Identification of key gene modules and transcription factors for human osteoarthritis by weighted gene co-expression network analysis

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Abstract. Osteoarthritis (OA) is one of the most prevalent causes of joint disease. However, the pathological mechanisms of OA have remained to be completely elucidated, and further investigation into the underlying mechanisms of OA development and the identification of novel therapeutic targets are urgently required. In the present study, the dataset GSE114007 was downloaded from the Gene Expression Omnibus database. Based on weighted gene co-expression network analysis (WGCNA) and the identification of differentially expressed genes (DEGs), the microarray data were further analyzed to identify hub genes, key transcription factors (TFs) and pivotal signaling pathways involved in the pathogenesis of OA. A total of 1,898 genes were identified to be differentially expressed between OA samples and normal samples. Based on WGCNA, the present study identified 5 hub modules closely associated with OA, and the potential key TFs for hub modules were further explored based on CisTargetX. The results demonstrated that B-Cell Lymphoma 6, Myelin Gene Expression Factor 2, Activating Transcription Factor 3, CCAAT Enhancer Binding Protein γ, Nuclear Factor Interleukin-3-Regulated, FOS Like Antigen-2, FOS-Like Antigen-1, Fos Proto-Oncogene, JunD Proto-Oncogene, Transcription Factor CP2 Like 1, RELA proto-oncogene NF-kB subunit, SRY-box transcription factor 3, V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog 2, Interferon Regulatory Factor 4 and REL proto-oncogene, NF-kB subunit were the potential key TFs. In addition, osteoclast differentiation, FoxO, MAPK and PI3K/Akt signaling pathways were revealed to be imperative for the pathogenesis of OA, as these 4 pivotal signaling pathways were observed to be tightly linked through 4 key TFs Fos Proto-Oncogene, JUN, JunD Proto-Oncogene and MYC, and 4 DEGs Vascular Endothelial Growth Factor A, Growth Arrest and DNA Damage Inducible α, Growth Arrest and DNA Damage Inducible β and Cyclin D1. The present study identified a set of potential key genes and signaling pathways, and provided an important opportunity to advance the current understanding of OA.

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Introduction

Osteoarthritis (OA) is one of the most prevalent joint diseases, and has been predicted to be the greatest cause of disability among patients aged >40 years by 2040 (1). One study have revealed that the economic and humanistic burden of OA is high worldwide (2). A patient with OA typically presents with cartilage degeneration and synovial inflammation (3). Clinically, OA is characterized by pain, swelling, joint deformity and disability (4). In addition to operative treatment, the most common pharmacological therapies for OA are analgesic and non-steroidal anti-inflammatory drugs (5). Although a considerable amount of studies investigating OA have been published (6), a systematic understanding of the pathogenesis of OA and its potential therapeutic targets would be of great clinical significance.

With the development of high-throughput genomics technology, gene expression profiles obtained from microarrays have been extensively used to explore and examine genes associated with the progression of various diseases, including the pathogenesis of OA (7,8). However, these studies may have been more relevant if they had considered the correlation of the expression between multiple genes, rather than just focused on the screening of differentially expressed genes (DEGs). Based on the idea that genes with similar expression patterns may be functionally associated, a novel biological methodology, weighted gene co-expression network analysis (WGCNA), has been developed (9). Based on WGCNA, complex microarray data may be simplified into several functional modules, which are composed of co-expressed genes. Subsequently, hub modules may be identified through the associations between modules and clinical traits. Due to the limited access to normal cartilage to serve as control groups, the majority of Bioinformatics studies are focused on knee synovial tissue. However, to the best of our knowledge, WGCNA has not been previously

applied to analyze the cartilaginous expression profiles of OA.

The primary objective of the present study was to obtain data which will aid in providing a more detailed understanding of the pathogenesis of OA. The methodological approach taken in the present study is a mixed methodology based on WGCNA and the screening of DEGs. Microarray data with the accession number GSE114007 were downloaded in the present study, which were subjected to Bioinformatics analysis with the attempt to identify hub genes from a protein-protein interaction (PPI) network, and construct a co-expression network through WGCNA in order to identify hub modules associated with the pathogenesis of OA. Considering that the co-expressed genes in the same module may participate in the same biological function or signaling pathway, and from the point of view of co-expressed genes having the potential to be regulated by the same transcription factors (TFs), the present study attempted to identify key TFs involved in the pathogenesis of OA (10,11).

Materials and methods

Data collection. The dataset GSE114007 [deposited by Fisch *et al* (11)], which is based on the Illumina HiSeq 2000 and Illumina HiSeq 500 platforms (Illumina, Inc.), was downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) (12). The GSE114007 dataset was selected for analysis as it contained the complete information comprising gene expression data and detailed clinical data in this database. RNA-sequencing (RNA-seq) was performed on 18 normal and 20 OA human knee cartilage tissues. Subsequently, the gene expression data were subjected to identical processing using the Robust Multichip Average function within the limma R package (version 3.24.15) (13) for background correction, log2 transformation, quantile normalization and median polish algorithm summarization. The microarray data probe was transformed to gene symbols with Bioconductor Annotation Data software packages (version 1.20.0) (14). In the case that 1 single gene symbol was captured by several probes, the final gene expression level was calculated from the average value of those probes. When one probe was mapped to multiple gene sets, information about the probe was deleted. A principal component analysis (PCA) of normalized data was performed in the present study using the prcomp algorithm in R software. PCA was used for unsupervised multivariate analyses in order to determine the directions of maximum covariance without referring to class labels (Normal/OA).

Identification of DEGs. The DEGs between OA and normal samples were identified through the limma R package using the Student's t-test (13). A $|\mathrm{Log}_2(\text{fold change})|>1$ and adjusted P-value of <0.05 were set as the cut-off criteria. To control the false discovery rate, adjusted P-values were computed for multiple testing corrections of the raw P-value through the Benjamini-Hochberg (BH) method (15). Volcano plots were also constructed to visually display the DEGs. DEGs were counted in the respective hub modules.

Weighted co-expression network construction. WGCNA is a widely used systems biology method, which may be used to construct a scale-free network from gene expression data. Based on the dataset GSE114007, which is comprised of 18 normal and 20 OA samples, a gene co-expression network was constructed through the WGCNA R package (version 1.68) (9). In order to determine whether there were any outlier samples, the present study performed sample clustering according to the distance between different samples in Pearson's correlation matrices. The correlation coefficient between genes 'm' and 'n' was defined as $s_{m,n}=|\mathrm{cor}(m,n)|$, with the gene expression correlation matrix being $S=[s_{mn}]$. The correlation matrix was subsequently translated into an adjacency matrix, $A=[a_{mn}]$, through a power function $a_{mn}=|s_{mn}|^{\beta}$. β was defined as the soft threshold parameter. The β-value was selected as long as scale-free topology fit index (R^2) reached 0.9. The adjacency matrix was then transformed into a topological overlap matrix (TOM) through the following algorithm:

$$\varpi_{mn} = \frac{l_{mn} + \alpha_{mn}}{k_m k_n + 1 - \alpha_{mn}}$$

l_{mn} indicates the product sums of the adjacency coefficients of the nodes connected to both m and n. α_{mn} indicates the correlation coefficient between m and n. k_m indicates the sum of the adjacency coefficients of the nodes only connected to m. k_n indicates the sum of the adjacency coefficients of the nodes only connected to n. Co-expressed gene modules were identified by the dynamic tree cut method, which performs adaptive branch pruning based on various criteria, including a minimum cluster size of 30 genes and a cut height of 0.25. Modules with highly correlated genes were detected and genes exhibiting weaker connectivity were left unassigned. Hierarchical clustering was used to produce a dendrogram of genes based on the corresponding dissimilarity of TOM, and to classify genes with similar expression patterns into gene modules. The module eigengene (ME) was calculated using the MEs function in the 'WGCNA' R package. Finally, hub modules were detected based on the correlation between ME and clinical traits. The correlation values >0.6 were set as cutoff criteria.

PPI network construction and identification of hub genes. The Search Tool for the Retrieval of Interacting Genes (STRING) database (http://www.string-db.org/; version 11.0) was used for the construction of the protein-protein interaction (PPI) network for DEGs and hub modules using default parameters in the STRING database. The types of connection included the following items: Textmining, Experiments, Databases, Co-expression, Neighborhood, Gene fusion and Co-occurrence. To extract valid interactions, a confidence score of >0.4 was set as the cut-off criterion. The nodes in the PPI network with the highest degrees of interactions were considered as hub proteins. The present study also used the Cytoscape plugin CytoHubba to calculate the degree of each node in the PPI network (version 1.5) (16). The top 10 genes in each module and top 10 DEGs were defined as hub genes. In addition, OA-associated genes were downloaded from Junior Doc (http://www.drwang.top/). By entering 'OA' in the search tool, 520 OA-associated genes were listed and downloaded.

Finally, OA-associated genes were counted in the respective hub modules.

Functional enrichment analysis of DEGs and hub modules. To determine the DEGs and hub modules involved in BP terms and pathways, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the ClusterProfiler R package (version 3.8.1) (17). An adjusted P-value <0.01 (to control the false discovery rate, adjusted P-values were computed for multiple testing corrections of the raw P-value through the BH method) was set as the cutoff criterion for the functional enrichment analysis.

Identification of key TFs. CisTargetX may be used to predict TFs involved in the regulation of co-expressed genes (18,19). Based on CisTargetX, the present study further explored the potential TFs for each hub module. CisTargetX was used with the following parameters: Mapping of genes in regions, '10 kb upstream, transcription start site, 10 kb downstream'; minimum fraction of overlap, '0.4'; normalized enrichment score threshold, '4'; receiver operating characteristic threshold for area under the curve calculation, '0.005'. Key TFs of each hub module were defined as predicted TFs, which were also DEGs in the present study. Common elements in DEGs and predicted TFs were compared using Microsoft Excel (version 14.5.1; Microsoft Corporation). By comparing the genes targeted by key TFs with DEGs, the DEGs that were targeted by the key TFs were counted and extracted for further study.

Identification of pivotal signaling pathways. To provide a deeper understanding of changes in gene regulation underlying OA, information on key TFs likely involved in regulating these DEGs was extracted. KEGG analysis was subsequently performed for key TFs and their target DEGs in order to identify pivotal signaling pathways. Based on STRING database and the pheatmap R package (https://cran.r-project.org/web/packages/pheatmap/index.html; version 1.0.12), a PPI network (confidence score >0.4 set as the cut-off criterion) and heatmap were constructed for key TFs and their target DEGs that were involved in key signaling pathways. The transcriptional regulatory network was visualized by Cytoscape.

Results

Identification of DEGs. To estimate false-positives, the procedure was performed using randomized versions of the sample labels and the outcomes were compared with the original results based on PCA (Fig. 1A) (20). PCA revealed clear differences between the normal and OA samples. Sample clustering revealed that no samples required removal from subsequent analysis due to outliers (Fig. 1B). With an adjusted P-value <0.05 and |log2fold change|>1 as the cut-off criteria, a total of 1,898 genes were identified to be differentially expressed between OA samples and normal samples, of which 966 were upregulated (Table SI) and 932 were downregulated (Table SII). The DEGs are highlighted in the volcano plot (Fig. S1). As illustrated in Fig. S2, unsupervised hierarchical clustering analysis of the obtained DEGs was able to distinctly classify all samples into normal and OA groups.

Weighted co-expression network construction. A weighted co-expression network was constructed using the 'WGCNA' R package. Construction of an appropriate scale-free network required an optimal soft-thresholding power to which co-expression similarity was raised to calculate adjacency. The analysis of network topology was performed for various soft-thresholding powers in order to obtain a relatively balanced scale independence and mean connectivity of the WGCNA (9). In Fig. 2A, the scale-free topology fitting index (R^2, y-axis) is presented as a function of the soft-thresholding power (x-axis) in the left panel, while the right panel displays the mean connectivity (degree, y-axis) as a function of the soft-thresholding power (x-axis). Red Arabic numbers in the panels denote different soft-thresholds. The red line in the left panel indicates R^2=0.9. There was a trade-off between maximization of the scale-free topology model fitting index (R^2) and maintenance of a high mean number of connections. Thus, β was set as 9. Subsequently, co-expressed gene modules were identified by the dynamic tree cut method, and each of the modules was marked by a color, indicated in the color band below the dendrogram (Fig. 2B). Genes that were not assigned to any of the modules were colored in grey. A total of 24 modules were identified in the GSE114007 dataset (Fig. 3), of which 5 modules were identified as hub modules based on the correlation coefficients between the ME and traits (>0.6). The genes in each module are listed in Table SIII. The purple, salmon, black, brown and magenta modules were close to 'OA' traits. Among them, the purple, black and brown modules were also close to 'age' traits. Previous studies have concluded that females are more susceptible to OA than males (21). However, the present study did not identify any modules that were closely associated with 'sex' traits. Detailed information of hub modules is listed in Table I.

GO enrichment function and pathway analysis. To further investigate the DEGs and hub modules involved in BP terms and signaling pathways, respective GO and KEGG analyses were performed using the clusterProfiler R package (Fig. 4). The results demonstrated that the DEGs were significantly enriched in key processes, including skeletal system development, ossification, extracellular structure organization, osteoblast differentiation, response to corticosteroid, focal adhesion, the Wnt signaling pathway, complement and coagulation cascades, extracellular matrix (ECM)-receptor interactions and rheumatoid arthritis (Fig. 4A). Fig. 4B-G presents the results obtained from the enrichment analysis of hub modules, which suggest that these BP terms and pathways may exert pivotal functions in the pathogenesis of OA.

PPI network analysis of DEGs and hub modules. A PPI network for the DEGs at the protein level was constructed using the STRING analysis tool. A total of 537 nodes and 1,218 edges were contained in the PPI network. Based on the plugin CytoHubba, the hub genes were defined as the genes with the top 10 with the highest degree of connectivity in the entire PPI network (Table II), including Jun Proto-Oncogene (JUN), Vascular Endothelial Growth Factor A (VEGFA), Fos

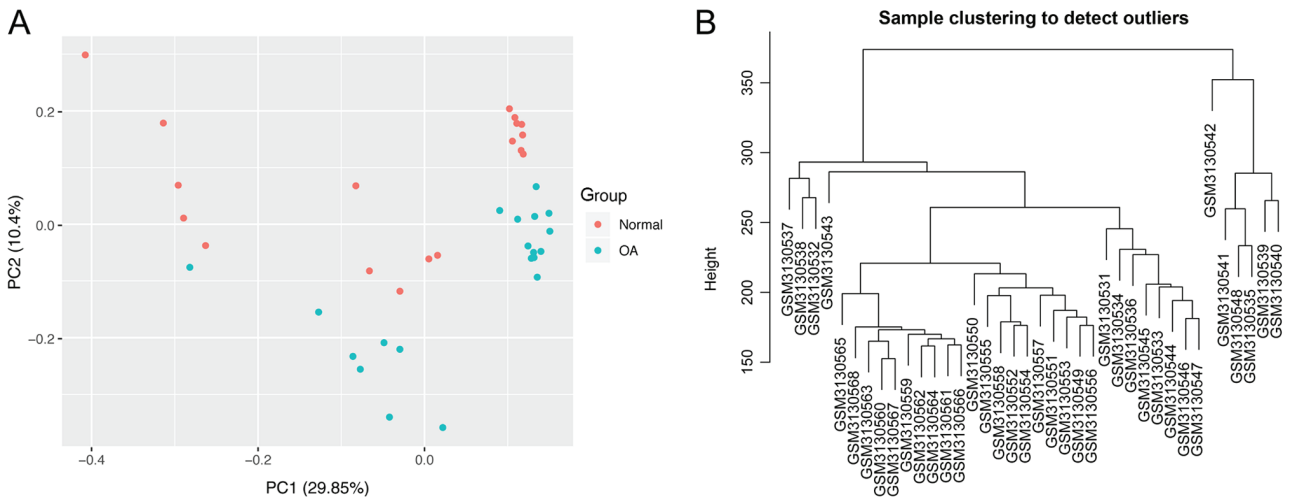

Figure 1. PCA and sample clustering. (A) PCA based on PC1 (proportion of contribution, 59.3%) and of PC2 (proportion of contribution, 23.1%) indicated a different distribution of normal and osteoarthritis samples. (B) Sample clustering indicated that no outliers were present that required removal from the subsequent analysis. FC, fold change; PCA, principal component analysis.

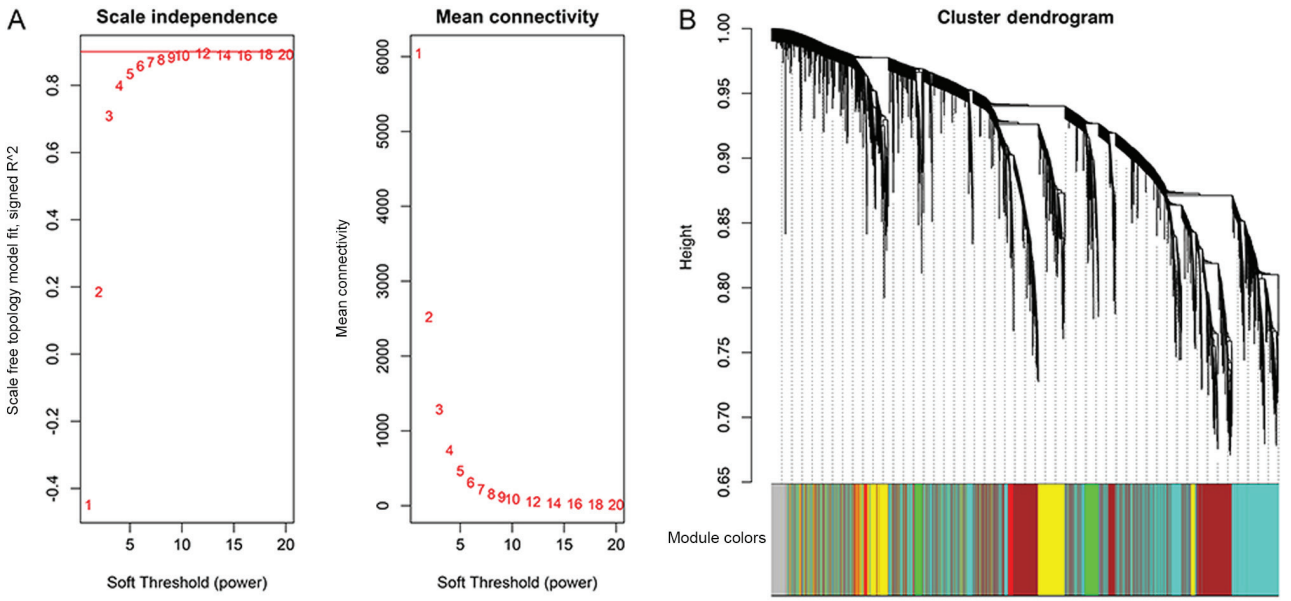

Figure 2. Weighted gene co-expression network construction. (A) Analysis of the scale free topology module fit and the mean connectivity for different soft-thresholding powers (β). The red line in the left panel indicates R^2=0.9. (B) Gene dendrogram obtained from hierarchical clustering. The row of colored bars below the dendrogram represents the module assignment identified by the dynamic tree cut.

Proto-Oncogene (FOS), DNA Topoisomerase IIα and Matrix Metallopeptidase 9. A PPI network of hub modules was constructed using the same method, and genes with the top 10 connectivity in the hub modules were defined as the hub genes (Table II), including Cell Division Cycle 42 (CDC42), PH Domain and Leucine Rich Repeat Protein Phosphatase 1 (PHLPP1), Protein Tyrosine Phosphatase Receptor Type C (PTPRC), JUN and Ribosome Production Factor 2 (RPF2).

Identification of key TFs for hub modules. Transcriptional regulation is a fundamental and pivotal process involved in the pathogenesis of various diseases (22). Co-expressed genes in each hub module may be involved in the same biological functions and regulated by the same TFs. Therefore, the present study further explored the potential TFs for hub modules based on CisTargetX (Table SIV). The results demonstrated that B-Cell Lymphoma 6 (BCL6), Myelin Gene Expression Factor 2 (MYEF2), Activating Transcription Factor 3 (ATF3), CCAAT Enhancer Binding Protein γ (CEBPG), Nuclear Factor Interleukin-3-Regulated (NFIL3), FOS Like Antigen-2 (FOSL2), FOS-Like Antigen-1 (FOSL1), Fos Proto-Oncogene (FOS), JunD Proto-Oncogene (JUND), Transcription Factor CP2 Like 1 (TFCP2L1), RELA proto-oncogene NF-kB subunit (RELA), SRY-box transcription factor 3, V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog 2, Interferon Regulatory Factor 4 (IRF4) and REL proto-oncogene NF-kB subunit (REL) were the potential regulators involved in managing hub modules (Table I). Detailed information of key TFs is listed in Table SV. These TFs may serve a key role in regulating the

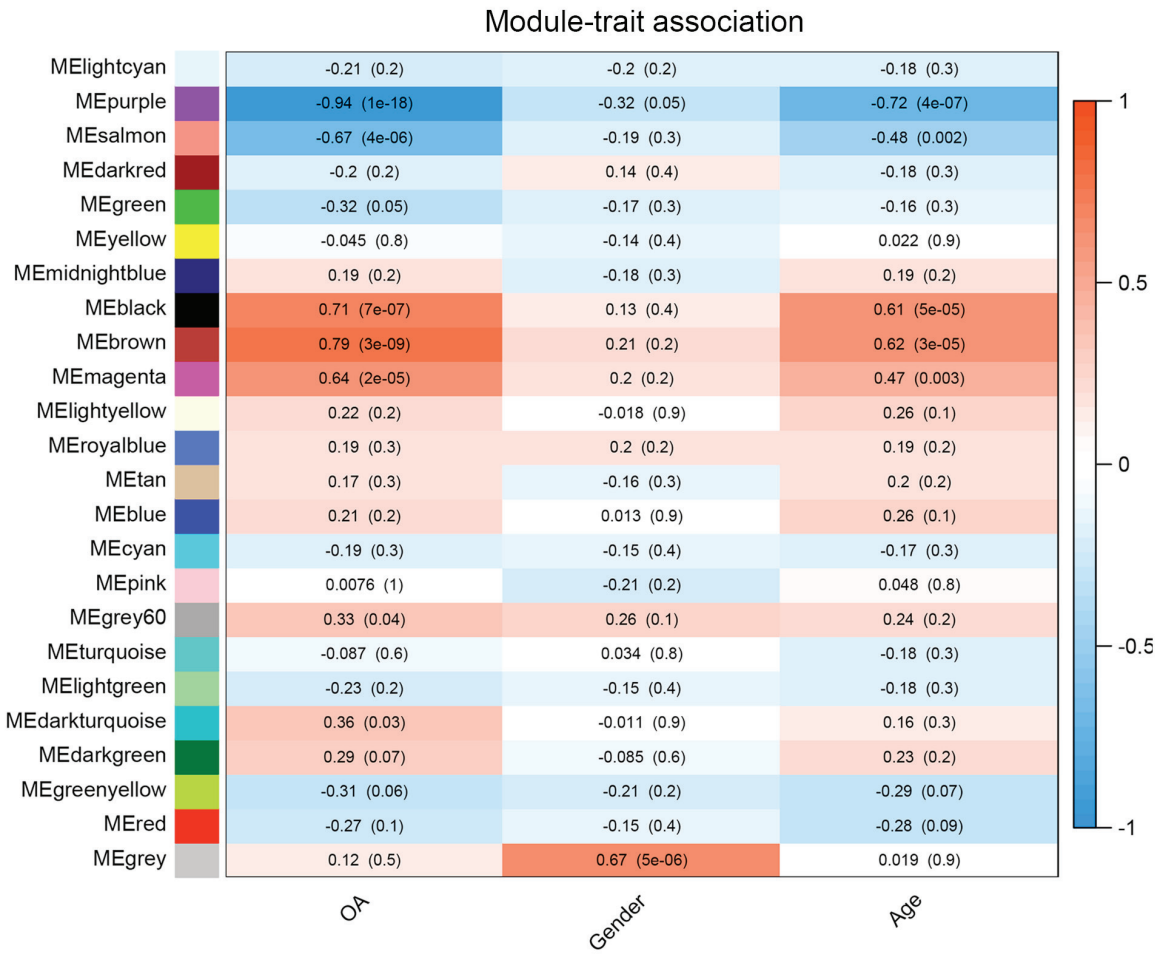

Figure 3. Module-trait matrixes were identified based on the correlation between clinical traits and ME. The number on the left of each cell illustrates the corresponding correlation coefficient and the number in bracket on the right is the P-value. High correlation coefficients were colored in red and low correlation coefficients in blue. The cutoff value for age was set as 60 years. ME, module eigengene; OA, osteoarthritis.

network of hub modules, and potentially affect the initiation and progression of OA.

Identification of pivotal signaling pathways. The key TFs that target the largest number of DEGs were in the purple, brown and magenta modules (Fig. 5A), and were therefore further investigated. Fig. 5B provides a heat map on 19 key TFs. KEGG analysis was subsequently performed for key TFs and their target DEGs in the purple, brown and magenta modules (Fig. 5C). Based on the results of this enrichment analysis, it was indicated that osteoclast differentiation (purple and magenta modules), forkhead box (Fox)O signaling pathway (brown and purple modules), mitogen-activated protein kinase (MAPK) signaling pathway (purple module) and PI3K/Akt signaling pathway (brown module) are imperative for the pathogenesis of OA. Based on analysis using the STRING database and the pheatmap R package, a PPI network and heatmap were respectively constructed for key TFs and their target DEGs that were involved in the pivotal signaling pathways (Fig. 6). This analysis highlights that these key TFs and DEGs serve a central role in the pivotal signaling pathways. It was also observed that the 4 pivotal signaling pathways are tightly linked through 4 key TFs, including FOS, JUN, JUND and MYC, as well as 4 DEGs, including VEGFA, GADD45A, GADD45B and CCND1. In addition to these 8 genes, the present study also revealed that BCL6 is the key TF that targets the largest number of DEGs, and that BCL6 is also involved in the FoxO signaling pathway. These results suggest that FOS, JUN, JUND, MYC, VEGFA, CCND1, GADD45A, GADD45B and BCL6 serve important roles in the pathogenesis of OA.

Discussion

OA is a common chronic joint disease among aging individuals, and is characterized by articular cartilage degeneration, thickening of synovial lining, subchondral bone sclerosis and the formation of osteophytes at the joint margin (23). However, there is currently no efficient treatment for OA. Therefore, it is imperative to understand the underlying molecular mechanisms and pathological processes governing OA. Due to the limited access to normal cartilage to serve as a control group, microarray data in the GEO database containing normal cartilage tissues are relatively sparse, and the sample size is frequently insufficient. For the present study, a total of 64 articles were screened out from the Pubmed database based on the following search terms applied to title and abstract only: 'Osteoarthritis' AND ('co-expression' OR 'coexpression' OR 'networks') AND gene expression. The abstract of these 64 studies was carefully read and these articles are summarized in Table SVI. As indicated in this table, the samples in most of

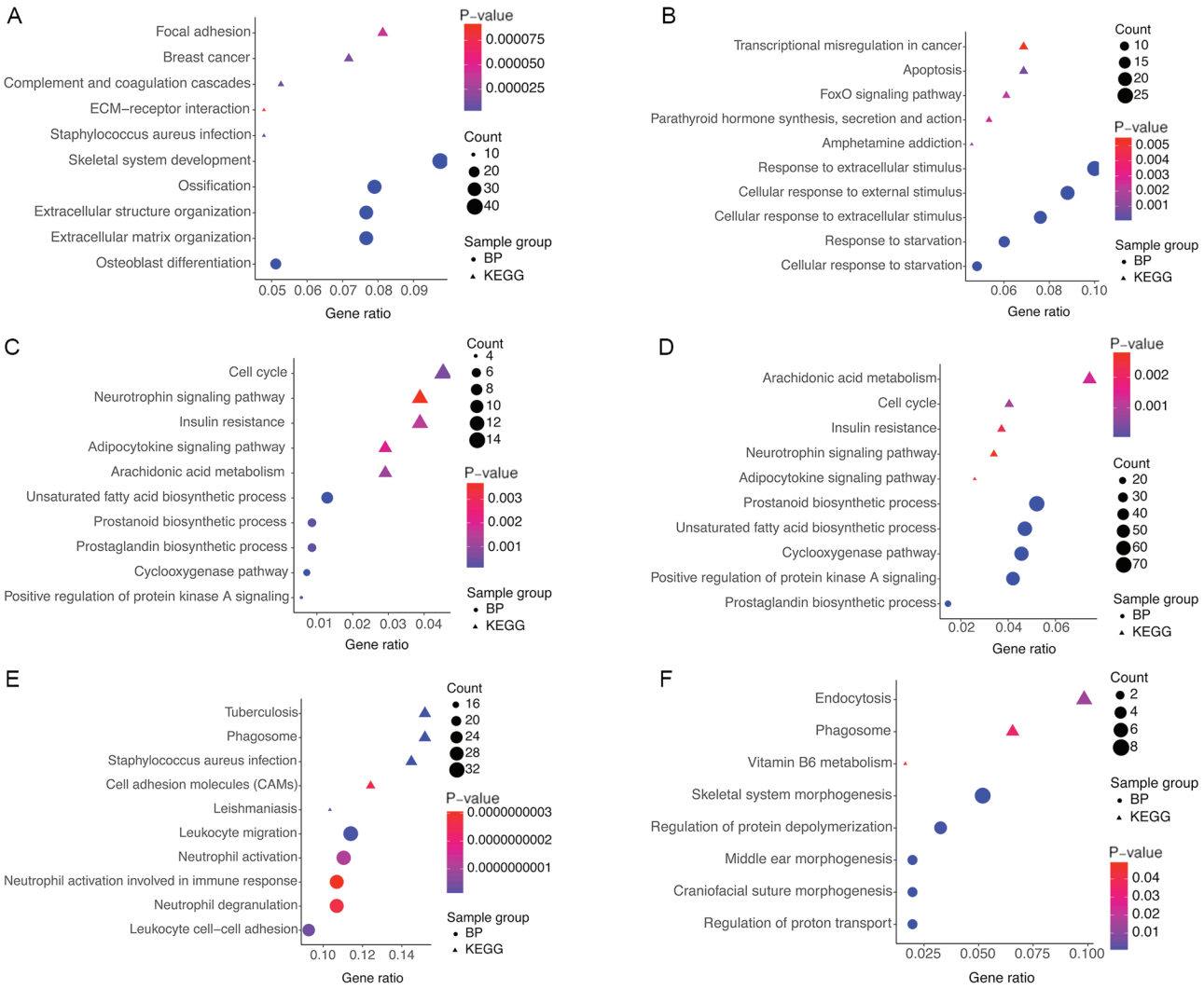

Figure 4. Functional enrichment analysis for DEGs and hub modules. (A) Enrichment analysis for all DEGs. (B-F) Enrichment analysis for individual modules. (B) Purple, (C) black, (D) brown, (E) magenta and (F) salmon module. Triangles represent GO terms and circles represent KEGG pathways. DEG, differentially expressed gene; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; ECM, extracellular matrix; Fox, forkhead box.

the studies are from knee synovial tissue, while three studies investigated the microarray data containing samples of human knee articular cartilage (11,24,25). The GSE114007 dataset contained 18 normal and 20 OA human knee cartilage tissues. The cartilage tissues were obtained from the weight-bearing regions on medial and lateral femoral condyles. The samples for the normal group were collected from joints that had no macroscopic or microscopic evidence of cartilage damage, so that the differences between normal and OA samples are obvious. Another two datasets in the GEO database are worth mentioning, namely GSE117999 and GSE113825, which contain data on human knee cartilage tissues. Degenerative OA tends to be initiated in weight-bearing regions. Therefore, the major drawback of GSE117999 is that the healthy-appearing cartilage was garnered from the non-weight-bearing site of the knee joint. The dataset GSE113825 is restricted by the amount of samples. On the basis of the above, it was we concluded that GSE114007 is a valuable microarray dataset, and is worthy of further in-depth analysis based on WGCNA. To the best of our knowledge, the WGCNA has not been previously applied to analyze the expression profiles of cartilaginous OA. The results of the present study revealed that there were a total of 1,898 DEGs, including 966 upregulated and 932 downregulated DEGs in human knee cartilage tissues of OA. In addition, 5 hub modules were identified to be closely associated with OA based on WGCNA. A PPI network was constructed in order to identify hub genes among DEGs and hub modules. Functional enrichment analysis was also performed for the DEGs and hub modules in order to identify significant BP terms and signaling pathways. It is well-accepted that co-expressed genes are more likely to be co-regulated (26), and transcriptional regulation is a basic and critical BP in eukaryotes (22). Therefore, the further analysis focused on TFs, and 19 potential key TFs were identified, which may be involved in the regulation of hub modules based on CisTargetX. Finally, 4 pivotal signaling pathways that may largely contribute to the molecular processes of OA were identified via KEGG enrichment analysis. These signaling pathways are i) Osteoclast differentiation; ii) FoxO signaling pathway; iii) the MAPK signaling pathway, and iv) the PI3K/Akt signaling pathway.

Based on the WGCNA performed in the present study, 5 hub modules with highly relevant expression patterns that

Table I. Detailed information of hub modules.

Module name	Trait	Gene count	Module association to trait	OA associated genes count	DEG count	Key TFs
Brown	OA/Age	1,741	0.79/0.62	77	594	BCL6, MYEF2
Purple	OA/Age	307	-0.94/-0.72	14	180	JUN, ATF3, CEBPG, NFIL3, FOSL2, FOSL1, FOS, MYC, JUND
Salmon	OA	202	-0.67	5	47	TFCP2L1, RELA, SOX3
Black	OA/Age	920	0.71/0.61	22	316	ETS2, SOX9, SOX10
Magenta	OA	328	0.64	18	159	IRF4, REL

OA, osteoarthritis; TFs, transcription factors; DEG, differentially expressed gene.

Table II. Top 10 hub genes in protein-protein interaction network of DEGs and hub modules.

DEGs/Modules	Top 10 hub genes
DEGs	JUN VEGFA FOS TOP2A MMP9 CDK1 BIRC5 PTPRC KIT KIF23
Black	CDC42 CDK2 ACTC1 EHHADH CDK5 INSR DLG4 ACSL4 ACE E2F4
Brown	PHLPP1 EGF MYC CALM1 CCND1 CDC20 IGF1 AURKA NOTCH1 APP
Magenta	PTPRC CDK1 KIF15 TOP2A BUB1 CSF1R TYROBP ITGAM MELK BUB1B
Purple	JUN FOS VEGFA ATF3 EGR1 CDKN1A DUSP1 YARS JUNB FOSB
Salmon	RPF2 NGDN RCL1 RSL24D1 AEN TRMT11 ZNRD1 PAN2 SRSF7 DDX39A

DEG, differentially expressed gene.

were closely associated with OA were identified. GO BP and KEGG analyses were subsequently performed to identify significant BP terms and pathways in each hub module. The genes in the black module were primarily enriched in monocarboxylic acid biosynthetic process, signal transduction by p53 class mediator, cell cycle, insulin signaling pathway and FoxO signaling pathway. Monocarboxylate transporter 4 is a potential therapeutic target for inflammatory arthritis (27) and Zhu *et al* (28) reported that p53 genes are correlated with the disease grade of knee OA. The brown module was primarily enriched in skeletal system development, extracellular structure organization, ECM organization, ossification, chondrocyte differentiation, collagen catabolic process, PI3K/Akt signaling pathway, Hippo signaling pathway and FoxO signaling pathway. It has previously been reported that the chondrocyte ECM serves an important role in cartilage function (29), and that the dynamic equilibrium between matrix synthesis and degradation maintains the stability and integrity of cartilage tissue (30). A recent study has reported that the Hippo signaling pathway and ECM-receptor pathway are involved in the process of osteoarthritic chondrocyte apoptosis (31). In addition, the results of the present study revealed that the purple module was primarily enriched in response to extracellular stimulus, FoxO signaling pathway and human T-cell leukemia virus 1 infection. Andriacchi *et al* (32) reported that a mechanical stimulus at the baseline may enhance the sensitivity of a biomarker to predict cartilage thinning in a 5-year follow-up in patients with knee OA through a systems model of OA. In addition, the magenta module was typically enriched in neutrophil-mediated immunity and neutrophil activation involved in immune response. The magenta module is principally associated with the immune response. A previous study has suggested that the innate immune response is a potentially modifiable process, which may subsequently augment the pathological changes observed in OA (33).

The pathogenesis of OA is multifactorial and intricate, and is influenced by inflammation and oxidative stress (34). Due to its complex mechanism, numerous genes are differentially expressed in OA. In order to efficiently identify the crucial candidate genes, the present study primarily focused on the TFs likely involved in the pathogenesis of OA. A holistic approach was utilized, in which hub genes, key TFs and pivotal signaling pathways were integrated to identify more effective targets, which may be assessed in subsequent research. This analysis identified 8 key TFs and 1 hub gene (JUN, JUND, FOS, FOSL1, FOSL2, MYC, BCL6, ATF3 and VEGFA) that exert major functions in regulating hub modules and pivotal signaling pathways. Among these genes, JUN, FOS, MYC, ATF3 and VEGFA are also the hub genes in the PPI network of DEGs and the hub modules (Table II). JUN, JUND, FOS, FOSL1 and FOSL2 are subunits of activator protein 1 (AP-1) (35). Previous studies have suggested that AP-1 is involved in the differentiation, hypertrophy and expression of matrix metalloproteinase (MMP)13 in chondrocytes (36-38). In addition, Papachristou *et al* (39) identified that AP-1 is involved in the signal transduction pathway of mechanical loading in condylar chondrocytes. The results of the present study indicate that JUN, JUND, FOS, FOSL1 and FOSL2 are

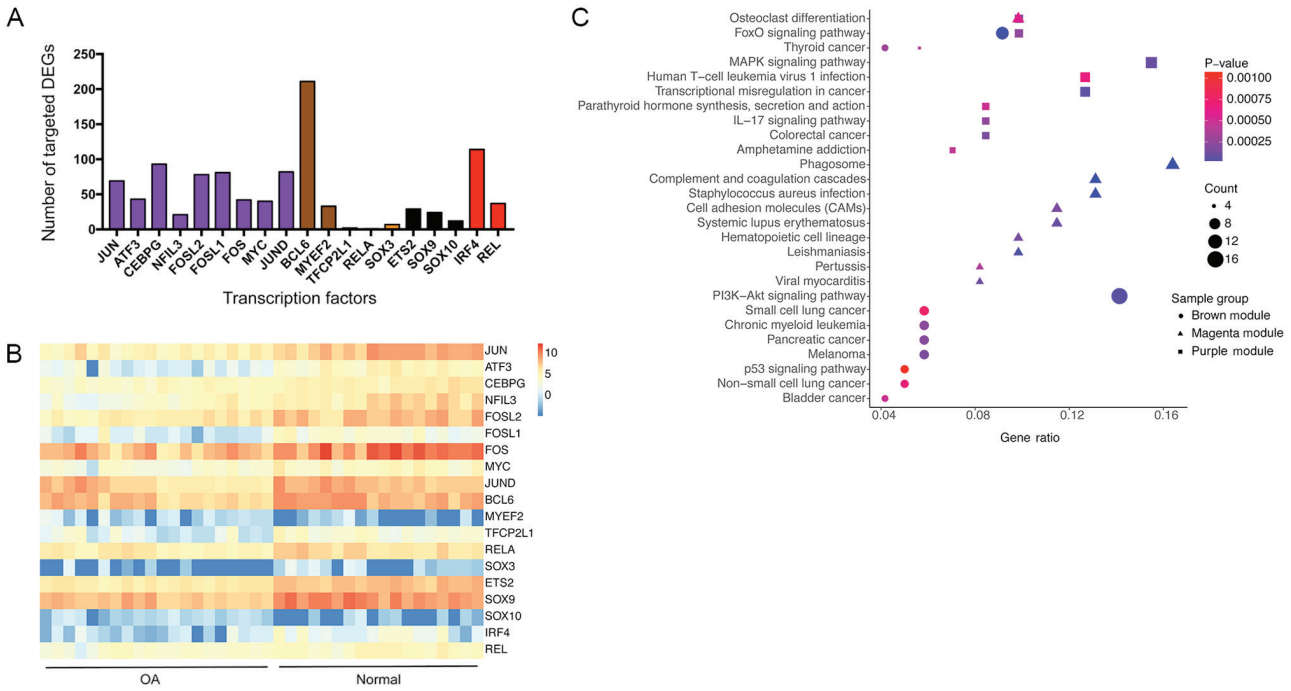

Figure 5. Identification of key TFs for hub modules. (A) Number of DEGs targeted by predicted TFss in each hub module. (B) Heatmap of predicted TFs based on the GSE114007 dataset between OA and normal samples. (C) Kyoto Encyclopedia of Genes and Genomes analysis for key TFs and their target DEGs. DEG, differentially expressed gene; OA, osteoarthritis; Fox, forkhead box; IL, interleukin; MAPK, mitogen-activated protein kinase; TF, transcription factor.

involved in osteoclast differentiation. In addition, previous research has suggested that AP-1 proteins are important inflammatory regulators in skin disease (40) and are hypothesized to be involved in regulating reactive oxygen species (ROS) in macrophages (41). There is evidence to suggest that C-myc suppresses apoptosis and promotes the proliferation of chondrocytes to prevent the occurrence and subsequent progression of OA via inactivating the MAPK signaling pathway (42). Consistent with the literature, the present study identified that MYC participates in the MAPK signaling pathway and is downregulated in OA samples. Lemos *et al* (43) demonstrated that inhibition of MYC *in vivo* as well as in human kidney organoids *in vitro* abrogated inflammation and fibrosis. The results of Anderton *et al* (44) suggest that MYC may serve a role in regulating a major anti-oxidant pathway downstream of glutamine. The present study identified BCL6 as the key TF that targeted the largest number of DEGs in the brown module. The precise association between OA and BCL6 has remained to be determined. However, a previous study reported that BCL6 attenuates lipopolysaccharide-induced inflammation in HK-2 cells via negative regulation of NLRP3 transcription (45). Iezaki *et al* (46) argued that ATF3 modulates the expression of MMP13 in human chondrocytes and indicated that ATF3 is implicated in the pathogenesis of OA through the modulation of inflammatory cytokine expression in chondrocytes, which may serve as a novel therapeutic target for the treatment of OA. ATF3 has been reported to be involved in regulating the inflammatory process in renal ischemia-reperfusion injury (47) and the central nervous system (48). With increasing severity of OA, greater vascular invasion into articular cartilage has been observed (49), and VEGFA has a fundamental role in angiogenesis (50). The present study observed that VEGFA is an integral DEG and in the center of a regulatory network (Fig. 6). CCND1 is an important cell cycle regulator, and CCND1 silencing may promote interleukin (IL)-1β-induced apoptosis in rat chondrocytes (51). SOX9, a TF known to regulate type II collagen and aggrecan, is a critical factor in the OA-associated downregulation of ECM molecules (52). According to a recent Bioinformatics study, VEGFA, JUN, ATF3, JUN and MYC were also significantly dysregulated in synovial membranes between the normal and OA group (53). These results suggest that dysregulation of these TFs and hub DEGs may participate in chondrocyte homeostasis and the pathogenesis of OA. Fisch *et al* (11) systematically analyzed the dataset GSE114007 based on a set of methods (identification of DEGs, TF enrichment analysis and network analysis), and obtained numerous significant results. They identified eight TFs (JUN, EGR1, JUND, FOSL2, MYC, KLF4, RELA and FOS) and 3 pivotal signaling pathways (hypoxia-inducible factor-1 signaling, pathways in cancer and FoxO signaling) that have a central role in the pathogenesis of OA. The present study used a different method, WGCNA, to further analyze the dataset GSE114007. The present analysis confirmed several of the key TFs identified by Fisch *et al* (11), including JUN, JUND, FOS, FOSL1, FOSL2, MYC and RELA, and suggested further key genes that may be involved in the pathogenesis of OA, including ATF3, BCL6, SOX9, VEGFA, CCND1, GADD45A and GADD45B. In addition to FoxO signaling, 3 further pivotal signaling pathways were identified (osteoclast differentiation, MAPK and PI3K/Akt signaling pathway).

In addition to the genes that have already been discussed, there are still other key TFs that target large numbers of DEGs and hub genes with high connectivity in PPI networks identified at present study that require further investigation, including GADD45A, GADD45B, CDC42, CEBPG, NFIL3, MYEF2, IRF4, REL, PHLPP1,

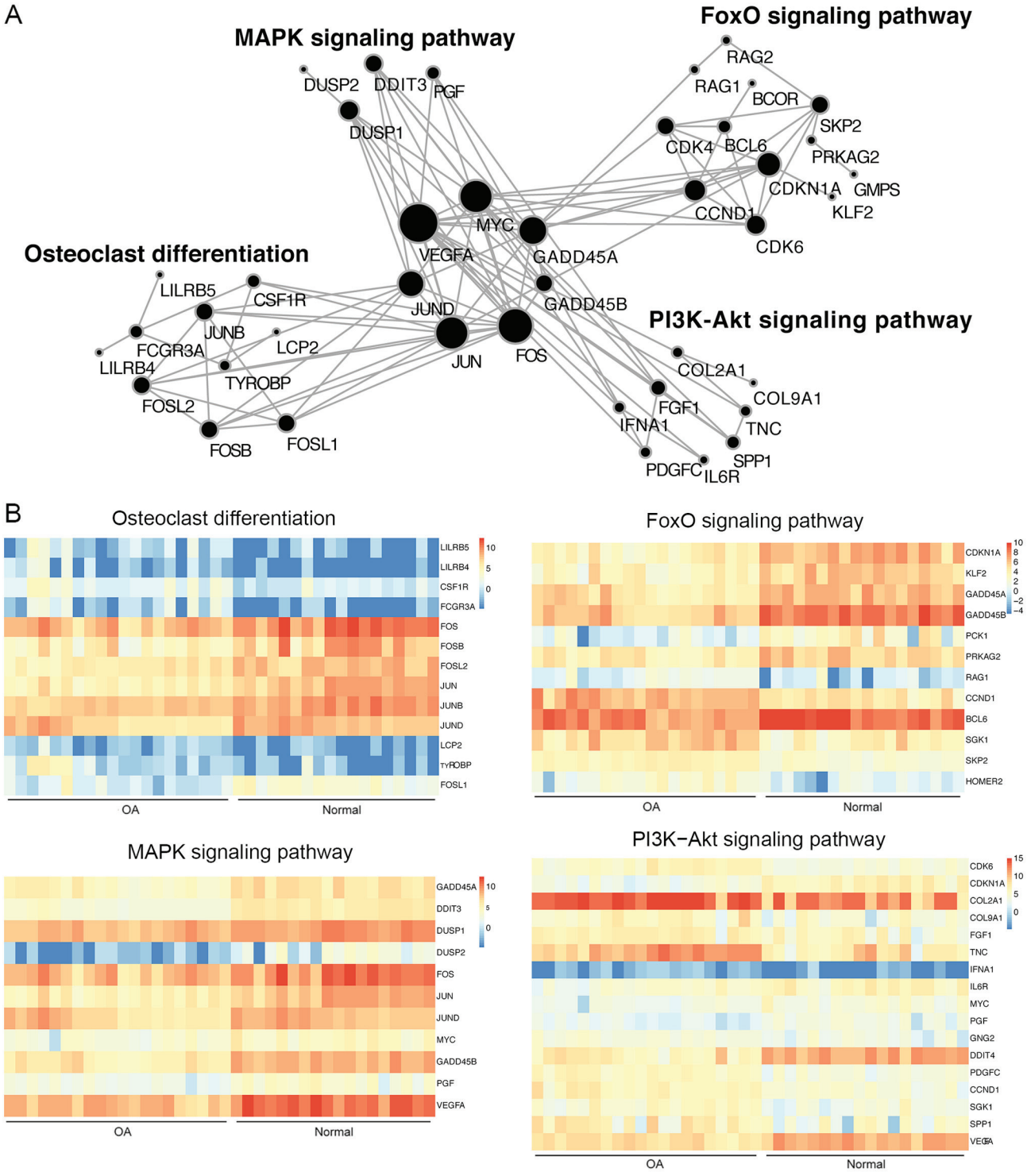

Figure 6. Identification of pivotal signaling pathways. (A) Protein-protein interaction network of key TFs and their target DEGs involved in the pivotal signaling pathways. The node size indicates the degree of interaction. (B) Heatmap of key TFs and their target DEGs involved in the pivotal signaling pathways. TF, transcription factor; DEG, differentially expressed gene; Fox, forkhead box; IL, interleukin; MAPK, mitogen-activated protein kinase; OA, osteoarthritis.

PTPRC, RPF2. GADD45A and GADD45B, members of the GADD45 family, have been implicated in numerous basic processes, and have been demonstrated to be associated with aging and age-associated diseases (54). GADD45A and GADD45B have been reported to interact with AP-1, ATF3 and MYC, which have been identified as key TFs in the present study (55). However, to the best of our knowledge, the roles of GADD45s in the pathogenesis of OA have not been previously assessed, and therefore, further studies are required in order to investigate this. Previous studies have demonstrated that CDC42 is closely associated with the chondrocyte phenotype (56) and age-associated OA (57). A previous study has also indicated that C/EBPβ represses the transcriptional activity of Col2A1 directly and indirectly in ATDC5 cells (58). However, there is currently no evidence that C/EBPγ directly correlates with OA.

REL, also termed C-Rel, is a subunit of NF-κB, and it is well established that NF-κB serves a key role in OA (59). Morrovati *et al* (60) demonstrated that treatment of chondrocytes with IL-1β resulted in a significant increase in the expression level of C-Rel. However, there is still uncertainty as to whether C-Rel may serve as a therapeutic target for OA. Bradley *et al* (61) reported that Phlpp1 controls chondrogenesis via multiple mechanisms and that Phlpp1 inhibition may be a strategy to promote cartilage regeneration and repair. However, no previous study has investigated other genes, including NFIL3, MYEF2, IRF4, PTPRC and RPF2, in articular cartilage, and the results of the present study suggest that they deserve further investigation. The degradation and loss of articular cartilage is a central feature in OA (29). Therefore, the present study utilized the GSE114007 dataset, in which samples were obtained from cartilage tissue. Subchondral bone has received particular attention in recent years (62). In one study, WGCNA, the same method as that used in the present study, was applied to analyze the dataset GSE51588, whose samples were obtained from subchondral bone. They identified another set of core genes, including COL6A3, COL6A1, ITGA11, BAMBI and HCK, to be associated with the pathological processes of subchondral bone in OA (63). However, these results are inconsistent with those obtained by the present study, which indicated that different pathological mechanisms may exist between cartilage tissue and subchondral bone in OA. As summarized by Loeser *et al* (62), it is indicated that each event (osteophyte formation, subchondral bone sclerosis and cartilage degeneration) is regulated by different mediators.

In addition to the key TFs and hub DEGs discussed above, the present study also identified 4 pivotal signaling pathways in the pathogenesis of OA, including osteoclast differentiation, FoxO, MAPK and the PI3K-Akt signaling pathway. It was also identified that GADD45A and GADD45B participated in the MAPK and FoxO signaling pathways, and that the PI3K/Akt and MAPK signaling pathways are closely associated with MYC and VEGFA. Furthermore, FOS, JUN and JUND were indicated to be involved in osteoclast differentiation and MAPK signaling pathways. It is now well established that in OA, the balance between osteoclast-induced bone resorption and osteoblast-induced remodeling is being progressively deregulated (34). A study by Durand *et al* (64), by comparing the osteoclastogenic capacity of peripheral blood mononuclear cells (PBMCs) from patients with OA to that of PBMCs from normal individuals, revealed that monocytes from patients with OA have increased osteoclastogenesis and bone resorption. In fact, numerous studies on OA have suggested that enhanced osteoclast-mediated bone resorption is a potential pathological mechanism for OA (65,66). The FoxO signaling pathway has been reported to be a regulator of the pathological processes of OA (67) and the function is perhaps realized by autophagy (68,69). A recent study revealed that FoxO as essential TFs regulating postnatal articular cartilage growth and homeostasis (68). Activation of the MAPK signaling pathway increases the expression of MMPs in chondrocytes (70). In addition, the MAPK signaling pathway is involved in the apoptosis and expression of pro-inflammatory cytokines in human OA chondrocytes (71). Therefore, MAPK signaling is closely associated with the pathological changes of OA, and is a potential therapeutic target for OA. The PI3K signaling pathway is paramount for cell growth and survival (72), with a previous study revealing that the influence of the PI3K/Akt signaling pathway on OA is realized by autophagy (73). The PI3K/Akt pathway may be suppressed by elevated levels of ROS in OA chondrocytes so that inflammatory processes are promoted (74). Furthermore, oxidative stress may cause chondrocyte senescence by activating MAPK and PI3K/Akt signaling pathways. In general, there is a close association between abnormal regulation of pivotal signaling pathways and the disequilibrium of cellular homeostasis and cartilage degeneration.

In summary, the present study identified the hub genes and 5 hub modules associated with OA based on WGCNA. In addition, the present study was the first to identify key TFs through co-expressed gene modules. Although there have been a multitude of academic theses on the pathogenic mechanism of OA, the pathogenesis of OA has remained to be completely elucidated. Novel potential targets and 4 pivotal signaling pathways for therapeutic intervention. The present study provides an important opportunity to advance the understanding of OA. However, the present results are based on a Bioinformatics analysis and further validation is required in order to verify the potential key roles of these genes in the development of OA *in vitro* and *in vivo*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL conceived and designed the study. XG performed data analyses and wrote the manuscript. YS contributed to data acquisition and manuscript revision.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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