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ORIGINAL RESEARCH

Single-Cell Analysis Reveals Aspirin Restores Intervertebral Disc Integrity via Ferroptosis Regulation

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Background: Low back pain represents a major global health issue, with intervertebral disc degeneration (IVDD) being one of its primary causes. Disc degeneration involves complex processes such as inflammation, matrix degradation, and cell death, yet the underlying mechanisms remain poorly understood. Single-cell RNA sequencing offers a powerful approach to elucidate cellular heterogeneity and dynamic changes in IVDD, providing valuable insights for early diagnosis and targeted therapeutic strategies.

Methods: The Harmony algorithm was used to integrate four independent single-cell sequencing datasets. Subtype identification, differential expression analysis, enrichment analysis, and cell proportion analysis were conducted to explore functional alterations in various nucleus pulposus cell (NPC) subpopulations. Molecular docking was employed to evaluate the stability of aspirin targeting GPX4. In vitro and in vivo experiments were performed to assess the therapeutic effects of aspirin on IVDD.

Results: Eight distinct NPC subtypes were identified based on cellular heterogeneity and their associated marker genes. The CDKN1A$^+$aNPC subtype increased progressively with disease severity, while the matrix-supporting ABI3BP$^+$mNPC and SOD3$^+$mNPC subtypes significantly decreased in advanced degeneration. Concurrently, there was an increase in ECM remodeling-related LTBP1$^+$mNPCs. Within the CDKN1A$^+$aNPC, GPX4 was notably downregulated, suggesting the activation of ferroptosis. Molecular docking results revealed a high affinity of aspirin for GPX4. Additionally, aspirin inhibited ferroptosis and ameliorated disc structural damage.

Conclusion: The increased proportion of CDKN1A$^+$aNPC cells serves as an early warning feature for the progression of IVDD. Aspirin stabilizes the targeting of GPX4, thereby inhibiting ferroptosis and exerting therapeutic effects on IVDD.

Keywords: low back pain, intervertebral disc degeneration, single-cell analysis, cell death, glutathione peroxidase 4

Introduction

Low back pain (LBP) adversely impacts individuals across all age groups and socioeconomic strata, with a lifetime prevalence ranging from 11% to 84% worldwide.[1–3] LBP significantly compromises quality of life, is the leading cause of disability, and results in substantial losses in societal productivity while directly escalating healthcare costs.[1] Although the etiology of LBP is multifactorial and complex, intervertebral disc degeneration (IVDD) has been identified as its primary driver.[4,5] IVDD represents an age- and injury-related pathological process within the intervertebral disc (IVD), driven by a series of intricate molecular mechanisms that evolve over time and ultimately lead to severe clinical manifestations.[6,7] The bidirectional crosstalk between the IVD and the adjacent bone marrow critically shapes the microenvironment and determines the progression of pathology.[8,9] Loss of homeostatic balance within the disc microenvironment triggers IVDD, characterized by a catabolic, hypoxic milieu and cellular senescence, which in turn drives immunometabolic alterations.[10,11] Current treatments for IVDD include conservative management and surgical interventions tailored to alleviate patient symptoms. However, these approaches primarily provide symptomatic relief and fail to reverse IVDD or restore the mechanical functionality of the spine.

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The development of intervertebral disc degeneration is influenced by several factors, including genetic predisposition, aging, mechanical injury, and nutritional deficiencies. Dysfunction of nucleus pulposus cells (NPCs), which reside in the gel-like central region of the intervertebral disc, is recognized as a key initiating factor in IVDD.[12,13] Pathological changes associated with NPC dysfunction primarily include cellular senescence and apoptosis, progressive extracellular matrix (ECM) degradation, fibrosis of the annulus fibrosus (AF), and inflammatory responses. These processes are marked by elevated levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1α/β, IL-6, and IL-17, which drive matrix degradation, chemokine production, and alterations in cell phenotype.[7] The resultant imbalance between catabolic and anabolic activities leads to disc degeneration, herniation, and nerve root pain. Recent studies suggest that conditions observed in type 2 diabetes mellitus (T2DM), such as nutrient deprivation and hyperglycemia within the intervertebral disc, impose significant cellular stress on disc cells. This stress activates death receptors and triggers apoptosis via endoplasmic reticulum and mitochondrial pathways.[14–16] Elevated glucose levels further induce reactive oxygen species (ROS) production and mitochondrial damage in AF,[17] NPCs, and notochordal cells. Oxidative stress, a key mediator of ferroptosis, has been implicated in various diseases. Ferroptosis is a form of oxidative damage characterized by iron-dependent lipid peroxidation, driven by intracellular iron or lipoxygenase-catalyzed oxidation of unsaturated fatty acids in cell membranes. Hallmarks of ferroptosis include glutathione (GSH) depletion and inactivation of GPX4, the central regulator of the glutathione antioxidant system.[18,19] However, the mechanistic interplay between oxidative stress and ferroptosis in IVDD remains poorly understood. Elucidating the roles of oxidative stress and regulated cell death in the catabolic and injury processes of IVDD may unveil novel therapeutic targets for the treatment of symptomatic disc diseases.

This study aims to elucidate the critical mechanisms underlying IVDD and explore potential new therapeutic targets. By integrating single-cell sequencing data from four independent IVDD cohorts and analyzing the heterogeneity of NPCs, we have conducted an in-depth investigation into the dynamic changes of NPCs during the progression of IVDD. The study focuses on the potential role of ferroptosis in IVDD, proposing that targeting ferroptosis through the regulation of GPX4 could represent a novel therapeutic strategy. Our findings highlight the significance of cellular heterogeneity in IVDD, particularly in processes such as matrix remodeling and cell death. Furthermore, we suggest that ferroptosis may play a pivotal role in the progression of IVDD, offering new insights for future therapeutic approaches.

Methods

Pre-Processing of Single-Cell Data

Single-cell transcriptomic data were obtained from previous studies.[20–23] All samples underwent stringent quality control following standard protocols implemented in R (version 4.4.0). The raw count matrix was imported using the "Read10X" function from the Seurat package and converted into a sparse matrix format. Individual datasets were integrated into a single combined object using the "merge" function, and unique cell labels were generated using the "RenameCells" function. Potential doublets were identified and removed using the Scrublet algorithm. Cells with fewer than 100 detected genes or genes expressed in fewer than three cells were excluded during the quality filtering process. Gene expression normalization across cells was performed using the "LogNormalize" method with a scaling factor of 10,000. To identify the top 2000 most variable genes, we used the "FindVariableFeatures" function. To account for unwanted technical variation, including UMI and mitochondrial gene content, these factors were regressed out using the "ScaleData" function. Principal component analysis was conducted on the highly variable features to reduce dimensionality, and the first 30 principal components were selected for subsequent analyses. Batch effects were corrected using the Harmony algorithm. For further dimensionality reduction, UMAP was employed. Clustering was performed based on the similarity of edge weights between cells using the Louvain method, with different resolution parameters (ranging from 0.1 to 2) tested to determine the optimal clustering configuration. A resolution of 0.6 produced the most robust and distinct clusters. Cell clusters were annotated by identifying differentially expressed genes within each cluster using the "FindAllMarkers" function, applying a nonparametric Wilcoxon rank-sum test with Bonferroni correction. Annotation of cell types was based on known surface markers, published gene lists, and the cell taxonomy database (https://ngdc.

cncb.ac.cn/celltaxonomy/), ensuring an accurate and comprehensive classification. Finally, only nucleus pulposus cells were extracted for further analysis.

Enrichment Analysis

Enrichment analysis of differentially expressed genes was performed using the "GSEABase" "ClusterProfiler" packages", and "org.Hs.eg.db" database. The analysis utilized data from the GO and KEGG databases. Pathways with a P-value < 0.05 were considered significantly enriched.

Analysis of Transcription Factor Activity

The transcription factor activity was assessed using the SCENIC approach. First, GENIE3 was applied to construct a regulatory network by identifying co-expression patterns between transcription factors and their potential target genes. This network was then enhanced by integrating motif relationships and ranking the regulatory potential of motifs binding to genes, which led to the formation of a "regulon." A regulon is defined as a set of target genes that are regulated by transcription factors through direct binding to upstream motifs. Finally, the activity of each regulon was evaluated across all cells using AUCell, which assesses the enrichment of regulon activity in individual cells.

Establishment of a Rat Tail Intervertebral Disc Degeneration Model by Needle Puncture and Intervention Administration

Twelve male Sprague-Dawley rats, eight weeks old and weighing approximately 300 grams, were used to establish a caudal intervertebral disc degeneration model. Inclusion criteria required the absence of congenital spinal deformities, prior trauma, infection, metabolic disease, immunodeficiency, and abnormal physiological parameters. Exclusion criteria included: (1) fractures, deformities, or congenital defects of the caudal vertebrae or intervertebral discs; skin damage, infection, or scarring in the surgical area; (2) recent exposure (within two weeks) to surgery, drug administration, or radiation, or prior participation in experiments; (3) restricted mobility, abnormal gait, pain behavior, significant weight loss (>10%), or abnormal food and water intake; and (4) procedural accidents during modeling, such as nerve or vascular injury from needle insertion. Following a two-week acclimatization period, the rats were randomly assigned to three groups: control, needle puncture + aspirin, and needle puncture only. Under 4% isoflurane anesthesia (maintained at 2% via a face mask), rats were placed in a prone position and immobilized. The intervertebral disc spaces at C6/7, C7/8, and C8/9 were identified and marked. The skin was sterilized with povidone-iodine, and a 21-gauge hypodermic needle was inserted dorsally into the C7/8 and C8/9 discs to a depth of 5 mm, guided by a depth-limiting cap. The needle was rotated 360° for 30 seconds before withdrawal to induce degeneration. For intervention, a 31-gauge microsyringe was used to inject 2 μL of aspirin solution into the C7/8 disc and 2 μL of phosphate-buffered saline (PBS) into the C8/9 disc along the puncture track. A similar procedure was performed at the C6/7 disc, where 2 μL of PBS was injected as a control. Following the procedure, injection sites were examined for bleeding and re-sterilized. Isoflurane was discontinued, and animals were monitored until full recovery before being returned to standard housing with free access to food and water.

Radiographic and MRI Evaluation of Rat Tail Vertebrae

Four weeks after the induction of intervertebral disc degeneration and corresponding interventions, radiographic (X-ray) and magnetic resonance imaging (MRI) examinations of the rat tail vertebrae were performed. The rats were anesthetized with 4% isoflurane for induction and maintained with 2% isoflurane inhalation. They were positioned in the prone position and secured with adhesive tape to ensure the tail remained straight during imaging. Sequential X-ray and MRI scans were conducted.

Preparation of Rat Intervertebral Disc Tissue Sections

Following radiographic and MRI evaluations, rats were euthanized by CO_2 inhalation, and tail vertebrae were harvested for histological analysis. The tail was transected at the upper vertebral body of the C6/7 intervertebral space. Skin was removed, and the tail was rinsed thoroughly with phosphate-buffered saline (PBS) before fixation in 4%

paraformaldehyde for 48 hours. After fixation, residual paraformaldehyde was removed by repeated PBS washes, and the tail was decalcified in freshly prepared 10% EDTA-2Na solution, with the decalcification solution replaced every 2–3 days for a total of 8–12 weeks. Decalcification was considered complete when a 21G needle could penetrate the vertebral body without resistance. The intervertebral discs at C6/7, C7/8, and C8/9, along with adjacent vertebral bodies, were isolated and trimmed into tissue blocks. Each block was oriented with the transverse process trimmed to create a flat surface and placed into embedding cassettes with appropriate labeling. Tissue Processing and Embedding: Decalcified samples were dehydrated and embedded in paraffin using the following sequence: 70% ethanol (48 h) → 80% ethanol (2 h) → 90% ethanol (2 h) → 95% ethanol (2 h) → absolute ethanol I (2 h) → absolute ethanol II (2 h) → absolute ethanol III (1.5 h) → xylene I (1 h) → xylene II (1 h) → xylene III (0.5 h) → molten paraffin I (3 h) → molten paraffin II (3 h).

Tissue blocks were oriented with the trimmed surface at the mold base, embedded in paraffin, and cooled on a refrigeration unit until fully solidified. Sectioning: Paraffin blocks were sectioned at a thickness of 5 μm using an ultramicrotome. Sections were floated on 40°C water, stretched, and mounted onto slides. Slides were dried at 60°C for 2 hours to complete preparation.

Hematoxylin and Eosin (H&E) Staining

Paraffin-embedded tissue sections (5 μm) were deparaffinized in xylene, rehydrated through a graded ethanol series, and rinsed in distilled water. Sections were stained with hematoxylin for 3–5 minutes, differentiated in 1% acid alcohol, blued in tap water, and counterstained with eosin for 1–3 minutes. After dehydration through graded ethanol and clearing in xylene, coverslips were mounted with a resin-based medium. Stained sections were examined under a light microscope.

Safranin O–Fast Green Staining

Paraffin-embedded tissue sections (5 μm) were deparaffinized in xylene and rehydrated through a graded ethanol series. Sections were stained with Fast Green solution (0.1%) for 3 minutes and rinsed briefly in 1% acetic acid to remove excess stain. Safranin O solution (0.1%) was then applied for 5 minutes. After rinsing in distilled water, sections were dehydrated through graded ethanol, cleared in xylene, and mounted with a resin-based medium. Stained sections were observed under a light microscope.

Cell Culture

Human intervertebral disc nucleus pulposus cells were purchased from Procell (#CP-H097, Wuhan, China) and passed to the third generation for experimental purposes. Cells were cultured in DMEM/F12 medium (#C11330500BT, Gibco) supplemented with 10% FBS (#10099141C, Gibco) and 100 μg/mL penicillin/streptomycin (#C0222, Beyotime). Cells were maintained in a humidified incubator at 37°C with 5% CO_2. The cell model of Intervertebral disc degeneration (IVDD) was established using LPS (#L-2880, Sigma-Aldrich) according to the scheme proposed by Fan et al.[24]

CCK-8

Cells were plated onto a 96-well plate at a density of 1×10^4 cells per well, with a medium volume of 100 μL. After 48 hours of treatment with LPS, 10μL CCK-8 solution was added to each well and incubated at 37°C for 1 hour. Absorbance was measured at 450 nm using a VersaMax microplate reader (TECAN M1000 Pro).

Flow Cytometry Assay

For cell death assay, cells were stained with 1 μM Propidium Iodide (#ST511, Beyotime) and analyzed by flow cytometry after incubation.

For ROS assay, cells were incubated with 1 μM DCFH-DA (#S0033S, Beyotime) at 37°C for 30 min, followed by flow cytometry analysis.

Immunofluorescent Staining

Cells were anchored to the coverslip and washed thrice in PBS before being fixed in 4% paraformaldehyde for 15 min, than rinsed in PBS, stained with BODIPY 493/503 (#C2053S, Beyotime) for 30 min at 37°C, and imaged using confocal microscopy (OLYMPUS FV3000).

Western Blot

Proteins were extracted using RIPA lysis buffer (#P0013B, Beyotime). Total protein level quantified using the BCA method (#P0009, Beyotime), the denatured protein was separated by SDS-PAGE, transferred to PVDF membranes, blocked with 5% fat-free milk, and probed with primary antibodies overnight at 4°C. Membranes were then incubated with HRP-conjugated secondary antibodies and visualized using chemiluminescent reagents. The following primary antibodies were used in this study: GPX4 Rabbit mAb (#59735), GAPDH Rabbit mAb (#2118), Anti-rabbit IgG, HRP-linked Antibody (#7074), all purchased from CST.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. A *t*-test was applied to analyze group differences for normally distributed variables, with the Mann–Whitney *U*-test used for non-normally distributed variables. A p-value of less than 0.05 was considered statistically significant.

Results

Multi-Cohort Single-Cell Analysis Reveals Functional Remodeling of Nucleus Pulposus Cells in Intervertebral Disc Degeneration

We integrated single-cell sequencing data from NPCs samples of intervertebral discs with varying degrees of degeneration across four independent cohorts.[20,21,23] A total of 24 samples passed quality control, comprising 91,131 NPCs. Based on the Pfirrmann grading system, samples were categorized into mild degenerative disc (MDD, grades I–II, n=9) and severe degenerative disc (SDD, grades III–V, n=15) groups. Differential gene expression analysis between SDD and MDD NPCs identified 521 differentially expressed genes (DEGs) ($|\log_2 FC| > 0.25$, adj. p-value < 0.05), including 284 upregulated and 237 downregulated genes. GO enrichment analysis revealed significant enrichment of these DEGs in pathways related to apoptosis, vascular development, skeletal system development, and collagen fibril organization (Figure 1A). Consistent with the activation of angiogenesis during disc degeneration, our results further confirmed the vascularization processes in SDD. Furthermore, disruptions in collagen and fiber metabolism indicated ongoing ECM remodeling. KEGG pathway analysis showed significant enrichment of DEGs in pathways such as protein processing in the endoplasmic reticulum, cytoskeleton organization, ferroptosis, focal adhesion, and ECM-receptor interactions (Figure 1B). Focal adhesion, a primary interface for cell-ECM interactions, involves the interplay between cytoskeletal structures and ECM receptors. Our findings suggest that NPCs in the SDD group exhibit matrix-fibrotic characteristics. Additionally, the protein processing pathway in the endoplasmic reticulum may regulate ferroptosis-related protein degradation, accelerating ferroptosis.[25] Gene set enrichment analysis further demonstrated the activation of matrix-fibrotic features in NPCs of SDD, including processes related to cell adhesion, collagen fibril organization, and cell junction assembly (Figure 1C). In contrast, GSEA revealed significant suppression of cellular oxidant detoxification in SDD, potentially impairing ROS-induced stress responses and reducing p53-mediated ferroptosis inhibition,[26] thereby promoting ferroptosis. Our study reveals substantial remodeling of NPC characteristics during disc degeneration, involving key biological processes such as matrix reconstruction, cell adhesion, and ferroptosis.

Single-Cell Atlas of NPCs via Cellular Heterogeneity Analysis

To further explore the heterogeneity of NPCs, we performed cellular subclustering, identifying three major types: matrix-NPCs (mNPC), apoptosis-NPCs (aNPC), and inflammation-NPCs (iNPC) (Figure 2A). Based on specific cell marker genes, these NPCs were further categorized into eight subtypes: COL9A3+mNPC, SOD3+mNPC, COL14A+mNPC, MMP14+mNPC, CDKN1A+aNPC, ABI3BP+mNPC, LTBP1+mNPC, and SLC7A2+iNPC (Figure 2A). UMAP analysis

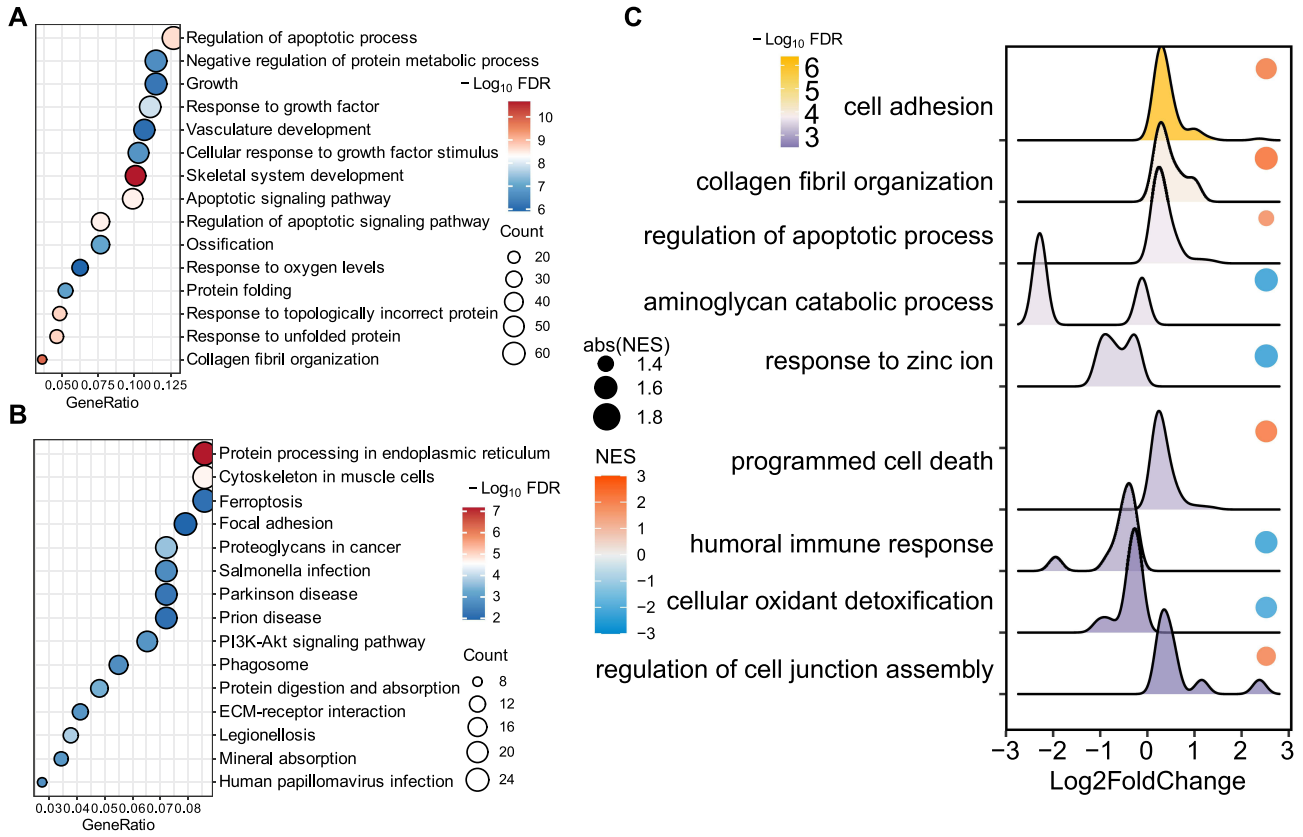

Figure 1 (**A**) GO enrichment analysis of differentially expressed genes. (**B**) KEGG pathway enrichment analysis of differentially expressed genes. (**C**) GSEA enrichment analysis.

revealed the distribution patterns of these subtypes under different degenerative conditions (Figure 2B), with differential gene expression identified for each subtype (Figure 2C). We observed that ABI3BP+mNPCs exhibited upregulation of COL2A1 expression, accompanied by downregulation of COL1A2 and COL3A1. COL2A1, encoding type II collagen, is a key structural component of cartilage, with degradation mediated by matrix metalloproteinases (MMPs), contributing to cartilage degeneration. In contrast, COL1A2 and COL3A1 regulate collagen degradation via interaction with MMPs (eg, MMP-1, MMP-2). This suggests that ABI3BP+mNPCs may help maintain ECM balance and homeostasis in the early stages, thus protecting the nucleus pulposus structure and disc ecology. We also found aberrant expression of multiple CXCL family proteins in mNPCs such as COL14A+mNPC and LTBP1+mNPC.These proteins play crucial roles in ECM remodeling during IVDD, primarily affecting tissue repair and regeneration through modulation of cell migration, inflammation, angiogenesis, and fibrosis. This indicates that COL14A+mNPC and LTBP1+mNPC may regulate ECM remodeling during IVDD progression. Furthermore, we observed significant downregulation of GPX4 expression in late-stage apoptosis-type NPCs (CDKN1A+aNPC). GPX4, a key antioxidant enzyme, regulates cellular redox homeostasis to prevent ferroptosis.[27,28] The reduced expression of GPX4 suggests the activation of ferroptosis in CDKN1A+aNPCs.In summary, our study highlights the heterogeneous characteristics of NPCs in IVDD samples, including ECM remodeling and the activation of ferroptosis. These findings provide new insights into the complex matrix degeneration and apoptotic responses in nucleus pulposus tissue, offering a novel perspective on the pathogenesis of IVDD.

Dynamic Shifts in Cell Subtypes Reflect Intervertebral Disc Degeneration Progression

We analyzed the proportional changes of different NPC subtypes across various stages of IVDD (Figure 3A). We found that the ferroptosis-associated subtype CDKN1A+aNPC increased progressively with disease progression, while matrix-supporting cells like ABI3BP+mNPC and SOD3+mNPC significantly decreased in the late degeneration stages. Conversely, the proportion of ECM remodeling-related cells, such as LTBP1+mNPC, increased, suggesting that dynamic

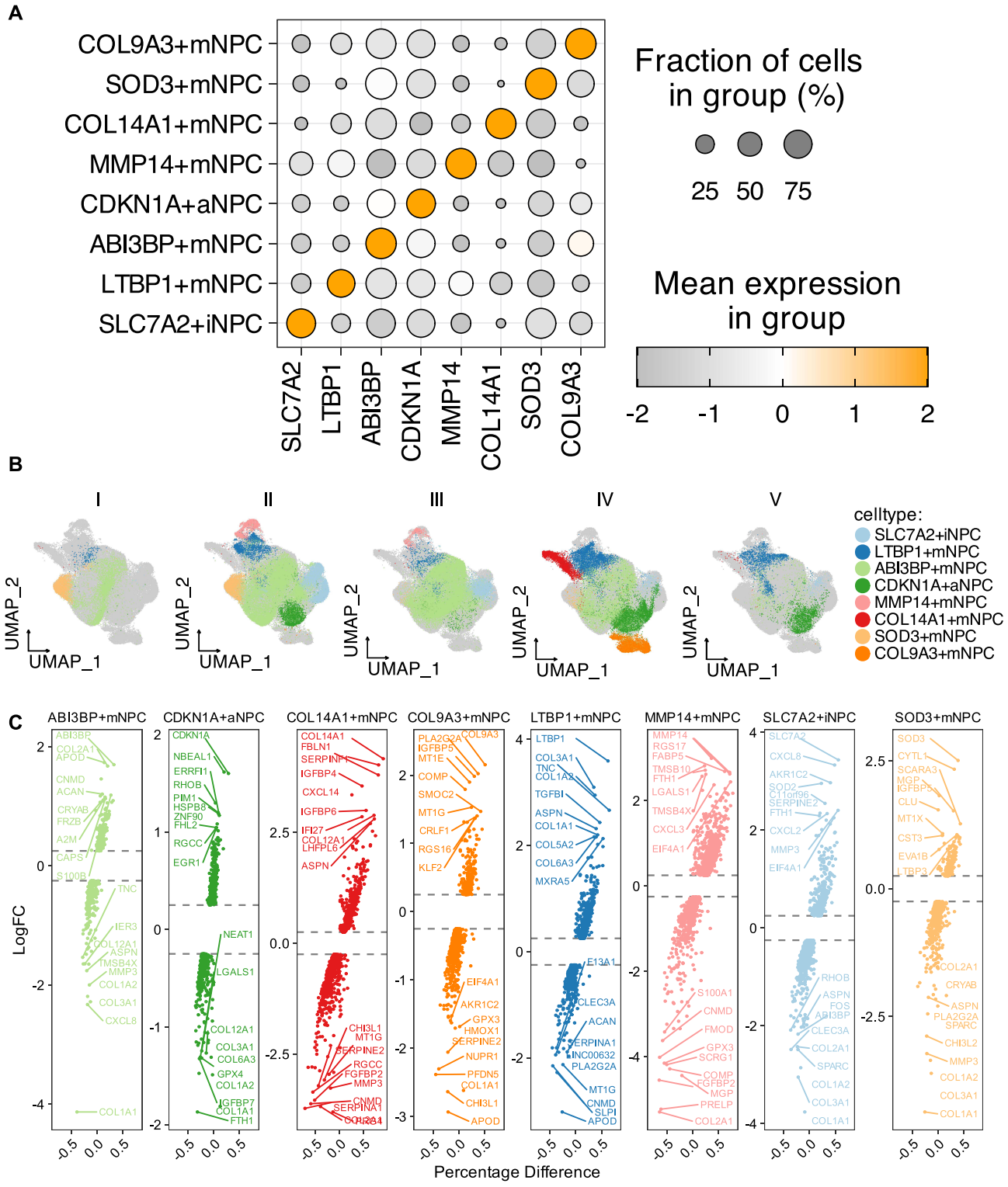

Figure 2 (**A**) Gene expression bubble plot for cell annotation. (**B**) Dimensionality reduction landscape of different cell subgroups at various disease stages. (**C**) Differentially expressed genes in each cell subgroup.

changes in NPC proportions are key features of IVDD. Cell preference analysis (Ro/e)[29] revealed that CDKN1A+aNPC and LTBP1+mNPC predominated in stages IV–V of degeneration, while ABI3BP+mNPC and SOD3+mNPC were concentrated in stages I–III (Figure 3B), consistent with the observed proportional trends. Functional enrichment analysis showed that ABI3BP+mNPC and SOD3+mNPC were enriched in growth factor and skeletal development pathways,

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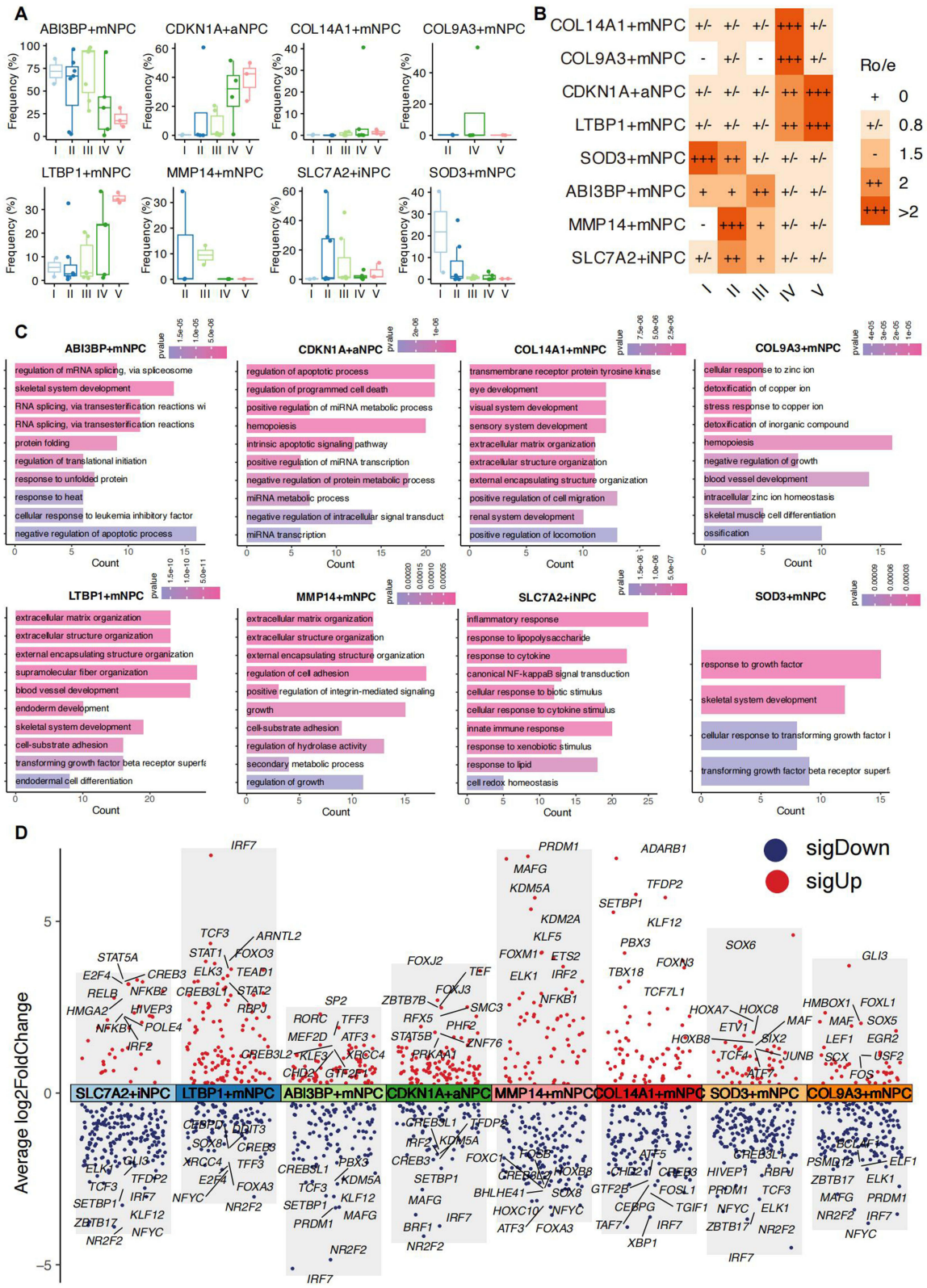

Figure 3 (**A**) Analysis of cell proportions in different disease stages for each cell subgroup. (**B**) Ro/e analysis of cell proportion preferences. (**C**) Enrichment analysis of the top 100 upregulated genes in each cell subgroup. (**D**) Differential transcription factor activity analysis across cell subgroups.

highlighting their role in maintaining disc homeostasis. CDKN1A+aNPC was enriched in apoptosis and programmed cell death pathways, potentially driving degenerative progression by disrupting tissue structure. Matrix-related subtypes (eg, COL14A+mNPC and MMP14+mNPC) were primarily involved in ECM synthesis and degradation, emphasizing the importance of matrix remodeling (Figure 3C). Further analysis of transcription factor activity revealed that KLF12 and ELK1 were downregulated in early degeneration-associated cells (eg, ABI3BP+mNPC and SLC7A2+iNPC), suggesting suppressed matrix function and stress responses. In contrast, NR2F2 was downregulated in late degeneration-associated cells, indicating enhanced inflammatory and apoptotic responses. Additionally, the angiogenesis factor RBPJ was activated in LTBP1+mNPC but inhibited in SOD3+mNPC, revealing dynamic changes in angiogenesis during degeneration (Figure 3D).

Ferroptosis Is Significantly Activated in SDD

We observed an increased proportion of CDKN1A+aNPC in SDD. Pathway enrichment analysis of these cells further highlighted a significant activation of ferroptosis pathways. To further validate this, we analyzed the expression of key ferroptosis-regulating factors in NPCs from stages I–V (Figure 4A). Our results revealed a significant downregulation of GPX4 and upregulation of CHAC1, TGFBR1, SMAD7, and CTSB as degeneration progressed. Glutathione, a cofactor for GPX4, is reduced by CHAC1-mediated degradation, thereby impairing GPX4's antioxidant function.[30,31] TGFBR1 activation modulates oxidative and inflammatory responses, indirectly influencing ferroptosis.[32] SMAD7, through TGF-β inhibition, increases oxidative stress, potentially promoting ferroptosis. CTSB contributes to lipid peroxidation and membrane damage, amplifying iron accumulation and oxidative stress, thus accelerating ferroptosis. In summary, our findings suggest that ferroptosis predominantly drives the characteristics of CDKN1A+aNPC. We initially stimulated NPCs with different concentrations of LPS. Cell viability assays revealed a gradual decrease in cell viability with increasing LPS concentrations (Figure 4B). Flow cytometry further demonstrated a significant increase in apoptotic cells as LPS concentrations increased, particularly when the concentration reached 10 μg/mL (Figure 4C). Lipid peroxidation and elevated intracellular reactive oxygen species (ROS) levels are fundamental to ferroptosis. BODIPY staining showed a marked increase in lipid peroxidation upon stimulation with 10 μg/mL LPS (Figure 4D), a finding corroborated by flow cytometric analysis of intracellular ROS levels (Figure 4E).

Aspirin Alleviates Intervertebral Disc Degeneration

Based on our previous clinical observations, aspirin effectively alleviates low back pain associated with intervertebral disc degeneration. Using an acupuncture-induced rat model of disc degeneration, we imaged the tail discs of rats in the control, acupuncture, and aspirin-treated groups using X-ray (Figure 5A). Our results showed that the disc height index was significantly lower in the acupuncture model group compared to the control, while aspirin treatment improved the disc height index after acupuncture (Figure 5B). MRI scans further revealed that the mean disc signal intensity was significantly higher in the aspirin-treated group compared to the acupuncture group (Figure 5C and D).The standard deviation of disc signal intensity in the three groups showed that the aspirin-treated group had the highest standard deviation, the acupuncture model group the lowest, and the control group in between (Figure 5E).This indirectly suggests that signal intensity was more uniform in the control and acupuncture model groups, with control disc signals fluctuating within the normal range and acupuncture model signals within the degenerative range. However, aspirin treatment after acupuncture led to recovery of some degenerative disc tissue, resulting in a larger SD of signal intensity in the aspirin group.

Subsequently, mid-sagittal paraffin sections of the caudal intervertebral discs from three groups of rats were subjected to HE staining and Safranin O–Fast Green staining (Figure 5F and G). In the control group, the stained intervertebral disc tissue displayed a smooth, ring-like annulus fibrosus with orderly and continuous arrangement, abundant and evenly distributed extracellular matrix, plump and numerous nucleus pulposus cells, and an intact, undamaged cartilage endplate. In the degeneration group induced by needle puncture, the annulus fibrosus appeared disrupted and disorganized, with a marked reduction in nucleus pulposus cells, severe extracellular matrix degradation, pronounced fibrosis in the nucleus pulposus region, and incomplete endplate integrity with varying degrees of fibrosis. The histological degeneration score of the intervertebral disc was significantly increased. In the aspirin-treated group following needle

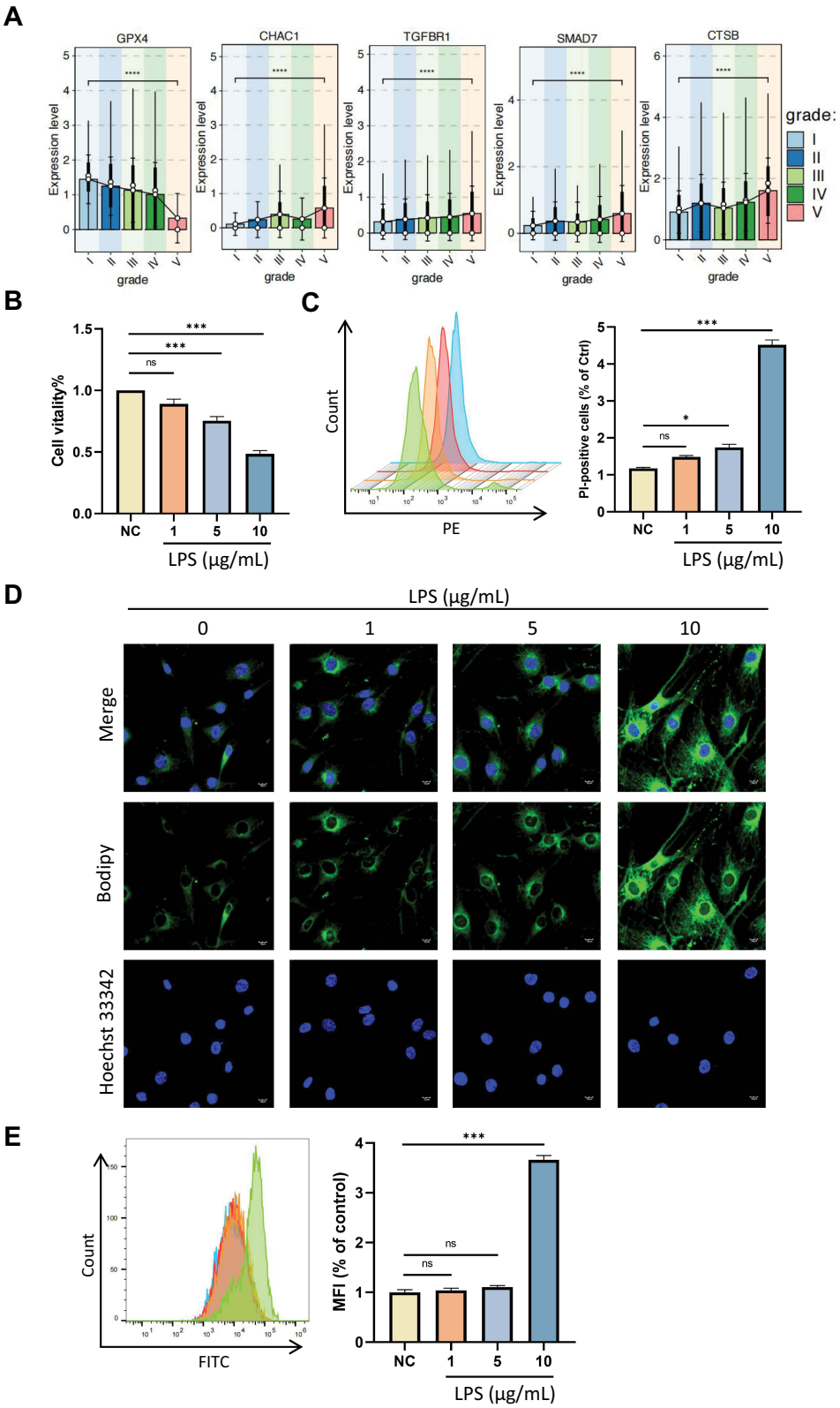

Figure 4 (**A**) Expression levels of ferroptosis inhibitory gene GPX4 and ferroptosis-activating genes CHAC1, TGFBR1, SMAD7, and CTSB at different stages. (**B**) Cell viability of human intervertebral disc nucleus pulposus (NPC) cells treated with varying concentrations (1, 5, and 10 mg/L) of LPS for 48 hours, measured by CCK-8 assay. (**C**) Representative PE fluorescence histograms of NPCs showing necrotic cells stained with PI, along with the quantitative results of necrotic percentages. (**D**) Representative fluorescent images depicting lipid peroxidation levels in NPCs, stained green with BODIPY, and nuclei stained blue with Hoechst 33342. (**E**) Representative FITC fluorescence histograms of NPCs showing cytosolic ROS production using DCFH-DA dye, with quantitative analysis of mean fluorescence intensity (MFI). *p < 0.05, ***p < 0.001 and ****p < 0.0001.

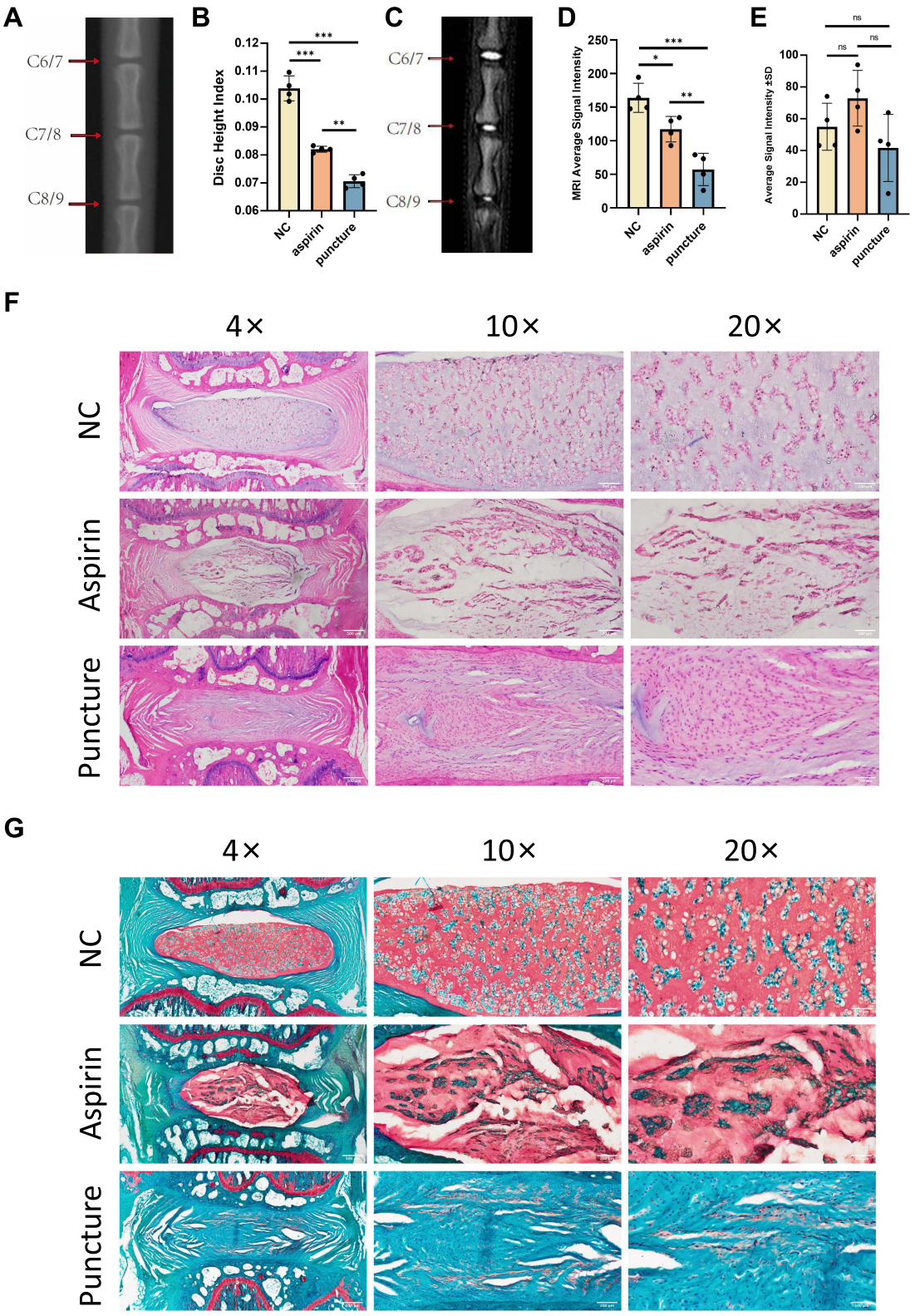

Figure 5 (**A** and **B**) X-ray images of the intervertebral disc in the rat coccygeal vertebra. (**C–E**) MRI images of the intervertebral disc in the rat coccygeal vertebra. (**F**) Representative HE-stained image. (**G**) Representative SO/FG-stained image. *p < 0.05, **p < 0.01, ***p < 0.001.

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puncture-induced degeneration, partial fibrosis and discontinuity of the annulus fibrosus were observed, exhibiting a serpentine appearance. The nucleus pulposus cells were reduced and shrunken, with partial degradation of the extracellular matrix. Fibrosis was evident in the nucleus pulposus region, and the cartilage endplate showed partial discontinuity and damage.

Aspirin Inhibits Ferroptosis by Targeting GPX4

Our prior bioinformatics analysis revealed significant downregulation of GPX4 expression in patients with severe nucleus pulposus degeneration. Clinically, high-dose aspirin alleviates disease progression in these patients. To investigate whether aspirin targets GPX4 to inhibit ferroptosis, we employed molecular docking. Our results showed an affinity of −9.837 kcal/mol between aspirin and GPX4, suggesting stable binding. Aspirin primarily interacts with the TRP-136, PRO-155, and LYS-48 residues of GPX4 (Figure 6A). This was further validated in our cell model, where Western blot analysis confirmed reduced GPX4 levels (Figure 6B). Additionally, high-dose aspirin significantly reduced lipid peroxidation and intracellular ROS production (Figure 6C and D) while increasing GPX4 expression (Figure 6E). These findings confirm aspirin's inhibitory effects on ferroptosis and its protective role in nucleus pulposus cells.

Discussion

Intervertebral disc degeneration (IVD) is a common form of spinal degenerative disease, characterized by progressive deterioration of disc structure and function, leading to pathological changes.[33] IVDD results in a range of symptoms, including back or neck pain, disc herniation, and spinal stenosis, imposing a significant social and economic burden.[34] The degeneration process involves gradual structural changes accompanied by severe alterations in metabolic homeostasis,[35] including apoptosis and necrosis of NPCs, which not only reduces the number of functional cells but also releases inflammatory factors that further exacerbate the disc microenvironment, driving disease progression.[24,36] In this study, we integrated multi-cohort single-cell transcriptomic data to systematically analyze the dynamic changes of NPC subtypes during disc degeneration, with a focus on the potential role of ferroptosis. We reannotated NPCs into eight subtypes and found that the CDKN1A+aNPC, associated with ferroptosis, gradually increased during disease progression, while the number of matrix-associated cells significantly decreased in the later stages of degeneration, accompanied by an increase in ECM remodeling-associated cells. Our findings highlight the complex interplay between matrix degeneration and cell apoptosis in degenerated discs, revealing the central role of ferroptosis in disease pathogenesis and proposing for the first time the potential of ferroptosis modulation as an intervention for IVDD, offering new perspectives for molecular mechanism research and targeted therapy.

The intervertebral disc consists of the nucleus pulposus, annulus fibrosus, and cartilage endplate,[37] which together effectively buffer stress and distribute loads to maintain spinal function under normal conditions. However, during degeneration, a series of changes, including reduced water content in the nucleus pulposus, degradation of the annulus fibrosus, and calcification of the endplate, gradually impair the mechanical function of the disc.These degenerative processes lay the foundation for IVDD, whose primary molecular mechanisms involve cellular senescence and apoptosis, changes in matrix components, inflammation, mechanical loading and damage, and microcirculatory disorders.[38–40] Studies have shown that inflammation and oxidative stress significantly accelerate apoptosis or necroptosis of IVD cells, particularly NPCs, leading to irreversible disc dysfunction. Meanwhile, chemokines released from degenerated discs attract the infiltration and activation of T cells, B cells, macrophages, neutrophils, and mast cells, amplifying the inflammatory cascade.[41,42] As immune cells migrate to the intervertebral disc, the formation of new microvessels and nerve fibers in the dorsal root ganglion (DRG) occurs.[43] In this inflammatory microenvironment, both IVD and immune cells secrete neurotrophic factors, particularly nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which promote the expression of pain-related ion channels in the DRG. The depolarization of these channels exacerbates both discogenic and radicular pain, while also amplifying the cytokine-mediated degenerative cascade. Furthermore, inflammation directly alters the microenvironment of NPCs, inducing apoptosis or necroptosis and driving the progression of IVDD. However, the exact underlying mechanisms remain unclear. Recent studies have showed that Grem1 accelerates NPC apoptosis and disc degeneration by inhibiting TGF-β-mediated Smad2/3 phosphorylation.[44] Cell aging impairs the repair of necrotic and apoptotic cells, leading to a reduction in metabolically active cells and a decline in

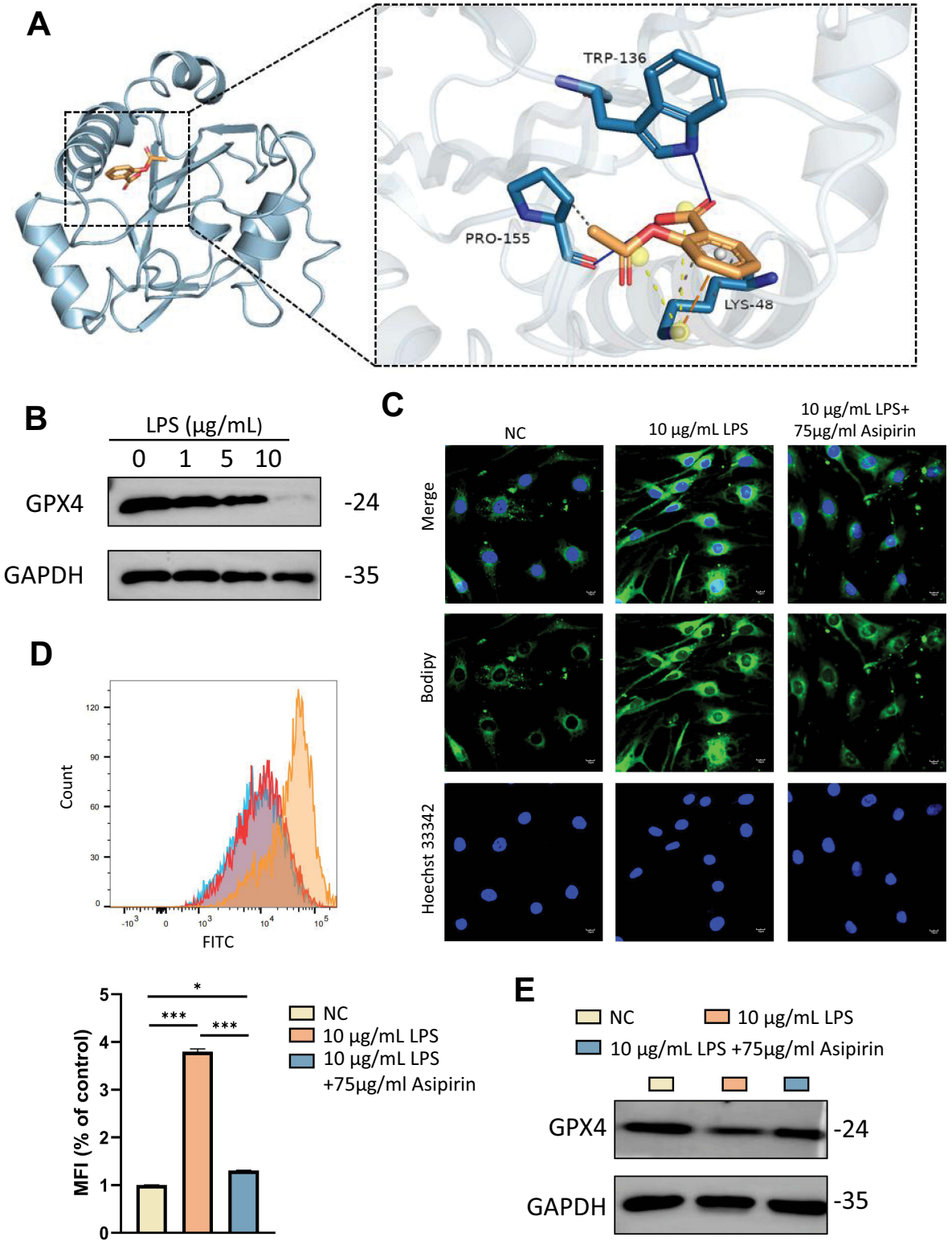

Figure 6 (**A**) Schematic model of aspirin binding to GPX4. (**B**) Western blot analysis of ferroptosis biomarker protein GPX4 expression in NPCs. (**C**) Representative fluorescent images of lipid peroxidation levels in NPCs, stained green with BODIPY and nuclei stained blue with Hoechst 33342. (**D**) Representative FITC fluorescence histograms of NPCs showing cytosolic ROS production using DCFH-DA dye, with quantitative analysis of mean fluorescence intensity (MFI). (**E**) Western blot analysis of GPX4 expression in NPCs as a ferroptosis biomarker. *p < 0.05, **p < 0.01, ***p < 0.001.

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ECM turnover. This shift toward catabolism progressively causes ECM degradation, structural failure, and ultimately, complete disc degeneration.[45] Additionally, weight gain and increased BMI are confirmed independent risk factors for IVDD.[46,47] Adipokines, initially identified as regulators of energy metabolism, are now recognized for their critical roles in immune responses and inflammation.[48,49] Excessive secretion of adipokines correlates significantly with accelerated apoptosis, autophagy, and ECM degradation in IVDD.[50] Our study first proves ferroptosis as a central mechanism in the progression of IVDD, providing new insights into the molecular mechanisms of disc degeneration. We found that in severely degenerated nucleus pulposus tissue, expression of the key ferroptosis enzyme GPX4 was markedly downregulated, while lipid peroxidation levels were elevated. Lipid peroxidation generates lipid peroxides, which disrupt membrane structures and degrade into toxic byproducts, such as 4-HNE and MDA, further aggravating cellular damage and triggering ferroptosis. GPX4, a crucial inhibitor of ferroptosis,[51] reduces lipid peroxides to harmless lipid alcohols, thereby protecting cells from ferroptosis. Inactivation or downregulation of GPX4, however, leads to uncontrolled lipid peroxidation, triggering ferroptosis.

The clinical management of IVDD currently involves both conservative and surgical treatments, such as physical therapy, pharmacological interventions, and surgical procedures, including discectomy, spinal fusion, and artificial disc replacement.[52] While these approaches can effectively alleviate symptoms and reduce pain, and may prevent degeneration of adjacent segments in some cases, they do not reverse the degenerative process of the intervertebral disc. Consequently, the treatment of IVDD remains challenging, necessitating further investigation into its underlying pathological mechanisms and the development of novel technologies and therapeutic strategies aimed at halting or reversing disc degeneration. Recent research has highlighted mesenchymal stem cell (MSC) transplantation as a promising therapeutic avenue,[53] showing potential through the promotion of nucleus pulposus cell differentiation, secretion of anti-inflammatory factors, and enhancement of matrix repair. Furthermore, the use of nanoscaffold materials and bioactive molecule complexes for reconstructing the structure and function of degenerated discs has been explored. Alongside this, the identification of highly sensitive and specific molecular biomarkers for early IVDD screening has become a key research focus. Personalized treatment strategies, informed by individual genetic profiles and pathological conditions, are expected to be a central component of future therapeutic approaches. At the molecular level, disc degeneration involves complex biological processes, including the upregulation of matrix-degrading metalloproteinases (MMPs) and ADAMTS proteins, as well as heightened oxidative stress.[54] While anti-inflammatory drugs and oxidative stress inhibitors have demonstrated some therapeutic potential, the regulation of apoptosis, particularly ferroptosis, remains underexplored. The role of ferroptosis in IVDD progression is not yet fully understood. Compared with traditional anti-inflammatory or antioxidant therapies, ferroptosis regulation offers distinct advantages, as it is a cell-specific form of death with a well-defined mechanism and a single target, thereby offering a new opportunity for precise therapeutic intervention. This study elucidates the significant role of ferroptosis in IVDD and explores the potential repurposing of aspirin, a commonly used drug, providing new insights for clinical intervention strategies.

Aspirin, a well-established non-steroidal anti-inflammatory drug (NSAID), is known to inhibit cyclooxygenase (COX) and platelet aggregation.[55] Recent studies suggest that aspirin may also modulate ferroptosis,[56] through mechanisms such as inhibition of lipid peroxidation and regulation of inflammatory responses, thereby protecting cells from ferroptosis-induced damage. However, the precise role of aspirin in IVDD remains unclear. In this study, we found significant downregulation of GPX4, a key regulator of ferroptosis, in severely degenerated nucleus pulposus tissue, potentially leading to increased lipid peroxidation and accelerated cell death. To further explore aspirin's potential in IVDD, we developed a rat model of disc degeneration and treated it with aspirin. Results indicated that aspirin treatment significantly reduced disc fibrosis and degeneration compared to controls. Additionally, in vitro experiments demonstrated that high-dose aspirin effectively inhibited ferroptosis by upregulating GPX4 expression, reducing lipid peroxidation and reactive oxygen species (ROS) accumulation, thus protecting the function of nucleus pulposus cells. GPX4 plays a central role in ferroptosis by reducing lipid peroxides, thereby preventing ferroptosis.[27,28] Our findings suggest that aspirin, by targeting GPX4 to regulate ferroptosis, can attenuate IVDD progression, providing new evidence for its potential use in disc degeneration.

Although our findings provide valuable insights into the role of ferroptosis in IVDD, several limitations should be acknowledged. First, our experimental models are primarily based on in vitro systems and animal studies, which may

limit the generalizability of the results to human clinical settings. Second, while ferroptosis responses are implicated as a key pathological mechanism in IVDD, the degenerative cascade involves multifaceted interactions among molecular pathways, cellular stress responses, and biomechanical factors. Targeting a single pathway, such as GPX4-mediated antioxidant defense, may be insufficient to address the complexity of IVDD progression. Third, the therapeutic potential of aspirin remains incompletely understood. Common side effects, such as gastrointestinal irritation, bleeding tendency, and allergic reactions, as well as dose-related and long-term adverse effects including salicylate toxicity and hepatic or renal impairment, limit its widespread clinical application. Therefore, its optimal dosing regimen, long-term biosafety, and translational applicability across different patient populations require further investigation. Future studies should prioritize multidimensional approaches to address these limitations. For instance, elucidating the interplay between GPX4 and other ferroptosis-regulating factors may uncover novel combinatorial therapeutic targets. Moreover, robust clinical validation through multicenter randomized controlled trials is essential for assessing the efficacy, safety, and dose-response relationships of ferroptosis-targeted interventions in humans. Despite these challenges, modulating ferroptosis represents a promising frontier in IVDD therapy. By integrating mechanistic and translational research, we anticipate that targeted interventions could attenuate pathological disc degeneration while preserving tissue homeostasis, ultimately improving clinical outcomes for patients with IVDD.

Conclusion

The increased proportion of CDKN1A^{+}aNPC cells serves as an early warning feature for the progression of IVDD. Aspirin stabilizes the targeting of GPX4, thereby inhibiting ferroptosis and exerting therapeutic effects on IVDD.

Ethical Approval and Informed Consent

The animal experiment conducted in this study were approved by the Ethics Committee of Hebei Medical University Third Hospital. The ethical review follows the "Guidelines for Ethical Review of Laboratory Animal Welfare GB/T35892" of the People's Republic of China (Ethics approval number: Z2024-054-1).

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Disclosure

The authors declare that there are no conflicts of interest in this study.

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