

Identification of Kukoamine A as an Osteogenic Suppressor and Anti-osteoporosis Drug Target

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Abstract

Osteoporosis (OP) is a major and growing public health problem characterized by decreased bone mineral density and destroyed bone microarchitecture. Previous studies found that *Lycium chinense* Mill (LC) has a potent role in inhibiting bone loss. Kukoamine A (KuA), a bioactive compound extract from LC which was responsible for the anti-osteoporosis effect. This study aimed to investigate the anti-osteoporosis effect of KuA isolated from LC in treating OP and its potential molecular mechanism. In this study, network pharmacology and molecular docking were investigated firstly to find the active ingredients of LC such as KuA and the target genes of OP. And then, the anti-osteoporotic effect of KuA in vivo and vitro were investigated and the potential molecular mechanism including inflammation level, cell apoptosis, and oxidative stress were analyzed by dual-energy X-ray absorptiometry (DXA), micro-CT, ELISA, RT-PCR, and Western Blot. A total of 22 active compounds were screened, and we found KuA was identified as the highest active ingredient. PYGM was the target gene associated with a maximum number of active ingredients of LC and regulated KuA. In vivo, KuA treatment significantly increased the bone mineral density and bone structure which promote bone formation and inhibit bone resorption. In vitro, KuA significantly improves osteogenic differentiation and mineralization in cells experiment. In addition, KuA also attenuated inflammation levels, cell apoptosis and oxidative stress level. The results suggest that KuA could protect against the development of OP in osteoblast cells and ovariectomized OP model mice.

Introduction

Osteoporosis (OP) is a major and growing public health problem characterized by decreased bone mineral density and destroyed bone microarchitecture¹. With the inevitable consequence of aging, osteoporotic fractures are becoming more and more common in women over 55 and men over 65, causing huge costs in mortality and health care². Ironically, despite great advances in treatment of OP, treatment gaps varied widely for patients at high risk for OP fractures both between and within countries³. Considering the complicated mechanisms and different treatment effects of OP, a large and growing body of literature showed that anti-osteoporosis targets have become a promising research field in recent years.

In China, many traditional Chinese medicines are used to treat diabetes and have been proved to be effective. For example, *Lycium chinense* Mill. (Lycii Cortex, LC) has great potential in preventing diabetes and glucocorticoid-induced bone loss^{4,5}. However, relatively little information is available on the properties of potential hypoglycemic compounds of LC⁶. Kukoamine A (KuA), a spermidine alkaloid, is a critical bioactive component extracted from the root bark of LC. KuA has several pharmacological effects such as anti-inflammatory, anti-pain, antibacterial, neuroprotective, autoimmune enhancing and hypotensive effect⁷. Other bioactive component such as betaine, scopoline, Kukoamine B extracted from LC also showed significant effectiveness in the treatment of OP^{4,5,8,9}. However, a systematic investigation for the main bioactive components of LC contributed to OP remains unexplored.

To date, network pharmacology integrates pharmacological, bioinformatics, and other scientific analyses into a systematic network and interprets the therapeutic mechanisms of different drug components and the targets of gene delivery. Network pharmacology is a promising approach for understanding multicomponent drug systems such as Traditional Chinese Medicine (TCM) formulae¹⁰. By analyzing the components and targets of diseases, we can provide biological processes and pathways that TCM may play a role in, helping us to analyze the mechanism of TCM treating diseases. Molecular docking is a drug development method that mimics the interaction between receptors and drugs. In recent years, the use of molecular docking to elucidate the appropriate mechanism has become a global trend in drug development¹¹. Molecular function and signaling pathways by constructing a “disease-phenotype-genetic” network can suitably interpret the relationship among different bioactive components in traditional Chinese medicine^{12,13}.

The current study aimed to investigate the relationship between the potential bioactive components in LC with OP. In this regard, we firstly used network pharmacology to analyze the effective ingredients of LC, then screened the bioactive ingredients in the treatment of OP. These selected targets were evaluated by pathways of action in functional enrichment pharmacology, genetic selection (GO), biological pathways (KEGG), and molecular docking technology¹⁴. Finally, the ovariectomized OP mice with different doses of KuA and gene silencing experiments at the cell level further verified the results of network pharmacologic analysis. This study provided a theoretical basis for investigating the molecular mechanism of LC against OP. The workflow is shown in Supplement Fig. 1.

Materials And Methods

This study was approved by the ethics committee and the institutional review board of The Shanghai Tenth People's Hospital of Tongji University (IRB) (SHDSYY-2021-6420, data:2021.5.6).

Ovariectomized OP Model Mice with different doses of KuA

The experimental protocol was approved by the Department of Laboratory Animal Science of the Shanghai Tenth People's Hospital of Tongji University (SHDSYY-2021-6420, data:2021.5.6). A total of forty-five C57BL/6N female mice (8 weeks) were housed in the same animal room with a controlled temperature (22 °C) and light cycle (12 h light, 12 h dark) with free access to fresh water and food. The mice were divided randomly into five groups (n=9): (1) Sham (n=9), (2) Ovariectomized (OVX, n=9), (3) OVX + 5mg/kg/day of KuA (KuA5, n=9), (4) OVX + 10mg/kg/day of KuA (KuA10, n=9), (5) OVX + 20mg/kg/day of KuA (KuA20, n=9). The mice treated with osteoporotic intervention measures were administered one week after surgery¹⁵ during 11 weeks of administration. The bone mineral density (BMD) of the right tibia and spine was measured at 0, 6, and 12 weeks respectively after ovariectomy (0 weeks is the time of ovariectomy). All operations were performed to minimize animal suffering and reduce the number of mice used. KuA (purity $\geq$ 98%,

Liaoning University, Shenyang, China) was dissolved in normal saline and administered by the intravenous route as previous studies¹⁶. All related reagents were of analytical or pharmaceutical grade.

Each mouse used in these studies was euthanized with pentobarbital (50mg/kg, intraperitoneal injection). Meanwhile, the protocol of mice sham surgery or bilateral oophorectomy was described as before¹⁷. All mice underwent intraperitoneal anesthesia with the injection of pentobarbital (50 mg/kg), and the sham group was exposed to both sides of the ovary and raised the fatty tissue around the ovary, leaving the ovary intact, but bilateral ovariectomy for the other eighteen mice performed under the premise of complete ovarian exposure resection. After the surgery, an intraperitoneal injection of penicillin was used to prevent infection twice a day for two days.

DXA and Micro-CT analysis

At the end of 12 weeks after treatment, Lumbar spine (L4) and right tibia bone mineral density (BMD) were determined in small animals using a high-resolution soft X-ray collimator (Faxitron® LX-60 Cabinet radiography system, US). The sample was then placed on the base of the PBS scanner using μ -CT (Inveon, Siemens, Erlangen, Germany) at a spatial resolution of 55 kVp, 145 mA, integration time 300 ms, 720 views, 20 mm voxel resolution. The region of interest (ROI) was 0.36 to 2.1 mm from the right proximal epiphyseal growth plate of the tibia, 1.5 mm long, and 0.5 mm from the L4 proximal growth plate. We analyzed trabecular and cortical bone microarchitecture by measuring bone volume (BV) over total volume (TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular spacing (Tb.Sp) in the medial tibial trabecular bone and lumbar vertebrae, total cross-sectional area inside the periosteal envelope (Tt.Ar), cortical bone area (Ct.Ar), bone marrow area (Ma.Ar), average cortical thickness (Ct.Th) and cortical area fraction (Ct.Ar/Tt.Ar) in medial tibial cortical bone which has been describing in previous studies^{18,19}. μ -CT did not analyze surrounding osteophytes.

Biomechanical analysis

Following radiographic measurements, three-point flexion testing was performed on these right tibias using a mechanical testing system (Landmark, MTS, Inc., Eden Prairie, MN) to determine the mechanical properties. The main support section was 9 mm and the load range was 5 mm. The tibia was placed in a bracket with a stretched medial surface, and the distal portion of the tibiofibular junction was placed directly into the leftmost fixation device. Each tibia should be loaded at 0.01 mm/s until rupture and the load and displacement recorded. Data was automatically recorded by the material testing device. According to load-displacement curve, the biomechanical properties were evaluated to analyze compressive maximum load [a measure of the maximum force that the sample tibia withstood before fracture (N)], stiffness [the slope on the linear portion of the load-deformation curve related to the tibia's flexural rigidity (N/mm)], displacement of maximum force [a measure of the maximum displacement that the sample tibia withstood before fracture (mm)] and the energy of maximum force (area under the linear portion of the load-deformation curve (mJ) [the slope on the linear portion of the load-deformation curve related to the tibia's flexural rigidity (N/mm)]).

Western Blot

To analyze the oxidative stress level, we extracted mitochondrial and cytosolic protein. The mitochondria were isolated from cortical neurons using a Mitochondria Isolation Kit according to the manufacturer's instructions. Protein concentration was quantified using the BCA Reagent.

We performed western blot analysis with rabbit anti-caspase-3 (1:1000), rabbit anti-cytochrome c (1:500), rabbit anti-Bax (1:1000), and rabbit anti-Bcl-2 (1:1000). The protein load of each channel was detected using an anti-GAPDH antibody (1:5000) and beta-actin antibody (1:2000). Goat anti-rabbit or anti-mouse secondary antibodies (1:12,000) were used before chemiluminescent detection. Immunoblots were visualized using BeyoECL Plus. Results were expressed as a percentage of control.

ELISA analysis

We collected serum in coagulation tubes and centrifuge (3000 rpm, 15 min), and Collected the plasma and stored it at -80 °C. Determination of serum osteocalcin (OCN), Tartrated Resistant Acid Phosphatase (TRAP), C-terminal telopeptide II (CTX-II), A Lkaline Phosphatase (ALP), Procollagen I Intact N-Terminal (PINP), Interleukin-6 (IL-6), C-reactive protein(CRP), tumor necrosis factor- α (TNF- α), and Interleukin-1 β (IL-1 β)(Meimian Biotechnology, Yancheng, Jiangsu, China) were performed using commercial enzyme-linked immunosorbent assay (ELISA) kits.

Osteoblast Cells experiment

Preosteoblast MC3T3-E1 cells were cultured overnight in 48-well plates and treated with 50 g/ml ascorbic acid and 10 mM α -glycerophosphate cells for 3 weeks with or without KuA (5, 10, and 20 M). Cells were fixed with cold 70% ethanol for 10 min at room temperature, then rinsed with water. Calcium precipitation in mineralized cells was determined by staining with Alizarin Red S (Sigma-Aldrich). Alizarin S red staining was positive under light microscopy. To quantify this, cells were extracted with 10% cetylpyridinium chloride for 1 hr and seeded into 96-well plates. We measured the absorbance of the extract at 550 nm (BIO-RAD, Hercules, CA, USA)²⁰.

RT-PCR analysis and oxidative stress

Muscle and connective tissue of the distal left tibia was washed, frozen in liquid nitrogen, and stored at -80 °C. Frozen tibiae were sprayed with a Bessman tissue sprayer under liquid nitrogen. (Spectrum Laboratories, Rancho Dominguez, CA, USA). Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The expression levels of bone metabolism and inflammation-related genes, including OCN, RANKL, OPG, IL-6, and Osterix (Supplement Table1). The relative change in gene expression was analyzed by the 2- $\Delta\Delta$ CT method.

The levels of MDA, H2O2, cytochrome and the activities of MnSOD and CuZnSOD were measured using commercially available kits (CAK) according to the manufacturer's instructions.

Transient transfections of siRNA molecules

The transient transfection of siRNA molecules PYGM (Life Technologies) was performed using RNAiMAX reagent as instructed by the manufacturer (Life Technologies). Briefly, MC3T3-E1 cells were transfected into 6-well plates containing 10 nM siRNA molecule or RNAiMAX shuffling control. Cells were then maintained in 2% horse serum, differentiated for 3 days, and 1 ml Tri reagent was collected from three RNA extraction wells or 1 ml RIPA buffer (Thermo Scientific) (containing Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Scientific) for RT-PCR analysis ²¹.

Bioactive ingredients and target genes of LC

Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, <https://tcmbspw.com/index.php>) was used to analyze the ingredients of LC such as oral bioavailability (OB), drug-likeness (DL), intestinal epithelial permeability, blood-brain barrier penetrability, and water solubility. After screening the TCMSP dataset, 22 bioactive ingredients were obtained ²². In preparation for molecular adaptation, constituent SDF files of 13 active molecules (<http://pubchem.ncbi.nlm.nih.gov>) were downloaded from the PubChem database after calculation in Chemdraw 3D Ultra software.

Differential Genes analysis of OP

The genetic samples (GSM1369766) of patients with low BMD and high BMD were obtained from the GEO dataset. The 'limma' package was installed in Perl and the sample values were patched and converted to log2 (logFC). Samples with P-value < 0.005 and |log2 fold change| >1 were considered to have statistically significant and selected as differential genes. Then we created a volcano gene map from the sample and selected the top 20 most important up-down corrected genes for the heatmap.

Protein-Protein Interaction (PPI) Network

In a PPI network, the concentration (DC) of each node is the number of edges per node. The higher the degree, the higher the center position of the node. The relay center (BC) receives the location of the node among other nodes. Specifically, it is the ratio of the number of shortest paths through this node to the total number of shortest paths in the network. DC and BC reflect the influence of individual nodes on the entire network. They describe topological centrality in terms of network connectivity and controllability.

The 'biogenetic, cytoNAC' package was installed in Cytoscape 3.8.0 and was used to enter the crossover gene, and select 'Homo sapiens' parameter. Data for constructing the PPI network were sourced from six main experimental research databases: Human Protein Reference Database, Biological General Repository for Interaction Datasets, Database of Interacting Proteins, IntAct molecular interaction database, Molecular INTERaction Database, and Biomolecular Interaction Network Database. We selected this method "input nodes and its neighbors" to obtain a PPI network and performed topology analysis based on the central location of the network

LC-OP-Potential target genes network

LC-related target genes were selected from the TCMSP database based on chemical similarity and pharmacophore models. We calibrated LC-related target names to default names using the UniProt database (<https://www.uniprot.org/>) ²³. The TCMSP formula was adapted using Cytoscape web page generation software.

GO and KEGG enrichment

Go enrichment analysis examines gene function at three levels: biological process (BP), cellular component (CC), and molecular function (MF). BP mainly involves aspects of response to a steroid hormone, response to oxygen levels, and regulation of lipid metabolic process. CC is mainly related to the integral component of the postsynaptic membrane, GABA receptor complex, and GABA-A receptor complex. MF is remarkably linked with neurotransmitter receptor activity, steroid hormone receptor activity, and GABA-A receptor activity.

Firstly, we changed the names of the potential target genes from R-package (org.Hs.eg.db, version 3.8) to entrezID which helps to exclude errors caused by capitalization or abbreviations of the target names. Then, we used the R-package 'DOSE', 'clusterProfiler' and 'pathview' to visualize the biological functions of graphene oxide and analyze the enrichment of the KEGG pathway for which the p-value was <0.05 for further analysis.

Molecular Docking analysis

We used CB-Dock Internet Molecular Docking Technology to select active components of potential LC-OP target gene networks and Docked with PYGM receptors (<http://cao.labshare.cn/cbdock/>)²⁴. PYGM (protein ID is 6y5o) and the active ingredients were uploaded to the CB-Dock website. After determining the coordinates of the docking pocket, molecular docking and conformational assessment were performed using the CB docking station. The lower the VINA score, the more stable the ligand binding. Finally, receptors are screened for the binding activity of compounds to the target.

Statistical Analysis

All measurements were presented as the mean $\pm$ standard deviation (SD) and a P-value of ≤ 0.05 was considered statistically significant. The bodyweight of the time-course study was analyzed by two-way repeated-measures analysis of variance (ANOVA). Data were analyzed for the main effect and timing of the intervention. One-way ANOVA followed by Tukey multiple analysis was performed using GraphPad Prism 8.02 (La Jolla, CA, USA).

Results

KuA administration protects against OVX induced bone loss

KuA isolated from LC extracts was identified by magnetic resonance imaging (NMR) and mass spectrometry (Figure 1A). As expected, the OVX mice showed a significantly reduction of BMD in lumber 4 and right tibia at 6 weeks and 12 weeks after surgery. KuA 10mg and 20 mg administration inhibited the reduction of BMD at 12 weeks (Figure 1B, C, Supplement Figure 5).

The bone microarchitecture of the right tibia analysis revealed that KuA administration with 5mg, 10mg, and 20mg prevented the tibia-BV/TV than the OVX group at 12 weeks after treatment. Tibia-Tb.N also showed a remarkably improvement by KuA 10 mg compared with OVX group at 6 weeks and 12 weeks. However, at 12 weeks after treatment, KuA 10 mg can decrease Tibia-Tb.Sp significantly than the OVX group. In addition, KuA 5mg, 10mg, and 20mg can also increase tibia-Tb.Th significantly than the OVX group 12 weeks after treatment (Figure 2A-E, Supplement Figure 6).

Lumber 4 microarchitecture was also shown in Figure 3A. For spine Tb.N and BV/TV, KuA 5mg, 10mg, and 20mg improved significantly than the OVX group at 12 weeks, but for spine Tb.Sp and Tb.Th, KuA showed no significant difference in the OVX group at 12 weeks after treatment (Figure 3B, C, D, E).

As shown in Table 1, there was a significant reduction in Ct.Th, Tt.Ar and Ct.Ar/Tt.Ar in the OVX group compared to the control. After KuA intervention, Ct.Th improved significantly in the KuA 20 group than the OVX group. The KuA 10 and KuA 20 groups improved significantly relative to the OVX group in Ct.Ar/Tt.Ar.

For bone turnover biomarkers, KuA treatment can improve the osteogenesis-related indicators including serum OCN and ALP but decrease osteoclast-related indicators TRAP and CTX-II compared with OVX group (Table 1).

Biomechanical analysis of the right tibia at 12 weeks after treatment was shown in Figure 6F, KuA 5mg, 10mg, and 20mg administration can significantly improve the maximum of force, stiffness, displacement than OVX group (Figure 6G, Supplement Figure3A, B). however, only KuA 5mg administration can improve the energy absorption than OVX group (Supplement Figure3C).

Identification of KuA as an Osteogenic Suppressor

LC contains 22 active ingredients totally in the TCMS dataset. The target genes were then screened from these active ingredients of LC by DrugBank dataset as the conditions. There were 321 target genes obtained for LC active ingredients. The removal of duplicates after verification yielded 242 target genes (Table 2).

By comparing 30 low BMD samples and 30 high BMD samples in the GEO database. A total of 12548 differential genes were required, including 7167 up-regulated genes and 5381 down-regulated genes. After screening with a P-value < 0.005 and $|\log_2(\text{fold change})| > 1$, gene volcano map was analyzed in Figure 4A, B. PCA analysis, UMAP, and heatmap analysis was showed in Figures 1C, D, E. The differential genes in the disease samples were normally distributed, and the number of up-regulated genes was greater than the number of down-regulated genes. Table 3 listed the 20 most important up-and down-regulated genes. Using Venny 2.1 software to hybridize OP target genes and LC target genes, 24 potential target genes were obtained, as shown in Figure 5A. We found that PYGM has the highest LogFC value.(Table 4)

PPI Network and Topological Analysis

The combination of DC and BC values is an effective method for reliable monitoring important proteins²⁵. As shown in Figure 2, 723 protein nodes and 8743 edges were obtained for intersection genes. After screening with DC > 61 and a BC range of 20–113.2, the first 20 proteins were selected in Table 5 (in descending order of degree), with a total of 322 edges. Among the 20 proteins, five proteins were predicted targets of the active ingredients, with their corresponding genes including NTRK1, MCM2, CUL3, NPM1, and FN1 (Figure 5B, C, Table 5).

Construction and analysis of the LC-OP-potential Target gene network

The gene and miRNA prediction network is an important method to predict miRNA and thus has certain significance to analyze the relationship between LC-OP (Figure 6). The LC-OP-potential Target gene network was constructed by Cytoscape software (version 3.7.1). From the network of potential LC-OP target genes, a total of 42 nodes and 266 lines were derived, and coumarin A had the highest level in the process, which also explains its important role in the network.

GO and KEGG enrichment analysis

According to the KEGG enrichment results, the mechanism of active ingredients of LC in the treatment of OP mainly focuses on the interaction of neuroactive ligands with receptors, postoperative cancer, and small cell lung cancer (Figure 6B).

GO enrichment analysis also included heatmap-related select GO patients, GO cell-type signatures, GO disease, GO trust, GO paGenbase and GO transcription factor. Heatmap related select GO patients were associated with the pathway in cancer and response to the hormone. GO patents were linked with response to stimulus, metabolic process and biological regulation. SP1, RELA and NFKB1 pathway also played a key role in GO TRRUST (Figure 6C, Supplement Figure2). Additionally, the PYGM-related glycogene synthesis and degrade pathway were shown in Supplement Figure 4.

Molecular Docking

From the network of potential LC-OP target genes, five active ingredients were selected, namely KuA, Emodin, Kulactone, Alexandrine, and Acacetin (Table 6). A low Vina score indicates a stronger and more stable interaction between the compound and the receptor. Molecular docking between Active ingredients and target genes were shown in Table 7. The Vina score results of KuA, Linarin, aurantiamide acetate, and acacetin increased steadily, indicating that KuA has the strongest and most stable binding affinity for PYGM. These results suggest that KuA may be the most suitable starting material for PYGM. 3D images of acacetin, alexandrine, emodin, KuA, and Kulactone to PYGM were shown in Figure 7C.

KuA increased mRNA expression of osteoblastic differentiation-related genes in OVX mice

Figure 8A showed the mRNA expression in tibia treated with different concentrations of KuA in OVX mice. We first analyzed the expression of osteogenesis-related genes and found that KuA significantly increased OCN expression compared with the OVX group. What's more, KuA treatment reducing osteoclast expression such as RANKL, TRAP, OPG than OVX group. Finally, the PYGM was also significantly reduced in KuA group than OVX group.

KuA protects against OVX induced cell apoptosis and oxidative stress level

According to the previous study, the neuroprotective effects of KuA inhibited oxidative stress in brain injury ²⁶. In this study, we analyzed the effect of KuA administration on cellular processes such as apoptosis and oxidative stress in the OVX mice. The Bax/Bcl-2, cytochrome c, and caspase-3 levels were lower significantly in the control group and KuA administration with 20mg group than the OVX group. In addition, we also analyzed the MnSOD activity, CuZnSOD, H₂O₂ and MDA level, and it showed that the OVX group can increase MnSOD activity and CuZnSOD level but reduce H₂O₂ and MDA level than the control group. After treatment with KuA, MnSOD activity and CuZnSOD levels increased significantly than OVX group (Figure 8B-H).

KuA protects against OVX induced inflammation

KuA can also prevent inflammation in hippocampal Neurogenesis ²⁷. So we investigated the anti-inflammatory role of KuA administration in the OVX mice and found that IL-6, CRP, TNF- α , and IL-1b levels were increased significantly in OVX group than the control group. Additionally, KuA treatment can inhibit inflammation levels such as IL-6, CRP, TNF- α , and IL-1b than OVX group (Supplement Figure 3D, E, F, G).

KuA increased osteoblastic differentiation and formation of mineralized nodules in MC3T3-E1 cells

We further examined the effects of KuA on osteoblast differentiation such as cell proliferation, matrix maturation, and matrix mineralization in the MC3T3-E1 cell line ²⁸. Several conventional methods have been used to assess the effects of KuA on osteoblast differentiation, including ALP activity, cell viability, and mineralization. ALP activity plays a key role in new bone mineralization and osteoblast differentiation ^{29,30}. ALP activity in KuA treatment cells (20 μ m) was increased significantly at five days but administration with 5 and 10 μ m KuA had no significant difference at five days. After 5 days of incubation with KuA (5, 10, and 20 μ M), cell viability improved significantly compared with control group.

The bone matrix is mineralized by osteoblast differentiation, leading to the induction of calcium and phosphorus-based minerals. Therefore, bone mineralization develops with different matrix proteins. ³¹. Alizarin Red S is a commonly used histochemical method to assess calcium-rich deposits in osteoblast mineralization ³². After simultaneous treatment with KuA (20 μ M) and induction reagent, positive colonies stained with Alizarin Red S were

larger than untreated control cells. These results suggested that KuA promotes osteoblast differentiation and the formation of mineralized nodules (Figure 9).

Transient transfections of siRNA molecules

The increased differentiation of osteoblasts is closely related to the high expression of the main osteoblast marker gene ALP, OCN and Osterix. Osteoblastic MC3T3-E1 cell line treated with 20 μ M of KuA significantly increased the expression of OCN and Osterix compared to the OVX group. In addition, the PYGM mRNA level was significantly lower in the OVX group than in the control group and after treatment with KuA, the PYGM mRNA level decreased significantly than OVX group (Figure 9D).

To further explore the PYGM gene functions in osteoblast differentiation, transient transfections of PYGM siRNA molecules were investigated and we found that KuA reduced the PYGM mRNA expression level. After transfection of PYGM siRNA, the OCN and Osterix mRNA level decreased significantly than the control group. In addition, after treatment with KuA, we found that the KuA with PYGM siRNA group decreased the OCN, and Osterix mRNA levels significantly than the PYGM siRNA group (Figure 9D).

Finally, to further clarify the influence of KuA intervention on bone loss, we further verified that KuA intervention would significantly improve the progress by principal cause analysis (Supplement Figure 3H).

Discussion

OP is a systemic bone disease characterized by decreased bone mass and deterioration of bone microarchitecture with associated bone fragility and increased risk of fracture³³. Clinical vertebral and femoral fractures are the most devastating consequences of OP and are associated with morbidity and mortality^{34,35}. The mechanism of OP is related to multiple factors such as the regulation by the adaptive immune response³⁶, genetic determination³⁷, oxidative stress, apoptotic mechanisms, sex-steroid deficiency, and macroautophagy³⁸. In our study, the firstly obvious finding to emerge from the analysis was that 22 active ingredients of LC we investigated in treatment with OP were associated with a variety of proteins and signaling pathways, indicating that active ingredients play a potential role in the development of OP. And then we found that KuA plays important role in the treatment of OP through PYGM pathway. In vivo and vitro experiment, we also found that KuA improves bone loss via inflammation and oxidative stress.

The role of LC has been widely studied and has been confirmed to exert beneficial effects on improving insulin resistance, lipid metabolism, bone metabolism, and tumor progress by inhibiting inflammation and immunity^{39–41}. The bioactive ingredients included KuA and B, scopolin, aurantiamide acetate, and others which has been uploaded into Table 1. In diabetic rats, LC treatment also altered key enzymes in glucose metabolism and has been shown to improve insulin resistance, insulin sensitivity, glucose and blood metabolism, lipids and lipid cytokines, and liver function. In particular, it protects the liver from lipid degeneration⁴². Administration of aurantiamide acetate suppresses the growth gliomas by blocking autophagic flux⁴³. Scopolin also contains bioactive components used to treat and prevent OP⁸. Anxiolytic and anticonvulsant potential of stigmasterol have the positive modulation of GABA receptors and were considered to be candidates for steroidal drugs in the treatment of neurological disorders⁴⁴. A total of 22 therapeutic compounds have varying degrees of therapeutic effects on OP, mediated by a variety of cytokines and signaling pathways.

For OP, LC extract prevents OVX-induced BMD loss in mice by promoting osteoblast differentiation⁴⁰. In addition, Kukoamine B has anti-osteoporotic effects in osteoblasts, osteoclasts, and ovariectomized OP mouse models²⁰. What's more, a combined extract of *Lycii Radicis* and *Achyranthes japonica* also has an anti-osteoporotic effect⁵. However, there are few studies to analyze which active components in LC may play an important role in OP. Previous study proved the role of LC-derived substances KuA and KuB in inhibiting amyloid aggregation in Alzheimer's disease and type II diabetes⁴⁵. These findings suggested that several LC active ingredients have synergistic effects in the treatment of OP.

Analysis of GO and KEGG enrichment revealed that steroid hormone, oxygen levels, and lipid metabolic may be the mechanism of LC in treating OP, which are according with previous research^{46,47}. The pathways with the best correlation were selected here for a discussion on the mechanism of the LC treatment for OP. Our results indicated a potential mechanism for treatment LC with OP.

As can be seen from the network of potential LC-OP target genes, many target genes can be regulated by β bonds, including but not limited to PYGM, RBM47, VNN1, TSFM, and ICAM1. An online meta-analysis examining polygene expression profiles in women finds that PYGM is associated with BMD⁴⁸. These results indicated that LC has the biological characteristics of multi-component and multi-target in the treatment of OP. In addition, the PPI results showed that the 89 target proteins were not independent of each other, but were linked and interacted with each other⁴⁹. These results also suggested that LC may participate in the remission and treatment of OP by regulating various proteins, and KuA may be the most critical target.

As previously mentioned, KuA is a major bioactive component extracted from the root barks of LC which can upregulate Srebp-1c and inhibit insulin-stimulated glucose uptake and lipid accumulation in hepatic steatosis⁵⁰. It also has an anti-oxidative effect and anti-apoptosis stress in protecting the brain against injury by pMACO oxidative effect and anti-apoptosis stress¹⁶. We further investigated the effects of KuA on osteoporotic mice and cell lines. Radiographic results and mechanical tests showed that KuA significantly improved the bone microstructure and mechanical strength of osteoporotic mice and osteoblast activity.

Inflammation status regulated the progress of OP in many previous studies^{51–53}. In the absence of estrogen, fracture healing is hindered by an increase in proinflammatory cytokines such as IL-6, which may lead to poor healing⁵⁴. The progressive increase in the secretion of IL-1 β and TNF- α contributes to postmenopausal bone loss^{55,56}. In previous studies, KuA attenuated the pro-inflammatory cytokines such as IL-1 β and TNF- α levels in radiation-induced neuroinflammation²⁷. There are similarities between the attitudes expressed in our study and found that KuA alleviated the inflammation level in treatment OP.

Oxidative stress is thought to be a causative factor in many disease states, possibly including a reduction in bone mineral density in OP⁵⁷. Oxidative stress serves as potential biomarkers such as superoxide dismutase (SOD) in erythrocytes, catalase (CAT), total antioxidant status (TAS), hydroperoxides (HY), advanced oxidation protein products (AOPP), malondialdehyde (MDA), and vitamin B12 (VB12) in the etiopathophysiology and clinical course of OP⁵⁸. Following the present results, previous studies have demonstrated that KuA has the ability to anti-oxidative stress via attenuated LDH release, ROS production, MDA level, MMP loss, and intracellular Ca²⁺ + overload⁵⁹. These results are consistent with our studies that KuA reduces the oxidative stress level in OP.

Cell apoptosis was also involved in the progress of OP⁶⁰. Expression of the pro-apoptotic factor caspase-3 or the anti-apoptotic factor Bcl-2 has been shown to affect osteoblast apoptosis⁶¹. OP reduces the expression of caspase-3 and Bcl-2 in osteoblasts, thereby preventing bone loss⁶². Mitochondrial dysfunction in osteoblasts contributes to glucocorticoid-induced bone loss⁶³. KuA inhibited apoptosis induction by decreasing the level of Bax, and caspase-3 in human glioblastoma cell growth⁶⁴. Their results match with observation in our study that KuA attenuated the cell apoptosis level in OP.

PYGM plays a potential role in glycogenolysis which affects glycogen metabolism, skeletal muscle, and bone metabolism^{48,65}. In our study, PYGM also play important role in KuA treatment of OP. In vivo and in vitro experiments, the PYGM mRNA level was regulated by KuA. What's more, to further clarify the role of PYGM in KuA in the treatment of OP, we used transient transfections of siRNA molecules to study the role of PYGM in the treatment of OP. Interestingly, PYGM may be a novelty discovered biologically meaningful functional modules in the progress of KuA treatment of OP.

Conclusion

Taken together, KuA extract from LC has obvious advantages in the treatment of OP. The biological activity of the active substance of LC and the signal transduction pathway of the target OP gene was studied by network pharmacology method and molecular binding test. Meanwhile, this is the first study to investigate the anti-osteoporotic effect of KuA in vivo and vitro. The results suggest that KuA could be a good candidate for treating and preventing OP.

Declarations

Ethics approval and consent to participate

No application

Consent for publication

We all agree to publication.

Data Availability statement

The data used to support the findings of this study are included within the article.

Competing interests

The authors declare no conflict of interest.

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Author Contributions

Conception and design: ZYG, SFL

Acquisition, analysis, and interpretation of the data: ZYG, SFL, LYL, ZQG

Drafting and writing: ZYG, KT

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Tables

Table 1. Changes in serological markers and cortical bone indicators in KuA-intervened osteoporotic mice

Characteristics	Groups	Control	OVX	KA5	KA10	KA20
Bone turnover biomarkers	OCN(ng/ml)	3.22±0.25 [^] &	1.16±0.15	1.65±0.21 [^] #	2.36±0.24 [^] # [^]	3.62±0.28 [^] &
	TRAP(ng/ml)	10.62±0.698 [*]	12.64±1.59	11.96±1.85#	10.12±2.61 [*]	10.06±1.82 [*]
	CTX-II (ng/ml)	0.081±0.001 [*]	0.086±0.002	0.085±0.003	0.084±0.001	0.084±0.002 [*]
	ALP(ng/ml)	1.26±0.26 [^] &	0.88±0.14	0.95±0.08#	0.99±0.21#	1.11±0.19 [^]
	PINP(ng/ml)	0.86±0.11 [*]	0.61±0.09	0.67±0.03#	0.72±0.12	0.78±0.15 [^]
Cortical bone indicators	Ct.Th(mm)	0.14±0.06 [*]	0.11±0.03	0.12±0.05#	0.12±0.04#	0.13±0.02 [*]
	Tt.Ar(mm ²)	1.35±0.36 [*]	1.62±0.15	1.58±0.23	1.54±0.13	1.42±0.42 [^]
	Ct.Ar(mm ²)	0.68±0.03	0.72±0.08	0.69±0.04	0.69±0.07	0.68±0.05
	Ct.Ar/Tt.Ar(%)	62.88±11.62 [*]	42.69±12.95	51.67±12.86#	58.61±14.62 [*]	62.59±21.61 [^] &

#compare with control group. * compare with OVX group. ^ compare with KA5 group. & compare with KA10 group. Tt.Ar, total cross-sectional area inside the periosteal envelope, Ct.Ar, cortical bone area, Ct.Th, average cortical thickness, Ct.Ar/Tt.Ar, cortical area fraction, KuA5, OVX + 5mg/kg/day of KuA, KuA10, OVX + 10mg/kg/day of KuA, KuA20, OVX + 20mg/kg/day of KuA,

Table 2. The total available compounds of Lycium chinense Mill.												
Index	Mol ID	Molecule Name	MW	AlogP	Hdon	Hacc	OB(%)	Caco-2	BBB	DL	FASA-	HL
9	MOL002224	aurantiamide acetate	444.57	4.526	2	6	58.38079	0.40909	-0.22162	0.5883	84.5	7.035985
5	MOL002218	scopolin	354.34	-0.288	4	9	56.44689	-1.05198	-1.75118	0.38714	138.82	3.006583
1	MOL001552	OIN	289.41	1.721	1	4	45.97058	0.43143	0.09193	0.19317	49.77	4.469495
11	MOL002228	Kulactone	452.74	6.233	0	3	45.43808	0.85591	0.1607	0.81578	43.37	5.521435
16	MOL000449	Stigmasterol	412.77	7.64	1	1	43.82985	1.44458	1.00045	0.75665	20.23	5.574595
2	MOL001645	Linoleyl acetate	308.56	6.847	0	2	42.10077	1.35826	1.08413	0.19845	26.3	7.478521
7	MOL002221	Kukoamine A	530.74	1.78	8	10	42.0846	-0.20726	-2.11576	0.56398	163.18	0
4	MOL001790	Linarin	592.6	-0.178	7	14	39.84373	-1.68135	-2.76521	0.70925	217.97	16.06778
22	MOL000953	CLR	386.73	7.376	1	1	37.8739	1.43101	1.12678	0.67677	20.23	4.518834
14	MOL000296	hederagenin	414.79	8.084	1	1	36.91391	1.31876	0.96428	0.75072	20.23	5.347511
15	MOL000358	beta-sitosterol	414.79	8.084	1	1	36.91391	1.32463	0.98588	0.75123	20.23	5.355491
8	MOL002222	sugiol	300.48	4.987	1	2	36.11353	1.14054	0.69922	0.27648	37.3	14.61994
3	MOL001689	acacetin	284.28	2.585	2	5	34.97357	0.67146	-0.04689	0.24082	79.9	17.24847
6	MOL002219	Atropine	289.41	1.996	2	4	34.52789	0.15341	-0.29661	0.21417	60.77	3.117746
18	MOL000472	emodin	270.25	2.492	3	5	24.39832	0.22289	-0.66096	0.23916	94.83	0
20	MOL000008	apigenin	270.25	2.334	3	5	23.06216	0.4256	-0.6109	0.21306	90.9	0
19	MOL000476	Physcion	284.28	2.743	2	5	22.2864	0.52191	-0.40229	0.26659	83.83	0
13	MOL000295	alexandrin	576.95	6.337	4	6	20.63194	-0.1993	-0.80971	0.62697	99.38	0
10	MOL002226	lyciumin A	874.02	-0.238	10	20	10.07929	-2.51813	-2.66337	0.1977	306.7	0
21	MOL000880	Tricosane	324.71	10.864	0	0	8.33048	1.84718	1.6799	0.2088	0	0
12	MOL002229	HEPTACOSANE	380.83	12.689	0	0	8.18071	1.87942	1.80044	0.36155	0	0
17	MOL000458	campesterol	400.76	7.972	1	1	5.568613	1.59665	1.40847	0.71577	20.23	0

Table 3. The first 20 genes that are upregulated and downregulated.

Gene names	LogFC	P value	Regulation direction
LCN2	-0.92606	0.003277	Down
CAMP	-0.89523	0.00297	Down
OLFM4	-0.88312	0.007425	Down
DEFA4	-0.88296	0.00825	Down
CEACAM8	-0.82947	0.024539	Down
LTF	-0.8221	0.013364	Down
CRISP3	-0.80266	0.008933	Down
CD24	-0.77216	0.017416	Down
MMP8	-0.72018	0.008758	Down
ELANE	-0.67617	0.001989	Down
HAB1	-0.66318	2.75E-05	Down
NCKAP1L	-0.57676	1.55E-07	Down
EGR2	-0.55255	0.012038	Down
HP	-0.5232	0.000704	Down
ARG1	-0.4947	0.007518	Down
PGLYRP1	-0.49116	0.000471	Down
GRINA	-0.49006	0.001152	Down
MFSD10	-0.4782	6.16E-07	Down
HSPB1	-0.4754	4.22E-05	Down
CTSD	-0.46251	2.28E-05	Down
YIPF5	0.308376	0.00068	Up
PARP8	0.310742	0.001162	Up
HIST1H2AC	0.313757	0.077778	Up
IFRD1	0.315896	0.006762	Up
ERGIC2	0.316251	0.000205	Up
FOXO3	0.318552	5.93E-07	Up
LARP7	0.319056	8.96E-05	Up
NCOA1	0.327933	5.32E-08	Up
KIAA1551	0.345275	0.001193	Up
MAN2A1	0.345422	1.54E-05	Up
GZMB	0.349538	0.022463	Up
TAOK3	0.35036	8.64E-06	Up
RUFY3	0.350575	0.000429	Up
PPWD1	0.360551	5.56E-05	Up
PPIG	0.376994	0.000203	Up
CACNA2D3	0.377052	8.98E-05	Up
ZNF91	0.380684	5.14E-05	Up
ERAP2	0.394709	0.11964	Up
DPP8	0.401369	3.81E-07	Up
FOLR3	0.59427	0.001779	Up

Table 4: The 20 intersection genes sorted by logFC.

Intersection gene	LogFC	P value
BTBD18	-0.000555667	0.983394254
DRD3	0.002213333	0.932333004
SYCE1L	0.002595333	0.885291809
ATP1A1	0.010409333	0.839478969
POLR2D	-0.010996333	0.746612769
ICAM1	-0.020975667	0.696896549
TNFAIP8	-0.041541333	0.569759794
RBM47	-0.045589667	0.455734347
VNN1	0.193443667	0.337112369
TSFM	0.047431667	0.256808881
NRXN1	0.039595	0.148943914
FAM186A	0.040678	0.059556681
MFNG	-0.109743	0.051251272
TMEM212	-0.052631333	0.040565698
TPST2	-0.154919333	0.030568761
RPS11	-0.095648333	0.014220426
GABRD	-0.096749333	0.012048201
NTS	-0.08813	0.008618444
TMEM120B	-0.080779	0.005985507
PYGM	0.102682333	0.000930636

Table 5. Topological analysis results by degree—the first 20 proteins

Gene names	Annotation	ClosenessCentrality	Degree
NTRK1	neurotrophic receptor tyrosine kinase 1	0.582762	218
MCM2	minichromosome maintenance complex component 2	0.574324	210
CUL3	cullin 3	0.557638	197
NPM1	nucleophosmin	0.555556	181
FN1	fibronectin 1	0.555037	188
HNRNPU	heterogeneous nuclear ribonucleoprotein U	0.5494	170
ESR1	estrogen receptor 1	0.537975	167
CDK2	cyclin dependent kinase 2	0.537975	154
RPS11	ribosomal protein S11	0.534591	171
RPS3A	ribosomal protein S3A	0.534111	148
ITGA4	integrin subunit alpha 4	0.533632	164
RPS3	ribosomal protein S3	0.533632	150
CAND1	cullin associated and neddylation dissociated 1	0.533154	165
RPS4X	ribosomal protein S4, X-linked	0.530303	144
COPS5	COP9 signalosome subunit 5	0.524691	148
RPS14	ribosomal protein S14	0.524691	146
RPS16	ribosomal protein S16	0.524229	143
VCAM1	vascular cell adhesion molecule 1	0.523307	146
CUL1	cullin 1	0.519197	153
ICAM1	intercellular adhesion molecule 1	0.510292	161

Table 6. Molecular docking parameters and results of seven active ingredients in LC binding with PYGM.

Molecule name	Vina scores	Cavity size	Center			Size		
			x	y	z	x	y	z
OIN	-7.8	1837	5	23	29	21	21	27
Kukoamine A	-12.3	1598	31	24	30	36	36	36
Linarin	-11.3	1598	31	24	30	24	24	24
aurantiamide acetate	-10.1	1598	31	24	30	29	23	23
acacetin	-10	1598	31	24	30	29	21	21
apigenin	-9.8	1598	31	24	30	29	21	21
Kulactone	-8.9	1484	26	3	51	24	24	24
emodin	-8.9	1598	31	24	30	29	19	19
scopolin	-8.6	1598	31	24	30	29	21	21
Physcion	-8.6	1598	31	24	30	29	20	20
Stigmasterol	-8.5	1484	26	3	51	25	25	25
lyciumin A	-8.4	1484	26	3	51	28	28	28
hederagenin	-8.2	1484	26	3	51	25	25	25
alexandrin	-7.9	1837	5	23	29	29	29	29
Atropine	-7.8	1837	5	23	29	21	21	27
sugiol	-7.7	1484	26	3	51	26	29	20
beta-sitosterol	-7.7	1484	26	3	51	23	29	23
campesterol	-7.7	1484	26	3	51	24	24	24
Linoleyl acetate	-7	1598	31	24	30	29	29	29
HEPTACOSANE	-7	1598	31	24	30	31	31	31
CLR	-6.7	1598	31	24	30	36	36	36
Tricosane	-6.4	1598	31	24	30	28	28	28

Table 7. Vina score of LC active components to the target gene molecules

active components	Genes- Vina score			
	PYGM	TPST2	RPS11	GABRD
acacetin	-10	-9	-7.4	-7.8
alexandrin	-7.9	-7.6	-8.4	-7.7
apigenin	-9.8	-8.2	-7.3	-7.7
Atropine	-7.8	-8.2	-6.8	-7.3
aurantiamide acetate	-10.1	-7.4	-8.2	-7.3
beta-sitosterol	-7.7	-7.3	-7.7	-7.3
campesterol	-7.7	-7.4	-7.3	-7.6
CLR	-6.7	-5.7	-6.2	-5.9
emodin	-8.9	-9.4	-8.3	-8.1
hederagenin	-8.2	-7.6	-8	-7.5
HEPTACOSANE	-7	-5.4	-5.6	-5.5
Kukoamine A	-12.3	-7.3	-6.5	-6.8
Kulactone	-8.9	-7.9	-8.5	-9.4
Linarin	-11.3	-9	-9.1	-8.8
Linoleyl acetate	-7	-6.1	-5.9	-5.4
lyciumin A	-8.4	-8.5	-9.8	-8.7
OIN	-7.8	-8.2	-6.9	-7.4
Physcion	-8.6	-9.6	-7.5	-8.3
scopolin	-8.6	-9	-7.9	-7.2
Stigmasterol	-8.5	-7.4	-8.1	-7.3
sugiol	-7.7	-6.8	-7.4	-7.5
Tricosane	-6.4	-5.3	-4.6	-5.4

Figures

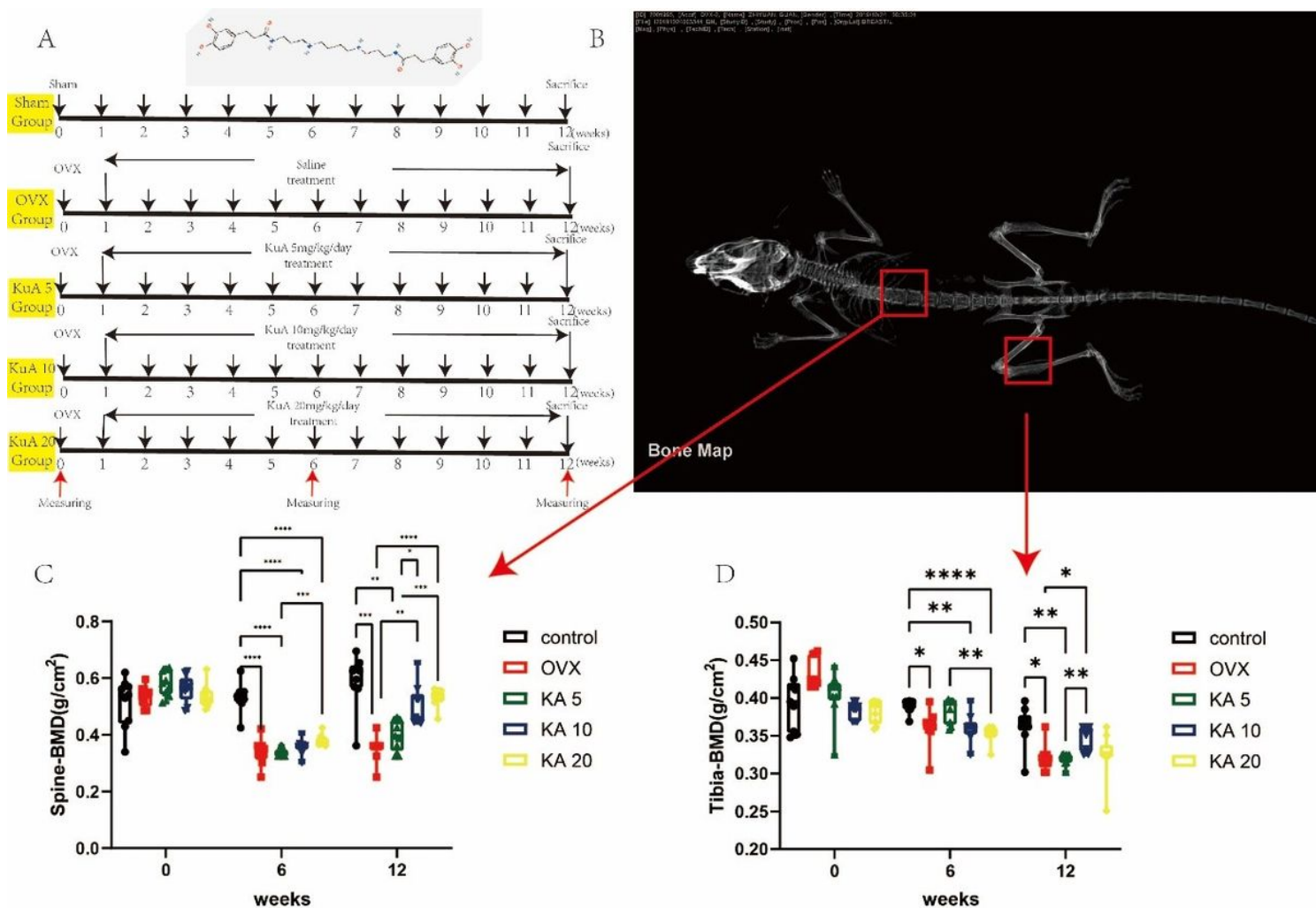


Figure 1

KuA significantly increased the bone mineral density in OVX mice. (A) KuA spectrometry analyses and experiment flowchart. (B) bone mineral density (BMD) of spine (Lumber 4). (C) bone mineral density (BMD) of tibia.

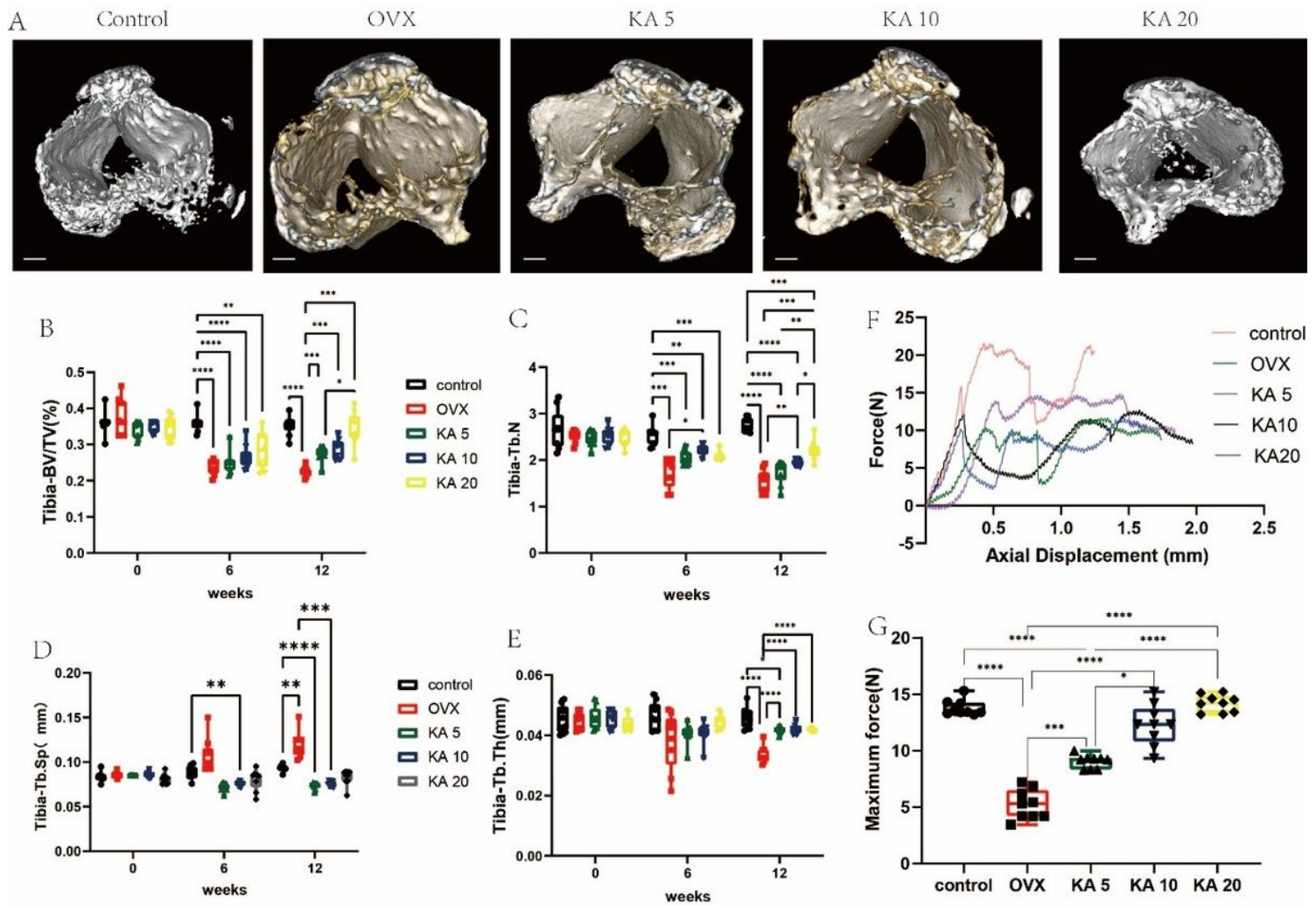


Figure 2

KuA significantly increased the tibia bone microstructure and mechanical properties in OVX mice. (A) Representative figure of 3D in tibia. (B) BV/TV of tibia. (C) Tb.N of tibia.(D) Tb.Sp of tibia.(E) Tb.Th of tibia.(F,G) maxium force of tibia. The bar is 0.7mm. *:P<0.05, **:P<0.01,*** :P<0.001,****:P<0.0001.

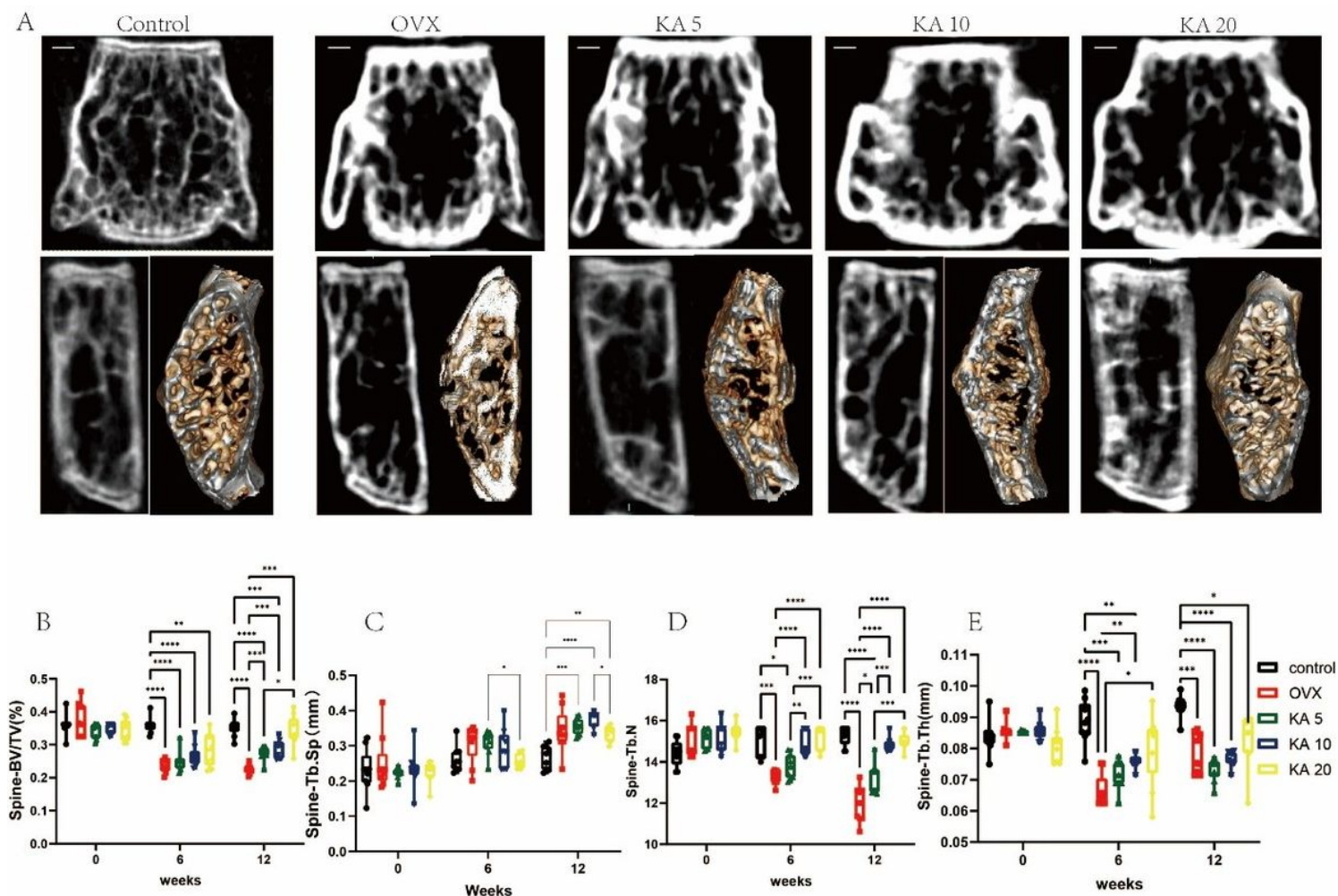


Figure 3

KuA significantly increased the bone microstructure of spine in OVX mice. (A) Representative figure of 3D in lumbar 4. (B) BV/TV of lumbar 4. (C) Tb.Sp of lumbar 4.(D) Tb.N of lumbar 4. (E) Tb.Th of lumbar 4. The bar is 0.3mm. *:P<0.05, **:P<0.01,*** :P<0.001,****:P<0.0001.

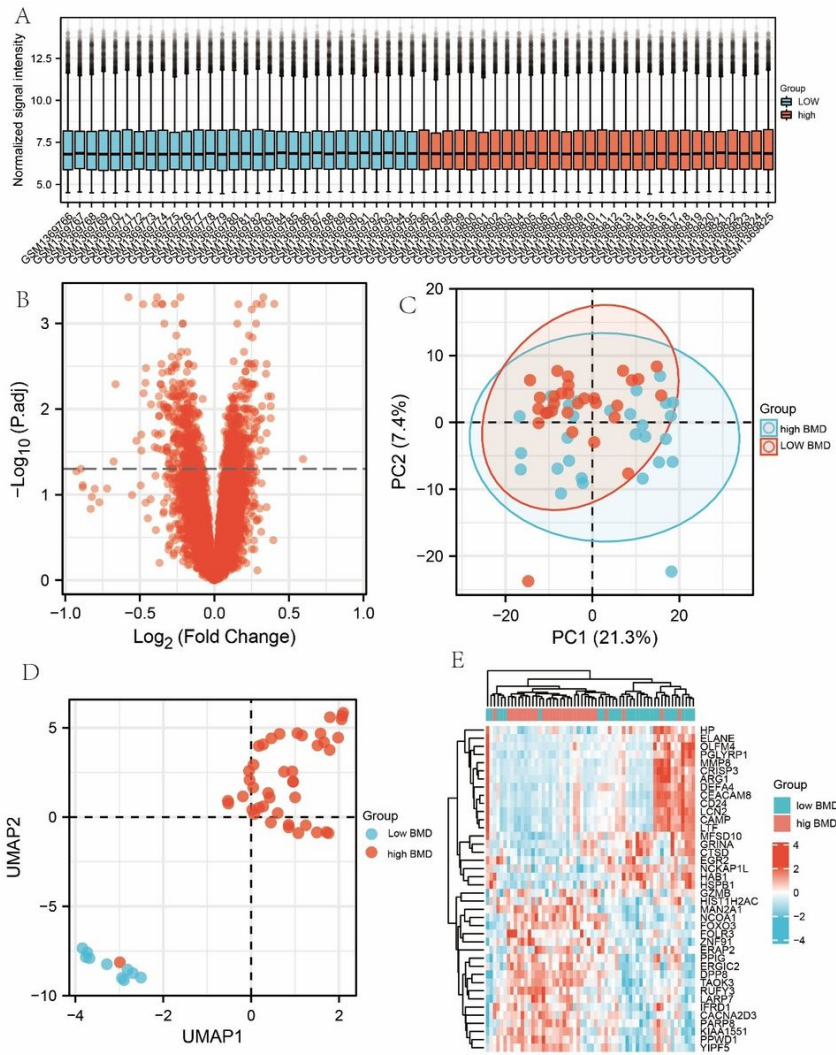


Figure 4

The different gene expression analyses in osteoporosis samples from the GEO dataset. (A) gene normalization diagram. (B) Gene volcano map. (C) Principal genetic analysis. (D) Uniform Manifold Approximation and Projection. (D) heatmap analysis.

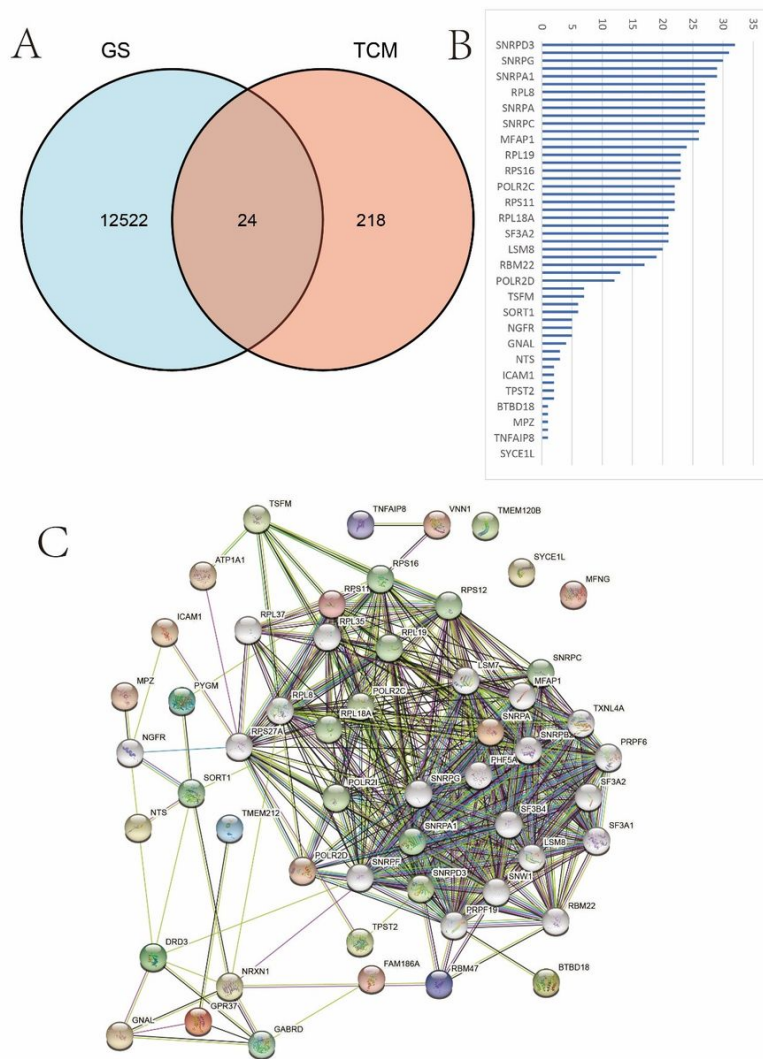


Figure 5

Potential target genes and PPI network map of LC therapy for OP. (A) The Venn results of potential genes of LC therapy for OP. (B) Counts and list of the top genes of PPI network map. (C) the PPI network map of 24 target genes.

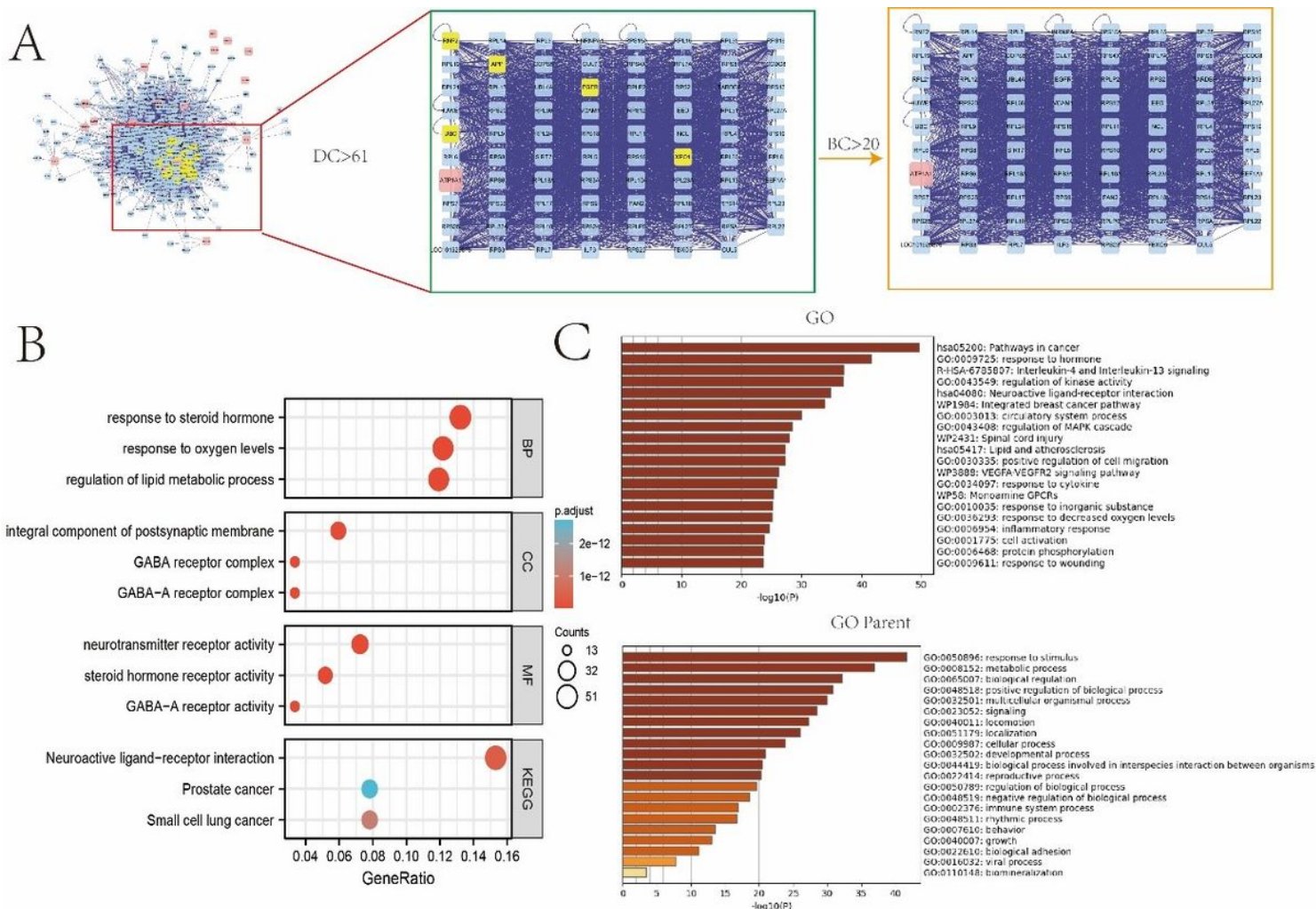


Figure 6

Topological analysis of the protein-protein interaction network (A) and GO/KEGG enrichment analysis (B, C).

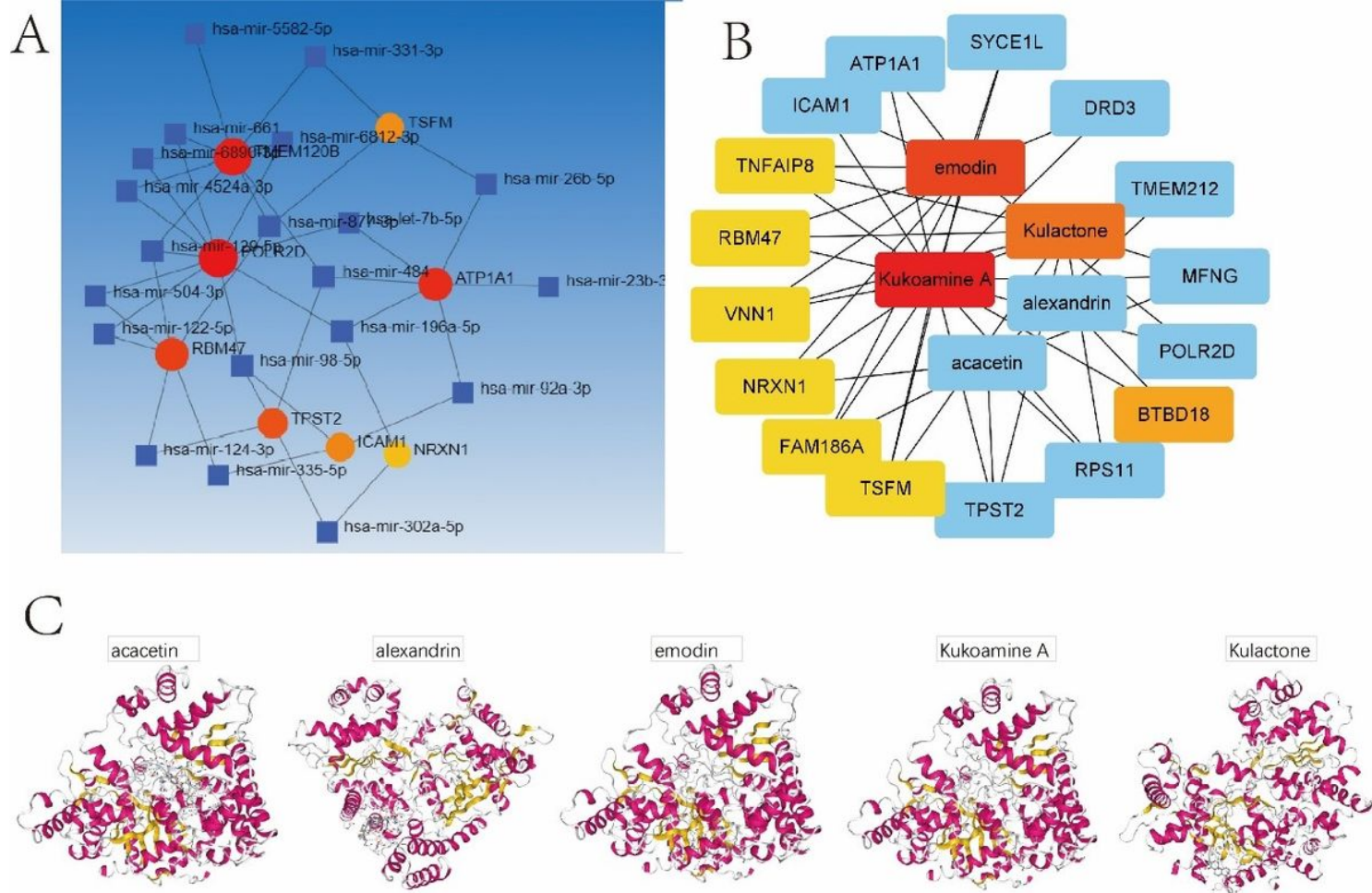


Figure 7

(A) Target genes-miRNA. (B) TCM compound-disease regulatory network. (C) the 3D map of binding of Kua.

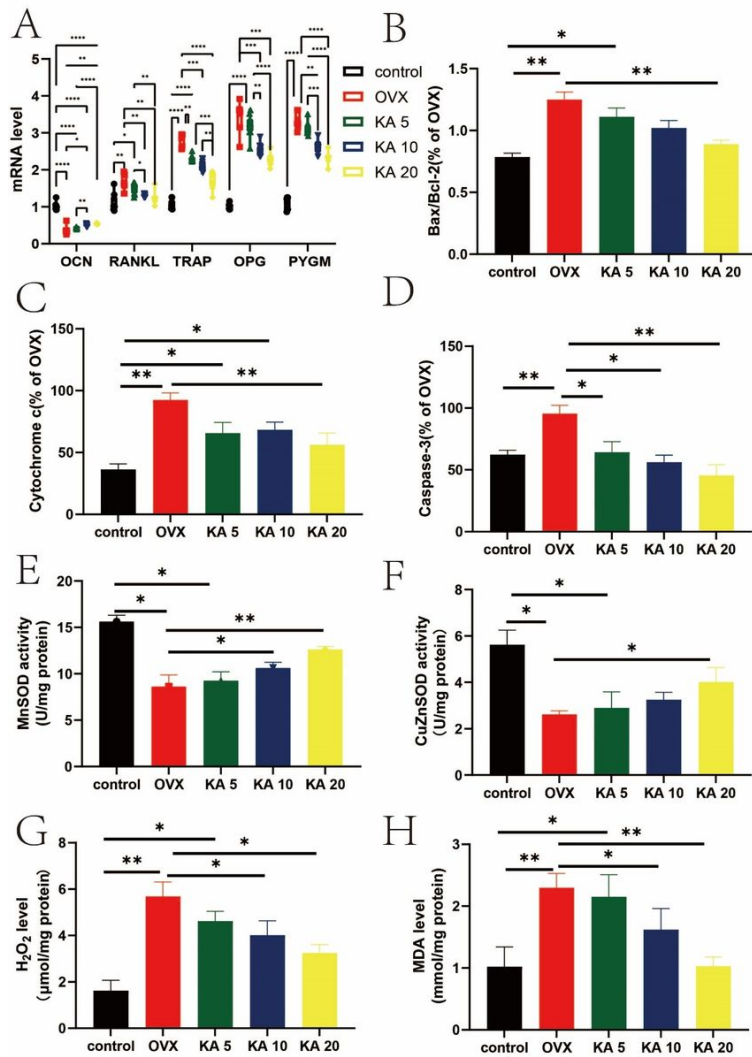


Figure 8

KuA protect against the cell apoptosis and oxidative stress level in OVX mice. (A) mRNA expression in tibia. (B) Bax/BCL-2 level. (C) cytochrome c level. (D) Caspase-3. (E) MnSOD. (F) CuZnSOD. (G) H₂O₂ level. (H) MDA

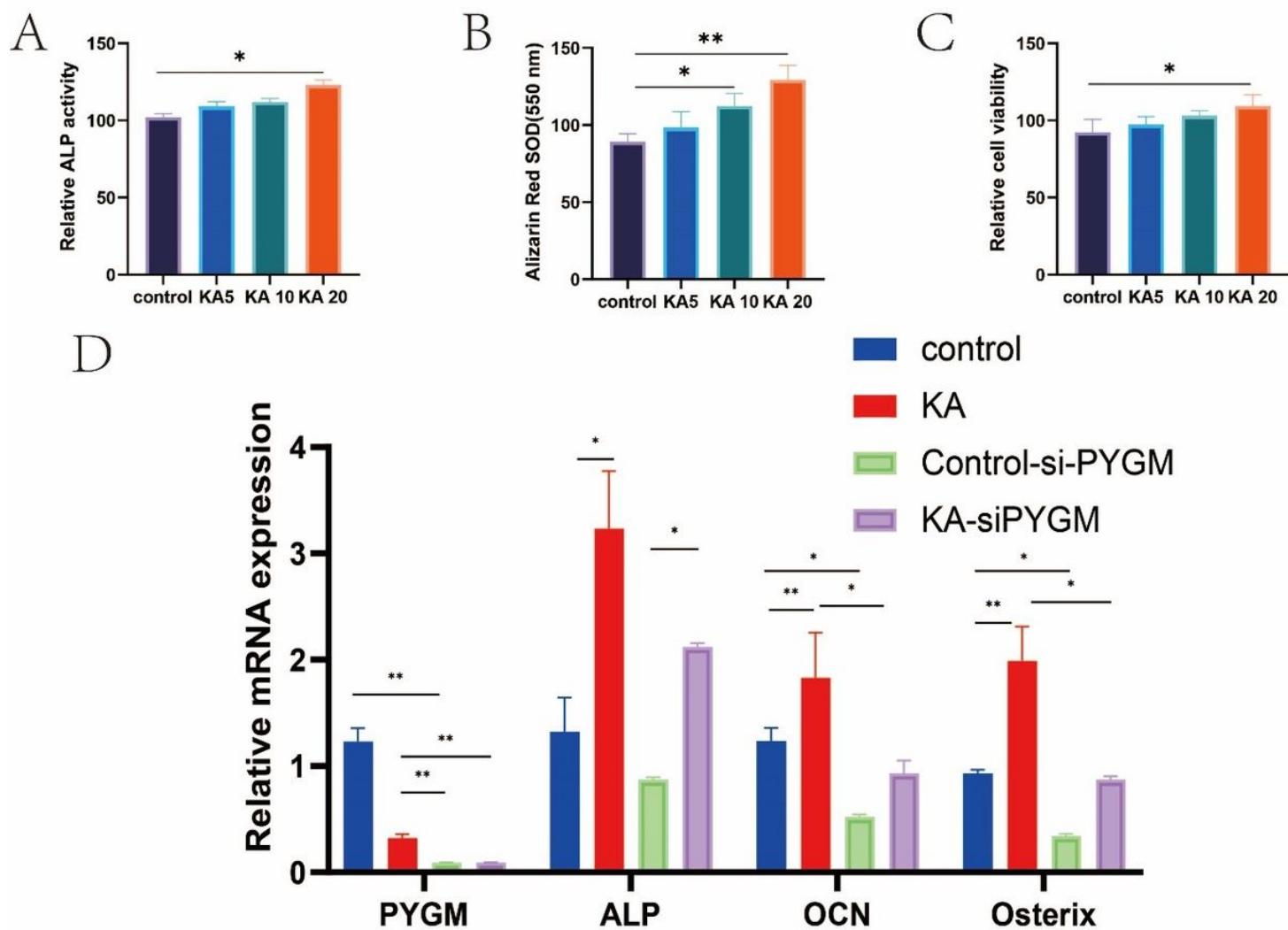


Figure 9

KuA increased the osteoblastic differentiation and mineralized nodule formation of osteoblastic MC3T3-E1 cells. (A) relative ALP activity. (B) Alizarin Red SOD. (C) relative cell viability. (D) mRNA level in MC3T3-E1 cells.

Supplementary Files

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- [SupplementTable1.docx](#)
- [SupplementFigure.docx](#)