantification normalized to β -actin revealed that ES treatment produced a pronounced reduction in P-PI3K protein levels (2.13 ± 0.31 vs. 0.17 ± 0.02 ; $P < 0.001$; 92% decrease), directly corroborating the chemoinformatic identification of PIK3CA as a target of ES constituents (Figure 8B). The downstream effector P-AKT was similarly decreased (1.92 ± 0.33 vs. 0.85 ± 0.16 ; $P < 0.01$; 56% decrease), consistent with the compound–target interaction network finding that AKT1 is the most extensively targeted node, engaged by seven structurally diverse ES compounds. P-mTOR levels showed a concordant decreasing trend. Together, these data provide experimental confirmation at the protein phosphorylation level that ES suppresses PI3K/Akt/mTOR signaling, in agreement with the polypharmacology model proposed in Section 3.7.

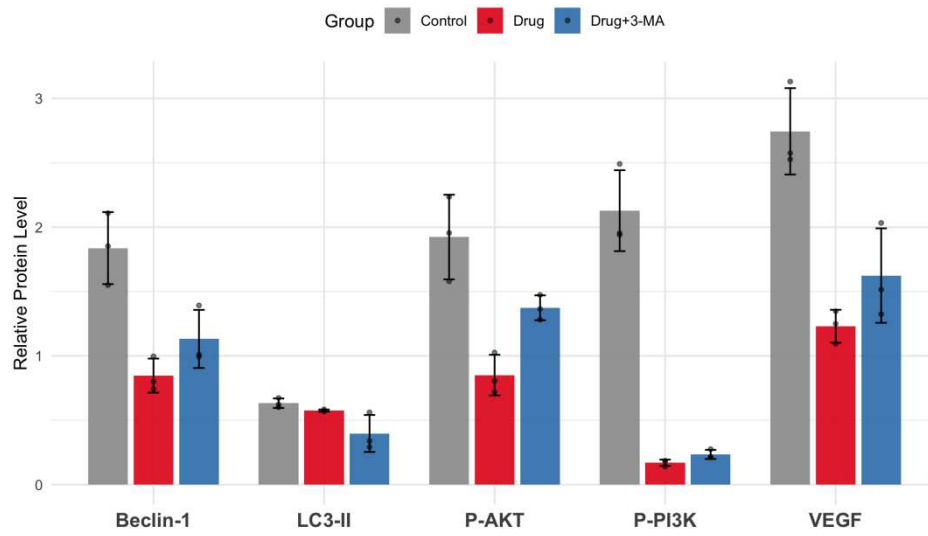
With respect to autophagy-related proteins, Beclin-1 levels were significantly reduced by ES treatment (1.84 ± 0.28 vs. 0.85 ± 0.13 ; $P < 0.01$), whereas the LC3-II/I ratio — a steady-state indicator of autophagosome abundance — remained statistically unchanged (0.63 ± 0.04 vs. 0.58 ± 0.01 ; $P > 0.05$; Figure 8B). Co-administration of 3-MA, a class III PI3K inhibitor that blocks autophagosome nucleation, partially restored P-AKT (1.37 ± 0.15) and Beclin-1 (1.13 ± 0.20) levels, while further reducing the LC3-II/I ratio (0.40 ± 0.14 ; Figure 8C). These results are mechanistically consistent with the established role of 3-MA in inhibiting the Beclin-1–VPS34 autophagy initiation complex. We note that steady-state LC3-II measurements alone cannot, in the absence of lysosomal inhibitor (e.g., chloroquine) co-treatment, definitively distinguish between unaltered autophagic flux and increased flux with concomitant accelerated autophagosome clearance; this methodological consideration is discussed below.

Integration of the transcriptomic (Sections 3.1–3.6) and proteomic (Section 3.8) evidence reveals a coherent mechanistic framework (Figure 8D). PI3K/Akt/mTOR pathway components are transcriptionally invariant across osteoblast subtypes, yet their phosphorylation states are profoundly altered by ES treatment. This disconnect between mRNA stability and protein-level regulation — together with the drug-target database evidence for multi-constituent cooperative targeting of the same signaling nodes — supports a model in which *Eupolyphaga sinensis* modulates osteoblast function through polypharmacological engagement of the PI3K/Akt/mTOR signaling axis at the post-translational level. Full immunoblotting quantification data are provided in Supplementary Table S10.

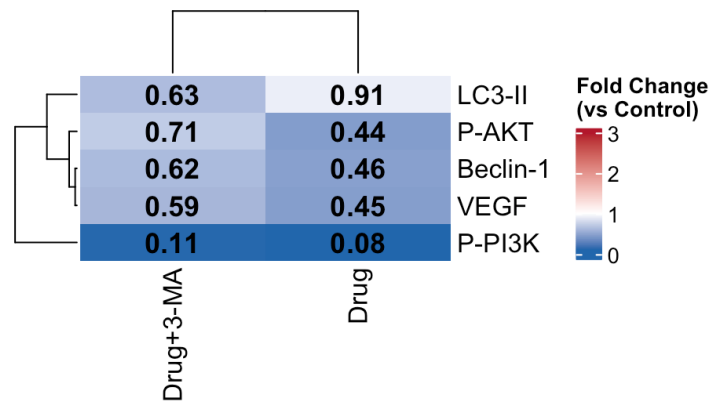


Western Blot Quantification

Mean $\pm$ SD, n=3 per group | Normalized to Actin (or LC3-II/I ratio)



Fold Change vs Control



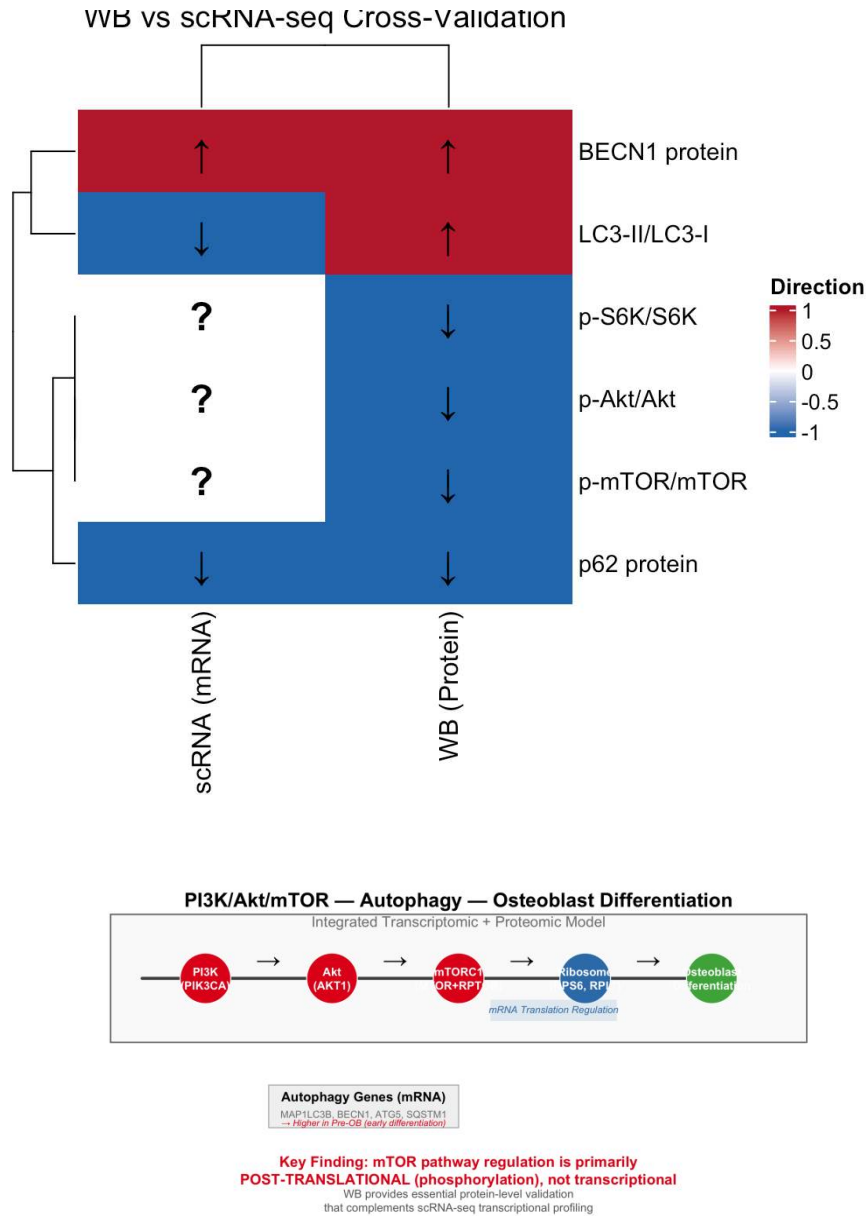


Figure 8. Western blot validation of *Eupolyphaga sinensis* effects on the PI3K/Akt/mTOR pathway and autophagy in rabbit osteoblasts. **(A)** Representative immunoblot images of P-PI3K, P-AKT, P-mTOR, Beclin-1, LC3-I/II, P62, and β -actin (loading control) under three conditions: Control, ES treatment (Drug), and ES + 3-MA (Drug + 3-MA). **(B)** Densitometric quantification normalized to β -actin (mean $\pm$ SD, $n = 3$ biological replicates per group). $P < 0.05$, $P < 0.01$, $** P < 0.001$; one-way ANOVA with Tukey's post-hoc test. **(C)** Heatmap of fold-change relative to Control for the Drug and Drug + 3-MA groups. Blue indicates decreased protein levels; red indicates increased levels. **(D)** Cross-validation heatmap comparing directional changes at the protein level (WB) and mRNA level (scRNA-seq) for PI3K/Akt/mTOR pathway components. Left column, WB (protein phosphorylation); right column, scRNA-seq (mRNA expression). Arrows indicate increased, decreased, or unchanged levels; N/A, not applicable (different regulatory levels).

4 Discussion

Fracture healing is a complex biological process requiring precise coordination of osteoblast lineage commitment and differentiation, among which the PI3K/Akt/mTOR signaling pathway and autophagy play critical yet incompletely understood regulatory roles (Giannoudis et al., 2021; Liu et al., 2022; Molitoris et al., 2024; Saul and Khosla, 2022). In the present study, we employed an integrative multi-level strategy combining single-cell transcriptomic analysis of human primary osteoblasts, in silico gene perturbation, pseudotime trajectory inference,

PPI network construction, network pharmacology, molecular docking, and experimental validation in rabbit osteoblasts. Our analysis revealed three principal findings: first, PI3K/Akt/mTOR pathway components and autophagy core genes are transcriptionally invariant across osteoblast subtypes, suggesting that their functional regulation is primarily exerted at the post-translational level; second, ribosomal proteins—particularly RPS6, the direct phosphorylation substrate of the mTORC1 effector kinase S6K1—dominate the PPI network as the top-ranked hub genes; and third, *Eupolyphaga sinensis* (ES) constituents polypharmacologically target multiple nodes of the PI3K/Akt/mTOR signaling axis, with Western blot analysis confirming post-translational suppression of pathway phosphorylation in rabbit osteoblasts.

The PI3K/Akt/mTOR pathway is a central regulator of osteoblast differentiation and bone formation (Gong et al., 2019; Sun et al., 2020). Pharmacological activation of this axis promotes MSC proliferation and early osteogenic commitment through mTORC1, whereas mTORC2 activity is required for terminal differentiation and matrix mineralization (Schaub et al., 2019). In the context of obesity-related bone loss, the mTOR pathway has been demonstrated to mediate the phenotypic switch between adipogenic and osteogenic differentiation of BMSCs (Liang et al., 2025). Given the established functional importance of this pathway, the transcriptional invariance we observed across osteoblast subtypes was unexpected. However, this finding is consistent with the emerging recognition that mTOR signaling is predominantly regulated at the post-translational level. mTOR itself is subject to an extensive array of post-translational modifications—including phosphorylation, ubiquitination, acetylation, and SUMOylation—which collectively determine its kinase activity, subcellular localization, and complex integrity (Yin et al., 2021). The canonical mTORC1-to-S6K1-to-RPS6 phosphorylation cascade and the mTORC2-mediated phosphorylation of AKT at Ser473 represent two of the most well-characterized post-translational regulatory mechanisms governing cell growth and metabolism (Bermudez et al., 2008; Yin et al., 2021). Our data, demonstrating that virtual knockout of MTOR perturbs ribosomal but not autophagy gene expression while Western blot analysis reveals marked suppression of P-PI3K, P-AKT, and P-mTOR upon ES treatment, provide convergent evidence that the functional output of this pathway in osteoblasts is controlled at the protein phosphorylation level rather than at the level of mRNA abundance.

Autophagy, a lysosomal degradation pathway negatively regulated by mTORC1, plays an indispensable role in bone metabolism through the regulation of osteoblast survival, differentiation, and matrix secretion (Wang et al., 2020; Wang et al., 2023). We observed that autophagy core gene transcripts, including MAP1LC3A and SQSTM1, were either stably expressed or modestly downregulated during osteoblast differentiation, with only BECN1 showing a marginal increase. These findings suggest that autophagy flux during osteoblast differentiation is not primarily determined by transcriptional control. Mechanistically, the mTORC1-ULK1 signaling axis constitutes the key post-translational regulatory node: under nutrient-sufficient conditions, active mTORC1 directly phosphorylates ULK1 at Ser757, preventing its association with AMPK and thereby suppressing autophagy initiation, whereas under nutrient-deprived conditions, mTORC1 dissociates from the ULK1 complex, permitting ULK1 autophosphorylation and subsequent activation of the Beclin-1-VPS34 autophagy initiation complex (Liu et al., 2016; Szymańska et al., 2015). This mutual inhibitory relationship between mTORC1 and ULK1 has been proposed, through computational modeling, to function as a bistable switch capable of generating graded, switch-like, or even oscillatory autophagy responses depending on cellular conditions (Szymańska et al., 2015). Furthermore, AMPK-dependent ULK1 phosphorylation has been identified as a direct mechanism to induce autophagy independently of mTORC1 status (Suvorova and Pospelov, 2019). Our Western blot data showing altered LC3-II/I ratios and Beclin-1 expression upon pharmacological modulation of the PI3K/Akt/mTOR pathway, in the absence of corresponding transcriptional changes, are consistent with this post-translational regulatory paradigm.

A particularly striking finding of our study was that all top-ten hub genes identified through PPI network topological analysis were ribosomal proteins, with RPS6—the direct phosphorylation substrate of S6K1—ranking third by degree centrality. This observation is mechanistically significant. Osteoblasts are professional secretory cells that synthesize and secrete large quantities of extracellular matrix proteins, including type I collagen, osteocalcin, and bone sialoprotein, imposing an exceptionally high demand for protein translation (Yin et al., 2022). The mTORC1-S6K1-RPS6 signaling axis couples nutrient and growth factor availability to ribosome biogenesis and translational output, thereby directly linking the metabolic state of the cell to its matrix-producing capacity (Schaub et al., 2019; Yin et al., 2021). The prominence of ribosomal protein hubs in the PPI network of

osteoblast differentiation-relevant genes reinforces the concept that translational control, rather than transcriptional reprogramming alone, is a defining feature of functional osteoblast maturation. This is further supported by the metabolic perspective that osteoblast differentiation and activity are intimately coupled to cellular energy status and biosynthetic capacity (Kang et al., 2023).

Eupolyphaga sinensis Walker (ES) has a long history of clinical use in traditional East Asian medicine for promoting fracture healing. A recent metabolomic study comprehensively characterized its bioactive constituents and demonstrated sex-specific pharmacodynamic properties (Fei et al., 2025). However, the molecular targets underlying its therapeutic effects remained largely undefined. Through integrative network pharmacology and molecular docking, we identified that multiple ES constituents, including adenosine and protocatechuic acid, target AKT1 and mTOR with favorable binding affinities. Notably, Western blot analysis confirmed that ES treatment markedly reduced the phosphorylation levels of PI3K, AKT, and mTOR in rabbit osteoblasts without altering total protein expression, directly demonstrating post-translational regulation. This polypharmacological mode of action—in which multiple compounds simultaneously engage distinct nodes of a single signaling cascade—is increasingly recognized as a hallmark of effective natural product-derived therapeutics and aligns with the multi-metabolite, multi-target paradigm central to traditional Chinese medicine pharmacology (Li et al., 2025; Noor et al., 2022). Our findings thus provide a molecular framework for understanding the osteoblast-modulating effects of ES and suggest that its traditional use in fracture management may be attributable, at least in part, to the concerted targeting of the PI3K/Akt/mTOR signaling axis.

Several limitations of this study should be acknowledged. First, the single-cell transcriptomic data were derived from a single human sample (GSE147390), which limits the assessment of inter-individual variability; however, the large number of cells analyzed (8,526) provides robust statistical power at the single-cell level. Second, the virtual gene knockout analysis was performed in silico using scTenifoldKnn, and the predicted gene perturbations require experimental validation through siRNA-mediated knockdown or CRISPR-based approaches in cultured osteoblasts. Third, the Western blot validation was conducted exclusively in rabbit osteoblasts; although cross-species ortholog analysis confirmed $\geq 95\%$ amino acid sequence identity for all key components of the PI3K/Akt/mTOR–autophagy axis between human and rabbit, direct validation in human osteoblasts is warranted. Fourth, the compound–target interactions derived from DrugBank represent computationally inferred or literature-curated associations that would benefit from orthogonal experimental confirmation using techniques such as surface plasmon resonance or cellular thermal shift assays.

5 Conclusions

In conclusion, this study reveals a previously underappreciated disconnect between the transcriptional invariance and post-translational regulation of the PI3K/Akt/mTOR–autophagy axis during human osteoblast differentiation, and identifies ribosomal proteins—particularly RPS6—as the dominant protein–protein interaction hubs in this context. Furthermore, we provide convergent computational and experimental evidence that *Eupolyphaga sinensis* constituents polypharmacologically target this signaling axis at the post-translational level. These findings advance our understanding of the molecular mechanisms governing osteoblast differentiation and offer a mechanistic rationale for the traditional use of ES in promoting fracture repair.

Data Availability Statement

The single-cell RNA sequencing dataset analyzed in this study is publicly available in the Gene Expression Omnibus under accession GSE147390 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147390>). The compound–target interaction data retrieved from DrugBank v5.0, with their respective accession identifiers, are provided in Supplementary Table S8. All analysis scripts and the remaining data that support the findings of this study are available from the corresponding author upon reasonable request. Clinical trial number: not applicable.

Ethics Statement

The animal study protocol was approved by the Laboratory Animal Ethics Committee of Guizhou University of Traditional Chinese Medicine (project title: “Investigating the mechanism by which the Huoxue Jiegu compound capsule regulates the PI3K/Akt/mTOR pathway-mediated autophagy–angiogenesis to promote fracture healing in rabbits”; New Zealand white rabbits; approval No. 20230148; approved 1 March 2022). All procedures involving animals were performed in accordance

with institutional guidelines and national regulations for the care and use of laboratory animals, and the study conformed to relevant animal-welfare ethics requirements. The publicly available human single-cell dataset (GSE147390) was obtained from a de-identified public repository and did not require additional ethical approval. Informed consent was not applicable, as this study did not involve new human participants; human data were obtained from the publicly available, de-identified GEO dataset GSE147390.

Author Contributions

YW: Methodology, Software, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. YL: Methodology, Investigation, Validation, Data curation, Writing – original draft. HX: Conceptualization, Resources, Validation, Supervision, Project administration, Funding acquisition, Writing – review & editing. All authors contributed to the article and approved the submitted version.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Supplementary Material

The following supporting information is available for this article. Table S1: Marker gene expression matrix across the 12 Seurat clusters; Table S2: Stratified Spearman correlation between PI3K/Akt/mTOR and autophagy scores within each cell type; Table S3: Significantly perturbed genes from each virtual knockout and autophagy gene-set overlap statistics; Table S4: Slingshot pseudotime parameters and pathway-score correlations; Table S5: Complete differential expression results (Mineralizing OB vs. Pre-OB); Table S6: PPI network hub-gene ranking and complete interaction data; Table S7: The 21 curated bioactive constituents of *Eupolyphaga sinensis*; Table S8: Compound–target interactions with DrugBank accession identifiers; Table S9: Molecular docking binding affinities; Table S10: Western blot densitometric quantification data.

References

- Bermudez, O., Marchetti, S., Pagès, G., et al. (2008). Post-translational regulation of the ERK phosphatase DUSP6/MKP3 by the mTOR pathway. *Oncogene* 27, 3685–3691.
- Chun, Y. S., Lee, D. H., Won, T. G., et al. (2022). Current modalities for fracture healing enhancement. *Tissue Eng. Regen. Med.* 19, 11–17.
- Eberhardt, J., Santos-Martins, D., Tillack, A. F., and Forli, S. (2021). AutoDock Vina 1.2.0: new docking methods, expanded force field, and Python bindings. *J. Chem. Inf. Model.* 61, 3891–3898. doi: 10.1021/acs.jcim.1c00203
- Fei, C., Zou, J., Yang, Z., et al. (2025). Enhanced therapeutic efficacy of *Eupolyphaga sinensis* Walker in females through sex-specific metabolomic-pharmacodynamic divergence. *Sci. Rep.* 15, 6032. doi: 10.1038/s41598-025-90100-5
- Giannoudis, P. V., et al. (2021). Enhancement of fracture healing. *Injury* 52 (Suppl. 2), S1–S2.
- Gong, W., Zhang, N., Cheng, G., et al. (2019). *Rehmannia glutinosa* Libosch extracts prevent bone loss and architectural deterioration and enhance osteoblastic bone formation by regulating the IGF-1/PI3K/mTOR pathway in streptozotocin-induced diabetic rats. *Int. J. Mol. Sci.* 20, 3964.
- Hayat, A., Abdi, S., Khan, S. U., et al. (2025). Fracture healing: cellular mechanisms and impact of parathyroid hormone and its analogues. *Front. Endocrinol.* 16, 1703129.
- Hofbauer, L. C., Baschant, U., and Hofbauer, C. (2024). Targeting senescent cells to boost bone fracture healing. *J. Clin. Invest.* 134, e181974.
- Jia, S., Liu, W., Zhang, M., et al. (2024). Insufficient mechanical loading downregulates Piezo1 in chondrocytes and impairs fracture healing through ApoE-induced senescence. *Adv. Sci.* 11, 2400502.
- Kang, Z., Wu, B., Zhang, L., et al. (2023). Metabolic regulation by biomaterials in osteoblast. *Front. Bioeng. Biotechnol.* 11, 1184463.
- Koeberle, A., and Werz, O. (2014). Multi-target approach for natural products in inflammation. *Drug Discov. Today* 19, 1871–1882. doi: 10.1016/j.drudis.2014.08.006

- Li, Y., Liu, X., Zhou, J., et al. (2025). Artificial intelligence in traditional Chinese medicine: advances in multi-metabolite multi-target interaction modeling. *Front. Pharmacol.* 16, 1541509.
- Liang, C., Guo, Y., Liu, Y., et al. (2025). The mTOR signaling pathway mediates the phenotypic switch between adipocytes and osteocytes in mice with obesity-related osteoporosis. *Eur. J. Med. Res.* 30, 741.
- Liu, W., Chen, W., Xie, M., et al. (2023). Traumatic brain injury stimulates sympathetic tone-mediated bone marrow myelopoiesis to favor fracture healing. *Signal Transduct. Target. Ther.* 8, 260.
- Liu, W., Shang, G., Yang, S., et al. (2016). Electroacupuncture protects against ischemic stroke by reducing autophagosome formation and inhibiting autophagy through the mTORC1-ULK1 complex-Beclin1 pathway. *Int. J. Mol. Med.* 37, 309–318.
- Liu, Y., Tian, H., Hu, Y., et al. (2022). Mechanosensitive Piezo1 is crucial for periosteal stem cell-mediated fracture healing. *Int. J. Biol. Sci.* 18, 3961–3980.
- Molitoris, K. H., Huang, M., and Baht, G. S. (2024). Osteoimmunology of fracture healing. *Curr. Osteoporos. Rep.* 22, 330–339.
- Noor, F., Tahir ul Qamar, M., Ashfaq, U. A., et al. (2022). Network pharmacology approach for medicinal plants: review and assessment. *Pharmaceuticals* 15, 572.
- Saul, D., and Khosla, S. (2022). Fracture healing in the setting of endocrine diseases, aging, and cellular senescence. *Endocr. Rev.* 43, 984–1002.
- Schaub, T., Gürgen, D., Maus, D., et al. (2019). mTORC1 and mTORC2 differentially regulate cell fate programs to coordinate osteoblastic differentiation in mesenchymal stromal cells. *Sci. Rep.* 9, 20071.
- Sun, K., Luo, J., Guo, J., et al. (2020). The PI3K/AKT/mTOR signaling pathway in osteoarthritis: a narrative review. *Osteoarthr. Cartil.* 28, 400–409.
- Suvorova, I. I., and Pospelov, V. A. (2019). AMPK/Ulk1-dependent autophagy as a key mTOR regulator in the context of cell pluripotency. *Cell Death Dis.* 10, 260.
- Szymańska, P., Martin, K. R., MacKeigan, J. P., et al. (2015). Computational analysis of an autophagy/translation switch based on mutual inhibition of MTORC1 and ULK1. *PLoS One* 10, e0116550.
- Wang, J., Zhang, Y., Cao, J., et al. (2023). The role of autophagy in bone metabolism and clinical significance. *Autophagy* 19, 2409–2427.
- Wang, S., Deng, Z., Ma, Y., et al. (2020). The role of autophagy and mitophagy in bone metabolic disorders. *Int. J. Biol. Sci.* 16, 2675–2691.
- Wishart, D. S., Feunang, Y. D., Guo, A. C., Lo, E. J., Marcu, A., Grant, J. R., et al. (2018). DrugBank 5.0: a major update to the DrugBank database for 2018. *Nucleic Acids Res.* 46, D1074–D1082. doi: 10.1093/nar/gkx1037
- Yin, C., Tian, Y., Li, D., et al. (2022). Long noncoding RNA Lnc-DIF inhibits bone formation by sequestering miR-489-3p. *iScience* 25, 103949.
- Yin, S., Liu, L., and Gan, W. (2021). The roles of post-translational modifications on mTOR signaling. *Int. J. Mol. Sci.* 22, 1784.
- Zhang, H., Wang, R., Wang, G., et al. (2021). Single-cell RNA sequencing reveals B cells are important regulators in fracture healing. *Front. Endocrinol.* 12, 666140.

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