

# Investigation of Anti-osteoporosis Mechanisms of Rehmanniae Radix Preparata Based on Network Pharmacology and Experimental Verification

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## Research Article

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# Abstract

**Background:** *Rehmanniae Radix Preparata* (RRP) can effectively improve the symptoms of osteoporosis, but its molecular mechanism for treating osteoporosis is still unclear. The objective of this study is to investigate anti-osteoporosis mechanisms of RRP through network pharmacology.

**Methods:** The overlapping targets of RRP and osteoporosis were screened out using online platforms. A visual network diagram of PPI was constructed and analyzed by Cytoscape 3.7.2 software. Molecular docking was used to evaluate the binding activity of ligands and receptors, and some key genes were randomly verified through pharmacological experiments.

**Results:** According to topological analysis results, AKT1, MAPK1, ESR1, SRC, and MMP9 are key genes for RRP to treat osteoporosis, and they have high binding activity with stigmasterol and sitosterol. The main signal pathways of RRP in the treatment of osteoporosis, including Estrogen signaling pathway, HIF-1 signal pathway, MAPK signal pathway, PI3K-Akt signal pathway, etc. Results of animal experiments showed that RRP could significantly increase the expression levels of Akt1, ESR1 and SRC-1 mRNA in bone tissue to promote bone formation.

**Conclusion:** This study explained the coordination between multiple components and multiple targets of RRP in the treatment of osteoporosis, and provided new ideas and basis for its clinical application and experimental research.

## Introduction

Osteoporosis is a common bone metabolic disease in middle-aged and elderly, which often leads to sprout, bone deformity and even fracture, and seriously affects the health and quality of life of middle-aged and elderly people [1, 2]. With the aging of the global population, its incidence is increasing year by year, so there is an urgent need to explore effective treatment methods [3, 4]. At present, the clinical treatment of osteoporosis is mainly through the application of three types of drugs: bone formation promoters, bone resorption inhibitors and bone mineral agents to improve the clinical symptoms of patients, but these treatment methods have certain limitations [5, 6]. Chinese herbal medicine has a long history of preventing and treating osteoporosis, with good curative effects and fewer side effects [7, 8]. *Rehmanniae Radix Preparata* (RRP) is a commonly used Chinese herbal medicine for the treatment of osteoporosis. Its main chemical components include sterol, styrene glycosides, amino acids, carbohydrates, etc., which can reduce bone loss and slow down aging [9, 10]. However, the material basis and molecular mechanism of RRP in the treatment of osteoporosis are still unclear.

Based on systems biology and bioinformatics, network pharmacology explores the interaction between biomolecules and targets in the body, so as to effectively predict the efficacy and mechanism of drugs [11]. This study integrated information such as active ingredients, drug targets and disease targets through network pharmacological methods to explore the material basis and mechanism of RRP in the treatment of osteoporosis.

This study explained the coordination between multiple components and multiple targets of RRP in the treatment of osteoporosis, and provided new ideas and basis for its clinical application and experimental research. First, overlapping targets of RRP and osteoporosis were screened out using online platforms. Next, a visual network diagram of PPI was constructed and analyzed by Cytoscape 3.7.2 software. Finally, molecular docking was used to evaluate the binding activity of ligands and receptors, and some key genes were randomly verified through pharmacological experiments. Network pharmacology research flow chart for RRP in the treatment of osteoporosis is shown in Fig. 1.

## Methods

### Screening of anti-osteoporosis targets of RRP

Osteoporosis-related targets were collected from online-accessible databases of DisGeNET (<https://www.disgenet.org/>), TTD (<http://db.idrblab.net/ttd/>) and Drugbank (<https://www.drugbank.ca/>) [12-14]. In addition, we used three online platforms: SEA (<http://sea.bkslab.org/>), PharmMapper (<http://www.lilab-ecust.cn/pharmmapper/>) and SwissTargetPrediction (<http://www.swisstargetprediction.ch/>) to search for the target of RRP, and used UniProt database (<https://www.Uniprot.org/>) to standardize the gene ID [15-17]. Furthermore, all overlapping targets of RRP and osteoporosis were assayed by Venn diagrams to identify the targets for RRP-treated osteoporosis.

### Construction and analysis of protein interaction network

The overlapping targets of RRP and osteoporosis were imported into STRING (<https://string-db.org/>) to obtain the protein-protein interaction (PPI) [18]. Then we used Cytoscape 3.7.2 software to construct a visual network diagram of PPI and further identified the targets for RRP-treated osteoporosis using cluster analysis [19].

### GO and pathway enrichment analysis for key targets

The key genes were imported into several online biological information databases such as DAVID (version: 6.8) and STRING (version: 11.0), and GO and KEGG pathway enrichment analysis were performed [20,21].

### Molecular docking of RRP and key targets

The 3D structure of the target protein was downloaded from PDB (<https://www.rcsb.org/>), and the water molecules and small molecule ligands of the target protein were removed using Pymol software [22]. Then we used AutoDock Tools software to prepare the hydrogenated protein and calculate the docking score.

### Establishment of the experimental model of osteoporosis

Female SD rats weighing  $200\pm 20$ g were randomly divided into three groups: sham operation group, model group and RRP group, with 10 rats in each group. The experimental animals were purchased from Sichuan Chengdu Dashuo Experimental Animal Co., Ltd. (Chengdu, China), and the license number is SCXK 2019-028. Rats were kept in a well-ventilated environment with a room temperature of 22-25°C and relative humidity of 50%-60%. Animal experiments were carried out in accordance with the principles of the Care and Use of Laboratory Animal and the protocol was approved by the Animal Ethics Committee of Shaanxi University of Traditional Chinese Medicine (ethics approval number: AEC-19-002).

The rats in the model group and the RRP group underwent ovariectomy, while the ovaries in the sham operation group were not removed. After the operation, the vaginal secretions of the rats were collected, and the keratinized epithelial cells were not observed as a key indicator of successful ovariectomy. Both the sham operation group and the model group were intragastrically administered with distilled water, and the RRP group was intragastrically administered with a dose of 5.4 g/kg of RRP daily. The rats were dissected and their femurs were taken after 16 weeks.

### **Bone density examination**

The rats were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (1 ml/kg), and the right femur and the third lumbar vertebra were peeled off. A dual-energy X-ray bone densitometer (Lunar, United States) was used to detect the bone mineral density of the femur and lumbar spine of rats.

### **Validation of key targets through qRT-PCR**

Three key targets were randomly verified by Real-Time quantitative reverse transcription (RT-PCR). Primers were designed and synthesized by the solid-phase phosphoramidite triester method, and the sequence of primer was as follows: AKT1 forward primer: 5'- GGCCCAGATGATCACCATCAC-3'; AKT1 reverse primer: 5'-CTATCGTC CAGCGCAGTCCA-3'; ESR1 forward primer: 5'- CCAACCAGTGCACCATTGAT-3'; ESR1 reverse primer: 5'-TTTGATCATGAGCGGGCTTG-3-3'; SRC-1 forward primer: 5'- CAACCAGCAAAGGCTGAGTCCA-3'; SRC-1 reverse primer: 5'- AGTACCTCCTGAGGGTTAGAG-3'. RNA of rats left femur were extracted with EasyPure™ RNA Kit (TransGen, China). TransScript first-strand cDNA synthesis SuperMix kit (TransGen, China) was used for reverse transcription reaction. The program used consisted of a pre-denaturation step of 95°C for 3 min, 40 cycling of denaturation 94°C for 15 s, annealing temperature 50°C for 30 s, extension 72°C for 1 min, and a final extension step of 72°C for 5 min. The gene expression data was analyzed by using the  $2^{-\Delta\Delta CT}$  method.

### **Statistical Analysis**

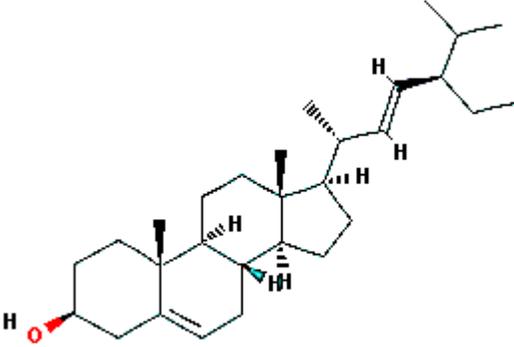
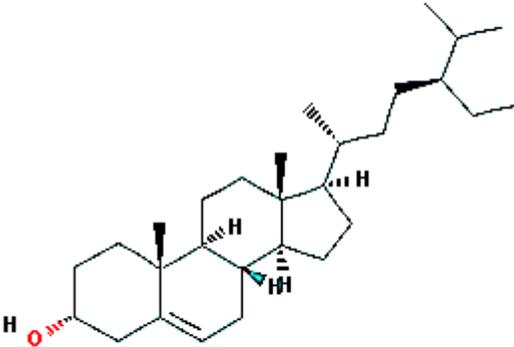
All statistical analyses were performed using SPSS19.0 software. All data were expressed as mean  $\pm$  standard deviation ( $\pm s$ ). One-way analysis of variance was used to analyze the data from multiple groups. P-Values of 0.05 or less were regarded as statistically significant.

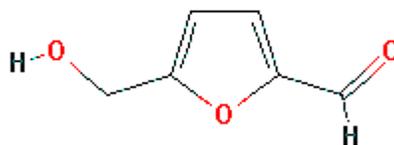
## **Results**

## Active ingredients and targets of RRP in the treatment of osteoporosis

A total of 76 active ingredients of RRP were searched, and 2 active ingredients were screened based on oral bioavailability (OB)>30% and drug-likeness (DL)>0.18, including  $\beta$ -sitosterol (MOL000359) and stigmasterol (MOL000449). It was reported in the literature that 5-HMF could promote osteoblast production and might be one of the components of RRP in the treatment of osteoporosis. Therefore, although the DL value of 5-HMF (MOL000748) did not meet the standard, it was also included as an active ingredient (Table 1). We searched three online Platforms with the keyword "Osteoporosis" and identified 1179 osteoporosis-related targets. And we also obtained 428 RRP targets after removing duplicates. Finally, we found that there were a total of 118 overlapping targets for RRP and osteoporosis.

### **Table 1** Active ingredients of RRP

Molecule ID	Molecule Name	Structure	OB (%)	DL
MOL000449	stigmasterol		43.83	0.76
MOL000359	sitosterol		36.91	0.75
MOL000748	5-HMF		45.07	0.02



Note: RRP: *Rehmanniae Radix Preparata*; OB: oral bioavailability; DL: drug-likeness.

## Network construction and analysis

After the overlapping targets were uploaded to STRING (at 70% confidence), the PPI network with 98 nodes and 378 edges was constructed using Cytoscape 3.7.2 software (Fig. 2). In the generated network, nodes represented targets, and edges represented the interaction between targets. We used the Cytohub plug-in to analyze the network topology properties. The degree value of node reflected the importance of the node in the network. In the PPI network, the node color changed from yellow to green reflected the degree value changed from low to high. The top 10 genes were MAPK1, MAPK3, AKT1, MAPK8, ESR1, PTG stigmasterol, EGFR, FGF2, SRC, MMP9. Their degree values were more than two fold of the median degree of all nodes in the network [23].

The MCODE plug-in was used to decompose the PPI network, and seven closely connected sub-modules in the network were identified, including two 16-cores (the connectivity of each node in the module is at least 16), one 7-cores, one 6-cores, two 4-cores and one 3-cores (Fig. 3). This sub-module reflected the closely related proteins interaction that completed specific molecular functions. The genes in these sub-modules were closely related to the following molecular functions: enzyme binding, phosphotransferase activity, signaling receptor binding, protein kinase binding, protein tyrosine kinase activity, ion binding, steroid hormone receptor activity, phosphatidylinositol-4,5-bisphosphate 3-kinase activity, heme binding, G protein-coupled receptor activity. And these genes were involved in many important biological processes related to osteoporosis, such as regulation of cell population proliferation, positive regulation of nitrogen compound metabolic process, activation of protein kinase activity, positive regulation of reactive oxygen species metabolic process, regulation of phosphorylation, steroid metabolic process, vitamin D metabolic process, bone development, regulation of protein binding and G protein-coupled receptor signaling pathway.

## Enrichment analysis of key targets

In the results of the enrichment of KEGG pathways, the pathways of basic biological processes were screened with false discovery rate (FDR) less than 0.01, and an enriched cluster containing 162 pathways was obtained (enrichment score = 2.12). According to the FDR value of these pathways, 10 pathways related to osteoporosis were screened out, including Estrogen signaling pathway, HIF-1 signaling pathway, VEGF signaling pathway, TNF signaling pathway, Ras signaling pathway, FoxO signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, Osteoclast differentiation and Inflammatory mediator regulation of TRP channels (Table 2). Then we classified and visualized the pathways based on the number of key genes in these pathways (Fig. 4). The classification of these pathways belongs to endocrine system, signal transduction, development and regeneration and sensory system, which were the key target pathways of RRP to interfere with the biological process of osteoporosis.

**Table 2** KEGG signaling pathways regulated by important targets

Category	Pathway	Number of genes	Mapped targets	FDR
Endocrine system	Estrogen signaling pathway	99	12	$8.91 \times 10^{-7}$
Signal transduction	HIF-1 signaling pathway	96	10	$4.82 \times 10^{-5}$
Signal transduction	VEGF signaling pathway signaling pathway	61	8	$1.33 \times 10^{-4}$
Signal transduction	TNF signaling pathway	107	9	$4.21 \times 10^{-4}$
Signal transduction	Ras signaling pathway	226	12	$5.69 \times 10^{-4}$
Signal transduction	FoxO signaling pathway	134	9	0.001
Signal transduction	MAPK signaling pathway	253	12	0.001
Signal transduction	PI3K-Akt signaling pathway	345	14	0.001
Development and regeneration	Osteoclast differentiation	131	8	0.004
Sensory system	Inflammatory mediator regulation of TRP channels	98	7	0.004

Note: KEGG: Kyoto Encyclopedia of Genes and Genomes; FDR: False discovery rate.

## Verification of molecular docking

Molecular docking could effectively predict whether the ligand and the receptor could interact with each other through the complementarity of the spatial structure and the principle of energy minimization in the

region of the receptor active site [24,25]. The lower the docking score between the ligand and the receptor, the greater the docking activity of the two and the more stable the structure. The molecular docking results showed that the molecular docking score between the active ingredients of RRP and the key targets was all less than -4.2 kcal/mol, suggesting that these active ingredients have a certain affinity and binding activity with the key targets (Table 3). Docking score of the ligand and the receptor was less than -7.0 kcal/mol, which indicated that they had strong binding activity. A total of 14 binding conformations have docking scores less than -7.0. The top 9 binding relationships with the highest docking activity are AKT1-stigmasterol, AKT1-sitosterol, MAPK1-stigmasterol, MAPK1-sitosterol, ESR1-stigmasterol, SRC-stigmasterol, MMP9-stigmasterol, ESR1-sitosterol, MMP9-sitosterol (Fig. 5).

**Table 3** Docking score of the active ingredients of RRP and key targets

Molecule name	PDB ID	Docking score(kcal/mol)		
		stigmasterol	sitosterol	5-HMF
MAPK1	4s33	-9.7	-9.5	-4.3
MAPK3	6ges	-5.3	-5.0	-4.2
AKT1	6hhf	-10.3	-10	-4.7
MAPK8	3pze	-8.2	-8.3	-4.4
ESR1	2iok	-9.6	-8.7	-4.3
PTGS2	4cox	-7.9	-8.1	-5.0
EGFR	5y9t	-6.3	-5.9	-3.8
FGF2	4fgf	-5.0	-4.6	-3.2
SRC	4u5j	-9.1	-8.0	-4.3
MMP9	6esm	-8.9	-8.6	-5.6

### Bone densitometry results

Compared with the sham operation group, the femur and vertebral bone mineral density of the model group were significantly decreased ( $P < 0.01$ ). Compared with the model control group, the RRP group could significantly increase the bone mineral density of the femur of ovariectomized rats ( $P < 0.01$ ), and could significantly increase the bone density of the vertebral body ( $P < 0.05$ ), as shown in Fig. 6-7.

### Effect on AKT1, ESR1 and SRC-1 mRNA expression

The relative quantitative expression levels of AKT1, ESR1 and SRC mRNA were calculated by the  $2^{-\Delta\Delta CT}$  method. The results showed that compared with the sham operation group, the expression levels of AKT1, ESR1 and SRC mRNA in the bone tissue of the model group decreased significantly ( $P < 0.05$ ). Compared with the model group, the expression of AKT1, ESR1 and SRC mRNA in the bone tissue of the

RRP group increased significantly ( $P < 0.05$ ). The results showed that RRP could increase the expression levels of AKT1, ESR1 and SRC mRNA in the bone tissue of osteoporotic rats (Fig. 8).

## Discussion

The high morbidity and mortality of osteoporosis and osteoporotic fractures not only seriously affect the quality of life of the elderly, but also cause a huge economic and social health burden [26]. RRP can effectively prevent and treat osteoporosis [27], but the material basis and molecular mechanism of its treatment of osteoporosis are still unclear. A total of 76 active ingredients of RRP were searched, and 3 active ingredients were screened:  $\beta$ -sitosterol, stigmasterol and 5-HMF. After removing the duplication, we obtained 428 RRP targets and 1,179 targets related to osteoporosis, of which 118 overlapping targets were the common targets of RRP and osteoporosis. We used the Cytohub plug-in to analyze the PPI network topology properties. According to the node degree value, the top 10 genes are MAPK1, MAPK3, AKT1, MAPK8, ESR1, PTGS2, EGFR, FGF2, SRC, MMP9. Results of molecular docking showed that AKT1, MAPK1, ESR1, SRC, MMP9 and Stigmasterol had strong binding activity. AKT1, MAPK1, ESR1, MMP9 and Sitosterol also had high binding activity. The main signal pathways of RRP in the treatment of osteoporosis, including Estrogen signaling pathway, HIF-1 signal pathway, MAPK signal pathway, PI3K-Akt signal pathway, VEGF signal pathway, osteoclast differentiation of TRP channel and regulation of inflammatory mediators, etc. The classification of these pathways belongs to endocrine system, signal transduction, development and regeneration and sensory system, which were the key target pathways of RRP to interfere with the biological process of osteoporosis.

MAPK is the main carrier for signal transmission from the cell surface to the nucleus, mainly involved in the growth, differentiation and apoptosis caused by extracellular stimulation, and is a positive regulator of osteoblast differentiation and bone formation [28]. MAPK1, MAPK3 and MAPK8 are important members of the mitogen-activated protein kinase family. MAPK protein binds to the receptor complex, leading to inactivation of the cytosolic complex and degradation of  $\beta$ -catenin. The  $\beta$ -congenial protein can accumulate in the cytoplasm, and then transferred to the nucleus to promote the expression of specific genes in the bone, ultimately resulting in osteogenic differentiation to reduce and promote osteoblasts [29, 30]. Activation of the MAPK pathway increases the proliferation and migration of osteoblasts, which can promote bone healing. Inhibition of MAPK signaling reduces the expression of specific genes in mature osteoblasts [31, 32]. Estrogen is an important factor in increasing bone density and preventing bone loss after menopause. ESR1 is a nuclear biological macromolecule that mediates the biological effects of estrogen. Under the stimulation of EGF or IGF, the activated MAPK phosphorylates the serine of ESR1, allowing the receptor to bind to the specific coactivator of ESR1 to activate target genes [33].

When estrogen is insufficient, TNF- $\alpha$  promotes the proliferation and differentiation of osteoclast precursor cells and inhibits the formation of osteoblasts. Estrogen can also biphasically activate nitric oxide synthase in endothelial cells through MAPK and PI3K/Akt pathways [34]. In osteoblasts and osteoclasts, estradiol rapidly activates MAPK, which may be involved in cell proliferation and anti-apoptotic effects to prevent osteoporosis [35]. VEGF is an important downstream gene of the HIF-1 signaling pathway, which

can promote the formation of osteoclasts and increase the activity of osteoclasts [36, 37]. The VEGF produced by mature osteoblasts is essential for the angiogenesis-osteogenesis coupling. VEGF can promote the production of osteochondral progenitor cells during bone repair and endochondral bone formation [38, 39]. PI3K/Akt signaling pathway is also involved in regulating the proliferation, differentiation and apoptosis of osteoclasts and osteoblasts [40, 41]. Studies have found that the lack of Akt1 in osteoclasts can lead to cellular dysfunction and impaired bone resorption [42, 43]. SRC-1 is an important nuclear receptor co-activator, which can enhance the effect of estrogen in many tissues and positively regulate the bone formation related to estrogen [44]. Experimental results showed that RRP could significantly increase the expression levels of Akt1, ESR1 and SRC-1 mRNA in bone tissue.

## Conclusions

This study applied network pharmacology and molecular docking methods to study the material basis and potential mechanism of RRP in the treatment of osteoporosis. RRP interferes with the biological process of osteoporosis through the endocrine system, signal transduction, development and regeneration, and sensory system. The experimental animal study showed that RRP could significantly increase the expression levels of Akt1, ESR1 and SRC-1 mRNA in bone tissue to promote bone formation. This study explained the coordination between multiple components and multiple targets of RRP in the treatment of osteoporosis, and provided new ideas and basis for its clinical application and experimental research.

## Abbreviations

RRP: *Rehmanniae Radix Preparata*; PPI: Protein-protein interaction; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; OB: Oral bioavailability; DL: Drug-likeness; RT-PCR: Real-Time quantitative reverse transcription; FDR: False discovery rate

## Declarations

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### Authors' contributions

O.L, L.ZY, K.WQ designed the study, analyzed the experiments, and wrote the paper. G.F, D.TW, W.PF, and L.M carried out the data collection and data analysis and revised the paper. The authors read and approved the final manuscript.

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### **Availability of data and materials**

All the data will be available upon motivated request to the corresponding author of the present paper.

### **Ethical approval and consent to participate**

This study was conducted in agreement with the Declaration of Helsinki and its later amendments or comparable ethical standards and had been approved by the ethics board of Shaanxi University of Traditional Chinese Medicine (No: AEC-19-002).

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors declare that they have no competing interests.

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## Figures

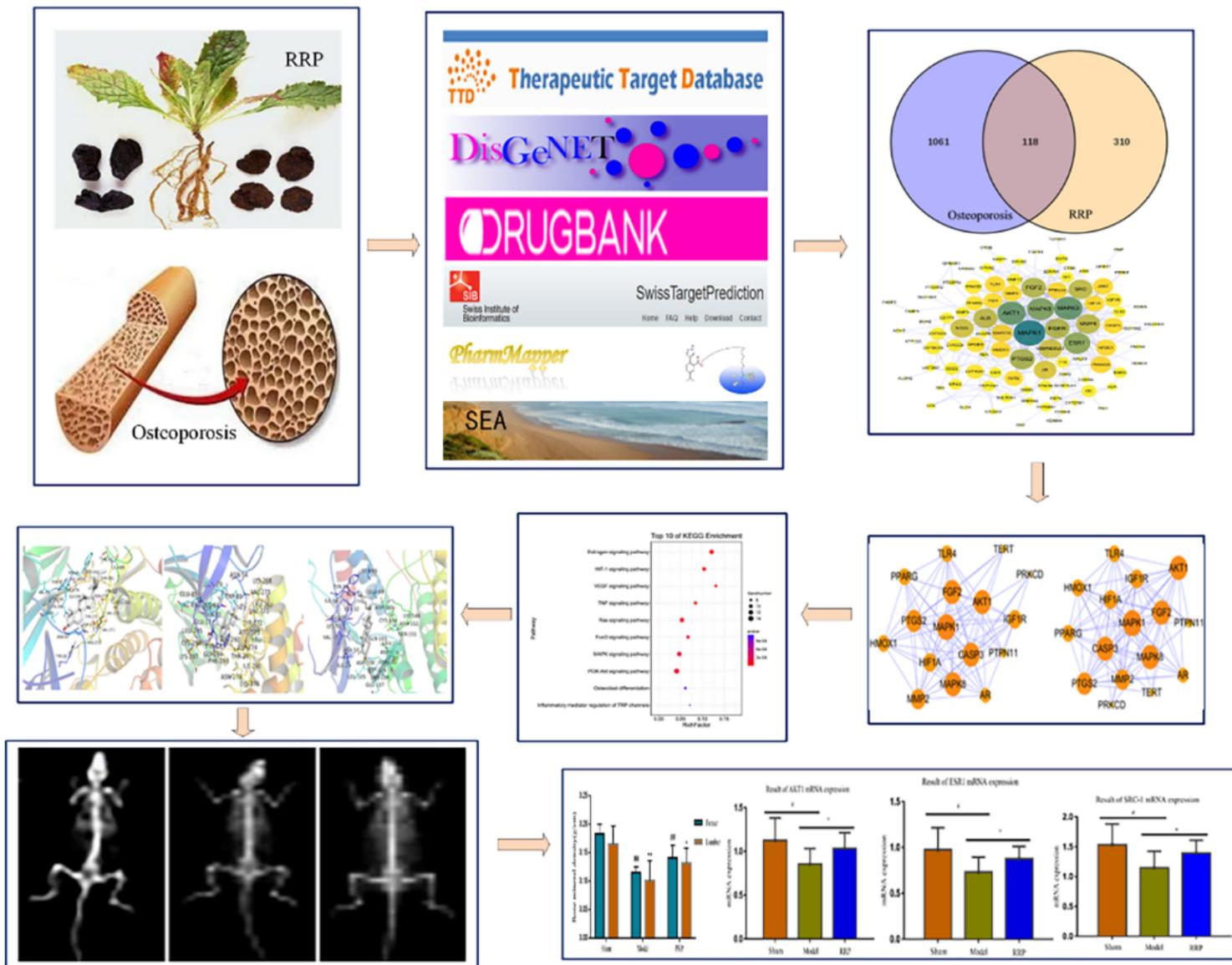


Figure 1

Network pharmacology research flow chart

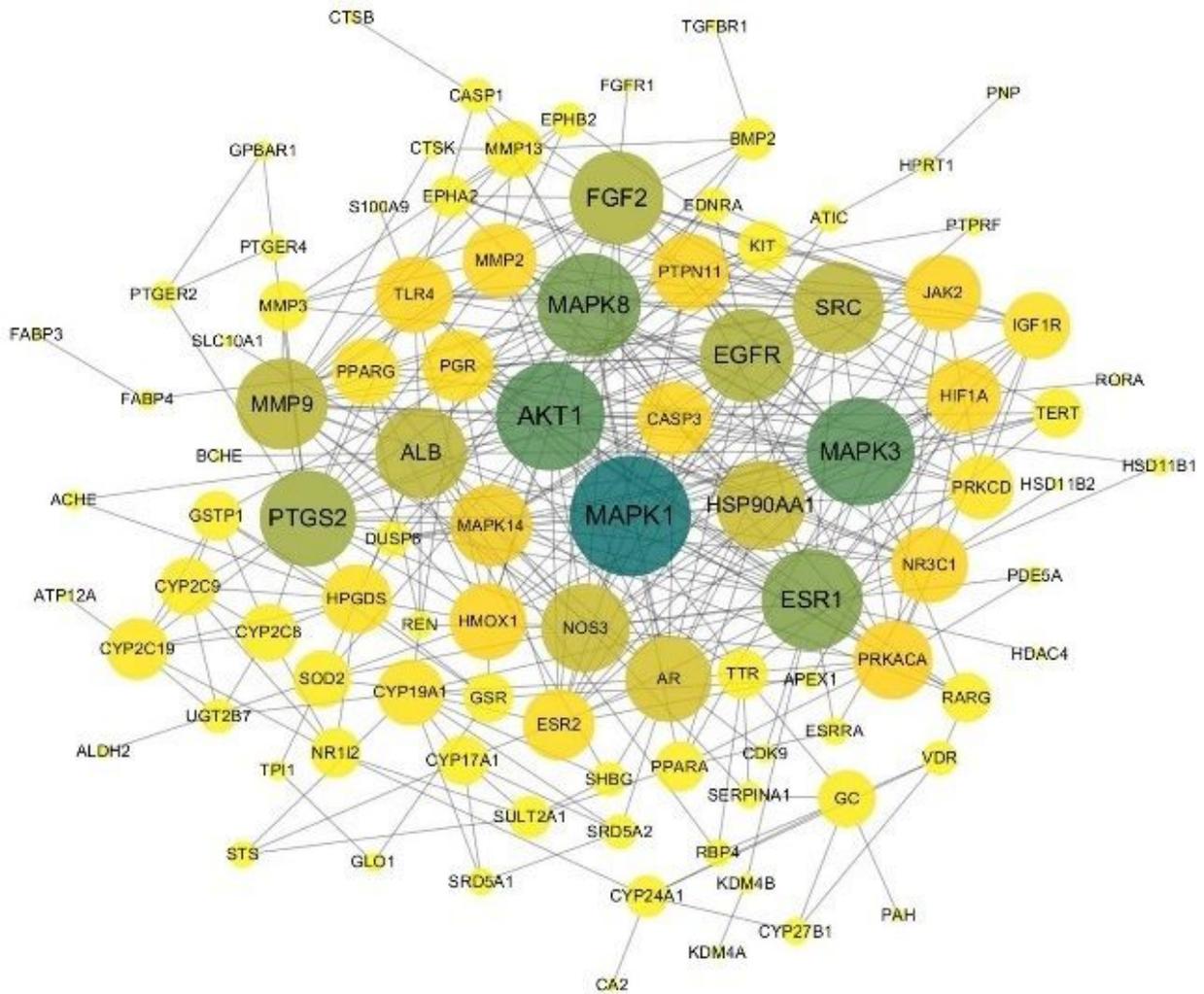


Figure 2

PPI network. PPI: Protein-protein interaction

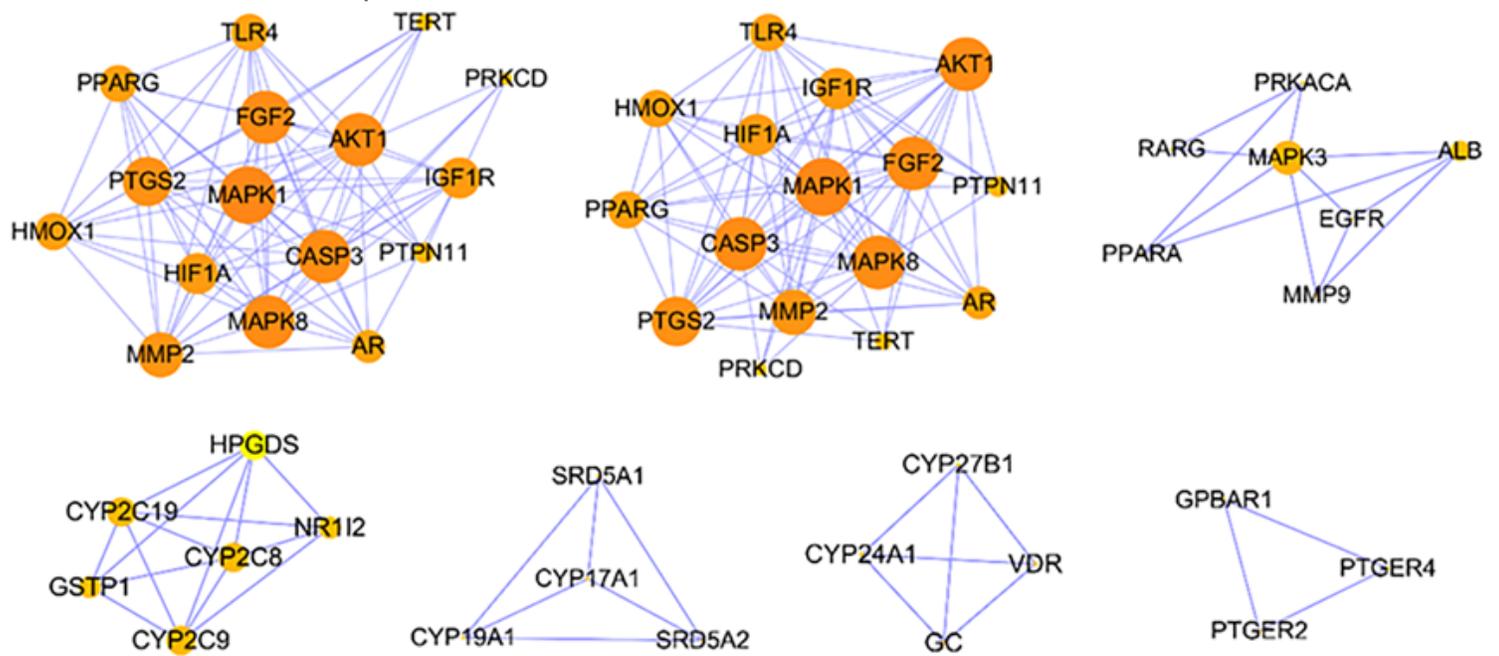


Figure 3

Densely linked modules included in target network of RRP

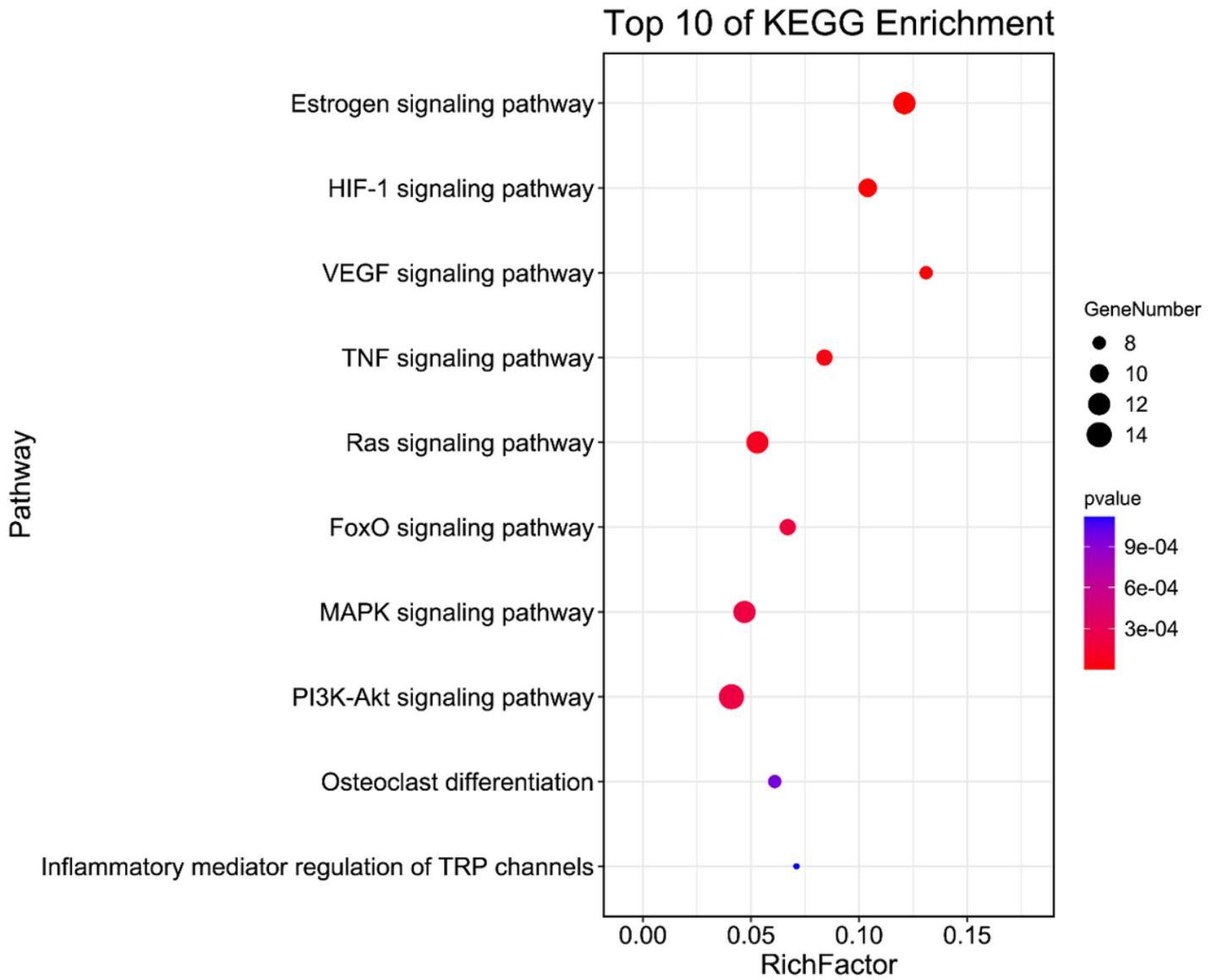
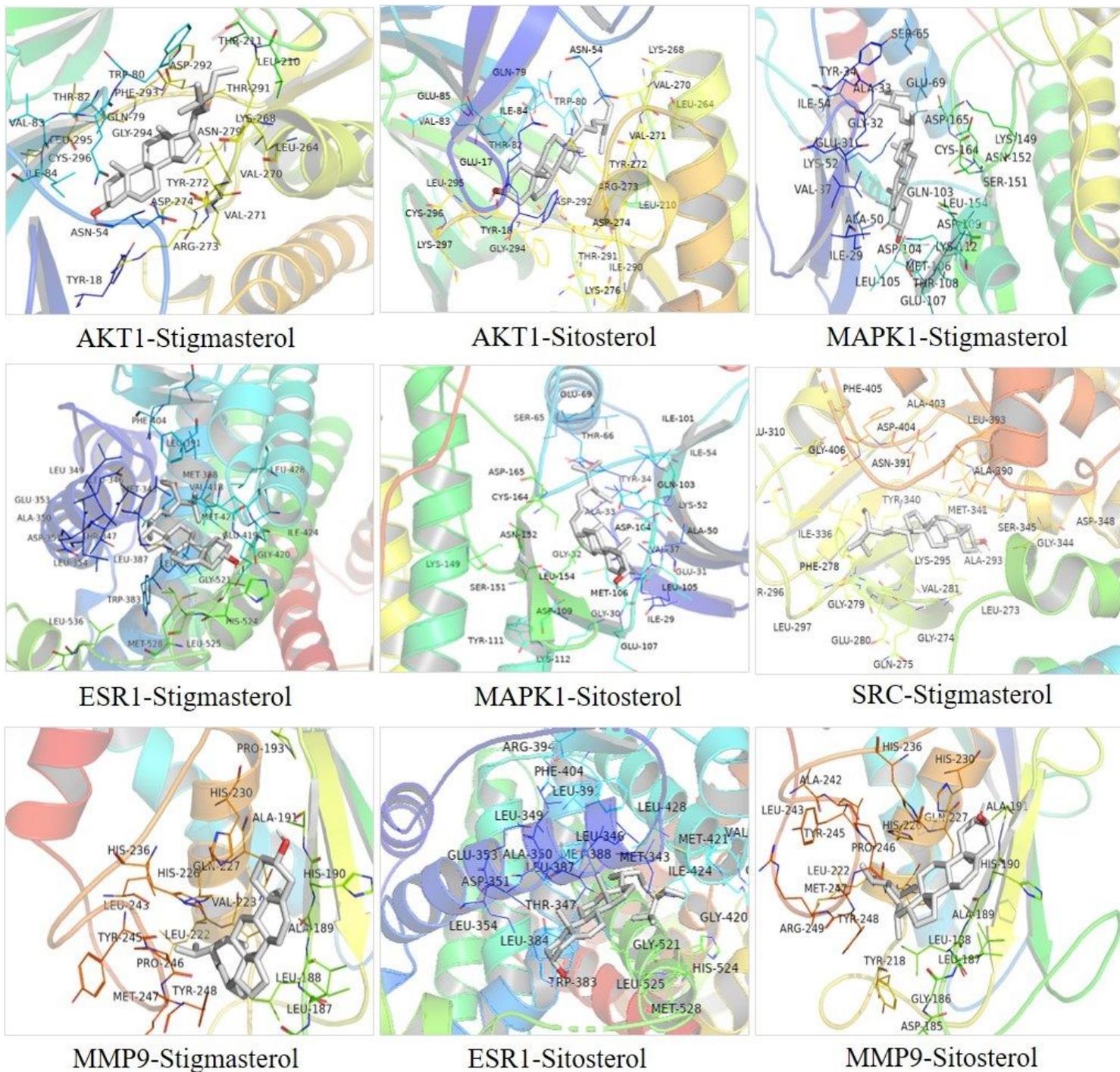


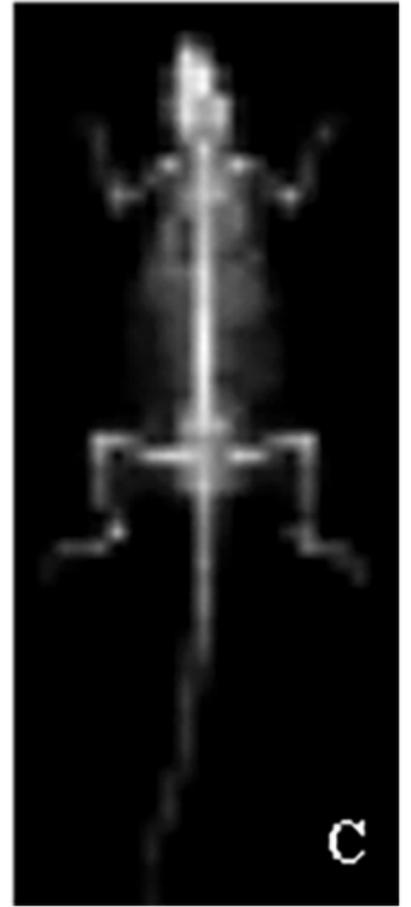
Figure 4

Bubble diagram of top 10 KEGG enrichment pathways



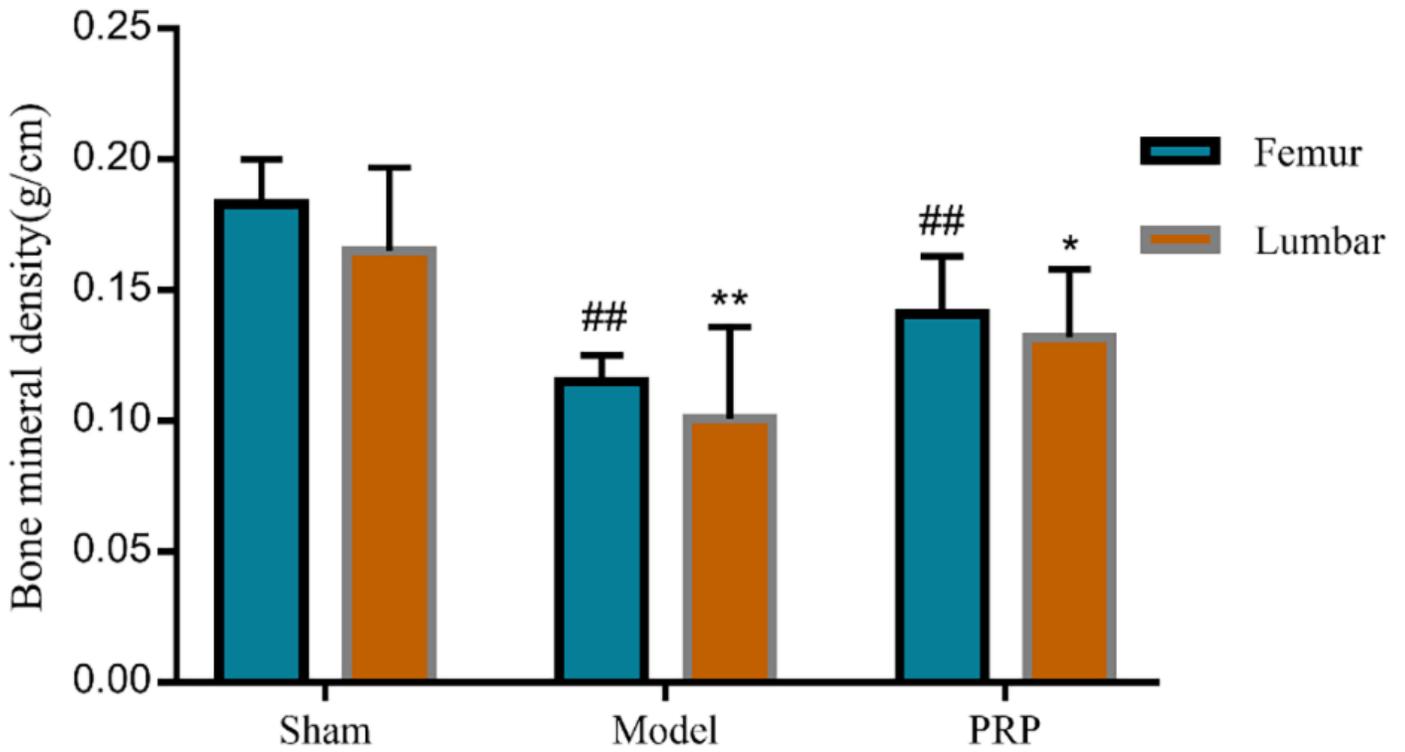
**Figure 5**

Molecular docking model diagram



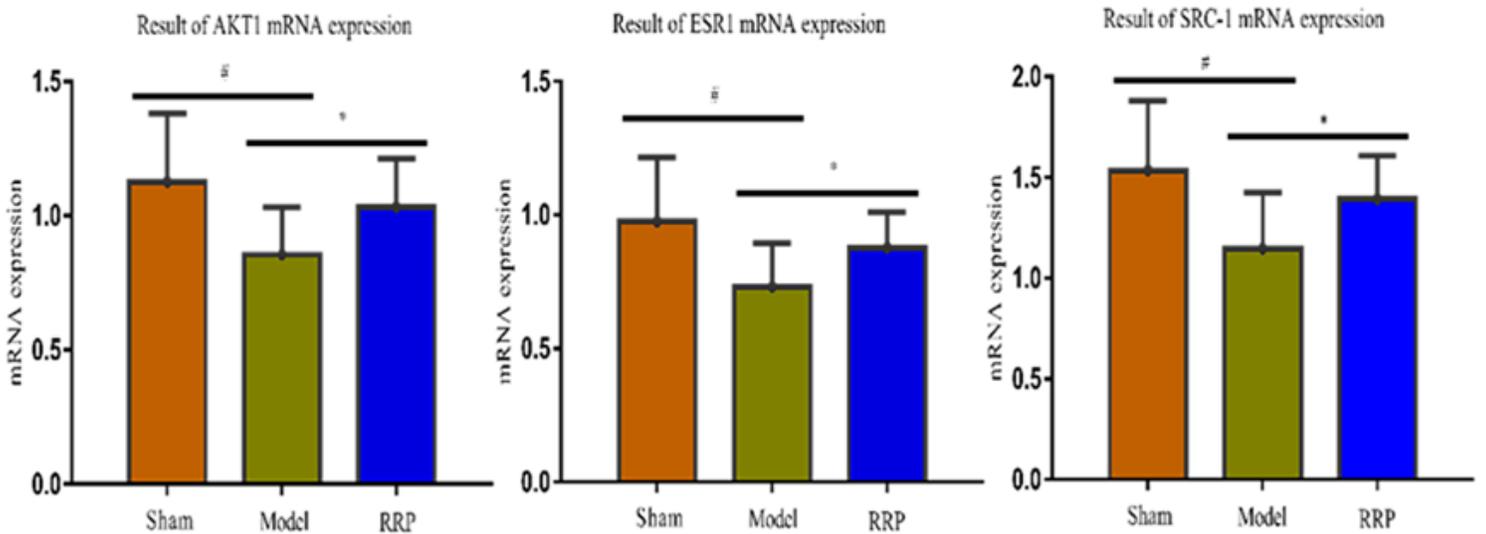
**Figure 6**

X-ray image of rat. A) Sham operation group. B) Model group. C) RRP group



**Figure 7**

Effect on bone mineral density. Compared with the sham operation group,  $##P<0.01$ ; Compared with the model group,  $**P<0.01$ ,  $*P<0.05$



**Figure 8**

Effect on AKT1, ESR1 and SRC mRNA expression. Compared with the sham operation group,  $#P<0.05$ ; Compared with the model group,  $*P<0.05$