Identification of Key Genes in Intervertebral Disc Degeneration Regulated by Acupuncture: An In Vivo Rat Model

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Running title: Tai Liu et al: KEY GENES IN INTERVERTEBRAL DISC DEGENERATION REGULATED BY ACUPUNCTURE

Abstract

In the present study, we established a rat model of intervertebral disc degeneration (IDD) to ivestigate the mechanism by which acupuncture attenuates IDD. Acupuncture were performed on IDD model rats for 14 days. RNA sequencing was used to identify differentially expressed genes (DEGs) in the intervertebral discs after acupuncture. Bioinformatics analysis was performed to identify the key genes among the DEGs. The expression levels of key genes were examined via qRT–PCR and immunofluorescence staining. IDD model was confirmed by HE staining. A total of 102 DEGs were identified. These DEGs were enriched in 18 GO MF terms, 13 GO CC terms, 60 GO BP terms and 5 KEGG pathways. Cidec, Retn, Angptl4, and Pdk4 were the key genes among the DEGs. Both qRT–PCR and immunofluorescence staining revealed that acupuncture increased the expression of Cidec and Retn and decreased that of Angptl4 and Pdk4 in degenerated disc tissues. In conclusion, Cidec, Retn, Angptl4 and Pdk4 play key roles in the response to acupuncture intervention in IDD patients. Acupuncture may ameliorate IDD by decreasing Angptl4 to inhibit inflammation and extracellular matrix degradation. The significance of the alteration of Cidec, Retn, Angptl4 and Pdk4 expression in IDD by acupuncture needs further study.

Key words

Intervertebral disk degeneration, rat model, HE staining, differentially expressed genes, bioinformatics analysis, qRT- PCR, immunofluorescence staining.

Introduction

Degenerative disc disease resulting from intervertebral disc degeneration (IDD) is believed to be the main cause of low back pain(Deyo et al., 2001; Hartvigsen et al., 2018), which is a medical challenge worldwide(Clark et al., 2018). Acupuncture is widely used to treat low back pain caused by disc diseases(Tang et al., 2018; Vickers et al., 2014), and is effective for alleviating pain and improving the mental and physical health of patients with disc disease(Huang et al., 2021). Moreover, acupuncture is recommended for all stages of low back pain by the American College of Physicians (Qaseem et al., 2017). Acupuncture relieves pain by regulating bioactive factors such as TNF-α, NF-κB, IL-6, IL-1β, PI3K, p38 MAPK, corticosterone, COX-2, PGE2, and substance P, among others(Kuo et al., 2014; Pan et al., 2024). Although the mechanism by which acupuncture attenuates IDD has been studied extensively, there remain many unknowns in the field. Thus, we conducted animal experiments to obtain and analyze gene expression data" from degenerated intervertebral discs before and after acupuncture, along with bioinformatics analyses and molecular biology experiments, to investigate the effects of acupuncture on IDD.

Materials & Methods

Animal experiments

Twelve healthy male 8-week-old SD rats (weighing 220 ± 30 g; SPF level) were purchased from Chengdu Dasuo Experimental Animal Company. SD rats were housed

with standard rat food and water for one week to adapt to the environment of the animal center. A model of IDD was established in eight SD rats via anterior approach surgery following a modification of the procedure introduced by Huang(Huang et al., 2024). Eight model SD rats were reared for 8 weeks, and then, 4 of the model SD rats were randomly selected to verify the success of model establishment. The remaining 4 model SD rats were administered acupuncture daily for 14 days before sampling. The control SD rats were reared normally for 10 weeks before sampling. The model SD rats were gently restrained under a soft cloth jacket before acupuncture. The ShenShu (BL23) and DachangShu (BL25) acupoints were selected according to the atlas of acupoints in rats(Xu et al., 2019). Stainless steel acupuncture needles (Huatuo acupuncture needles, 0.30 × 25 mm in size, stainless steel, Suzhoushi Hualun Acupuncture Supplies Co. Ltd., lot 230201) were disinfected and then inserted into the acupoints on both sides of the spine to a depth of 5 mm, with needle retention times of 20 min. The rats were allowed to move freely after acupuncture. The present study was approved by the Ethics Committee of Sichuan Province Orthopedic Hospital.

Hematoxylin–eosin (HE) staining

The discs were fixed with 4% paraformaldehyde, and then ethylene diamine tetraacetic acid was added and replaced every 3 days. Decalcification was performed for 1 month prior to paraffin embedding and sectioning. Hematoxylin–eosin (HE) staining was performed according to standard protocols.

RNA extraction and sequencing

Total RNA was extracted separately from 4 acupuncture group and 4 IDD model group samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance

with the manufacturer's instructions. Then, DNase I was added to remove contaminating genomic DNA. The RNA quality and quantity were measured with a NanoDrop spectrophotometer (Thermo Scientific, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, USA), respectively. RNA integrity was determined by 1% gel electrophoresis. Equal amounts of total RNA from the intervertebral disc samples were pooled into a model group and a control group.

For high-throughput sequencing, ribosomal RNA (rRNA) was depleted from total RNA via the Ribo-Zero™ rRNA Removal Kit (Human/Mouse/Rat; Epicenter, USA) according to the manufacturer's protocol. The cDNA libraries were prepared with a ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicenter, USA) and sequenced on an Illumina HiSeq X ten paired-end reads at GeneX Health, Beijing, China. RNA-seq read mapping and transcriptome assembly were performed by GeneX Health (Beijing, China).

Differentially expressed genes(DEGs)

The fragments per kilobase per million reads (FPKM) method was used to normalize the RNA sequence reads of all the samples. R package DESeq 2 (version v4.3.3) was used to identify DEGs between the disc tissues of acupunctured IDD rats and IDD model rats ($p < 0.05$, log 2-fold change (FC) ≥ 1.5 or ≤ -1.5).

Functional analysis of DEGs

We performed enrichment analyses, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment, to explore the

functions of the DEGs. The clusterProfiler package of R for Bioconductor was used to perform the enrichment analysis.

Construction of the protein–protein interaction (PPI) network

We mapped the DEGs in the Search Tool for the Retrieval of Interacting Genes (STRING) using the default instructions and then constructed a protein–protein interaction (PPI) network of the nodes with a combined score >0.15. The PPI network was visualized in Cytoscape software (version 3.7.1), and we calculated the degree centrality for each node in the PPI network with the CentiScaPe plug-in.

Validation by quantitative real-time polymerase chain reaction (qRT–PCR)

The expression levels of key DEGs from the RNA-seq data analysis were validated by qRT–PCR using the 2-ΔΔCq method. GAPDH was used as an internal control. Specific primers for the DEGs were designed with Primer 5.0 and synthesized as previously described by our group(Wang et al., 2021).

Immunofluorescence staining

Immunofluorescence staining was performed to identify the proteins associated with the key DEGs. Disc tissue samples were fixed in 4% paraformaldehyde for 48 h, embedded in paraffin, and sectioned into 5-μm-thick sections. The sections were antigen-repaired in antigen repair buffer in a staining rack, washed with PBS, and blocked in 5% BSA for 30 min. Then, the disc tissue slices were incubated with the following antibodies: anti-Cidec (1:100 dilution; Abcam, DF8913), anti-Retn (1:100 dilution; Affinity, DF8288), anti-Angptl4 (1:100 dilution; Affinity, DF6751), and anti-

Pdk4 (1:100 dilution; Affinity, DF7169) overnight in a humid box at 4°C. The slides were washed with PBS and then incubated with a FITC-labeled goat anti-rabbit secondary antibody (1:100 dilution; Servicebio, GB22303) for 30 min at room temperature. The nuclei were stained by incubation with 2 g/mL 2-(4-amidinophenyl)indole-6-carbamidine dihydrochloride (DAPI; Servicebio, Wuhan, China) for cellular localization and then washed extensively with PBS.

Results

Histological staining with HE

HE staining revealed that the intervertebral discs of the normal rats were morphologically healthy, with layered rings and no signs of rupture or disorder, and that the central part of the nucleus pulposus was oval, full in volume(Figure 1 A1), and had no obvious inflammatory cell infiltration good continuity(Figure 1 A2). In the model group, the intervertebral disc tissue was stained unevenly, with disordered structures and glass changes and cracks in the annulus(Figure 1 B1). The nucleus pulposus was shrunken or absent, and the collagen content was significantly reduced, resulting in a large amount of chondrocyte-like necrosis, nuclear fragmentation or lysis, and enhanced cytosolic eosinophilia(Figure 1 B2). In the acupuncture group, the disorder of the annulus fibers significantly improved, the collagen distribution in the matrix was essentially uniform(Figure 1 C1), the number of cells was significantly increased, and the number of cell fissures was decreased(Figure 1 C2).

DEGs

There were 102 DEGs in the intervertebral disc tissues between the acupuncture group and the model group, including 62 upregulated genes and 40 downregulated genes. Figure 2 shows a heatmap of DEG expression in both groups.

DEG enrichment analysis

The DEG enrichment analysis revealed 18 GO MF terms, 13 GO CC terms, 60 GO BP terms (Figure 3), and 5 KEGG pathways (Figure 4). The most significantly enriched GO terms were regulation of lipid metabolic process (BP), transporter complex (CC) and channel activity (MF), and the most significantly enriched KEGG pathway was the AAPR signaling pathway.

PPI network of DEGs

The PPI network of the DEGs comprised 221 edges and 79 connected nodes (Figure 5). The six submodules identified by MCODE analysis are shown in different colors in Figure 5. The genes in the PPI network were sorted by the centrality degree of each node as calculated by using the CentiScaPe plug-in, and the top 4 genes with the highest degree centrality were identified as key genes of IDD. These key genes are involved in cell death-inducing DFFA-like effector c (Cidec, centrality degree 8), resistin (Retn, centrality degree 6), angiopoietin-like 4 (Angptl4, centrality degree 3), and pyruvate dehydrogenase kinase 4 (Pdk4, centrality degree 3).

Validation of the mRNA expression levels of the hub genes

The expression levels of Cidec, Retn, Angptl4, and Pdk4 were verified by qRT–PCR **(Figure 6).** The expression levels of Cidec and Retn were greater in the acupuncture group than in the model group. The expression levels of Angptl4 and

Pdk4 were lower in the acupuncture group than in the model group. The primers used for qRT–PCR are listed in Table 1.

Immunofluorescence staining

To explore the protein expression of key DEGs in the intervertebral disc tissue of both groups, Cidec, Retn, Angptl4, and Pdk4 were detected in rat disc tissue samples via immunofluorescence staining. As shown in Figure 7, Cidec- and Retn-positive cells (green fluorescence) were detected in the discs of the acupuncture group but were rarely observed in the model group; Angptl4- and Pdk4-positive cells (green fluorescence) were detected in the discs of the model group but rarely found in the acupuncture group. These results demonstrated that acupuncture increased Cidec and Retn protein expression and decreased Angptl4 and Pdk4 protein expression in degenerated intervertebral discs.

Discussion

IDD is influenced by multiple factors (Karchevskaya et al., 2023; Kirnaz et al., 2022). Although the pathophysiology of IDD has been widely studied(Xia et al., 2024), tthe mechanism by which acupuncture affects IDD is not completely clear. Animal experiments, in which the influence of external factors can be reduced, are an important tool for investigating how acupuncture affects IDD. Successful animal experiments are highly dependent on effective and suitable animal models. In the present study, we followed the instructions and surgical video for an improved anterior approach to establish a rat puncture model of IDD(Huang et al., 2024) and explored the mechanism by which acupuncture affects IDD in this model.

In the present study, we identified 102 DEGs between disc tissues before and after acupuncture. These DEGs were significantly enriched in GO terms including regulation of lipid metabolic process (BP), transporter complex (CC) and channel activity (MF), and the most significantly enriched KEGG pathway was the AAPR signaling pathway. We identified Cidec, Retn, Angptl4 and Pdk4 as the key genes in the PPI network of the genes regulated by acupuncture, suggesting that these genes play crucial roles in acupuncture-induced IDD. Acupuncture increased Cidec and Retn expression and decreased Angptl4 and Pdk4 expression in degenerated intervertebral discs.

Cidec is expressed mainly in fat. It is a member of the cell death-inducing DFFA-like effector family, which promotes apoptosis(Zheng et al., 2021). The protein encoded by Cidec plays a role in lipid droplet formation and adipocyte apoptosis(Balakrishnan et al., 2023). Cidec expression is regulated by insulin(Song et al., 2022). Insulin sensitivity is positively correlated with Cidec expression, and insulin-resistant diabetes is related to mutations in this gene(Zhou et al., 2021). The short arm of chromosome 3 contains a Cidec pseudogene, and different isoforms of this gene are encoded by alternatively spliced transcript variants. However, the role of Cidec in IDD has not been reported, and how Cidec is upregulated after acupuncture and how this upregulation ameliorates IDD needs further study.

Retn is a mouse resistin-like protein that is secreted by adipocytes. The Retn gene is a potential hub between obesity and type II diabetes(Steppan et al., 2001). The protein encoded by Retn exhibits antibacterial properties against both gram-negative and gram-positive bacteria in the skin(Adeghate 2004). Retn is expressed by human monocytes and macrophages(Patel et al., 2003). and has significant proinflammatory

properties(Bokarewa et al., 2005). Retn expression may be upregulated by proinflammatory factors, including IL-1β, IL-6, IL-12, TNF-α and lipopolysaccharide(Anderson et al., 2007; Kaser et al., 2003; Lehrke et al., 2004). Although many physiological and pathological processes have been demonstrated to involve Retn(Filkova et al., 2009), the specific role of Retn in mediating the effects of acupuncture on IDD has never been reported and needs further study.

Angptl4 is related to the regulation of inflammation, glucose homeostasis, insulin sensitivity and lipid metabolism(Zuo et al., 2023). Peroxisome proliferation activators (PPARs) induce the expression ofthe protein encoded by Angptl4, which inhibits vascular growth and vascular endothelial cell apoptosis(Aryal et al., 2019). Downregulation of Angptl4 was reported in type 2 diabetes(Gusarova et al., 2018). Both the mRNA and protein expression of Angptl4 in intervertebral discs increase with increasing IDD severity(Liu et al., 2021). Both in vitro and in vivo experiments have confirmed the increased expression of Angptl4 in IDD, and Angptl4 was identified as the hub gene involved in the crosstalk between IDD and type 2 diabetes mellitus(Chen et al., 2023). The inhibition of inflammation and extracellular matrix degradation in chondrocytes by Angptl4 knockdown suggests that Angptl4 has proinflammatory and matrix degradation-promoting effects(Jia et al., 2022). In our study, qRT–PCR and immunofluorescence confirmed that Angptl4 in degenerated discs was downregulated by acupuncture. We hypothesized that acupuncture blocks IDD by decreasing Angptl4 to inhibit inflammation and extracellular matrix degradation.

Pdk4 is related to glucose metabolism by inhibiting the pyruvate dehydrogenase complex and is regulated by insulin, retinoic acid and glucocorticoids. The expression

of Pdk4 was decreased in the cartilage of patients with osteoarthritis. Pdk4 has anti-inflammatory and antiapoptotic effects on hepatocytes(Wu et al., 2018), inhibiting inflammation by downregulating TNF-α, IL-8, and IL-6; inhibiting extracellular matrix degradation by downregulating MMP-3, MMP-13, and ADAMTS-4; and restoring chondrocyte viability (Li et al., 2024). Previous studies have suggested that Pdk4 has a protective effect on cells, but the exact role of Pdk4 in IDD has not been reported. The qRT–PCR and immunofluorescence analyses in our study revealed that Pdk4 in degenerated discs was downregulated by acupuncture. The significance of acupuncture-mediated downregulation of Pdk4 in IDD should be studied further.

Conclusions

In this work, we identified Cidec, Retn, Angptl4 and Pdk4 as the hubs in the PPI network of the genes regulated by acupuncture, suggesting that these genes play crucial roles in the effect of acupuncture intervention in IDD. Acupuncture increased the expression of Cidec and Retn but decreased the expression of Angptl4 and Pdk4 in degenerated intervertebral discs. Acupuncture may ameliorate IDD by decreasing Angptl4 to inhibit inflammation and extracellular matrix degradation. The significance of the changes in Cidec, Retn and Pdk4 expression in IDD by acupuncture needs further study.

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Availability of data and material: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: Tai Liu: Conceptualization, Research design, Project administration, Resources, Methodology, Software, Validation, Investigation, Formal analysis, Writing - Review & Editing. **Yi Wang:** Conceptualization, Research design, Project administration, Investigation, Validation, Writing - Original Draft, Visualization, Supervision, Funding acquisition. **Ling Jiang, Yan Xu, Guogang Dai and Ji Wu:** Methodology, Software, Validation, Methodology, Data Curation, Formal analysis, Investigation, Visualization, Writing - Original Draft. **Bunyun Liu, Yanjie Wang and Hai Shen:** Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft. All authors read and approved the final manuscript.

Ethics approval and consent to participate: Ethical approval for this study was obtained from the Ethics Committee of the Sichuan Province Orthopedic Hospital (approve NO. 2022YFS0420). All methods were carried out in accordance with relevant guidelines and regulations.

Patient consent for publication: Not applicable.

Competing interests: The authors declare that they have no conflict of interest.

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Table

Table 1. Sequences of primers used for quantitative real-time polymerase chain reaction.

Gene	Sequence (5’ to 3’)
Cidec	F: ATGGACTACGCCATGAAGTCT
	R: CGGTGCTAACACGACAGGG
Retn	F: ACAAGACTTCAACTCCCTGTTTC
	R: TTTCTTCACGAATGTCCCACG3
Angptl4	F: TCACAGCCTGCAGACACAACT
	R: CCATCTCGGGCAGCCTCTTT
Pdk4	F: TCCACTGCACCAACGCCT
	R: TGGCAAGCCGTAACCAAAA

Figures

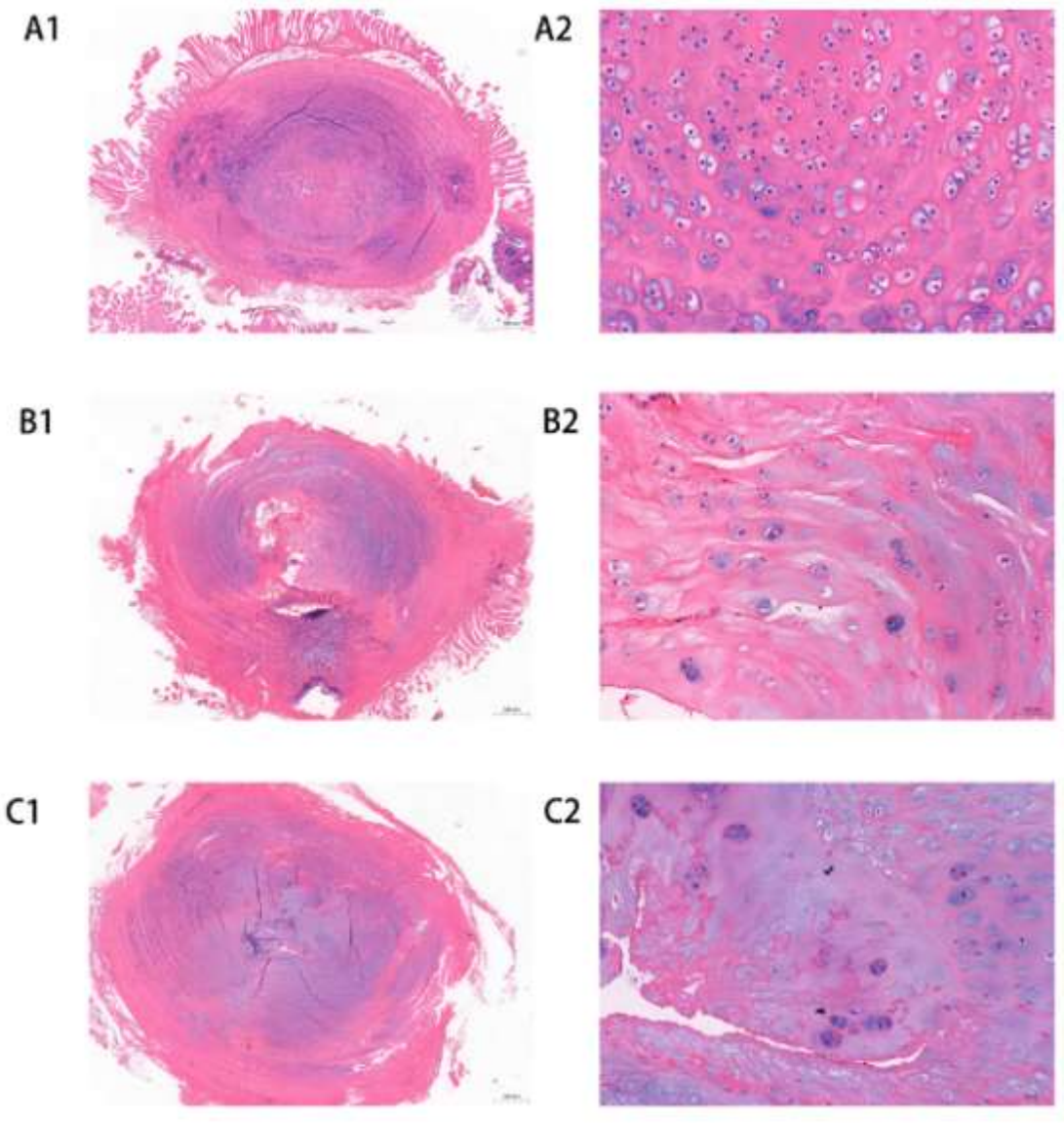

Figure 1. HE staining of intervertebral discs. A: Normal group. A-a: disc tissue was morphologically healthy, with layered rings and no signs of rupture or disorder, and that the central part of the nucleus pulposus was oval, full in volume; A-b: disc tissue had no obvious inflammatory cell infiltration good continuity. B: IDD model group. B-a: disc tissue was stained unevenly, with disordered structures and glass changes and cracks in the annulus; B-b: The nucleus pulposus was shrunken or absent, and the collagen content was significantly reduced, resulting in a large amount of chondrocyte-like necrosis, nuclear fragmentation or lysis, and enhanced cytosolic

eosinophilia. C: Acupuncture group. C-a: the disorder of the annulus fibers significantly improved, the collagen distribution in the matrix was essentially uniform; C-b: the number of cells was significantly increased, and the number of cell fissures was decreased.

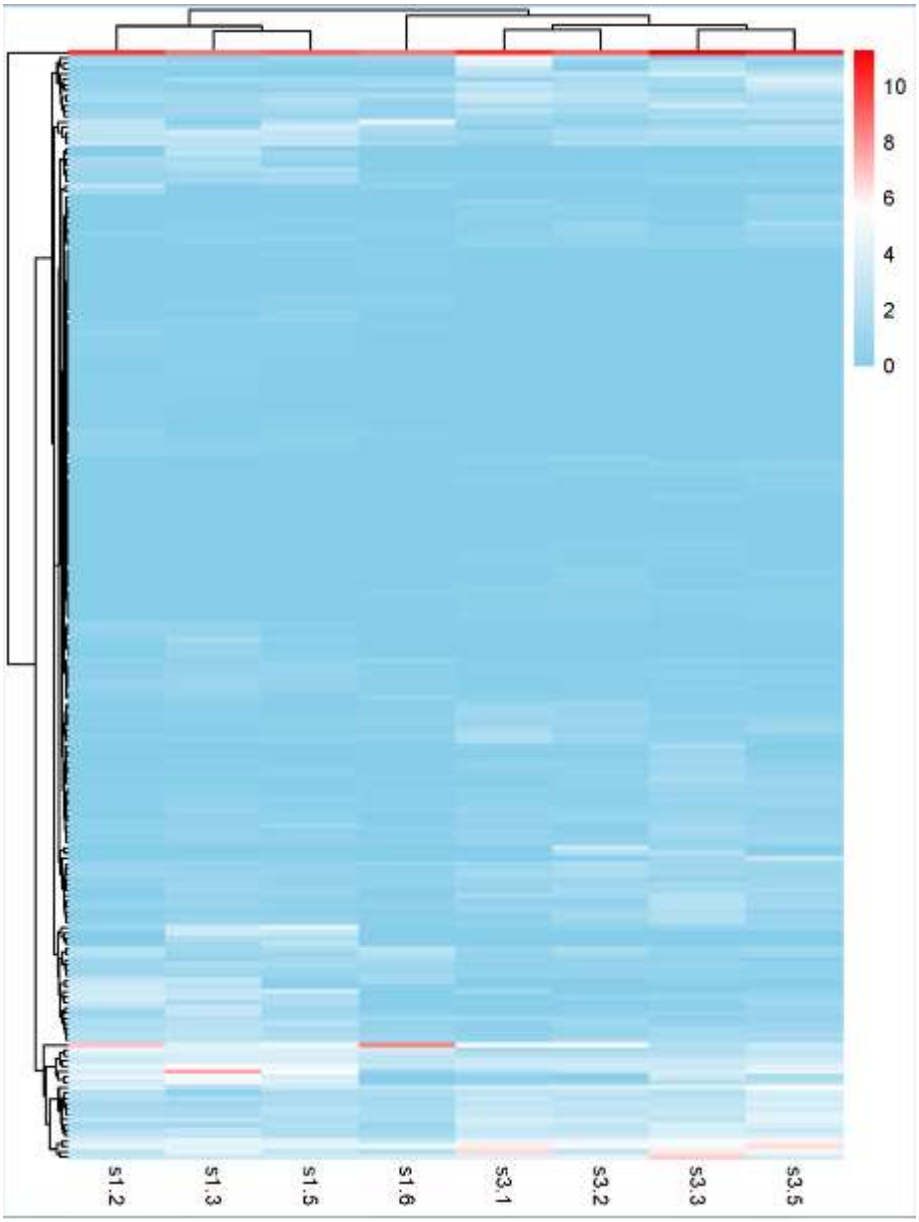

Figure 2. Heatmap of differentially expressed genes in the intervertebral disc tissues between the acupuncture group and the model group.

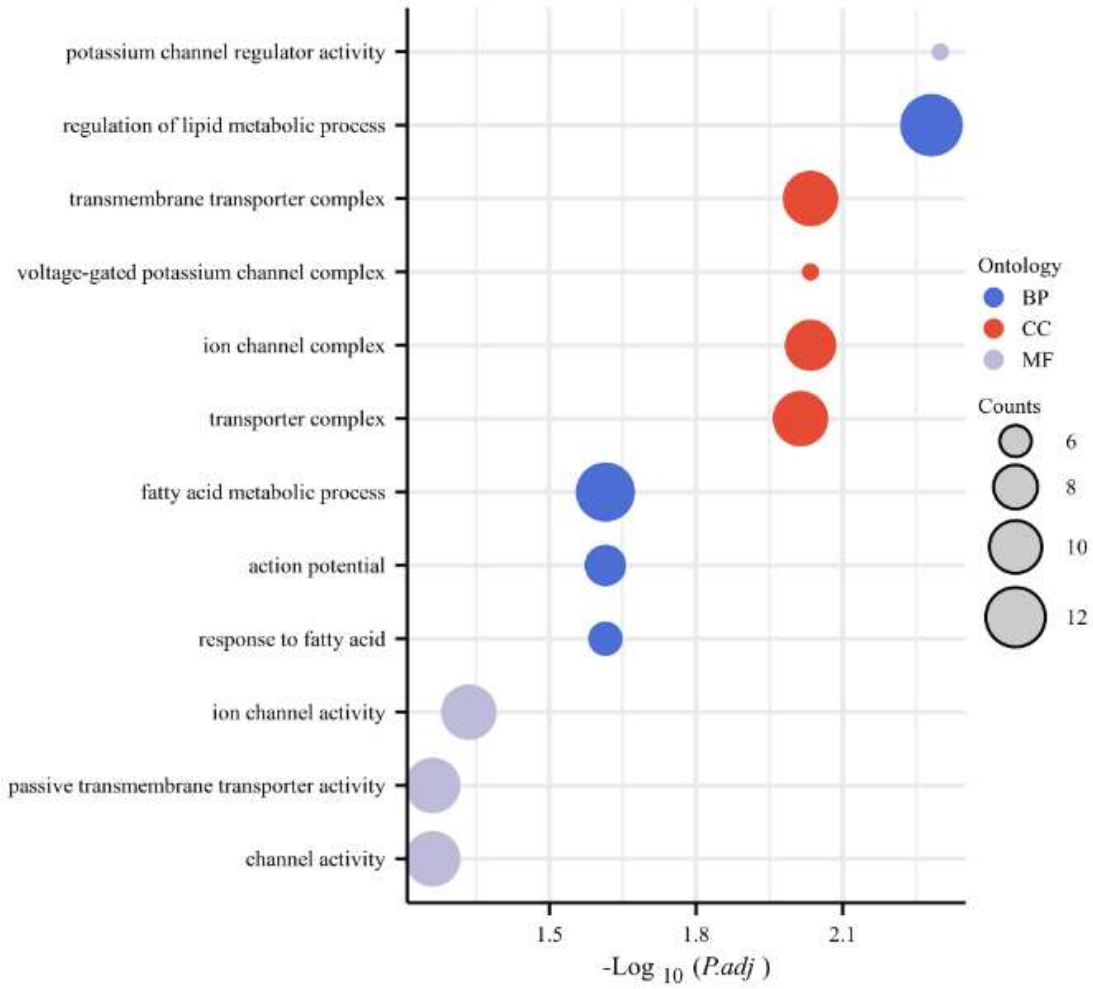

Figure 3. Gene Ontology enrichment of the differentially expressed genes.

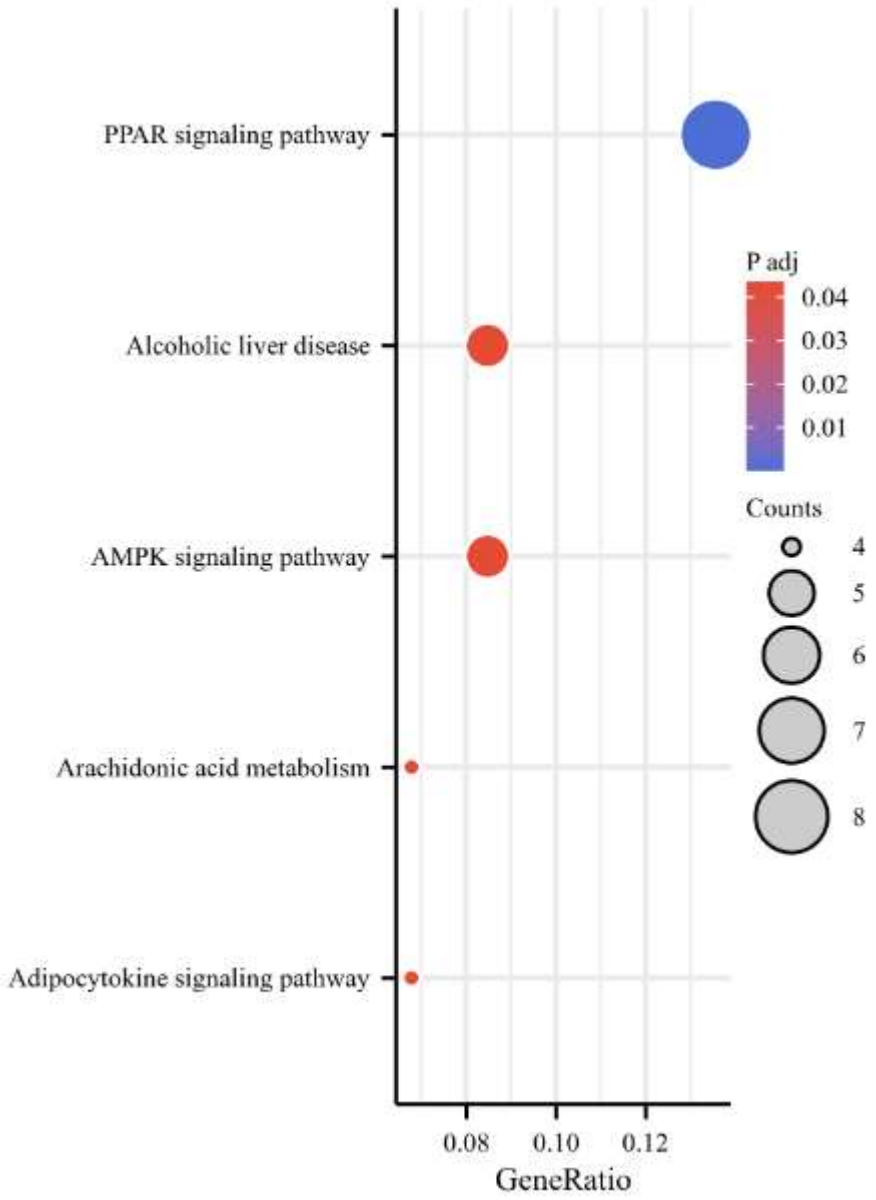

Figure 4. KEGG pathway enrichment of the differentially expressed genes

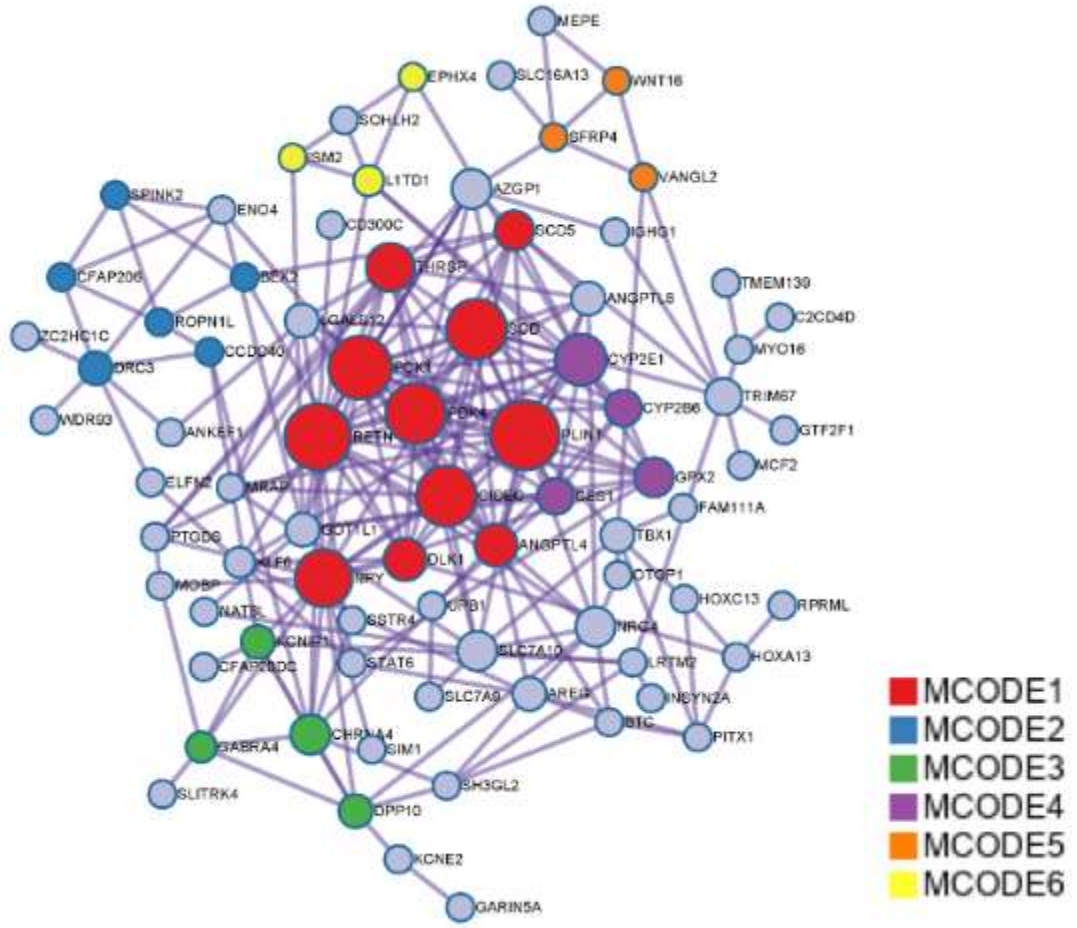

Figure 5. PPI network of differentially expressed genes. MCODE analysis identified 6 clusters.

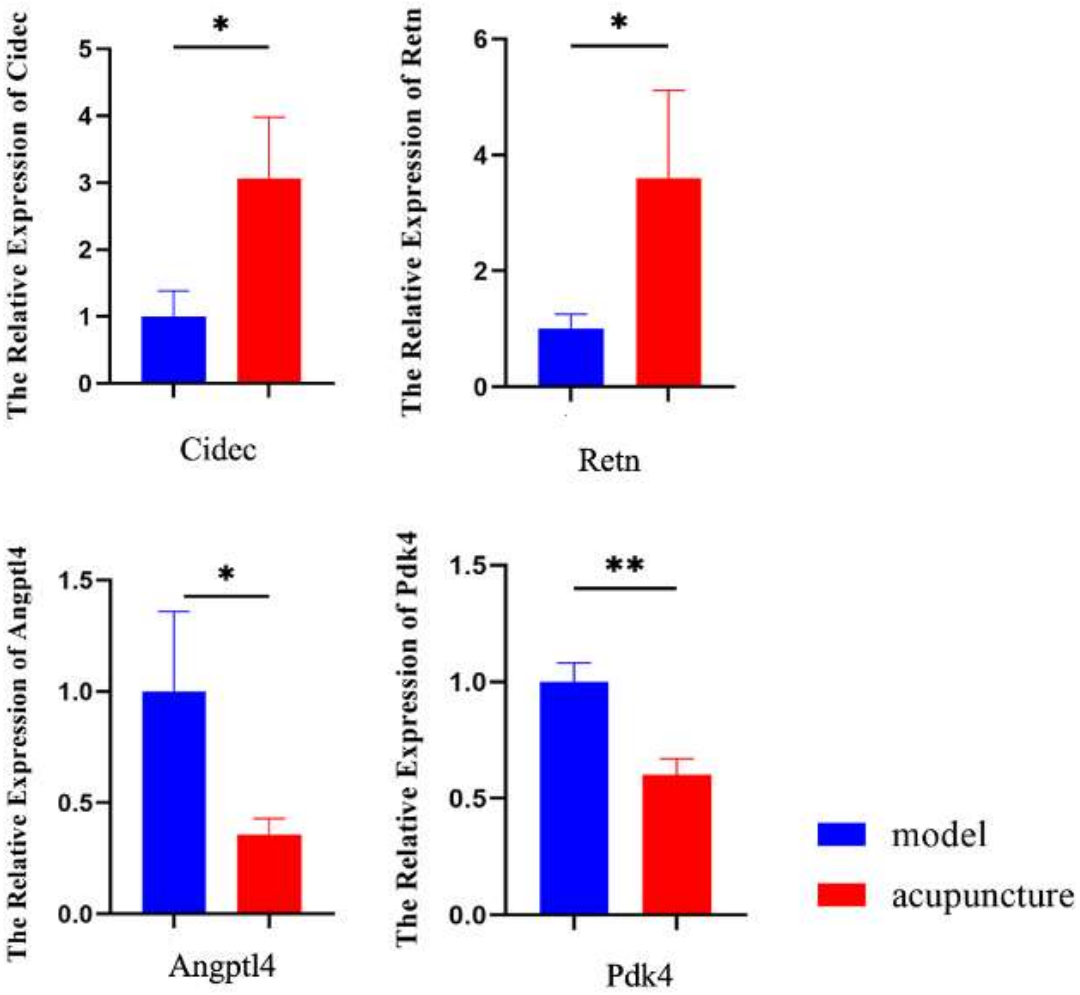

Figure 6. Expression of key genes.

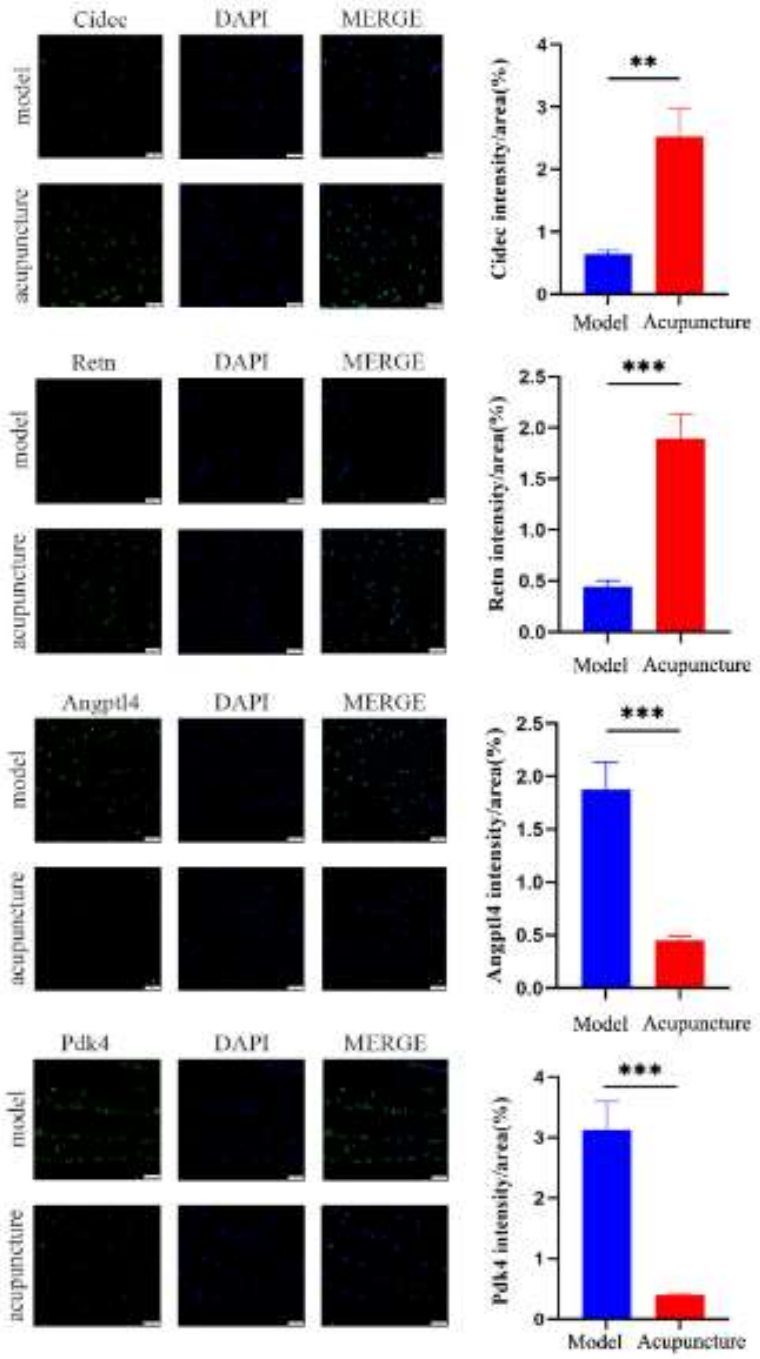

Figure 7. Key genes were detected by immunofluorescence combined with DAPI staining.

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