Integrate analysis and identification for different expression genes in chondrogenesis

Keda Liu
School of Stomatology, China Medical University
Nanjue Cao
The Fourth Affiliated Hospital, Zhejiang University School of Medicine
Yuhe Zhu
School of Stomatology, China Medical University
Wei Wang (wwang75@cmu.edu.cn)
School of Stomatology, China Medical University https://orcid.org/0000-0003-2798-0400

Research article

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Abstract

Background: The intricate mechanisms of articular chondrogenesis are largely unknown. Gradually, with the help of high-throughput platforms, microarrays have become an important and useful method to testify hub genes in desease. Today, advanced bioinformatic analysis of available microarray data can provide more reliable and accurate screening results by duplicating related data sets.

Results: Microarray datasets GSE9451 and GSE104113 were downloaded from Gene Expression Omnibus (GEO) database. The differentially expressed genes (DEGs) were performed, and function enrichment analyses were demonstrated. The protein-protein interaction network (PPI) was constructed and the module analysis was performed by using STRING and Cytoscape. Quantitative PCR was used to confirm the results of bioinformatics analysis.

Conclusion: Compared to individual studies, this study can provide extra reliable and accurate screening results by duplicating relevant records. Additional molecular experiments are required to confirm the discovery of candidate genes identified by chondrogenesis. S100A4 is predicted to integrate with miR-325-3p to promote osteogenesis.

Background

Chondrogenesis happens in fracture healing and skeleton development. The intricate mechanisms of articular chondrogenesis are largely unknown. It starts with proliferation, condensation and recruitment of mesenchymal progenitor cells which finally cause the form of a precartilaginous primordium [1]. Additionally, this process is programmed in genomic sequences [2–4].

Gradually, with the help of high-throughput platforms, microarrays have become an important and useful method to testify hub genes in casino and screen biomarkers of cancer [5]. Some specific genes influence chondrogenesis [1]. Gene expression profiling microarrays of chondrogenesis have been made to screen multiple differentially expressed genes (GES9451, GSE104113) [6]. However, individual studies using a limited number of overlapping gene profiles have high false-positive rates, not sufficient to identify pathways and important genes involved in various cellular processes and biological functions. Today, advanced bioinformatic analysis of available microarray data can provide more reliable and accurate screening results by duplicating related data sets.

So far, chondrogenic microarrays have no gene expression profiling results together with the other. In this study, using a series of bioinformatic tools analyzed microarray data. Gene expression profile biochips (GSE9451) and (GSE104113) were integrated and analyzed. In chondrogenesis, DEG pathways have been identified. Networks of protein-protein interaction was established and essential genes were revealed. By using PCR technology for verification to find diagnostic markers, we fought new molecules and pathways in cartilage formation and reveal the potential molecular mechanisms that regulate cartilage formation.

Results

Normalization of aberrantly expressed genes in chondrogenesis

The data of each microarray were respectively analyzed by GEO2R to identify GSE9451 (1515 up-regulated, 1564 down-regulated) and GSE104113 (425 up-regulated, 328 down-regulated)(Fig. 1 & Fig. 2).Subsequently, by overlapping DEGs respectively, we acquired 42 DEG-down expression genes and 38 DEG-up expression genes (Fig. 3)

Functional and pathway enrichment analysis

Terms of GO analysis were demonstrated in Fig. 4. The results showed that changes were most enriched in striated muscle cell development, keratinization, muscle cell development, keratinocyte differentiation, skin development, regulation of cardiac muscle tissue growth.

KEGG enrichment analysis in Fig. 5 suggested that DMRs were majorly enriched in Estrogen signaling pathway, Staphylococcus aureus infection, Hematopoietic cell lineage, Thiamine metabolism, Primary bile acid biosynthesis, Growth hormone synthesis, secretion and action, Signaling pathways regulating pluripotency of stem cells, Cell adhesion molecules (CAMs), Wnt signaling pathway, Neuroactive ligand-receptor interaction.

PPI network construction

PPI network of different expression genes was shown in Fig. 6 and disconnected nodes were hidden in the network for further visualization. The DEG-up and down expressed gene PPI network, showing a functional association between them and we selected the major genes: KRT19, LGR5, KRT18, KRT34, S100A4(Fig. 7) by using Cytohubba in Cytoscape.

Hub gene analysis and QRT-PCR confirmation

A total of 5 genes were identified as hub genes. Names, full names and functions of these hub genes are shown in Table 1. The forward and reverse primers for each gene are listed in Table 2. Two genes (LGR5, S100A4) were upregulated and the other 3 were downregulated. The expression hub genes were consistent with our integrated analysis (Fig. 8).

Table 1

Functional roles of 5 hub genes

Name	Official Full Name	Function
KRT19	Keratin, type I cytoskeletal 19	Involved in the organization of myofibers. Together with KRT8, helps to link the contractile apparatus to dystrophin at the costameres of striated muscle.
LGR5	Leucine rich repeat-containing G-protein coupled receptor 5	Involved in the development and/or maintenance of the adult intestinal stem cells during postembryonic development.
KRT18	Keratin, type I cytoskeletal 18	Involved in the uptake of thrombin-antithrombin complexes by hepatic cells. Together with KRT8, is involved in interleukin-6 (IL-6)-mediated barrier protection.
KRT34	Keratin, type I cuticular Ha4	The protein encoded by this gene is a member of the keratin gene family.
S100A4	Protein S100-A4	Involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. S100 genes include at least 13 members which are located as a cluster on chromosome 1q21. This protein may function in motility, invasion, and tubulin polymerization.

Table 2

Primer sequence for qRT-PCR

gene	Primers	Sequences (5' to 3')
LGR5	Forward Reverse	GAGGATCTGGTGAGCCTGAGAA CATAAGTGATGCTGGAGCTGGTAA
S100A4	Forward Reverse	TCAGAACTAAAGGAGCTGCTGACC TTTCTTCCTGGGCTGCTTATCTGG
KRT19	Forward Reverse	CAGCTTCTGAGACCAGGGTT GACTGGCGATAGCTGTAGGA
KRT18	Forward Reverse	GGCCCTGCTGAACATCAAG CTGCTGTCCAAGGCATCAC
KRT34	Forward Reverse	TTAACCGCAGGGAAGTGGAGC GCTGGATACCACCTGCTTGTT
GAPDH	Forward Reverse	CCTGGTATGACAACGAATTTG CAGTGAGGGTCTCTCTCTTCC

Discussion

Elucidation of the underlying mechanisms of chondrogenesis contribute to the diagnosis, and prognosis evaluation. In this research, by analyzing data of gene expression GSE9451 and GSE104113 microarrays, we screened 297 DMRs-up expression genes and 373 DMRs-down expression genes, using several bioinformatics tools. Enrichment of these genes indicates specific pathways and central genes affected by aberrant methylation, and could provide novel information on the pathogenesis of chondrogenesis.

KRT19, Involved in the organization of myofibers. Together with KRT8, helps to link the contractile apparatus to dystrophin at the costameres of striated muscle[7]. Intervertebral disc degeneration has been treatment by using Mesenchymal stem cell (MSC)-based therapies [8]. Intervertebral discs receiving chondroprogenitor cells exhibited higher expression of nucleus pulposus-specific human markers KRT19[9]. Research has shown that perspectives of biomaterials and cellular treatments combining chondrocytes, chondrogenesis and MSC are optimistic [10]. Therefore, KRT19 can be the master regulators of chondrogenesis. This potentially provides new cell candidates for chondrogenesis treatment.

LGR5, Receptor for R-spondins that potentiates the canonical Wnt signaling pathway and acts as a stem cell marker of the intestinal epithelium and the hair follicle [11]. Upon binding to R-spondins, associates with phosphorylated LRP6 and frizzled receptors that are activated by extracellular Wnt receptors, triggering the canonical Wnt signaling pathway to increase expression of target genes. Involved in the development and/or maintenance of the adult intestinal stem cells during postembryonic development [12–14]. Recently study has shown that inhibition of Bone morphogenetic protein (BMP) signaling activate LGR5 $^{+}$ cells during inflammation [15, 16]. Thus, LGR5 can be a novel marker of chondrogenesis.

KRT18 was involved in the uptake of thrombin-antithrombin complexes by hepatic cells. When phosphorylated, plays a role in filament reorganization. Involved in the delivery of mutated CFTR to the plasma membrane. Together with KRT8, is involved in interleukin-6 (IL-6)-mediated barrier protection [17, 18]. A study showed that nucleus pulposus derived stem cells (NPDCs) keep the regeneration ability similar to BMSCs [19]. Research has confirmed that KRT18 was nucleus pulposus specific gene [20]. So KRT18 can be a key growth factor of chondrogenesis

KRT34, the protein encoded by this gene is a member of the keratin gene family. As a type I hair keratin, it is an acidic protein which heterodimerizes with type II keratins to form hair and nails. The type I hair keratins are clustered in a region of chromosome 17q12-q21 and have the same direction of transcription [21, 22]. KRT19, KRT34 can be regulated by the depolymerization of F-actin, triggering keratin intermediate filament formation. This finding provides a new insight in MSC-based tissue engineering [23].

S100A4, S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. S100 genes include at least 13 members which are located as a cluster on chromosome 1q21. This protein may function in motility, invasion, and tubulin polymerization. Chromosomal

rearrangements and altered expression of this gene have been implicated in tumor metastasis. Multiple alternatively spliced variants, encoding the same protein, have been identified [24, 25]. S100A4 has been used as osteoarthritis markers in research [26]. By using Target Scan (v7.2) [27], S100A4 is predicted to integrate with miR-325-3p to promote osteogenesis. But the molecular mechanisms require further research.

Conclusion

In summary, our study united bioinformatics analysis of gene expressed microarrays to disclose the following factor: organization of myofibers, the development and/or maintenance of the intestinal stem cells, interleukin-6 (IL-6)-mediated barrier protection, the regulation of a number of cellular processes. Functions of hub genes act as an abnormal biomarker for accurate diagnosis and treatment of future chondrogenesis. Compared to individual studies, this study can provide extra reliable and accurate screening results by duplicating relevant records. Additional molecular experiments are required to confirm the discovery of candidate genes identified by chondrogenesis.

Methods

Microarray data

In this study, the gene expression profiling datasets GSE9451 and GSE104113 were obtained from the National Center for Biotechnology Information gene expression omnibus (NCBI-GEO). GSE9451(platform: GPL570) includes 3 MSC-chondrogenesis samples and 3 MSC samples. GSE104113(platform: GPL6244) includes 2 MSC-chondrogenesis samples and 2 MSC samples.

Data processing

Normalization and background correction of the initial raw data were performed by using R software sva and limma package. Next, probe names were annotated gene symbols according to annotation files, and probe without a corresponding gene symbol was filtered. Subsequently, the average value of gene symbols with multiple probes were calculated and data were log2-transformed. The differently expressed mRNAs were filtered applying R software limma package, with the criterion of $|\log_2$ (fold change) $| > 1$ and *P*-value < 0.05. Finally, hierarchical cluster analysis was used R software pheatmap package.

Functional and pathway enrichment analysis of DEGs

To carry out functional and pathway enrichment analysis, an R package called clusterProfiler was used for Note, Visualization and Enrichment Discovery [28]. GO is a professional bioinformatics tool for gene annotation and biological process analysis of these genes [29]. *P*.adjust< 0.05 was thought as statistically significant. KEGG is a database for understanding of advanced functions and biological

systems of molecular data generated by high-throughput experimental techniques [30]. The software performs an enrichment analysis of top 10 target genes to all known GO and KEGG pathways.

PPI network constructions, module analysis and Hub genes analysis.

Using a search tool (STRING; http://string-db.org) predicted the network of interacting genes [31]. The hub genes were ranked by EPC method. Explore important hubs and fragile motifs in an interactome network by several topological algorithms including Degree, Edge Percolated Component (EPC). The PPI network and Hub genes network were visualized by using Cytoscape v3.7.2 [32].

Cell culture

BMSCs were purchased from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China). The cells were grown to the third passage (P3) were inoculated into 6-well plates, and chondrogenic induction solution (L-DMEM, 10% FBS, 10 μg/L TGF-β1, 37.5 μg/mL Vc, 0.1 μmol/L Dex, 6.25 μg/mL transferrin, 6.25 μg/mL insulin, 1.25 μg/mL BSA, 6.25 μg/mL sodium selenite, and 1 mmol/L sodium pyruvate) was added to the chondrogenic induction group. At the same time, BMSCs in accordance with the routine culture were prepared as the control group, cultured in ordinary solution (L-DMEM, 10% FBS, 100U/ml penicillin and 100μg/ml streptomycin) at a density of 1×10^5 cells. Both groups were maintained at 37°C in 5% CO2. The supplemented medium was changed every 3-4 days and the cells were passaged when 80% confluent using 0.05% trypsin to lift them from their surface attachment.

QRT-PCR confirmation

With RNAiso Plus (Takara, Shiga, Japan) reagent, total RNA was isolated and cDNA was obtained from isolated RNA with PrimeScript RT reagent Kit With gDNA Eraser (Takara, Shiga, Japan). In a real-time PCR system, quantitative PCR was performed with SYBR Premix Ex Taq II (Takara, Shiga, Japan). Relative gene expressions were analyzed by using $2^{-\Delta\Delta Ct}$ method. GAPDH were served as endogenous controls for mRNA expression in analysis.

Statistical analysis

Data were calculated using the IBM SPSS Statistics Version 23.0 (IBM, Armonk, NY, USA), and presented with GraphPad Prism 8.0 (GraphPad Software, CA, USA). All qRT-PCR results are expressed as the mean ± standard deviation (SD). The data were analyzed by Independent Samples *t*-test. All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

Declarations

Availability of supporting data

The Microarray data are publicly available in Gene Expression Omnibus, accession number GSE29087 and GSE54695.

Abbreviations

DEG: different expression gene

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Wei Wang and Yuhe Zhu designed the study. Nanjue Cao and Keda Liu implemented the analysis. Keda Liu wrote the manuscript. All authors read and approved the final manuscript.

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Figures

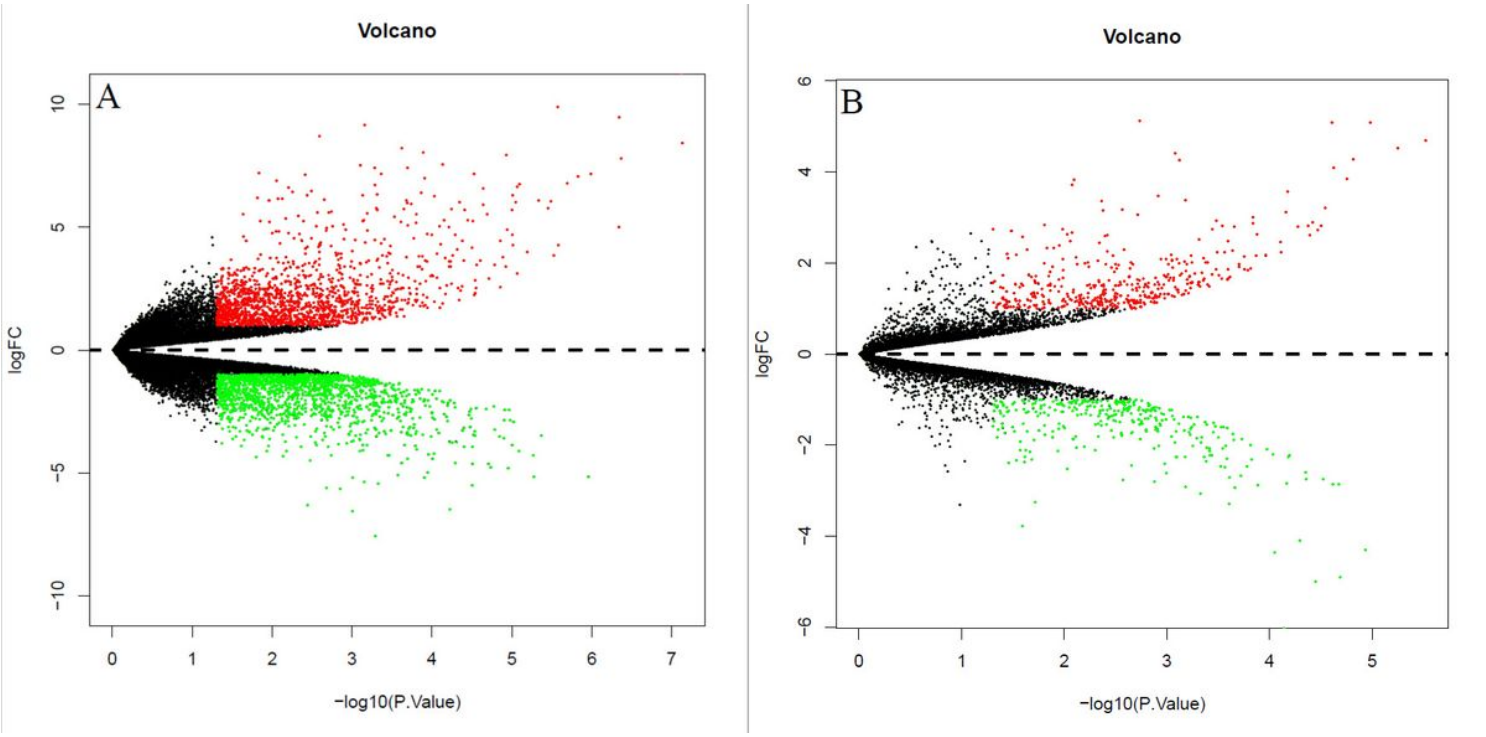


Figure 1

Volcano plot of DEGs in gene expression datasets (A GSE9451; B GSE104113). Red represents up-regulated genes, green represents down-regulated genes and black represents genes that are not significantly different.

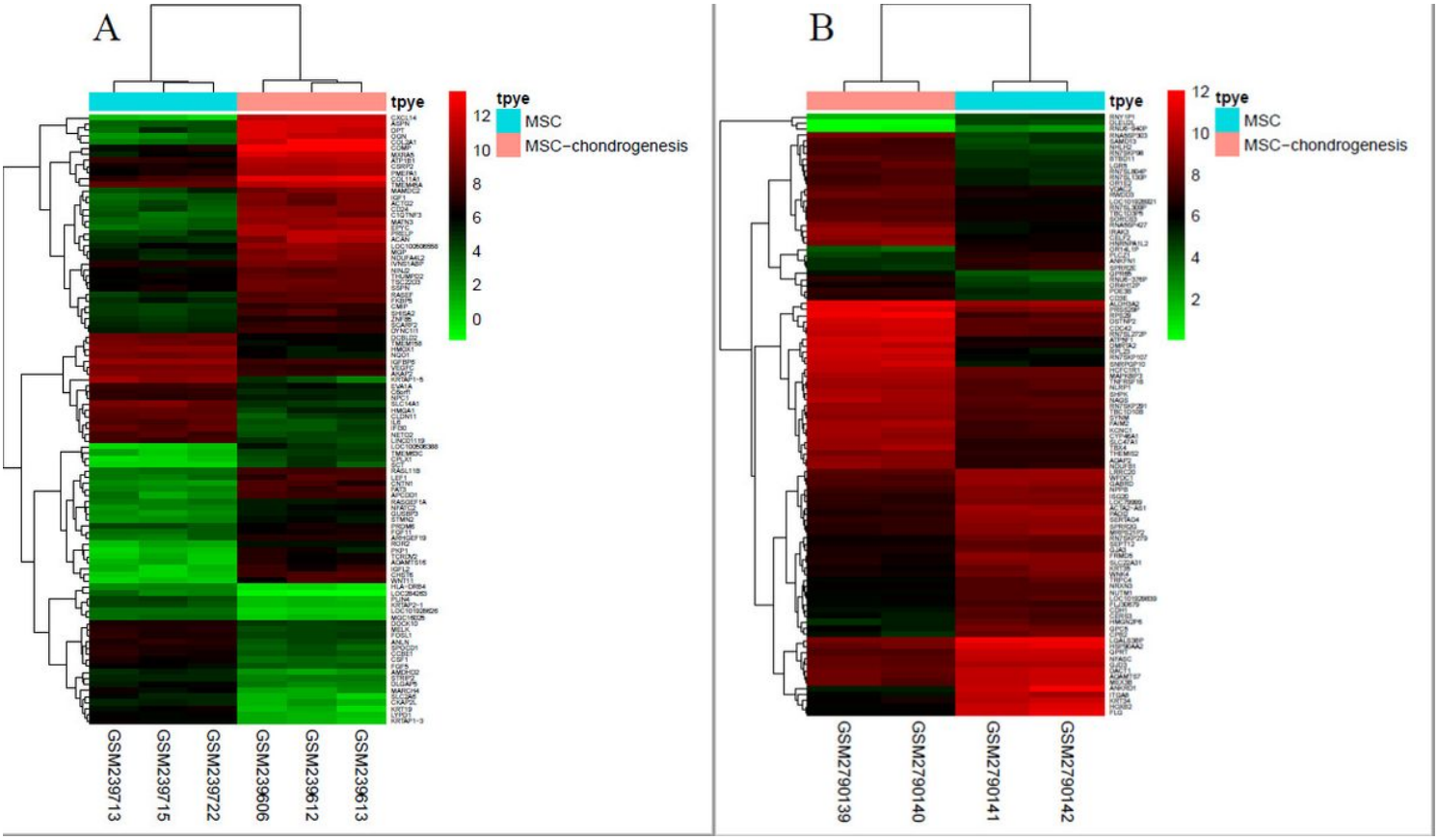


Figure 2

Hierarchical clustering of DEGs in gene expression datasets (A GSE9451; B GSE104113). Rows represent different expression gene and columns represent samples. Red represents up-regulated genes, green represents down-regulated genes and black represents genes that are not significantly different for all samples. For the sample, pink means chondrogenesis adjacent tissues and blue means normal tissue.

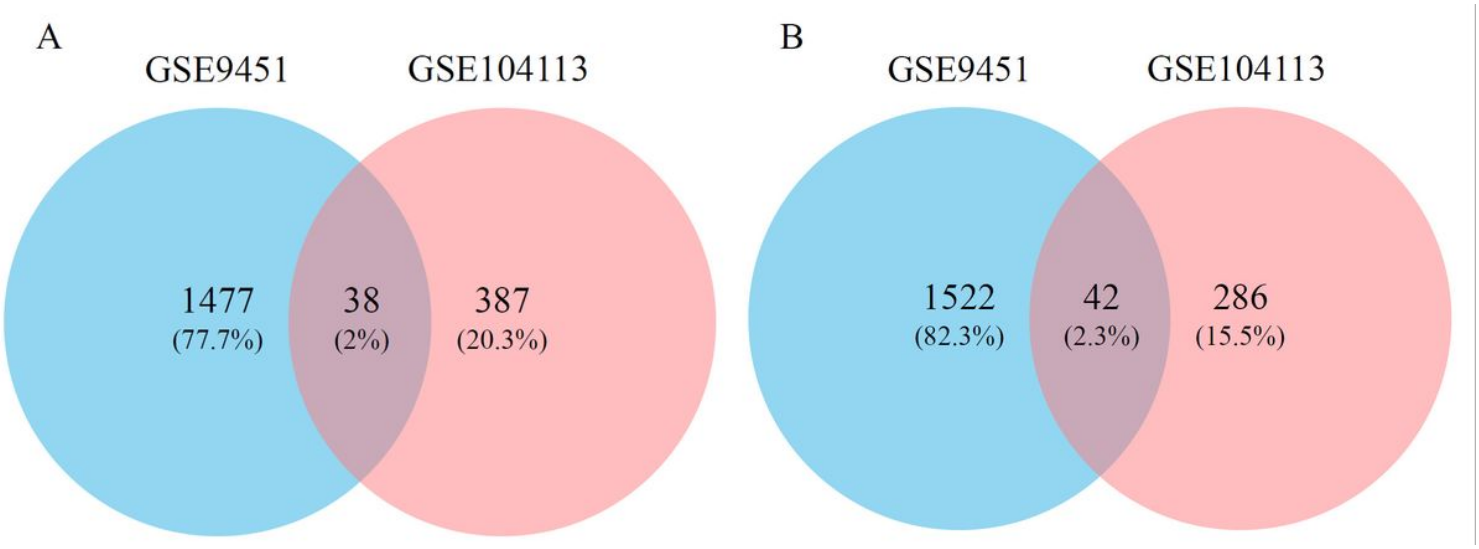
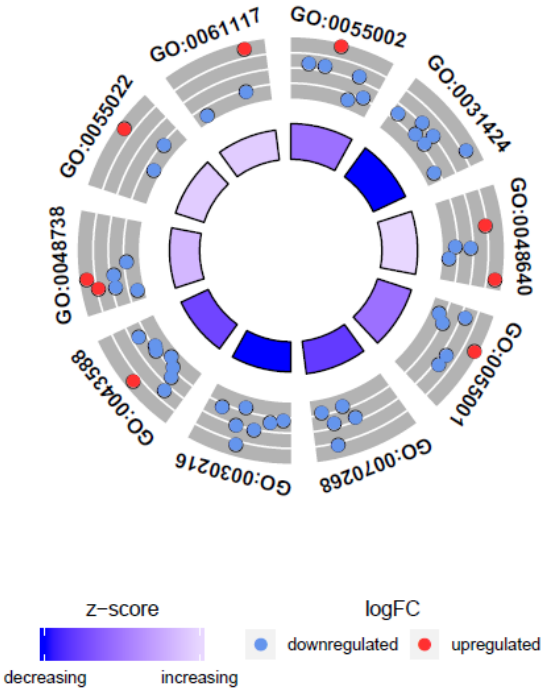


Figure 3

Venn diagram for different expression genes by overlapping gene expression datasets (A up-regulated genes; B down-regulated genes).



ID	Description
GO:0055002	striated muscle cell development
GO:0031424	keratinization
GO:0048640	negative regulation of developmental growth
GO:0055001	muscle cell development
GO:0070268	cornification
GO:0030216	keratinocyte differentiation
GO:0043588	skin development
GO:0048738	cardiac muscle tissue development
GO:0055022	negative regulation of cardiac muscle tissue growth
GO:0061117	negative regulation of heart growth

Figure 4

Gene ontology analysis of aberrantly expressed genes in chondrogenesis.

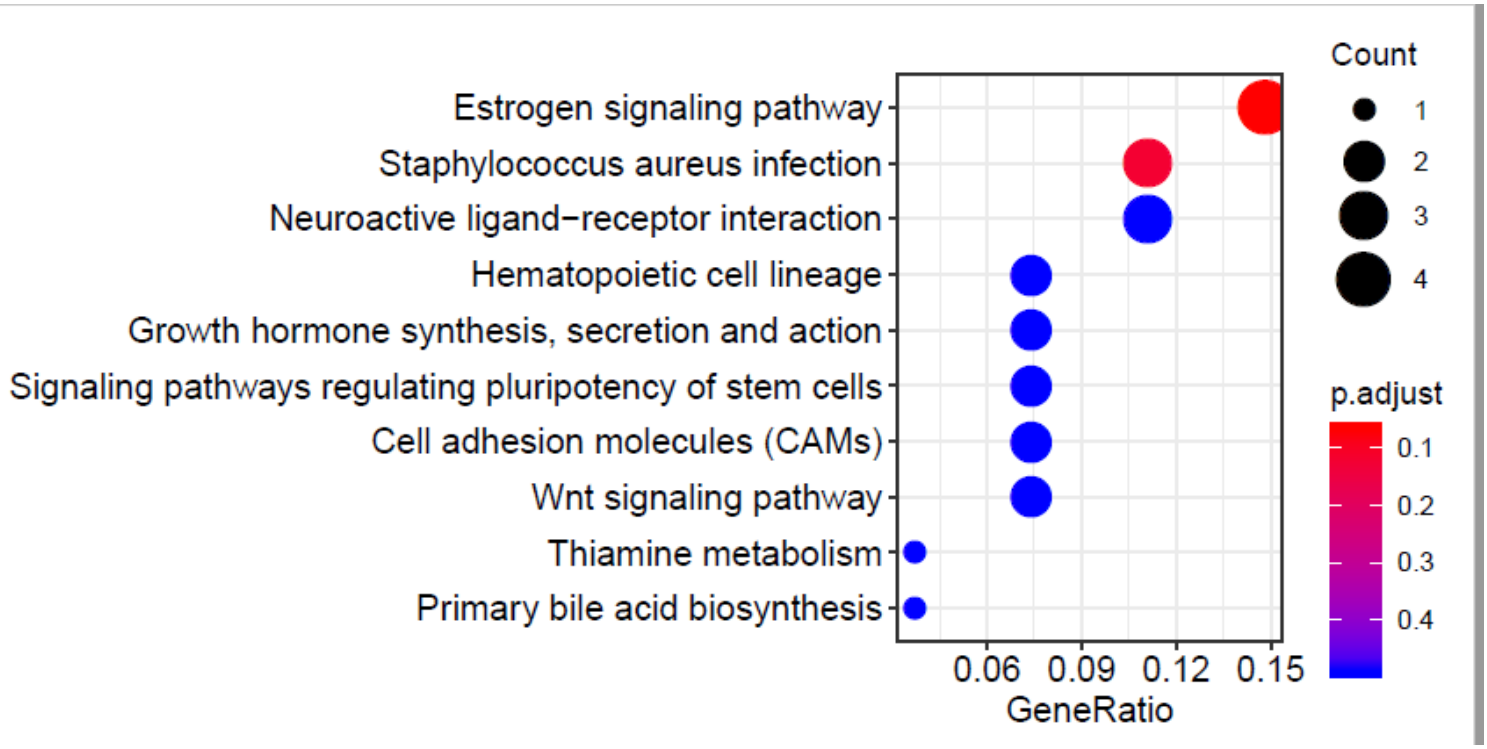


Figure 5

KEGG pathway analysis of aberrantly expressed genes in chondrogenesis.

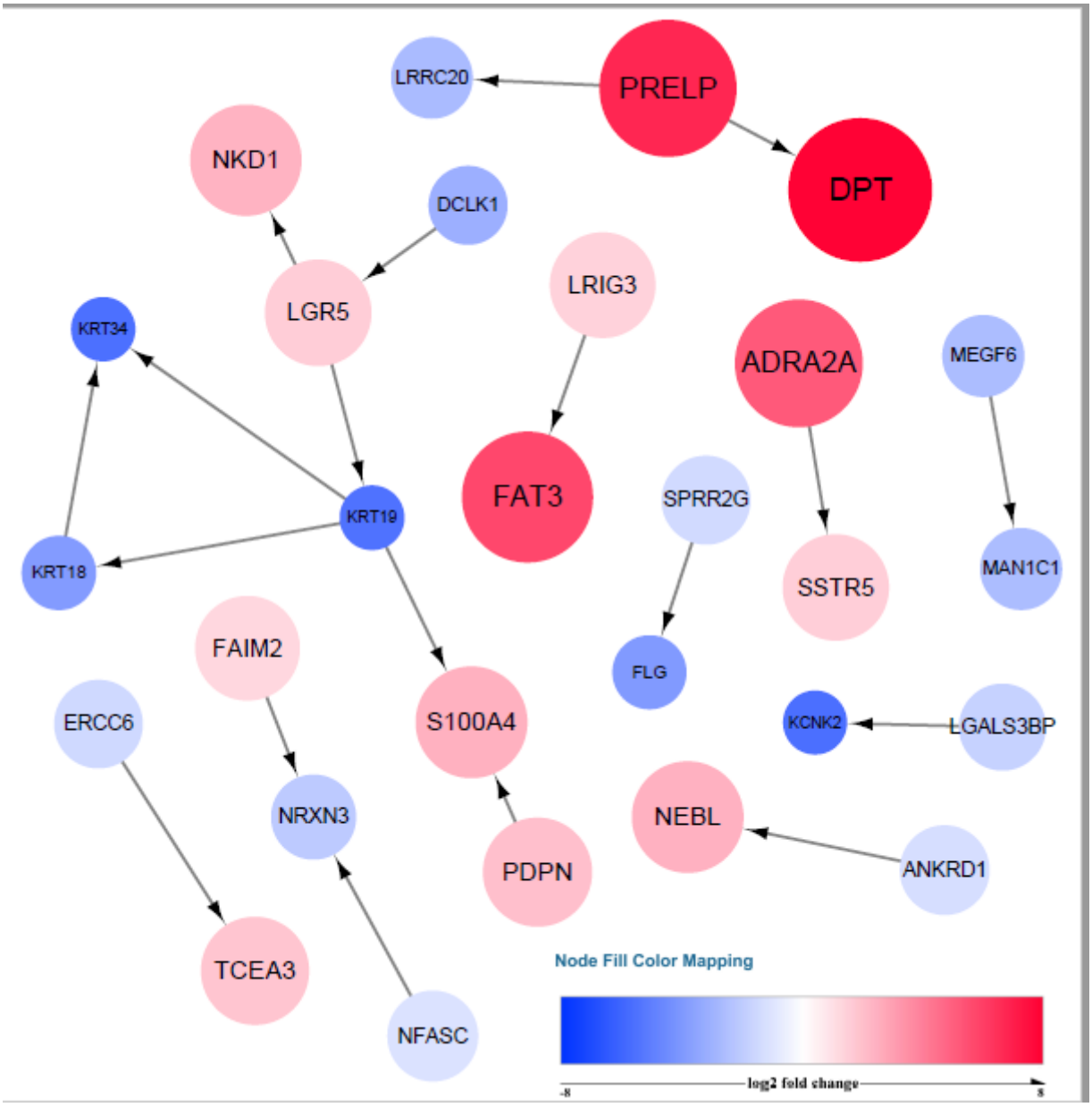


Figure 6

PPI network. Red represents up-regulated genes, blue represents down-regulated genes.

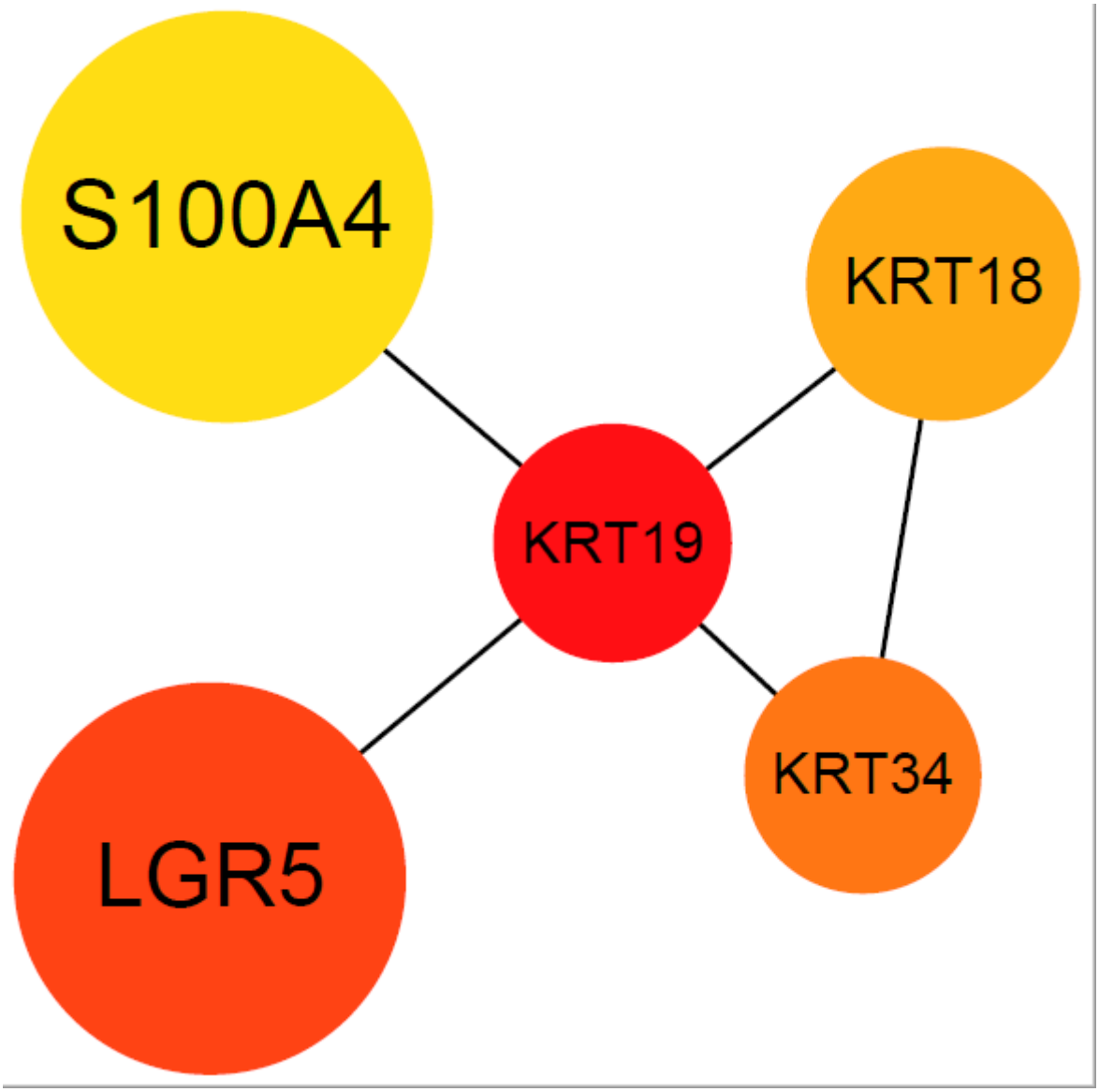


Figure 7

PPI network of 5 hub genes.

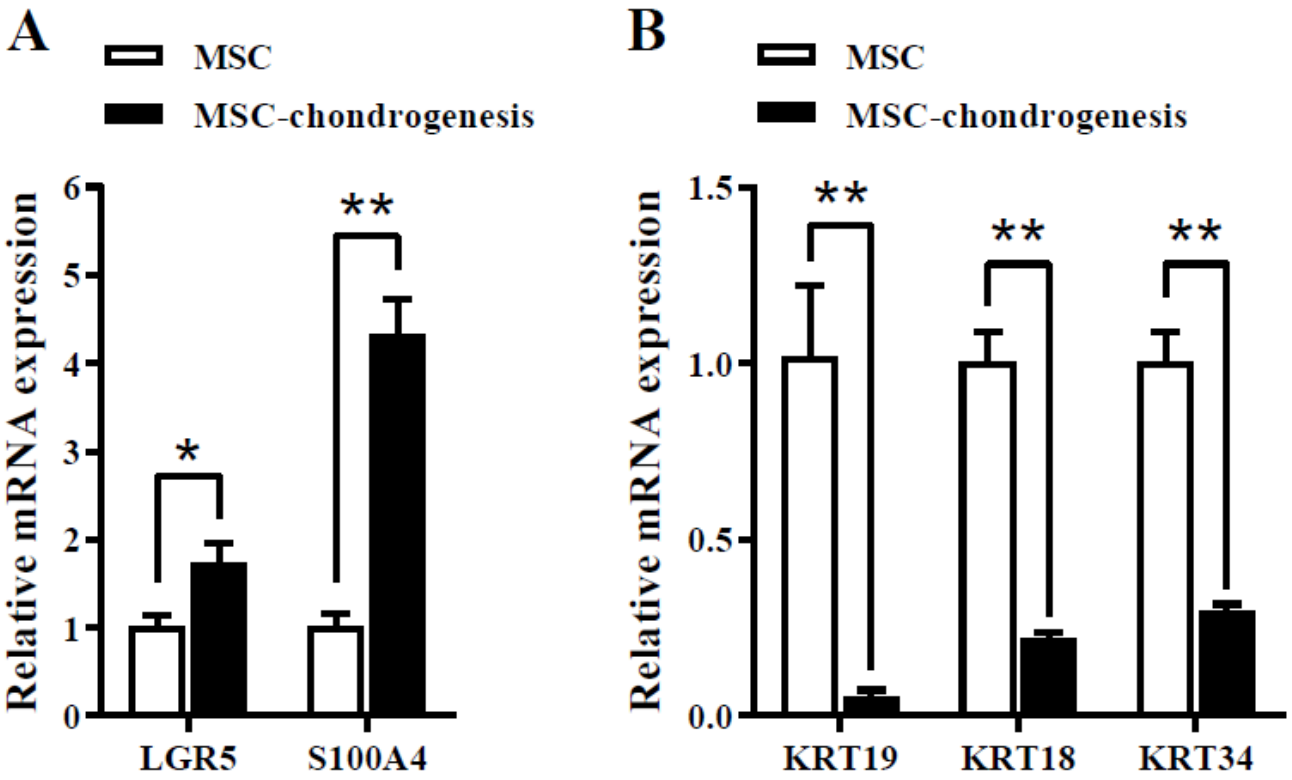


Figure 8

qRT-PCR results of selected DEGs in chondrogenesis. A represented up-regulated genes. B represented down-regulated genes. “∗” was represented P<0.05 and “∗∗” was represented P<0.01.

Supplementary Files

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