Identification of critical genes in nucleus pulposus cells isolated from degenerated intervertebral discs using bioinformatics analysis

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Received March 15, 2016; Accepted February 28, 2017

DOI: 10.3892/mmr.2017.6662

Abstract. Intervertebral disc (IVD) degeneration is a pathological process, which may lead to lower back pain. The present study aimed to investigate the pathogenesis of IVD degeneration. GSE42611 was downloaded from Gene Expression Omnibus, including 4 nucleus pulposus samples isolated from degenerated IVDs and 4 nucleus pulposus samples separated from normal IVDs. The differentially expressed genes (DEGs) between the degenerated and normal samples were screened using the limma package in R. Functional and pathway enrichment analyses were conducted separately for the upregulated and downregulated genes, using Database for Annotation, Visualization and Integrated Discovery software. In addition, protein-protein interaction (PPI) networks were constructed using the Search Tool for the Retrieval of Interacting Genes database and Cytoscape software. Finally, module analyses were conducted for the PPI networks using the MCODE plug-in in Cytoscape. A total of 558 DEGs were identified in the degenerated nucleus pulposus cells: 253 upregulated and 305 downregulated. Pathway enrichment analysis revealed that downregulated thrombospondin 1 (THBS1) was enriched in extracellular matrix-receptor interaction. Interleukin (IL)-6 in the PPI network for the upregulated genes and vascular endothelial growth factor A (VEGFA) in the PPI network for the downregulated genes had higher degrees. Additionally, four modules (μM1, μM2, μM3 and μM4) were identified from the PPI network for the upregulated genes. Four modules (dM1, dM2, dM3 and dM4) were identified from the PPI network for the downregulated genes. In the dM2 module, collagen genes and integrin subunit α4 (ITGA4) may interact with each other. Additionally, functional enrichment indicated that collagen genes were enriched in extracellular matrix organization. In conclusion, IL-6, VEGFA, THBS1, ITGA4 and collagen genes may contribute to the progression of IVD degeneration. These results suggested that the manipulation of these genes and their products may have potential as a novel therapeutic strategy for the treatment of patients with IVD.

Introduction

Intervertebral disc (IVD) degeneration, also termed degenerative disc disorder or degenerative disc disease, is a pathological process that may induce acute or chronic lower back pain (1,2). Lower back pain is one of the primary health problems in developed countries (3). The risk factors for disc degeneration include genetic inheritance and environmental risk factors, including smoking cigarettes and repetitive and high mechanical loading (4). IVD degeneration is a rapidly progressing disease without an effective therapeutic method (5). Therefore, it is necessary to explore the mechanisms of IVD degeneration in order to be able to develop a novel treatment scheme.

IVD degeneration and the underlying molecular mechanisms have been previously investigated. The aggrecanases ADAM metallopeptidase with thrombospondin type 1 motif (ADAMTS)-1, -4, -5, -9 and -15 may promote extracellular matrix (ECM) alterations during IVD degeneration, and may be used for preventing IVD degeneration and its morbidity (6). In disc cells, reduced expression of SRY-type high mobility group box 9 (*SOX9*) may be associated with disc degeneration and disc ageing via inhibition of type II collagen expression (7). The growth differentiation factor-5 (*GDF-5*) cDNA and the recombinant GDF-5 protein may promote the expression of ECM protein-coding genes in mouse IVD cells (8). Previous studies have detected overexpressed tumor necrosis factor α (*TNF-α*) and interleukin (*IL*)-1 in aged and degenerative IVDs obtained from human and animal models (9,10). *IL-1* has been identified to be involved in IVD degeneration via directly inhibiting matrix synthesis and promoting matrix

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Key words: intervertebral disc degeneration, differentially expressed genes, functional and pathway enrichment analysis, protein-protein interaction network

degradation (11,12). Cytokines of *IL-1* and *TNF-α* may be associated with the pathogenesis of IVD degeneration; however, *IL-1* may have a greater contribution to IVD degeneration and may be a more suitable therapeutic target for the disease (13).

In 2013, Markova *et al* (14) established a rat disc organ culture model that mimicked IVD degeneration via culturing rat IVDs in the presence of IL-1β, TNF-α and serum-limiting conditions. They obtained 1036 differentially expressed genes (DEGs) between experimental and control groups following gene expression analysis for microarray data. The present study used the data from Markova *et al* (14) and the DEGs between degenerated and normal nucleus pulposus cells were identified, and their possible functions were predicted using enrichment analysis. Additionally, protein-protein interaction (PPI) networks were visualized and module analysis was conducted to screen for key genes in degenerated nucleus pulposus cells.

Materials and methods

Microarray data. Microarray data obtained from GSE42611 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42611), which was downloaded from the database of Gene Expression Omnibus (GEO), were sequenced on the platform of GPL6247 Affymetrix Rat Gene 1.0 ST Array [transcript (gene) version]. GSE42611 included 4 nucleus pulposus samples isolated from degenerated IVDs and 4 nucleus pulposus samples separated from normal IVDs. The procedure that had been used to obtain the rat lumbar disc specimens (n=4 specimens/group) was as follows, according to the method of Ponnappan *et al* (8): Whole lumbar IVDs with endplates had been dissected and preserved in organ culture. Lumbar discs in the experimental group had been cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 100 ng/ml TNF-α, 10 ng/ml IL-1β, 50 μg/ml L-ascorbate, 40 mM NaCl, 1% fetal bovine serum (FBS), antibiotics and antimycotics. Lumbar discs in the control group had been cultured in DMEM containing 50 μg/ml L-ascorbate, 40 mM NaCl, 10% FBS and antibiotics without cytokines. The discs had been cultured for a total of 10 days (14). GSE42611 used in this study was downloaded from a public database; therefore, patient consent or ethics committee approval were not required.

Data preprocessing and DEGs screening. GSE42611 was downloaded and the microarray data was preprocessed using the Affy package (15) in R. The process of data preprocessing included background correction, quantile normalization, summarization and probe ID to gene symbol transformation. Linear models for microarray data in the limma package (16) in R were used to analyze the DEGs between degenerated and normal nucleus pulposus cells. P-values of the DEGs were calculated separately and adjusted using the t-test method and the Benjamini & Hochberg method (17). $P<0.05$ and $|\log_2$ fold-change (FC)$|>1$ were used as the thresholds.

Functional and pathway enrichment analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID; david.abcc.ncifcrf.gov) software was used to interpret functions of extensive genes obtained from previous genome studies (18). The Gene Ontology database (GO; www.geneontology.org) contained structured ontologies or vocabularies that depict basic characteristics of genes and gene products (19). The Kyoto Encyclopedia of Genes and Genomes database (KEGG; www.genome.jp/kegg/) synthesizes information of biological systems from genomic, chemical and systemic functional aspects (20). Using the DAVID software, functional and pathway enrichment analyses were conducted separately, for upregulated and downregulated genes. $P\leq0.05$ and >2 enriched genes were set as the thresholds.

PPI network construction and module analysis. The Search Tool for the Retrieval of Interacting Genes (STRING; string-db.org) database provide comprehensive and easily accessible interaction information derived from experiments and predictions (21). Cytoscape software (www.cytoscape.org) was used to integrate high-throughput expression data and biomolecular interaction networks into a unified framework (22). The PPIs obtained for the DEGs were searched using the STRING database (21), with the required confidence (combined score) >0.4 as the threshold. Using Cytoscape software version 2.8 (22), the PPIs were used to established a PPI network. In the network, the proteins were termed nodes and the number of edges involved were their degrees. Finally, the MCODE plug-in (23) in Cytoscape was used to perform module analysis of the PPI networks. The parameters were set at the default thresholds.

Results

DEG analysis. $P<0.05$ and $|\log_2FC|>1$ were set as thresholds and the DEGs between degenerated and normal nucleus pulposus cells were analyzed. There were 558 DEGs identified in the degenerated nucleus pulposus cells compared with normal nucleus pulposus cells, including 253 upregulated and 305 downregulated genes. There were more downregulated genes compared with upregulated genes.

Functional and pathway enrichment analysis. The upregulated genes in the degenerated nucleus pulposus cells were significantly enriched in 255 GO terms and 9 KEGG pathways. The top 10 functions are presented in Table IA, including response to wounding ($P=2.35\times10^{-8}$), inflammatory response ($P=5.99\times10^{-8}$) and response to organic substance ($P=1.56\times10^{-7}$).

The downregulated genes in the degenerated nucleus pulposus cells were significantly enriched in 263 GO terms and 10 KEGG pathways. The top 10 functions included M phase ($P=9.18\times10^{-12}$), cell cycle phase ($P=2.81\times10^{-10}$) and response to steroid hormone stimulus ($P=2.95\times10^{-9}$; Table IB).

Additionally, the upregulated genes were significantly enriched in cytokine-cytokine receptor interaction ($P=2.86\times10^{-4}$), apoptosis ($P=3.95\times10^{-4}$) and chemokine ($P=1.60\times10^{-3}$; Table IIA) signaling pathways.

The pathways enriched for the downregulated genes included ECM-receptor interaction [$P=1.17\times10^{-11}$, involving thrombospondin 1 (*THBS1*)], focal adhesion ($P=1.90\times10^{-9}$) and hematopoietic cell lineage ($P=3.12\times10^{-3}$; Table IIB).

PPI network construction and module analysis. PPI networks were constructed by Cytoscape software following a PPI

Table I. The top 10 enriched functions for the differentially expressed genes in the degenerated nucleus pulposus cells

A, Top 10 functions enriched for the upregulated genes in the degenerated nucleus pulposus cells

ID	Description	P-value	Number of genes	Gene
GO:0009611	Response to wounding	2.35×10^{-8}	24	*KNG1, CXCL1, NFKBIZ, IL6, GIP, KNG2, OLR1, C3, CXCL3, KNG1L1, CXCL2, CLU, HP, GLI3, TIMP1, SOD2, ORM1, CASP4, HIF1A, CCL20, PTGES, HMOX1, JAK2, TFPI2*
GO:0006954	Inflammatory response	5.99×10^{-8}	17	*KNG1, CXCL1, NFKBIZ, IL6, KNG2, OLR1, C3, CXCL3, CXCL2, KNG1L1, HP, ORM1, CASP4, HIF1A, CCL20, PTGES, HMOX1*
GO:0010033	Response to organic substance	1.56×10^{-7}	35	*FOSL2, OSMR, IL6ST, TLR2, NFKBIA, HP, GNG12, MMP3, GLI3, TIMP1, GCH1, IRAK3, PTGES, HMOX1, CSF2RB, ANGPT1, PPP3CA, SKIL, PIK3R3, NR1H3, IL6, SGK1, GIP, BCKDHB, MMP14, CYP7B1, HIF1A, ATP2A2, ABCB1B, CXCL16, JAK2, CTSC, PTPN1, CAR4, STEAP2*
GO:0006952	Defense response	1.68×10^{-7}	22	*KNG1, CXCL1, NFKBIZ, IL6, KNG2, OLR1, FGR, C3, CXCL3, KNG1L1, CXCL2, TLR2, HP, GCH1, ORM1, CASP4, HIF1A, CCL20, PTGES, CXCL16, HMOX1, NOS2*
GO:0042311	Vasodilation	2.48×10^{-6}	7	*KNG1, EDNRB, KNG2, KNG1L1, ITGA1, SOD2, GCH1*
GO:0034097	Response to cytokine stimulus	3.62×10^{-6}	11	*IRAK3, IL6, FOSL2, OSMR, IL6ST, PTGES, CXCL16, SKIL, MMP3, TIMP1, GCH1*
GO:0009719	Response to endogenous stimulus	4.73×10^{-6}	24	*SGK1, IL6, FOSL2, GIP, BCKDHB, TLR2, HP, GNG12, MMP14, MMP3, GLI3, TIMP1, HIF1A, ATP2A2, ABCB1B, HMOX1, ANGPT1, JAK2, PTPN1, PPP3CA, PIK3R3, STEAP2, CAR4, NR1H3*
GO:0009725	Response to hormone stimulus	8.64×10^{-6}	22	*SGK1, IL6, FOSL2, GIP, BCKDHB, TLR2, HP, GNG12, MMP14, GLI3, TIMP1, HIF1A, ATP2A2, ABCB1B, HMOX1, ANGPT1, JAK2, PTPN1, STEAP2, CAR4, PIK3R3, NR1H3*
GO:0055066	Di-, tri-valent inorganic cation homeostasis	1.15×10^{-5}	14	*KNG1, IL6ST, HEXB, SOD2, SLC11A2, EDNRB, HIF1A, MT1A, ATP2A2, HMOX1, MT2A, PKD2, JAK2, CP*
GO:0055080	Cation homeostasis	1.82×10^{-5}	15	*KNG1, SGK1, IL6ST, HEXB, SOD2, SLC11A2, EDNRB, HIF1A, MT1A, ATP2A2, HMOX1, MT2A, PKD2, JAK2, CP*

B, Top 10 enriched functions for the downregulated genes in the degenerated nucleus pulposus cells

ID	Description	P-value	Number of genes	Gene
GO:0000279	M phase	9.18×10^{-12}	21	*KIF11, MKI67, SGOL2, DLGAP5, HAUS1, NUF2, NUSAP1, CENPF, BIRC5, NDC80, CEP55, TACC3, CCNB1, KIF2C, PLK1, TUBB5, BUB1B, MNS1, SKA3, STMN1, CDCA3*

Table I. Continued.

ID	Description	P-value	Number of genes	Gene
GO:0048545	Response to steroid hormone stimulus	2.95×10^{-9}	23	*SOCS2, AIF1, CRYAB, IL1RN, TGFB3, IGF1, BIRC5, AQP1, MMP2, ADIPOQ, TIMP3, H19, CCND1, KRT19, CD36, SERPINF1, ADM, AVPR1A, FABP4, RARA, COL1A1, CD24, CCNA2*
GO:0009628	Response to abiotic stimulus	1.09×10^{-8}	26	*RBP4, APOBEC1, GCLC, AIF1, LXN, IL18, COL3A1, MMP2, CXCL12, TIMP3, KRT8, THBS1, COL11A1, MYOF, PTPRC, CRYAB, ATP1A3, IGF1, SNAI2, CCND1, CD36, ADM, FYN, AVPR1A, TGFB1I1, COL1A1*
GO:0022610	Biological adhesion	1.23×10^{-8}	28	*IBSP, COL3A1, LMO7, KITLG, ITGBL1, FAT3, CD93, COMP, ACAN, COL12A1, TNN, CD4, EMB, CD24, THBS1, COL11A1, THBS4, PTPRC, ACTN1, ITGA4, PCDH18, THY1, OMD, COL14A1, CD36, PECAM1, DSC2, CDH11*
GO:0007155	Cell adhesion	1.23×10^{-8}	28	*IBSP, COL3A1, LMO7, KITLG, ITGBL1, FAT3, CD93, COMP, ACAN, COL12A1, TNN, CD4, EMB, CD24, THBS1, COL11A1, THBS4, PTPRC, ACTN1, ITGA4, PCDH18, THY1, OMD, COL14A1, CD36, PECAM1, DSC2, CDH11*
GO:0051301	Cell division	2.09×10^{-8}	16	*RBP4, HAUS1, NUF2, NUSAP1, BIRC5, CEP55, CCNB1, CCND1, CCNB2, PLK1, BUB1B, SKA3, TOP2A, CCNA2, ASPM, CDCA3*
GO:0022402	Cell cycle process	2.49×10^{-8}	24	*GAS2L3, KIF11, MKI67, SGOL2, DLGAP5, HAUS1, NUF2, NUSAP1, CENPF, BIRC5, NDC80, CEP55, TACC3, CDKN3, CCNB1, KIF2C, CCND1, PLK1, TUBB5, BUB1B, MNS1, SKA3, STMN1, CDCA3*
GO:0030199	Collagen fibril organization	4.36×10^{-8}	8	*COL3A1, COL1A2, ACAN, COL1A1, COL11A1, COL5A2, SERPINH1, DPT*
GO:0007049	Cell cycle	4.42×10^{-8}	27	*GAS2L3, S100A6, HAUS1, CEP55, KIF2C, TUBB5, MNS1, SKA3, CCNA2, CDCA3, KIF11, MKI67, SGOL2, DLGAP5, NUF2, CENPF, NUSAP1, BIRC5, NDC80, TACC3, CDKN3, CCNB1, CCND1, CCNB2, PLK1, BUB1B, STMN1*

Table II. Enriched pathways for the differentially expressed genes in the degenerated nucleus pulposus cells.

A, Pathways enriched for the upregulated genes

ID	Description	P-value	Number of genes	Gene
rno04060	Cytokine-cytokine receptor interaction	2.86×10^{-4}	12	*TNFRSF9, IL6, ZCCHC2, IL23R, TNFSF11, OSMR, IL6ST, CXCL16, MET, CXCL2, CSF2RB, IL13RA1*
rno04210	Apoptosis	3.95×10^{-4}	8	*CFLAR, IRAK3, CSF2RB, NFKBIA, NFKB1, PPP3CA, BIRC3, PIK3R3*
rno04062	Chemokine signaling pathway	1.60×10^{-3}	10	*CXCL1, FGR, CCL20, CXCL16, CXCL2, NFKBIA, JAK2, NFKB1, GNG12, PIK3R3*
rno04621	NOD-like receptor signaling pathway	3.04×10^{-3}	6	*CXCL1, IL6, CXCL2, NFKBIA, NFKB1, BIRC3*
rno04630	Jak-STAT signaling pathway	6.77×10^{-3}	8	*IL6, IL23R, OSMR, IL6ST, CSF2RB, JAK2, PIK3R3, IL13RA1*
rno04620	Toll-like receptor signaling pathway	1.45×10^{-2}	6	*IL6, MAP3K8, TLR2, NFKBIA, NFKB1, PIK3R3*
rno05200	Pathways in cancer	3.02×10^{-2}	11	*IL6, HIF1A, EPAS1, MET, NFKBIA, NFKB1, NOS2, RUNX1, BIRC3, PIK3R3, GLI3*
rno00230	Purine metabolism	3.71×10^{-2}	7	*XDH, GDA, PDE7A, PDE4B, PDE10A, AMPD3, NT5E*
rno05222	Small cell lung cancer	4.41×10^{-2}	5	*NFKBIA, NFKB1, NOS2, BIRC3, PIK3R3*

B, Pathways enriched for the downregulated genes

rno04512	ECM-receptor interaction	1.17×10^{-11}	16	*IBSP, COL3A1, ITGA4, COL5A2, HMMR, CD36, COMP, COL6A3, COL1A2, COL6A2, COL6A1, TNN, COL1A1, THBS1, COL11A1, THBS4*
rno04510	Focal adhesion	1.90×10^{-9}	20	*IBSP, COL3A1, IGF1, ACTN1, ITGA4, COL5A2, CCND1, FYN, COMP, VEGFA, COL6A3, COL1A2, COL6A2, COL6A1, TNN, COL1A1, THBS1, COL11A1, FIGF, THBS4*
rno04640	Hematopoietic cell lineage	3.12×10^{-3}	7	*CD36, KITLG, CD4, ANPEP, CD24, ITGA4, CSF1R*
rno05200	Pathways in cancer	5.58×10^{-3}	14	*FGF7, TGFB3, EGLN3, KITLG, IGF1, BIRC5, FZD4, MMP2, CCND1, VEGFA, RARA, FGF1, FIGF, CSF1R*
rno04670	Leukocyte transendothelial migration	1.97×10^{-2}	7	*CYBB, PECAM1, ACTN1, ITGA4, MMP2, CXCL12, THY1*
rno05219	Bladder cancer	2.86×10^{-2}	4	*CCND1, VEGFA, MMP2, FIGF*
rno04110	Cell cycle	2.93×10^{-2}	7	*CCNB1, CCND1, CCNB2, PLK1, TGFB3, BUB1B, CCNA2*
rno04115	p53 signaling pathway	3.38×10^{-2}	5	*CCNB1, CCND1, CCNB2, SERPINE1, IGF1*
rno04610	Complement and coagulation cascades	4.07×10^{-2}	5	*C1QA, C3AR1, C5AR1, MASP1, SERPINE1*
rno03320	PPAR signaling pathway	4.25×10^{-2}	5	*LPL, CD36, FABP4, ADIPOQ, PLTP*

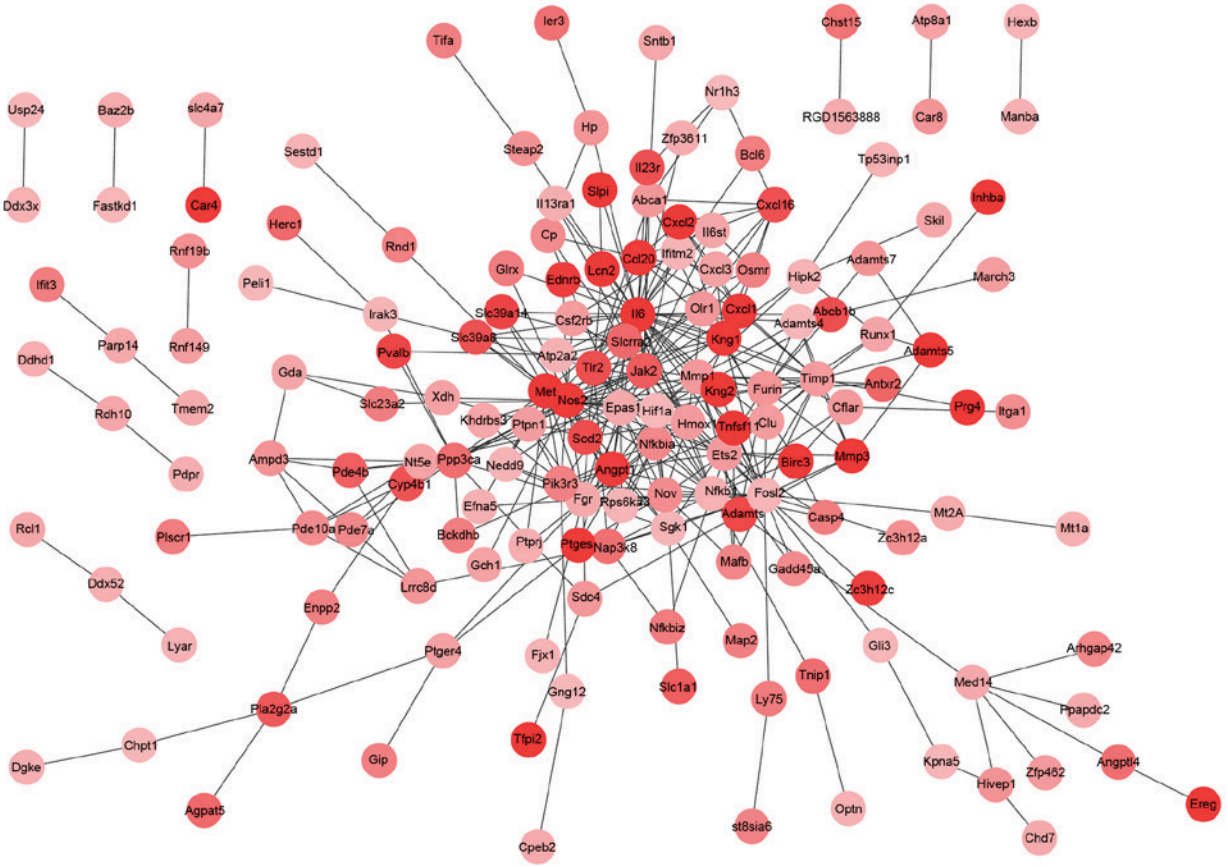

Figure 1. Protein-protein interaction network constructed for the upregulated genes.

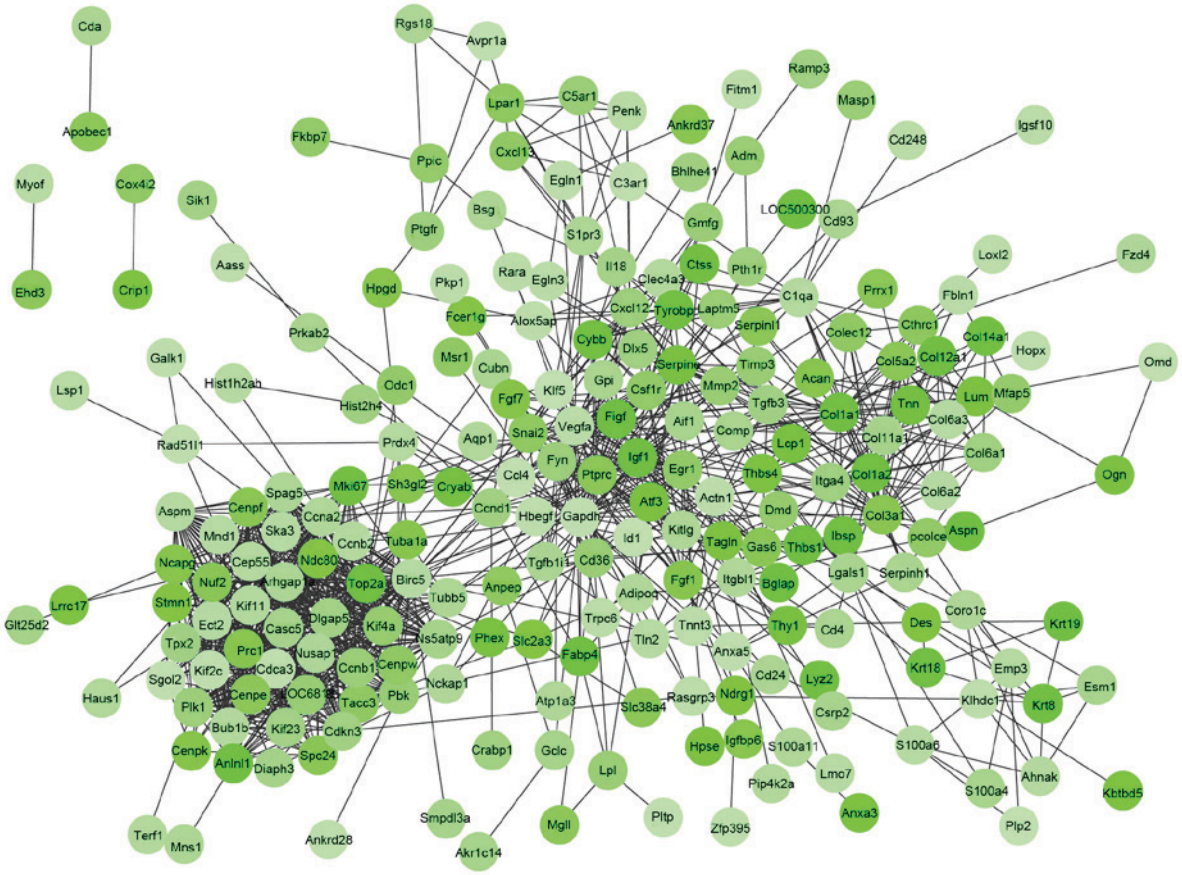

Figure 2. Protein-protein interaction network constructed for the downregulated genes.

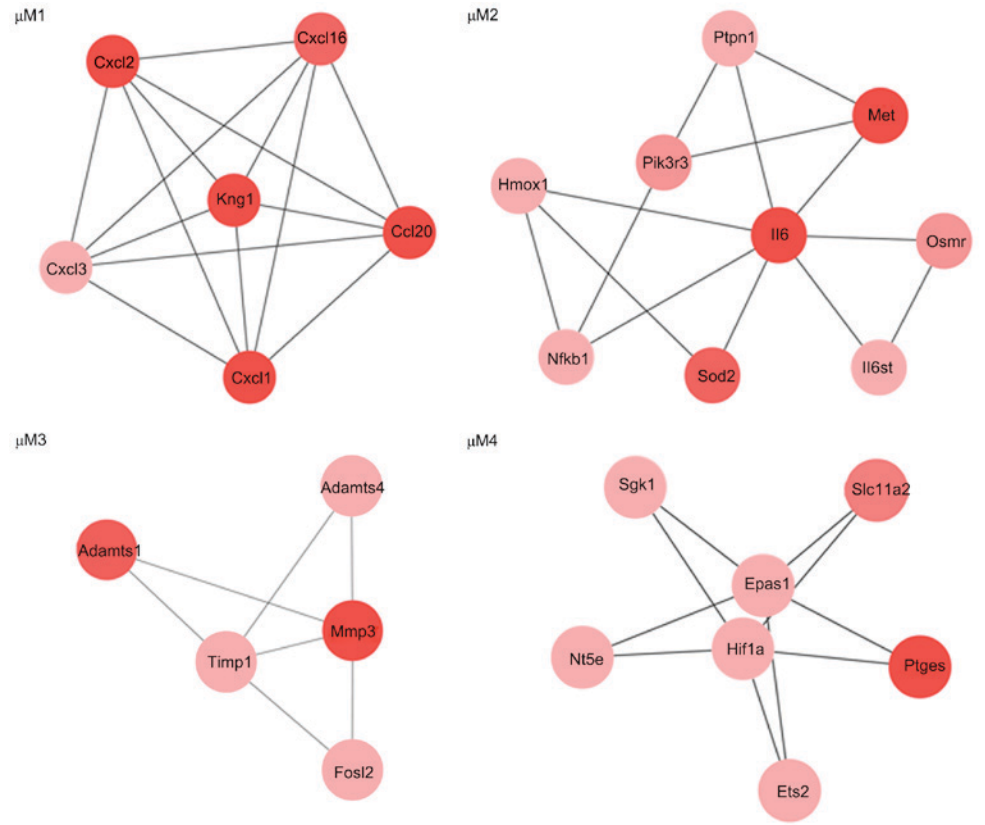

Figure 3. Four modules (μM1, μM2, μM3 and μM4) identified from the protein-protein interaction network constructed for the upregulated genes.

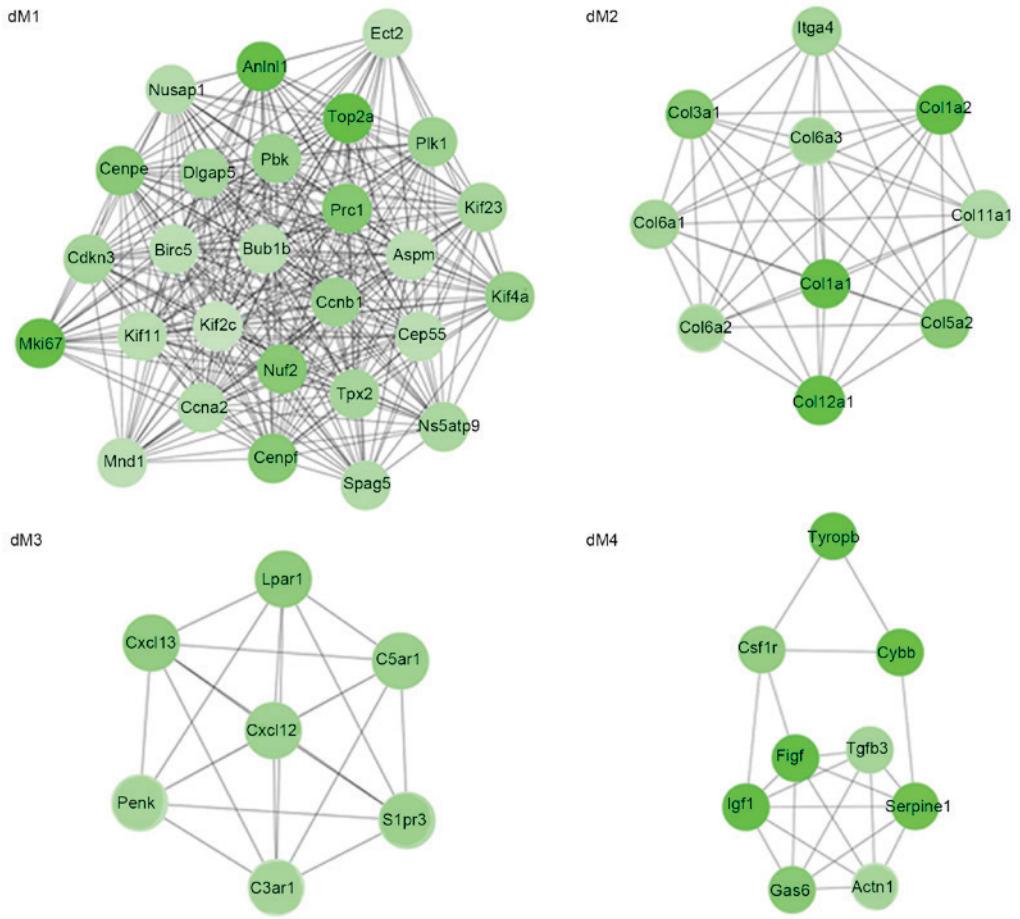

Figure 4. Four modules (dM1, dM2, dM3 and dM4) identified from the protein-protein interaction network constructed for the downregulated genes.

Table III. Top 5 functions and pathways enriched for the upregulated genes in μM1, μM2, μM3 and μM4 modules.

A, Top 5 functions enriched for the upregulated genes in μM1, μM2, μM3 and μM4 modules

Module	ID	Description	P-value	Number of genes	Gene
μM1	GO:0042330	Taxis	1.03×10^{-8}	5	*CXCL1, CCL20, CXCL16, CXCL3, CXCL2*
	GO:0006935	Chemotaxis	1.03×10^{-8}	5	*CXCL1, CCL20, CXCL16, CXCL3, CXCL2*
	GO:0006952	Defense response	3.82×10^{-8}	6	*KNG1, CXCL1, CCL20, CXCL16, CXCL3, CXCL2*
	GO:0007626	Locomotory behavior	4.20×10^{-7}	5	*CXCL1, CCL20, CXCL16, CXCL3, CXCL2*
	GO:0006954	Inflammatory response	4.97×10^{-7}	5	*KNG1, CXCL1, CCL20, CXCL3, CXCL2*
μM2	GO:0010033	Response to organic substance	1.21×10^{-4}	6	*IL6, OSMR, IL6ST, HMOX1, PTPN1, PIK3R3*
	GO:0042127	Regulation of cell proliferation	5.31×10^{-4}	5	*IL6, OSMR, IL6ST, HMOX1, SOD2*
	GO:0010035	Response to inorganic substance	5.55×10^{-4}	4	*IL6, HMOX1, NFKB1, SOD2*
	GO:0007167	Enzyme-linked receptor protein signaling pathway	6.31×10^{-4}	4	*IL6ST, MET, PTPN1, PIK3R3*
	GO:0031667	Response to nutrient levels	6.44×10^{-4}	4	*IL6, IL6ST, HMOX1, SOD2*
μM3	GO:0034097	Response to cytokine stimulus	5.04×10^{-4}	3	*FOSL2, MMP3, TIMP1*
	GO:0009719	Response to endogenous stimulus	1.26×10^{-2}	3	*FOSL2, MMP3, TIMP1*
	GO:0006508	Proteolysis	2.26×10^{-2}	3	*ADAMTS1, MMP3, ADAMTS4*
	GO:0010033	Response to organic substance	3.18×10^{-2}	3	*FOSL2, MMP3, TIMP1*
	GO:0007568	Aging	4.87×10^{-2}	2	*FOSL2, TIMP1*
μM4	GO:0048878	Chemical homeostasis	1.22×10^{-3}	4	*SLC11A2, SGK1, HIF1A, EPAS1*
	GO:0043619	Regulation of transcription from RNA polymerase II promoter in response to oxidative stress	2.48×10^{-3}	2	*HIF1A, EPAS1*
	GO:0043618	Regulation of transcription from RNA polymerase II promoter in response to stress	2.97×10^{-3}	2	*HIF1A, EPAS1*
	GO:0043620	Regulation of transcription in response to stress	2.97×10^{-3}	2	*HIF1A, EPAS1*
	GO:0042592	Homeostatic process	3.58×10^{-3}	4	*SLC11A2, SGK1, HIF1A, EPAS1*

B, Pathways enriched for the upregulated genes in μM1 and μM2 modules

Module	ID	Description	P-value	Number of genes	Gene
μM1	rno04062	Chemokine signaling pathway	1.10×10^{-4}	4	*CXCL1, CCL20, CXCL16, CXCL2*
	rno04621	NOD-like receptor signaling pathway	4.36×10^{-2}	2	*CXCL1, CXCL2*
μM2	rno04630	Jak-STAT signaling pathway	7.69×10^{-4}	4	*IL6, OSMR, IL6ST, PIK3R3*

Table III. Continued.

Module	ID	Description	P-value	Number of genes	Gene
μM2	rno04060	Cytokine-cytokine receptor interaction	2.12×10^{-3}	4	*IL6*, *OSMR*, *IL6ST*, *MET*
	rno04620	Toll-like receptor signaling pathway	6.74×10^{-3}	3	*IL6*, *NFKB1*, *PIK3R3*
	rno05200	Pathways in cancer	8.17×10^{-3}	4	*IL6*, *MET*, *NFKB1*, *PIK3R3*

search of the DEGs. The PPI networks for the upregulated (Fig. 1) and the downregulated (Fig. 2) genes separately had 360 and 1,112 interactions. Notably, IL-6 (degree=39) in the PPI network for the upregulated genes and vascular endothelial growth factor A (VEGFA; degree=37) in the PPI network for the downregulated genes had higher degrees. Using the MCODE plug-in in Cytoscape, four modules (μM1, μM2, μM3 and μM4) were identified from the PPI network for the upregulated genes (Fig. 3). Meanwhile, four modules (dM1, dM2, dM3 and dM4) were identified from the PPI network for the downregulated genes (Fig. 4). It is of note that collagen, type I, α1 (COL1A1), COL1A2, COL3A1, COL5A2, COL6A1, COL6A2, COL6A3, COL11A1, COL12A1 and integrin α4 (ITGA4) may interact with each other in the dM2 module.

The top 5 functions enriched for the upregulated genes in modules included taxis (μM1; $P=1.03\times10^{-8}$), response to organic substance (μM2; $P=1.21\times10^{-4}$), response to cytokine stimulus (μM3; $P=5.04\times10^{-4}$) and chemical homeostasis (μM4, $P=1.22\times10^{-3}$; Table IIIA). The pathways enriched for the upregulated genes in modules included the chemokine signaling pathway (μM1; $P=1.10\times10^{-4}$) and the Jak-STAT signaling pathway (μM2; $P=7.69\times10^{-4}$; Table IIIB). Additionally, the top 5 functions enriched for the downregulated genes in modules, included M phase (dM1; $P=2.70\times10^{-16}$), extracellular matrix organization (dM2; $P=1.79\times10^{-7}$, including *COL3A1*, *COL1A2*, *COL1A1*, *COL11A1* and *COL5A2*), G-protein coupled receptor protein signaling pathway (dM3; $P=7.27\times10^{-4}$) and wound healing (dM4; $P=1.56\times10^{-4}$; Table IVA). The pathways enriched for the downregulated genes in modules included cell cycle (dM1; $P=4.40\times10^{-5}$), ECM-receptor interaction (dM2; $P=1.37\times10^{-15}$, including *COL3A1*, *COL6A3*, *COL1A2*, *COL6A2*, *COL6A1*, *ITGA4*, *COL1A1*, *COL11A1* and *COL5A2*) and neuroactive ligand-receptor interaction (dM3; $P=9.49\times10^{-4}$; Table IVB).

Discussion

The present study identified a total of 558 DEGs in degenerated nucleus pulposus cells compared with normal nucleus pulposus cells, including 253 upregulated and 305 downregulated genes. Using the MCODE plug-in in Cytoscape, four modules (μM1, μ0M2, μM3 and μM4) were identified from the PPI network for the upregulated genes. Additionally, four modules (dM1, dM2, dM3 and dM4) were identified from the PPI network for the downregulated genes.

A previous study demonstrated that genetic variations of *IL-6* may be associated with IVD degeneration, accompanied by sciatica (24). *VEGFA* was overexpressed in the nucleus pulposus and affects the survival of nucleus pulposus cells in an autocrine/paracrine manner (25). Injuries of IVDs may lead to increased VEGF levels, indicating that VEGF may be associated with discogenic back pain (26). Under co-culture conditions, VEGF induction may contribute to neo-vascularization of IVD tissue and may function in the resorption of herniated discs (27). The findings of the present study indicated that IL-6 (degree=39) in the PPI network for the upregulated genes and VEGFA (degree=37) in the PPI network for the downregulated genes had higher degrees. Therefore, *IL6* and *VEGFA* may be key genes involved in IVD degeneration. A previous study observed the immunolocalization of THBS in

Table IV. Top 5 functions and pathways enriched for the downregulated genes in dM1, dM2, dM3 and dM4 modules.

A, Top 5 functions enriched for the downregulated genes

Module	ID	Description	P-value	Number of genes	Gene
dM1	GO:0000279	M phase	2.70×10^{-16}	12	*CCNB1, KIF2C, KIF11, MKI67, PLK1, DLGAP5, NUF2, NUSAP1, BUB1B, CENPF, BIRC5, CEP55*
	GO:0007049	Cell cycle	6.52×10^{-15}	14	*KIF11, MKI67, DLGAP5, NUF2, NUSAP1, CENPF, BIRC5, CEP55, CDKN3, CCNB1, KIF2C, PLK1, BUB1B, CCNA2*
	GO:0022403	Cell cycle phase	7.11×10^{-15}	12	*CCNB1, KIF2C, KIF11, MKI67, PLK1, DLGAP5, NUF2, NUSAP1, BUB1B, CENPF, BIRC5, CEP55*
	GO:0022402	Cell cycle process	1.48×10^{-14}	13	*KIF11, MKI67, DLGAP5, NUF2, NUSAP1, CENPF, BIRC5, CEP55, CDKN3, CCNB1, KIF2C, PLK1, BUB1B*
	GO:0000087	M phase of mitotic cell cycle	1.70×10^{-14}	10	*CCNB1, KIF11, PLK1, DLGAP5, NUF2, NUSAP1, BUB1B, CENPF, BIRC5, CEP55*
dM2	GO:0030199	Collagen fibril organization	5.72×10^{-10}	5	*COL3A1, COL1A2, COL1A1, COL11A1, COL5A2*
	GO:0030198	Extracellular matrix organization	1.79×10^{-7}	5	*COL3A1, COL1A2, COL1A1, COL11A1, COL5A2*
	GO:0043588	Skin development	6.89×10^{-7}	4	*COL3A1, COL1A2, COL1A1, COL5A2*
	GO:0043062	Extracellular structure organization	1.10×10^{-6}	5	*COL3A1, COL1A2, COL1A1, COL11A1, COL5A2*
	GO:0001501	Skeletal system development	1.45×10^{-5}	5	*COL3A1, COL1A2, COL1A1, COL11A1, COL5A2*
dM3	GO:0007186	G-protein coupled receptor protein signaling pathway	7.27×10^{-4}	6	*S1PR3, C3AR1, C5AR1, PENK, LPAR1, CXCL12*
	GO:0007610	Behavior	7.91×10^{-4}	4	*C3AR1, C5AR1, PENK, CXCL12*
	GO:0002430	Complement receptor mediated signaling pathway	9.92×10^{-4}	2	*C3AR1, C5AR1*
	GO:0007204	Elevation of cytosolic calcium ion concentration	1.03×10^{-3}	3	*C3AR1, C5AR1, LPAR1*
	GO:0051480	Cytosolic calcium ion homeostasis	1.29×10^{-3}	3	*C3AR1, C5AR1, LPAR1*
dM4	GO:0042060	Wound healing	1.56×10^{-4}	4	*SERPINE1, TGFB3, IGF1, GAS6*
	GO:0040007	Growth	2.91×10^{-4}	4	*SERPINE1, TGFB3, IGF1, GAS6*
	GO:0042246	Tissue regeneration	3.74×10^{-4}	3	*SERPINE1, IGF1, GAS6*
	GO:0007167	enzyme linked receptor protein signaling pathway	6.31×10^{-4}	4	*TGFB3, IGF1, FIGF, CSF1R*
	GO:0051094	Positive regulation of developmental process	8.57×10^{-4}	4	*TGFB3, IGF1, FIGF, CSF1R*
dM1	rno04110	Cell cycle	4.40x10-5	4	CCNB1, PLK1, BUB1B, CCNA2
	rno04914	Progesterone-mediated oocyte maturation	1.41x10-3	3	CCNB1, PLK1, CCNA2

Table IV. Continued.

B, Pathways enriched for the downregulated genes

Module	ID	Description	P-value	Number of genes	Gene
dM2	rno04512	ECM-receptor interaction	$1.37x10^{-15}$	9	*COL3A1*, *COL6A3*, *COL1A2*, *COL6A2*, *COL6A1*, *ITGA4*, *COL1A1*, *COL11A1*, *COL5A2*
	rno04510	Focal adhesion	$1.91x10^{-12}$	9	*COL3A1*, *COL6A3*, *COL1A2*, *COL6A2*, *COL6A1*, *ITGA4*, *COL1A1*, *COL11A1*, *COL5A2*
dM3	rno04080	Neuroactive ligand-receptor interaction	$9.49x10^{-4}$	4	*S1PR3*, *C3AR1*, *C5AR1*, *LPAR1*
dM4	rno05200	Pathways in cancer	$5.33x10^{-3}$	4	*TGFB3*, *IGF1*, *FIGF*, *CSF1R*
	rno04510	Focal adhesion	$2.26x10^{-2}$	3	*IGF1*, *ACTN1*, *FIGF*

human IVD (28). *THBS1* and *THBS2* are promising susceptibility genes in lumbar-disc herniation (LDH) that mediate the expression levels of matrix metalloproteinases (MMPs) 2 and 9, which are critical effectors of ECM remodeling (29). Mice with *THBS1* or *THBS2* deficiency exhibit abnormal spine curvature (30). Pathway enrichment performed in the present study revealed that downregulated *THBS1* was enriched in ECM-receptor interactions, suggesting that *THBS1* may have an important role in IVD degeneration.

The sequence variation of the regulatory region of *COL1A1* is closely associated with lumbar disc disease (LDD) in young military recruits who are newly diagnosed (31). Ribosomal protein L8, ribosomal protein S16 and ribosomal protein S23 have been identified to contribute to protein synthesis, and *COL3A1* was involved in skeletal system processes in disc degeneration (DD), indicating that they may be used for diagnosis and therapy of DD (32). Polymorphisms of the *COL9* and *COL11* genes contribute to the progression of degenerative lumbar spinal stenosis (33). *COL11A1* expression level was reduced in the IVD of patients with LDH and it had a negative association with the severity of disc degeneration in patients with LDH (34). In the dM2 module identified by the present study, COL1A1, COL1A2, COL3A1, COL5A2, COL6A1, COL6A2, COL6A3, COL11A1, COL12A1 and ITGA4 may interact with each other. Functional enrichment indicated that collagen genes were enriched in ECM organization. Therefore, collagen genes may contribute to the progression of IVD degeneration. Additionally, *ITGA4* may also be implicated in IVD degeneration via interaction with collagen genes.

In conclusion, the present study investigated the underlying mechanisms of IVD degeneration via bioinformatics analysis. A total 558 DEGs were screened in the degenerated nucleus pulposus cells. *IL6*, *VEGFA*, *THBS1*, *ITGA4* and collagen genes may be involved in the progression of IVD degeneration. These results suggested that the manipulation of these genes and their products may have potential as a novel therapeutic strategy for the treatment of patients with IVD. However, these findings were obtained by bioinformatics prediction and require further confirmation via further experimental studies.

Acknowledgements

The present study was supported by the Shandong Province Pharmaceutical Technology Development Program (grant no. 2015-261), the Projects of Medical and Health Technology Development Program in Shandong Province, China (grant no. 2014WS0502), the Taishan Medical University Cultivate High-level Task Projects (grant no. 2014GCC02) and the Projects of Health Science and Technology Association in Shandong Province, China (grant no. 2016BJ0009).

References

1. Sakai D, Mochida J, Yamamoto Y, Nomura T, Okuma M, Nishimura K, Nakai T, Ando K and Hotta T: Transplantation of mesenchymal stem cells embedded in Atelocollagen gel to the intervertebral disc: A potential therapeutic model for disc degeneratio. Biomaterials 24: 3531-3541, 2003.
2. Le Maitre CL, Pockert A, Buttle DJ, Freemont AJ and Hoyland JA: Matrix synthesis and degradation in human intervertebral disc degeneration. Biochem Soc Trans 35: 652-655, 2007.

3. Richardson SM, Walker RV, Parker S, Rhodes NP, Hunt JA, Freemont AJ and Hoyland JA: Intervertebral disc cell-mediated mesenchymal stem cell differentiation. Stem Cells 24: 707-716, 2006.
4. Battié MC, Videman T and Parent E: Lumbar disc degeneration: Epidemiology and genetic influences. Spine 29: 2679-2690, 2004.
5. Sakai D, Mochida J, Iwashina T, Hiyama A, Omi H, Imai M, Nakai T, Ando K and Hotta T: Regenerative effects of transplanting mesenchymal stem cells embedded in atelocollagen to the degenerated intervertebral disc. Biomaterials 27: 335-345, 2006.
6. Pockert AJ, Richardson SM, Le Maitre CL, Lyon M, Deakin JA, Buttle DJ, Freemont AJ and Hoyland JA: Modified expression of the ADAMTS enzymes and tissue inhibitor of metalloproteinases 3 during human intervertebral disc degeneration. Arthritis Rheum 60: 482-491, 2009.
7. Gruber HE, Norton HJ, Ingram JA and Hanley EN Jr: The SOX9 transcription factor in the human disc: Decreased immunolocalization with age and disc degeneration. Spine (Phila Pa 1976) 30: 625-630, 2005.
8. Ponnappan RK, Markova DZ, Antonio PJ, Murray HB, Vaccaro AR, Shapiro IM, Anderson DG, Albert TJ and Risbud MV: An organ culture system to model early degenerative changes of the intervertebral disc. Arthritis Res Ther 13: R171, 2011.
9. Weiler C, Nerlich AG, Bachmeier BE and Boos N: Expression and distribution of tumor necrosis factor alpha in human lumbar intervertebral discs: A study in surgical specimen and autopsy controls. Spine (Phila Pa 1976) 30: 44-54, 2005.
10. Bachmeier BE, Nerlich AG, Weiler C, Paesold G, Jochum M and Boos N: Analysis of tissue distribution of TNF-alpha, TNF-alpha-receptors and the activating TNF-alpha-converting enzyme suggests activation of the TNF-alpha system in the aging intervertebral disc. Ann N Y Acad Sci 1096: 44-54, 2007.
11. Le Maitre CL, Freemont AJ and Hoyland JA: The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. Arthritis Res Ther 7: R732-R745, 2005.
12. Hoyland J, Le Maitre C and Freemont A: Investigation of the role of IL-1 and TNF in matrix degradation in the intervertebral disc. Rheumatology (Oxford) 47: 809-814, 2008.
13. Le Maitre CL, Hoyland JA and Freemont AJ: Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1beta and TNFalpha expression profile. Arthritis Res Ther 9: R77, 2007.
14. Markova DZ, Kepler CK, Addya S, Murray HB, Vaccaro AR, Shapiro IM, Anderson DG, Albert TJ and Risbud MV: An organ culture system to model early degenerative changes of the intervertebral disc II: Profiling global gene expression changes. Arthritis Res Ther 15: R121, 2013.
15. Gautier L, Cope L, Bolstad BM and Irizarry RA: affy-analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20: 307-315, 2004.
16. Smyth GK: Limma: Linear Models for Microarray Data. In: Bioinformatics and Computational Biology Solutions Using R and Bioconductor Springer, pp397-420, 2005.
17. Benjamini Y and Hochberg Y: Controlling the false discovery rate: A practical and powerful approach to multiple testing. J Royal Statistical Soci 57: 289-300, 1995.
18. Huang DW, Sherman BT, Tan Q, Kir J, Liu D, Bryant D, Guo Y, Stephens R, Baseler MW, Lane HC and Lempicki RA: DAVID bioinformatics resources: Expanded annotation database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Res 35: W169-W175, 2007.
19. Carbon S, Ireland A, Mungall CJ, Shu S, Marshall B and Lewis S; AmiGO Hub; Web Presence Working Group: AmiGO: Online access to ontology and annotation data. Bioinformatics 25: 288-289, 2009.
20. Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T and Yamanishi Y: KEGG for linking genomes to life and the environment. Nucleic Acids Res 36 (Database issue): D480-D484, 2008.
21. Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P, Doerks T, Stark M, Muller J, Bork P, *et al*: The STRING database in 2011: Functional interaction networks of proteins, globally integrated and scored. Nucleic Acids Res 39 (Database issue): D561-D568, 2011.
22. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B and Ideker T: Cytoscape: A software environment for integrated models of biomolecular interaction networks. Genome Res 13: 2498-2504, 2003.
23. Bader GD and Hogue CW: An automated method for finding molecular complexes in large protein interaction networks. BMC bioinformatics 4: 2, 2003.
24. Noponen-Hietala N, Virtanen I, Karttunen R, Schwenke S, Jakkula E, Li H, Merikivi R, Barral S, Ott J, Karppinen J and Ala-Kokko L: Genetic variations in IL6 associate with intervertebral disc disease characterized by sciatica. Pain 114: 186-194, 2005.
25. Fujita N, Imai J, Suzuki T, Yamada M, Ninomiya K, Miyamoto K, Iwasaki R, Morioka H, Matsumoto M, Chiba K, *et al*: Vascular endothelial growth factor-A is a survival factor for nucleus pulposus cells in the intervertebral disc. Biochem Biophys Res Commun 372: 367-372, 2008.
26. Sato J, Sakuma Y, Yamauchi K, Orita S, Kubota G, Oikawa Y, Inage K, Sainoh T, Fujimoto K, Takahashi K, *et al*: Elevated VEGF in degenerative intervertebral discs in rats with injured intervertebral discs of the caudal vertebrae. Global Spine J 4: po. 165, 2014.
27. Haro H, Kato T, Komori H, Osada M and Shinomiya K: Vascular endothelial growth factor (VEGF)-induced angiogenesis in herniated disc resorption. J Orthop Res 20: 409-415, 2002.
28. Gruber HE, Ingram JA and Hanley EN Jr: Immunolocalization of thrombospondin in the human and sand rat intervertebral disc. Spine (Phila Pa 1976) 31: 2556-2561, 2006.
29. Hirose Y, Chiba K, Karasugi T, Nakajima M, Kawaguchi Y, Mikami Y, Furuichi T, Mio F, Miyake A, Miyamoto T, *et al*: A functional polymorphism in THBS2 that affects alternative splicing and MMP binding is associated with lumbar-disc herniation. Am J Med Genet 82: 1122-1129, 2008.
30. Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H and Hynes RO: Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101: 982-992, 1998.
31. Tilkeridis C, Bei T, Garantziotis S and Stratakis CA: Association of a COL1A1 polymorphism with lumbar disc disease in young military recruits. J Med Genet 42: e44, 2005.
32. Yang Z, Chen X, Zhang Q, Cai B, Chen K, Chen Z, Bai Y, Shi Z and Li M: Dysregulated COL3A1 and RPL8, RPS16, and RPS23 in disc degeneration revealed by bioinformatics methods. Spine (Phila Pa 1976) 40: E745-E751, 2015.
33. Noponen-Hietala N, Kyllönen E, Männikkö M, Ilkko E, Karppinen J, Ott J and Ala-Kokko L: Sequence variations in the collagen IX and XI genes are associated with degenerative lumbar spinal stenosis. Ann Rheum Dis 62: 1208-1214, 2003.
34. Mio F, Chiba K, Hirose Y, Kawaguchi Y, Mikami Y, Oya T, Mori M, Kamata M, Matsumoto M, Ozaki K, *et al*: A functional polymorphism in COL11A1, which encodes the alpha 1 chain of type XI collagen, is associated with susceptibility to lumbar disc herniation. Am J Med Genet 81: 1271-1277, 2007.