

Bioinformatics analysis of differentially expressed genes involved in human developmental chondrogenesis

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Abstract

Osteoarthritis (OA), also known as degenerative arthritis, affects millions of people all over the world. OA occurs when the cartilage wears down over time, which is a worldwide complaint. The aim of this study was to screen and verify hub genes involved in developmental chondrogenesis as well as to explore potential molecular mechanisms.

The expression profiles of GSE51812 were downloaded from the Gene Expression Omnibus (GEO) database, which contained 9 samples, including 6-week pre-chondrocytes (PC, 6 independent specimens) and 17-week fetal periarticular resting chondrocytes (RC, 3 independent specimens). The raw data were integrated to obtain differentially expressed genes (DEGs) and were further analyzed with bioinformatics analysis. The Gene Ontology (GO) and pathway enrichment of DEGs were conducted via Database for Annotation, Visualization, and Integrated Discovery (DAVID). The protein-protein interaction (PPI) networks of the DEGs were constructed based on data from the search tool for the retrieval of interacting genes (STRING) database. An intersection figure was provided to show the relationship between the DEGs identified in this study and genes from any existed related studies.

A total of 9486 DEGs, including 4821 upregulated genes and 4665 downregulated genes were observed. The top 30 developmental chondrogenesis associated genes were identified, including matrix metalloproteinase (MMP)1, MMP3, MMP13, prostaglandin-endoperoxide synthase 2 (PTGS2), and so on. The majority of DEGs, including PTGS2, CCL20, CHI3L1, LIF, CXCL8, and CXCL12 were intensively enriched in immune-associated biological process terms, including inflammatory, and immune responses. Additionally, the majority of DEGs were mainly enriched in NF-kappa β (NF- κ B) signaling pathway and tumor necrosis factor (TNF) signaling pathway. The hub genes identified in STRING and Cytoscape databases included MMP1, MMP3, MMP13, PTGS2 and so on. Among the top 30 upregulated and downregulated DEGs, there were 15 genes have been reported to be associated with OA or developmental chondrogenesis.

This large scale gene expression study observed genes associated with human developmental chondrogenesis and their relative GO function, which may offer opportunities for the research for cartilage tissue engineering and novel insights into the prevention of OA in the near future.

Abbreviations: DAVID = Database for Annotation, Visualization, and Integrated Discovery, DEGs = differentially expressed genes, GEO = gene expression omnibus, GO = gene ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes, MMP = matrix metalloproteinase, NF- κ B = NF-kappa β , OA = osteoarthritis, PC = prechondrocytes, PPI = protein-protein interaction, PTGS2 = prostaglandin-endoperoxide synthase 2, RC = resting chondrocytes, STRING = search tool for the retrieval of interacting genes, TNF = Tumor necrosis factor.

Keywords: bioinformatics analysis, enrichment analysis, human developmental chondrogenesis, PPI network module

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1. Introduction

Articular cartilage protects bones of diarthrodial joints from load bearing and impact. It promotes nearly frictionless motion between the articular surfaces.^[1,2] Osteoarthritis (OA) is a common clinical disease involving degradation of joints,^[3–5] which was usually caused by cartilage injury and the lack of cartilage regeneration. This disease can occur in any joint, but mainly in the hip joint, knee joint, hand joint and spine facet joint. OA currently affects about 20 million people in the United States alone, which makes joint-surface restoration a major priority in modern medicine.^[6]

Microarrays play an important role in the analysis of gene expression, which served as key tools in medical oncology with great clinical application.^[7–9] Recently, a large number of gene expression profiling researches have been reported with the use of microarray technology. Many differentially expressed genes (DEGs) involved in different biological processes, pathways, or molecular functions have been revealed.^[10,11] Comparative analysis in independent studies indicated a relatively limited degree of overlap. However, expression profiling techniques combining with integrated bioinformatics methods might solve the disadvantages.^[12–14]

The present study is aimed to identify biomarkers and pathways involved in human developmental chondrogenesis with microarray gene expression profile and bioinformatics analysis. Based on the results of biological functions and pathways, these key genes and pathways involved in human developmental chondrogenesis could make substantial progress in cartilage tissue engineering, which may be helpful for the prevention of OA.

2. Materials and methods

2.1. Microarray data

The gene expression profiles GSE51812 were downloaded from the National Center for Biotechnology Information Gene Expression Omnibus DataSets (<https://www.ncbi.nlm.nih.gov/gds>) and were based on the Affymetrix Human Genome U133 Plus 2.0 Array. As described by Wu et al,^[2] adult articular chondrocytes (N=6) were obtained from National Disease Research Interchange. Postnatal, healthy, paraffin-embedded joint and growth plate specimens (N=3) were kindly donated by Dr Marcel Karperien from the University of Twente (Netherlands). A total of 9 chips was used in this study, including 6 laser-capture microdissection (LCM) isolated pre-chondrocytes in chondrogenic condensations at week 6 of embryogenesis and 3 articular chondrocytes at week 17 of development. Background corrected signal intensities were determined by the MAS 5.0 software (Affymetrix). The normalization of datasets obtained on Affymetrix arrays and top 30 upregulated and downregulated DEGs were conducted by the preprocessCore package in R.^[15] The results can provide insights into molecular markers during the process of pre-chondrocyte maturation.

2.2. Data preprocessing and DEGs screening

The raw data were preprocessed via Affy package of R language. Multiple Linear Regression limma was used for DEGs analysis.^[16] We used the ComBat function of sva package to remove known batch effects from microarray data (2). The DEGs of each series was analyzed by the same method without sva package.

Volcano plot was used to display both total DEGs and top 30 upregulated and downregulated DEGs. That was generated with ggplots package of R language. DEGs were identified using classical *t* test and statistically significant DEGs were defined with log₂ fold change (log₂ FC) >1 and *P* <.01 as the cut-off criterion.

2.3. Hierarchical clustering analysis

A bidirectional hierarchical clustering heatmap of total DEGs and top 30 upregulated and downregulated DEGs were constructed using gplots package of R language after extracting the expression values from the gene expression profile.^[17]

2.4. Functional and pathway enrichment analysis

Gene ontology (GO) defines concepts or classes used to describe gene function and relationships. It classifies functions along 3 aspects: biological process (BP), cellular component (CC) and molecular function (MF). Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource to understand high-level functions and utilities of biological system. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/tools.jsp>) was used to classify significant DEGs by their biological processes, cellular components or molecular functions via GO and the significant trans cripts (Benjamini–Hochberg false discovery rate <0.05) were identified using the Functional Annotation clustering tool.^[18] The DAVID database was also used to perform pathway enrichment analysis with reference from KEGG database website and Benjamini–Hochberg false discovery rate (FDR) <0.05 was considered as a cut-off point.

2.5. Protein-protein interaction network construction and module analysis

The search tool for the retrieval of interacting genes (STRING) version 10.5 (<http://www.string-db.org/>), a web biological database for prediction of known and unknown protein interaction relationships, was used to construct the protein-protein interaction (PPI) networks.^[19] The top 30 upregulated and downregulated DEGs were selected to construct the PPI network. The results were visualized by Cytoscape software version 3.5.^[20]

2.6. Cross-validation of top 30 DEGs and genes from related studies

The Bioinformatics & Evolutionary Genomics (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) was used to construct the Venn diagram containing the top 30 DEGs and genes from existed related studies.

3. Results

3.1. Normalization of datasets

The data of gene expression was downloaded from gene expression omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) (GEO accession no. GSE51812). Normalization of the data of gene expression was performed using the preprocessCore package in R and presented in a boxplot (Fig. 1). The normalization of total differentially expressed genes was shown in Figure 1A and 1B, while the normalization of the

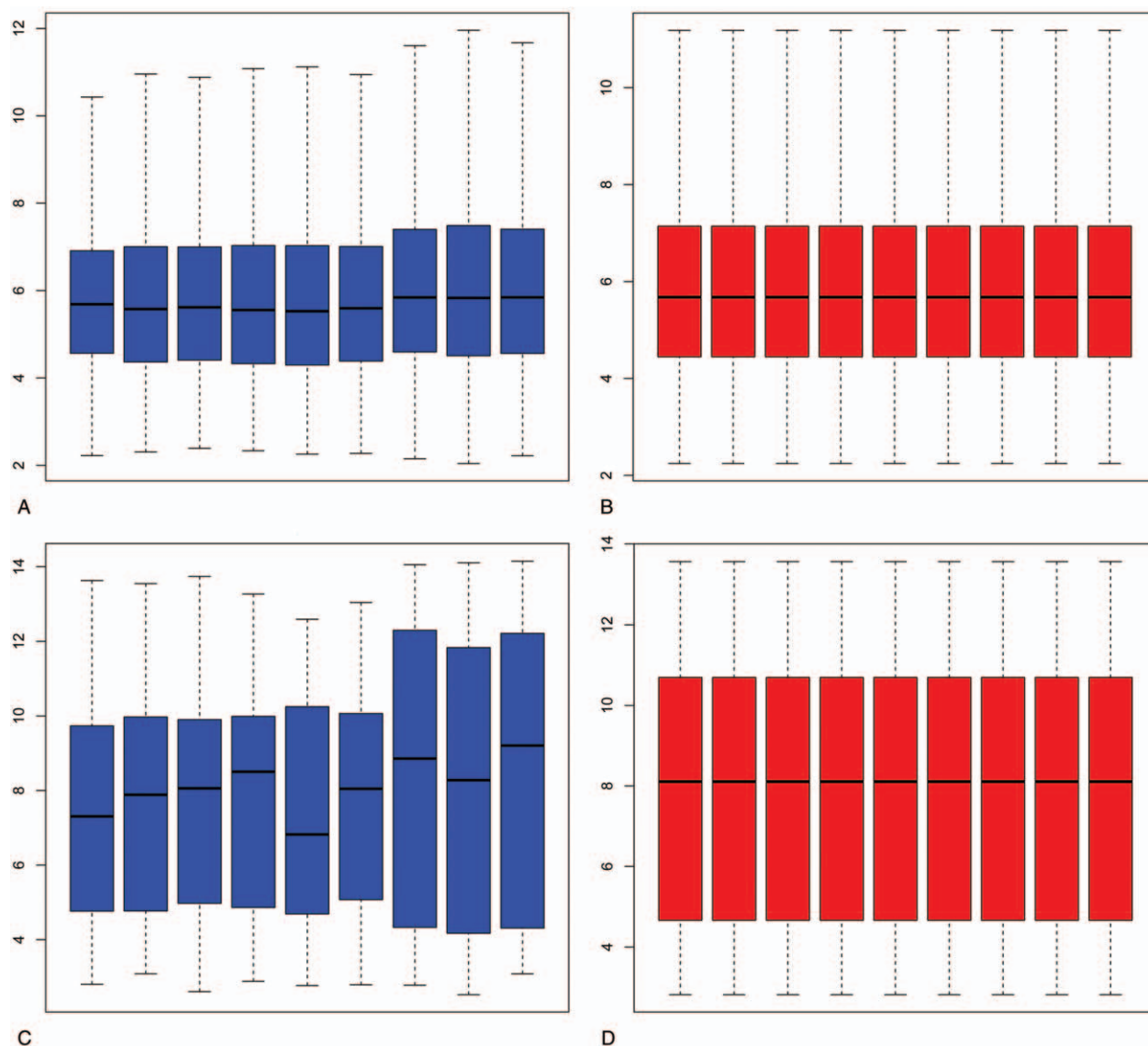


Figure 1. Normalization of samples. (A) Before normalization of total DEGs and (B) following normalization of total DEGs, (C) Before normalization of top 30 upregulated and downregulated DEGs and (D) following normalization of top 30 upregulated and downregulated DEGs. 1-6 = 6-week PC, 6 independent specimens, 7-9 = 17-week RC, 3 independent specimens. DEGs=differentially expressed genes, PC=prechondrocytes, RC=resting chondrocytes.

top 30 differentially expressed genes was shown in Figure 1C and 1D. The black lines in Figure 1B and Figure 1D are basically at the same level, indicating a high consistency.

3.2. Differentially gene expression

Gene expression in 6-week pre-chondrocytes (PC) was compared with that in 17-week resting chondrocytes (RC). A total of 9486 differentially expressed genes were observed. Among them, 4821 genes were upregulated and 4665 genes were downregulated in 6-week PC compared with 17-week RC (Fig. 2A and Fig. 3A). An expression volcano plots and heat map of the top 30 upregulated and downregulated DEGs were indicated in Fig. 2B and Fig. 3B.

3.3. Functional annotation of candidate genes

The pathways and associated biological processes of top 30 developmental chondrogenesis associated candidate genes were

detected via the GO BP terms and KEGG pathway analyses. The top 10 GO BP terms were presented in Table 1. the majority of the top 30 upregulated and downregulated DEGs, including prostaglandin-endoperoxide synthase 2 (PTGS2), CCL20, CHI3L1, LIF, CXCL8, and CXCL12 were intensively enriched in immune-associated biological process terms, including inflammatory and immune responses. Moreover, ICAM1, COMP, and CNTN3 were involved in cell adhesion. In addition, the top 10 KEGG pathway were presented in Table 2. The majority of the top 30 upregulated and downregulated DEGs were mainly enriched in NF- κ B signaling pathway and tumor necrosis factor (TNF) signaling pathway.

3.4. PPI network construction

According to the information in STRING and Cytoscape databases, the PPI relationships of top 30 upregulated and downregulated DEGs were obtained (Fig. 4). The hub genes

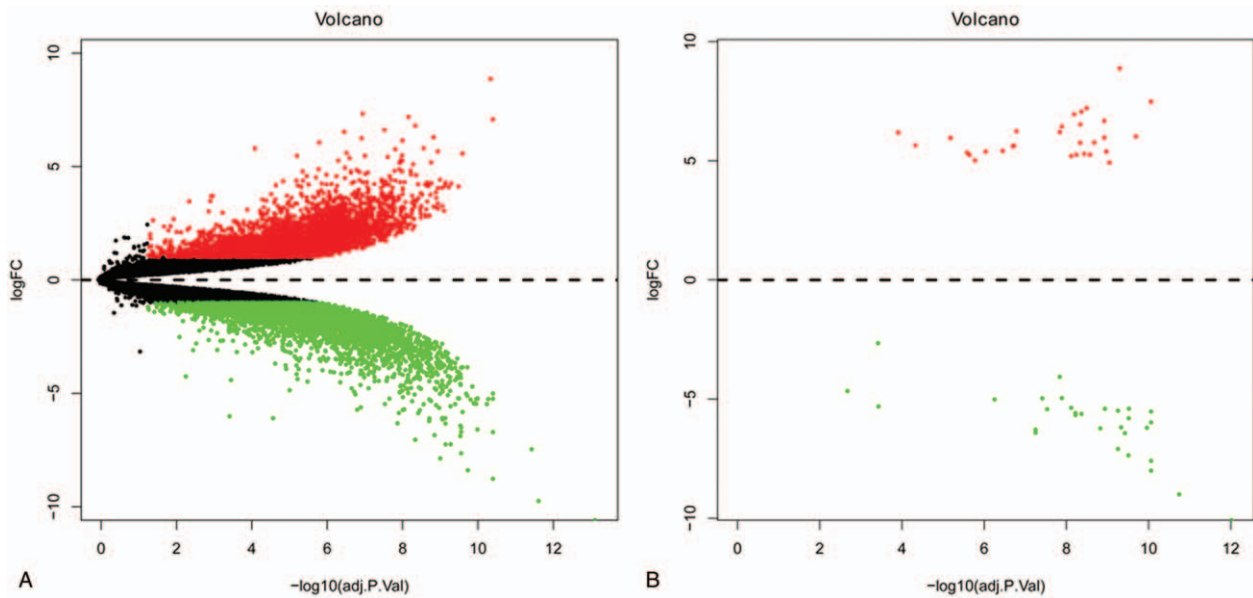


Figure 2. Volcano plot showing A. all DEGs and B. top 30 upregulated and downregulated DEGs in 17-week RC compared to 6-week PC. Grey represents no change in expression. Green represents down-regulation. Red represents up-regulation. DEGs=differentially expressed genes, PC=prechondrocytes, RC=resting chondrocytes.

included matrix metalloproteinase (MMP)1, MMP3, MMP13, PTGS2, and so on. Among these genes, MMP1, MMP3, and MMP13 possessed the highest node degree.

3.5. Venn diagram containing the top 30 DEGs and genes from existed related studies

Many related studies have reported some genes involved in OA or developmental chondrogenesis. Cross-validation containing the top 30 DEGs and genes from related studies would be helpful for further research of OA. Among the top 30 upregulated and downregulated DEGs, there were 15 genes have been reported to be related to OA or developmental chondrogenesis (Fig. 5).

4. Discussion

OA is a common chronic disease that afflicts the middle-aged and elderly individuals. According to statistics, the probability of this disease in male is approximately 40% while in female it is around 47%. Moreover, the incidence of people over 50 years old is rapidly increasing over time.^[21] Osteoarthritis is characterized by a decrease in articular cartilage tissue, thickening of subchondral bone, and formation of callus.^[22] At present, the etiology of osteoarthritis has not yet been clarified. On the surface of existing research, the occurrence of osteoarthritis is related to factors such as age, gender, family history, obesity, trauma and increased weight bearing of the joints.^[23] The incidence of osteoarthritis is positively correlated with age, and the study of its etiology and pathogenesis plays an important role in early diagnosis, termination of disease progression and effective treatment. Gene expression studies have been widely used to analyze DEGs and identify novel pathways. In this study, DEGs in 6-week PC compared with 17-week RC were identified based on gene expression profiling, among which 4821 upregulated genes and 4665 downregulated genes were observed.

Furthermore, functional enrichment analysis of the top 30 genes associate with chondrogenesis was performed to demonstrate the possible biological mechanisms. The top 10 pathways and associated biological processes were detected via the GO BP terms and KEGG pathway analyses. The majority of the top 30 upregulated and downregulated DEGs, including PTGS2, CCL20, CHI3L1, LIF, CXCL8, and CXCL12 were intensively enriched in immune-associated biological process terms, including inflammatory and immune responses. Moreover, ICAM1, COMP, and CNTN3 were involved in cell adhesion. In addition, the majority of the top 30 upregulated and downregulated DEGs were mainly enriched in NF-kappa B signaling pathway and TNF signaling pathway.

The PPI network of top 30 upregulated and downregulated DEGs was constructed. The results indicated that MMP1, MMP3, MMP13, and PTGS2 were hub genes. According to previous study, PTGS2, MMP1, MMP3, and MMP13 play a crucial role in the degeneration of articular cartilage, which accelerates the destruction of articular cartilage. MMP-1, also known as collagenase-1, is the first MMPs to be characterized and isolated from human fibroblasts. It is a fibroblastic collagenase that hydrolyzes collagen, gelatin such as I, II, III, VII, VIII, cell adhesive and aggrecan. It is abundantly expressed in OA cartilage and acts on newly synthesized type II collagen. In the case of compression or damage of chondrocytes, chondrocytes synthesize MMP-1 in a large amount, and the site of action is located at the Gly775-Leu776 site on the newly formed type II collagen alpha chain in the cartilage matrix, which is cleaved into 2. The A and B segments, which are 1/4 and 3/4 long, respectively, can be further decomposed by other MMPs. Type II collagen cleavage, destruction of collagen network structure, decomposition or loss of proteoglycans in ECM accelerated the degradation of cartilage matrix, destruction of cartilage and defects, leading to OA. Additionally, Freemont et al^[24] found that the expression sites of MMP1 and MMP3 in human knee OA cartilage were

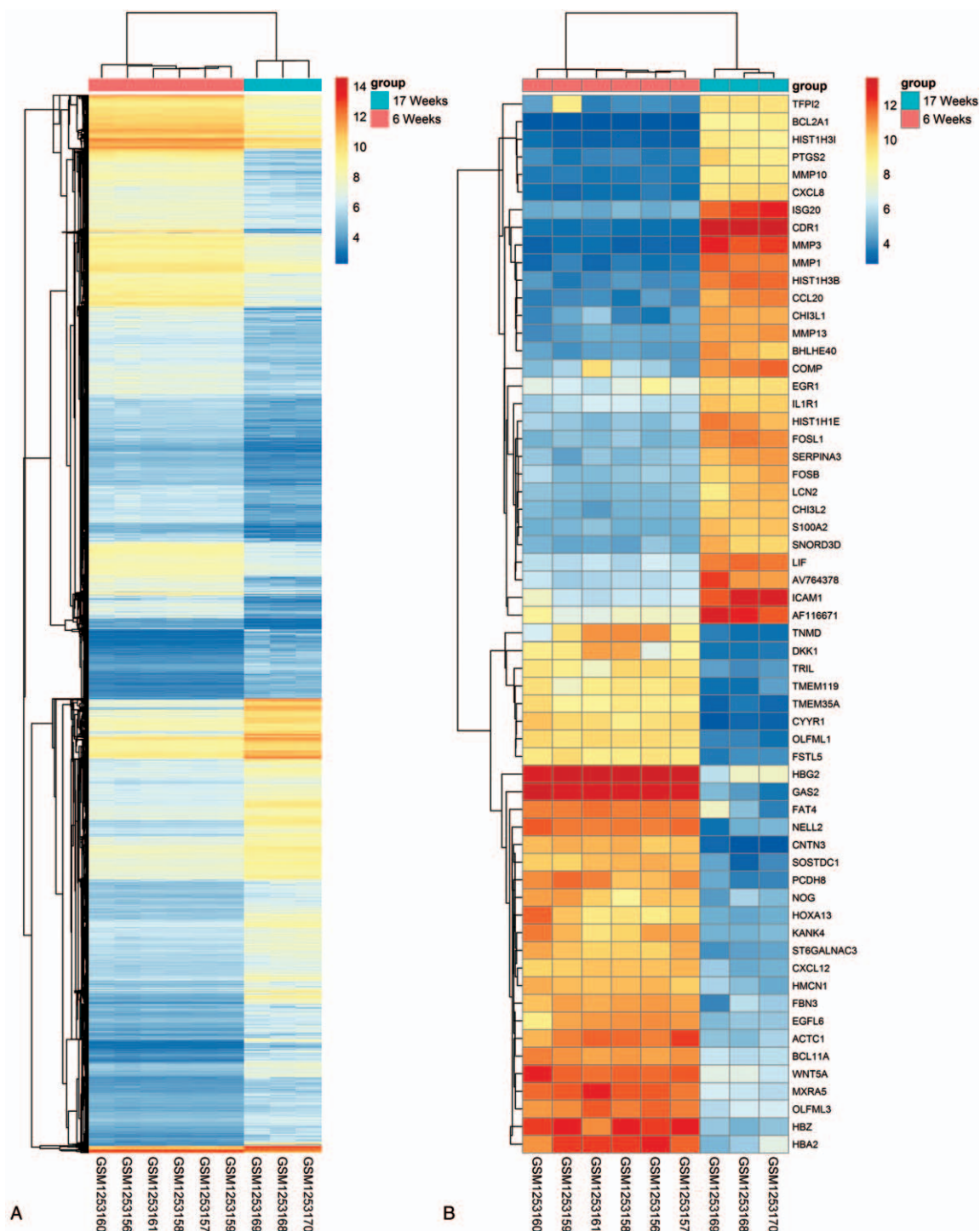


Figure 3. Heat map showing A. all and B. top 30 up-regulated and down-regulated differentially expressed genes in 17-week RC compared to 6-week PC. The expression values are log₂ fold changes (>1 or <-1). Blue represents down-regulation and red represents up-regulation. PC=prechondrocytes, RC=resting chondrocytes.

specific, and the abnormal expression period was not the same. MMP1 was mainly abnormal in the superficial layer of cartilage, and MMP3 was abnormally expressed in the deep layer of cartilage. MMP3 increased significantly in the early and late stages of OA. Moreover, MMP13, also known as collagenase 3, is mainly secreted by chondrocytes and is the most effective type II collagen degrading enzyme in the MMPs family.^[2,5] Its degrada-

tion ability is 5 to 10 times stronger than that of MMP1. MMP13 can directly degrade the most abundant and characteristic type II collagen in ECM. Compared with MMP1, MMP13 plays a more significant role during cartilage degeneration. Although MMP1 and MMP13 cannot directly degrade proteoglycans, proteoglycans are mainly linked with type II collagen and hyaluronic acid in the form of polymers. MMP1 and MMP13

Table 1
GO analysis of top 30 upregulated and downregulated DEGs.

GO-ID	Description	Count	P value	Genes
0030514	Negative regulation of BMP signaling pathway	4	1.27E-04	WNT5A, NOG, DKK1, SOSTDC1
0006954	Inflammatory response	5	.002005629	PTGS2, CCL20, CHI3L1, CXCL8, CXCL12
0048570	Notochord morphogenesis	2	.010647782	WNT5A, NOG
0072307	Regulation of metanephric nephron tubule epithelial cell differentiation	2	.013292484	LIF, FAT4
0045766	Positive regulation of angiogenesis	3	.019231634	WNT5A, CHI3L1, CXCL8
0090090	Negative regulation of canonical Wnt signaling pathway	3	.021547563	WNT5A, NOG, SOSTDC1
0070374	Positive regulation of ERK1 and ERK2 cascade	3	.048290109	ICAM1, CCL20, CHI3L1
0007155	Cell adhesion	3	.052955663	ICAM1, COMP, CNTN3
0006955	Immune response	3	.080033228	LIF, CXCL8, CXCL12
0000122	Negative regulation of transcription from RNA polymerase II promoter	4	.084577085	HIST1H1E, NOG, DKK1, BCL11A

DEGs=differentially expressed genes, GO= gene ontology.

Table 2
Top 10 KEGG pathway of top 30 upregulated and downregulated DEGs.

Database	Pathway name	Count	P value	Genes
KEGG	Rheumatoid arthritis	6	2.94E-06	ICAM1, CCL20, CXCL8, MMP3, CXCL12, MMP1
KEGG	NF-kappa B signaling pathway	6	3.29E-06	ICAM1, IL1R1, PTGS2, BCL2A1, CXCL8, CXCL12
KEGG	TNF signaling pathway	5	1.73E-04	LIF, ICAM1, PTGS2, CCL20, MMP3
KEGG	Malaria	4	3.52E-04	ICAM1, COMP, CXCL8, HBA2
KEGG	Cytokine-cytokine receptor interaction	5	.001909056	LIF, IL1R1, CCL20, CXCL8, CXCL12
KEGG	HTLV-I infection	5	.004701526	EGR1, WNT5A, ICAM1, IL1R1, FOSL1
KEGG	Pathways in cancer	5	.0196971	WNT5A, PTGS2, CXCL8, CXCL12, MMP1
KEGG	ptr04310:Wnt signaling pathway	3	.054447544	WNT5A, DKK1, FOSL1
KEGG	Transcriptional misregulation in cancer	3	.076010333	BCL2A1, CXCL8, MMP3
KEGG	Chemokine signaling pathway	3	.082381079	CCL20, CXCL8, CXCL12

DEGs=differentially expressed genes, KEGG = Kyoto Encyclopedia of Genes and Genomes.

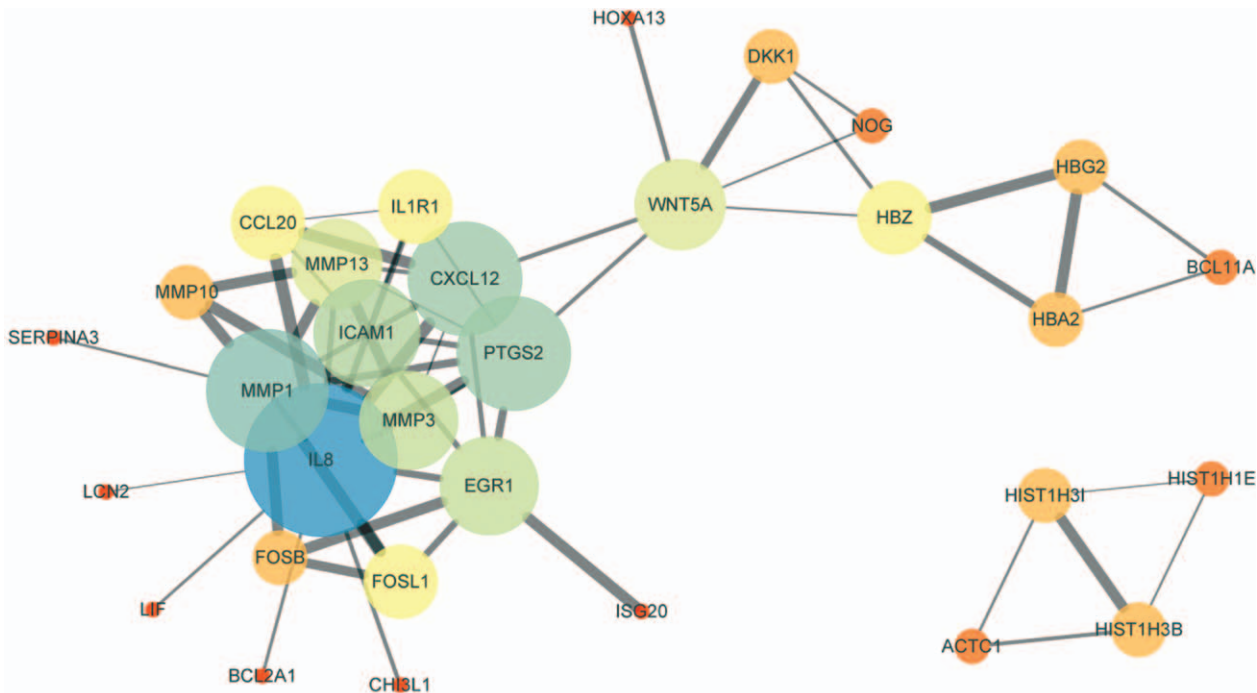


Figure 4. PPI network of top 30 DEGs was constructed and visualized by Cytoscape software. Low value of combined score to circle with small size or bright colors and line with small sizes. DEGs=differentially expressed genes, PPI=Protein-protein interaction.

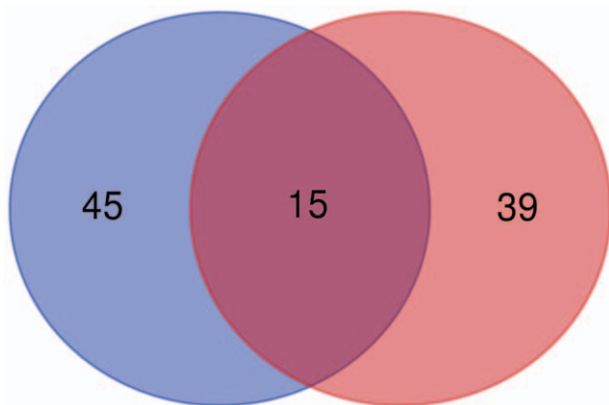


Figure 5. Venn diagram containing the top 30 DEGs and genes from existed related studies. A. Top 30 DEGs, B. Genes from existed related studies. Among the top 30 DEGs, there were 15 genes have been reported to be related to OA or developmental chondrogenesis (MMP-1, MMP-3, MMP-13, CCL20, LIF, CHI3L2, CXCL12, WNT5A, SERPINA3, IL1R1, LCN2, CHI3L1, ICAM1, DKK1, and COMP). DEGs = differentially expressed genes, OA = osteoarthritis.

directly degrade type II collagen, which leads to the loss of the connection of proteoglycans and type II collagen. It further leads to the loss of proteoglycan, indirectly reduces the content of proteoglycan and causes the mechanical properties and deformation ability of cartilage to decrease, which accelerates the degeneration process of articular cartilage.^[26] Construction of PPI network of DEGs may be helpful for understanding the relationship of developmental chondrogenesis and OA. Additionally, cross-validation was performed to explore the relationship between the top 30 DEGs and genes from related studies. Among the top 30 upregulated and downregulated DEGs, there were 15 genes have been reported to be related to OA or developmental chondrogenesis. For instance, DKK1 levels correlate with osteoarthritis and are regulated by OA associated factors.^[27] CXCL12 levels are correlated with disease severity in patients with knee OA.^[28] Analysis of relationship between the top 30 DEGs and genes from related studies would be helpful for further study of OA and human developmental chondrogenesis.

In conclusion, the present study provides significant information that may aid in understanding the molecular mechanisms of developmental chondrogenesis. The top 30 upregulated and downregulated DEGs associated with developmental chondrogenesis were observed in this study, which may facilitate the research for cartilage tissue engineering and prevention of OA in the near future.

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