**Molecular characterization of intervertebral disc tissue**

**by next generation RNA sequencing**

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**Running Title:** RNA-seq characterization of intervertebral disk

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**Abstract**

INTRODUCTION:

Degenerative disc disease of the spine is a leading cause of back pain and disability in patients. Current medical therapies are focused on treating pain symptoms, but are not able to regenerate damaged spine tissues. Regenerative medicine therapies are being investigated as treatments for degenerative disc disease. The goal of this investigation is to provide a comprehensive overview of gene expression data in annulus fibrosis and nucleus pulposis tissues that can be used to guide ongoing initiatives in tissue engineering, therapeutic drug discovery, and cell based therapies.

METHODS:

In this investigation we performed high throughput next generation RNA sequencing on 60 spinal disc specimens (39 annulus fibrosis and 21 nucleus pulposis samples). Specimens were collected from patients undergoing surgical discectomy for the treatment of degenerative disc disease. Annulus fibrosis and nucleus pulposis tissues were dissected from one another at the time of surgery and snap frozen in liquid nitrogen prior to RNA extraction and sequencing. Computational methods for weighted gene correlation analysis were used to define gene associations and candidate regulatory networks in spine tissues.

RESULTS:

We observed statistically significant enrichment of 1399 genes in annulus fibrosis tissue and 373 genes with a statistically significant enrichment in nucleus pulposis tissue (Figure 1). Next generation RNA sequencing studies confirm the expression of known annulus fibrosis and nucleus pulposis specific genes (Figure 2). Studies also identify novel extracellular matrix proteins, and associated transcription factors, and growth factors with potential regulatory functions in spinal disc tissue. Notable genes associated with NOTCH signaling that are enriched in annulus fibrosis tissue include NOTCH 3, NOTCH4, JAG1, JAG2, HEY1, HEYL, CNTN1, DDL1, and MAML3. The nucleus pulposis samples show enrichment in mRNAs associated with proteoglycan extracellular matrix synthesis, including genes associated with the endoplasmic reticulum and golgi apparatus. The nucleus pulposis samples show enrichment in mRNAs associated with proteoglycan extracellular matrix synthesis, including genes associated with the endoplasmic reticulum and golgi apparatus.

DISCUSSION:

These findings are also consistent with the functional role of the nucleus pulposis as a hydrostatic cushion to reduce pressure and impact between the intervertebral bodies of the spine. Our results also implicate the NOTCH signaling as a potentially important regulatory pathway in annulus fibrosis tissue, which is known to impact cellular adhesion and tissue integrity. Utilizing our large dataset of clinical specimens we have been able to identify candidate gene regulatory networks using computational analysis, that act in annulus fibrosis and nucleus pulposis tissues to regulate extracellular matrix synthesis, an important determinant of intervertebral disc integrity.

SIGNIFICANCE:

These studies provide valuable information that can be used to optimize and validate therapeutics and tissue engineering strategies currently under development. They can also be applied to develop novel therapeutic approaches for the treatment of degenerative disc disease.

**Key words:** RNA sequencing, nucleus pulposus, annulus fibrosus, intervertebral disk, extracellular matrix

**Introduction:**

Back pain is among the leading global causes of disability1, 2, with degenerative disk disease and osteoarthritis being important causes of disease. Disk degeneration is caused by a dysregulation of extracellular matrix homeostasis, characterized by dehydration of the central nucleus, reduced proteoglycan content, decreased cellularity, diminished endplate density, and disruption of the annulus3-5. Environmental exposures, as well as genetic and epigenetic factors have been associated with disk degeneration and altered extracellular matrix synthesis in disk tissues6,7. Novel molecular approaches that can target molecular factors regulating extracellular matrix synthesis in disk tissue have the potential to be used as therapeutic agents to slow or reverse disk degeneration in patients.

The molecular phenotype of intervertebral spinal disk tissue, including the annulus fibrosus (AF) and nucleus pulposus (NP), has been studied extensively in non-human animal models for degenerative disk disease8-14. Studies evaluating transcriptome data using microarrays have provided us with an initial understanding of the molecular mechanisms underlying disk biology and have played a major role in helping to identify important biologic markers specific for AF and NP disk tissues15-19. However knowledge regarding the regulatory role of molecular factors and how they contribute to tissue homeostasis still requires further study.

In this investigation we seek to identify molecular regulatory factors whose transcriptional profiles correlate with the expression of extracellular matrix proteins important for the structural phenotype of human AF and NP tissues. To achieve this objective we evaluated transcriptome profiles of a cohort of human cervical disk tissue samples utilizing high throughput next generation RNA sequencing. We obtained complete gene expression profiles for 60 surgically harvested cervical disk specimens (AF and NP), and evaluated the main molecular landscapes of these two principal disc tissues. The large cohort of samples analyzed in this study allowed us to successfully perform weighted gene correlation analysis to identify gene regulatory clusters in disk tissues and assess gene relationships.

The molecular regulators that show relationships with extracellular matrix gene expression represent promising candidates for future study and therapeutic validation. The findings in this investigation also serve to support

regenerative medicine therapies currently under development for the treatment of intervertebral disk disease, including stem cell therapies and tissue engineering strategies to regrow disk tissue for surgical transplantation and disk replacement procedures20,21. Both of these strategies require a comprehensive definition of the molecular phenotype of the human intervertebral disk to evaluate the efficacy of strategies to differentiate stem cells or engineer tissue disk tissue in vivo. The transcriptional signatures and gene relationships identified in this study have broad applicability in both the stem cell and tissue engineering fields.

**Methods:**

*Surgical tissue collection*

A total of 60 tissue specimens were collected for research use from 48 adult patients undergoing cervical discectomy. Patients ranged in age from 32 to 77 years of age and included a balanced distribution of male and female patients (**Supplemental Table 1**). Patients in this study underwent surgery for the treatment of symptomtic degenerative disk disease presenting with or without myelopathy. Subjects were enrolled in the study in the period between January 2011 and April 2015. Cases in which discectomy was performed in the setting of acute trauma or infection were excluded from this study. At the time of tissue collection, the AF and NP were carefully dissected from one another in the operating room by the staff surgeon. In cases where disc degeneration was severe, NP tissue could not always be readily identified and distinguished from the AF tissue and therefore could not be collected for some patients. At the time of surgical harvest, tissues were snap frozen in liquid nitrogen and stored at -80°C until ready for RNA extraction. All samples were frozen within 40 minutes of removal from the patient. Grade of disk degeneration was evaluated on preoperative lateral radiographs and was characterized using the classification described by Lane et al.22,23. Clinical data available for each disk sample is provided in **Supplemental Table 1**. The specimens used in this investigation were collected under institutional review board approved protocols (IRB#10-005713). Written informed consent was obtained for all biospecimens that were analyzed.

*RNA extraction from intervertebral disk tissue*

Frozen tissue biopsies were ground into a powder using a mortar and pestle and homogenized in Qiazol reagent (Qiagen, Hilden, Germany) and homogenized. Total RNA was extracted from research biopsies based on previous methods24, 25 using the miRNeasy minikit (Qiagen, Hilden, Germany) and quantified using the NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Wilmington, Delaware). For samples selected for next generation sequencing, RNA integrity was assessed using the Agilent Bioanalyzer DNA 1000 chip (Invitrogen, Carlsbad, CA).

*Next generation mRNA sequencing, statistics and bioinformatics*

RNA sequencing and bioinformatics analyses were performed as previously described26-29. In brief, library preparation was performed using the TruSeq RNA library preparation kit (Illumina, San Diego, CA). Polyadenylated mRNAs were selected using oligo dT magnetic beads. TruSeq Kits (12-Set A and 12-Set B) were used for indexing to permit multiplex sample loading on the flow cells. Paired-end sequencing reads were generated on the Illumina HiSeq 2000 sequencer. Quality control for concentration and library size distribution was performed using an Agilent Bioanalyzer DNA 1000 chip and Qubit fluorometry (Invitrogen, Carlsbad, CA). Sequence alignment of reads and determination of normalized gene counts were performed using the MAP-RSeq (v.1.2.1) workflow30, utilizing TopHat 2.0.631, 32, and HTSeq33.

RNA sequencing data were analyzed to assess relevant genes that differ between AF and NP specimens. Genes with a minimal expression value (RPKM > 0.01) were included in subsequent computational analysis. Fold-change differences in gene expression were evaluated using the Mann-Whitney U test with a 1% false discovery rate (FDR), and statistical significance was set at p < 0.05. Unsupervised hierarchical clustering was performed using the Pearson correlation method. Weighted gene correlation analysis was performed using the R package WGCNA (Weighted Gene Correlation Analysis)34. Genes with an average RPKM expression > 0.01 across all specimens were included in the computational analysis. Functional gene annotation classification of WGCNA clusters was performed using DAVID Bioinformatics Resources 6.7 database (DAVID 6.7)35.

**Results:**

RNA sequencing was performed using 60 unique cervical spine disk tissue samples (39 AF and 21 NP specimens). High quality sequencing reads were obtained for 57 of the 60 samples. The 3 samples with abnormally low read counts were excluded from further analysis. To detect sample outliers, an unbiased assessment of transcriptome data using unsupervised hierarchical clustering was performed. This analysis revealed 10 disk samples that clustered independently from the majority of the disk specimens (**Supplemental Figure 1**). A comparison of these samples with specimens in the primary cluster show that outlier samples express higher levels of blood related genes including genes linked to the erythroid, lymphoid, and myeloid lineages. A selective evaluation of the blood specific hemoglobin genes, hemoglobin subunit beta (HBB), hemoglobin subunit alpha 1 (HBA1), and hemoglobin subunit alpha 2 (HBA2), confirmed that these 10 samples express the highest levels of blood related genes (**Supplemental Table 2**). To ensure the comparability of samples in our study these outliers were removed from subsequent analysis. Repeated unsupervised hierarchical clustering after removal of outliers showed an expected trend toward independent clustering of AF and nucleus puplosus tissue samples (**Figure 1a**). Genes that are not strictly linked to a tissue phenotype such as hematopoietic and inflammation related genes, as well as tissue heterogeneity, played a role in defining the clustering dendrogram. This observation explains why the clustering dendrogram showed a trending, but not completely independent clustering of the AF and NP specimens, despite the distinct biological phenotypes of AF and NP tissues.

An examination of the most highly expressed genes (expression > 100 RPKM) commonly expressed in AF and NP expectedly showed common enrichment of genes associated with housekeeping functions (i.e. translation, protein ubiquitination) (**Figure 1b**). The AF and NP samples share many ECM related genes in common among their highest expressed genes, however the abundance of each gene and their ratios are quite different between AF and NP samples. Of the genes that are commonly enriched in AF and NP that are not associated with house-keeping functions, the AF samples showed higher expression of mRNAs encoding ECM proteins associated with a fibrous matrix including type I collagen (COL1A2), and type VI collagen (COL6A1, COL6A2, COL6A3) (**Table 1**). In contrast, the NP samples showed increased mRNA levels of genes encoding extracellular matrix proteins associated with a proteoglycan rich chondrogenic matrix, including cartilage oligomeric protein (COMP), lumican (LUM), type II collagen (COL2A1), cartilage intermediate layer protein (CILP), biglycan (BGN), aggrecan (ACAN), type III collagen (COL3A1), chondroadherin (CHAD), and others (**Table 2**).

To determine genes that are differentially expressed between AF and NP tissues irrespective of their overall abundance, a fold-change comparison of gene expression data in AF and NP was performed. We observed statistically significant enrichment of 1399 genes in AF tissue and 373 genes with enrichment in NP tissue. Analysis revealed differential gene expression consistent with the biological properties and function of each tissue type. The AF showed enrichment in genes linked to adhesion and regulation of cell contact, consistent with its fibrous structural properties (**Figure 1c**). In contrast, the NP samples showed enrichment in mRNAs associated with proteoglycan extracellular matrix synthesis, including genes associated with the endoplasmic reticulum and Golgi apparatus (**Figure 1d**). These findings are consistent with the functional role of the NP, which acts as a hydrostatic cushion to reduce contact pressure between the bony vertebral bodies of the spine. We also observed preferential expression of the notochord specific transcription factor brachyury (T) in NP tissues at low, but detectable levels in about half of the samples. This indicates that residual notochord cell populations, detectable when highly sensitive molecular techniques are applied, may be present in degenerative adult disc tissue.

The AF and NP specimens both showed statistically significant enrichment in known, as well as novel, extracellular matrix proteins and signaling molecules. The AF specimens showed expression of phenotypically important genes such as type IV collagen (COL4A1), multiple laminins important for cell adhesion (LAMA3, LAMA4, LAMA5), and genes linked to NOTCH signaling (DLL1, JAG1, JAG2, NOTCH3, NOTCH4). In NP specimens we observed expression of genes promoting a proteoglycan rich ECM including aggrecan (ACAN), type XI collagen (COL11A1), glypican 6 (GPC6), lumican (LUM), among others in NP specimens (**Table 3**).

Given the heterogeneous nature of spinal tissues, statistical methods used to assess simple fold-change analyses may not always be able to identify all important biological gene relationships. To overcome this challenge and identify novel gene regulatory networks with a functional role in regulating extracellular matrix production, we performed weighted gene correlation network analysis for spine tissues using the R package WGCNA34. Gene correlation analysis identified 46 regulatory gene clusters present in our intervertebral disk samples (**Figure 2**). We observed gene regulatory clusters associated with housekeeping functions (i.e. translation, transcription, mitochondrion, nuclear homeostasis), cellular infiltration including blood and inflammatory cells. We also observed gene regulatory clusters associated with non-disk tissue including processes related to muscle, bone, and adipogenesis, which likely represent small quantities of tissue mixed in with disk tissue at the time of surgical harvesting.

To identify novel extracellular matrix proteins and regulatory molecules that control tissue specific phenotypes, we examined clusters containing genes associated extracellular matrix synthesis. The related clusters “paleturquoise”, “darkorange2”, and “darkslateblue” each show enrichment in extracellular matrix proteins and adhesive proteins associated with a fibrous matrix, which is typically characteristic of AF tissue. These clusters contain genes that promote a strong fibrous matrix, including collagens, fibulins, integrins, lamamins, elastin, and others (**Table 4**). These gene clusters were notably associated with a three diffusible growth factors, fibroblast growth factor 9 (FGF9), platelet-derived growth factor beta polypeptide (PDGFB), and vascular endothelial growth factor C (VEGFC). These findings suggest that these growth factors may play a regulatory roles in maintenance of the AF phenotype and warrant further investigation. Additionally, these clusters also exhibited strong enrichment in genes linked to cell-cell signaling interactions, including the Wnt signaling and NOTCH signaling pathways. Both of these pathways are known to be involved in mediating cell-cell interactions and cellular adhesion in various tissues outside of intervertebral disk36, 37. Given the paucity of diffusible growth factors and the fact that AF cells are in close contact with one another, these data suggest that AF ECM production may be regulated or strongly influenced by direct cell-cell signaling mechanisms, possibly mediated through the Wnt and NOTCH signaling pathways.

The clusters “black”, “grey60”, and “lightyellow” show enrichment in genes associated with a proteoglycan rich extracellular matrix. Genes included in these clusters include the known NP markers type II collagen (COL2A1), type IX collagens (COL9A2, COL9A3), type XI collagen (COL11A2), aggrecan (ACAN), as well as other genes associated with a proteoglycan rich ECM that have not previously been associated with NP phenotype (**Table 5**). In contrast to the gene clusters previously discussed that were associated with synthesis of a fibrous matrix, these gene clusters express a diverse array of diffusible growth factors, with many being associated with the TGFβ signaling cascade. Associated growth factors include transforming growth factor alpha (TGFA), inhibin beta A (INHBA), inhibin alpha (INHA), growth differentiation factors (GDF5, GDF6), and bone morphogenetic proteins (BMP2, BMP6) and others (**Table 5**). The reliance on diffusible growth factors to mediate ECM homeostasis in a proteoglycan rich matrix such as that observed in the NP is logical since cells are usually separated by a thick matrix and have limited direct cell to cell contact.

**Discussion:**

The molecular phenotype of intervertebral spinal disk tissue, including the AF and NP, has been studied extensively over the past several years, primarily in animal models. The disk periphery is comprised of the fibrous annulus, derived from the scleroderm, while NP is derived from the notochord. However, notochordal cells in humans decrease in abundance with age, and are largely absent after adolescence38, 39, although visible notochord tissue is present at maturity in other species. NP cells make predominantly type II collagen, whereas AF cells make both type I and type II collagen40. The findings in our investigation utilizing high throughput RNA sequencing approaches are consistent with these findings in previous investigations, and also identify associations with other novel extracellular matrix proteins and associated regulatory factors.

Our initial clustering analysis performed using AF and NP specimens (**Figure 1**) demonstrates that blood content is an important consideration in the evaluation of surgically collected spinal disk tissues. Disk tissues have a very low density of cells, and the few cells that are present are usually encased in a thick extracellular matrix that makes RNA extraction technically challenging. Even the presence of small quantities of blood, from which RNA is much more easily extracted, can profoundly impact RNA content and resulting transcriptome data analyses if not carefully considered.

Our analysis reveals increased expression of known AF and NP markers within corresponding tissue types including enrichment of type I collagen in AF and a proteoglycan associated extracellular matrix enriched in genes such as ACAN, COMP, LUM, and others in the NP. We note that there is some overlap in mRNA expression between annulus and nucleus specimens. This overlap may reflect similarities in the developmental origin of these tissues or could be due to technical issues, for example, because there is some intermixing of annulus and nucleus cells during tissue harvest (e.g., in degenerative disk tissues with altered structural morphology).

These studies also implicate the WNT and NOTCH signaling pathway as a potentially important regulators of cell adhesion and matrix synthesis in AF tissue. These pathways are mediated by direct cell to cell interactions and have been shown to impact cellular adhesion and tissue integrity in various tissue types41. Golgi and ER related genes enriched in NP tissue may contribute to the production of the proteogylcan rich matrix associated with the NP environment. Therapeutic strategies that can increase protein output and upregulate the expression of NP specific genes have the potential to help disk tissue retain fluid and appropriate hydrostatic pressure, thus preventing disk space degeneration and associated disk space narrowing and osteoarthritis.

Recent studies have identified several novel AF and NP markers, our study shows support for many of these markers42. In our analyses, the proposed NP markers desmocollin 2 (DSC2)18, lubricin (PRG4)43, and paired box 1 (PAX1)20, showed co-regulation with networks enriched in NP related genes supporting their classification as NP markers. The novel AF markers brain abundant membrane attached signal protein 1 (BASP1), sclerostin domain containing 1 (SOSTDC1)18, glypican 3 (GPC3), and pleiotrophin (PTN)44 also showed co-regulation with AF related ECM gene networks. Our study did not show a clear link to either AF or NP phenotypes for several published markers including CD24 antigen (CD24), keratin 8 (KRT8), keratin 18 (KRT18), keratin 19 (KRT19), cadherin 2 (CDH2)17, carbonic anhydrase 12 (CA12)45, and hypoxia inducible factor 1 alpha subunit (HIF1A)13, 14, 46, all of which showed co-regulation with gene networks unrelated to disk phenotype. Protein levels do not always correlate with mRNA expression, which could explain some of the differences between our study and previous investigations. Discrepancies could also be related to interspecies differences, as many of these published studies were carried out using non-human tissues. In addition, our study focused on evaluation of degenerative disc tissue, and it is possible that many of these markers may be present during early disk development and are gradually lost over time with aging and degeneration.

It is important to note that the gene relationships defined by network analyses in this study may exclude important functional/regulatory genes when a gene has a stronger relationship to another network. This was observed for the known AF related gene type I collagen (COL1A1, COL1A2), which showed stronger co-regulation with bone related genes (gene cluster “royalblue”) rather than AF related genes. Despite this limitation, we were still able to identify large gene regulatory networks associated with ECM production in AF and NP tissues. Our analysis also does not take in account the numerous regulatory mechanisms that act in coordination with transcriptional mechanisms including protein phosphorylation and acetylation, histone modifications, microRNAs, and others. Future studies that integrate intervertebral disk transcriptomic profiles with various types of molecular data including microRNA profiles, and mass spectroscopy data may further help to elucidate novel molecular pathways involved intervertebral disk homeostasis.

This investigation provides a comprehensive overview of mRNA expression in annulus fibrosus and nucleus pulposus intervertebral disk tissue, including extracellular matrix components. By applying computational analyses to our large dataset of human clinical specimens, we have been able to identify candidate gene regulatory networks that act in AF and NP tissues to regulate extracellular matrix synthesis, an important determinant of intervertebral disk integrity. The transcriptome data generated in this study also serves as an important reference data set and has the potential to help solve many biological questions related to disk tissues. For example, our data can be used to evaluate the efficacy of tissue engineering strategies for intervertebral disk development. The data can also be applied to optimize stem cell differentiation strategies for therapeutic disk regeneration, as a variety of stem cell therapies are just beginning to be investigated in new clinical trials. Information generated in this study can also potentially be applied to identify novel therapeutic targets to enhance extracellular matrix synthesis and restore the normal mechanical properties of intervertebral disk tissue.

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**Tables:**

**Table 1**: Extracellular matrix related genes highly expressed in annulus fibrosus

**Table 2**: Extracellular matrix related genes highly expressed in nucleus pulposus

**Table 3:** Significant extracellular matrix related genes enriched in annulus fibrosus and nucleus pulposus

**Table 4:** Annulus fibrosus co-regulatory gene networks

**Table 5:** Nucleus pulposus co-regulatory gene networks

**Figures Legends:**

**Figure 1**: (a) Unsupervised hierarchical clustering of RNA sequencing data after removal of sample outliers. In this clustering scheme there is a trend for AF and NP samples to preferentially cluster separately. These findings suggest that there are tissue specific differences contained within the transcriptome data, representing the known biological differences that exist between these two tissue types. Our unbiased approach also incorporates various factors that are not directly related to the disc phenotype such as tissue heterogeneity, blood content, and inflammation, which can drive some of the biological variation between specimens, thus precluding a perfect clustering dendogram in which AF and NP specimens cluster as completely independent groups. (b) Genes expressed > 100 RPKM in surgically isolated AF and NP tissue with equal expression levels (Fold change <1.5 between AF and NP). This analysis shows that AF and NP both share common expression of a large number of housekeeping genes as well as a small number of extracellular matrix proteins and growth factor binding associated proteins. (c) Gene ontology analysis reveals enrichment in pathways that promote cellular adhesion including genes linked to notch signaling (vasculature development, GTPase regulator activity) in AF tissue. (d) The NP shows enrichment in genes linked to extracellular matrix protein synthesis, including in genes controlling the extracellular matrix protein synthesis machinery (golgi complex and endoplasmic reticulum).

**Figure 2:** Gene correlation networks predicted using weighted genes correlation analysis (WGCNA). Gene networks are associated with a variety of cellular activities including cellular housekeeping, mitosis, tissue heterogeneity, extracellular matrix synthesis as well as numerous others. Gene clusters “paleturquoise”, “darkorange2”, and “darkslateblue” are enriched in known extracellular matrix protein markers in AF, while the clusters “black”, “grey60”, and “lightyellow” are associated with extracellular matrix protein markers characteristic of NP.

**Supplemental Tables:**

**Supplemental Table 1:** Clinical data associated with each disk specimen

**Supplemental Table 2:** Hemoglobin expression in sample outliers

**Supplementary Figures:**

**Supplemental Figure 1**: Unsupervised hierarchical clustering of spine samples was performed to detect sample outliers. In total 10 samples were found to cluster independent from the majority of samples. These 10 samples expressed high levels of blood related mRNAs compared with the remaining samples.

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| **Table 1: Extracellular matrix related genes highly expressed in annulus fibrosus** | | | | |
| GeneID | Average expression (RPKM) in  annulus fibrosus |  | GeneID | Average expression (RPKM) in  annulus fibrosus |
| COMP | 3990.88 |  | SERPINA1 | 247.96 |
| FN1 | 3374.09 |  | TIMP2 | 246.71 |
| CLU | 2562.49 |  | CALR | 242.68 |
| TPT1 | 2502.16 |  | CRTAC1 | 242.53 |
| DCN | 2434.52 |  | DPT | 204.77 |
| MGP | 2270.18 |  | SERPINF1 | 198.73 |
| LUM | 2039.40 |  | SERPING1 | 186.09 |
| COL1A1 | 1393.37 |  | CILP2 | 185.94 |
| SPARC | 1386.58 |  | APOE | 179.84 |
| FMOD | 1325.29 |  | MMP14 | 175.88 |
| COL1A2 | 1255.57 |  | TGFBI | 174.91 |
| COL2A1 | 1161.98 |  | POSTN | 173.01 |
| CILP | 1126.00 |  | IBSP | 168.53 |
| COL3A1 | 1042.38 |  | IGFBP4 | 165.21 |
| CST3 | 972.51 |  | COL9A3 | 154.93 |
| HTRA1 | 871.24 |  | SOD3 | 152.31 |
| BGN | 865.06 |  | IGFBP6 | 145.98 |
| FGFBP2 | 860.36 |  | SPARCL1 | 142.91 |
| LGALS1 | 711.77 |  | SOD1 | 137.06 |
| SPP1 | 681.08 |  | BGLAP | 135.65 |
| PRELP | 640.12 |  | PLA2G2A | 134.42 |
| SCRG1 | 629.02 |  | APOD | 131.80 |
| CHAD | 614.98 |  | CHI3L2 | 130.44 |
| COL6A2 | 567.11 |  | ANGPTL2 | 127.09 |
| ACAN | 564.31 |  | TIMP3 | 126.93 |
| CTSK | 534.82 |  | FSTL1 | 126.74 |
| TIMP1 | 469.09 |  | SERPINE2 | 125.99 |
| GPX3 | 450.88 |  | ALDOA | 125.16 |
| CTGF | 433.32 |  | PRDX4 | 123.56 |
| MMP9 | 416.09 |  | CCDC80 | 121.68 |
| IGFBP7 | 352.81 |  | COL11A2 | 117.92 |
| PSAP | 349.13 |  | COL5A2 | 112.32 |
| COL6A1 | 313.96 |  | NUCB1 | 111.91 |
| ASPN | 308.16 |  | A2M | 111.41 |
| MFGE8 | 292.56 |  | COL6A3 | 106.22 |
| CYTL1 | 277.74 |  | LGALS3 | 105.95 |
| GSN | 277.71 |  | FXYD6 | 104.61 |
| OGN | 259.14 |  | ANXA2 | 100.74 |

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| **Table 2: Extracellular matrix related genes highly expressed in nucleus pulposus** | | | | |
| GeneID | Average expression (RPKM) in  nucleus pulposus |  | GeneID | Average expression (RPKM) in  nucleus pulposus |
| FN1 | 6385.17 |  | TIMP2 | 262.80 |
| COMP | 6014.23 |  | CILP2 | 261.34 |
| CLU | 4378.49 |  | CALR | 261.25 |
| DCN | 3295.38 |  | PLA2G2A | 254.91 |
| LUM | 3093.90 |  | COL9A3 | 245.78 |
| MGP | 2855.43 |  | TGFBI | 242.75 |
| TPT1 | 2341.70 |  | GSN | 242.39 |
| FMOD | 2199.02 |  | SERPING1 | 233.51 |
| COL2A1 | 1840.27 |  | SERPINE2 | 226.51 |
| CILP | 1626.80 |  | SOD3 | 201.91 |
| HTRA1 | 1482.74 |  | IBSP | 184.82 |
| FGFBP2 | 1220.04 |  | CHI3L1 | 184.64 |
| BGN | 1155.23 |  | COL11A2 | 181.05 |
| SPARC | 1065.99 |  | TIMP3 | 180.53 |
| ACAN | 1030.66 |  | CCDC80 | 175.94 |
| SCRG1 | 1028.47 |  | COL9A2 | 168.77 |
| PRELP | 989.54 |  | IGFBP6 | 160.61 |
| COL3A1 | 940.80 |  | POSTN | 158.17 |
| CHAD | 835.31 |  | SOD1 | 155.23 |
| GPX3 | 643.54 |  | FSTL1 | 153.32 |
| CTGF | 567.45 |  | SPP1 | 152.31 |
| CHI3L2 | 541.51 |  | PRDX4 | 150.92 |
| COL1A2 | 534.43 |  | FXYD6 | 147.34 |
| TIMP1 | 529.60 |  | ANGPTL2 | 145.53 |
| LGALS1 | 516.12 |  | IGFBP4 | 138.17 |
| CRTAC1 | 475.35 |  | RBP4 | 137.83 |
| CYTL1 | 452.88 |  | NUCB1 | 124.30 |
| CST3 | 437.51 |  | ALDOA | 122.41 |
| COL6A2 | 430.48 |  | APOE | 121.20 |
| SERPINA1 | 411.25 |  | COL11A1 | 118.90 |
| OGN | 411.25 |  | LGALS3 | 118.41 |
| PSAP | 402.65 |  | APOD | 116.58 |
| MFGE8 | 337.14 |  | COL5A2 | 116.18 |
| COL1A1 | 322.44 |  | COL6A3 | 111.84 |
| ASPN | 314.72 |  | CTSK | 111.52 |
| DPT | 306.39 |  | CRLF1 | 110.49 |
| COL6A1 | 285.52 |  | ANXA2 | 103.52 |
| IGFBP7 | 278.01 |  | MIA | 103.03 |

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| **Table 3: Significant extracellular matrix related genes enriched in annulus fibrosus and nucleus pulposus** | | | |
| Genes enriched in nucleus pulposus | | Genes enriched in nucleus pulposus | |
| CCBE1 | collagen and calcium binding EGF domains 1 | ACAN | aggrecan |
| CNTN1 | contactin 1 | CHI3L1 | chitinase 3 like 1 |
| CNTNAP3B | contactin associated protein-like 3B | CHRD | chordin |
| COL14A1 | collagen type XIV alpha 1 chain | COL10A1 | collagen type X alpha 1 chain |
| COL17A1 | collagen type XVII alpha 1 chain | COL11A1 | collagen type XI alpha 1 chain |
| COL18A1 | collagen type XVIII alpha 1 chain | COL8A2 | collagen type VIII alpha 2 chain |
| COL21A1 | collagen type XXI alpha 1 chain | COL9A2 | collagen type IX alpha 2 chain |
| COL24A1 | collagen type XXIV alpha 1 chain | CRTAC1 | cartilage acidic protein 1 |
| COL4A1 | collagen type IV alpha 1 chain | FMOD | fibromodulin |
| DLL1 | delta like canonical Notch ligand 1 | FN1 | fibronectin 1 |
| DTX1 | deltex E3 ubiquitin ligase 1 | GPC6 | glypican 6 |
| DTX4 | deltex E3 ubiquitin ligase 4 | HHIPL1 | HHIP like 1 |
| EGFLAM | EGF like, fibronectin type III and laminin G domains | HHIPL2 | HHIP like 2 |
| JAG1 | jagged 1 | LAMC3 | laminin subunit gamma 3 |
| JAG2 | jagged 2 | LTBP2 | latent transforming growth factor beta binding protein 2 |
| LAMA3 | laminin subunit alpha 3 | LUM | lumican |
| LAMA4 | laminin subunit alpha 4 | OGN | osteoglycin |
| LAMA5 | laminin subunit alpha 5 | PRG4 | proteoglycan 4 |
| NOTCH3 | notch 3 | SDC4 | syndecan 4 |
| NOTCH4 | notch 4 | SRPX2 | sushi repeat containing protein, X-linked 2 |
| PDGFB | platelet derived growth factor subunit B | WISP3 | WNT1 inducible signaling pathway protein 3 |

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| **Table 4: Annulus fibrosus co-regulatory gene networks** | | | | | | | |
| **ECM and cell adhesion related genes** | | | | **Signaling Associated Genes** | | | |
| Gene symbol | **Gene name** | **Function** | **Cluster** | **Gene symbol** | **Gene name** | **Function** | **Cluster** |
| ADAM12 | ADAM metallopeptidase domain 12 | ECM | "paleturquoise" | FGF9 | fibroblast growth factor 9 | Growth factor | "paleturquoise" |
| COL5A1 | collagen, type V, alpha 1 | ECM | "paleturquoise" | KREMEN1 | kringle containing transmembrane protein 1 | Wnt signaling | "paleturquoise" |
| COL5A2 | collagen, type V, alpha 2 | ECM | "paleturquoise" | PDGFRA | platelet-derived growth factor receptor, alpha polypeptide | Growth factor | "paleturquoise" |
| COL6A3 | collagen, type VI, alpha 3 | ECM | "paleturquoise" | WISP1 | WNT1 inducible signaling pathway protein 1 | Wnt signaling | "paleturquoise" |
| CDH5 | cadherin 5, type 2 | Adhesion | "darkorange2" | WNT5A | wingless-type MMTV integration site family, member 5A | Wnt signaling | "paleturquoise" |
| CDH6 | cadherin 6, type 2, K-cadherin | Adhesion | "darkorange2" | WNT6 | wingless-type MMTV integration site family, member 6 | Wnt signaling | "paleturquoise" |
| CDH24 | cadherin-like 24 | Adhesion | "darkorange2" | WNT9B | wingless-type MMTV integration site family, member 9B | Wnt signaling | "paleturquoise" |
| COL17A1 | collagen type XVII alpha 1 chain | ECM | "darkorange2" | CDH6 | cadherin 6 | NOTCH signaling | "darkorange2" |
| COL18A1 | collagen, type XVIII, alpha 1 | ECM | "darkorange2" | CDKN1B | cyclin dependent kinase inhibitor 1B | NOTCH signaling | "darkorange2" |
| COL21A1 | collagen, type XXI, alpha 1 | ECM | "darkorange2" | DNER | delta/notch like EGF repeat containing | NOTCH signaling | "darkorange2" |
| COL4A1 | collagen type IV alpha 1 chain | ECM | "darkorange2" | HES5 | hes family bHLH transcription factor 5 | NOTCH signaling | "darkorange2" |
| COL4A2 | collagen type IV alpha 2 chain | ECM | "darkorange2" | HEYL | hes related family bHLH transcription factor with YRPW motif-like | NOTCH signaling | "darkorange2" |
| COL4A5 | collagen type IV alpha 5 chain | ECM | "darkorange2" | HHEX | hematopoietically expressed homeobox | NOTCH signaling | "darkorange2" |
| CNTN4 | contactin 4 | Adhesion | "darkorange2" | HOXD3 | homeobox D3 | NOTCH signaling | "darkorange2" |
| ELN | elastin | ECM | "darkorange2" | IGFBP4 | insulin-like growth factor binding protein 4 | Wnt signaling | "darkorange2" |
| FBLN1 | fibulin 1 | ECM | "darkorange2" | IGFBP6 | insulin-like growth factor binding protein 6 | Wnt signaling | "darkorange2" |
| FBLN5 | fibulin 5 | ECM | "darkorange2" | IGFBP7 | insulin-like growth factor binding protein 7 | Wnt signaling | "darkorange2" |
| ICAM1 | intercellular adhesion molecule 1 | Adhesion | "darkorange2" | JAG1 | jagged 1 | NOTCH signaling | "darkorange2" |
| ICAM2 | intercellular adhesion molecule 2 | Adhesion | "darkorange2" | JAG2 | jagged 2 | NOTCH signaling | "darkorange2" |
| ITGA3 | integrin subunit alpha 3 | Adhesion | "darkorange2" | KCNA5 | potassium voltage-gated channel subfamily A member 5 | NOTCH signaling | "darkorange2" |
| ITGA6 | integrin subunit alpha 6 | Adhesion | "darkorange2" | MAML3 | mastermind like transcriptional coactivator 3 | NOTCH signaling | "darkorange2" |
| ITGA7 | integrin subunit alpha 7 | Adhesion | "darkorange2" | NEURL1B | neuralized E3 ubiquitin protein ligase 1B | NOTCH signaling | "darkorange2" |
| ITGA8 | integrin subunit alpha 8 | Adhesion | "darkorange2" | NOTCH3 | notch 3 | NOTCH signaling | "darkorange2" |
| ITGA9 | integrin subunit alpha 9 | Adhesion | "darkorange2" | NOTCH4 | notch 4 | NOTCH signaling | "darkorange2" |
| ITGB4 | integrin subunit beta 4 | Adhesion | "darkorange2" | NRARP | NOTCH-regulated ankyrin repeat protein | NOTCH signaling | "darkorange2" |
| JAM2 | junctional adhesion molecule 2 | Adhesion | "darkorange2" | PDGFB | platelet-derived growth factor beta polypeptide | Growth factor | "darkorange2" |
| LAMA3 | laminin subunit alpha 3 | Adhesion | "darkorange2" | PTP4A3 | protein tyrosine phosphatase type IVA, member 3 | NOTCH signaling | "darkorange2" |
| LAMA4 | laminin subunit alpha 4 | Adhesion | "darkorange2" | VEGFC | vascular endothelial growth factor C | Growth factor | "darkorange2" |
| LAMA5 | laminin subunit alpha 5 | Adhesion | "darkorange2" | WISP2 | WNT1 inducible signaling pathway protein 2 | Wnt signaling | "darkorange2" |
| LAMB1 | laminin subunit beta 1 | Adhesion | "darkorange2" | WISP3 | WNT1 inducible signaling pathway protein 3 | Wnt signaling | "darkorange2" |
| LAMB1 | laminin subunit beta 1 | Adhesion | "darkorange2" | ZNF423 | zinc finger protein 423 | NOTCH signaling | "darkorange2" |
| MYH9 | myosin heavy chain 9 | Adhesion | "darkorange2" |  |  |  |  |
| PCDH1 | protocadherin 1 | Adhesion | "darkorange2" |  |  |  |  |
| PCDH12 | protocadherin 12 | Adhesion | "darkorange2" |  |  |  |  |
| PCDH17 | protocadherin 17 | Adhesion | "darkorange2" |  |  |  |  |
| PCDH19 | protocadherin 19 | Adhesion | "darkorange2" |  |  |  |  |
| TINAGL1 | tubulointerstitial nephritis antigen like 1 | Adhesion | "darkorange2" |  |  |  |  |
| ADAMTS7 | ADAM metallopeptidase with thrombospondin type 1 motif, 7 | ECM | "darkslateblue" |  |  |  |  |
| CNTN1 | contactin 1 | Adhesion | "darkslateblue" |  |  |  |  |
| COL5A3 | collagen, type V, alpha 3 | ECM | "darkslateblue" |  |  |  |  |
| COL6A1 | collagen, type VI, alpha 1 | ECM | "darkslateblue" |  |  |  |  |
| LAMA1 | laminin, alpha 1 | Adhesion | "darkslateblue" |  |  |  |  |

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| **Table 5: Nucleus pulposus co-regulatory gene network** | | | | | | | |
| **ECM and cell adhesion related genes** | | | | **Signaling associated genes** | | | |
| Gene symbol | Gene name | Function | Cluster | Gene symbol | Gene name | Function | Cluster |
| CDH26 | cadherin-like 26 | Adhesion | "black" | CTGF | connective tissue growth factor | Growth factor | "black" |
| CDHR5 | mucin-like protocadherin | Adhesion | "black" | FGFR2 | fibroblast growth factor receptor 2 | Growth factor | "black" |
| CELSR3 | cadherin, EGF LAG seven-pass G-type receptor 3 | Adhesion | "black" | FGFR3 | fibroblast growth factor receptor 3 | Growth factor | "black" |
| CILP | cartilage intermediate layer protein, nucleotide pyrophosphohydrolase | ECM | "black" | BMP2 | bone morphogenetic protein 2 | Growth factor | "grey60" |
| COL27A1 | collagen, type XXVII, alpha 1 | ECM | "black" | BMP6 | bone morphogenetic protein 6 | Growth factor | "grey60" |
| COL2A1 | collagen, type II, alpha 1 | ECM | "black" | FGF1 | fibroblast growth factor 1 (acidic) | Growth factor | "grey60" |
| CRTAP | cartilage associated protein | ECM | "black" | FGF2 | fibroblast growth factor 2 (basic) | Growth factor | "grey60" |
| CTGF | connective tissue growth factor | ECM | "black" | GDF6 | growth differentiation factor 6 | Growth factor | "grey60" |
| DSCAML1 | Down syndrome cell adhesion molecule like 1 | Adhesion | "black" | HHIP | hedgehog interacting protein | Growth factor | "grey60" |
| GPC6 | glypican 6 | ECM | "black" | IGFBP3 | insulin-like growth factor binding protein 3 | Growth factor | "grey60" |
| ITGA10 | integrin, alpha 10 | Adhesion | "black" | INHBA | inhibin, beta A | Growth factor | "grey60" |
| LMLN | leishmanolysin-like | Adhesion | "black" | NGF | nerve growth factor (beta polypeptide) | Growth factor | "grey60" |
| PCDH20 | protocadherin 20 | Adhesion | "black" | NOG | noggin | Growth factor | "grey60" |
| TESK2 | testis-specific kinase 2 | Adhesion | "black" | PDGFC | platelet derived growth factor C | Growth factor | "grey60" |
| ADAMTS6 | ADAM metallopeptidase with thrombospondin type 1 motif, 6 | ECM | "grey60" | TGFA | transforming growth factor, alpha | Growth factor | "grey60" |
| CCDC80 | coiled-coil domain containing 80 | ECM | "grey60" | TGFBR1 | transforming growth factor, beta receptor 1 | Growth factor | "grey60" |
| CDH19 | cadherin 19, type 2 | Adhesion | "grey60" | TSHB | thyroid stimulating hormone, beta | Growth factor | "grey60" |
| CHI3L1 | chitinase 3-like 1 | ECM | "grey60" | VEGFA | vascular endothelial growth factor A | Growth factor | "grey60" |
| FN1 | fibronectin 1 | ECM | "grey60" | WNT1 | wingless-type MMTV integration site family, member 1 | Wnt signaling | "grey60" |
| HAPLN1 | hyaluronan and proteoglycan link protein 1 | ECM | "grey60" | WNT16 | wingless-type MMTV integration site family, member 16 | Wnt signaling | "grey60" |
| IMPG2 | interphotoreceptor matrix proteoglycan 2 | ECM | "grey60" | WNT9A | wingless-type MMTV integration site family, member 9A | Wnt signaling | "grey60" |
| ITGB5 | integrin, beta 5 | Adhesion | "grey60" | GDF5 | growth differentiation factor 5 | Growth factor | "lightyellow" |
| LAMB3 | laminin, beta 3 | Adhesion | "grey60" | HHIPL1 | HHIP-like 1 | Hedgehog signaling | "lightyellow" |
| LUM | lumican | ECM | "grey60" | HHIPL2 | HHIP-like 2 | Hedgehog signaling | "lightyellow" |
| PRG4 | proteoglycan 4 | ECM | "grey60" | INHA | inhibin, alpha | Growth factor | "lightyellow" |
| SERPINE1 | serpin peptidase inhibitor, clade E member 1 | ECM | "grey60" | NRG4 | neuregulin 4 | Growth factor | "lightyellow" |
| SERPINE2 | serpin peptidase inhibitor, clade E member 2 | ECM | "grey60" | NRTN | neurturin | Growth factor | "lightyellow" |
| SMOC1 | SPARC related modular calcium binding 1 | ECM | "grey60" |  |  |  |  |
| TIMP2 | TIMP metallopeptidase inhibitor 2 | ECM | "grey60" |  |  |  |  |
| TIMP3 | TIMP metallopeptidase inhibitor 3 | ECM | "grey60" |  |  |  |  |
| VCAN | versican | ECM | "grey60" |  |  |  |  |
| ACAN | aggrecan | ECM | "lightyellow" |  |  |  |  |
| ADAMTSL2 | similar to ADAMTS-like 2; ADAMTS-like 2 | ECM | "lightyellow" |  |  |  |  |
| BGN | biglycan | ECM | "lightyellow" |  |  |  |  |
| CHAD | chondroadherin | Adhesion | "lightyellow" |  |  |  |  |
| CILP2 | cartilage intermediate layer protein 2 | ECM | "lightyellow" |  |  |  |  |
| COL11A2 | collagen, type XI, alpha 2 | ECM | "lightyellow" |  |  |  |  |
| COL9A2 | collagen, type IX, alpha 2 | ECM | "lightyellow" |  |  |  |  |
| COL9A3 | collagen, type IX, alpha 3 | ECM | "lightyellow" |  |  |  |  |
| COMP | cartilage oligomeric matrix protein | ECM | "lightyellow" |  |  |  |  |
| EMILIN3 | elastin microfibril interfacer 3 | ECM | "lightyellow" |  |  |  |  |
| PRELP | proline/arginine-rich end leucine-rich repeat protein | ECM | "lightyellow" |  |  |  |  |
| SERPINA1 | serpin peptidase inhibitor, clade A member 1 | ECM | "lightyellow" |  |  |  |  |
| SPINT2 | serine peptidase inhibitor, Kunitz type, 2 | ECM | "lightyellow" |  |  |  |  |

**Figure 1**



**Figure 2**



**Supplemental Figure 1**



|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Supplemental Table 1: Clinical data associated with each disk specimen** | | | | | | | |
| **Patient ID** | **Sample Label** | **Tissue Label** | **Cervical Disk Level** | **Disk degen grade** | **Age** | **Gender** | **Analysis** |
| Patient 1 | Spine 1 | Annulus 1 | 4-5 | 2 | 71 | Female | Included in analysis |
| Patient 2 | Spine 2 | Annulus 2 | 5-6 | 3 | 57 | Male | Included in analysis |
| Patient 3 | Spine 3 | Annulus 3 | 4-5 | 2 | 77 | Female | Excluded  clustering outlier |
| Patient 4 | Spine 4 | Annulus 4 | 3-4 | 3 | 63 | Female | Excluded  clustering outlier |
| Patient 5 | Spine 5 | Annulus 5 | 6-7 | 2 | 60 | Male | Excluded  clustering outlier |
| Patient 6 | Spine 6 | Annulus 6 | 6-7 | 2 | 78 | Male | Included in analysis |
| Patient 7 | Spine 7 | Annulus 7 | 3-4 | 3 | 53 | Male | Excluded  clustering outlier |
| Patient 8 | Spine 8 | Annulus 8 | 3-4 | 3 | 70 | Male | Excluded  clustering outlier |
| Patient 9 | Spine 9 | Annulus 9 | 5-6 | 3 | 55 | Female | Excluded  clustering outlier |
| Patient 10 | Spine 10 | Annulus 10 | 4-5 | 2 | 77 | Female | Included in analysis |
| Patient 11 | Spine 11 | Annulus 11 | 6-7 | 2 | 34 | Female | Included in analysis |
| Patient 12 | Spine 12 | Annulus 12 | 4-5 | 2 | 54 | Male | Included in analysis |
| Patient 13 | Spine 13 | Annulus 13 | 5-7 | 3 | 49 | Male | Included in analysis |
| Patient 14 | Spine 14 | Annulus 14 | 5-6 | 2 | 32 | Female | Included in analysis |
| Patient 15 | Spine 15 | Annulus 15 | 6-7 | 3 | 48 | Male | Excluded  clustering outlier |
| Patient 16 | Spine 16 | Annulus 16 | 5-6 | 2 | 54 | Male | Included in analysis |
| Patient 17 | Spine 17 | Annulus 17 | 5-6 | 3 | 47 | Male | Included in analysis |
| Patient 18 | Spine 18 | Annulus 18 | 4-5 | 3 | 47 | Female | Included in analysis |
| Patient 19 | Spine 19 | Annulus 19 | 5-7 | 3 | 49 | Male | Included in analysis |
| Patient 20 | Spine 20 | Annulus 20 | 4-5 | 1 | 54 | Female | Included in analysis |
| Patient 21 | Spine 21 | Annulus 21 | 4-7 | 3 | 70 | Female | Included in analysis |
| Patient 22 | Spine 22 | Annulus 22 | 5-6 | 3 | 65 | Female | Included in analysis |
| Patient 23 | Spine 23 | Annulus 23 | 4-5 | 1 | 57 | Male | Included in analysis |
| Patient 24 | Spine 24 | Annulus 24 | 6-7 | 2 | 45 | Female | Included in analysis |
| Patient 25 | Spine 25 | Annulus 25 | 5-6 | 2 | 40 | Female | Included in analysis |
| Patient 26 | Spine 26 | Annulus 26 | 5-6 | 3 | 50 | Male | Excluded  clustering outlier |
| Patient 27 | Spine 27 | Annulus 27 | 3-4 | 3 | 69 | Female | Included in analysis |
| Patient 28 | Spine 28 | Annulus 28 | 5-6 | 3 | 47 | Female | Included in analysis |
| Patient 29 | Spine 29 | Annulus 29 | 6-7 | 3 | 35 | Male | Included in analysis |
| Patient 30 | Spine 30 | Annulus 30 | 7-1 | 2 | 45 | Male | Included in analysis |
| Patient 31 | Spine 31 | Annulus 31 | 5-6 | 3 | 41 | Female | Excluded low quality sequence reads |
| Patient 32 | Spine 32 | Annulus 32 | 6-7 | NA | 64 | Female | Included in analysis |
| Patient 33 | Spine 33 | Annulus 33 | 4-5 | 2 | 60 | Male | Excluded low quality sequence reads |
| Patient 34 | Spine 34 | Annulus 34 | 3-4 | 2 | 50 | Male | Excluded  clustering outlier |
| Patient 35 | Spine 35 | Annulus 35 | 5-6 | 2 | 55 | Male | Included in analysis |
| Patient 36 | Spine 36 | Annulus 36 | NA | NA | NA | NA | Included in analysis |
| Patient 37 | Spine 37 | Annulus 37 | 5-6 | 2 | 42 | Male | Excluded low quality sequence reads |
| Patient 38 | Spine 38 | Annulus 38 | 6-7 | 3 | 50 | Male | Included in analysis |
| Patient 39 | Spine 39 | Annulus 39 | 5-6 | 3 | 36 | Male | Included in analysis |
| Patient 40 | Spine 40 | Nucleus 1 | 5-6 | 3 | 64 | Male | Included in analysis |
| Patient 3 | Spine 41 | Nucleus 2 | 4-5 | 2 | 77 | Female | Included in analysis |
| Patient 4 | Spine 42 | Nucleus 3 | 3-4 | 3 | 63 | Female | Excluded  clustering outlier |
| Patient 41 | Spine 43 | Nucleus 4 | 4-5 | 3 | 64 | Female | Included in analysis |
| Patient 10 | Spine 44 | Nucleus 5 | 4-5 | 2 | 77 | Female | Included in analysis |
| Patient 7 | Spine 45 | Nucleus 6 | 3-4 | 3 | 53 | Male | Excluded  clustering outlier |
| Patient 42 | Spine 46 | Nucleus 7 | 5-6 | 2 | 57 | Male | Excluded  clustering outlier |
| Patient 43 | Spine 47 | Nucleus 8 | 5-6 | 1 | 52 | Female | Included in analysis |
| Patient 16 | Spine 48 | Nucleus 9 | 5-6 | 2 | 54 | Male | Included in analysis |
| Patient 44 | Spine 49 | Nucleus 10 | 4-5 | 2 | 67 | Female | Included in analysis |
| Patient 45 | Spine 50 | Nucleus 11 | 5-6 | 1 | 45 | Female | Excluded  clustering outlier |
| Patient 46 | Spine 51 | Nucleus 12 | NA | NA | NA | NA | Included in analysis |
| Patient 47 | Spine 52 | Nucleus 13 | 5-6 | 3 | 41 | Female | Included in analysis |
| Patient 22 | Spine 53 | Nucleus 14 | 5-6 | 3 | 65 | Female | Included in analysis |
| Patient 25 | Spine 54 | Nucleus 15 | 5-6 | 2 | 40 | Female | Included in analysis |
| Patient 48 | Spine 55 | Nucleus 16 | 4-5 | 3 | 69 | Male | Included in analysis |
| Patient 29 | Spine 56 | Nucleus 17 | 6-7 | 3 | 35 | Male | Included in analysis |
| Patient 30 | Spine 57 | Nucleus 18 | 7-1 | 2 | 45 | Male | Included in analysis |
| Patient 32 | Spine 58 | Nucleus 19 | 6-7 | NA | 64 | Female | Excluded  clustering outlier |
| Patient 31 | Spine 59 | Nucleus 20 | 5-6 | 3 | 41 | Female | Included in analysis |
| Patient 33 | Spine 60 | Nucleus 21 | 4-5 | 2 | 60 | Male | Included in analysis |

|  |  |  |  |
| --- | --- | --- | --- |
| **Supplemental Table 2: Hemoglobin expression in sample outliers** | | | |
| **GeneID** | **HBB** | **HBA1** | **HBA2** |
| Spine 5 | 231707.5 | 3354.966 | 8966.395 |
| Spine 6 | 76572.01 | 522.8024 | 1196.394 |
| Spine 7 | 50811.74 | 659.834 | 1610.641 |
| Spine 8 | 44365.89 | 1575.196 | 3635.422 |
| Spine 12 | 57485.11 | 808.8986 | 2270.575 |
| Spine 13 | 258146.7 | 2392.466 | 4954.019 |
| Spine 15 | 47861.13 | 418.4691 | 1670.217 |
| Spine 23 | 86260.39 | 937.9726 | 1797.626 |
| Spine 50 | 46268.13 | 499.5708 | 939.4183 |
| Spine 51 | 197426.2 | 2152.395 | 5041.469 |
| Spine 1 | 1109.845 | 44.02879 | 45.82063 |
| Spine 2 | 8757.846 | 197.9626 | 807.6419 |
| Spine 3 | 4265.278 | 353.1868 | 206.2838 |
| Spine 4 | 3454.75 | 193.3775 | 91.22973 |
| Spine 9 | 4937.788 | 899.793 | 848.0942 |
| Spine 10 | 13494.12 | 108.5997 | 447.424 |
| Spine 11 | 1003.909 | 63.22794 | 44.56979 |
| Spine 14 | 25955.6 | 2479.675 | 1770.855 |
| Spine 16 | 1657.235 | 37.59327 | 11.59994 |
| Spine 17 | 5852.255 | 152.9127 | 360.1547 |
| Spine 18 | 27429.79 | 59.42669 | 1222.876 |
| Spine 19 | 13270.62 | 83.28675 | 149.6529 |
| Spine 20 | 7279.07 | 121.2427 | 87.97746 |
| Spine 21 | 3972.358 | 143.5934 | 54.481 |
| Spine 22 | 18499.29 | 194.2335 | 257.0419 |
| Spine 24 | 1919.116 | 167.9213 | 117.3898 |
| Spine 25 | 156.7978 | 20.61771 | 11.80487 |
| Spine 26 | 1027.668 | 101.6721 | 66.29819 |
| Spine 27 | 23458.4 | 640.5297 | 2543.354 |
| Spine 28 | 2588.365 | 73.55155 | 74.61515 |
| Spine 29 | 3262.234 | 105.4299 | 221.363 |
| Spine 30 | 6884.308 | 50.92927 | 123.8167 |
| Spine 31 | 136.6845 | 8.134394 | 4.241025 |
| Spine 32 | 161.0688 | 19.98078 | 16.26885 |
| Spine 33 | 4825.275 | 51.86172 | 281.1946 |
| Spine 34 | 1404.696 | 292.1335 | 318.636 |
| Spine 35 | 1257.399 | 54.03949 | 22.67175 |
| Spine 36 | 13762.42 | 167.5623 | 399.1917 |
| Spine 37 | 3324.096 | 90.15185 | 54.04144 |
| Spine 38 | 8804.239 | 168.5112 | 161.1025 |
| Spine 39 | 1818.045 | 89.68212 | 49.96764 |
| Spine 40 | 95.0468 | 5.580626 | 2.072119 |
| Spine 41 | 1232.303 | 96.84991 | 65.06059 |
| Spine 42 | 32282.36 | 192.446 | 480.3754 |
| Spine 43 | 1757.439 | 135.5536 | 72.14403 |
| Spine 44 | 163.5198 | 10.18787 | 6.359292 |
| Spine 45 | 1609.998 | 48.66259 | 52.70395 |
| Spine 46 | 495.4961 | 18.95263 | 35.01638 |
| Spine 47 | 5753.63 | 260.5508 | 367.3439 |
| Spine 48 | 2458.166 | 28.63946 | 102.3593 |
| Spine 49 | 6885.874 | 252.6364 | 275.4919 |
| Spine 52 | 2146.561 | 212.1498 | 242.5093 |
| Spine 53 | 16374.02 | 667.3234 | 1551.613 |
| Spine 54 | 136.0263 | 8.007918 | 3.893141 |
| Spine 55 | 4308.499 | 311.0131 | 285.0663 |
| Spine 56 | 642.2064 | 290.6479 | 356.6957 |
| Spine 57 | 4473.854 | 145.7502 | 706.6027 |