

# Identification of potential specific biomarkers and key signaling pathways between osteogenic and adipogenic differentiation of hBMSCs for osteoporosis therapy

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## Research article

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# Abstract

**Background:** The differentiation of bone mesenchymal stem cells (BMSCs) into adipogenesis (AD) rather than osteogenesis (OS) is an important pathological feature of osteoporosis. Illuminating the detailed mechanisms of the differentiation of BMSCs into OS and AD would contribute to the interpretation of osteoporosis pathology.

**Methods:** To identify the regulated mechanism in lineage commitment of the BMSCs into OS and AD in the early-stages, the gene expression profiles with temporal series were downloaded to reveal the distinct fates when BMSCs adopt a committed lineage. For both OS and AD lineages, the profiles of day 2-4 were compared with day 0 to screen the differentially expressed genes (DEGs), respectively. Next, the functional enrichment analysis was utilized to find out the biological function, and Protein-Protein Interaction network to predict the central genes. Finally, experiments were performed to verify our finding.

**Results:** FoxO signaling pathway with central genes like FoxO3, IL6 and CAT, is the crucial mechanism of OS, while Rap1 signaling pathway of VEGFA and FGF2 enrichment is more significant for AD. Besides, PI3K-Akt signaling pathway might serve as the latent mechanism about the initiation of differentiation of BMSCs into multiple lineages.

**Conclusion:** Above hub genes and early-responder signaling pathways control osteogenic and adipogenic fates of BMSCs, which maybe mechanistic models clarifying the changes of bone metabolism in the clinical progress of osteoporosis. The findings provide a crucial reference for the prevention and therapy of osteoporosis.

## Introduction

Osteoporosis, characterized by increase of bone fragility and susceptibility to fracture owing to low bone mass and degeneration of bone microstructure, is a major social problem in the elderly population [1]. Currently, most therapeutic strategies for osteoporosis were administrated by anti-absorption drugs including bisphosphonates, calcitonin and estrogen [2-5]. Although these drugs have a certain effect on delaying the progress of osteoporosis, the serious side effects caused by long-term use are concerns of clinical application [6-8]. Thus, it's still an urgent need to discover a safer and more effective therapeutic strategy.

Growing evidences indicated that age-related microenvironmental changes including metabolic changes, immune system diseases and hormonal disorders, reduced the capacity of osteogenic differentiation and strengthened the ability in adipogenic differentiation of BMSCs, which were the paramount motivators in the initiation and progress of osteoporosis [9-12]. Therefore, a regenerative therapy has aroused much attention, which restores the balance of the normal intraosseous microenvironment of osteoporosis by artificially promoting OS and inhibiting AD from BMSCs. However, the molecular mechanisms of lineage commitment determinants during BMSCs differentiate into OS and AD are still unclear.

Adipogenesis and osteogenesis exists a competitive relation during the differentiation of BMSCs, and the signaling pathways between the adipo-osteogenesis could be altered to favor osteoblast for preventing osteoporosis. Adipo-osteogenic differentiation is a process regulated by multiple factors and signals. It has reported that the activation of glucagon-likepeptide1 (GLP-1) not only promotes osteogenic differentiation of BMSCs, but also inhibits adipogenic differentiation which is beneficial for osteoporosis [13]. Other osteogenic factors such as myocardin-related transcription factor A (MRTFA),  $\gamma$ -glutamyl carboxylase (GGCX) and transducin-like enhancer of split 3 (TLE3), inhibited adipogenic differentiation resulting in amelioration of bone formation [14-16]. Although previous studies have revealed possible molecular mechanisms involved in BMSCs differentiate into OS and AD, their results are limited to a single lineage and/or a differentiation in late-stages, and the mechanism in the differentiation of BMSCs into OS and AD were still unknown [17-19]. Thus, temporal expression profiles in the early stage of the BMSCs differentiate into OS and AD may better understand the mechanisms of their lineage commitment determinants.

The rapid development of high-throughput sequencing provides a more systematic and comprehensive approach to studying physiological or pathological mechanisms [20]. Characterizing the features of transcriptional regulation during the different stages of stem cell development would be contributed to clarifying the mechanism of lineage commitment differentiation. Zhang et al., had firstly reported the stage-specific interaction patterns between oocyte and granulosa cells during the development of folliculogenesis with the mRNA-sequencing (mRNA-Seq) technology [21]. Moreover, novel regulators of early cardiomyocytes development from human embryonic stem cells were identified with the temporal transcriptome and methylome [22]. Therefore, bioinformatics analysis is a powerful tool to discover the molecular-level biomarkers and related pathways in the osteogenic and adipogenic differentiation from BMSCs.

In the present study, we utilized the temporal gene expression profiles of an early stage in the *in vitro* induced (0-4 days) OS and AD from human BMSCs (hBMSCs), combined with bioinformatics and experiments, to explore the potential lineage commitment mechanisms of hBMSCs differentiate into OS or AD. By comparing the multiple profiles, the hub-genes and key signaling pathways that critical for osteogenic and adipogenic differentiation were identified. Our work might provide the key clues for the mechanism of stepwise osteogenesis and adipogenesis, which would provide a solid reference for prevention and therapy of osteoporosis.

## Materials And Methods

### Affymetrix microarray data source

GSE80614, a gene expression profile of hMSCs differentiated into OS or AD based on GPL6947 platform (Illumina HumanHT-12 V3.0 expression bead chip) were downloaded from the Gene Expression Omnibus (GEO) database. It contains 66 samples including 11 groups in OS or AD differentiation and 3 samples in each group. For the differentiation of BMSCs, the first 2 days are the lineage acquisition phase and the

first 2-4 days are lineage stable phase. So samples in day 0 (0d), day 2 (2d), day 3 (3d) and day 4 (4d) groups of OS or AD were selected to explore the crucial regulators involved in the initial stage of differentiation [23]. More information about the profile could be obtained from the online description (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE80614>). The study protocols were reviewed and approved by the Ethics Committee of Guangxi Medical University (Nanning, China).

### **Screening of differential expression genes (DEGs)**

All the data were normalized and analyzed with R-based web application in GEO dataset (GEO2R) [24]. The data was preprocessed with excluding the probes with no gene annotation and selecting the probes with maximum expression value if a gene is corresponding to multiple probes. DEGs were identified by the inclusion criteria with *P*. value <0.05 and fold change (FC)  $\geq 2$  [25]. The visualization of DEGs was drawn via volcano plot and venn diagram by using the ggplot2 and VennDiagram packages in R language (version 3.5.1) (<http://www.r-project.org/>) [26].

### **Function enrichment analysis of DEGs**

Gene Ontology (GO; <http://www.geneontology.org/>) analysis, a common and useful method for the computational analysis of large-scale molecular biology and transcriptome data in biomedical research, can classify the genes into three functional categories, including the Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) [27, 28]. Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/pathway.html>) is a well-knowledge database for understanding the molecular interaction and relation networks [29]. To expound the distinctive biological function, the identified DEGs were utilized to perform the GO term and KEGG pathway enrichment analysis with the Database for Annotation, Visualization, and Integrated Discovery (DAVID v6.8, <https://david.ncifcrf.gov/tools.jsp>) online tool [30]. The cut-off value of the result was set at *P*. value <0.05. Scatter plots were used to depict the top 10 and command KEGG pathways in the Image GP (<http://www.ehbio.com/ImageGP/>), and GO term enrichment analyses were visualized via the Bar plot and Cord plot with GOplot package in R [31].

### **Construction of protein-protein interaction (PPI) networks**

Search Tool for the Retrieval of Interacting Genes (STRING, v.10.5; <http://string-db.org/cgi/input.pl>), an online biological database and web resource, provides integrations and assessments of the PPI which could assess the relationships between DEGs [32]. Score (median confidence) >0.4 was set as the cut-off criterion of the selected PPIs, followed by PPI network construction via Cytoscape software (version 3.4.0; <http://www.cytoscape.org/>). The PPI networks, in which each node represents a protein and the lines denote direct interactions between proteins, were then used Between and Stress algorithm of cytoHubba in the Cytoscape to screen the top 20 hub genes in the OS and AD DEGs [33].

### **Cell isolation and culture**

The hMSCs were isolated from the marrows fluid that extracted from the patients with traumatic joint replacement based on informed consent. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, penicillin (100U/ml) and streptomycin (100mg/ml) underling 37°C and 5% CO<sub>2</sub>. The medium was replaced every 2 days and the cells at passage 2 were used for further studies.

### **Induction of hBMSCs into OS and AD.**

Prior to induction, hMSCs with a density of  $1.5 \times 10^4$ /cm<sup>2</sup> were seeded in growth medium until reach to 90%-100% confluence. Then, the medium was changed with the OS or AD induction medium for 4 days. The OS induction medium was prepared with  $\alpha$ - MEM that supplemented with 10 mmol/L of glycerol 2-phosphate, 100 nmol/L of dexamethasone and 50  $\mu$ mol/L of ascorbic acid. For AD induction medium, 5  $\mu$ g/ml insulin, 1  $\mu$ M dexamethasone, 500  $\mu$ M 3-isobutyl-1-methylxantine and 50  $\mu$ M indomethacin were added into DMEM [34].

### **RNA extraction and quantitative real-time PCR (qRT-PCR)**

Intracellular total RNA of the induced samples was purified via a Hipure Total RNA Mini kit (Magen, China) after 0, 2, 3 and 4 days of induction. Next, 1000 ng extracted RNA was reverse transcribed to cDNA using a cDNA synthesis kit (Takara, China). A LightCycler® 480 Sequence Detention System (Roche, Germany) with PCR Green Master Mix (Roche, Germany) was used to conduct the qRT-PCR for the detection of gene expression. Forkhead box O3 (FoxO3), phosphoinositide-3-kinase regulatory subunit 1(PIK3R1), catalase (CAT), interleukin 6 (IL6), alkaline phosphatase (ALP, an early maker of OS), vascular endothelial growth factor A(VEGFA), fibroblast growth factor 2 (FGF2), lipoprotein lipase (LPL, an early maker of AD) were normalized by Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control.

**Table 1** lists the primer sequences of the above genes.

### **Immunofluorescence staining**

The expression levels of proteins in the key signaling pathway were detected by immunofluorescence. The cells were removed from the well plate and cleared three times with PBS. After fixed with 4% (w/v) paraformaldehyde for 20 minutes, the cells were transparented in the Triton X100 for 15 minutes. Subsequently, endogenous peroxidase activity was blocked by the H<sub>2</sub>O<sub>2</sub> for 15 min at 25°C. Then, the cells were incubated with primary antibodies containing PIK3R1 (1:500), FoxO3 (1:100), CAT (1:100), IL6 (1:100), VEGFA (1:200) and FGF2 (1:100) at 4°C for 12h according to reagent instructions. After added with secondary antibodies for 1h at 37°C, the cells were observed and photographed by using an inverted phase-contrast microscope (OLYMPUS, Japan).

### **Statistical analysis**

Statistical analysis of the data was performed by one-way ANOVA and the quantitative data were expressed as means $\pm$ standard deviation of experiment. P<0.5 was set as the cutoff of significant differences.

# Results

## DEGs and KEGG pathway of OS induction

To systematically explore gene expression changes during the early stage of hMSCs differentiated into OS, we identified the DEGs by comparing the OS\_2d, OS\_3d, OS\_4d groups with the group OS\_0d. There were 452 DEGs in the OS\_2d group, including 262 up-regulations and 190 down-regulations while 656 DEGs in the OS\_3d group, of which 350 were up-regulated and 306 were down-regulated (**Figure 1A and B**). In addition, a total of 687 genes with 378 up-regulations and 309 down-regulations were detected to be differentially expressed in the OS\_4d group (**Figure 1C**). Moreover, the common upregulations were nearly twice compared to downregulations in these three time points, which might mean that the OS was the result of the upregulation of pro-differentiation genes and activation of related pathways (Figure S1A and B). To explore the potential mechanisms in the OS initiative differentiation, KEGG pathway enrichment analysis were performed with DAVID database. The top 10 pathways sorted by the number of DEGs enriched in every term were displayed via scatter plot (**Figure 1D-F**). Obviously, PI3K-Akt signaling pathway was the most significant pathway in each time point during the OS differentiation.

## DEGs and KEGG pathway of AD induction

There were a total of 729 DEGs in AD\_2d group, 870 DEGs in AD\_3d group and 905 DEGs in AD\_4d group, including 364, 441 and 461 up-regulations while 365, 429 and 444 down-regulations in the AD\_2d, AD\_3d and AD\_4d group, respectively (**Figure 2A-C**). The count of DEGs in AD induction showed little change in each time point as well, but increased significantly compared to DEGs OS, which might be due to hMSCs favor differentiate into AD rather than OS [35, 36]. As shown in the Venn diagrams, the number of the down-regulations was similar to the up-regulations in the AD, but was more than twice that in the OS, meaning the down-regulation play more significance in the AD. (Figure S1A and B). As the same as OS induction, the top 10 KEGG pathways enrichment at each time point were visualized in the scatter plot (**Figure 2D-F**). According to numbers of enriched DEGs, the PI3K-Akt signaling pathway was also ranked at first, ignoring the pathways in cancer usually enriched most genes in the DAVID. Therefore, we surmised that the PI3K-Akt signaling pathway also exerted a crucial effect on AD from BMSCs.

## Key KEGG pathway enrichment

We next investigated the key signaling pathway in these two lineages of differentiation. The PI3K-Akt signaling pathway, FoxO signaling pathway, Focal adhesion and Cell cycle were the common signaling pathways in OS differentiation (**Figure 3A**). In AD induction, PI3K-Akt signaling pathway, Rap1 signaling pathway, Focal adhesion, Regulation of actin cytoskeleton, Cell cycle and HTLV-I infection were all enriched in the three groups (**Figure 3C**). Three common pathways including PI3K-Akt signaling pathway, Focal adhesion and Cell cycle were all existed in the OS and AD induction. Therefore, we hypothesized that the PI3K-AKT signaling pathway, as well as Focal adhesion, and Cell cycle were the crucial mechanism for initiating the differentiation of hMSCs. For the differences between the two lineages differentiation, the FoxO signaling pathway was specific for OS induction and the Rap1 signaling

pathway belonged only to AD induction, indicating that FoxO signaling pathway and Rap1 signaling pathway were the specific signaling pathway for the OS and AD induction, respectively. And the expression level of DEGs enriched in the FoxO signaling pathway and Rap1 signaling pathway were showed at heatmap (**Figure 3B** and **D**).

### **Key genes in the OS induction**

Interactions between multiple genes could be well understood by PPI analysis. The DEGs OS were used to perform the PPI analysis with Between and Stress algorithm of cytoHubba, from which we choose the top 20 hub genes to construct the sub-network (**Figure 4A-C** and **Figure S2**). As shown in these six PPI networks, the common genes with high degree and clustering coefficients were FoxO3, IL-6, JUN and CAT, which might play important roles in the OS induction. At the same time, candidate genes enriched in the FoxO signaling pathway in each group were also used to perform the PPI network (**Figure 4D-F**). Similarly, FoxO1, IL-6 and CAT were localized at the center of network. These results indicated that FoxO3, IL-6, JUN and CAT maybe the key genes for early OS differentiation. Since FoxO1, IL-6 and CAT were all enriched in the FoxO signaling pathway, further indicating that FoxO signaling pathway may be the specific signaling pathway for early OS differentiation.

### **Key genes in the AD induction**

DEGs AD were used to perform the PPI networks, and sub-networks were formed by the top 20 hub genes. VEGFA, FGF2, MYC and PTEN had the highest degree and clustering coefficients in each group computed by Between algorithm (**Figure 5A-C**), while VEGFA, FGF2, MYC, CCND1 and PTEN were the core computed by Stress algorithm (**Figure S3**), which meant that these genes played core roles in the AD induction. Similarly, the DEGs related to Rap1 signaling pathway were selected to construct PPI network. VEGFA, FGF2 and PIK3R1 were identified as the core genes in the network (**Figure 5D-F**). These interactions relied strongly on VEGFA and FGF2, which may determine the tendency of BMSCs differentiate into AD. Therefore, hub genes VEGFA, FGF2 and Rap1 signaling pathway might be the crucial mechanisms for early AD differentiation.

### **GO analysis of OS induction**

Gene ontology enrichment analyses were performed by the DEGs from each group, and the significant top 15 of the GO terms including BP, CC and MF were displayed as the bar diagrams (**Figure 6A-C**). In the MF category, receptor binding, protein binding, growth factor activity were the common GO terms in the groups of OS\_2d, OS\_3d and OS\_4d, while cytoplasm, cytosol, extracellular exosome were the common GO terms in the CC category. In the BP category, signal transduction, positive regulation of transcription and positive regulation of cell proliferation were all enriched in the three time points of OS induction. To further investigate the relationship of the key genes and GO functional categories, the key genes from the FoxO signaling pathway and PI3K-Akt signaling pathway were put to perform the chord plots (**Figure 6D-F**). Key genes including FoxO3, IL-6, CAT and PIK3R1 mainly clustered into protein binding, membrane, cytosol, nucleus, extracellular space, plasma membrane, cytoplasm, negative and positive regulation of

apoptotic process. Suggesting that GO terms in OS induction were a series of biological responses that initiated by ligand-receptor binding and transcriptional information into the nucleus.

### **GO analysis of AD induction**

The top 15 of the GO terms performed by the DEGs from each group of AD were shown in the bar diagrams (**Figure 7A-C**). The results of the AD differentiation were similar to that in the OS, implied that there were similar biological effects on the two lineages differentiations from hMSCs. Chord plots of AD induction (**Figure 7D-F**) performed by the DEGs of Rap1 signaling pathway and top 15 of GO terms in each group, showed that FGF2 was mainly enriched in the protein binding, growth factor activity, cytoplasm, nucleus, extracellular space, extracellular region, signal transduction, positive regulation of cell proliferation and positive regulation of cell proliferation. Moreover, another target gene VEGFA was mainly related to protein binding, growth factor activity, membrane, extracellular space, extracellular region, cytoplasm and positive regulation of cell proliferation. This was consistent with previously reported that VEGFA was an extracellular signal molecule, which regulated Rap1 signaling pathway by combining some intracellular signal factors [37, 38].

### **Osteo-adipogenesis relative gene expression**

Expression level of OS (ALP) and AD (LPL) specific genes at day 2-4 was increased comparing to the day 0, suggesting that hBMSCs were successfully induced into OS and AD (**Figure 8A and C**). The expression levels of the key genes for OS (IL6 and CAT) or AD (PIK3R1, FGF2 and VEGFA) induction were changed dramatically at the first 2 days, but no obvious change was founded after that, indicating that the first 2 days maybe the critical period for the lineage commitment determinant. However, FoxO3 at day 2-4 was significantly increased in the OS induction, revealing that it may be key genes for osteogenic differentiation at a steady period of lineage-continuation (**Figure 8A**). In addition, the expression of PIK3R1 was higher in both OS and AD, suggesting PIK-AKT signaling pathway is a promoter in the differentiation of BMSCs.

### **Signaling pathway of OS and AD induction**

The expression of the key proteins in the signaling pathway relative to the OS and AD induction were detected using inflorescence staining (**Figure 8 B and D**). In consistent with the results of PCR, the expression of FoxO3 and CAT in OS induction at day 2-4 was higher compared to the day 0, while IL6 was conversely lower, suggesting that FoxO3 signaling pathway was involved in the osteogenic differentiation (**Figure 8B**). Additionally, the downregulation of VEGFA and FGF2 in AD induction indicated that Rap1 signaling pathway maybe crucial for AD differentiation. Meanwhile, the expression of PIK3R1 protein was also increased both in OS and AD.

## **Discussion**

Loss of bone mass and aggravation of micro-fractures increase the bone fragility and susceptibility to fractures in patients of osteoporosis [39]. Currently, most strategies involving drugs based on inhibiting osteoclast activity and then blocking bone resorption, were reported to cause serious adverse reactions. For example, denosumab, a new monoclonal antibody targeting RANKL, is an effective treatment of osteoporosis, but its serious side-effects including increased risk of spinal fracture and osteonecrosis, and the rebound effect after stopping denosumab exposure limit its clinical application [40-43]. Thus, there is currently a lack of enough safe and effective treatment strategy for dealing with this gap [44, 45]. Many studies have confirmed that the pathogenesis of osteoporosis is highly correlated with the tendency of increased adipocytes and decreased osteoblasts [46-48] and our analysis found the number of the DEGs in the AD was more than that of OS corresponding to previous studies (**Figure 1-2**). Therefore, clarifying the detailed mechanisms about the differentiation of MSCs into OS and AD would be contributed to develop a new strategy and treatment for osteoporosis. As the differentiation of MSCs into a specific lineage was determined at the early stage of differentiation [49, 50], we selected early OS and AD profiles to explore the early differentiation mechanisms that determined lineage fates of BMSCs.

After a series of integration analyses, we identified FoxO3, IL-6 and CAT as key genes for osteogenic differentiation, and these genes were all enriched in the FoxO signaling pathway (**Figure 4 and S2**). FoxO, an intracellular signaling factor belongs to transcriptional regulators family of forkhead box O, consisting of FoxO1, FoxO3, FoxO4 and FoxO6, plays a critical regulatory role in the multiple biological processes, including cell cycle, anti-apoptosis and anti-oxidation [51, 52]. Previous studies had discovered that FoxO3 could reduce ROS and promote OS differentiation of MSCs by activating autophagy [53-55] which demonstrated that the up-regulation of FoxO had an essential role on the activity of the OS in MSCs. Wen Sun had verified that Sirt1 overexpression promoted FoxO3a deacetylation and inhibited oxidative stress and resisted the apoptosis to increase the osteogenesis and partially restoring the defects of the skeletal system in osteoporosis [56]. However, lots of evidences also indicated that FoxO3 have a contradictory function in different tissues or expression levels. Anthony et al. had reported that the overexpression of FoxO3 could result in muscle atrophy while the ablation of FoxO3 may lead to defects in the regeneration of muscle by downregulating MyoD (a key myogenic regulator)[57-59]. Meanwhile, Lorenowicz et al have confirmed that activation of autophagy accelerated the osteogenic differentiation via the upregulation of FoxO3, consistent with our findings. On the contrary, stem cell lost a capacity to avoid rising ROS and osteogenic differentiation was impaired on the FoxO3 downregulation[60]. Therefore, we speculate that the function of FoxO may be dependent on the activation level and tissue location, of course, which requires further basic and animal experiments, and even future clinical experiments to discover the mechanism of the contradictory functions, revealing whether the activation of FoxO3 would bring other side effects when promoting OS in BMSCs, such as skeletal muscle atrophy. IL-6, a pleiotropic cytokine with multiple physiological functions including immune regulation, hematopoiesis and tissue regeneration, plays a paramount role in the tissue regeneration engineer, especially in the bone metabolism [61, 62]. It could stimulate the hepatocytes to return to the cell cycle progression through triggering the initiative signal of liver regeneration [63]. Research showed that HIF1A-AS2 could erase the antagonistic effect of IL-6 exerted by miR-665, and then promote osteogenic differentiation of the BMSCs

[64]. Zhongyu Xie also confirmed that IL-6 had an important role in the osteogenic differentiation of BMSCs [65]. As for CAT, Mao Li had illuminated that it could promote osteogenic differentiation through enhancing resistance to oxidative stress [66]. Next, we extracted the expression value of FOXO3, IL-6 and CAT (**Figure 8A**), suggesting FOXO3 and CAT were positively correlated with osteogenic differentiation, while IL-6 was negative. Based on the previous studies, we surmised that FOXO3, IL-6 and CAT were key genes in the OS and FoxO was a core signaling pathway, especially in the early stage of OS.

In the AD, we identified hub-genes of VEGFA and FGF2 in the Rap1 signaling pathway was the essential mechanisms involved in the early stage of adipogenic differentiation (**Figure 5 and S3**). Frank Yeung had indicated that Rap1, a mammalian telomere-binding protein, played a key role in the AD through its additional non-telomeric functions, which was known as co-factor of transcriptional cascade and regulator of NF- $\kappa$ B pathway[67]. VEGFA, a key angiogenic factor, was initially considered as an important molecular in the angiogenesis, and latterly was identified that it had multiple biological functions including bone cellular survival [68]. Wen Zhang pointed out that miR-128 promoted adipogenic differentiation of hMSCs by the suppression of the VEGF pathway [69]. As a heparin-binding growth factor stored in the extracellular matrix, FGF2 had been identified to be an important modulator in the early differentiation and development of cells, owing to its multiple biological functions including chemotaxis, angiogenesis and mitotic activity [70]. Kim, S had found that BMSCs with deficiency of FGF2 showed a strong capacity of adipogenic differentiation, which indicated that down-regulated FGF2 played an important role in the AD[71]. Thus, VEGFA and FGF2 were both negative factors during adipogenic differentiation, which was consistent with our analysis (**Figure.8B**). In the AD, FGF2 might increase the conduction of extracellular signals through molecular adhesion, and then regulate the Rap1 signaling pathway to promote the differentiation of BMSCs.

In the trigger phase of BMSCs differentiation, we speculated PI3K-Akt signaling pathway played a key role in triggering the differentiation of stem cells into various progenitor lines, at least in the OS and AD, based on the previous studies and our surveys (**Figure 1,2 D-F and Figure.3 A-C**). As shown in our analysis, the PI3K-Akt signaling pathway enriched the most DEGs during the whole process of the early stage in OS and AD (**Figure 1D-F and 2D-F**), which meant a paramount role in the differentiation of BMSCs. Activation of PI3K-Akt signaling pathway had been demonstrated not only to promote the OS and AD, but stimulate the chondrogenic differentiation of BMSCs, while it's antagonist could lead to inhibition of BMSCs differentiation [72-74]. Through the PI3K-Akt signaling pathway, BMSCs can also differentiate into other progenitor cells, such as endothelial cells (ECs), Vascular smooth muscle cells [75, 76]. Researches had confirmed that stimulation of PI3K-Akt signaling pathway had the ability to activate its downstream target of rapamycin-p70S6 kinase, and promote BMSCs differentiate into coulvascular smooth muscle. Proangiogenic bioscaffold composited of porous  $\beta$ -CaSiO(3)/PDLGA advanced the ECs differentiation of BMSCs via activating PI3K-Akt signaling pathway, which in turn promoted phosphorylation of endothelial nitric oxide synthase (eNOS), production of nitric oxide (NO) and increased secretion of vascular endothelial growth factor (VEGF). Therefore, we surmised activation of PI3K-Akt signaling pathway in the trigger phase of BMSCs differentiation might subsequently activate the FoxO and Rap1 signaling pathways, respectively, thereby promoting OS and AD. And expression level of

the key gene PIK3R1 from PI3K-Akt signaling pathway was up-regulated both in the OS and AD, which are in line with our speculation (**Figure 8A and B**).

Based on the GO enrichment analysis of OS, protein binding, transcription factor binding, nucleus, membrane, cytosol and Cytoplasm were clustered in the FoxO3 and IL-6. These aggregations were similar in the adipogenesis (**Figure 6D-F and 7D-F**). From these results, it seemed that osteogenic and adipogenic differentiations were consistent in biological processes, both through protein-protein binding and transmission of signaling molecules inside and outside the membrane.

## Conclusions

Taken together, this study analyzed the gene chip expression profile of hMSCs differentiation to identify the potential biomarkers and key pathways between the BMSCs into OS and AD by using the bioinformatic method. Our result indicates that FoxO signaling pathway was a key pathway in the OS and Rap1 signaling pathway is the key pathway in the AD, while the PI3K-Akt is a key signaling pathway with the key gene PIK3R1 in the initial stage of stem cell differentiation. Moreover, FOXO3, IL-6 and CAT are suggested as the potential biomarkers in the OS as well as VEGFA and FGF2 in the AD. Our research may provide a new insight into the study of BMSCs differentiation, contributing to the potential therapeutic targets of osteoporosis.

## Abbreviations

BMSCs

The differentiation of bone mesenchymal stem cells;

AD

Adipogenesis;

OS

Osteogenesis;

DEGs

Differentially expressed genes;

hBMSCs

The differentiation of human bone mesenchymal stem cells;

## Declarations

## Competing interests

The authors declare that they have no competing interests.

## Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the Gene Expression Omnibus repository, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE80614>).

## Ethics approval and consent to participate

Ethics approval was obtained from the Ethics Committee of Guangxi Medical University (Nanning, China)..

## Consent for publication

Not applicable.

## Competing interests

All the authors in this manuscript have read and approved the final version submitted, and there are no conflicts involved in this submission.

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## Authors' contributions

JZ, LZ and PC conceived and designed the study. JW performed the primary bioinformatics analysis and was a major contributor in writing the manuscript; ZZ, BZ and XH made substantial contributions to data analysis, including the biological significance of hub genes and figure editing. ZL and PC were involved in the edition of manuscript. All authors read and approved the final manuscript.

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## Figures

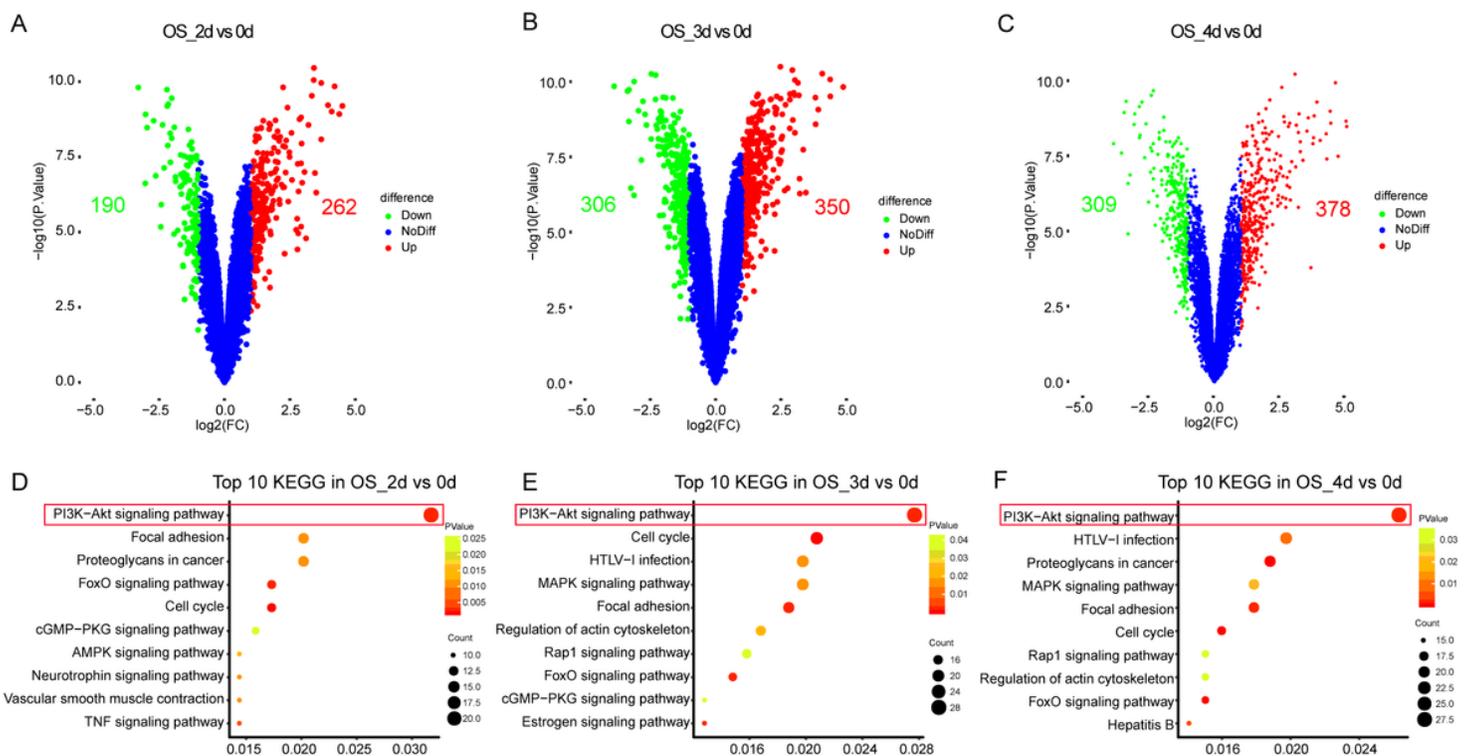
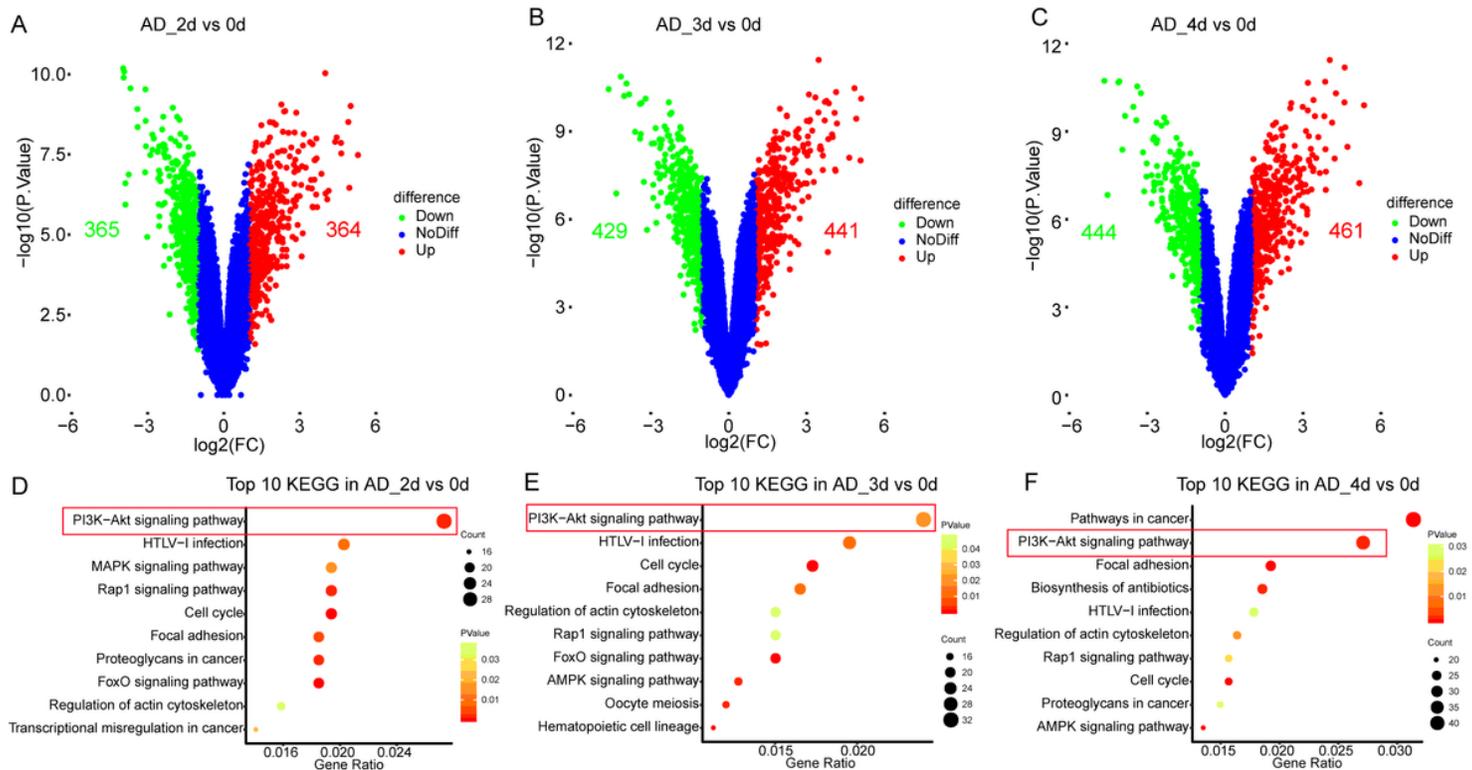


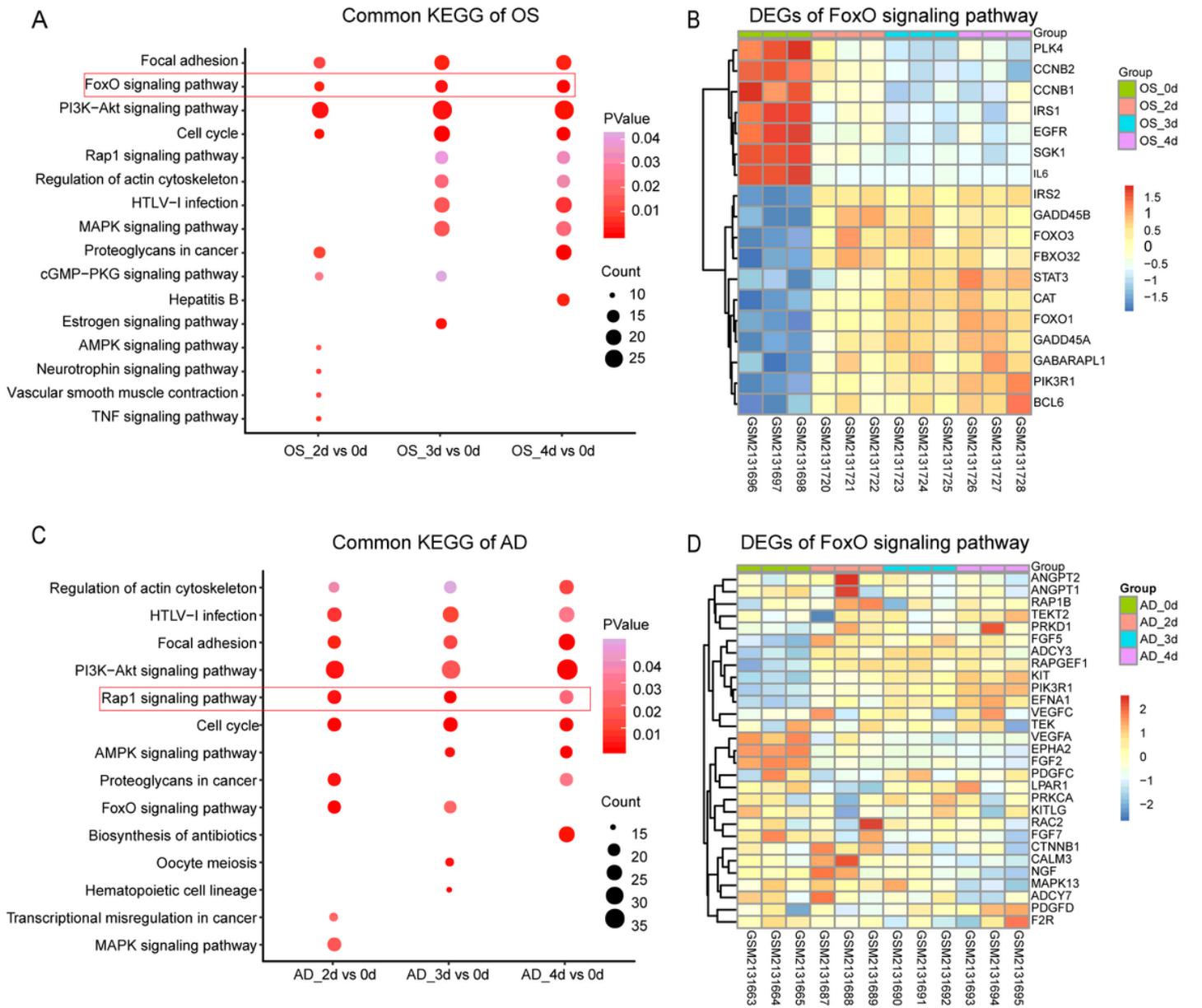
Figure 1

DEGs and KEGG pathway analysis in the three time points of the OS. (A-C) The volcano plot for DEGs of osteogenic differentiation at d2-d3 and d4, respectively. Red point indicated up-regulated genes and the green represented down-regulation while the blue meant no difference. (DEGs were identified as differentially expressed when P. value <0.05 and fold change (FC)  $\geq$  2). (D-F) Top 10 of DEGs Enrichment Pathway resulted at the at d2-d3 and d4 of the OS, respectively. Point size means the gene numbers while the point color represents the P. value (P. value <0.05). OS: osteogenesis; DEGs: differentially expressed genes; KEGG: Kyoto Encyclopedia of Genes and Genomes; d: day.



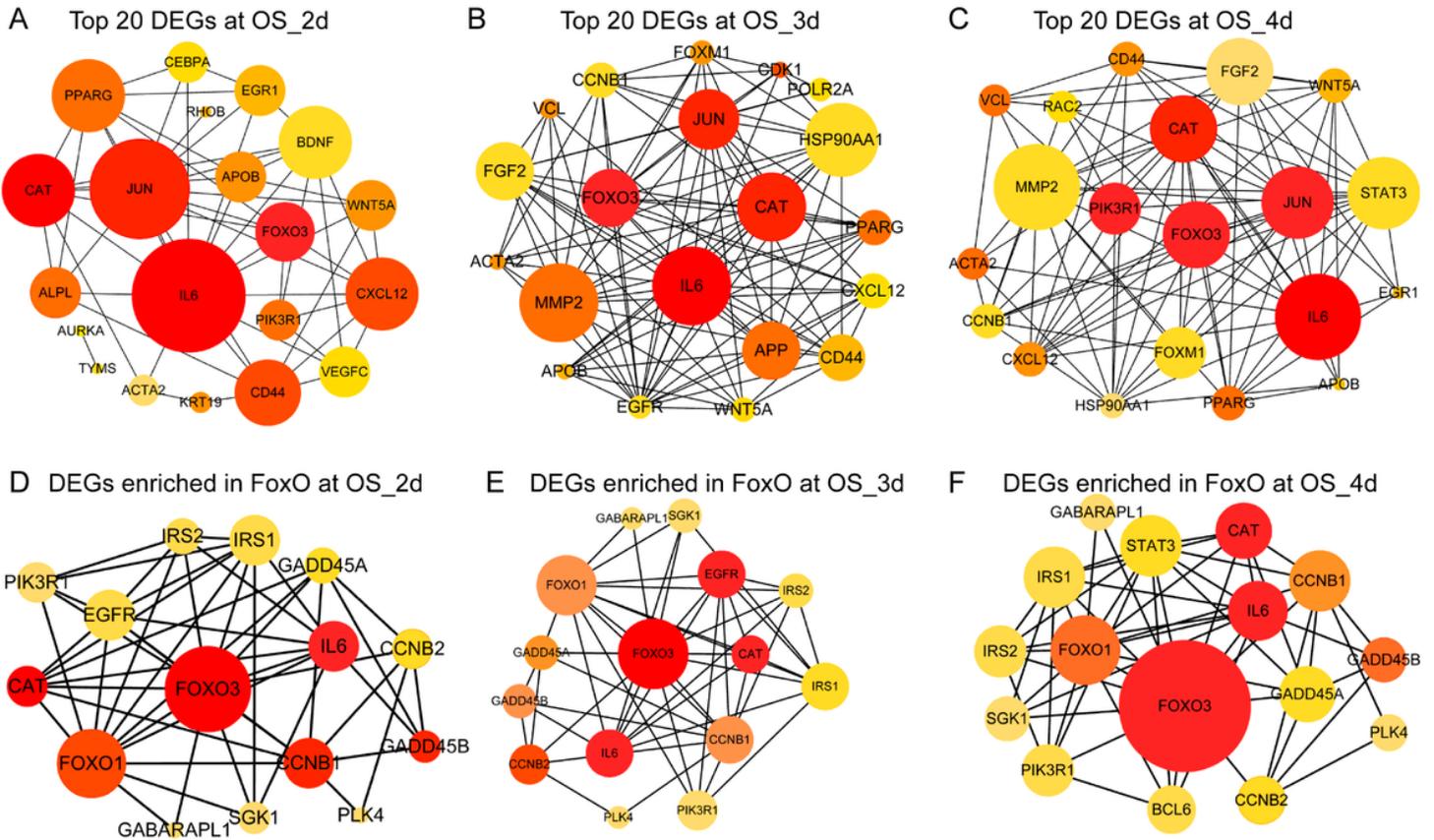
**Figure 2**

DEGs and KEGG pathway analysis in the three time points of the AD. (A-C) The volcano plot for DEGs of adipogenic differentiation at the d2-d2 and d4, respectively. Red point indicates up-regulated genes and the green represented downregulation while the blue meant no difference. (DEGs were identified as differentially expressed when P. value <0.05 and fold change (FC)  $\geq$  2). (D-F) Top 10 of DEGs Enrichment Pathway resulted at the d2-d3 and d4 of the AD, respectively. Point size meant the gene numbers while the point color represented the P. value (P. value <0.05). AD: adipogenesis.



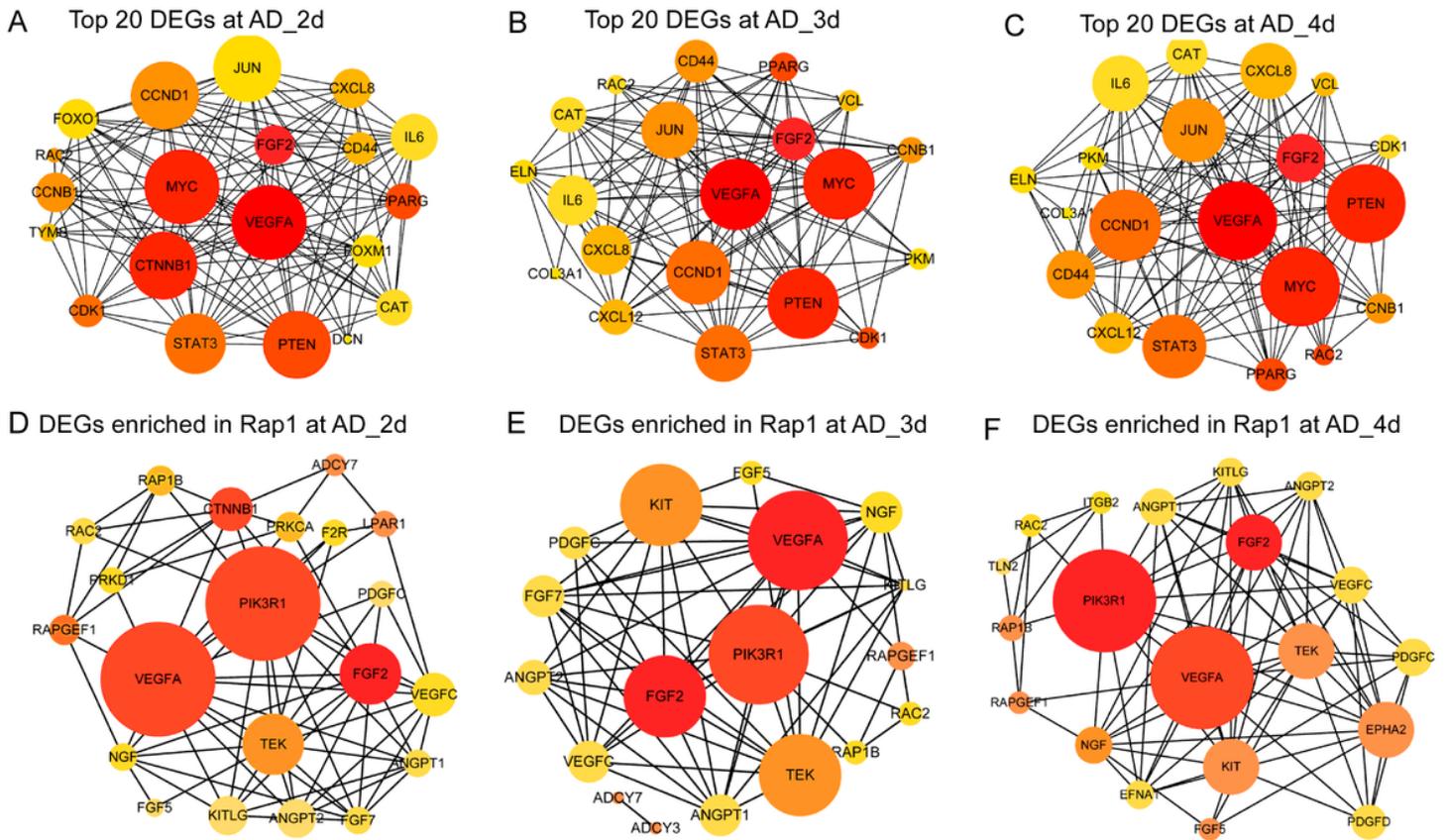
**Figure 3**

Command pathway analysis. (A) Common Enrichment Pathway results of the OS in each time point. (B) Hierarchical cluster of the DEGs enriched in the hub pathway (FoxO signaling pathway). The abscissa was the sample, the right-hand indicated gene name and the left-hand ordinate meant the clustering of DEGs enriched in the key pathway in the OS, while the color represented the log<sub>2</sub> fold change of expression value (range from bright red (upregulation) to bright blue (downregulation)). (C) Top 10 of each group's DEGs Pathway Enrichment results of the AD. (D) Hierarchical clustering of the differential genes enriched in the hub pathway (Rap1 signaling pathway). All icons represented the same meaning as above.



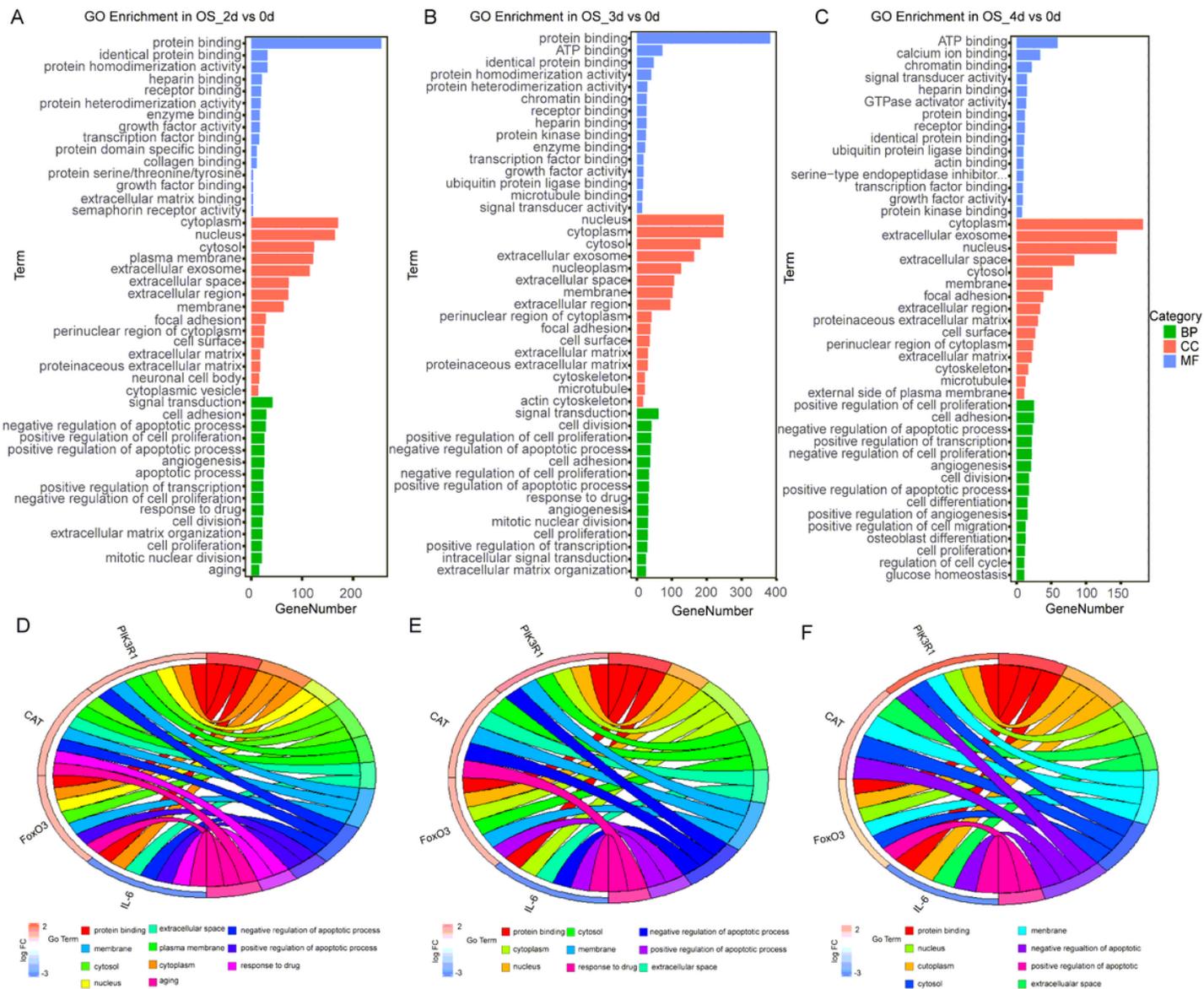
**Figure 4**

Key genes analysis in the OS. (A-C) Network representation of the protein-protein interactions (PPI) between the top 20 genes of the DEGs in different time points in the OS by the Between mode using cytoHubba app in the cytoscape. The node's colors donated the clustering coefficient, and the brighter the color, the higher the clustering, which meant stronger interaction (rang from red (high) to yellow (low)), while the size of each node represented the node degree distribution. (D-F) PPI visualization of networks with the DEGs enriched in the hub pathway (FoxO signaling pathway) of the OS, the color and size of each point meant as the same meaning as above. (A) (D) 2 d vs 0 d; (B) (E) 3 d vs 0 d; (C) (F): 4 d vs 0 d.



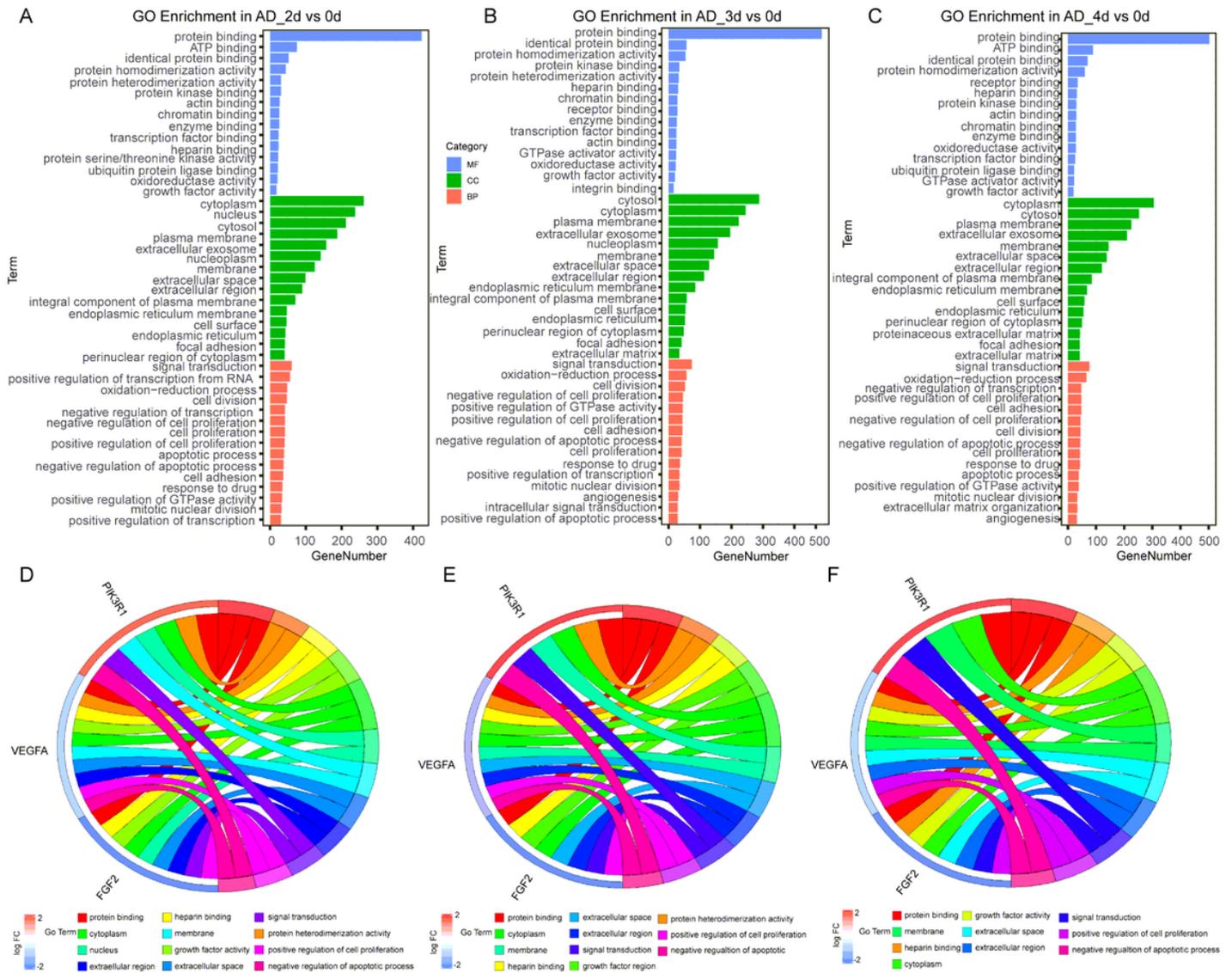
**Figure 5**

Key genes analysis in the AD. (A-C) Network representation of the protein-protein interactions (PPI) between the top 20 genes of the DEGs in different time points in the AD by the Between mode using cytoHubba app in the cytoscape. The node's colors donated the clustering coefficient, and the brighter the color, the higher the clustering, which meant stronger interaction (rang from red (high) to yellow (low)), while the size of each node represented node degree distribution. (D-F) PPI visualization of networks with the DEGs enriched in the hub pathway (Rap1 signaling pathway) of the AD, the color and size of each point meant as the same meaning as above. (A) (D) 2 d vs 0 d; (B) (E) 3 d vs 0 d; (C) (F) 4 d vs 0 d.



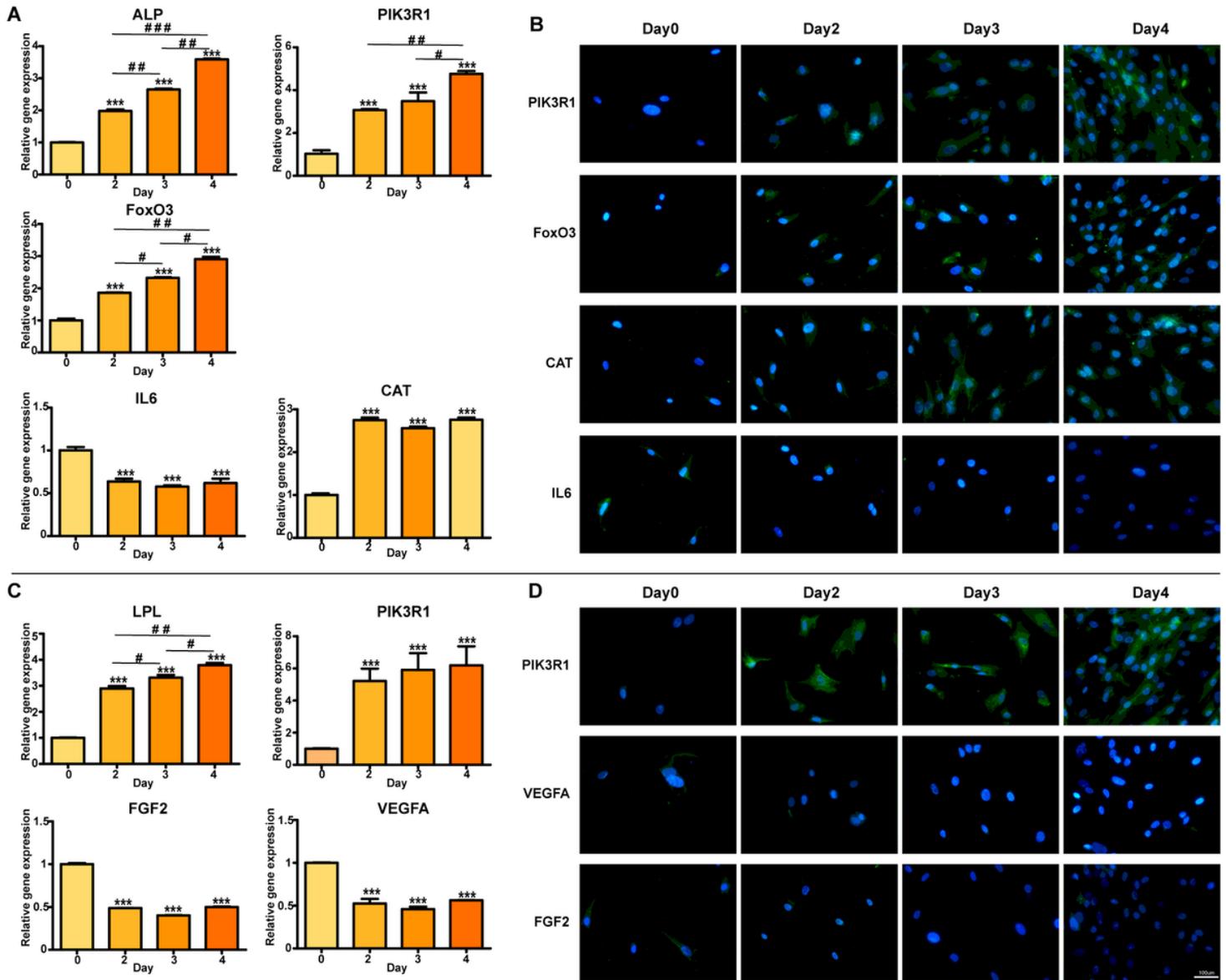
**Figure 6**

GO analysis of DEGs in the OS. (A-C) Top 15 of GO terms including MF, CC and BP in the three time points. The abscissa represented the gene numbers, and the ordinate was the GO terms. (P. value <0.05). (D-F) Cord plot displays of relationship between GO terms and key genes enriched in the hub pathway of the OS (FoxO signaling pathway). (A) (D) 2d vs 0d; (B) (E) 3d vs 0d; (C) (F) 4d vs 0d; GO Gene Ontology; MF molecular function; CC cellular component; BP biological process.



**Figure 7**

GO analysis of DEGs in the AD. (A-C) Top 15 of GO terms including MF, CC and BP in the three time points. The abscissa represented the gene numbers, and the ordinate was the GO terms. (P. value <0.05). (D-F) Cord plot displays of relationship between GO terms and key genes enriched in the hub pathway of the AD (Rap1 signaling pathway). (A) (D) 2d vs 0d; (B) (E) 3d vs 0d; (C) (F) 4d vs 0d.



**Figure 8**

PCR and immunofluorescence staining of the key genes and proteins in the relative signaling pathways. The expression of the key genes and proteins in day 0, day 2, day 3 and day 4 (FoxO3, CAT and IL6 in the FoxO3 signaling pathway; VEGFA and FGF2 in the Rap1 signaling pathway; PIK3R1 in PI3K-Akt signaling pathway).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterial.pdf](#)