Global proteomics identifies age-related changes in the secretome of mouse lumbar intervertebral discs

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ABSTRACT INTRODUCTION:
Pathologies of the intervertebral disc are a leading cause of chronic back pain. Aging is a major risk factor to develop disc pathologies and/or chronic low back pain. While these conditions are top global burdens of disease, they have no cure. The development of therapeutics is impeded by a limited understanding of the cellular and molecular basis of disc pathologies and how they contribute to chronic back or radiating pain. Each disc has a central core of proteoglycan-rich nucleus pulposus cells surrounded by collagenous annulus fibrosus cells. Degenerated discs lose their nucleus pulposus cells and extracellular matrix including proteoglycans that maintain disc hydration, height, and function. Degenerated and dehydrated discs of patients are short and bulge out, resulting in nerve compression and radiating pain. Moreover, while healthy discs are avascular and aneural, degenerated discs are vascularized, innervated, and inflammatory, which is thought to cause localized pain. Recent studies showed severe structural and degenerated pathology including innervation and vascularization in the lower lumbar discs of aging mice. Also, aged mice responded significantly more to pain and peripheral stimulation compared to younger mice. However, we do not know whether the age-related structural changes are associated with molecular changes that could lead to pain. Hence, the goal of the current study was to gain molecular insights into disc pathology by quantitatively profiling the secretory proteome of lumbar intervertebral discs from young and very aged mice.

METHODS: Both male and female mice used in the study were maintained in adherence with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experiments were conducted following approval and in adherence to institutional guidelines under Institutional Animal Care and Use Committee (IACUC). Wild-type young mice of around 3-4 months of age and very aged mice of about 22-27 months of age were used. Lumbar discs at L4 to S1 levels were individually dissected from the lumbar spine of each mouse. Each lumbar disc was cultured in a 48-well culture plate using serum-free DMEM Ham /F12 media containing antibiotics. After 24 hours the supernatant culture media along with secretory factors was collected from each well (n=6/cohort) and subjected to in-solution trypsin digestion, followed by stage-tip desalting and LC-MS/MS for quantitative global proteome analysis. Each sample was analyzed using a data-independent acquisition (DIA) method. The data were searched against a Uniprot protein database using the DIANN software. The intensity values were log-transformed, and data was filtered to keep only those with 2 valid values in at least one experimental group. *T*-test was performed to identify Differentially expressed proteins (DEP) between cohorts. Multiple hypothesis correction of p-values was performed using the BH method. Gene ontology (GO) analysis and Ingenuity Pathway Analysis (IPA) of DEP was carried out to gain molecular insights. Next, the lumbar discs from each cohort (n=6/cohort) were individually processed for RNA isolation and multi-plex RT-qPCR analysis using gene-specific TaqMan probes and *Gapdh* as internal control. Statistical differences between the qPCR results were analyzed using Student's *t*-test and GraphPad Prism software.

RESULTS SECTION: Quantitative global proteome analysis using label-free LC-MS/MS identified about 575 secretory proteins validated to be localized in the extracellular space using PANTHER database analysis. Principal component analysis (PCA) showed distinct variability in the secretome of discs from young and aged mice (Fig. 1). Volcano plot showed the number of proteins that were either up- or downregulated in the lumbar disc secretome with aging (Fig. 2). GO analysis showed that a major component of the secretome constituted the extracellular matrix components that were decreased with aging. IPA of the proteins revealed upregulation of inflammatory pathways including IFNG, TNF, and IL1beta in the secretome of the intervertebral discs of very aged mice. Multiplex RT-qPCR analysis of the discs for *Tnf* and *Il1b* using *Gapdh* as an internal control validated increased expression of these inflammatory genes in the discs of very aged mice. Moreover, the TGF-beta pathway was downregulated in the secretome of the very aged mouse discs. Decline in TGF-beta signaling in the intervertebral disc has been previously reported with aging.

DISCUSSION: The findings of current study indicate that with the severity of disc degeneration, the microenvironment of the degenerated lumbar discs of mice is significantly inflammatory. We know that the very aged mouse discs are innervated and vascularized. Hence, the increased nociceptive signaling and sensation of pain in very aged mice could be due to the increased expression of inflammatory molecules in the aged mouse disc. TGF-beta is an important developmental signaling pathway and previous studies have reported its importance in disc development and maintenance. We found that the TGF-beta pathway was downregulated in the secretome of very aged mouse discs, in line with previous studies.

SIGNIFICANCE: Intervertebral disc degeneration and associated chronic back pain are top global burdens of disease, and a better understanding of disc pathogenesis will help design therapeutics aimed at treating both conditions. Here we have identified numerous secretory proteins whose expression changes with aging in mice when the lumbar discs are pathological. Future studies aimed at understanding what leads to the molecular and cellular changes in the disc with aging may help develop approaches to prevent disc pathologies to combat chronic back pain at the root cause.

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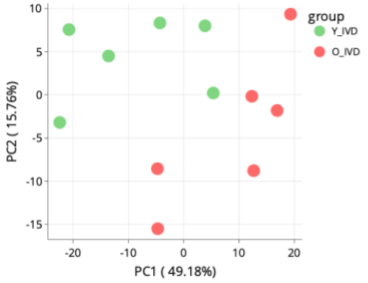

Fig. 1. Principal component analysis (PCA)

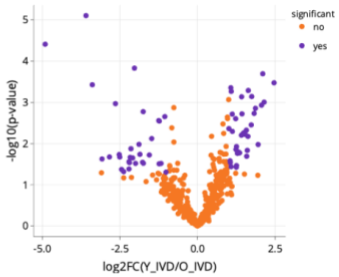

Fig. 2. Volcano plot