

Riociguat Suppresses Osteoclastogenesis and Ovariectomy-Induced Bone Loss by Attenuating MAPK Signaling and PPAR γ –PGC-1 β Coupling

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Research Article

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Riociguat suppresses osteoclastogenesis and ovariectomy-induced bone loss by attenuating MAPK signaling and PPAR γ –PGC-1 β coupling

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Abstract

Background: Postmenopausal osteoporosis is driven in part by excessive osteoclast-mediated bone resorption. We investigated whether the soluble guanylate cyclase stimulator riociguat regulates osteoclastogenesis and protects against estrogen-deficiency-induced bone loss.

Methods: Riociguat was evaluated in RANKL-stimulated bone marrow-derived macrophages and ovariectomized mice using viability, differentiation, cytoskeletal, resorption, immunoblotting, immunofluorescence, micro-computed tomography, and histomorphometric assays. Computational target prioritization, public single-cell transcriptomic reanalysis, co-immunoprecipitation, rosiglitazone interaction experiments, molecular docking, and molecular dynamics simulations were used to examine MAPK- and PPAR γ –PGC-1 β -associated responses. Group comparisons used analysis of variance or nonparametric alternatives as appropriate.

Results: Riociguat inhibited osteoclast formation without detectable cytotoxicity, disrupted F-actin-ring organization, reduced resorption-pit formation, and lowered the abundance of CTSK, integrin $\beta 3$, c-Fos, and NFATc1. Riociguat attenuated RANKL-induced phosphorylation of JNK, ERK, and p38 and reduced PPAR γ –PGC-1 β association. Rosiglitazone partially counteracted several cellular and biochemical effects associated with riociguat treatment. Structural simulations associated modelled riociguat occupancy of the PPAR γ ligand-binding pocket with reduced energetic favorability of a PPAR γ –PGC-1 β interface. In ovariectomized mice, riociguat preserved trabecular microarchitecture and reduced histological indices of osteoclast activity.

Conclusions: Riociguat suppressed osteoclastogenesis and preserved trabecular bone in an ovariectomy model. The data support involvement of early MAPK attenuation and reduced PPAR γ –PGC-1 β coupling, while direct target engagement, pathway necessity, and canonical sGC–cGMP–PKG dependence remain to be established.

Keywords: riociguat; postmenopausal osteoporosis; osteoclastogenesis; MAPK signaling; NFATc1; PPAR γ ; PGC-1 β

Background

Postmenopausal osteoporosis (PMOP) arises from uncoupled bone remodeling after estrogen withdrawal and is characterized by loss of bone mass, deterioration of trabecular microarchitecture, and increased fracture risk [1–3]. Osteoclasts are the principal bone-resorbing cells in this process. Their differentiation from monocyte/macrophage precursors is driven by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL). Downstream of RANKL, the ERK, JNK, and p38 mitogen-activated protein kinase (MAPK) branches induce c-Fos and NFATc1, which together establish the transcriptional program required for osteoclast maturation and bone

resorption [4–6]. Consequently, interventions that restrain this signaling cascade may limit the excessive osteoclast activity associated with estrogen deficiency.

Osteoclastogenesis is also coupled to metabolic and nuclear-receptor programs. Peroxisome proliferator-activated receptor gamma (PPAR γ) can promote osteoclast differentiation by regulating c-Fos, and its coactivator PPAR γ coactivator 1 beta (PGC-1 β) links osteoclastogenic transcription to mitochondrial biogenesis and cytoskeletal organization [7,8,10–14]. However, the role of PPAR γ is context dependent: genetic and pharmacological studies support a pro-osteoclastogenic PPAR γ –PGC-1 β program, whereas other work indicates that PPAR γ is more important for pharmacologically induced than for physiological or pathological osteoclast formation [7–9]. This distinction makes direct perturbation and appropriately bounded mechanistic claims essential when evaluating PPAR γ -directed effects.

Riociguat is an approved soluble guanylate cyclase (sGC) stimulator that increases cyclic guanosine monophosphate production and is used to treat pulmonary vascular disease [15,16]. The NO–sGC–cGMP–protein kinase G (PKG) network also contributes to skeletal homeostasis. Experimental stimulation of sGC has improved bone architecture in ovariectomized mice, and agents acting at several points in the cGMP network have altered osteoclast differentiation or bone resorption [17–22]. These effects are not uniform: vericiguat, for example, produces concentration-dependent bidirectional effects on osteoclastogenesis [18]. Whether riociguat directly regulates osteoclast differentiation and estrogen-deficiency-induced bone loss has not been established.

We therefore tested whether riociguat suppresses RANKL-induced osteoclast formation and function in vitro and protects against ovariectomy (OVX)-induced bone loss in vivo. We further integrated computational target prioritization, public single-cell transcriptomic data, biochemical assays,

pharmacological interaction experiments, and structural modelling to determine whether MAPK signaling and PPAR γ –PGC-1 β coupling are associated with the response to riociguat.

Methods

Materials and reagents

Riociguat (purity, 99.80%) and rosiglitazone were purchased from MedChemExpress (USA). Recombinant mouse M-CSF and RANKL were obtained from R&D Systems (USA). Fetal bovine serum and α -modified minimum essential medium (α -MEM) were obtained from Thermo Fisher Scientific. Cell Counting Kit-8 (CCK-8) was obtained from Engreen Biosystem (Beijing, China), and the tartrate-resistant acid phosphatase (TRAP) staining kit was obtained from Sigma-Aldrich. Primary antibodies against cathepsin K (CTSK), NFATc1, c-Fos, β -actin, and integrin β 3 were obtained from Santa Cruz Biotechnology. Antibodies against phosphorylated and total JNK, p38, and ERK1/2 were obtained from Cell Signaling Technology. Antibodies against PPAR γ , PGC-1 β , and lamin B1, together with the corresponding secondary antibodies, were obtained from Proteintech unless otherwise specified.

Bone marrow-derived macrophage culture and cell-viability assay

Bone marrow cells were isolated from the tibias and femurs of 6-week-old C57BL/6 mice and differentiated into bone marrow-derived macrophages (BMMs). Cells were maintained in α -MEM containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 50 ng/mL M-CSF at 37 °C in 5% CO₂. Osteoclast differentiation was induced with M-CSF (50 ng/mL) and RANKL (50 ng/mL), with riociguat, rosiglitazone, or their combination added as indicated. Unless otherwise stated, rosiglitazone was used at 1 μ M, a commonly used PPAR γ -activating concentration in mouse BMM osteoclast cultures, and was present from the onset of RANKL induction in differentiation and rescue experiments

[23]. Medium and compounds were refreshed every 2 days. For short-term signaling experiments, cells were serum-starved when required, pretreated with riociguat (10 μ M) or vehicle, and stimulated with RANKL for the stated intervals.

For cell-viability analysis, BMMs were seeded in 96-well plates at 1×10^4 cells per well and allowed to adhere for 24 h. Cells were exposed to riociguat (0–160 μ M) for 72 h and then incubated with CCK-8 reagent for 2 h. Absorbance at 450 nm was measured with an ELX800 microplate reader (Bio-Tek Instruments).

TRAP and F-actin-ring staining

BMMs were seeded in 96-well plates at 1×10^4 cells per well and differentiated for 6 days with M-CSF and RANKL in the presence of the indicated compounds. In rosiglitazone rescue experiments, rosiglitazone (1 μ M) was added from the onset of RANKL induction and replenished with each medium change. For time-window experiments, riociguat (10 μ M) was applied on days 1–6, 1–3, 3–5, or 5–6. Cells were fixed in 2.5% glutaraldehyde for 20 min and stained with the commercial TRAP kit. TRAP-positive multinucleated cells containing at least three nuclei were counted as mature osteoclasts. For F-actin visualization, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 3% bovine serum albumin, incubated with rhodamine–phalloidin, and counterstained with DAPI. Images were acquired by light or fluorescence microscopy and quantified in ImageJ.

Bone-resorption assay

BMMs were seeded on bovine bone slices at 1×10^4 cells/cm² and differentiated with M-CSF and RANKL. Mature osteoclasts were maintained on the slices for an additional 48 h with vehicle or riociguat. Cells were removed by mechanical agitation and brief sonication, and the slices were rinsed

and air-dried. Resorption pits were visualized with a SU8010 scanning electron microscope (Hitachi). Resorbed surface area was quantified from the micrographs and expressed relative to the imaged bone-slice area.

Immunoblotting

BMMs were induced with RANKL in the presence or absence of riociguat (10 μ M), rosiglitazone (1 μ M), or both compounds for the indicated intervals. Cells were lysed in RIPA buffer containing PMSF, DNase I, and protease/phosphatase inhibitors. Clarified lysates (12,000 $\times$ g, 15 min, 4 $^{\circ}$ C) were quantified by BCA assay. Equal protein amounts (30 μ g) were separated by 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% skim milk/TBST, incubated with primary antibodies overnight at 4 $^{\circ}$ C, and then incubated with horseradish-peroxidase-conjugated secondary antibodies for 2 h at room temperature. Signals were developed with BeyoECL Plus and captured with a ChemiDoc XRS+ system. Densitometry was performed in ImageJ. Osteoclastogenic proteins were normalized to β -actin, and phosphorylated MAPKs were normalized to their corresponding total proteins. Three biologically independent experiments were used for densitometric analysis.

Immunofluorescence and colocalization analysis

BMMs grown on coverslips or in 96-well plates were treated as indicated. After fixation with 4% paraformaldehyde, cells were permeabilized with 0.1% Triton X-100 and blocked with 3% bovine serum albumin. Cells were incubated overnight at 4 $^{\circ}$ C with antibodies against NFATc1, PPAR γ , or PGC-1 β , followed by Alexa Fluor-conjugated secondary antibodies. Nuclei were counterstained with DAPI. Images were acquired using a BZ-X800 fluorescence microscope (Keyence) or a confocal microscope under identical acquisition settings within each experiment. NFATc1 localization and the

spatial overlap between PPAR γ and PGC-1 β were assessed qualitatively from representative images; no quantitative colocalization coefficient was calculated.

Computational target prioritization and enrichment analysis

The simplified molecular-input line-entry system string for riociguat was obtained from PubChem. Putative human targets were assembled from SwissTargetPrediction and the retained local TargetNet/Similarity Ensemble Approach prediction tables [24,37,38]. PMOP- and osteoporosis-associated genes were collected from GeneCards and Online Mendelian Inheritance in Man (OMIM) [25,26]. After duplicate removal, the target sets represented in Figure 4 comprised 117 drug-associated genes and 1,697 disease-associated genes; 29 genes were shared. A compound–target–disease network was constructed in Cytoscape 3.9.0 [27]. The 29 intersecting genes were submitted to STRING (Homo sapiens; minimum interaction score, 0.900; disconnected nodes hidden) to construct the protein–protein interaction network [28].

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses were performed in DAVID using the default Homo sapiens annotated-gene backgrounds (8,865 genes for the retained KEGG export and 19,416 genes for the retained GO export) [29]. Benjamini–Hochberg-adjusted $P < 0.05$, rather than nominal P alone, defined the terms retained for interpretation. The complete 29-gene evidence matrix and FDR-filtered enrichment outputs are provided in Supplementary Tables S4–S6. These analyses were treated as candidate prioritization rather than evidence of direct target engagement.

Public single-cell transcriptomic contextualization

Public single-cell RNA-sequencing data from GSE147174 were reanalyzed to contextualize the candidate pathways during osteoclast differentiation [30]. Raw 10x Genomics matrices from day 0, day 1,

and day 3 were processed in R using Seurat [39]. Gene identifiers were mapped to official mouse symbols using the retained GENCODE annotation. Cells with 300–6,000 detected genes and no more than 15% mitochondrial reads were retained. This yielded 2,184 of 2,387 day-0 cells, 2,991 of 6,206 day-1 cells, and 2,448 of 5,752 day-3 cells (7,623 cells and 17,366 genes in total). Counts were normalized to 10,000 reads per cell and log1p-transformed. Dedicated empty-droplet, ambient-RNA, and doublet-removal algorithms were not available in the retained pipeline; consequently, rare RNA co-detection states were interpreted conservatively.

Prespecified module scores were calculated for canonical PPAR targets, osteoclast-context PPAR targets, MAPK–NFAT signaling, early and mature osteoclast programs, mitochondrial/fatty-acid oxidation, and coactivators. Exposure genes were excluded from the corresponding outcome modules: *Ppargc1b* was omitted from the coactivator score, and *Fos* was omitted from the MAPK–NFAT and early-osteoclast scores used in the robustness analysis. The complete module definitions are provided in Supplementary Table S2. Spearman correlations between *Ppargc1b* and the independent outcome modules were summarized both across all cells and separately within each time point (Supplementary Table S3).

The network analysis used human genes, whereas GSE147174 was generated in mouse cells. No human and mouse expression values were pooled. Cross-species comparison was restricted to prespecified conserved symbol mappings used for context, including *PPARG* to *Pparg* and *MAPK1* to *Mapk1*; *Ppargc1b* was evaluated as a literature-motivated coactivator rather than as a predicted riociguat target. Because one 10x library represented each time point, cell-level analyses and between-time patterns were considered descriptive and exploratory; cells were not treated as independent biological replicates. Low transcript abundance of sGC-pathway genes was not interpreted as pathway inactivity because riociguat signaling is primarily regulated at the protein and second-messenger levels.

Co-immunoprecipitation and subcellular fractionation

For co-immunoprecipitation, treated BMMs were lysed in ice-cold immunoprecipitation buffer containing protease and phosphatase inhibitors. Clarified lysates were reserved as input or incubated overnight at 4 °C with anti-PPAR γ or anti-PGC-1 β antibodies; normal IgG served as the negative control. Immune complexes were captured with magnetic beads, washed, eluted, resolved by SDS–PAGE, and immunoblotted with the reciprocal antibody. Association was quantified after normalization to the immunoprecipitated protein.

For nuclear–cytoplasmic fractionation, cells were lysed in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1% NP-40, and 1 mM DTT, with protease/phosphatase inhibitors). Following centrifugation at 1,000 $\times$ g for 5 min at 4 °C, the cytoplasmic supernatant was collected. Nuclear pellets were washed and extracted for 30 min in high-salt buffer (20 mM HEPES, pH 7.9, 300–420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 20% glycerol). Benzonase was used to reduce viscosity, and nuclear extracts were clarified at 16,000 $\times$ g for 10 min. Lamin B1 and β -actin served as nuclear and cytoplasmic markers, respectively.

Molecular docking and molecular dynamics simulations

The three-dimensional structure of riociguat was retrieved from PubChem, and available target-protein structures were obtained from the Protein Data Bank. Initial cavity-guided docking was performed with CB-Dock, and lower Vina scores were interpreted as more favorable predicted poses [31]. Docking results were used only to nominate structural hypotheses.

For interface modelling, the PPAR γ –SRC-1 structure (PDB 2PRG) was used as the structural reference for the AF-2 coactivator-binding surface. Two LXXLL-containing PGC-1 β motifs were modelled at the AF-2 groove and evaluated by 100-ns molecular dynamics simulations. The selected motif complex

was then modelled with riociguat in the PPAR γ ligand-binding pocket. Trajectories were evaluated using root-mean-square deviation (RMSD), radius of gyration (Rg), solvent-accessible surface area (SASA), and residue-level fluctuation (RMSF). Interface energetics were estimated by molecular mechanics/Poisson–Boltzmann surface-area (MM/PBSA) analysis and per-residue energy decomposition. These simulations were interpreted as model-dependent evidence of altered interface energetics rather than as direct evidence of binding or allostery.

Ovariectomy model and treatment

Animal procedures were approved by the Institutional Animal Ethics Committee of Wenzhou Institute, University of Chinese Academy of Sciences (approval WIUCAS23063001) and are reported with reference to the ARRIVE 2.0 framework [32]. Twelve-week-old female C57BL/6 mice were acclimatized for 1 week under specific-pathogen-free conditions and allocated to five groups (n = 5 per group): Sham, OVX, OVX plus riociguat (5 mg/kg per administration), OVX plus riociguat (10 mg/kg per administration), and OVX plus alendronate (1 mg/kg per administration). Bilateral ovariectomy was performed in the OVX groups; Sham animals underwent the same procedure without ovary removal. Seven days later, riociguat or alendronate was administered by oral gavage every 2 days for 6 weeks. Sham and OVX control animals received the corresponding vehicle. At study completion, mice were euthanized and femurs were collected for micro-CT and histological analysis.

Micro-computed tomography

Femurs were fixed in 4% paraformaldehyde for 48 h and scanned with a SkyScan micro-CT system (Bruker) using a 0.5-mm aluminum filter, 50-kV tube voltage, 500- μ A current, 0.4° rotation step, and 9- μ m isotropic voxel size. Images were reconstructed in NRecon. Trabecular bone volume fraction

(BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp) were quantified in a 1-mm region beginning 0.5 mm below the growth plate.

Histology and histomorphometry

Fixed femurs were decalcified in 14% EDTA (pH 7.4) for 10 days at 37 °C, embedded in paraffin, and sectioned sagittally at 5 µm. Sections were stained with hematoxylin and eosin, TRAP, or Masson's trichrome and scanned with a KFBIO digital pathology system. Osteoclast surface per bone surface (Oc.S/BS) and osteoclast number per bone surface (N.Oc/BS) were quantified with BIOQUANT OSTEO 2011.

Statistical analysis

Data are presented as mean ± standard deviation or mean ± standard error of the mean, as specified in the figure legends. Multiple-group comparisons were performed with one-way analysis of variance followed by Tukey's test or, when parametric assumptions were not met, with the Kruskal–Wallis test followed by Dunn's multiple-comparison test. Two-way analysis of variance was used for time-by-treatment analyses. $P < 0.05$ was considered statistically significant. The unit represented by n is specified in each figure legend.

Results

Riociguat inhibits osteoclast formation during early and intermediate differentiation

We first determined whether the anti-osteoclastogenic response could be separated from nonspecific cytotoxicity and whether it depended on the stage of differentiation. Riociguat did not detectably reduce BMM viability after 72 h at concentrations up to 160 µM (Figure 1A). In RANKL-stimulated cultures, however, riociguat reduced the number of TRAP-positive multinucleated osteoclasts in a concentration-dependent manner between 2.5 and 10 µM, with the largest reduction at 10 µM (Figure

244 1B,D). Time-window experiments showed that exposure during days 1–3 or 3–5 inhibited osteoclast
245 formation, while treatment restricted to days 5–6 had a smaller effect than continuous exposure (Figure
246 1C,E). Thus, the response was not explained by generalized cytotoxicity and was most pronounced
247 during early and intermediate differentiation.

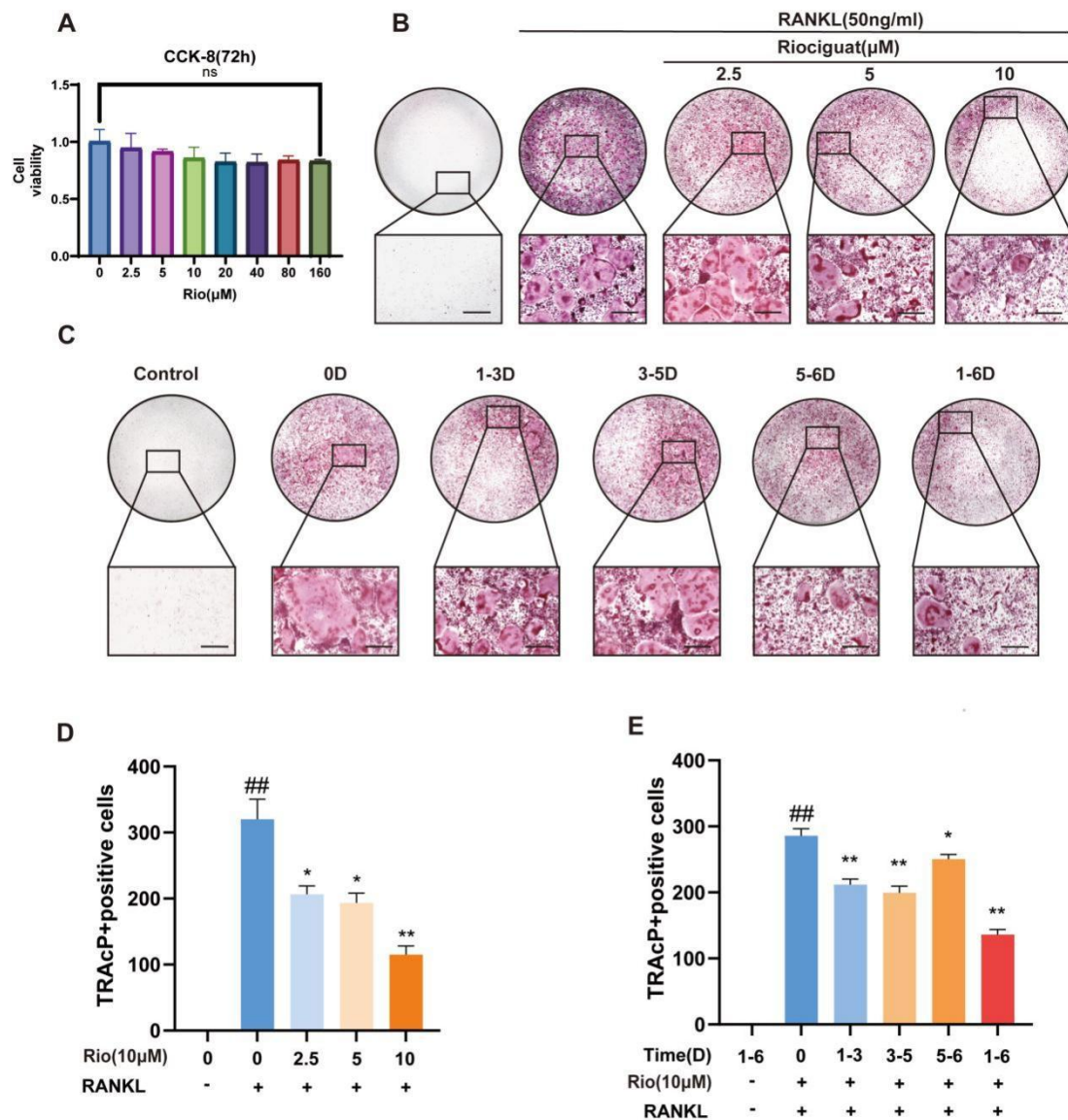
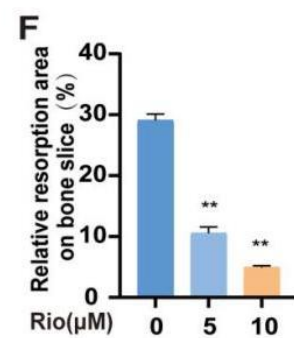
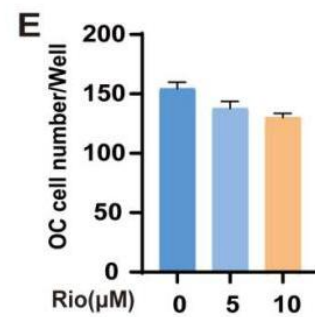
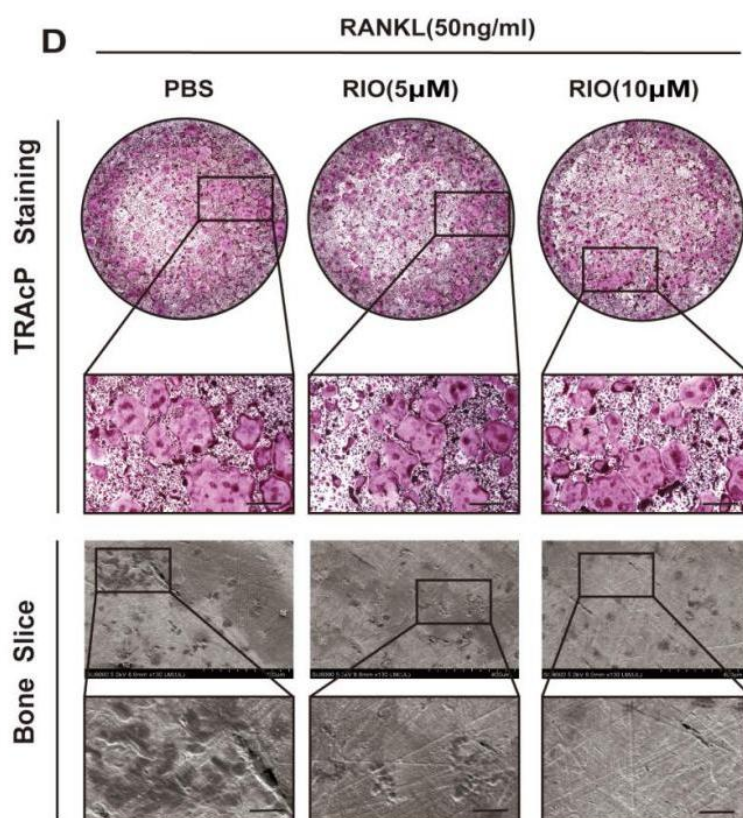
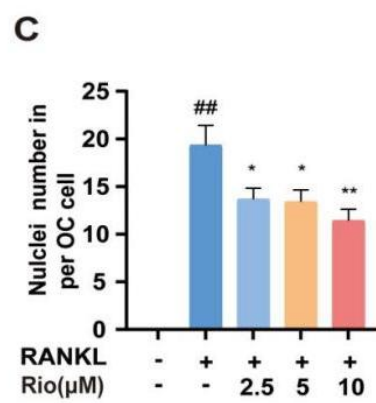
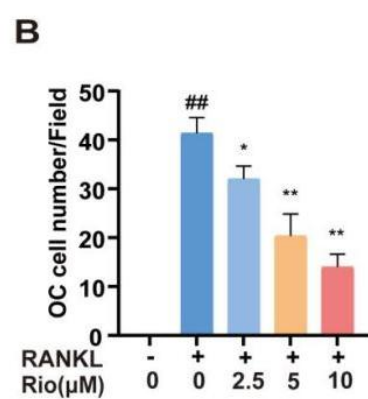
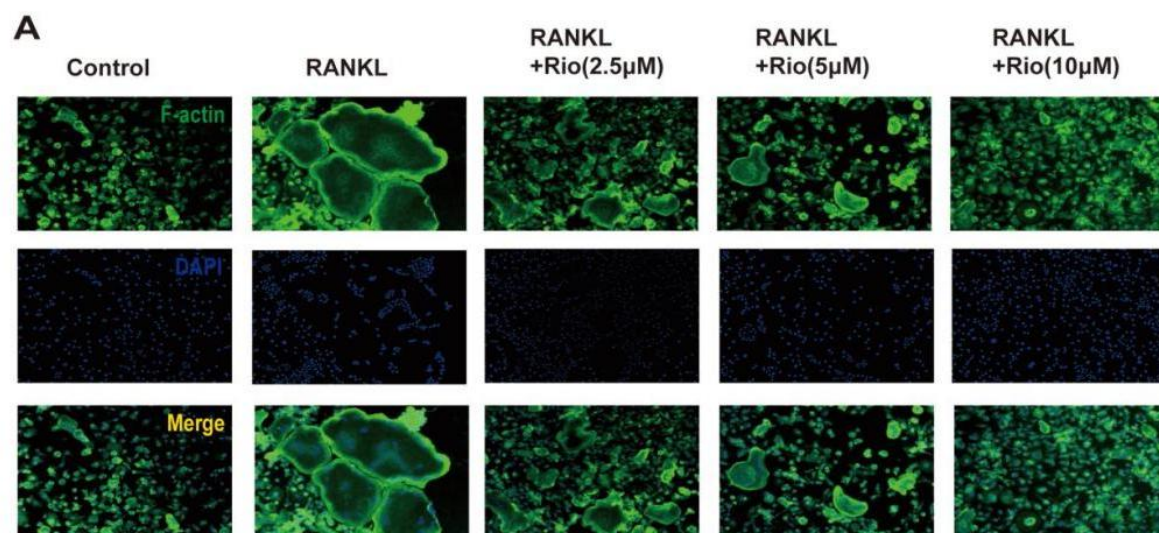


Figure 1. Riociguat inhibits RANKL-induced osteoclast formation. (A) CCK-8 viability assay after 72 h of riociguat exposure. (B) Representative TRAP staining after differentiation with RANKL and the indicated riociguat concentrations. (C) Representative TRAP staining after exposure to 10 μM riociguat during the stated differentiation windows. (D,E) Quantification of TRAP-positive multinucleated cells in the concentration- and time-window experiments. Data are mean ± SD from n = 3 biologically independent experiments. ##P < 0.01 versus untreated control; *P < 0.05 and **P < 0.01 versus RANKL alone.

258

259 **Riociguat impairs osteoclast cytoskeletal organization and resorptive function**

260 Because reduced TRAP-positive cell formation does not by itself establish loss of osteoclast function,
261 we next examined cytoskeletal organization and bone resorption. RANKL induced large multinucleated
262 cells with well-defined F-actin rings, whereas riociguat produced fewer and smaller osteoclasts with
263 attenuated ring structures (Figure 2A–C). On bone slices, the number of TRAP-positive cells was not
264 substantially altered over the 48-h functional assay, but riociguat at 5 or 10 μ M markedly reduced the
265 resorbed surface area (Figure 2D–F). These data distinguish an effect on bone-resorptive function from
266 the earlier reduction in osteoclast formation and show that riociguat also compromises the activity of
267 differentiated osteoclasts.



269 **Figure 2.** Riociguat disrupts F-actin organization and suppresses osteoclast-mediated bone resorption. (A) Representative
270 F-actin and nuclear staining after treatment with the indicated riociguat concentrations. (B,C) Osteoclast number per field and
271 mean nuclei per osteoclast. (D) TRAP staining of mature osteoclasts and scanning electron micrographs of resorption pits on
272 bone slices. (E) Osteoclast number during the functional assay. (F) Relative resorbed bone-slice area. Data are mean $\pm$ SD from
273 $n = 3$ biologically independent experiments. ### $P < 0.01$ versus untreated control; * $P < 0.05$ and ** $P < 0.01$ versus RANKL
274 alone. Scale bars, 200 μm for F-actin images and 400 μm for bone-slice images.

Riociguat suppresses the c-Fos/NFATc1 osteoclastogenic program

We then asked whether the morphological and functional effects were accompanied by suppression of the canonical osteoclast differentiation program. Immunoblotting showed that riociguat reduced the RANKL-induced accumulation of c-Fos and NFATc1 and decreased the late osteoclast markers integrin β 3 and CTSK during differentiation (Figure 3A–E). Representative immunofluorescence images showed stronger NFATc1 staining with apparent nuclear enrichment following RANKL stimulation, whereas riociguat-treated cells displayed weaker NFATc1 staining and less apparent nuclear enrichment (Figure 3F). Because nuclear localization was not quantitatively assessed, these images were interpreted descriptively. The immunoblotting results nevertheless support suppression of the c-Fos/NFATc1 osteoclastogenic program by riociguat.

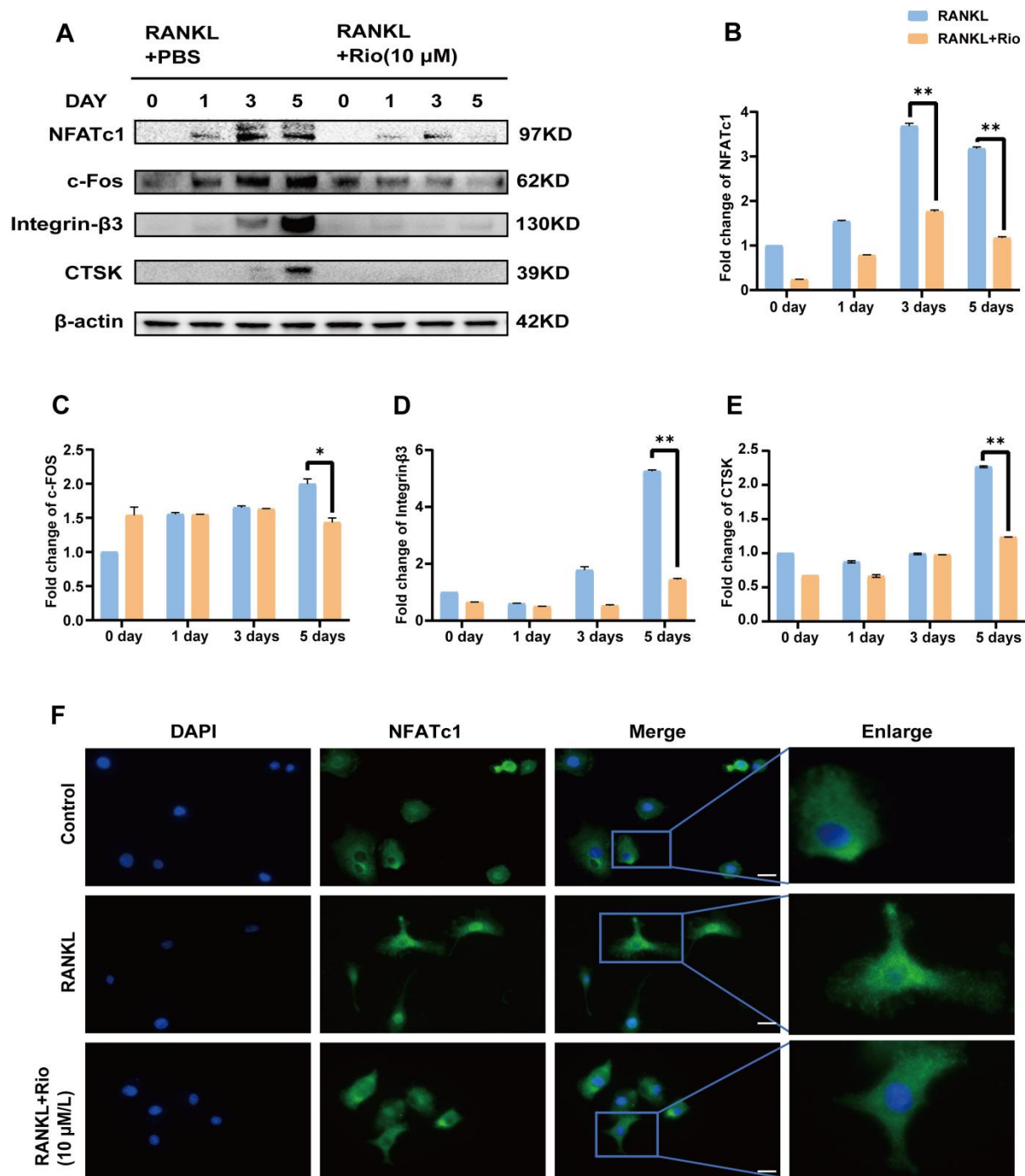


Figure 3. Riociguat reduces osteoclastogenic proteins and alters the apparent cellular distribution of NFATc1. (A) Immunoblots for NFATc1, c-Fos, integrin β 3, CTSK, and β -actin during differentiation. **(B–E)** Densitometric quantification normalized to β -actin. **(F)** Representative NFATc1 immunofluorescence images under the indicated conditions. The images were used for qualitative assessment of NFATc1 signal distribution; nuclear localization was not quantitatively analyzed. Data in B–E are mean $\pm$ SD from $n = 3$ biologically independent experiments. * $P < 0.05$ and ** $P < 0.01$ for the indicated comparisons.

Computational prioritization nominates MAPK signaling and PPAR γ for experimental follow-up

Having established the anti-osteoclastogenic phenotype, we next used computational target prioritization to select mechanisms for experimental follow-up. The Venn analysis represented 117 drug-associated and 1,697 disease-associated genes: 88 were drug-only, 1,668 were disease-only, and 29 were shared (Figure 4A–C; Supplementary Table S4). Thus, the numbers displayed in the non-overlapping regions of Figure 4C are not the total sizes of the two sets. The high-confidence protein–protein interaction network placed PPARG at the highest degree (17), followed by NFKB1 (13), HIF1A, PRKACA, IGF1R, MAPK1, and other connected candidates (Figure 4D,E).

After multiple-testing correction, the MAPK signaling pathway remained enriched (seven overlapping genes; Benjamini–Hochberg-adjusted $P = 0.0040$), as did signal transduction and positive regulation of DNA-templated transcription among the retained GO biological-process terms (adjusted $P = 0.0026$ and 0.0466 , respectively; Figure 4F,G; Supplementary Tables S5 and S6). The 29-gene evidence matrix shows which candidates originated from each prediction source, their disease support, network degree, enriched-term membership, and whether they received experimental follow-up. These analyses prioritized MAPK signaling and PPARG but did not establish that riociguat directly binds either target.

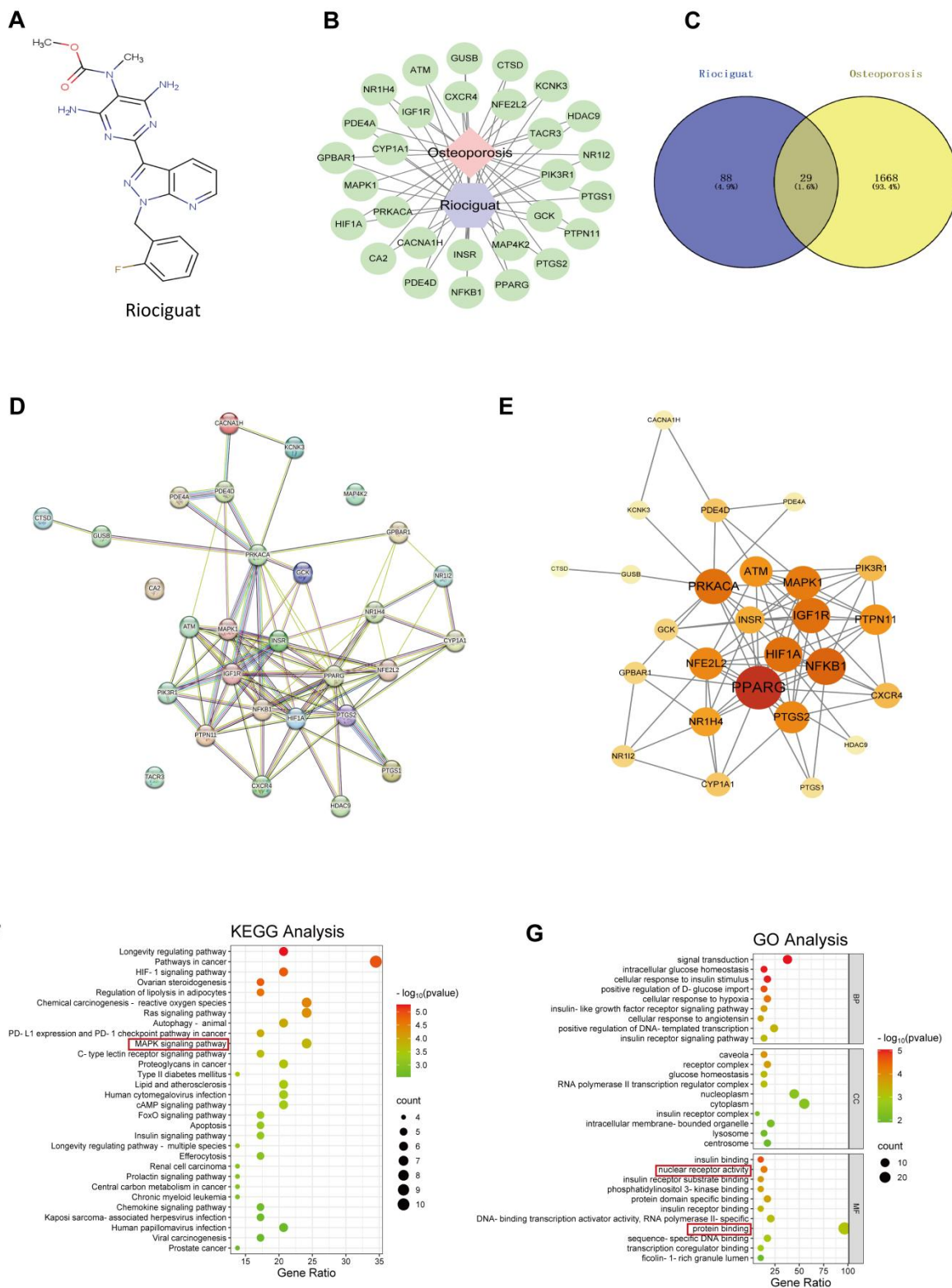


Figure 4. Computational target prioritization nominates MAPK and PPAR γ -related regulatory contexts for riociguat in osteoporosis. (A) Chemical structure of riociguat. (B) Compound–target–disease network. (C) Intersection of predicted riociguat targets and osteoporosis-associated genes; the displayed values denote 88 drug-only, 1,668 disease-only, and 29 shared genes. (D) High-confidence STRING interaction network for the 29 intersecting genes. (E) Hub visualization of the interaction network. (F) KEGG enrichment of intersecting genes. (G) GO enrichment by biological process, cellular component, and molecular function. Bubble colors in the legacy enrichment panels display nominal P values, whereas inferential interpretation in the text used Benjamini–Hochberg-adjusted $P < 0.05$.

Public single-cell reanalysis contextualizes a PGC-1 β -enriched differentiation state

We next reanalyzed GSE147174 to determine whether the nominated regulatory context was present during osteoclast differentiation. Quality control retained 2,184 day-0, 2,991 day-1, and 2,448 day-3 cells (Supplementary Table S1). *Pparg* and *Rxra* transcripts were relatively sparse, whereas *Rxrb* and *Ppargc1b* were detected in a larger fraction of day-1 and day-3 cells (Figure 5E). Module-score distributions documented a shift toward osteoclast and MAPK–NFAT programs across the three collection days (Figure 5A,B).

To avoid mathematical self-correlation, *Ppargc1b* and *Fos* were removed from the relevant outcome modules before robustness testing. Across all 7,623 cells, *Ppargc1b* correlated descriptively with the mature osteoclast program (Spearman $\rho = 0.49$), the early osteoclast program ($\rho = 0.33$), and the MAPK–NFAT program excluding *Fos* ($\rho = 0.26$), but not with the canonical PPAR-target module ($\rho = -0.11$). The corresponding within-day correlations were near zero at day 0 and stronger at day 3 (mature osteoclast $\rho = 0.59$; early osteoclast $\rho = 0.50$; MAPK–NFAT $\rho = 0.41$; Supplementary Table S3). This stratification shows that the pooled association partly reflects differentiation state and should not be interpreted as a treatment effect or a population-level inferential result.

334 UMAP visualization further showed that Ppargc1b was distributed more broadly than Pparg or Rxra
335 (Figure 6A–C). Within the Pparg⁺/Rxra⁺ RNA-defined population, the proportion of Ppargc1b-positive
336 cells increased from 3.4% on day 0 to 15.4% on day 1 and 20.2% on day 3 (Figure 6D–F). These
337 analyses support a PGC-1 β -enriched coactivator context during differentiation but do not demonstrate
338 physical PPAR γ –PGC-1 β complex formation, direct target engagement, or treatment response. The rare
339 triple-positive state remains sensitive to empty-droplet, ambient-RNA, and doublet handling, and all
340 between-time comparisons are descriptive because one 10x library represented each time point.

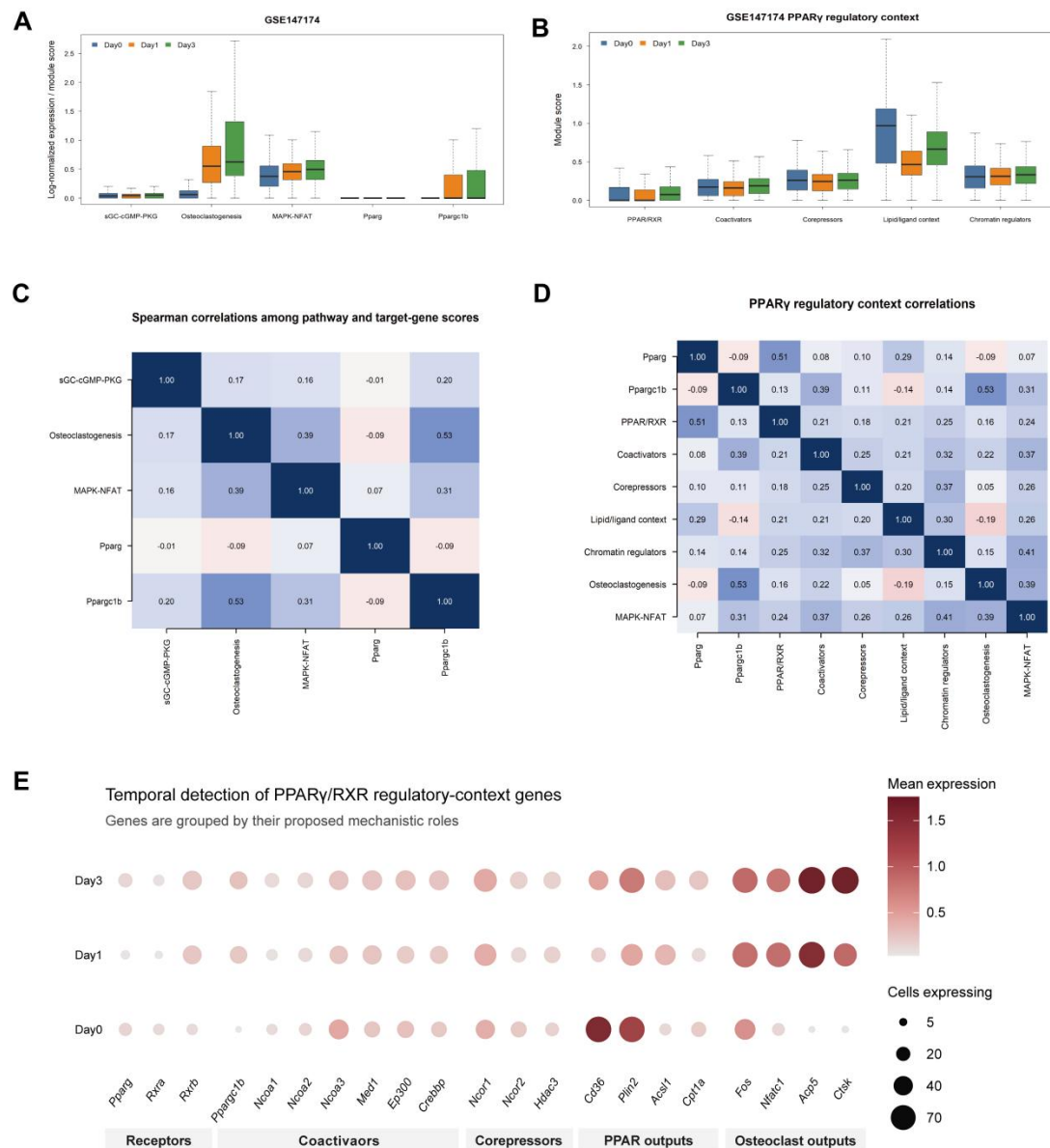


Figure 5. Single-cell transcriptomic context during osteoclast differentiation in GSE147174. (A) Distributions of sGC–cGMP–PKG, osteoclastogenesis, MAPK–NFAT, Pparg, and Ppargc1b expression or module scores across day 0, day 1, and day 3. (B) Distributions of PPAR/RXR, coactivator, corepressor, lipid/ligand-context, and chromatin-regulator module scores. (C,D) Legacy pooled Spearman correlation matrices across pathway, target-gene, and PPAR γ regulatory-context scores. Because some displayed modules contain the indexed genes, panels C and D are descriptive visualizations and were not used for the non-circular estimates reported in the text; corrected pooled and time-stratified correlations are provided in Supplementary Table S3. (E) Dot plot of PPAR γ regulatory-context genes; dot size denotes the fraction of cells expressing each gene and color denotes mean log-normalized expression. Each time point was represented by one 10x library; between-time comparisons are descriptive.

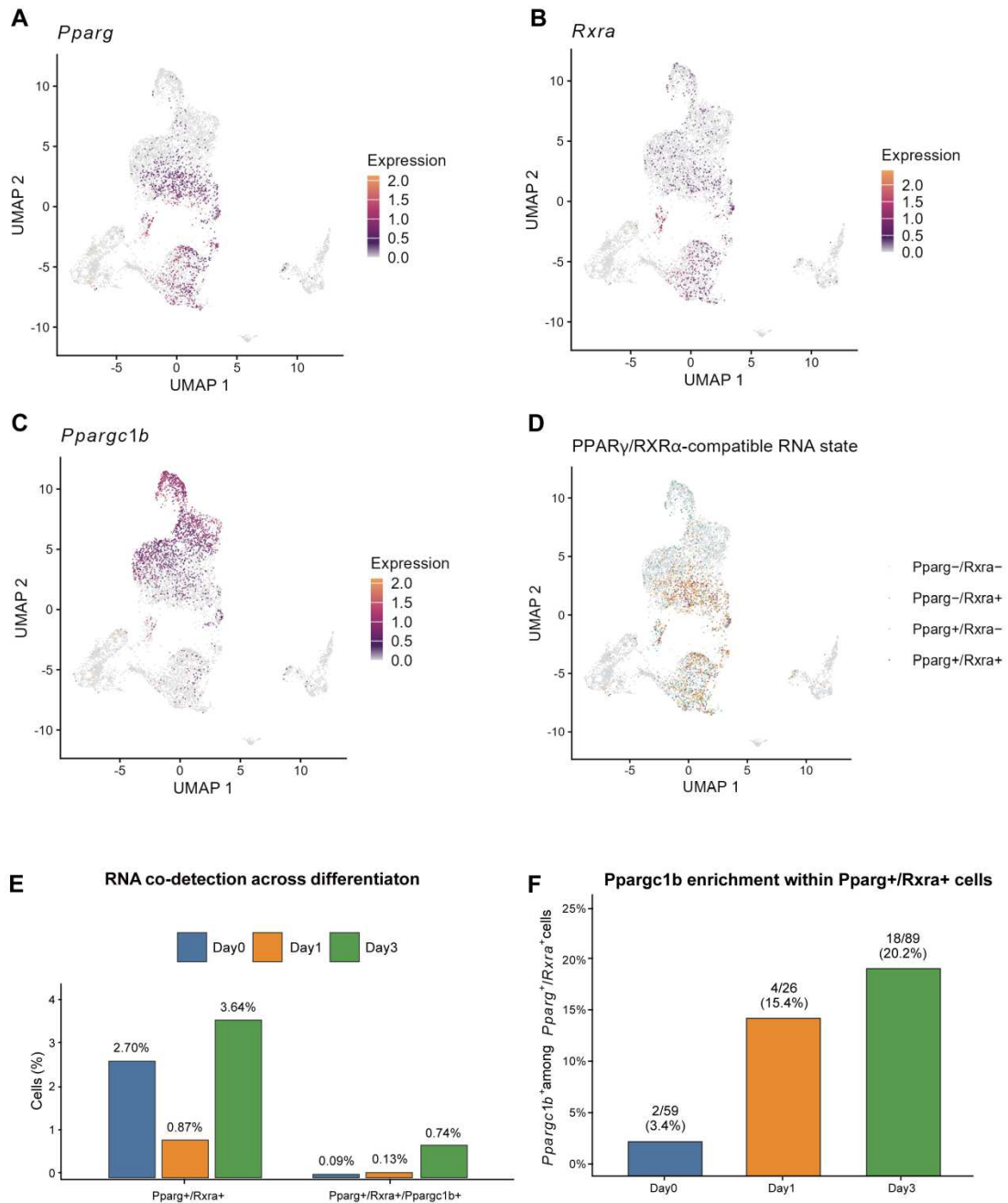


Figure 6. *Pparg1b* enrichment within a *Pparg*⁺/*Rxra*⁺ RNA-defined state during osteoclast differentiation. (A–C) UMAP plots of normalized *Pparg*, *Rxra*, and *Pparg1b* expression. (D) Cells classified by *Pparg* and *Rxra* RNA co-detection; double-positive cells were designated *Pparg*⁺/*Rxra*⁺. (E) Frequencies of *Pparg*⁺/*Rxra*⁺ and *Pparg*⁺/*Rxra*⁺/*Pparg1b*⁺ cells at each time point. (F) Fraction of *Pparg1b*⁺ cells within the *Pparg*⁺/*Rxra*⁺ population. Data are descriptive; each time point was represented by one 10x library.

358

359 **Riociguat attenuates MAPK activation and weakens PPAR γ –PGC-1 β coupling**

360 RANKL rapidly increased phosphorylation of JNK, ERK, and p38. Riociguat reduced phosphorylation
361 of all three MAPKs, with the clearest differences between 10 and 30 min after stimulation (Figure 7A–
362 D). This early effect preceded the later reduction in c-Fos and NFATc1 and is consistent with attenuation
363 of the MAPK/c-Fos/NFATc1 cascade.

364 Because PPARG was prioritized by the interaction network and the single-cell data identified a
365 PGC-1 β -enriched context, we next examined the PPAR γ –PGC-1 β complex experimentally. RANKL
366 increased PGC-1 β co-immunoprecipitation with PPAR γ , whereas riociguat reduced the normalized
367 association (Figure 7F,G). Immunofluorescence showed a corresponding reduction in PPAR γ /PGC-1 β
368 colocalization after riociguat treatment (Figure 7H). A separate cavity-guided docking model placed
369 riociguat in the PPAR γ ligand-binding pocket with a Vina score of -8.2 (Figure 7E); this prediction was
370 treated only as a structural hypothesis and not as evidence of binding. Together, the experimental data
371 associate riociguat with both early MAPK attenuation and weaker PPAR γ –PGC-1 β coupling.

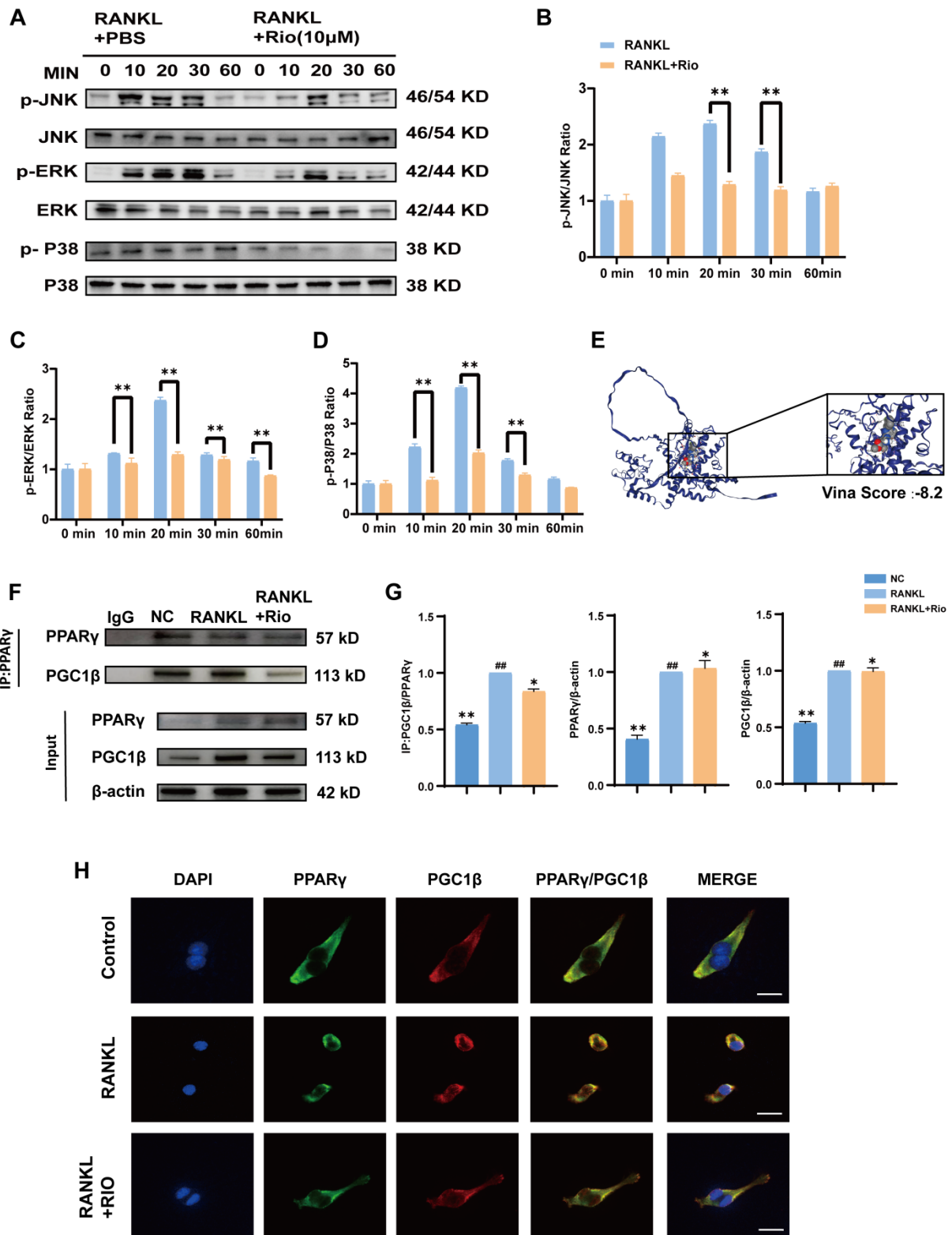


Figure 7. Riociguat attenuates RANKL-induced MAPK activation and reduces PPAR γ -PGC-1 β association. (A) Immunoblots for phosphorylated and total JNK, ERK, and p38 from 0 to 60 min after RANKL stimulation. (B–D) Quantification of p-JNK/JNK, p-ERK/ERK, and p-p38/p38. (E) Predicted docking pose of riociguat in PPAR γ . (F) PPAR γ immunoprecipitation and PGC-1 β immunoblotting. (G) Quantification of the normalized PPAR γ -PGC-1 β association and input proteins. (H) Representative PPAR γ and PGC-1 β immunofluorescence. Data are mean $\pm$ SEM from n = 3 biologically independent experiments. *P < 0.05 and **P < 0.01; ###P < 0.01 for the comparisons indicated in the panels. Scale bars, 10 μ m.

393

394 **Rosiglitazone partially counteracts the effects of riociguat**

395 To assess the pharmacological interaction between rosiglitazone and riociguat, RANKL-stimulated
396 BMMs were treated with either compound alone or in combination. Representative TRAP and F-actin
397 staining suggested partial recovery of osteoclast formation and actin-ring organization with
398 co-treatment compared with riociguat alone (Figure 8A). Co-treatment also increased the abundance
399 of NFATc1, c-Fos, integrin $\beta 3$, and CTSK (Figure 8B,C). The p-JNK/JNK, p-ERK/ERK, and
400 p-p38/p38 ratios were higher with co-treatment than with riociguat alone (Figure 8D,E). Together,
401 these findings indicate that rosiglitazone partially counteracted several cellular and biochemical
402 effects associated with riociguat treatment.

403 Rosiglitazone increased nuclear PPAR γ abundance and PPAR γ -PGC-1 β co-immunoprecipitation under
404 RANKL stimulation, whereas riociguat reduced both readouts. Co-treatment with rosiglitazone
405 partially restored nuclear PPAR γ abundance and the normalized PPAR γ -PGC-1 β association relative to
406 riociguat treatment alone (Figure 9B-E). Representative immunofluorescence images showed
407 treatment-associated differences in PPAR γ and PGC-1 β localization and spatial overlap (Figure 9A).
408 Because colocalization was not quantitatively assessed, these images were interpreted descriptively.
409 Together, the biochemical measurements support an association between riociguat treatment and
410 reduced PPAR γ -PGC-1 β coupling, whereas the immunofluorescence images provide qualitative spatial
411 context.

412

413

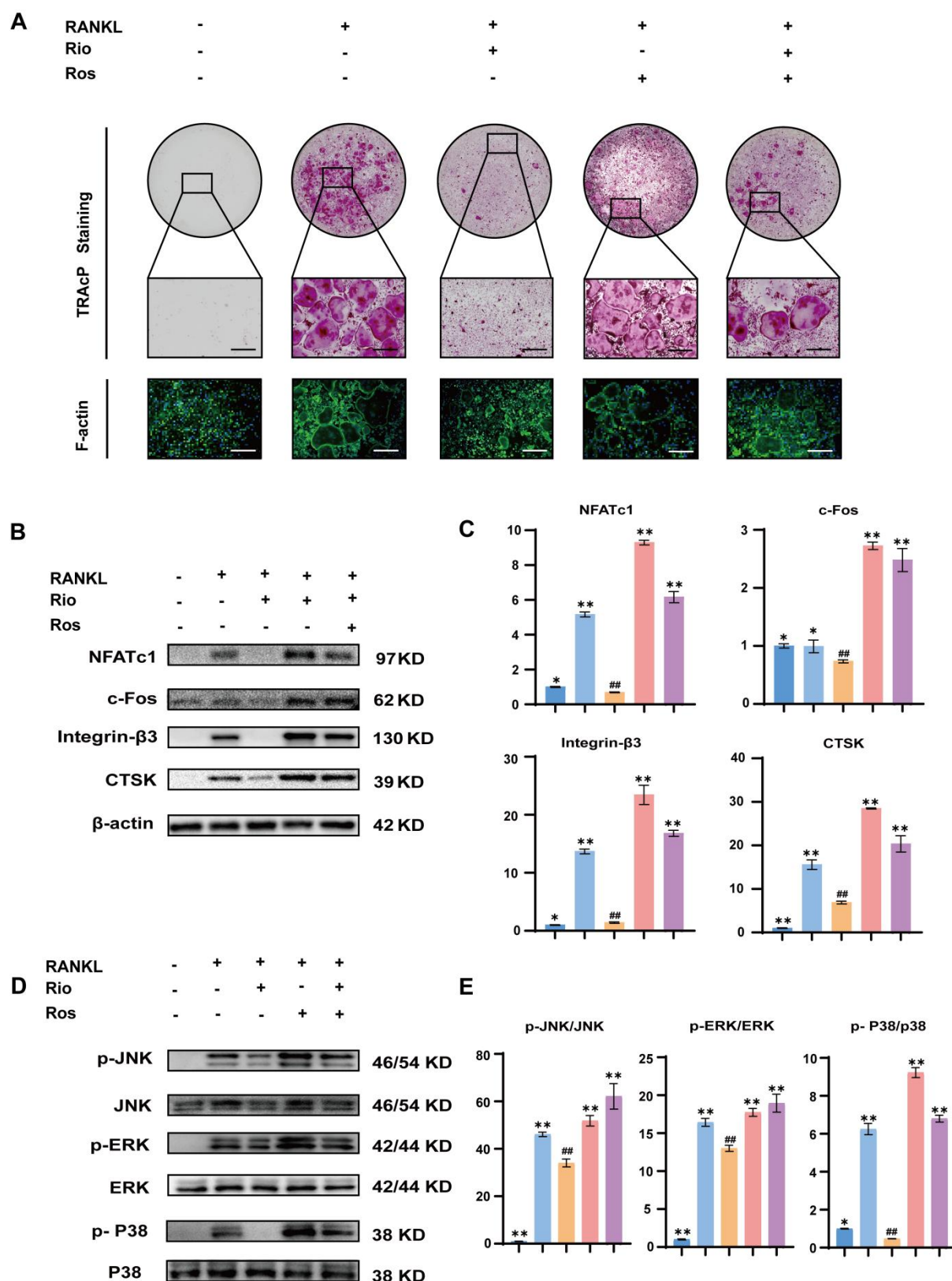


Figure 8. Rosiglitazone partially counteracts riociguat-associated changes in osteoclastogenesis, osteoclastogenic proteins, and MAPK phosphorylation. (A) Representative TRAP and F-actin-ring staining after treatment with RANKL, riociguat (10 μ M), rosiglitazone (1 μ M), or their combination. (B) Immunoblots for NFATc1, c-Fos, integrin

418 β 3, CTSK, and β -actin. (C) Densitometric quantification of the proteins in B. (D) Immunoblots for phosphorylated and
419 total JNK, ERK, and p38. (E) Quantification of MAPK phosphorylation ratios. Data are mean $\pm$ SD from n = 3
420 biologically independent experiments. *P < 0.05 and **P < 0.01 versus the RANKL + riociguat group; ###P < 0.01
421 versus RANKL alone. Scale bars, 200 μ m.

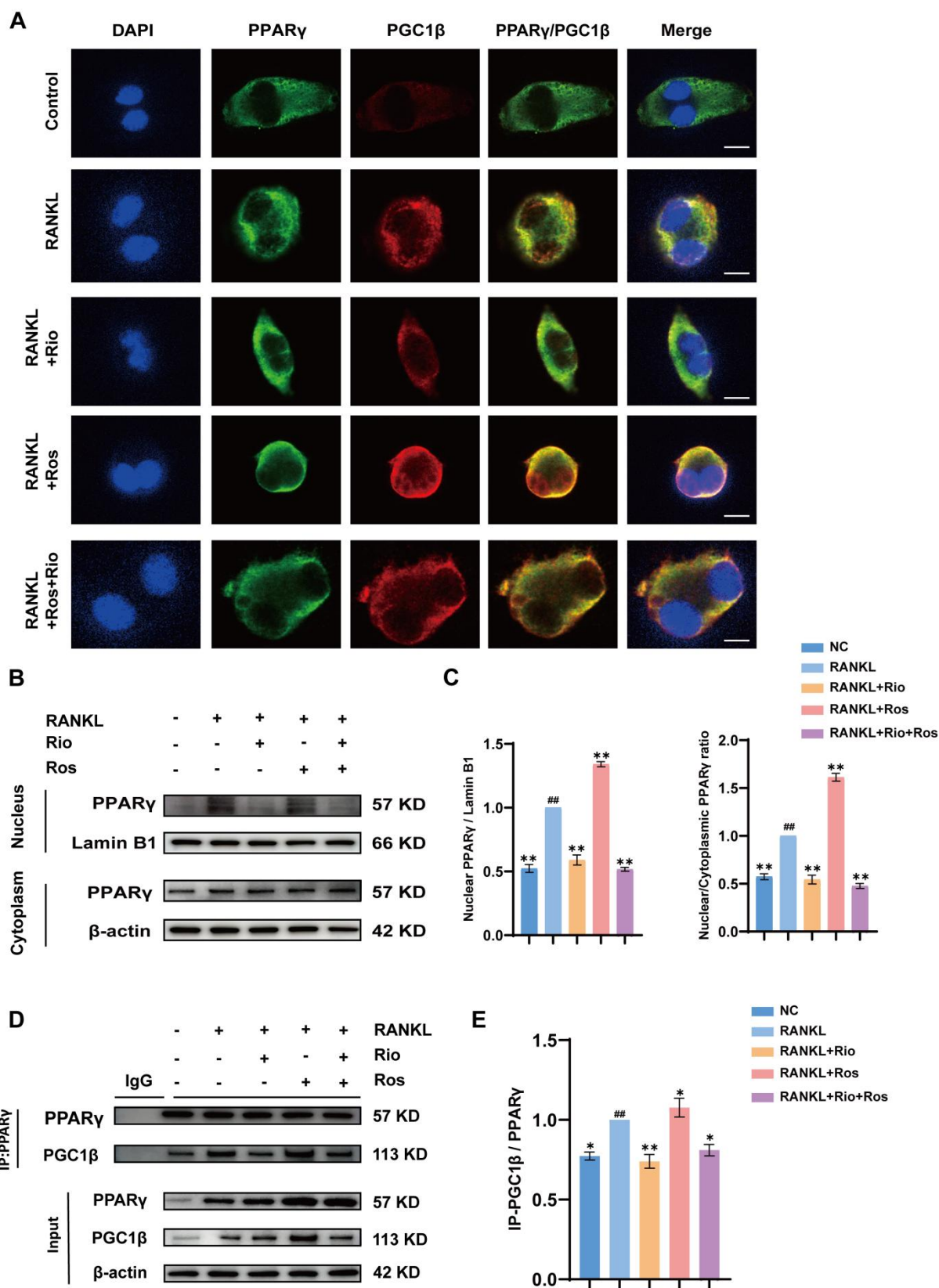


Figure 9. Rosiglitazone modifies riociguat-associated changes in PPAR γ localization and PPAR γ –PGC-1 β biochemical association. (A) Representative immunofluorescence images showing PPAR γ and PGC-1 β localization and their qualitative spatial overlap after treatment with RANKL, riociguat (10 μ M), rosiglitazone (1 μ M), or their combination. Spatial overlap was assessed descriptively, and no quantitative colocalization coefficient was calculated. (B) Nuclear and cytoplasmic PPAR γ immunoblots; lamin B1 and β -actin served as nuclear and cytoplasmic markers, respectively. (C) Quantification of nuclear PPAR γ abundance and the nuclear-to-cytoplasmic PPAR γ ratio. (D) PPAR γ immunoprecipitation followed by PGC-1 β immunoblotting; IgG served as a negative control. (E) Quantification of the normalized PPAR γ –PGC-1 β association. Data in C and E are mean $\pm$ SEM from n = 3 biologically independent experiments. *P < 0.05 and **P < 0.01; ###P < 0.01 for the indicated comparisons. Scale bars, 10 μ m.

Riociguat is associated with reduced energetic favorability of a modelled PPAR γ –PGC-1 β interface

We used the PPAR γ –SRC-1 complex (PDB 2PRG) to model two LXXLL-containing PGC-1 β motifs at the AF-2 coactivator surface (Figure 10A,B). Both complexes remained stable during 100-ns simulations, with similar second-half RMSD values (Motif 1, 0.286 ± 0.015 nm; Motif 2, 0.282 ± 0.017 nm). Motif 1 had a lower mean Rg (1.905 ± 0.008 versus 1.922 ± 0.011 nm) and a more favorable MM/PBSA total binding energy (-58.5 versus -46.5 kcal/mol), supporting its selection for the ternary-complex analysis (Figure 10C–E).

Riociguat was then positioned in the PPAR γ ligand-binding pocket, spatially separated from the AF-2-bound PGC-1 β motif (Figure 10F). The ternary complex remained globally stable, with only a modest RMSD increase relative to the ligand-free complex (0.305 ± 0.011 versus 0.286 ± 0.015 nm), nearly unchanged Rg (1.902 ± 0.006 versus 1.905 ± 0.008 nm), and slightly lower SASA (147.084 ± 2.213 versus 148.903 ± 2.762 nm²; Figure 10H–J). Despite this global stability, the modelled PPAR γ –PGC-1 β interface energy shifted from -58.5 to -40.1 kcal/mol after riociguat addition, with local changes in residue flexibility and energy contribution (Figure 10G,K,L). The simulations therefore support an interface-weakening hypothesis but do not demonstrate direct binding or long-range allosteric coupling.

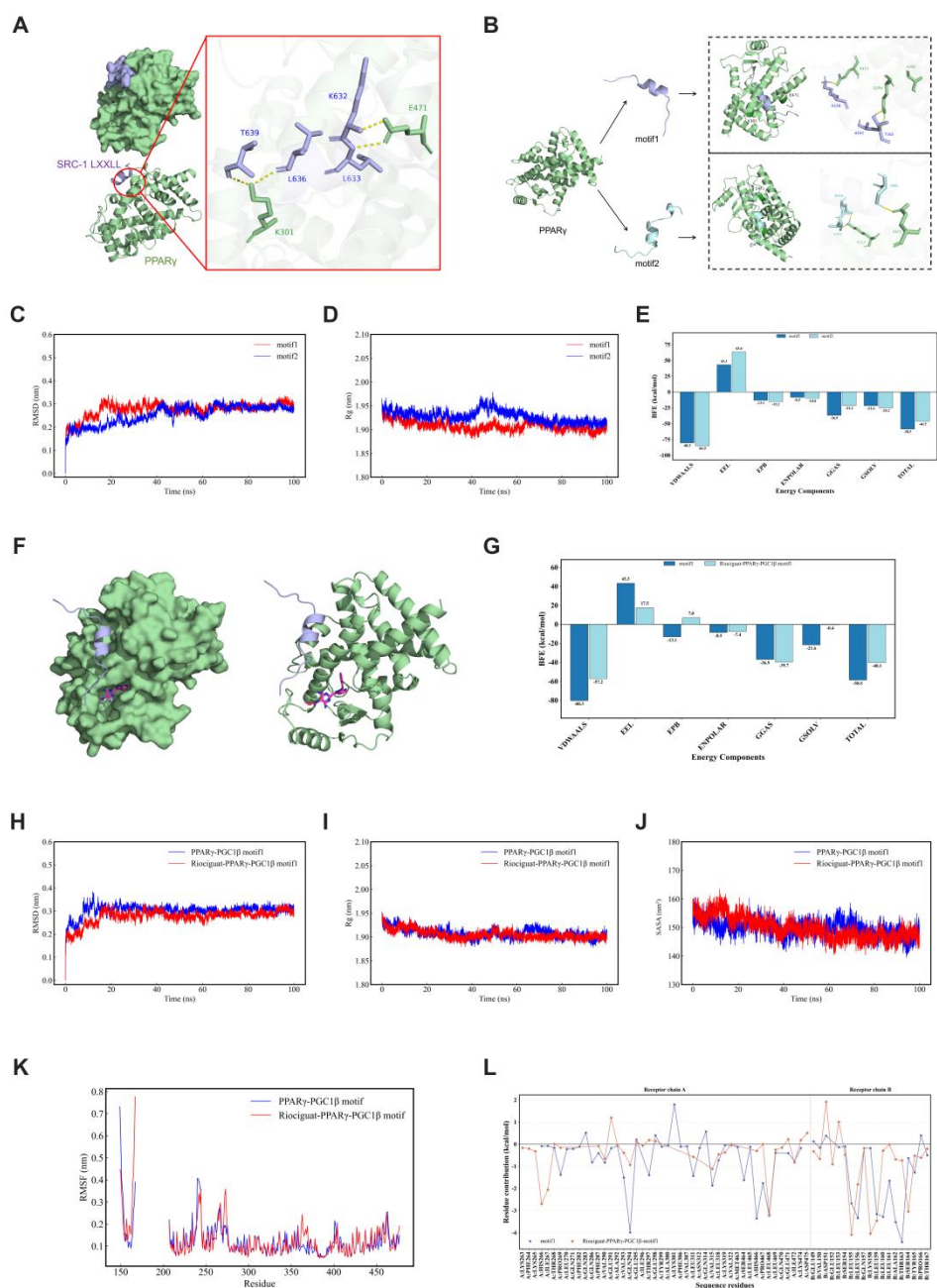


Figure 10. Structural modelling and molecular dynamics analysis of the PPAR γ -PGC-1 β complex. (A) PPAR γ -SRC-1 interface in PDB 2PRG. (B) Predicted poses of two PGC-1 β LXXLL-containing motifs. (C,D) RMSD and Rg of the motif complexes during 100-ns simulations. (E) MM/PBSA energy components of the two interfaces. (F) Modelled riociguat-PPAR γ -PGC-1 β Motif 1 ternary complex. (G) Interface MM/PBSA energy components before and after riociguat addition. (H-J) RMSD, Rg, and SASA for ligand-free and riociguat-containing complexes. (K) Residue-level RMSF. (L) Per-residue energy decomposition. Values are model dependent and are not evidence of direct target engagement.

Riociguat preserves trabecular bone and reduces osteoclast indices in ovariectomized mice

We finally assessed whether the in vitro response translated to an estrogen-deficiency model. OVX reduced trabecular bone volume and disrupted distal femoral microarchitecture relative to Sham surgery. Riociguat at 5 or 10 mg/kg per administration preserved trabecular connectivity, with the 10-mg/kg group showing the larger overall response; alendronate served as a positive control (Figure 11A). Both riociguat doses increased BV/TV and Tb.N and reduced Tb.Sp relative to OVX, whereas Tb.Th was not significantly changed (Figure 11B–E).

Histological analyses were concordant with the micro-CT findings. Riociguat reduced TRAP-positive osteoclast surface and number and improved Masson-positive and trabecular area indices relative to untreated OVX mice (Figure 11F–J). These results establish preclinical bone-protective activity in the OVX model but do not distinguish direct osteoclast effects from systemic or osteoblast-, osteocyte-, immune-, or vascular-cell-mediated actions.

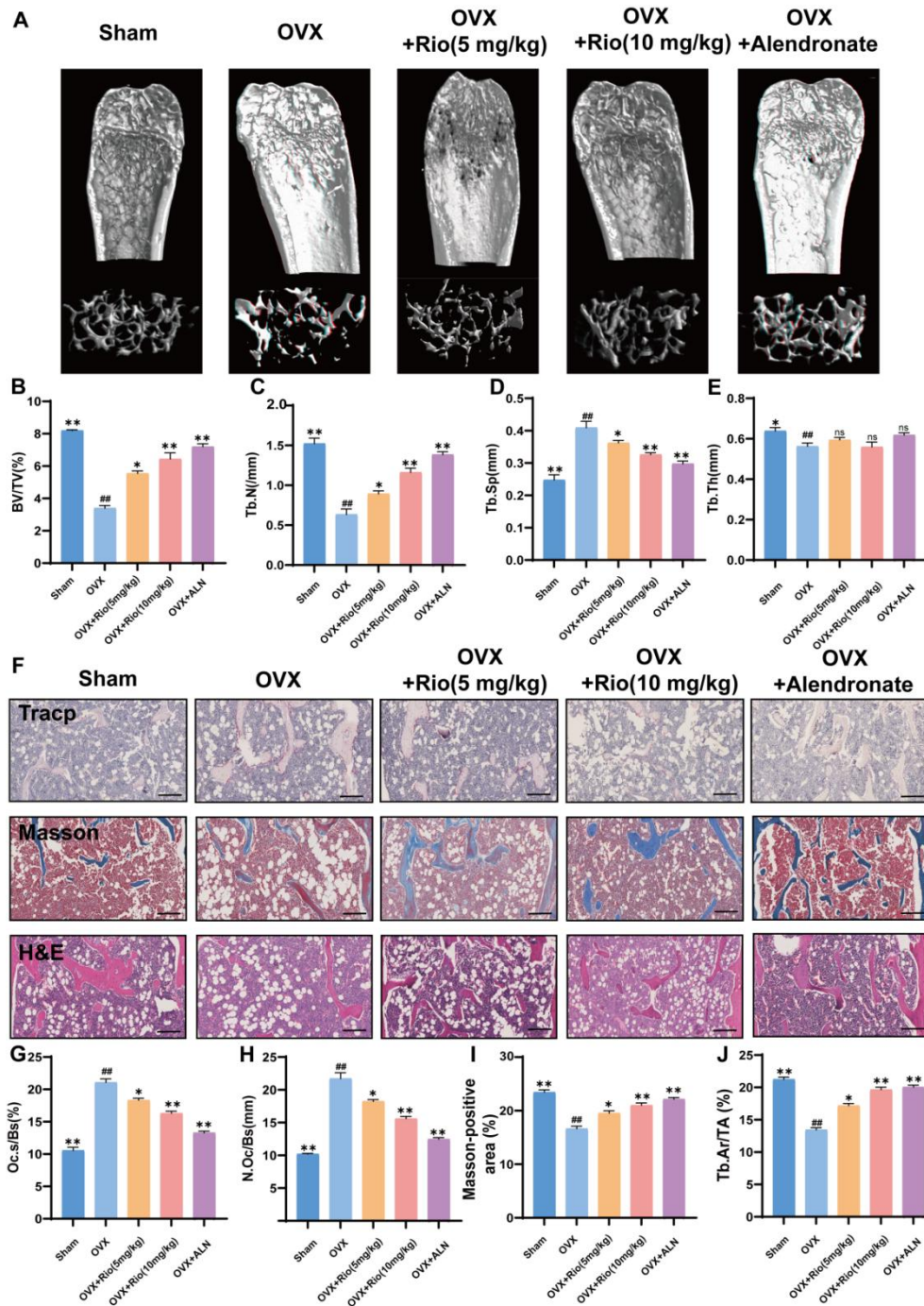


Figure 11. Riociguat attenuates OVX-induced trabecular bone loss. (A) Representative micro-CT reconstructions of distal femurs from Sham, OVX, OVX plus riociguat (5 or 10 mg/kg per administration), and OVX plus alendronate (1 mg/kg per administration) groups. (B–E) BV/TV, Tb.N, Tb.Sp, and Tb.Th. (F) Representative TRAP, Masson's trichrome, and hematoxylin-and-eosin staining. (G–J) Oc.S/BS, N.Oc/BS, Masson-positive area, and trabecular area/tissue area. Data are mean $\pm$ SEM or SD, as shown, from $n = 5$ mice per group. ### $P < 0.01$ versus Sham; * $P < 0.05$ and ** $P < 0.01$ versus OVX. Scale bars, 100 μ m.

488

489 **Discussion**

490 **Principal findings and biological significance**

491 This study establishes a coherent preclinical evidence chain linking riociguat to reduced osteoclast
492 differentiation, resorptive function, and OVX-induced trabecular bone loss. In BMMs, riociguat acted
493 without detectable cytotoxicity, was most effective during early and intermediate differentiation,
494 disrupted the F-actin apparatus, and reduced both resorption and the c-Fos/NFATc1 program. The in
495 vivo findings extended these observations by showing preservation of trabecular bone and lower
496 osteoclast indices after OVX. Together, these results support riociguat as a suppressor of pathological
497 osteoclast activation rather than merely a compound that reduces cell viability, consistent with
498 established osteoclast-centered mechanisms of estrogen-deficiency-induced bone loss [33,34].

499 **Relationship to sGC biology and the MAPK/c-Fos/NFATc1 cascade**

500 The findings add an osteoclast-focused dimension to the skeletal biology of sGC stimulation. Previous
501 work showed that the sGC activator cinaciguat improved bone architecture in ovariectomized mice,
502 with prominent effects on osteoblast-lineage cells and osteocyte survival [17]. Other interventions in the
503 NO–cGMP network have altered osteoclast differentiation, motility, or resorption [19–22]. Importantly,
504 vericiguat enhanced osteoclastogenesis at low concentrations and inhibited it at higher concentrations
505 through a balance between VASP and NF- κ B signaling [18]. Riociguat therefore should not be assumed
506 to represent a uniform class effect. Its dose window, exposure schedule, and signaling dependence
507 require direct comparison across sGC stimulators and activators.

508 The temporal data place MAPK attenuation upstream of the later differentiation phenotype. ERK, JNK,
509 and p38 are established drivers of c-Fos and NFATc1 induction during RANKL-stimulated

osteoclastogenesis [4–6]. Riociguat reduced phosphorylation of all three kinases within 10–30 min and subsequently reduced c-Fos, NFATc1, integrin β 3, and CTSK. This sequence is consistent with suppression of the MAPK/c-Fos/NFATc1 cascade. It does not, however, identify the immediate upstream event. Measurements of cGMP, PKG activity, and VASP phosphorylation, together with sGC and PKG inhibition or rescue, are needed to determine whether the MAPK response is mediated by canonical riociguat pharmacology.

PPAR γ –PGC-1 β coupling and pharmacological rescue

The PPAR γ –PGC-1 β findings require a similarly graded interpretation. Computational prioritization placed PPARG at the center of the retained interaction network, while FDR-controlled enrichment retained MAPK signaling. In the public single-cell data, Ppargc1b tracked independently defined differentiation programs, particularly at day 3, but did not correlate positively with the canonical PPAR-target module. This pattern supports a differentiation-associated coactivator context rather than generalized PPAR γ activation, and it cannot show a riociguat response because the public dataset contains no riociguat treatment. Network prediction and single-cell expression therefore remain hypothesis-generating; docking and molecular dynamics are model dependent; colocalization indicates proximity rather than binding. More directly, co-immunoprecipitation showed reduced PPAR γ –PGC-1 β association after riociguat exposure, and rosiglitazone partially counteracted the biochemical and cellular effects of riociguat. Although rosiglitazone increased MAPK phosphorylation in riociguat-treated cells, this pharmacological interaction does not establish whether the MAPK changes are upstream of, downstream of, or parallel to PPAR γ –PGC-1 β regulation. This convergence supports involvement of the complex but does not establish direct target engagement or necessity. Genetic perturbation of Pparg or Ppargc1b, PGC-1 β rescue, and orthogonal binding assays will be required to determine whether the complex is causal.

Such caution is particularly important because the endogenous role of PPAR γ in osteoclasts remains context dependent. PPAR γ deletion and rosiglitazone studies support a PPAR γ –PGC-1 β circuit that induces c-Fos and coordinates mitochondrial activation [7,8]. PGC-1 β also links mitochondrial biogenesis, fatty-acid use, and cytoskeletal organization to the high energy demands of cell fusion and bone resorption [10–14]. Conversely, PPAR γ has been reported to regulate pharmacological but not physiological or pathological osteoclast formation [9]. The present rosiglitazone interaction therefore supports pharmacological modulation of this axis but should not be read as proof that endogenous PPAR γ –PGC-1 β signaling is the sole driver of the RANKL response.

The skeletal effects of PPAR γ agonism also extend beyond osteoclasts. Thiazolidinediones reduce bone formation and increase fracture risk, particularly in women, through combined effects on bone-cell lineages and remodeling [35,36]. Riociguat cannot yet be described as a PPAR γ antagonist, and the current data do not establish that it avoids all skeletal or systemic risks associated with long-term pathway modulation. Nevertheless, the reduction in PPAR γ –PGC-1 β association distinguishes the observed response from sustained pharmacological PPAR γ activation and provides a testable mechanistic direction.

Limitations and priorities for further validation

Several limitations define the boundary of the conclusions. First, the study lacks direct biochemical target-engagement measurements and genetic tests of PPAR γ –PGC-1 β necessity. Second, canonical sGC–cGMP–PKG signaling was not perturbed or rescued. Third, the public single-cell comparison was based on one library per time point and is descriptive; cells are not biological replicates, and the retained pipeline did not apply dedicated empty-droplet, ambient-RNA, or doublet algorithms. The rare Pparg/Rxr/Ppargc1b co-detection state therefore requires independent confirmation. Fourth, the human target network and mouse single-cell data were connected only through prespecified ortholog context

and were not quantitatively pooled. Fifth, the molecular-dynamics analysis is a single model whose force-field and replication details require complete reporting before the estimates can be independently reproduced. Sixth, the OVX experiment was small and did not include dynamic bone-formation indices, systemic pharmacodynamic measurements, or cell-specific models; thus, direct osteoclast effects cannot be separated from actions on osteoblasts, osteocytes, immune cells, or the vasculature. Finally, dose, exposure, and long-term skeletal and cardiovascular safety must be defined before considering clinical translation.

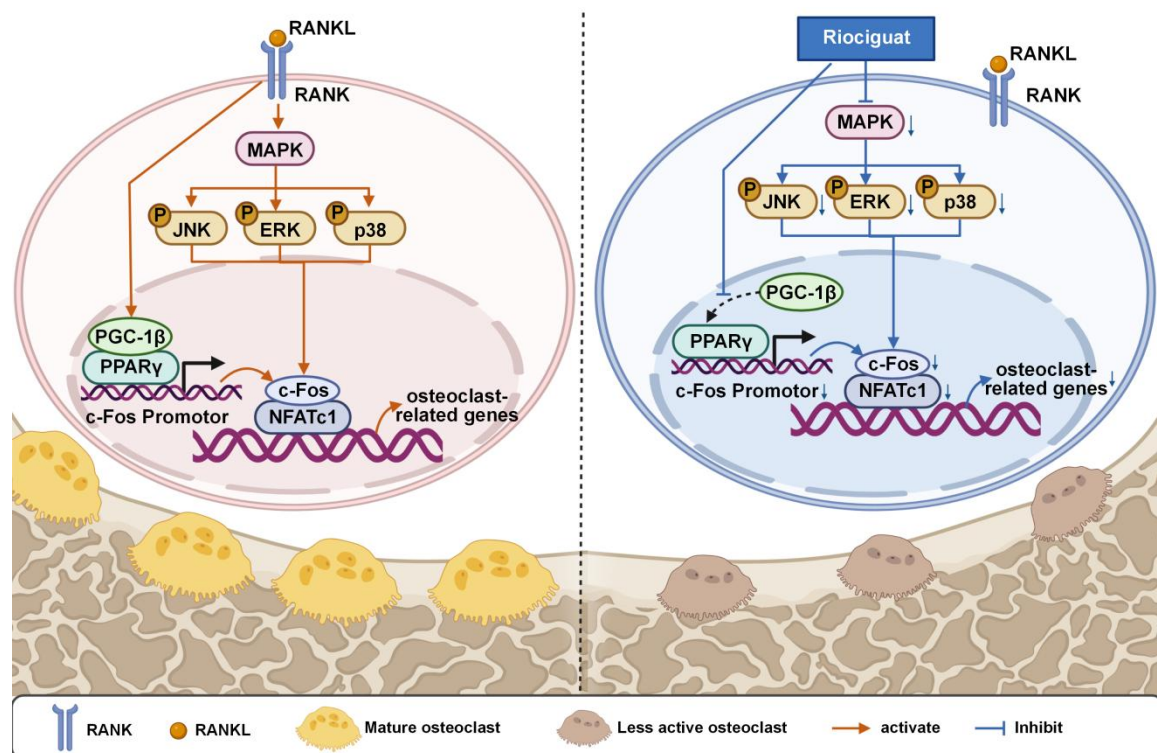


Figure 12. Proposed working model of riociguat-mediated suppression of RANKL-induced osteoclastogenesis. RANKL binding to RANK activates MAPK signaling, including the phosphorylation of JNK, ERK, and p38. In parallel, the nuclear PGC-1 β -PPAR γ complex promotes c-FOS transcription. Increased c-FOS/AP-1 activity induces NFATc1 expression, thereby activating osteoclast-related genes and promoting osteoclast differentiation and bone resorption. Riociguat attenuates MAPK phosphorylation and may reduce PGC-1 β -PPAR γ complex formation or recruitment, resulting in decreased c-FOS/NFATc1 signaling, reduced expression of osteoclast-related genes, and the formation of fewer, less-active osteoclasts. Arrows indicate activation, blunt-ended lines indicate inhibition, and dashed lines indicate proposed or indirect regulatory relationships. Created in Biorender.

Conclusions

Riociguat inhibited osteoclast differentiation and resorptive function in vitro and preserved trabecular bone in an OVX mouse model. The evidence links this response to early MAPK attenuation and reduced PPAR γ -PGC-1 β coupling, with rosiglitazone providing a pharmacological interaction and structural simulations providing a model-dependent interface hypothesis. These data support further evaluation of riociguat as a preclinical anti-resorptive repurposing candidate while defining the key next tests: direct target engagement, genetic or biochemical rescue, canonical sGC-cGMP-PKG dependence, and longer-term in vivo efficacy and safety.

List of abbreviations

α -MEM, alpha-modified minimum essential medium; AF-2, activation function-2; BCA, bicinchoninic acid; BMM, bone marrow-derived macrophage; BV/TV, bone volume fraction; CCK-8, Cell Counting Kit-8; cGMP, cyclic guanosine monophosphate; CTSK, cathepsin K; DAPI, 4',6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; FDR, false discovery rate; GEO, Gene Expression Omnibus; GO, Gene Ontology; IgG, immunoglobulin G; JNK, c-Jun N-terminal

589 kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase;
 590 M-CSF, macrophage colony-stimulating factor; micro-CT, micro-computed tomography; MM/PBSA,
 591 molecular mechanics/Poisson–Boltzmann surface area; NFATc1, nuclear factor of activated T cells 1;
 592 NO, nitric oxide; OMIM, Online Mendelian Inheritance in Man; OVX, ovariectomy; PGC-1 β , PPAR γ
 593 coactivator 1 beta; PKG, protein kinase G; PMOP, postmenopausal osteoporosis; PPAR γ , peroxisome
 594 proliferator-activated receptor gamma; PVDF, polyvinylidene fluoride; RANKL, receptor activator of
 595 nuclear factor- κ B ligand; Rg, radius of gyration; RMSD, root-mean-square deviation; RMSF,
 596 root-mean-square fluctuation; SASA, solvent-accessible surface area; SD, standard deviation; SEM,
 597 standard error of the mean; sGC, soluble guanylate cyclase; TRAP, tartrate-resistant acid phosphatase;
 598 UMAP, uniform manifold approximation and projection.

599 Declarations

600 Ethics approval and consent to participate

601 All animal procedures were approved by the Institutional Animal Ethics Committee of Wenzhou
 602 Institute, University of Chinese Academy of Sciences (approval WIUCAS23063001). Consent to
 603 participate was not applicable.

604 Consent for publication

605 Not applicable.

606 Availability of data and materials

607 The public single-cell RNA-sequencing dataset analysed during the current study is available in the
 608 Gene Expression Omnibus under accession GSE147174
 609 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147174>). Auditable quality-control

610 summaries, module-gene definitions, stratified correlations, the 29-gene target evidence matrix, and
611 FDR-filtered enrichment outputs are included in Additional file 1.

612 Competing interests

613 The authors declare that they have no competing interests

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619 Authors' contributions

620 QL ,HJ ,BX and ZX conceived and designed the study. QL , YX , YN , MS , JL , LW , HL , RW , JM ,YT , ZX
621 and BX performed the experiments and collected the data. QL, YX and YN analyzed and interpreted
622 the data. QL performed the bioinformatics and structural analyses and drafted the manuscript. YX
623 and HJ supervised the study and critically revised the manuscript. All authors read and approved the
624 final manuscript.

625 Acknowledgments

626 Not applicable.

627 Additional files

628 Additional file 1 (XLSX): Supplementary bioinformatics analyses. Contains quality-control summaries,
629 module-gene definitions, stratified correlations, the 29-gene target evidence matrix, and FDR-filtered
630 enrichment outputs.

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