Isopsoralen suppresses RANKL-induced osteoclastogenesis by inhibiting the NF-κB signaling

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13 **Abstract**

14   Osteoporosis is a serious systemic metabolic bone system disease.This study aimed to identify

15 the target genes of isopsoralen and the signaling pathways involved in the differential expression

16 of the genes involved in osteoclast differentiation. We hypothesized that isopsoralen may inhibit

17 osteoclast differentiation by blocking the NF-κB signaling pathway and verified our hypothesis

18 through basic experiments. An MTT kit was used to detect the effect of isopsoralen on the

19 proliferation and viability of primary mouse bone marrow monocytes (BMMCs). The effect of

20 isopsoralen on receptor activator of nuclear factor (NF)-κB ligand (RANKL)-induced osteoclast

21 differentiation was determined by using tartrate-resistant acid phosphatase (TRAP) staining.

22 Quantitative real-time PCR (qRT-PCR) and Western blot were used to detect the expression of the

23 related genes and proteins. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of
24 isopsoralen target genes were obtained through comprehensive analysis using the STITCH
25 database, Cytoscape 3.8.2, and R-Studio software. Differentially expressed genes (DEGs) were
26 found in osteoclasts induced by RANKL before and after 3 days using R-Studio, following which
27 KEGG analysis was performed. Next, enrichment analysis was performed on the KEGG pathway
28 shared by the target genes of isopsoralen and the differentially expressed genes during osteoclast
29 differentiation to predict the signaling pathway underlying the inhibition of osteoclast
30 differentiation by isopsoralen. Finally, Western blot was used to detect the effect of isopsoralen on
31 the activation of signaling pathways to verify the results of our bioinformatics analysis. Based on
32 the enrichment analysis of isopsoralen target genes and differentially expressed genes during
33 osteoclastogenesis, we believe that isopsoralen can inhibit RANKL-induced osteoclastogenesis by
34 inhibiting the NF-κB signaling pathway.

35 **1. Introduction**

36   Osteoporosis is a common bone metabolic disease, which is mainly characterized by the
37 destruction of bone microstructure, the reduction of bone trabecular number, the increase of bone
38 brittleness, and the fracture can occur under the action of minor external forces (*Cherubino et al.,*
39 *2011*). Osteoporosis can be divided into primary osteoporosis and secondary osteoporosis, among
40 which postmenopausal osteoporosis and senile osteoporosis are more common in primary
41 osteoporosis. Secondary osteoporosis refers to osteoporosis that occurs secondary to certain other
42 diseases or after taking certain drugs, such as diabetes, hyperthyroidism, kidney disease and other
43 systemic metabolic diseases or taking a large amount of drugs such as glucocorticoid and
44 immunosuppressive drugs (*Yong & Logan, 2021*).

45   Bone remodeling is a tightly coupled process that mainly involves osteoclasts and osteoblasts.
46 Osteoclasts are the only bone-resorbing cells in the human body, and they are essential for bone
47 remodeling (*Boyle, Simonet & Lacey, 2003*). They are multinucleated cells generated from the
48 fusion of bone marrow-derived monocyte-macrophages that are induced in the resorption zone in
49 response to macrophage colony-stimulating factor (M-CSF) and RANKL. RANKL induces the
50 secretion of H^+, Cl^-, cathepsin K (CTSK) and matrix metalloproteinase (MMP) to dissolve the
51 bone matrix (*Cappellen et al., 2002*; *Lorenzo, 2017*). Once RANKL binds to its cognate receptor
52 RANK, it binds to the adaptor protein tumor necrosis factor receptor-associated factor 6 (TRAF6)

53   (*Tanaka et al., 2005*) and then rapidly triggers a signaling cascade including nuclear factor NF-κB

54   and mitogen-activated protein kinases (MAPKs) (*Matsumoto et al., 2000*; *Li et al., 2003*; *Wada et*

55   *al., 2006*; *Lee et al., 2016*), which then activate c-Fos (*Grigoriadis et al., 1994*). Activated NF-κB

56   or c-Fos can induce the activation and expansion of nuclear factor of activated T cell cytoplasmic

57   1 (NFATc1), thereby initiating the expression of osteoclast-related genes.

58   Isopsoralen is the main active component of psoralen, a coumarin compound with antibacterial,

59   anti-inflammatory, and antitumor properties (*Wei et al., 2016*; *Lu et al., 2014*). Isopsoralen can

60   promote bone and cartilage formation (*Ge et al., 2019*), and it can stimulate the differentiation of

61   bone marrow mesenchymal stem cells into osteoblasts and inhibit their differentiation into

62   adipocytes (*Wang et al., 2017*). In addition, isopsoralen can inhibit the apoptosis of osteoblasts

63   induced by hydrogen peroxide (H_2O_2) (*Li et al., 2019*). In vivo, isopsoralen also ameliorates

64   osteoporosis caused by sex hormone deficiency in female and male mice (*Yuan et al., 2016*). This

65   suggests that isopsoralen has potential clinical value in the prevention and treatment of

66   postmenopausal osteoporosis. However, the molecular mechanism of isopsoralen is not clear.

67   Therefore, it is necessary to further elucidate the mechanism of isopsoralen in the treatment of

68   osteoporosis from the perspective of molecular biology.

69   In recent years, with the development of sequencing technology, a variety of genes and their

70   products can be associated with diseases through analysis and visualization of gene expression

71   profiles (*Zhang et al., 2022*). Gene expression varies greatly in different diseases or different

72   subtypes of the same disease. DEGs (between healthy people and those suffering from a disease)

73   can be identified through bioinformatics, and the key genes and their related molecular

74   mechanisms can also be predicted based on protein interaction and gene enrichment analysis.

75   Bioinformatic analysis has been widely used to identify disease mechanisms and search for target

76   genes.

77   In this study, we used bioinformatic analysis to identify the KEGG pathway shared by

78   isopsoralen target genes and differentially expressed genes involved in osteoclast differentiation,

79   and to obtain the core genes. We investigated the potential role of isopsoralen in the inhibition of

80   osteoclastogenesis and the specific molecular mechanisms underlying this process.

81

2. Materials and methods

2.1. Reagents

Isopsoralen (purity > 98%, **Figure 1A**) and the MTT kit were purchased from Solebao Company (Beijing, China). Alpha-modified Eagle's medium (α-MEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco (Rockville, MD, USA). TRIzol reagent was purchased from Tiangen (Beijing, China). The SYBR Green Master Mix was purchased from Imgenex (Littleton, CO, USA). Recombinant M-CSF and RANKL were obtained from R&D Systems (Minneapolis, MN, USA). The TRAP staining kit was purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-P65, anti-phospho-P65, anti-IκBα, and anti-phospho-IκBα antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-MMP-9, anti-P50/P105, and anti-phospho-P50/P65 antibodies were purchased from Abcam (Cambridge, UK). Anti-GAPDH antibody was obtained from ABclonal Technology (Wuhan, Hubei, China). Anti-NFATc1 antibody was obtained from AiFang Biological (Changsha, Hunan, China). The anti-CTSK antibody was obtained from Proteintech Group (Wuhan, Hubei, China).

2.2 Ethical use of animal

4 to 5 week-old male C57BL/6 mice were purchased from the Comparative Medical Center of Yangzhou University. All the animal experiments were designed and approved by the Yangzhou University Laboratory Animal Ethics Committee (yzu-lcyxy-n059). All animals were raised in the Yangzhou University Center for Comparative Medicine, with different groups raised in different cages. The experimental environment was managed by the Animal Center: a room temperature of 26 °C and 12 h light-dark cycles were maintained, and the breeding environment was cleaned regularly by professionals. All mice were euthanized with anesthetic at the end of the experimental. After freezing treatment, the experimental animal carcasses were sealed and handed over to the Animal Carcass Processing Center of Yangzhou University for centralized harmless treatment. There were no redundant experimental animals in this experiment.

2.3. Cell culture and cell viability assay

110   BMMCs were extracted from whole bone marrow of the mice as described previously (*Hu et*
111   *al., 2008*). In brief, femurs of 4 to 5 week-old male C57BL/6 mice were taken. After the bone
112   marrow cavity was exposed, the medium was aspirated with a sterile 1mL syringe, and the bone
113   marrow cavity was thoroughly flushed until it turned white. After collecting the bone marrow
114   cavity rinse solution, the rinse solution was filtered into a new 50mL centrifuge tube using a 100μm
115   filter. After centrifugation at 2000rpm for 5min, the cells were resuspended using primary
116   osteoclast medium supplemented with 30ng/mL M-CSF (*Yang et al., 2022*; *Liu et al., 2017*), and
117   the cells were evenly spread into a 24-well plate. Unadherent cells were washed with PBS after
118   48h, and the remaining cells were BMMCs. BMMCs were supplemented with 10% (v/v) FBS, 1%
119   (w/v) penicillin/streptomycin and 30 ng/mL M-CSF in α-MEM complete medium in an incubator
120   at 37°C and 5% CO_2 until ready for further applications. To verify the cytotoxic effect of
121   isopsoralen on BMMCs, cell proliferation and viability were measured using an MTT assay.
122   BMMCs were seeded in 96-well plates at a density of 3×10^3 cells/well and incubated with
123   different concentrations of isopsoralen (10, 30, 50, and 100 μM) and M-CSF (30 ng/mL) for 24,
124   48, and 72 h. MTT solution (10 μL/well) was added to each well, and cells were incubated for 4
125   h. The absorbance was measured at 490 nm using a microplate reader (Thermo, USA).

126

127   2.4. In vitro osteoclast formation assay

128   Whether isopsoralen affects the differentiation of BMMCs was observed using TRAP staining.
129   After the BMMCs were extracted into 24-well plates, and the cells had proliferated to 70%
130   confluence, the differentiation of BMMCs was stimulated with 50 ng/mL RANKL (*Momiuchi et*
131   *al., 2021*; *Yin et al., 2019*) and cultured with different concentrations (10, 20, and 30 μM) of
132   isopsoralen to generate mature multinucleated osteoclasts. The cells were then fixed with 4%
133   paraformaldehyde for 1 min, washed with sterile double-distilled water, and TRAP incubation
134   solution was added, followed by incubation at 37 °C for 60 min in the dark. Cells that stained dark
135   red, containing three or more nuclei, were mature osteoclasts.

136

137   2.5. Quantitative real-time PCR (qRT-PCR)

138   Real-time quantitative PCR was used to study the expression of specific genes involved in

139   osteoclast differentiation induced by isopsoralen. BMMCs were lysed with TRIzol and total RNA

140   was extracted. One microgram of total RNA was reverse-transcribed into single-stranded cDNA

141   using oligo-dT primers (Promega Corporation, Madison, WI, USA). qRT-PCR was performed

142   using the SYBR Green Master Mix. The relative mRNA levels were consistent with the expression

143   levels of the housekeeping gene, GAPDH. All data were analyzed using the $2^{-\Delta\Delta CT}$ method. Primer

144   sequences were shown in **Table 1**.

145

146   **2.6. Western blot**

147   In order to analyze the effect of protein related to osteoclast differentiation, BMMCs were

148   extracted from 24-well plates. After cell proliferation reached 70%, BMMCs differentiation was

149   stimulated by 50 ng/ml RANKL and cultured with isopsoralen at different concentrations (10, 20,

150   and 30 μM). After reaching this time point, cell lysates were collected. Whole cell lysates were

151   separated by SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding was

152   blocked with 5% nonfat milk for 1 h, and the membrane was incubated with the appropriate

153   primary antibody overnight at 4 °C. The membrane was washed three times with TBS-Tween

154   (TBST) and incubated with the appropriate HRP-conjugated secondary antibody for 1.5 h. The

155   color was developed and exposed, and the gray value of the bands was analyzed using Image J

156   software with ECL reagents and a chemiluminescence imaging system.

157

158   **2.7. Bioinformatics Analysis**

159   After predicting the target genes of isopsoralen using the default settings of STITCH, Cytoscape

160   3.8.2 software was used to construct the interaction network of isopsoralen and its target genes.

161   The enrich KEGG language package in the R-Studio software was used to enrich and analyze the

162   signaling pathways of isopsoralen target genes. We extracted the data from the GSE176265

163   database (this database uses RANKL to induce primary mouse mononuclear macrophages, and

164   collects cells for RNA-seq on the 0th, 1st, 2nd, and 3rd days of induction), we converted the gene

165   ID, DEGs were screened according to $P < 0.05$ and $|LogFC| > 2$. KEGG signaling pathway

166   enrichment analysis of DEGs during osteoclast-induced differentiation was performed using R-

167 Studio software. Subsequently, the Venn diagram online tool (Venny 2.1,

168 http://bioinfogp.cnb.csic.es/tools/venny/index.html) was used to obtain isopsoralen-targeted genes

169 shared with DEGs during the osteoclast-induced differentiation KEGG pathway analysis. The

170 enrichment information for the top five KEGG pathways with the smallest p-value was presented

171 using GOplot, an R language package that combines the visualization of expression data with

172 functional analysis. The genes involved in all five KEGG pathways were regarded as core genes.

173 We searched the osteoclast differentiation signaling pathway map on the KEGG official website

174 to identify the signaling pathways involved in the core genes.

175

176 **2.8. Statistical analysis methods**

177 All basic experiments were performed independently at least three times. SPSS software

178 (version 22.0) was used for statistical analysis, and GraphPad Prism 8 software was used to draw

179 graphs. Quantitative data were described as mean ± standard deviation, independent samples t-test

180 was used for comparison between two groups, and one-way analysis of variance was used for

181 comparison between multiple groups. Statistical significance was set to $p < 0.05$ *, $p < 0.01$**, or

182 $p < 0.001$***.

183

184 **3. Results**

185 **3.1. Effects of isopsoralen on the proliferation of BMMCs**

186 To probe the effect of isopsoralen on the proliferation and viability of BMMCs, we used an

187 MTT kit to detect cell proliferation and viability (**Figure 1B**). The results show that treatment with

188 isopsoralen (< 50 μM) for 24, 48, or 72 h did not reduce the viability of BMMCs ($P > 0.05$). When

189 the concentration of isopsoralen was greater than or equal to 50 μM, cell viability decreases

190 gradually with increasing isopsoralen concentration ($P < 0.05$). The results of this experiment

191 determined the maximum concentration of isopsoralen required for the subsequent detection of the

192 effect of isopsoralen on RANKL-induced osteoclast differentiation.

193

194 **3.2. Isopsoralen inhibits RANKL-induced osteoclast differentiation**

195   To explore whether isopsoralen could inhibit osteoclast differentiation, we plated BMMCs in
196   24-well plates in the presence of 0, 10, 20, and 30 μM isopsoralen by adding 30 ng/mL M-CSF;
197   BMMCs were treated with 50 ng/mL RANKL. In vitro osteoclast differentiation was assessed by
198   TRAP staining after five days, and TRAP-positive cells were quantified. Compared to the
199   RANKL-induced group, isopsoralen reduced the formation of mature osteoclasts in a dose-
200   dependent manner (**Figure 2A,B**).

201   To further verify the effect of isopsoralen on osteoclast differentiation, BMMC cells treated as
202   described above. After five days of induction, cell proteins were extracted for Western blot
203   experiments. After five days of induction, isopsoralen had a stronger inhibitory effect on the
204   expression of NFATc1, CTSK, and MMP9 proteins in osteoclasts at increasing concentrations
205   (**Figure 2C**). We also extracted cellular RNA to further verify that isopsoralen inhibited the mRNA
206   expression of NFATc1, CTSK, and MMP9 in osteoclasts (**Figure 2D**). The results were similar to
207   those of Western blot experiments. Isopsoralen inhibited the expression of osteoclast-related genes
208   induced by RANKL in a concentration-dependent manner.

209

210   **3.3. Target genes, interaction networks and KEGG analysis of isopsoralen**

211   To explore the mechanism by which isopsoralen inhibits osteoclast differentiation, we used the
212   default search method of the STITCH database to find 30 proteins and compounds that may act on
213   isopsoralen, 28 of which are isopsoralen target genes. Cytoscape 3.8.2 software was used to
214   establish an interaction network (**Figure 3A**). The first layer, from the inside to the outside, was
215   the interaction between isopsoralen and protein, and the second layer was the interaction between
216   protein and protein or other compounds. Cytoscape 3.8.2 software analysis obtained the degree,
217   closeness, and betweenness of each target gene in the interaction network. The degree of freedom
218   represents the degree to which a node is related to all other nodes in the network, closeness
219   centrality represents the distance between a node and other nodes in the network (*Yu et al., 2020*),
220   and spacing centrality represents the shortest node between the other two nodes (*Lee, Choi & Cho,*
221   *2018*). The next step of visualization and analysis was performed according to the degree of
222   freedom (**Figure 3B**) (the darker the color and the larger the circle, the higher the degree of
223   freedom). HDAC1, NFκB1, and NFκB2 are the three genes with the highest degrees of freedom.

224   We used the language package in the R-Studio software to perform KEGG signaling pathway
225   enrichment analysis for isopsoralen target genes and draw bubble plots (**Figure 3C**). A total of 87
226   signaling pathways (P < 0.05) were enriched. Ranking the from small to large, the top five were
227   the NF-κB signaling pathway, Toll-like receptor signaling pathway, C-type lectin receptor
228   signaling pathway, osteoclast differentiation signaling pathway, and Epstein-Barr virus infection
229   signaling pathway.

230

231   **3.4. DEGs and KEGG Analysis of Induced Differentiation of Osteoclasts**

232   We downloaded the GSE176265 dataset (transcriptomic analysis of osteoclastogenesis) from
233   the GEO database and analyzed RNA-Seq data on days 0 and 3 after induction of BMMCs with
234   RANKL through the language package in R-Studio software. After setting the screening criteria
235   at P < 0.05, and |LogFC| > 2, we obtained 174 differentially expressed genes, of which 111 were
236   downregulated and 63 were upregulated; they were heatmapped using the pheatmap R language
237   package (**Figure 3D**).

238   We used the language package in the R-Studio software to analyze the KEGG signaling pathway
239   enrichment of DEGs during osteoclast-induced differentiation and drew a bubble plot (**Figure 3E**).
240   A total of 41 signaling pathways (P < 0.05) were enriched. Ranking the p-value from small to
241   large, the top five are the neutrophil extracellular trap formation signaling pathway, systemic lupus
242   erythematosus signaling pathway, cell cycle signaling pathway, alcoholism signaling pathway and
243   programmed necrosis signaling pathway.

244

245   **3.5. KEGG pathway shared by isopsoralen target genes and DEGs during osteoclast-induced**
246   **differentiation**

247   We obtained 17 isopsoralen target genes and DEGs in the osteoclast-induced differentiation
248   process using the Venn Diagram online tool from the previously obtained isopsoralen target gene
249   KEGG pathway and KEGG pathway of osteoclast-induced differentiation process DEGs.
250   According to the order of p-value from small to large (**Table 2**), the first five were the EB virus
251   infection signaling pathway, TNF signaling pathway, IL-17 signaling pathway, human T-cell
252   leukemia virus 1 infection signaling pathway and apoptosis signaling pathway.

253

3.6. Isopsoralen inhibits RANKL-induced osteoclastogenesis by inhibiting NF-κB signaling pathway

We analyzed the top five shared KEGG signaling pathways and related genes through GOplot R package visualization and enrichment analysis to obtain the NFKB1 (P50), TNF, RELA (P65), NFKBIA (IκBα), IKBKB (IKK–β), and CHUK (IKK–α) genes that are involved in all five KEGG pathways, and therefore, the core genes (**Figure 4A**). After bringing the above six core genes into the osteoclast signaling pathway map, we found that the focus was on the NF-κB signaling pathway (**Figure 4B**); therefore, we predicted that isopsoralen inhibits osteoclasts generation by inhibiting the NF-κB signaling pathway.

After RANKL binds to its receptor, RANK, it recruits TRAF6 to its cytoplasmic domain, which subsequently activates signaling pathways such as MAPK and NF-κB to induce the expression of osteoclastogenesis genes (*Lee et al., 2005*). We examined the effect of isopsoralen on activation of the NF-κB signaling pathway. The analysis of experimental results shows (**Figure 4C**) that the levels of p-P65, p-P50, and p-IκBα in RANKL-treated BMMCs were significantly increased, while isopsoralen significantly decreased p-P65, p-P65 and p-IκBα in a time-dependent manner. These data suggested that isopsoralen inhibits RANKL-induced osteoclastogenesis by inhibiting the NF-κB signaling pathway.

271

4. Discussion

Osteoporosis is a global health concern. It is characterized by osteopenia and deterioration of the bone microarchitecture, leading to increased bone fragility and fracture risk (*Chen et al., 2021*). Osteoporosis mostly occurs in middle-aged and elderly individuals. As China has entered an aging stage, the number of osteoporosis patients has also increased annually; therefore, it is of great significance to identify relevant diagnostic and treatment actions (*Lin et al., 2015*). Osteoporosis is usually caused by an imbalance in bone metabolism in the body; therefore, osteoporosis treatment is mainly achieved by promoting bone formation and inhibiting bone resorption (*Son et al., 2019*). Many experimental studies have been conducted to treat osteoporosis by promoting the differentiation of bone marrow-derived mesenchymal stem cells into osteoblasts (*Zhi et al., 2021*)

282   and enhancing osteogenic ability (*Chen et al., 2021*); however, research on the curative effect of

283   the inhibition of the generation and activation of osteoclasts is still relatively limited. Therefore, it

284   is important to actively search for preventive drugs that can promote bone homeostasis and inhibit

285   the bone loss caused by overactive osteoclasts.

286   Isopsoralen, an important component of the traditional Chinese medicine, Psoralea, has been

287   extensively studied. Previous research on isopsoralen has focused on its antibacterial, osteogenic,

288   and anti-inflammatory effects (*Li et al., 2018*). However, there are no relevant research reports on

289   the effect of isopsoralen on osteoclastogenesis. Our results demonstrate for the first time that

290   isopsoralen does not inhibit the proliferation and viability of primary mouse mononuclear

291   macrophages when the concentration of isopsoralen is lower than 50 μM, but can significantly

292   inhibit RANKL at increasing concentrations. The induced osteoclasts differentiated, and the

293   transcription and expression of osteoclast-specific genes (NFATc1, MMP9, and CTSK) was

294   significantly inhibited.

295   Osteoclasts are derived from bone marrow-derived mononuclear macrophages and are the only

296   cells in the body that can absorb bone (*Asagiri & Takayanagi, 2007*). The survival, proliferation,

297   differentiation, and activation of osteoclasts requires the stimulation and induction of M-CSF and

298   RANKL, and the binding of RANKL to its receptor RANK which triggers the fusion and

299   differentiation of osteoclast precursor cells into mature osteoclasts (*Infante et al., 2019*). The gene

300   encoding RANKL is TNFRSF11, and when it is completely absent, mice exhibit severe

301   osteosclerosis with concomitant defects in tooth eruption owing to the complete absence of mature

302   osteoclasts (*Li et al., 2000*). RANKL signaling is also negatively regulated by osteoprotegerin

303   (encoded by TNFRSF11B), a soluble decoy receptor for RANKL that prevents RANKL from

304   binding to RANK (*Yasuda et al., 1998*).

305   Binding of RANKL to RANK induces trimerization of RANK and recruits the adaptor protein

306   TRAF6 through three TRAF6-binding sites in its C-terminal cytoplasmic tail, thereby initiating

307   downstream signaling cascades such as MAPK, NF-κB, and AP-1 (*Wang et al., 2019*). NF-κB is

308   a pleiotropic transcription factor; normally, the classical NF-κB dimer (P50/RELA) is concentrated

309   in the cytoplasm due to the inhibition of the IκB (IκBα, IκBβ, and IκBε) proteins (*An et al., 2019*).

310   TRAF6 can phosphorylate the IKK complex, and the activated IKK complex catalyzes the

311   phosphorylation of IκB protein, leading to its ubiquitination. Ubiquitinase degrades IκB protein,

312 thereby releasing the NF-κB dimer into the nucleus to regulate, in osteoclasts, the expression of

313 specific transcription factors, such as NFATc1 (*Wu et al., 2019*). NF-κB signaling is critical for

314 osteoclastogenesis, and P50/P52 double-knockout mice exhibit a pronounced osteosclerotic

315 phenotype due to the failure of osteoclastogenesis (*Franzoso et al., 1997*). Using bioinformatic

316 correlation methods, after enriching the KEGG pathway of isopsoralen target genes and

317 differentially expressed genes involved in osteoclast differentiation, we predicted that isopsoralen

318 might inhibit osteoclasts via the NF-κB signaling pathway. Subsequently, we experimentally

319 verified this prediction and demonstrated for the first time that 30 μM isopsoralen can significantly

320 inhibit the phosphorylation of P50, P65, and IκBα induced by RANKL, thereby inhibiting the

321 activation of the NF-κB signaling pathway and preventing the differentiation and generation of

322 osteoclasts.

323   There have been extensive studies on the use of isopsoralen in the treatment of osteoporosis, but

324 the mechanism of its therapeutic effect is not fully understood. Through basic experiments and

325 bioinformatics analysis, we demonstrated that isopsoralen can inhibit the differentiation and

326 generation of osteoclasts by inhibiting the activation of the NF-κB signaling pathway. Therefore,

327 isopsoralen may be a reference data for treatment of osteoporosis.

328 **5.Conclusion**

329   Collectively, our study demonstrated that isopsoralen did not affect cell proliferation and

330 viability when the concentration of isopsoralen was less than 50 μM, and that isopsoralen inhibited

331 the expression of osteoclast-related genes induced by RANKL in a concentration-dependent

332 manner. Our bioinformatics analysis verified that isopsoralen inhibited RANKL-induced

333 osteoclastogenesis by inhibiting the NF-κB signaling pathway.

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Figure 1

Effects of isopsoralen on the proliferation of BMMCs.

(A) Chemical structure of isopsoralen.**(B)**Effects of isopsoralen on the proliferation of BMMCs.The cytotoxic effect of isopsoralen was evaluated using the MTT method. BMMCs were cultured for 24, 48 and 72h in the presence or absence of varying concentrations of isopsoralen. Optical density was measured at 490 nm. *** $p < 0.001$ in comparison with the negative control group. All assays were repeated three times in triplicate.

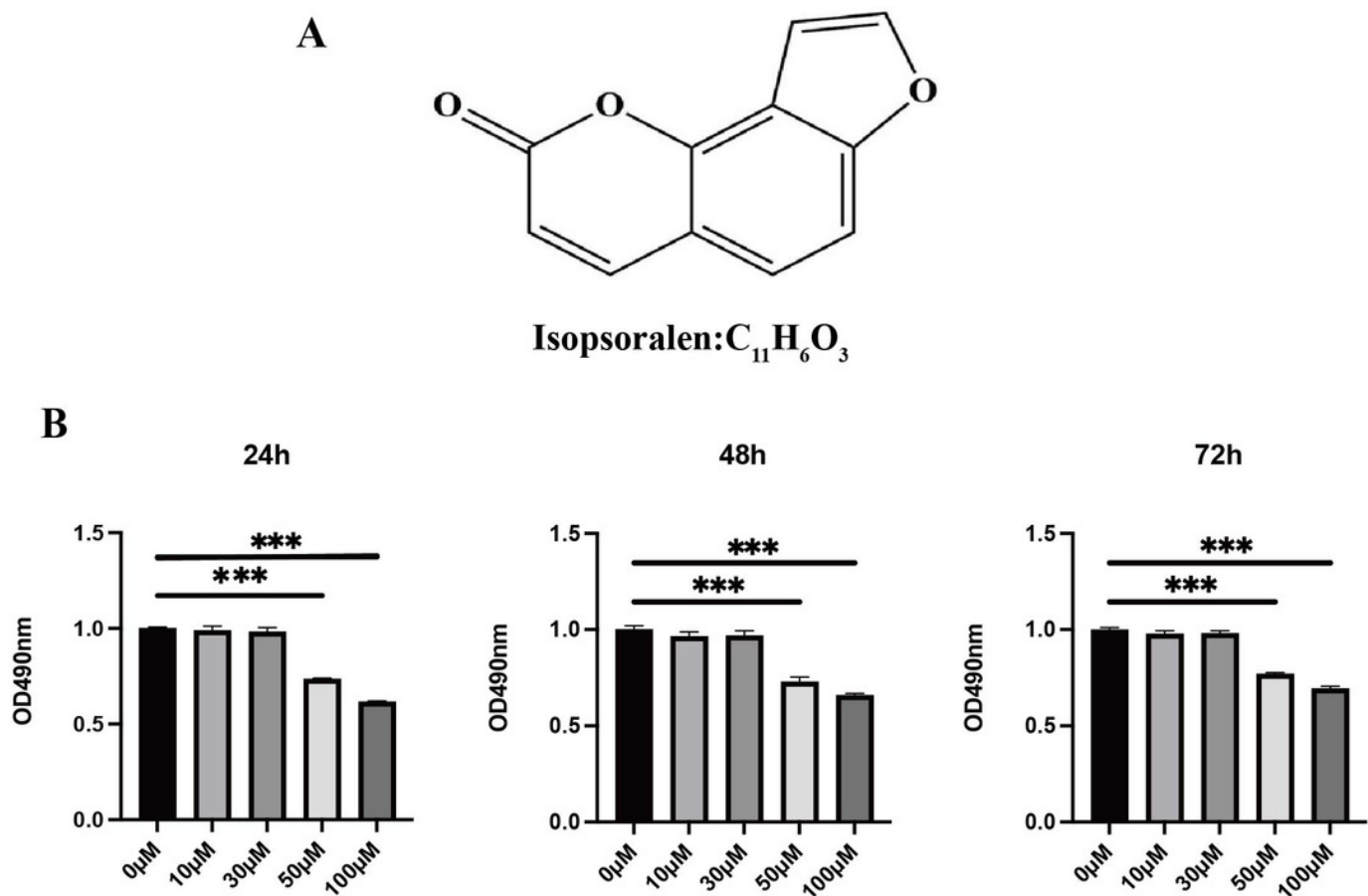



Figure 2

Isopsoralen inhibits RANKL-induced osteoclast differentiation.

(A,B) Isopsoralen reduces the formation of mature osteoclasts in a dose-dependent manner compared with the RANKL-induced group.**(C,D)** Effects of different doses of isopsoralen on the protein and mRNA expression of BMMCs with RANKL-induced.* p < 0.05,** p < 0.01,*** p < 0.001 in comparison with the negative control group. All assays were repeated three times in triplicate. Original magnification, 20×. Scale bar: 100 μm.

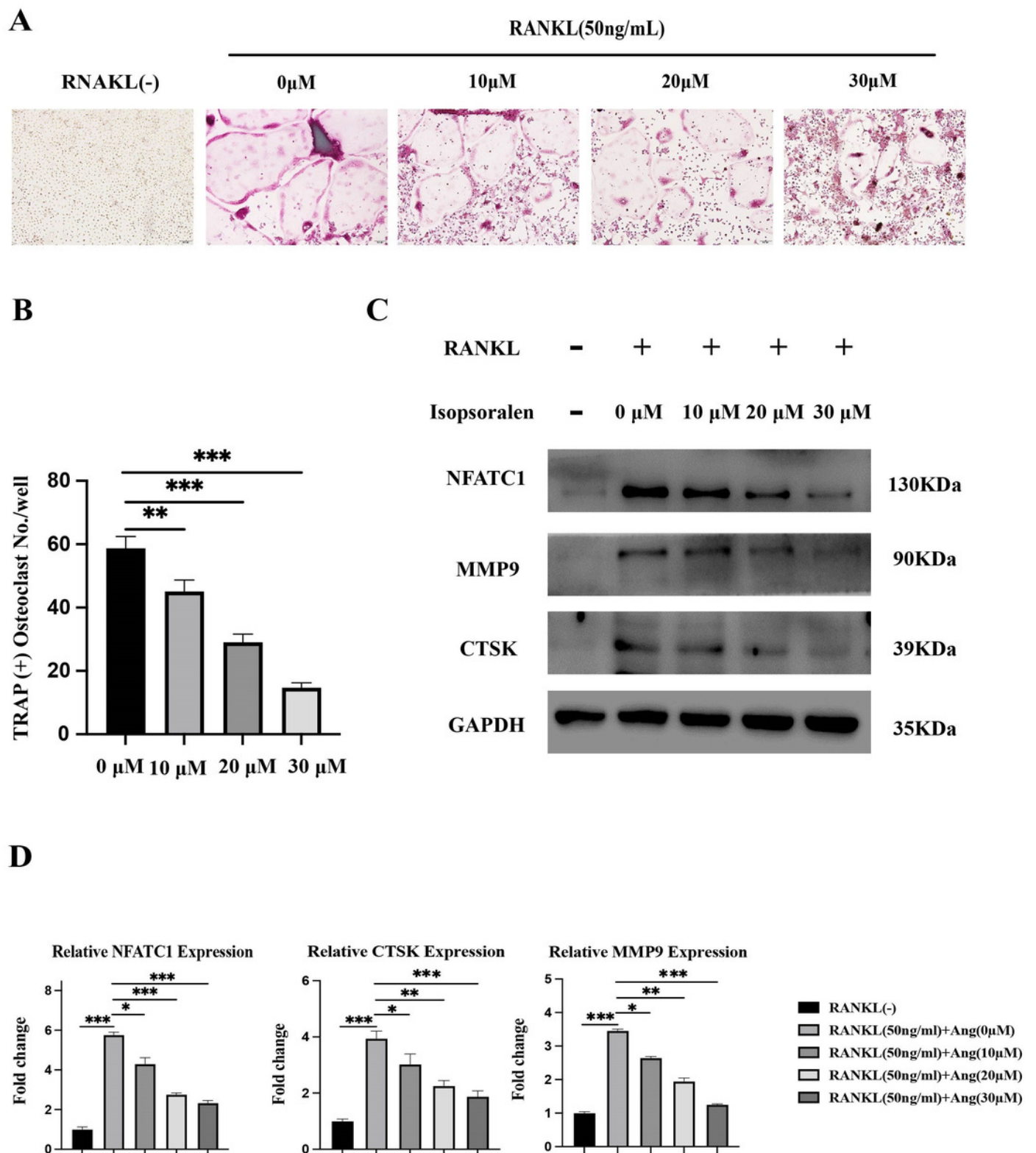
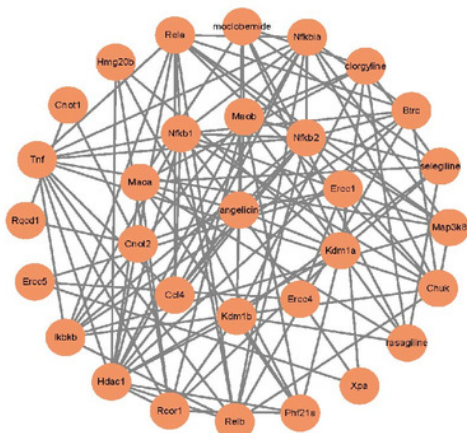
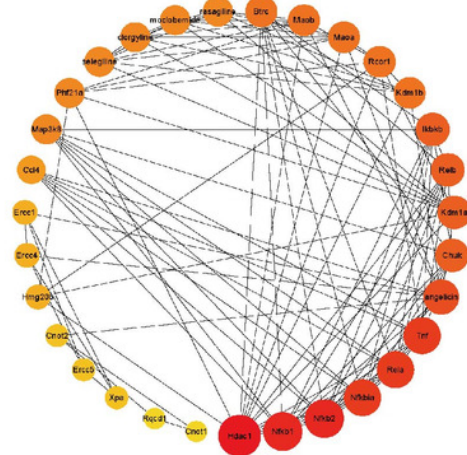
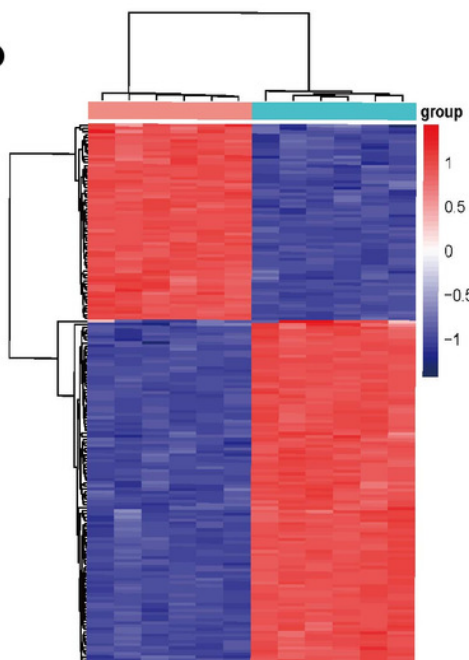



Figure 3

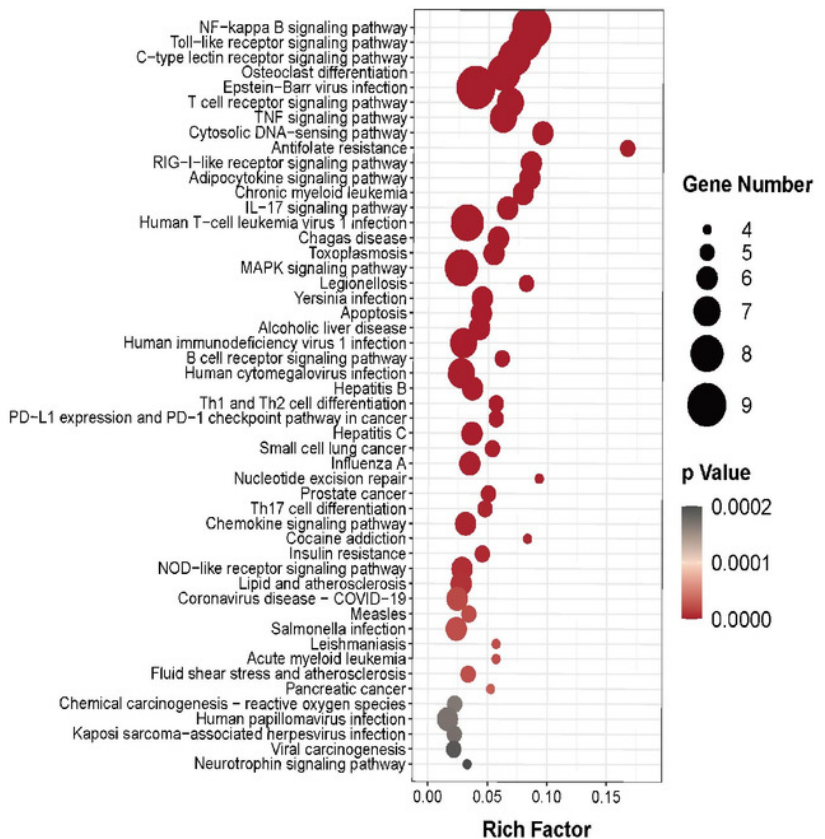
Bioinformatics analysis of isopsoralen and DEGs's KEGG analysis of induced differentiation of osteoclasts.

(A) Interaction network of isopsoralen target genes.**(B)**Visualization of the degrees of freedom of isopsoralen target genes.**(C)**KEGG analysis of isopsoralen's target gene.**(D)**Heatmap of DEGs during osteoclast-induced differentiation.**(E)**KEGG analysis of DEGs during osteoclast-induced differentiation.

A

B

C

KEGG Pathway Enrichment

D

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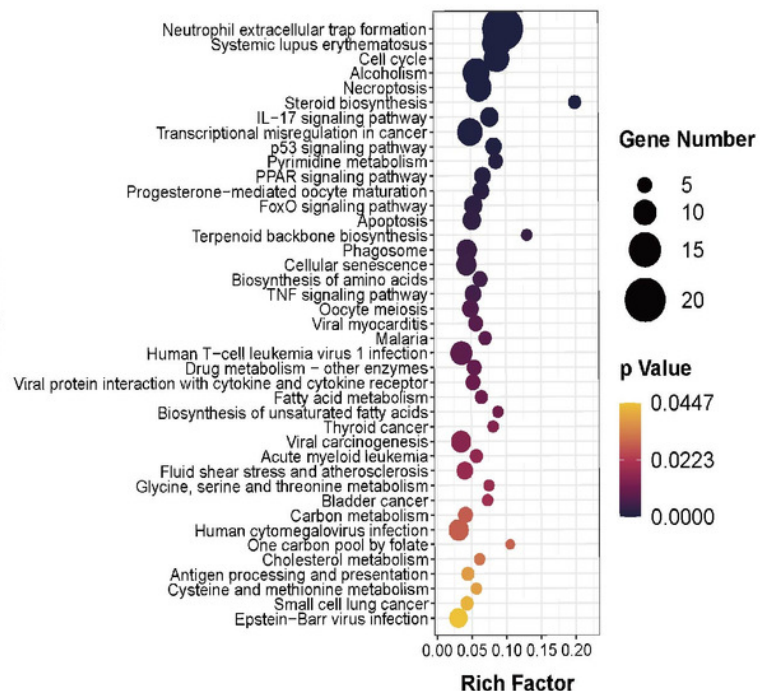


Figure 4

Isopsoralen inhibits RANKL-induced osteoclastogenesis by inhibiting NF-κB signaling pathway.

(A) Gene enrichment analysis of KEGG signaling pathway.**(B)**Positional distribution of core genes in osteoclast differentiation signaling pathways.**(C)**Representative images of Western blot used to analyze the expression of NF-κB pathway related proteins and semiquantification of the data.

A

KEGG Pathway

Degree
7 — 14

rmu05169   rmu04668   rmu04657   rmu05166
rmu04210

B

OSTEOCLAST DIFFERENTIATION

C

| | RANKL(50ng/ml) | | | | RANKL(50ng/ml)+ Iso (30μM) | | | | KDa |

(Mins)   0   10   30   60   0   10   30   60   KDa

P-IκBα — 40

IκBα — 39

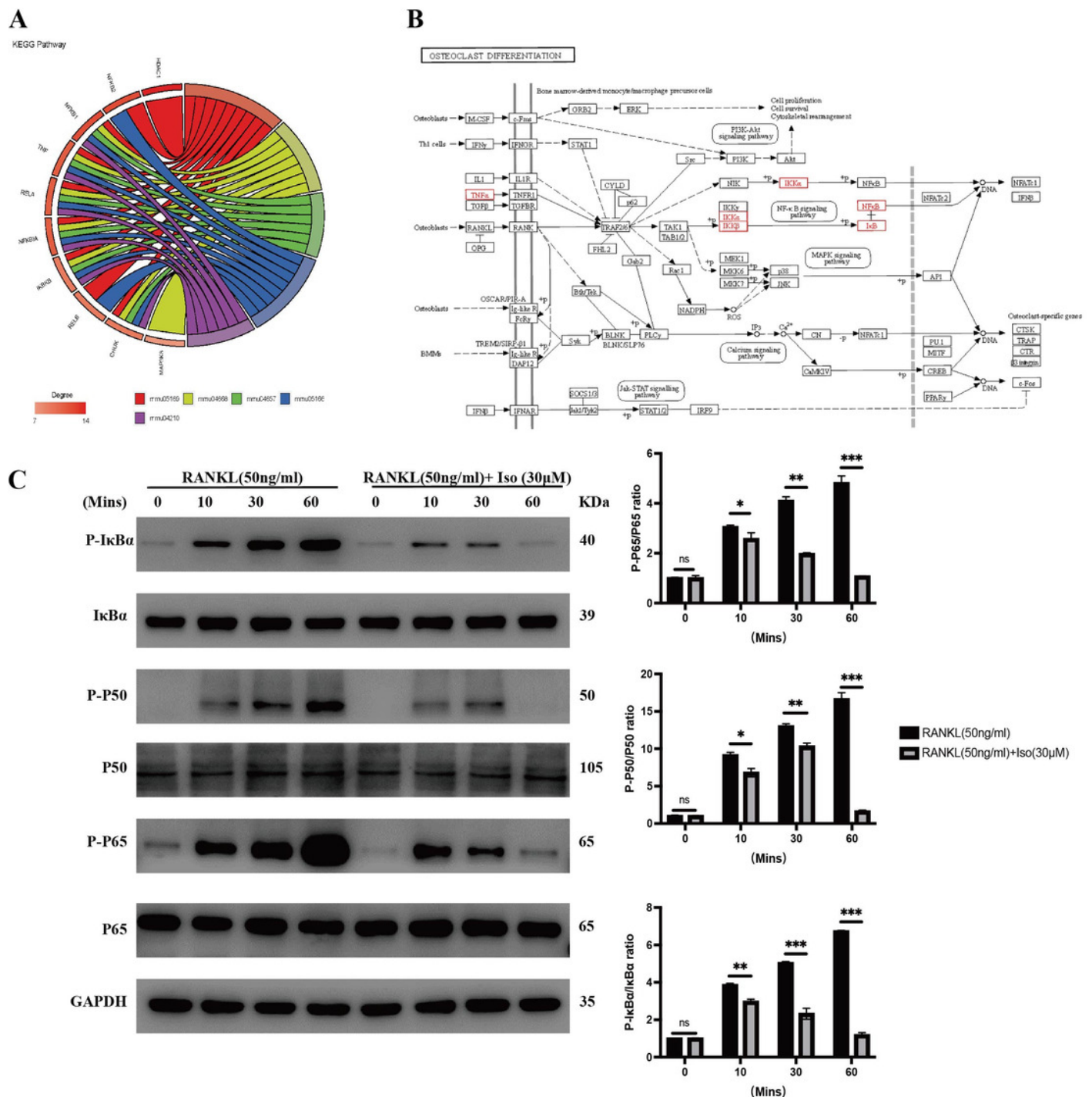
P-P50 — 50

P50 — 105

P-P65 — 65

P65 — 65

GAPDH — 35

RANKL(50ng/ml)
RANKL(50ng/ml)+Iso(30μM)

qRT-PCR primer sequences.

qRT-PCR primer sequences.

TABLE 1 | qRT-PCR primer sequences.

Primer name	Primer sequences(5'--3')
RT-ACTIN-F	GATCATTGCTCCTCCTGAGC
RT-ACTIN-R	GTCATAGTCCGCCTAGAAGCAT
RT-MMP9-F	CTGGACAGCCAGACACTAAAG
RT-MMP9-R	CTCGCGGCAAGTCTTCAGAG
RT- NFATc1-F	CAACGCCCTGACCACCGATAG
RT- NFATc1-R	GGGAAGTCAGAAGTGGGTGGA
RT-CTSK-F	GAAGAAGACTCACCAGAAGCAG
RT-CTSK-R	TCCAGGTTATGGGCAGAGATT

Shared KEGG pathways and related genes.

Shared KEGG pathways and related genes.

1 **TABLE 2 |** Shared KEGG pathways and related genes.

KEGG Pathway	Term	Isopsoralen-targeted Genes	*p* value
mmu05169	Epstein-Barr virus infection	IKBKB,TNF,RELA,NFKBIA,RELB,HDAC1,NFKB2,NFKB1,CHUK	*p*<0.001
mmu04668	TNF signaling pathway	IKBKB,MAP3K8,TNF,RELA,NFKBIA,NFKB1,CHUK	*p*<0.001
mmu04657	IL-17 signaling pathway	IKBKB,TNF,RELA,NFKBIA,NFKB1,CHUK	*p*<0.001
mmu05166	Human T-cell leukemia virus 1 infection	IKBKB,TNF,RELA,NFKBIA,RELB,NFKB2,NFKB1,CHUK	*p*<0.001
mmu04210	Apoptosis	IKBKB,TNF,RELA,NFKBIA,NFKB1,CHUK	*p*<0.001