

Protective effects of PDGF-AB/BB against cellular senescence in human intervertebral disc

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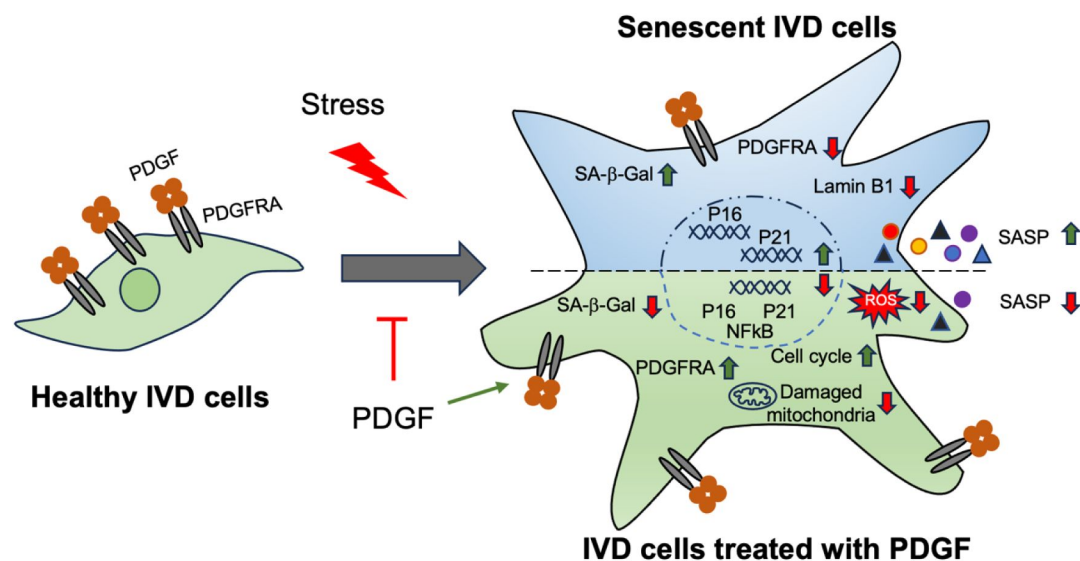
This **important** study demonstrates the therapeutic potential of recombinant human PDGF-AB/BB proteins in reducing the senescent signatures of primary human intervertebral disc cells. The study represents a significant advance in the treatment of intervertebral disc degeneration and can be applied broadly to other degenerative musculoskeletal tissues through the suppression of senescence. **Solid** evidence, primarily based on transcriptomic analysis and direct protein measurements from relatively homogeneous cell populations, supports the therapeutic potential of PDGF, but more experimental details would be needed to make the evidence stronger.

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Abstract

Cellular senescence, characterized by a permanent state of cell cycle arrest and a secretory phenotype contributing to inflammation and tissue deterioration, has emerged as a target for age-related interventions. Accumulation of senescent cells is closely linked with intervertebral disc (IVD) degeneration, a prevalent age-dependent chronic disorder causing low back pain. Previous studies have highlighted that platelet-derived growth factor (PDGF) mitigated IVD degeneration through anti-apoptosis, anti-inflammation, and pro-anabolism. However, its impact on IVD cell senescence remains elusive. In this study, human NP and AF cells derived from aged, degenerated IVDs were treated with recombinant human (rh) PDGF-AB/BB for 5 days and changes of transcriptome profiling were examined through mRNA sequencing. NP and AF cells demonstrated similar but distinct responses to the treatment. However, the effects of PDGF-AB and BB on human IVD cells were comparable. Specifically, PDGF-AB/BB treatment resulted in downregulation of gene clusters related to neurogenesis and response to mechanical stimulus in AF cells while the downregulated genes in NP cells were mainly associated with metabolic pathways. In both NP and AF cells, PDGF-AB and BB treatment upregulated the expression of genes involved in cell cycle regulation, mesenchymal cell differentiation, and response to reduced oxygen levels, while downregulating the expression of genes related to senescence associated phenotype,

including oxidative stress, reactive oxygen species (ROS), and mitochondria dysfunction. Network analysis revealed that PDGFRA and IL6 were the top hub genes in treated NP cells. Furthermore, in irradiation-induced senescent NP cells, PDGFRA gene expression was significantly reduced compared to non-irradiated cells. However, rhPDGF-AB/BB treatment increased PDGFRA expression and mitigated the senescence progression through increased cell population in the S phase, reduced SA- β -Gal activity, and decreased expression of senescence related regulators including P21, P16, IL6, and NF- κ B. Our findings reveal a novel anti-senescence role of PDGF in the IVD, demonstrating its ability to alleviate the senescent phenotype and protect against the progression of senescence. This makes it a promising candidate for preventing or treating IVD degeneration by targeting cellular senescence.



1 Introduction

Low back pain (LBP), ranked as the first cause of years lived with disability, is a prevalent condition. Although the etiology of LBP is multifactorial, a major contributor of LBP is intervertebral disc (IVD) degeneration, which is increasing exponentially due to a growing aged population worldwide (1). Current treatments, such as medicine, physical therapy, and surgical excision of the herniated tissues, only temporally alleviate painful symptoms (2). In fact, patients often experience persistent LBP after surgery (3). The fundamental cause of this is the inadequate knowledge of the underlying pathophysiology mechanisms leading to IVD degeneration. Therefore, it is crucial to understand the pathogenesis of IVD degeneration and develop novel therapeutic interventions to mitigate the degenerative processes.

The IVD consists of three compartments: the gelatinous nucleus pulposus (NP), fibrous annulus fibrosus (AF), and cartilaginous endplate (4). NP and AF tissues play distinct yet complementary roles in maintaining the spinal function. The NP has high content of proteoglycans and water, absorbing and distributing mechanical forces. In contrast, AF consists of multiple layers of concentric lamellae, which provides strength and stability to the disc and withstands compressive forces (4,5). To date, the main focus of IVD cell studies has been on the NP. It is often the first site impacted during aging and degeneration, indicating the crucial role of NP cells in the initiation of IVD degeneration. In contrast, degenerated alterations in the AF are more closely linked to pain and disability. As a result, effective therapeutic strategies for IVD degeneration should aim to restore the functionality of the entire disc unit.

Cellular senescence, triggered by normal cells in response to various intrinsic and extrinsic stressors, is a fundamental mechanism underlying age-related chronic diseases. Senescent cells are featured by irreversible growth arrest and acquire a senescent-associated secretory phenotype (SASP) and the secretion of pro-inflammatory cytokines, chemokines, and tissue-damaging proteases (6). Activation of P53/P21 and P16 pathways play a critical role in regulating senescence (7). In addition, NF- κ B pathway is a major pathway involved in the expression of pro-inflammatory mediators in senescent cells (8). Factors within the SASP can act in an autocrine manner to reinforce the senescent state and induce senescence in neighboring cells via paracrine signaling (9). This mechanism elucidates the accumulation of senescent cells with aging and its associated detrimental effects. In the IVD, it has been well established that the number of senescent cells increases with aging and IVD degeneration (10–12), as demonstrated by increased senescence-associated β -galactosidase (SA- β -Gal) positive cells and decreased cell proliferation. Growing research also demonstrates that targeting cellular senescence holds promise in alleviating the progression of IVD degeneration. Long-term systemic or local clearance of senescent cells by senolytic drugs mitigated the age-related IVD degeneration in mice (13) and reduced the expression of pro-inflammatory cytokines and matrix proteases and restored IVD structure in an injury-induced IVD degeneration model in rats (14). Cherif et al. and Mannarino et al. demonstrated that in humans, senolytic drugs o-Vanillin (15) and RG-7112 (16) attenuated NP cellular senescence, where a combination of these two senolytic drugs reduced the release of inflammatory factors and pain mediators in degenerated NP cells from low back pain patients (17).

PDGF is a major constituent of platelet rich plasma (PRP), which is widely used in the clinical setting for tissue regeneration and repair. It can be made of a homodimer of A, B, C, and D polypeptide chains, or an AB heterodimer (18,19). Among these, PDGF-AB and -BB are the predominant forms in PRPs (20). Two types of PDGFR (PDGFRA and PDGFRB) were identified that differ in ligand-binding specificity. PDGFRA binds to all PDGF chains but D chain while PDGFRB only binds to B and D chains (18,19). Upon binding, PDGF-PDGFR network induces a cascade of phosphorylation in pathways including cell proliferation, migration, and survival (21). We previously demonstrated that recombinant human PDGF-BB (rhPDGF-BB) inhibited cell apoptosis while promoting cell proliferation and matrix production in human IVD cells (22). Furthermore, when delivered via hydrogel, rhPDGF-BB successfully inhibited IVD degeneration and restored IVD biomechanical function in a rabbit preclinical model of puncture-induced IVD degeneration (23). In the current study, we hypothesize that PDGF-AB and -BB mitigated IVD degenerating through targeting cellular senescence. To test it, aged, degenerated human IVD cells were treated with rhPDGF-AB or -BB and transcriptome profiling was performed on treated NP and AF cells. We found similar but distinct responses to the treatment between NP and AF cells. In NP cells, PDGF-AB and -BB treatment upregulated the expression of PDGFRA and genes involved in cell cycle progression while downregulating the expression of genes related to ROS production, oxidative stress, and mitochondrial function. The alterations in these pathways indicated that the senescent phenotype was alleviated in human degenerated IVD cells treated with PDGF-AB/BB. Interestingly, in human senescent NP cells induced by irradiation, PDGFRA was significantly reduced while PDGF-AB/BB treatment prevented the progression of senescence through increased cell cycle progression and reduced SA- β -Gal staining and the expression of P21, P16, NF- κ B, and IL6. In contrast, AF cells treated with PDGF-AB/BB showed alleviated senescent phenotype independent of PDGFRA.

2 Methods

2.1 Sample collection and cell extraction

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. All experiments and procedures were reviewed and approved by the institutional review board of Emory University (IRB #00099028) approval. Human degenerated NP and AF tissues (Grade IV or V on Pfirrmann grade) were obtained from donors after discectomy surgeries for discogenic pain, with each donor providing written informed consent. Healthy NP and AF cells were gifted by Professor Lisbet A. Haglund from McGill University (Tissue Biobank #2019-4896). The donor information was listed in [Table 1](#). NP and AF cells were extracted as previously described (24). Isolated cells were expanded in growth medium containing low glucose DMEM, 10% FBS, 1X antibiotic-antimycotic (ThermoFisher Scientific, 15240062), and 50 µg/ml L-Ascorbic acid 2-phosphate (Sigma, A8960). Cells were grown at 37°C under 5% CO₂ and 20% O₂. To minimize the variability between experiments that may be caused by changes in cell behavior with each passage, cells of passage 3 were used for each experiment.

2.2 RNA-sequencing

To synchronize cells and enhance the sensitivity of cells to PDGF stimulation, degenerated NP (n = 5/each group) and AF cells (n = 6/each group) were serum-deprived in low glucose medium containing 0.2% FBS for 1 day before treating with PDGF-AB (40ng/ml) or -BB (20ng/ml) for 5 days. Total RNA was isolated with TRIzol (ThermoFisher Scientific, 15596018) and purified using miRNeasy kit (Qiagen, 217084). Genomic DNA contamination was eliminated through on-column DNase digestion. The concentration and quality of RNA were determined using Nanodrop and RNA integrity was assessed by Agilent 2200 Bioanalyzer. RNA samples (NP: n = 5, AF: n = 6) with RIN > 7 were shipped in dry ice to Novogene for RNA-sequencing, which was carried out using NovaSeq 6000 (Novogene Corporation Inc). Libraries were constructed from 0.2 µg RNA using TruSeq Stranded Total RNA Sample Prep Kit (Illumina). Quality control was performed for distribution of sequencing error rate and GC content and data filtering was performed to remove the low-quality reads and reads containing adapters. The resulting sequences were uploaded into the NCBI Sequence Read Archive (SRA) database (PRJNA1150962). The clean reads were mapped to human reference genome sequence using DNASTAR.

2.3 Differential gene expression analysis of RNA-seq data

Unnormalized raw counts exported from DNASTAR were used for pairwise gene expression comparison between untreated and PDGF-AB/BB samples. Differential expression genes (DEGs) were identified using DESeq2 package in R and DEGs with fold change greater than 1.5 and adjusted p value less than 0.05 were considered statistically significant. Heatmap and volcano plots and principal component analysis were performed to visualize the DEGs between untreated and PDGF-AB/BB samples.

2.4 Pathway enrichment analysis

To identify the biological process that DEGs were enriched, Gene ontology (GO) analysis was performed using upregulated and downregulated DEGs separately in R. The significantly enriched GO terms were visualized by dot plot analysis. GeneRatio in X-axis was calculated by the ratio of gene counts enriched in a particular GO term to all the gene counts annotated in the GO term.

Gene Set Enrichment Analysis (GSEA) was performed on all the genes to determine whether a predefined set of genes is significantly enriched in untreated or PDGF-BB samples. GSEA was conducted based on KEGG (Kyoto Encyclopedia of Genes and Genomes) database using clusterProfiler package in R (25) and a gene set was considered significantly enriched when the false discovery rate (FDR) was less than 0.05. Enrichment score (ES) was calculated to reflect the degree to which a gene set is overrepresented in our dataset, which was ranked based on fold change. Normalized enrichment score (NES) is the ES normalized to the gene set size.

Grade	Age	Sex	Cell type
4	67	M	NP & AF
5	61	M	NP & AF
5	61	M	NP & AF
4 or 5	65	F	NP & AF
4 or 5	81	F	AF
4	53	F	AF
4	64	F	NP
1	19	M	NP & AF
1	21	M	NP & AF
1	25	F	NP & AF
1	27	F	NP & AF

Table 1

Donor information.

2.5 Protein-protein interaction network analysis

The interactions among DEGs were performed using the Search Tool for the Retrieval of Interacting Genes (STRING) database (v12.0) with an interaction score greater than 0.4. Discrete proteins were removed, and the node files were exported and visualized in Cytospace (v3.10.0). To identify essential nodes in the interaction network, the betweenness centrality among the nodes was calculated using CytoNCA plugin (v2.1.6) in Cytospace (26 [↗](#)) and the top 10 hub nodes were identified using CytoHubba plugin (v0.1)(27 [↗](#)).

2.6 Cellular senescence induction by irradiation

To induce cellular senescence, healthy NP and AF cells (n=4/each group) were exposed to X-ray in a CellRad benchtop X-ray irradiator (Precision X-Ray) at a rate of 2.6 Gy/min. The cells were cultured in growth medium and exposed to indicated doses of ionizing radiation (IR) at 50% confluency. Irradiated cells were immediately treated with PDGF-AB/BB (20ng/ml) for 10 days.

2.7 Senescence-associated β -galactosidase (SA- β -Gal) staining

SA- β -Gal staining was performed according to the manufacturer's protocol (Cell Signaling Technology, #9860). In brief, cells cultured in 6-well plates were washed in PBS and fixed with 1X fixative solution for 10 min. 1 ml of β -Gal staining solution (containing 1 mg/ml X-Gal) was added and the cells were incubated at 37 °C in the absence of CO₂ overnight. Cells were washed in PBS and observed under bright-field microscope (ECHO Revolve).

2.8 Immunocytochemistry

Cells cultured on glass coverslips were washed in 1 X PBS and fixed in 4% (v/v) formaldehyde for 10 min. After washing in 1 X PBS, cells were premetallized in PBS with 0.1% Triton X-100 for 15 min. Non-specific binding sites were blocked in 0.1% Tween-20/PBS with 1% bovine serum albumin for 30 min, followed by incubating with primary antibodies against P21 (1:500, Cell Signaling Technology, #2947), P16 (1:500, Cell Signaling Technology, #80772), or Lamin B1 (1:500, Cell Signaling Technology, #68591S). After overnight incubation at 4 °C, the cells were washed and incubated with the corresponding host-specific secondary antibodies goat anti mouse Alex Fluor 488 (1:1000, #ab150113), or goat anti rabbit Alex Fluor 647 (1:1000, #ab150079) for 1 hour. Cells were counterstained and mounted with mounting medium with DAPI (Abcam, #ab104139) and visualized using an Olympus BX63 automated fluorescence microscope.

2.9 Quantitative real-time PCR assays

Total RNA was extracted from cells collected in TRIzol reagent (ThermoFisher Scientific, #15596018) and RNA quality was measured using NanoDrop-1000 spectrophotometer (ThermoFisher Scientific, #ND-2000). 1 μ g of RNA was reverse transcribed using Verso cDNA synthesis kit (ThermoFisher Scientific, #AB-1453B). Quantification of mRNA expression was determined by SYBR-based real-time PCR using PowerUp SYBR Green Master Mix (Applied Biosystems, #A25742). PCR primer sequences were listed in **Table 2** [↗](#). Values were normalized to *ACTB*. mRNA levels were presented as fold change compared to untreated cells according to the 2^{- $\Delta\Delta$ CT} method.

2.10 Immunoblotting

Whole-cell protein extraction and immunoblotting analysis were performed as previously described (24 [↗](#)). Briefly, cells were lysed using RIPA lysis buffer with 1 X protease and phosphatase inhibitor (ThermoFisher Scientific, #PI78440). Protein concentration was determined using Pierce BCA protein assay kit (ThermoFisher Scientific, #23225) and 20 μ g of cell lysate from

Gene	Primer sequence
<i>P21</i>	Forward: 5'-GAC ACCACT GGA GGG TGA CT-3' Reverse: 5'-CAGGTC CAC ATG GTC TTC CT-3'
<i>P16</i>	Forward: 5'-CCAACGCACCGAATAGTTACG-3' Reverse: 5'-GCGCTGCCCATCATCATG-3'
<i>IL6</i>	Forward: 5'-CCGGGAACGAAAGAGAAGCT-3' Reverse: 5'-GCGCTTGTGGAGAAGGAGTT-3'
<i>CCNB1</i>	Forward: 5'-ACTGGGTCGGGAAGTCACTG-3' Reverse: 5'-CATTCTTAGCCAGGTGCTGC-3'
<i>CCND1</i>	Forward: 5'-CTGTGCTGCGAAGTGGAAC-3' Reverse: 5'-TCTGTTTGTCTCCTCCGCC-3'
<i>CASP3</i>	Forward: 5'-TGG TTC ATC CAG TCG CTT TG-3' Reverse: 5'-ATT CTG TTG CCA CCT TTC G-3'
<i>MKI67</i>	Forward: 5'-ATTTGCTTCTGGCCTTCCCC-3' Reverse: 5'-CCAAACAAGCAGGTGCTGAG-3'
<i>ACTB</i>	Forward: 5'-CTC TTC CAG CCT TCC TTC CT-3' Reverse: 5'-AGC ACT GTG TTG GCG TAC AG-3'
<i>PDGFRA</i>	Forward: 5'-AGAAAACAACAGCGGCCTTT -3' Reverse: 5'-CTACATCTGGGTCTGGCACA -3'

Table 2

Sequence of primers.

each sample was used to determine the protein expression of P21 (1:2000, Cell Signaling Technology, #2947) and NF- κ B (1:2000, Cell Signaling Technology, #8242S). All proteins were normalized to b-actin (1:2000, Cell Signaling Technology, #5125S).

2.11 Statistics

One-way analysis of variance (ANOVA) testing was performed to assess the effects of treatment on NP and AF cells with subsequent Dunnett post hoc testing. To analyze the effects of ionizing radiation on the expression of senescence markers in cells, t-test was utilized. The statistical analyses were performed using GraphPad Prism9. A p-value < 0.05 was considered statistically significant.

3 Results

3.1 Transcriptomics of AF and NP cells in response to PDGF-AB/BB

Bulk RNA sequencing was performed to investigate the global changes in gene expression of degenerated AF and NP cells in response to rhPDGF-AB/BB treatment.

Principal component analysis (PCA) on all detected genes revealed distinct clusters between untreated and rhPDGF-AB/BB treated samples while clusters of rhPDGF-AB and rhPDGF-BB treated samples were overlapping (**Supplementary figure 1** [↗](#)). As shown in volcano plots, differential expression analysis ($|FC| > 1.5$ and $FDR < 0.05$) revealed 880 downregulated and 740 upregulated genes in AF cells treated with rhPDGF-AB and 996 downregulated and 821 upregulated genes induced by rhPDGF-BB treatment after 5 days exposure (**Figure 1A** [↗](#)). In contrast, NP cells had 563 downregulated and 423 upregulated genes in rhPDGF-AB treated group and 409 downregulated and 404 upregulated genes in rhPDGF-BB treated group. The top differentially expressed genes (DEGs) were annotated according to FDR and most of these genes exhibited similarity between treatment groups in both AF and NP cells (**Figure 1A+B** [↗](#)). Furthermore, a heat map visualizing the top 50 DEGs by fold change revealed variability among donors while demonstrating an overall consistent response to rhPDGF-AB/BB treatment across the samples (**Figure 1C+D** [↗](#)). Tissue-specific changes were observed in AF and NP cells when treated with rhPDGF-AB/BB. For example, *SLC26A4* (Pendrin) and *CA2*, regulators of the process of transporting and producing bicarbonate, are involved in the homeostatic control of pH in various type of cells ([28](#) [↗](#), [29](#) [↗](#)) and their expression was upregulated in NP cells when treated with rhPDGF-AB/BB (**Figure 1D** [↗](#)). *MAMDC2* and *COL21A1*, involved in extracellular matrix organization ([30](#) [↗](#), [31](#) [↗](#)), were also upregulated in NP cells while *WISP2* ([32](#) [↗](#)) and *RARRES2* ([33](#) [↗](#)), modulators of the induction of inflammatory mediators and immune response, were downregulated after treatment. In contrast, in addition to the commonly upregulated *CA2*, *MDMC2* and *RARRES2*, AF cells showed alterations in genes involved in mechanical stress, pain sensation, and angiogenesis, such as *CSRP3* ([34](#) [↗](#)), *PI16* ([35](#) [↗](#)), *EFEMP1* ([36](#) [↗](#)) and *SLC26A4-AS1* ([37](#) [↗](#)) (**Figure 1C** [↗](#)). Notably, *PRELP*, potentially associated with Hutchinson-Gilford progeria ([38](#) [↗](#)), was also downregulated in rhPDGF-AB/BB treated AF cells (**Figure 1A** [↗](#)).

3.2 Functional analysis of AF cells treated with PDGF-AB/BB

To investigate the enriched biological processes (BP) induced by rhPDGF-AB/BB treatment in AF cells, gene ontology (GO) analysis was performed using upregulated and downregulated DEGs separately. As expected, the biological processes between rhPDGF-AB and -BB treated samples were mostly overlapping (**Figure 2A+B** [↗](#)). In AF cells, upregulated DEGs were significantly enriched in biological processes including regulation of cell cycle and chromosome segregation. Furthermore, AF cells demonstrated upregulated pathways including mesenchymal cell differentiation and response to oxygen levels and downregulated pathways including response to

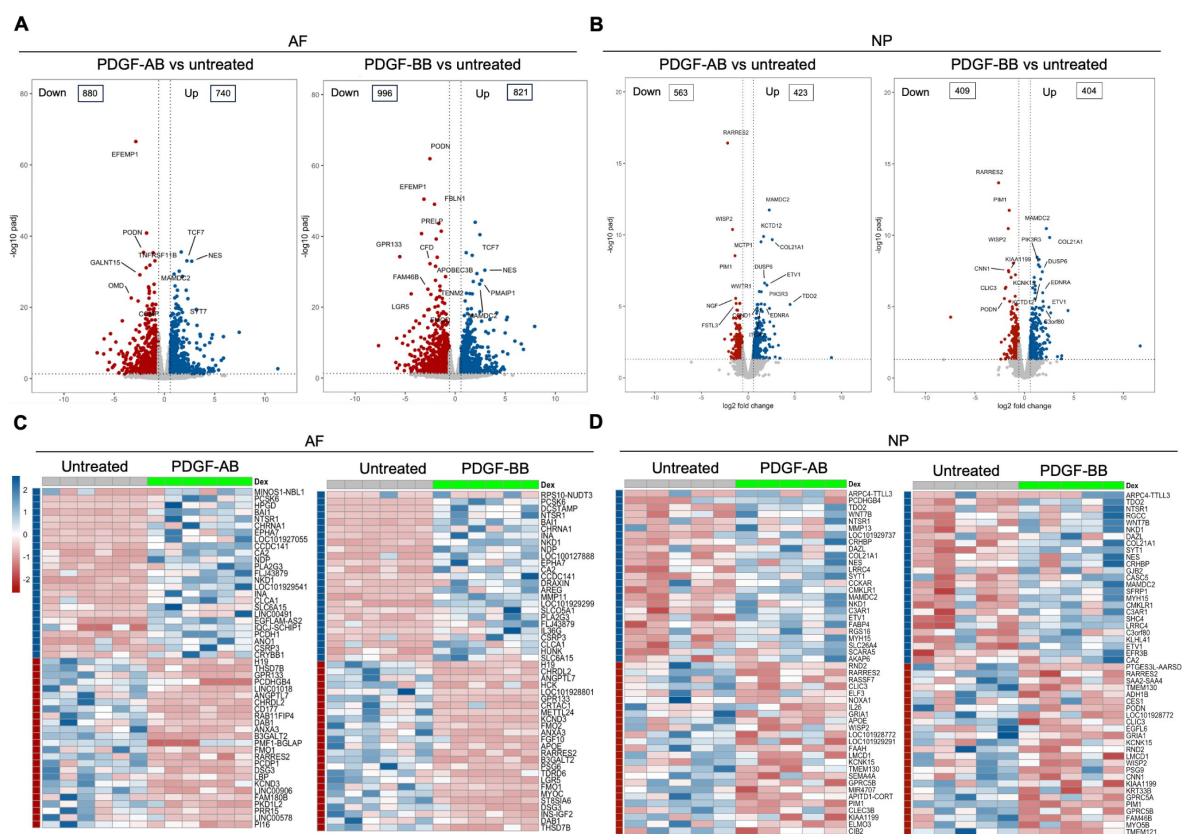


Figure 1.

Transcriptomics of AF and NP cells in response to PDGF-AB/BB treatment.

A + B Volcano plots showing the differentially expressed genes (DEGs) in AF (**A**) and NP (**B**) cells treated with rhPDGF-AB/BB for 5 days (cutoff: $|FC| > 1.5$ and $FDR < 0.05$). DEGs were annotated in the plot according to FDR. **C+D** Heatmap plots showing the top 50 DEGs in rhPDGF-AB/BB treated AF (**C**) and NP (**D**) cells based on fold change. NP: n = 5 samples. AF: n = 6 samples.

ROS and oxidative stress (**Figure 2A** [↗](#)). Moreover, the treatment downregulated groups of genes related to neurogenesis and response to mechanical stimulus (**Figure 2B** [↗](#)). In line with the results of GO analysis, GSEA analysis revealed an overrepresentation of cell cycle pathway in rhPDGF-AB/BB treated cells (**Figure 2C** [↗](#)). Additionally, the ErbB signaling pathway, proteasome pathway, and cytosolic DNA-sensing pathway were enriched, suggesting that PDGF-AB/BB treatment promoted cell growth, protein turnover, and maintenance of cellular homeostasis. The leading-edge genes that contributed most significantly to these pathways include *CCNB1*, *CCND1*, *AREG*, *PSMB9*, *TREX1*, and *POLR3A*, as shown in **Figure 2D** [↗](#).

3.3 Functional analysis of NP cells treated with PDGF-AB/BB

GO analysis revealed similar yet distinct responses to rhPDGF-AB/BB treatment between NP and AF cells. In NP cells, rhPDGF-AB and -BB treatment upregulated groups of genes related to cartilage development, cell-matrix adhesion, Wnt signaling pathway, and response to decreased oxygen levels, which were all important to maintaining NP phenotype (**Figure 3A** [↗](#)). Downregulated DEGs were enriched in GO terms associated with fatty acid metabolism, cellular respiration, mitochondrial function, and reactive oxygen species production (**Figure 3B** [↗](#)), suggesting alterations in cellular metabolism and oxidative stress. Gene set enrichment analysis (GSEA) on the entire transcriptomics also revealed that Wnt signaling pathway and neuroactive ligand-receptor interaction were upregulated while the pathways related to oxidative phosphorylation and ribosome were downregulated in rhPDGF-BB treated NP cells (**Figure 3C** [↗](#)). The core enrichment genes associated with these pathways were visualized in a chord diagram (**Figure 3D** [↗](#)). Specifically, the leading-edge DEGs enriched in Wnt signaling pathway include *WNT7B*, *WNT5A*, *NKD1*, and *SFRP1* and oxidative phosphorylation pathway showed enrichment in *NDUFB7*, *NDUFA11*, *COX5B*, and *CYC1*.

To better understand the regulatory mechanisms of PDGF-AB/BB in degenerated NP cells, DEGs between untreated and treated groups were used to construct protein-protein interaction (PPI) networks through STRING and Cytoscape software. The network of the top 10 hub nodes based on betweenness was demonstrated in **Figure 3E** [↗](#) and it revealed the strong correlation between *PDGFRA* (PDGF receptor alpha) and *IL6*, *MDM2*, *CCND1*, *PIK3R1*, *NOP53*, *FN1*, and *IDH2*. The enrichment of hub gene *IDH2*, which plays a role in generating NADPH within mitochondria, further validated the alterations in oxidative phosphorylation pathway and subsequent ROS production, as indicated by GSEA and GO analysis in rhPDGF-AB/BB treated NP cells. *MDM2* (a negative regulator of P53) and predicted *NOP53* (a regulator of P53 binding activity) suggested that PDGF-BB interfered with P53 signaling pathway. The interaction between these genes and inflammatory cytokine *IL6* indicated a potential involvement of PDGF-AB/BB in cellular senescence. The gene expression of top hub genes *PDGFRA* and *IL6* was further verified in both NP (**Figure 3F** [↗](#)) and AF (**Figure 3G** [↗](#)) cells by qPCR. PDGF-AB/BB increased the gene expression of *PDGFRA* in only NP cells and reduced the *IL6* gene expression in both type of cells. Taken together, NP and AF cells responded differently to PDGF-AB/BB treatment, but both cell types showed a positive regulation of cell cycle and inhibited inflammation, response to ROS, oxidative stress, and mitochondrial dysfunction, indicating a role of PDGF-AB/BB in IVD cell senescence.

3.4 Induction of NP cellular senescence through X-ray radiation

To test our hypothesis that PDGF-AB/BB provides protection against the progression of cellular senescence in the IVD, we first created a cellular senescence model using X-ray radiation. The healthy NP cells were exposed to 5, 10 and 15 Gy of irradiation and cultured for 7-10 days to develop senescent phenotype. SA- β -gal staining was performed to detect senescent cells. We found that non-irradiated, proliferating NP cells showed elongated, spindle-shaped morphology and faint SA- β -gal staining at day 7 (**Figure 4A** [↗](#)). In contrast, cells exposed to 5 and 10 Gy of irradiation developed characteristics of a senescent cell phenotype — enlarged cell size, low cell number, and a higher percentage of SA- β -gal positive cells. However, cells exposed to 15 Gy of radiation

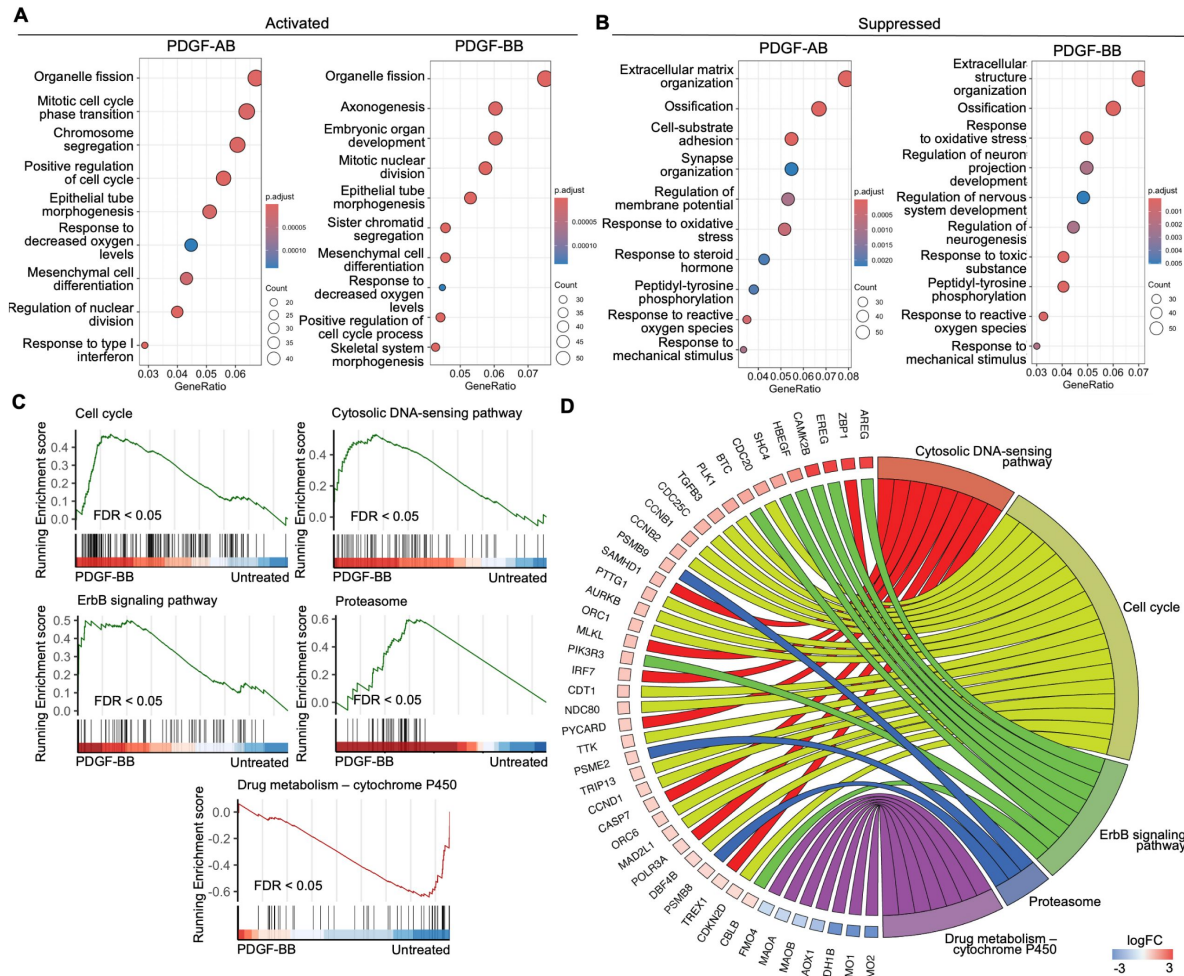


Figure 2.

Functional analysis of AF cells treated with PDGF-AB/BB.

A + B Gene ontology (GO) analysis for biological process using upregulated (**A**) and downregulated (**B**) DEGs in AF cells. Y-axis label represents GO terms, and x-axis represents geneRatio (geneRatio refers to the proportion of genes that are annotated in specific GO term within all the input genes). The size of bubble represents the gene counts enriched in a particular GO term and the color indicates the FDR of GO terms. **C** Gene set enrichment analysis (GSEA) on the entire transcriptome profile demonstrated an upregulation of cell cycle, cytosolic DNA-sensing pathway, ErbB signaling pathway, and proteasome and a downregulation of drug metabolism-cytochrome P450 pathway in rhPDGF-BB treated NP cells. **D** The leading-edge genes associated with these pathways were shown using a chord diagram.

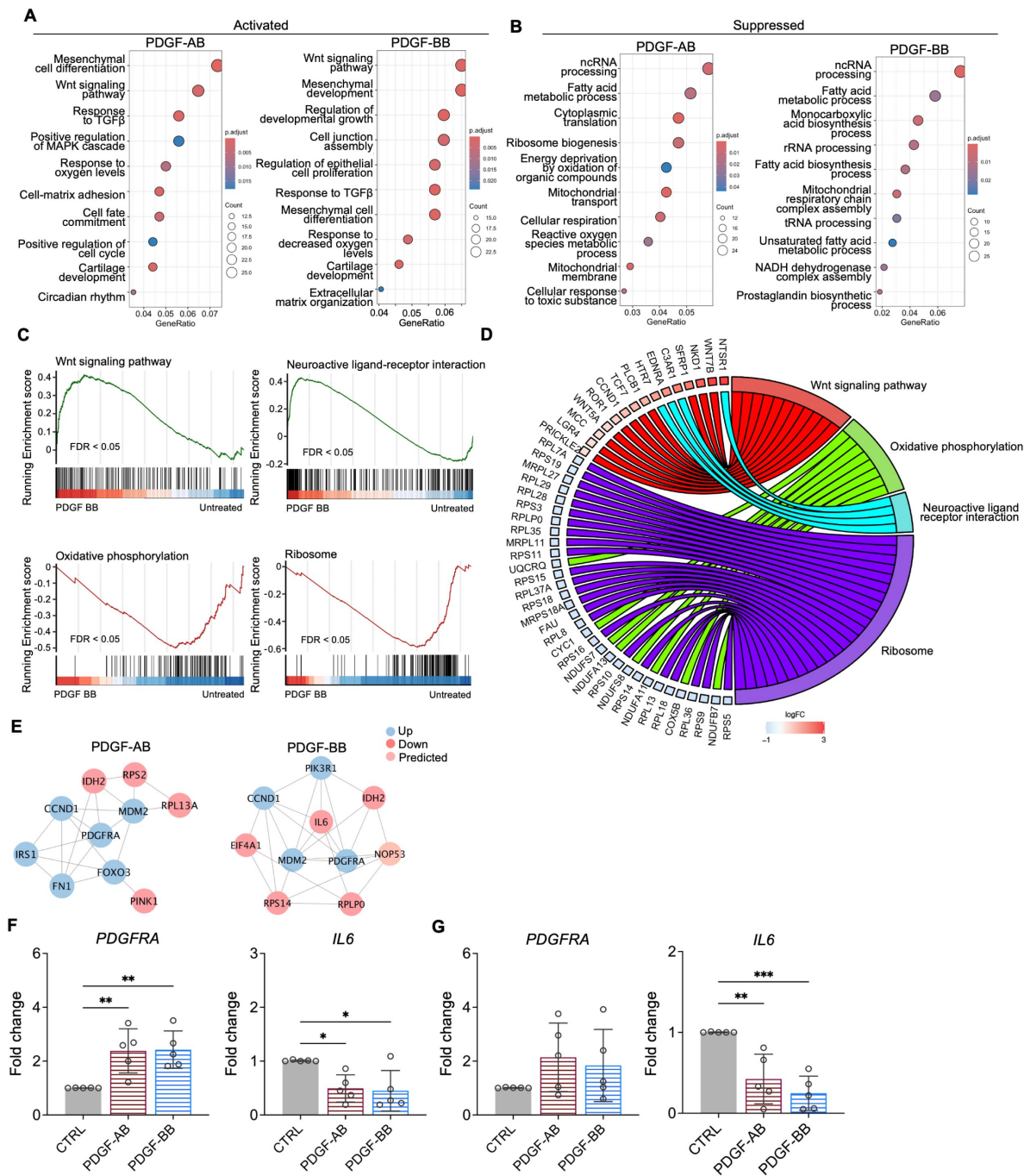


Figure 3.

Functional analysis of NP cells treated with PDGF-AB/BB.

A + B) Gene ontology (GO) analysis for biological process using upregulated (**A**) and downregulated (**B**) DEGs in NP cells. Y-axis label represents GO terms, and x-axis represents geneRatio (geneRatio refers to the proportion of genes that are annotated in specific GO term within all the input genes). The size of bubble represents the gene counts enriched in a particular GO term and the color indicates the FDR of GO terms. **C**) Gene set enrichment analysis (GSEA) on the entire transcriptome profile demonstrated an upregulation of Wnt signaling pathway and a downregulation of oxidative phosphorylation and ribosome in rhPDGF-BB treated NP cells. **D**) The leading-edge genes associated with these pathways were shown using a chord diagram. **E**) Protein-protein interaction network using DEGs was constructed in STRING and visualized in Cytoscape. **F+G**) The changes in the gene expression of PDGFRA and IL6 were verified in NP (**F**) and AF (**G**) cells treated with rhPDGF-AB (40ng/ml) or -BB (20ng/ml) for 5 days. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

underwent significant cell death, showing negligible SA- β -gal staining. As a result, we excluded the 15 Gy dosage group in subsequent studies. To quantify cell growth, MTT assay was performed, which revealed reduced cell proliferation under 5 and 10 Gy of irradiation compared to the non-irradiated group (**Figure 4B**). Furthermore, we examined the expression of senescence related markers — Lamin B1, P21 and P16. Lamin B1 is a structural component of the nucleus, and its loss is a senescence-associated biomarker (39). Non-irradiated cells displayed nuclear Lamin B1 expression while its expression was lost in healthy NP cells exposed to 5 and 10 Gy of irradiation for 7 days (**Figure 4C**). Meanwhile, nuclear localization of P21 (**Figure 4C**) and P16 (**Figure 4D**) was observed in irradiated cells, with little immunopositivity in non-irradiated cells at day 7. However, the transcripts of *P21* and *P16* were not significantly decreased until day 10 (**Figure 4E-F**). Caspase 3 (*CASP3*) gene expression was reduced at both timepoints, suggesting that the irradiated cells were resistant to apoptosis. Furthermore, the gene expression of *PDGFRA* was significantly decreased in cells under 10 Gy of irradiation at day 10. Taken together, when exposed to irradiation for 10 days, healthy human NP cells developed senescent phenotype, accompanied with decreased *PDGFRA* expression.

3.5 PDGF-AB/BB inhibited senescent phenotype in NP and AF cells

To investigate the protective effects of PDGF-AB/BB against cell senescence in the IVD, healthy human NP and AF cells were exposed to 10Gy of irradiation, followed by immediate treatment with 20 ng/ml rhPDGF-AB/BB for 10 days. We first examined the cell cycle progression using DAPI stained cells and found that rhPDGF-BB significantly increased the percentage of cell population in the S phase in irradiated NP (**Figure 5A-B**) and AF (**Figure 5C-D**) cells at day 10. In rhPDGF-AB treated cells, the percentage of cell population in the S phase was increased in AF cells, but not significantly in NP cells. These findings suggested that PDGF-AB/BB rescued IVD cells from cell cycle arrest in irradiation induced senescent cells. Additionally, SA- β -gal staining demonstrated an increase in the number of senescent cells after irradiation at day 10, whereas a decrease was examined in rhPDGF-AB/BB treated NP and AF cells (**Figure 5E**).

At day 10, we analyzed the expression of *PDGFRA* and key senescence regulators of cell cycle inhibitors *P21* and *P16*, *CASP3*, and SASP mediator *IL6*. The treatment of rhPDGF-BB to irradiated NP cells significantly increased the gene expression of *PDGFRA* and decreased the gene expression of *P21*, *P16*, and *IL6*, while playing no effect in the gene expression of *CASP3* (**Figure 6A**). Comparably, the treatment of rhPDGF-AB to irradiated NP cells significantly decreased the gene expression of *P21* and *IL6* and played no effect on the gene expression of *PDGFRA*, *P16*, and *CASP3* (**Figure 6A**). In AF cells, the results show that the treatment of rhPDGF-BB and rhPDGF-AB reduced the gene expression of *P21* as those of NP cells (**Figure 6B**). However, the treatment of either rhPDGF-BB or rhPDGF-AB had no effect in the gene expression of other genes examined (**Figure 6B**). The lack of change in the gene expression of *PDGFRA* from the treatment of either rhPDGF-BB or rhPDGF-AB suggested that PDGF-BB reduced senescent phenotype through other receptors in AF cells.

Furthermore, compared to irradiation only group, the protein levels of P21 and NF- κ B, a major regulator stimulating SASP, were inhibited in rhPDGF-BB treated NP cells (**Figure 6C**). In irradiated AF cells, the protein expression of P21 was significantly reduced by rhPDGF-BB treatment. The expression of NF- κ B showed a decreasing trend in rhPDGF-BB treated cells. rhPDGF-AB had no effects on the protein expression of P21 and NF- κ B. Overall, PDGF-BB mitigated the progression of senescent phenotype in both human NP and AF cells.

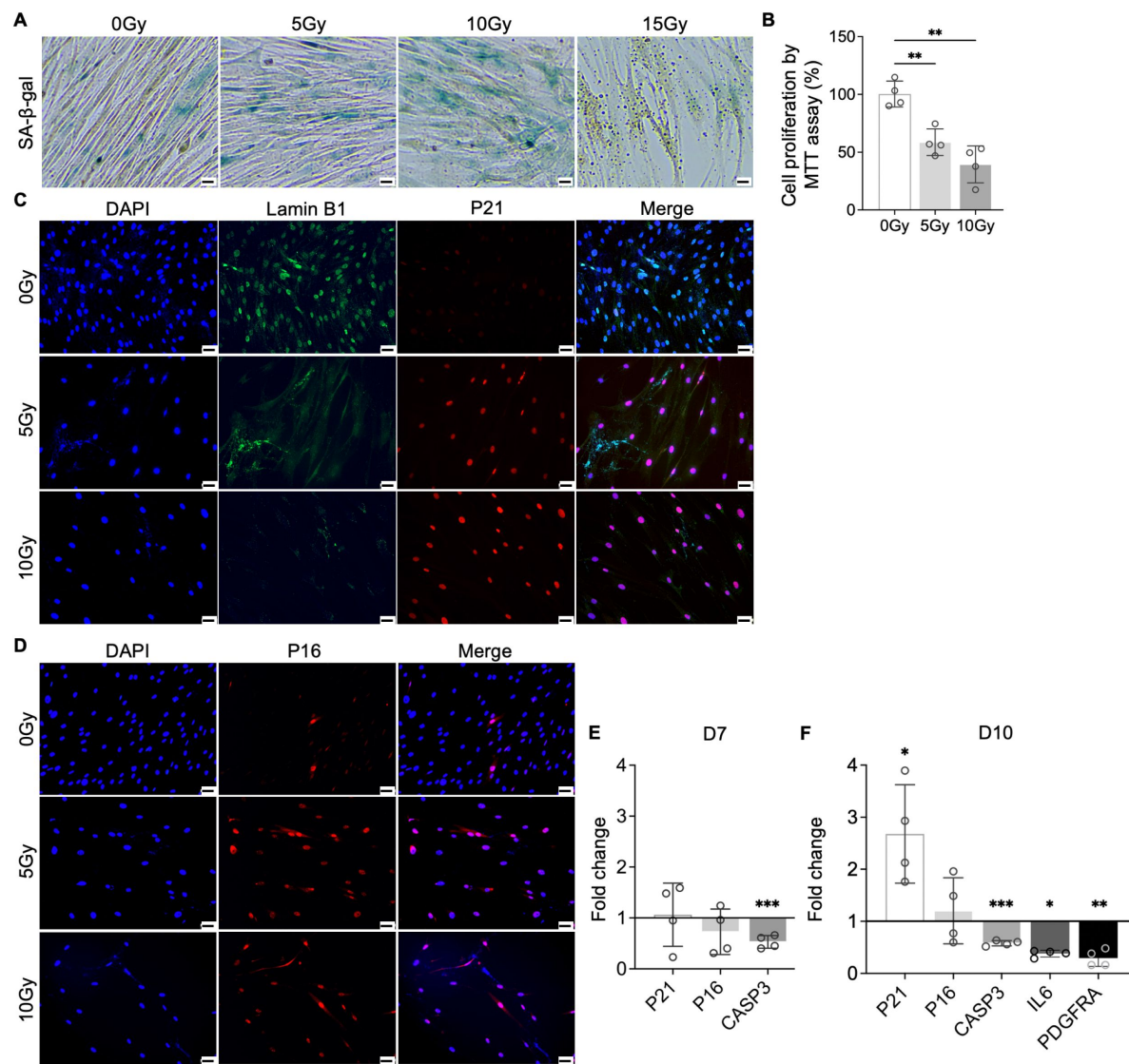


Figure 4.

Cellular senescence induction by irradiation.

A) SA-β-gal staining in healthy NP cells under different doses of irradiation at day 7. **B)** MTT assay showed decreased cell proliferation after irradiation at day 7. **C, D)** Immunocytochemistry staining on Lamin B1 and P21 (**C**) and P16 (**D**) in irradiated cells at day 7. Scale bar = 100uM. **E)** Changes in the gene expression levels of *P21*, *P16*, and *CASP3* in NP cells under 10 Gy of irradiation at day 7. **F)** The gene expression of *P21*, *P16*, *CASP3*, *IL6*, and *PDGFRA* was altered in NP cells under 10 Gy of irradiation at day 10. n = 4 per group. Statistical significance is analyzed using t-test. The data is presented as mean with SD. * $p < 0.05$. *** $p < 0.001$. **** $p < 0.0001$.

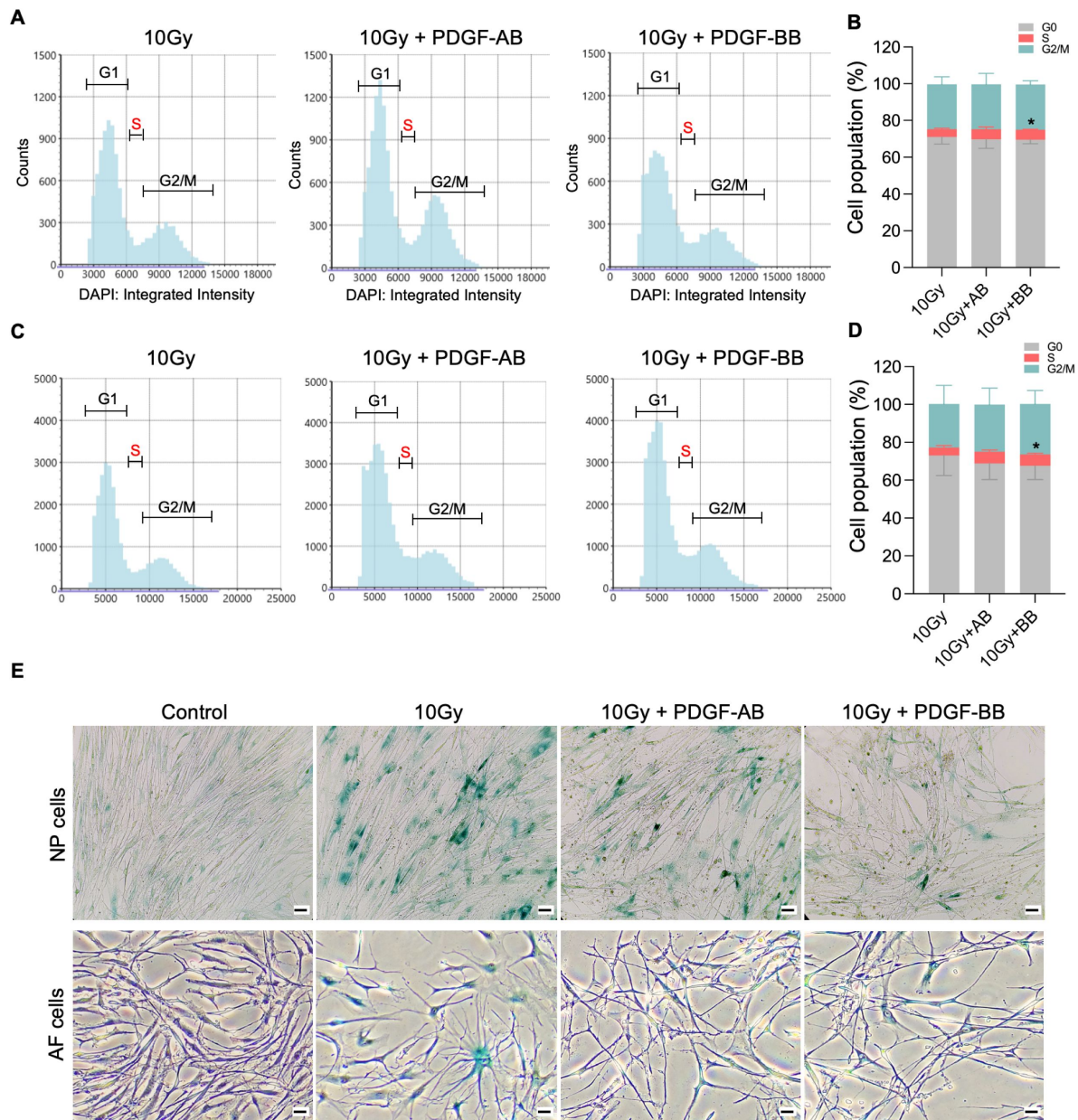


Figure 5.

PDGF-AB/BB treatment protected healthy NP and AF cells against senescence progression.

A-F Cell cycle analysis by DAPI staining in irradiated healthy NP (**A-B**) and AF (**C-D**) cells treated with rhPDGF-AB/BB for 10days. The upper panel shows the data obtained from NP cells, and lower panel shows the data from AF cells. Representative graphs (**A+C**) showing the changes in cell cycle progression in NP and AF cells after treatment. Quantification of cell cycle analysis showed the cell percentage in each phase (**B+D**). **E** SA-β-gal staining in irradiated healthy NP (upper panel) and AF cells (lower panel) showing the reduced SA-β-gal positive cells after treatment. Scale bar = 100μm. Two-way ANOVA with Dunnett post hoc testing was performed. $n = 4$ each group. The data is presented as mean with SD. $*p < 0.05$.

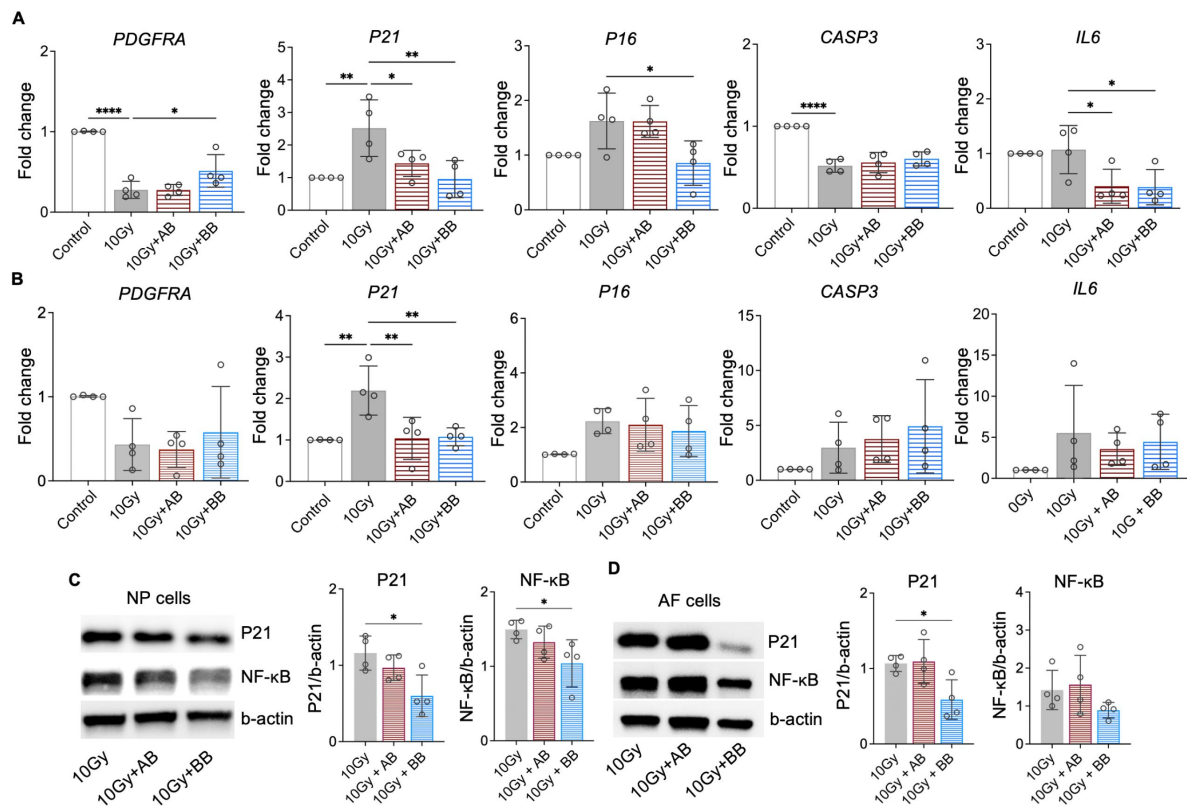


Figure 6.

PDGF-AB/BB treatment suppressed the expression of senescence associated regulators.

A-B) Changes in the gene expression of *PDGFRA*, *P21*, *P16*, *CASP3*, and *IL6* in irradiated NP (**A**) and AF (**B**) cells treated with rhPDGF-AB/BB. **C+D)** Protein expression levels of *P21* and *NF-κB* in irradiated NP (**C**) and AF (**D**) cells treated with rhPDGF-AB/BB. $n = 4$ each group. One-way ANOVA with Dunnett post hoc testing was performed. The data is presented as mean with SD. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.0001$.

4 Discussion

The current study has shown for the first time that PDGF-AB and -BB treatment not only alleviated the senescent phenotype, but also suppressed the progression of cellular senescence in the IVD. Specifically, in aged, degenerated human NP and AF cells, PDGF-AB/BB treatment suppressed the ROS accumulation, mitochondrial dysfunction, and the resultant oxidative stress, while stimulating cell cycle progression. In the NP, *PDGFRA* was among the top hub genes that showed strong interaction with other genes. Moreover, its expression was significantly reduced in irradiation induced senescent cells. Importantly, immediate PDGF-BB treatment after irradiation promoted the expression of *PDGFRA* and provided protection to IVD cells against acquiring senescence associated phenotype. This novel function of PDGF-BB/*PDGFRA* signaling is likely linked to their ability to inhibit NF- κ B, P16, and P21 pathways in NP cells. In contrast, PDGF-AB/BB alleviated the senescent phenotype in AF cells independent of *PDGFRA*.

Senescence is a key contributor to the onset and the progression of IVD degeneration (40). It can occur naturally with aging or can be triggered by growth factor deficiency, radiation, DNA damage, oxidative stress, and inflammatory stimuli (41,42). Senescent cells exit the cell cycle and cannot divide; their accumulation reduce the IVD's ability to generate new cells to replace apoptotic or necrotic cells. It has been well established that increased number of senescent cells was positively correlated with the IVD aging and the severity of degeneration while negatively correlated with the percentage of proliferating cells in human NP and AF tissues (10–12). Since PDGF is a mitogenic growth factor and promotes cell proliferation in various cell types (21,43,44), it is intriguing to explore whether PDGF-AB/BB could mitigate or prevent against the cellular senescence in IVDs. We observed groups of upregulated genes regulating cell cycle progression and cell proliferation in PDGF-AB/BB treated NP cells. AF cells also had an upregulation of chromosome segregation and mitotic nuclear division pathways, suggesting that these cells were actively proliferating. Cell cycle progression is controlled by cyclins, and the expression of cyclin D1 and B1, regulators of the G1/S and G2/M phase transition respectively, was increased in degenerated NP cells after PDGF-AB/BB treatment. Transcriptomic analysis also revealed that increased cyclin D1 was among the top hub nodes, tightly regulated by PDGF-AB and BB. In addition, cell cycle analysis confirmed that PDGF-AB/BB induced the transition from G1 to S1 phase in senescent IVD cells. Our data suggested that PDGF treatment led to an increase in the proliferation of senescent IVD cells in the S phase likely through cyclin D1 and B1. These findings were in accordance with previous studies showing that PDGF stimulated cell proliferation and cyclin D1 expression in rat mesangial cells (45), rat adventitial fibroblasts (46), and human vascular smooth muscle cells (44).

The antisenesence of PDGF has been demonstrated in several studies. For example, PDGF-BB decreased SA- β -gal positive cells and the expression of P53 and P21 while enhancing proliferative potential in senescent human dermal fibroblasts (47) and mesenchymal stem cells (MSCs) derived from immune thrombocytopenia patients (48). PDGF exerts its function by binding to its receptors, thereby triggering a cascade of intracellular signaling events, such as cell proliferation and survival. The PDGF-*PDGFRA* signaling is often suppressed in premature aged or senescent cells (49–51), suggesting that PDGF signaling dysfunction might be involved in senescence or aging. Notably, our transcriptome data has shown that both PDGF-AB and -BB induced the expression of *PDGFRA* in aged, degenerated NP cells. This autoregulatory loop caused IVD cells to become more sensitive to PDGF treatment and augmented the signaling cascades triggered by PDGF binding, including senescence-associated P21, P16, P53, and NF- κ B pathways. In line with this thought, PPI analysis revealed that *PDGFRA* closely interacted with upregulated *MDM2*, a negative regulator of P53. NF- κ B signaling is a major signaling pathway that is involved in inflamm-aging (52) and induces the onset of SASP (8), such as IL6, IL8, and CXCL1. In our study, irradiation in NP cells led to decreased *PDGFRA* expression, which impaired the activity of

PDGF/PDGRFA signaling. However, PDGF-BB treatment promoted the expression of *PDGFRA* and decreased the expression of *P21*, *P16*, and *IL6* in senescent NP cells, likely by inhibiting NF- κ B signaling. These findings support the idea that PDGF treatment could stimulate the expression of *PDGFRA* and prevent healthy IVD cells from developing senescent phenotype. Further studies using preclinical animal models to validate the protectiveness of PDGF against the onset or progression of senescence in the IVD will offer more robust evidence.

Degenerated IVD cells experienced an imbalance between ROS accumulation and antioxidant capacity, leading to oxidative stress, which is involved in cellular senescence (53 [↗](#)). Mitochondrion is the primary location of intracellular ROS production and its dysfunction in degenerated IVD cells causes electron leakage, resulting in the formation of superoxide radicals (54 [↗](#)). PDGF-AB/BB treatment suppressed the activity of damaged mitochondria, leading to decreased ROS and oxidative stress in degenerated NP and AF cells, as indicated by GO BP analysis. Consistently, GSEA revealed the underrepresentation of oxidative phosphorylation in treated NP cells. The downregulation of genes related to mitochondria activity and oxidative phosphorylation may be indicative of a shift in metabolic pathways, leading cells to potentially prioritize alternative pathways such as glycolysis. Indeed, PDGF treatment upregulated groups of genes involved in response to decreased oxygen levels, suggesting that the treatment activated cellular mechanisms involving in sensing and adapting to low oxygen conditions. In line with this idea, it has been demonstrated that hypoxia-inducible factor 1 α is highly expressed in healthy NP cells to suppress oxidative phosphorylation and enhanced glycolysis, allowing cells to adjust to a hypoxia microenvironment (55 [↗](#), 56 [↗](#)). Conversely, degenerated NP cells show an opposite metabolic pattern (55 [↗](#), 56 [↗](#)).

Besides the anti-senescence, NP and AF cells responded differently to PDGF-AB/BB treatment. AF cells were more responsive to the treatment with twice as many DEGs as NP cells. The difference in tissue architecture and cellular contents between NP and AF contributes to their specialized functions (57 [↗](#)). The multiple layers of concentric lamellae in the AF enables it to withstand circumferential loads while this highly organized structure was disrupted with aging and degeneration (58 [↗](#)). In addition, damage to the AF has the potential to stimulate neovascularization and nerve ingrowth, provoking an inflammatory response and contributing to low back pain. PDGF-AB/BB treatment downregulated groups of genes related to neurogenesis and response to mechanical stimulus in AF cells while downregulated genes in the NP were more associated with metabolic pathways, providing evidence that the treatment enhanced IVD functionality in different aspects between the two compartments.

Senescence as a central hallmark of aging can be induced by several factors (40 [↗](#)). One limitation of this study is that we did not examine the protective effects of PDGF-AB/BB treatment on senescence using other senescence models. In addition, our findings were obtained in vitro experiments, so caution is needed when interpreting our data. Our future work will focus on establishing an appropriate experimental animal model to study the functions of PDGF on cellular senescence.

In this study, we demonstrated a novel function of PDGF in human NP and AF cells. PDGF-AB/BB treatment alleviated oxidative stress and mitochondria dysfunction while stimulating cell cycle progression in degenerated IVD cells. In addition, the treatment protected healthy NP and AF cells against the progression of senescence induced by irradiation. Our results suggest that PDGF-AB/BB could be a promising candidate for preventing or treating IVD degeneration by targeting cellular senescence.

Human ethics and consent to participate

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. All experiments and procedures were reviewed and approved by the Institutional Review Board (IRB #00099028) of Emory University for all experimental procedures. Human tissue

samples were collected after obtaining familial consent.

Additional information

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Conflict of interest

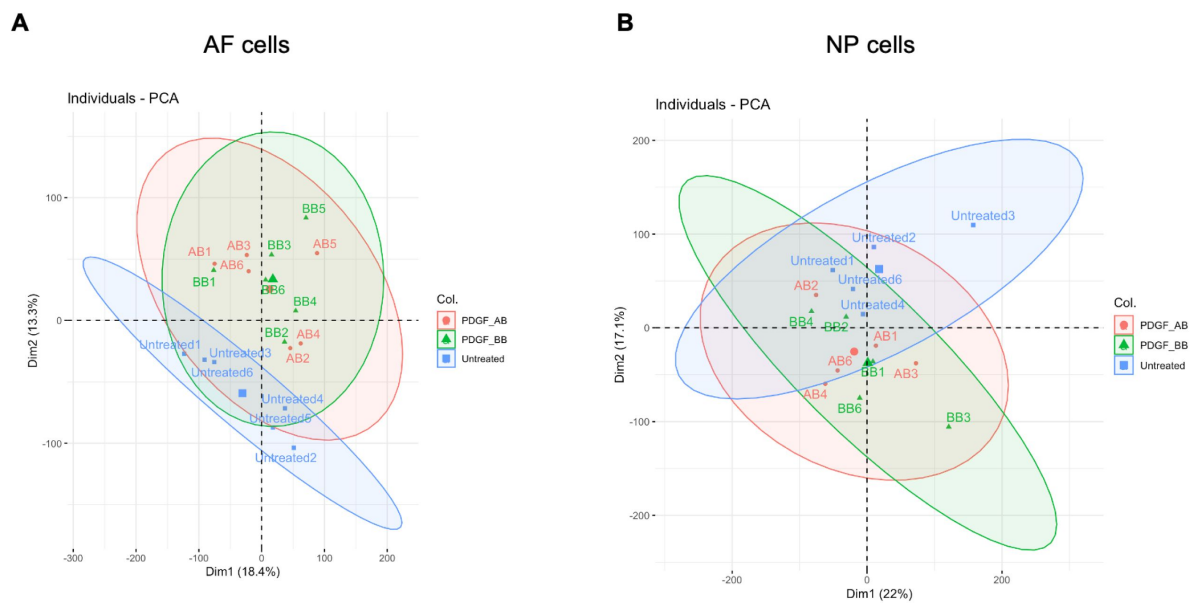
The authors have no conflict of interest associated with this work.

Authors' contributions

CZ designed and conducted the study, analyzed and interpreted the data, and drafted the manuscript. TF and MDH reviewed and revised the manuscript. SS assisted in data collection. SY provided diseased IVD tissues and reviewed the manuscript. LH provided healthy IVD cells and revised the manuscript. HD designed the study and interpreted the data. All authors have reviewed the manuscript and provided their consent for publication.

Availability of data and materials

The datasets and code used and analyzed during the current study are available from the corresponding author on reasonable request.



Supplementary figure 1

Principle component analysis (PCA) of NP and AF samples treated with rhPDGF-AB and BB.

A+B) PCA plot of NP (**A**) cells and AF cell (**B**) showed the distinct cluster between untreated and treated samples. The clusters of PDGF-AB and BB samples were overlapping. NP: n = 5 each group. AF: n = 6 each group.

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Reviewer #1 (Public review):

The authors, Zhang et al., demonstrate the beneficial effects of treating degenerate human primary intervertebral disc (IVD) cells with recombinant human PDGF-AB/BB on the senescence transcriptomic signatures. Utilizing a combination of degenerate cells from elderly humans and experimentally induced senescence in young, healthy IVD cells, the

authors show the therapeutic effects on mRNA transcription as well as cellular processes through informatics approaches.

One notable strength of this study is the use of human primary cells and recombinant forms of human PDGF-AB/BB proteins, which increases the translational potential of these in vitro studies. The manuscript is well-written, and the informatics analyses are thorough and clearly presented.

However, in its current form, the study does not provide sufficient experimental details, and clarifications are needed. These are as follows:

- (1) The source of PDGF-AB/BB proteins is not detailed.
- (2) The irradiation parameters are not adequately reported - the authors should consider (PMCID: PMC5495460) for the parameters that should be reported.
- (3) The criteria for young and old patient donors are not explicitly described - though from the table, one presumes the cut-off for young is 27 years old.
- (4) What is the rationale for using different concentrations of PDGF-AB/BB in the degenerate cell and irradiation experiments?

There are also a number of other issues the authors could consider. First, in the title and throughout the manuscript, the effects of PDGF-AB/BB are described as protective, yet in all the experiments, PDGF-AB/BB appears to be administered following either in vivo degeneration or in vitro irradiation, where protective effects (e.g., administration prior to insult) were not tested. Therefore, the effects of PDGF-AB/BB may be more accurately described as mitigating or therapeutic rather than protective.

The authors state that the focus on NP (nucleus pulposus) cell studies is due to NP being the first site impacted during degeneration. However, this reviewer believes that this is because changes in the NP are more clinically evident (by imaging methods), despite degeneration often initiating from the AF (annulus fibrosus), e.g. through tears/microtears.

A prior study has examined the effects of X-ray irradiation on NF- κ B signaling in young and aged IVDs (PMCID: PMC5495460), and the authors may wish to consider this work.

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Reviewer #2 (Public review):

Summary:

This work highlights a novel role for platelet-derived growth factor (PDGF) in mitigating cellular senescence associated with age-related and painful intervertebral disc degeneration. Prior literature has demonstrated the importance of the accumulation of senescent cells in mediating many of the pathological effects associated with the degenerate disc joint such as inflammation and tissue breakdown. In this study, the authors treat clinically relevant human nucleus pulposus and annulus fibrosus cells from patients undergoing discectomy with recombinant PDGF-AB/BB for 5 days and then deep phenotyped the outcomes using bulk RNA sequencing. In addition, they irradiated healthy human disc cells which they subsequently treated with PDGF-AB/BB examining the expression of SASP-related markers and also PDGFRA receptor gene expression. Overall PDGF was able to down-regulate many senescent-associated pathways and the degenerate phenotype in IVD cells. Altered pathways were associated with neurogenesis, mechanical stimuli, metabolism, cell cycle, reactive oxygen species, and mitochondrial dysfunction. Overall the authors achieved their aims and the results by and large support their conclusions although improvements could be made to enhance the rigor of the study and findings.

Strengths:

A major strength of this study is the use of human cells from patients undergoing discectomy for disc herniation as well as access to healthy human cells. Investigating the role of PDGF regarding cellular senescence in the degenerate disc joint is a novel and underexplored area of research which is a significant contribution to the field of spine. This study highlights a potential target for addressing cellular senescence where most of the prior focus has been on senolytic drugs. Such studies have broad implications for other age-related diseases where senescence plays a major role. The use of transcriptomics and therefore an unbiased approach to investigating the role of PDGF is also considered a strength as are the follow-up studies involving irradiating healthy human disc cells and treating these cells with PDGF. The combined assessment of both nucleus pulposus and annulus fibrosus cells in the context of these studies adds to the impact.

Weaknesses:

A weakness of these studies lies in the lack of experimental details provided in the methodology including the rationale for such methods/conditions. Many details such as the specific culture models utilized, substrates, cell density, and media components are missing which impacts rigor. Such details would strengthen the manuscript and the ability to replicate and build on such work/findings. An additional weakness relates to qualitative data presented such as the B-galactosidase assay and immunofluorescence of senescence-associated proteins such as P21 and P16. Quantification of such data sets would greatly strengthen the studies and lend further support to the hypotheses. The study in its current form could be strengthened by the inclusion of mechanistic studies probing the downstream PDGF receptor-associated pathways for example specifically targeting or modulating the activity of the PDGF receptor PDGFRA including validation of the gene data for PDGFRA with protein level data to determine if the transcripts are being translated to protein. The claim that in annulus fibrosus cells, PDGF do not mediate their effects via the PDGFRA does not appear to be supported by the current data as only gene expression for the receptor was assessed with no functional or mechanistic studies being performed. Further discussion, interpretation, and direct comparison of the nucleus pulposus and annulus fibrosus data sets would be helpful for the readers. The magnitude of changes related to the effects of PDGF-BB on the S-phase in irradiated NP and AF cells between control and treated groups seem small making interpretation of these findings challenging.

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