Transcriptomic Analysis of Murine Intervertebral Disc Reveals Anatomical Spine Level-Dependent Differences in Immune Regulatory Pathway

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INTRODUCTION: Intervertebral disc (IVD) degeneration (IDD), characterized by age- or injury-manifested changes leading to structural and functional tissue failure, is often associated with lower back pain (LBP), one the most prevalent and disabling conditions worldwide. The lack of therapeutic treatments of LBP are due to major gaps in current understanding of IVD biology. The IVD is a specialized connective tissue structure composed of interdependent tissues: central nucleus pulposus (NP), encapsulating annulus fibrosus (AF), and cartilage endplate. Different genetic and injury mouse models have been developed to understand the heterogeneity of IVD tissue types (reporter mice) and disease progression (aging, puncture injury, mechanical loading) in both lumbar and caudal IVDs. However, our recent studies and others show anatomical region differences in inflammation-induced disease progression[1] and physico-mechanical properties between lumbar and caudal IVDs[2-3]. Furthermore, how these differences can be resolved to model human IDD remains unclear, limiting the translational potential of mouse models. The objective of the current study was to use the next generation sequencing to provide a benchmark of genetic profiles between lumbar and caudal NP and AF tissues from young healthy mice.

METHODS: NP and AF tissues from 6 lumbar and 6 caudal IVDs were carefully dissected from C57BL/6 mice at 6-7 months-of-age (n=8). For each animal, IVD tissue types were pooled by anatomical region (lumbar NP, LNP; lumbar AF, LAF; caudal NP, CNP; caudal AF, CAF) and total RNA was extracted (average RIN = 8.6). Bulk RNA sequencing and initial data processing (read alignment) were performed by the Genome Center. STRPOLYA kit was used for library preparations and for each sample, a minimum of 20 million 100 base-pair paired end reads were sequenced using Illumina NovaSeq 6000. Gene count data were normalized using the variance stabilizing function using DESeq2. Differentially expressed genes were identified based on $|\log2FoldChange| \geq 1.5$ and adjusted p value (padj.) < 0.05. Analyses were performed in R to identify differences within NP or AF tissues from caudal versus lumbar regions (CNP vs. LNP and CAF vs. LAF). In order to explore the up- or down-regulated biological processes and pathways between spinal regions, gene set enrichment analysis (GSEA) was performed using the clusterProfiler package and the Molecular Signatures Database (MSigDB). Enriched biological processes were evaluated using C5 gene ontology database and pathway enrichment analysis was performed using C2 pathway database.

RESULTS: Comparison between CNP and LNP: We identified 1144 upregulated genes and 3538 downregulated genes in the CNP compared to LNP (**Fig. 1.A**). The heatmap of 25 top differentially regulated genes show clear distinction between the two spinal regions (**Fig. 1.B**). When assessed for changes in known NP markers, the expression of *Brachyury* (*T*), *Gpc3*, *Krt8*, and *Krt19* was significantly upregulated, while *Shh* and *Slc2a3* expression was significantly downregulated (**Fig. 1.C**). The 5 most enriched upregulated genes were those associated with cAMP signaling (*Pakap*), Rab GTPase signaling (*Rasef*), protein transport and folding (*Ssu2*), matrix remodeling (*Serpina1f*), and phospholipase A2 production (*Pla2g2e*), whereas 5 most enriched downregulated genes were genes involved in immunoglobulin production (*Ighv1-81, Igkv5-39, Ighv5-6, Ighv1-11*) and immune response (*Tlr9*). Gene set enrichment analysis showed motile cilium assembly, protein hydroxylation, regulation of cilium movement, vesicle transport, and nucleotide sugar metabolic process as top 5 upregulated biological processes, while top 5 downregulated biological processes included complement activation, membrane invagination, immunoglobulin immune response, phagocytosis recognition, and B cell receptor signaling (**Fig. 1.D**). Comparison between CAF and LAF: Among the differentially expressed genes, 25 genes were upregulated and 25 genes were downregulated (**Fig. 1.E**), with less clear distinction of lumbar and caudal samples when segregated with top differentially regulated genes (**Fig. 1.F**). The 5 most enriched upregulated genes were involved in cholinergic pathway and membrane depolarization (*Chrna10, Dmgdh, Kcnh3*), BMP signaling (*Gdf6*), and GTPase signaling (*Rin1*), whereas 5 most enriched downregulated genes were involved in immune response (*Igkv4-91, Igkv6-32*), ion binding and transport (*Slco1a4, Rph3a*), and synaptic vesicle exocytosis (*Cplx1*). Gene set enrichment analysis identified regulation of chondrocyte differentiation, negative regulation of cartilage development, connective tissue development, protein hydroxylation, and collagen fibril organization as top 5 upregulated biological processes, while top 5 downregulated biological processes included phagocytosis recognition, membrane invagination, cell surface receptor immune response, antigen receptor signaling, and B cell receptor signaling (**Fig. 1.G**). No significant difference in the expression of known AF markers (*Acan, Col1a1, Col2a1, Cilp, Cilp2, Comp, Chad, Dkk1, Prg4*) was observed between regions.

DISCUSSION: Our findings suggest that there are anatomical region differences in the transcriptomic profiles within the NP and AF tissues. Notably, large transcriptomic differences were detected in CNP compared to LNP. Increased expression of genes associated with notochordal cells (*Brachyruy, Krt8, Krt19*), suggests greater contributions of notochordal-like cells in the CNP. Among the upregulated genes in the CNP, *Serpina1f* and *Pla2g2e* have been associated with IDD. SERPINA1 expression has been shown to decrease with IDD in both human and rat NP[4] and overexpression of *SERPINA1* in human NP cells led to increased cell viability[5]. Phospholipase A2 (encoded by *Pla2g2e*) is involved in prostaglandin E2 production, which has been shown to increase in herniated IVD[6]. The observed transcriptomic differences in the CNP suggest differentially regulated pathophysiology of IDD compared to LNP. Interestingly, GSEA showed that most of the downregulated biological processes for both CNP and CAF were immune-related pathways, suggesting differentially regulated IVD tissue immune response by anatomical regions.

SIGNIFICANCE/CLINICAL RELEVANCE: This study establishes a transcriptomic landscape outlining the molecular differences in the lumbar and caudal IVD tissues and provides mechanistic insights to anatomical region-dependent IVD biology. Considering that both murine lumbar and caudal IVDs are utilized in spine research, defining how these molecular differences can better recapitulate human IDD will help establish more clinically relevant *in vivo* models.

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IMAGES AND TABLES:

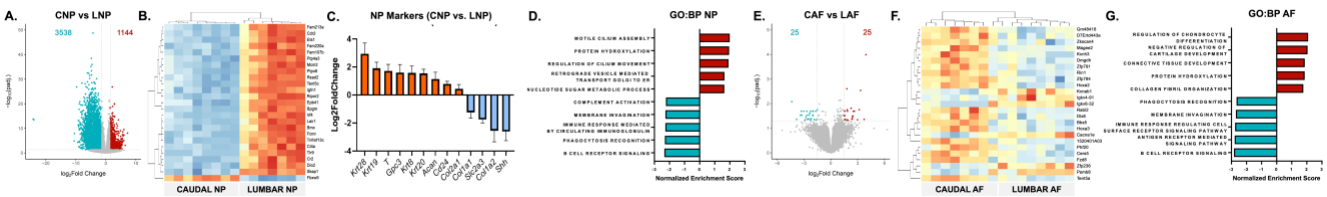

Figure 1. Summary of data processing and functional bioinformatics analysis for NP and AF. (A, E) Volcano plot outlining differentially expressed genes for NP (A) and AF (E) in different anatomical regions. (B, F) Heatmap of top 25 differentially regulated genes of NP (B) and AF (F) in caudal region relative to lumbar region. (C) Expression levels of NP marker genes in CNP compared to LNP. (D, G) Gene set enrichment analysis of enriched biological pathways in NP (D) and AF (G).