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RESEARCH

Effects of GDF6 on active protein synthesis by cells of degenerated intervertebral disc

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Abstract

Introduction Intervertebral disc degeneration (IVD) is a leading cause of low back pain, a prevalent musculoskeletal condition. IVD degeneration is characterized by the degradation of nucleus pulposus (NP), annulus fibrosus (AF), and cartilage endplates (EP). Growth Differentiation Factor 6 (GDF6), part of the bone morphogenetic protein family, has demonstrated potential in maintaining disc integrity. However, its precise role in cellular protein synthesis during IVD degeneration remains unclear.

Methods This study employed Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) to investigate the effects of GDF6 on protein synthesis in NP, AF, and EP cells isolated from degenerated human IVDs. Cells were cultured in SILAC media with and without GDF6 treatment. The proteomic profiles were analyzed via mass spectrometry, comparing newly synthesized "heavy" proteins with pre-existing "light" proteins.

Results GDF6 treatment altered protein synthesis in degenerated IVD cells. In NP cells, GDF6 reduced the synthesis of matrisome proteins, including collagens and proteoglycans, while promoting proteins associated with ECM stability, such as LOX, PCOLCE and HAPLN1/3. AF cells demonstrated an upregulation of ECM-stabilizing proteins like POSTN and FMOD. EP cells showed minimal changes, but GDF6 enhanced the synthesis of collagen type II, suggesting improved ECM integrity. Secretome analysis revealed that GDF6 modulated extracellular signalling by promoting ECM-stabilizing proteins and reducing inflammatory markers.

Conclusion GDF6 exerts compartment-specific effects on protein synthesis in degenerated IVDs, promoting ECM stability, reducing fibrosis, and potentially preserving hydration. These findings support the potential of GDF6 as a therapeutic agent in treating IVD degeneration, particularly in NP-targeted therapies. Future studies should optimize GDF6 dosing and delivery to maximize its regenerative potential.

Keywords Intervertebral Disc Degeneration · Growth Differentiation Factor 6 · Annulus Fibrosus · Nucleus Pulposus · Cartilage Endplate · Proteomics · SILAC

Introduction

Intervertebral disc degeneration (IVD) is a significant contributor to low back pain (LBP), affecting millions globally [1], making it the most frequent musculoskeletal condition in terms of the resultant years lived with a disability, as reported in a 30-year global study of 371 human diseases [2]. It is closely associated with the degeneration of the annulus fibrosus (AF), nucleus pulposus (NP) and cartilage endplates (EP) of vertebrae, whereby a meta-analysis indicated that out of 632 million people, 403 million people showed symptomatic disc degeneration as a cause of LBP [3]. As degeneration progresses, inflammation and changes in the extracellular matrix (ECM) further exacerbate the decline in cell health and function [4–7].

One of the most significant challenges in treating IVD degeneration is the development of therapies that effectively target and address the underlying biological mechanisms driving the degenerative process. One approach is to directly administer biological treatments intradiscally

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[8]. A promising candidate is Growth Differentiation Factor 6 (GDF6), a member of the bone morphogenetic protein (BMP) family, that plays a role in the development and maintenance of cartilage and intervertebral discs. A mutation in the pro-domain of GDF6 was first identified as a cause of congenital absence of the intervertebral disc in individuals with Klippel Feil Syndrome (KFS) [9]. The clinical features of KFS include a short neck with fused vertebral bodies (or absent disc), carpal tarsal fusions and possible ocular manifestations. Subsequent work in human embryos revealed that GDF6 is present in regions where discs form, and absent in areas where bone forms in the developing notochord to spinal column [10].

In-vitro studies have demonstrated that GDF6 exhibits anti-osteogenic and pro-chondrogenic properties [11]. Clarke et al. demonstrated that GDF6 is more effective than GDF5 in directing adipose-derived and bone marrow-derived mesenchymal stem cells towards a disc phenotype [12]. Further research has demonstrated that GDF6 has the potential to transform degenerate disc cells towards functional disc cells at a molecular level, even in degenerate human tissues [13]. GDF6 influences the behaviour of NP and AF cells by maintaining their phenotype and functionality, thereby counteracting degenerative changes. Additionally, GDF6 plays a crucial role in regulating the balance between anabolic and catabolic processes within the disc. It promotes the synthesis of ECM, which is essential for maintaining disc integrity, that we have demonstrated in a 12-month long sheep disc injury model [10]. This study is in continuation of our ISSLS award (2018) winning research that demonstrated improved disc height following GDF6 therapy, better pain relief and disc hydration in MRI scan in a composite rat-rabbit model [14] and subsequent work that showed improved pain response in multi-level rodent disc injury [15]. The positive impact of GDF6 in animal studies were seen in the nucleus pulposus, annulus fibrosus and endplates. However, the understanding of molecular pathways at a protein level that are involved in the regenerative cascade remain unclear.

Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC) is a robust method, offering insights into cellular processes and protein dynamics that are crucial for understanding biological functions and disease mechanisms. SILAC is based on direct addition of selected stable isotope amino acids into the cell culture medium, allowing analysis of the cellular proteome with quantitative accuracy and reproducibility in comparison to chemical labeling or label-free quantification strategies [16]. SILAC has been widely applied to characterize the proteomic changes between different biological samples, to investigate dynamic changes of protein post translation modifications (PTMs), to distinguish specific interacting proteins in interaction proteomic analysis, and to analyze protein turnover in the proteome-wide scale. Another interesting application of SILAC is the study of the secretome comprising of secreted proteins, cytokines, interleukins, growth factors, hormones, and others, all of which function as key messengers to coordinate body homeostasis.

In this study, we utilized SILAC to better understand the effect of GDF6 on NP, AF and EP cells that were isolated from degenerated IVDs. The understanding of this pathway will likely help select better candidates for therapy or alternatively develop strategies to augment the therapeutic response of GDF6.

Materials and methods

Cadaveric specimen

A degenerated clinical specimen was obtained with approval by the Institutional Review Board (reference UW 13–576) and with informed consent in accordance with the Helsinki Declaration of 1975 (revision 1983) from a female patient (aged 55 years) undergoing surgery for intervertebral disc degeneration at the Queen Mary Hospital (Hong Kong). L3/4 level was graded as Pffirmann level 5.

Intervertebral disc cell isolation

The NP, AF and EP tissues were identified by gross morphology, then dissected and cut to 1–2 mm small pieces. Tissues were digested with TrypLE™ Express enzyme (Thermofisher, cat# 12604021) and serum-free α-MEM (Gibco, Cat #11900-024) for 30 min at 37ºC on a rotary shaker, before centrifugation at 300 g for 5 min at room temperature. Supernatant was discarded and the cells were incubated with 0.025 mg/mL collagenase P (Roche, Cat# 11213857001) in serum-free α-MEM for 60 min for NP tissue, while AF and EP were digested for up to 90 min. Cells were centrifuged as above, and then cultured in α-MEM supplemented with 10% FBS (Gibco, cat#10270106) and penicillin/streptomycin (Gibco, cat#15140-122). Cells were cultured to passage 3 before the experiment.

SILAC cell culture

Cells from degenerated NP, AF and EP disc tissues were cultured at a concentration of 10^4 cells/cm^2 in triplicate assays for 4 days in unlabelled "light" media [α-MEM (Gibco, Cat #11900-024) supplemented with 10% dialysed FBS (10,000 MWCO, Biowest, Cat# S181D), penicillin/streptomycin, 2.2 g/L sodium bicarbonate (Sigma)] with or without the presence of 400ng/mL GDF6 (Preprotech, cat#120-04), and

then for a further 4 days with or without GDF6 in labelled "heavy" custom-made Arg- and Lys-free α-MEM (AthenaES) as per formulation of α-MEM (Gibco Cat #11900-024), supplemented with 10% dialysed FBS (10,000 MWCO, Biowest, Cat# S181D), penicillin/streptomycin, 2.2 g/L sodium bicarbonate (Sigma), 30 mg/L L-methionine (Sigma), 21 mg/L "heavy" isotope-labelled 13C6 L-arginine (Arg6, Cambridge Isotopes, Cat # CLM-2265-H), 146 mg/L "heavy" isotope-labelled 4,4,5,5-D4 L-Lysine (Lys4, Cambridge Isotopes, Cat # DLM-2640). Cells were cultured in hypoxia (1% O_2 and 5% CO_2 in air) at 37ºC. Media was collected and centrifuged to get rid of cell debris, and cells were washed with PBS x 2 before being lysed with 1% SDS with HALT protease inhibitor cocktail (Thermo Fischer Scientific). Collected media and cell lysate were snap frozen at stored at -80 C until processing.

Proteomic sample preparation

For the collected media, samples were concentrated using Amicon Ultra-0.5mL centrifugal filters with a 10,000 molecular cut off (Millipore, cat #UFC501024). For the cell lysate, the samples were mechanically dissociated by 10 freeze-thaw cycles and sonicated in a cold water bath. Samples were centrifuged at 15,000 g for 30 min at 4 °C and the supernatant was collected. SDS was removed from the samples using the PS removal spin column (Pierce detergent removal resin, Cat#87780) as per manufacturer's instructions. Samples were precipitated in four times the volume of ice-cold acetone and incubated at -20 °C overnight. Samples were centrifuged for 10 min at 15,000 g at 4ºC and the supernatant removed before being air dried and resuspended in 2 M urea in 50mM ammonium bicarbonate. Protein concentration was measured using the BCA assay (Biorad) as per manufacturers' instructions. 100 µg of sample underwent reduction with TCEP (5mM final concentration) at 60 °C for 1 h, and alkylation with iodoacetamide (10mM final concentration) for 20 min at RT. Samples were digested with mass spec grade Trypsin (Promega) as per manufacturers' instructions. Digested peptides were re-suspended in 0.1% formic acid in water, re-quantified and desalted prior to LC-MS/MS analysis. Samples were loaded onto the Dionex UltiMate 3000 RSLC nano Liquid Chromatography coupled to the Orbitrap Fusion Lumos Tribid Mass Spectrometer.

Data processing protocol

MS data obtained were processed using Proteome Discoverer (Ver 2.4), wherein data were searched using Sequest algorithm against Human Uniprot database (20,351 entries, April 2021), supplemented with sequences of contaminant proteins, using below search parameters settings: oxidized methionine (M), acetylation (Protein N-term), heavy Arginine (R6) and Lysine (K4) were selected as dynamic modifications, carboxyamidomethylation of cysteines was specified as a fixed modification, minimum peptide length of 7 amino acids was enabled, tolerance of 10 ppm for the parental peptide, and 20 ppm for fragmentation spectra, and trypsin specificity allowing up to 2 mis-cleaved sites. Confident proteins were identified using a target-decoy approach with a reversed database, strict false-discovery rate of 1% at peptide and PSM level. Newly synthesized proteins were heavy labelled with Arg6- and Lys4 and the data was expressed as the normalized protein abundance obtained from heavy (labelled)/light (un-labelled) ratio.

Raw data depository and software availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [17] repository with the following dataset identifier PXD029192.

Results

Overview of protein profiles of in vitro-cultured cells and secretome of degenerated disc

To understand the effects of GDF6 on degenerated cells of the IVD, we used the SILAC approach which uses heavy isotope labelling of lysine and arginine amino acids ('heavy labelled') to incorporate into newly synthesised proteins which can be used to compare to pre-existing unlabelled proteins ('light' proteins). From degenerated disc tissue, cells isolated from the AF, NP and EP were cultured for 4 days in 'light' media before being cultured in 'heavy' media with or without GDF6 treatment, and both the cellular proteins as well as the secreted proteins in the media (secretome) were collected and analysed (Fig. 1A). From the profiles of the cellular and media fractions (Fig. 1B), non matrisome proteins accounted for the majority of 'heavy' and 'light' proteins that were identified in each group, whilst media had larger proportions of the matrisome proteins. In all cells, the treatment with GDF6 reduced the numbers of detected proteins compared to the untreated controls (636 vs. 714 in AF cells; 825 vs. 1,332 in NP cells; 1,452 vs. 1,484 in EP cells). The numbers of proteins identified in the media fractions were consistent, regardless of whether they were treated with GDF6 or not, and were far fewer in number than their cellular counterparts (Fig. 1B).

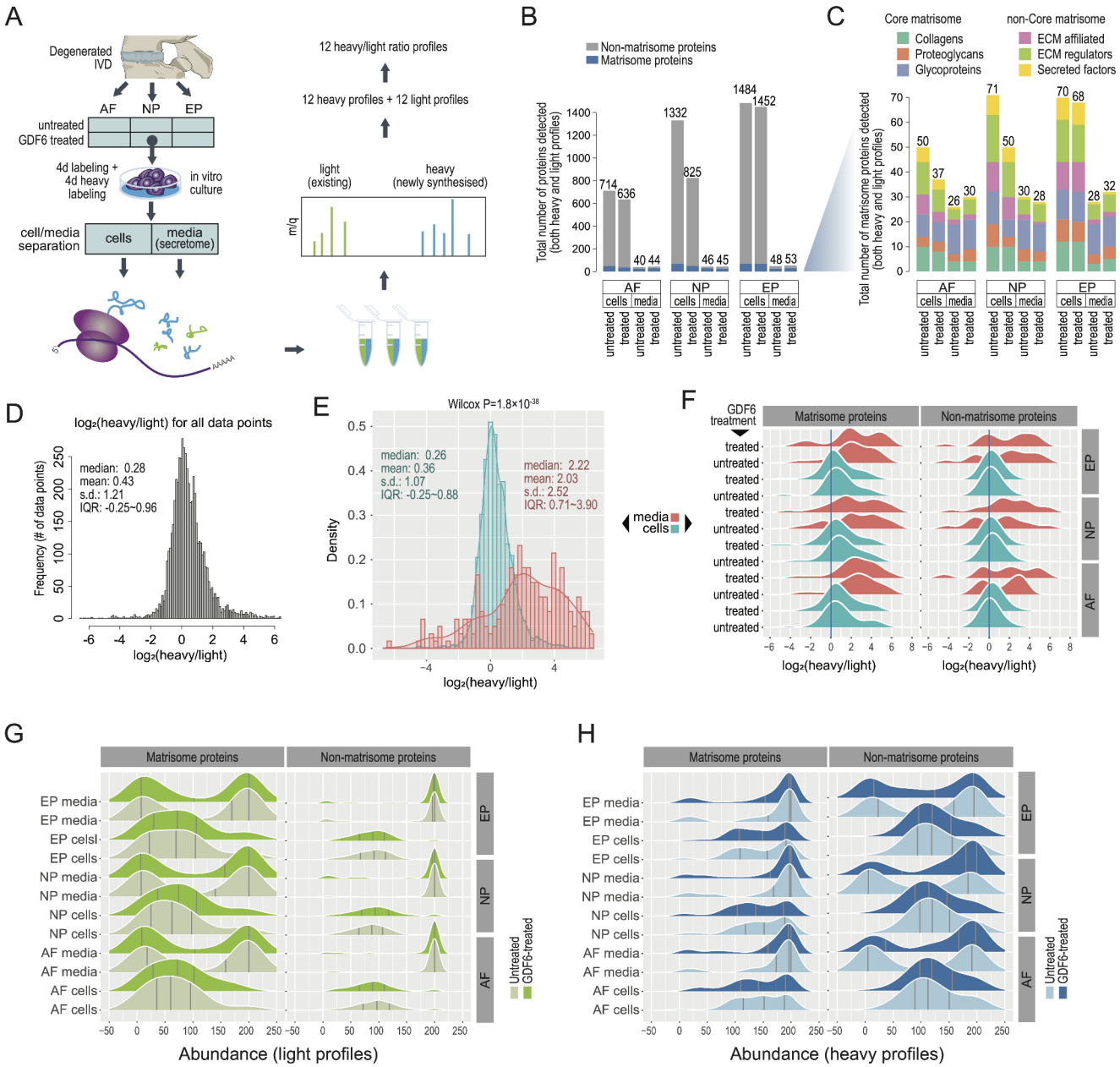

Fig. 1 Workflow of SILAC proteomics. AF, NP and EP tissues were excised from clinical samples and cultured in SILAC media with/without GDF6, and the proteins extracted from cells and media (secretome) and analysed by mass spectrometry for 'heavy' (newly-synthesised) and 'light' (pre-existing) proteins (**A**). Matrisome and non-matrisome profiles across all the samples (**B**). Categorisation of the matrisome proteins across all samples (**C**). Frequency of the synthesized proteins expressed as log2(heavy/light) ratios across all the samples (**D**). Abundance of the proteins expressed as the log2(heavy/light) ratios for media (secretome) and cells (**E**). Abundance of matrisome and non-matrisome proteins expressed as the log2(heavy/light) ratios across the samples (**F**). Abundance of pre-existing ('light') proteins (**G**). Abundance of newly-synthesised ('heavy') proteins (**H**)

GDF6 treatment downregulates protein synthesis in degenerated cells

We further divided the proteins into matrisome protein categories according to a matrisome classification database [18] (matrisomeproject.mit.edu) and found that in both cell and media fractions, ECM regulators and glycoproteins made up the majority of the matrisome subtypes, whilst proteoglycans and ECM-affiliated proteins accounted the for the least (Fig. 1C). It was also observed that whilst the numbers of

matrisome proteins decreased with GDF6 treatment in cells, the numbers between treated and untreated media remained similar (Fig. 1C).

We then expressed the amounts of proteins that were synthesised as the log2(heavy/light ratio, H/L ratio) regardless of whether the sample was from the cell or media, and plotted these on a histogram which showed a normal distribution and a mean of 0.43 H/L ratio (Fig. 1D). However, when the data for the cell and media were separated, the media had a higher mean (2.03) in comparison to the cells (0.36) which indicated that there were more proteins that were synthesised in higher abundance in the media (Fig. 1E).

We were further interested in gaining a better understanding of whether it was the non-matrisome or matrisome proteins that were contributing to the higher abundance proteins and subsequently separated the matrisome and non-matrisome proteins (Fig. 1F) which showed that media had consistently higher synthesis of proteins due to both the matrisome and non-matrisome proteins. The abundance of both the light and heavy profiles are shown in Fig. 1G and H, respectively.

Effects of GDF6 treatment on different cell types

To decipher an overall influence that GDF6 can exert on the cellular and secreted (secretome) matrisome and cellular (non-matrisome) components of the degenerate IVDs, this study analysed compartment-specific responses in NP, AF, and EP cells isolated from degenerate IVDs. Comparison of the cellular profiles of the NP, AF and EP cells of degenerated IVD showed a combined aggregate of 1,807 proteins, with 735 (40.7%) proteins shared between the 3 cell types (Fig. 2A). There were 911 proteins identified in AF cells, 1,470 proteins in NP cells, and 1,665 proteins in EP cells (Fig. 2B-D). In all cells, the treatment with GDF6 reduced the numbers of detected proteins compared to the untreated controls in AF and NP (636 vs. 714 in AF cells; 825 vs. 1,332 in NP cells, Fig. 2B & C), but not EP cells (1,478 vs. 1,484 proteins, Fig. 2D). In fact, the proportion of shared proteins between treated and untreated EP cells was much higher at 77.9% than AF cells and NP cells (48.2% and 46.7%, respectively).

Comparison of the secretome profiles of the NP, AF and EP showed far fewer protein numbers with 62 in EP media, 59 in NP media and 55 in AF media, of which 42 were shared between the three groups (Fig. 2E). In a similar manner to the cells, the media of AF and NP had similar proportions common between treated and untreated groups (52.7% and 54.2%, respectively, Fig. 2F & G), whilst EP media had 62.9% proteins common between treated and untreated groups (Fig. 2H).

To gain a better comprehension of differences between each of the groups, the log2(H/L ratios) were re-ranked and rearranged according to their level of abundance (Fig. 2I). Whilst GDF6-treatement resulted in overall lower number of proteins synthesised for AF and NP cells but not EP cells, we identified that treatment with GDF6 resulted in different types of ECM proteins being synthesised intracellularly. In AF cells, treatment with GDF6 increased production of core matrisome proteins COL8A1, FN1, ABI3BP, MFGE8, POSTN, FMOD, but suppression of ELN, LAMC1, EMILIN and IGFBP3. AF cells also had increase (ANXA11/2, LGALS1, CD109, CTSB, HTRA1, MMP14, PLOD1, FSTL1, S100A10/13/16) and reduction (ITH2/3, GDF6) in non-core matrisome proteins. In NP cells, there was a reduction in COL2A1, but increase in COL6A2, OGN, ACAN, HAPLN1/3, IGFBP3, PCOLCE, and CREDL2 core matrisome proteins. NP cells also had an increase (GREM1, ANXA7, CSPG4, GPC4, MUC5AC, SCD4, SERPINB1, CTSA/D, LOX, FSTL1, FGF2, HCFC1, S100A2) in non-core matrisome proteins. In EP cells, there was also a reduction in COL2A1, but an increase in OGN, and EMILIN1 core matrisome proteins, whilst there were both increases (ITH2/3, GDF6, S100A2) and decreases (LOXL2, FSTL1) in the non-core matrisome proteins (Fig. 2J).

Next, we compared the heavy and light profiles of all the groups divided into the matrisome or non-matrisome proteins (Fig. 3A) that clearly reflected a decrease of synthesised proteins in response to GDF6 across all the cell groups, but similar proportions in the media groups. Examination of the numbers of the different matrisome categories also reflected similarities in their proportions (Fig. 3B) of which glycoproteins and ECM regulators were dominant.

We performed geneset analysis on only the heavy profiles that represent all the newly synthesised proteins in cells (Fig. 3C). In AF cells, treatment with GDF6 resulted in the enrichment of genes associated with Myc targets, EMT, MTORC1 signalling and glycolysis, whilst the media was increased for MFGE8, CDH13, TIMP2, MMP2 and FBN1. In NP cells, there was enrichment of genes associated with Myc targets, EMT, MTORC1 signalling, and oxidative phosphorylation, whilst there was secretion of proteins including DCN, MFGE8, NOTCH2, TIMP1, IGF1, and FBN2 into the media. In EP cells, there was enrichment of similar genesets to NP cells, but the media had secreted HTRA1, IGFBP7, TIMP1 and NME1. Comparison of all the genes that were significant in the different after treatment showed that AF, NP and EP had very little overlap (Fig. 3C), with AF producing proteins such as ANXA5, PLOD2, ACAT2, whilst NP cells expressed proteins including TNC, PUF60, CIRBP, MXRA5, and FMOD. Common proteins between AF and NP included COL3A1, CLU, POLR2A, PDE4DIP.

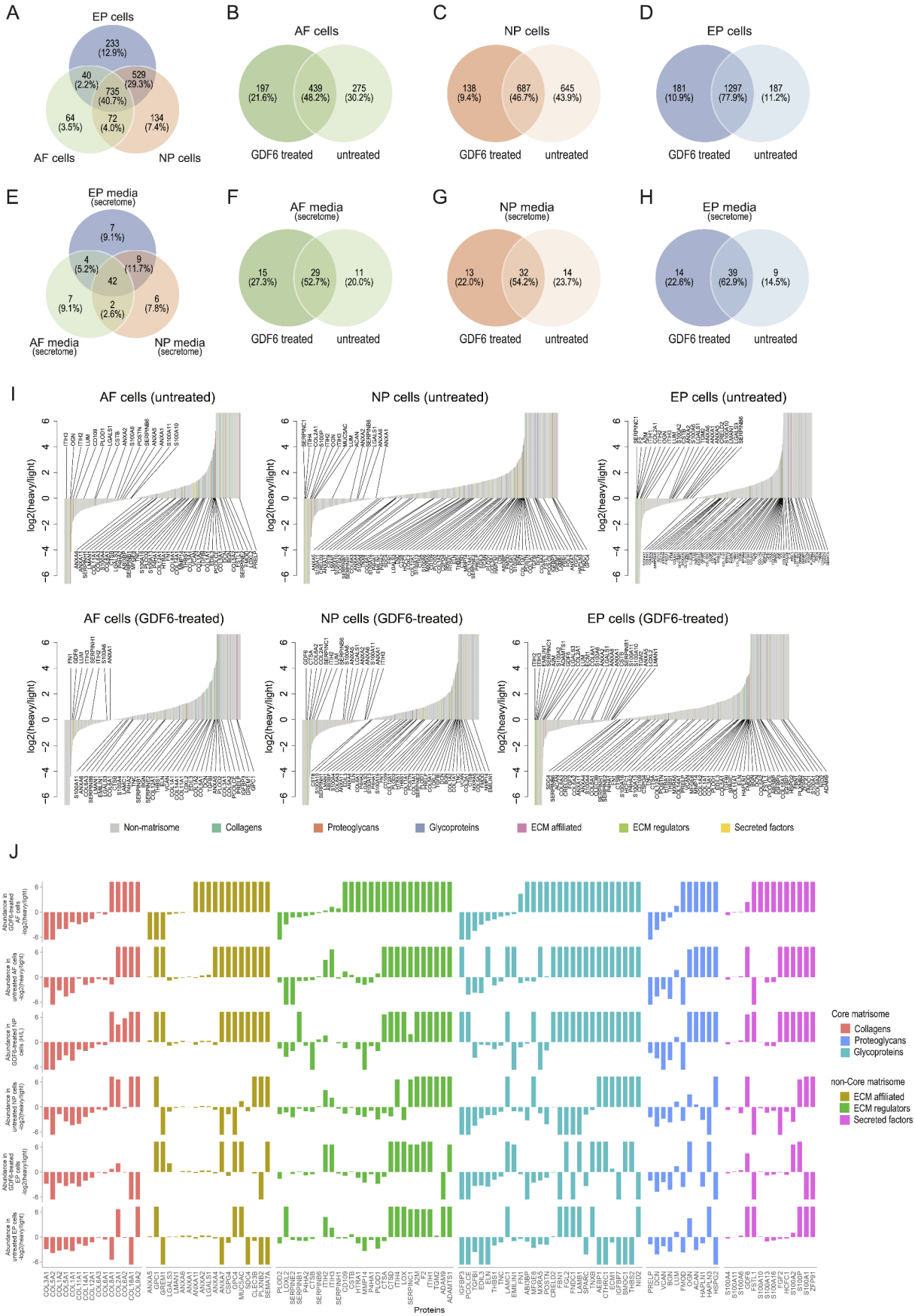
A
EP cells
233 (12.9%)
40 (2.2%)
529 (29.3%)
735 (40.7%)
64 (3.5%)
72 (4.0%)
134 (7.4%)
AF cells
NP cells
B
AF cells
197 (21.6%)
439 (48.2%)
275 (30.2%)
GDF6 treated
untreated
C
NP cells
138 (9.4%)
687 (46.7%)
645 (43.9%)
GDF6 treated
untreated
D
EP cells
181 (10.9%)
1297 (77.9%)
187 (11.2%)
GDF6 treated
untreated
E
EP media (secretome)
7 (9.1%)
4 (5.2%)
9 (11.7%)
42
7 (9.1%)
2 (2.6%)
6 (7.8%)
AF media (secretome)
NP media (secretome)
F
AF media (secretome)
15 (27.3%)
29 (52.7%)
11 (20.0%)
GDF6 treated
untreated
G
NP media (secretome)
13 (22.0%)
32 (54.2%)
14 (23.7%)
GDF6 treated
untreated
H
EP media (secretome)
14 (22.6%)
39 (62.9%)
9 (14.5%)
GDF6 treated
untreated
I
AF cells (untreated)
NP cells (untreated)
EP cells (untreated)
AF cells (GDF6-treated)
NP cells (GDF6-treated)
EP cells (GDF6-treated)
log2(heavy/light)
Non-matrisome
Collagens
Proteoglycans
Glycoproteins
ECM affiliated
ECM regulators
Secreted factors
J
Abundance in GDF6-treated AF cells -log2(heavy/light)
Abundance in untreated AF cells -log2(heavy/light)
Abundance in GDF6-treated NP cells (H/L)
Abundance in untreated NP cells -log2(heavy/light)
Abundance in GDF6-treated EP cells -log2(heavy/light)
Abundance in untreated EP cells -log2(heavy/light)
Proteins
Core matrisome
Collagens
Proteoglycans
Glycoproteins
non-Core matrisome
ECM affiliated
ECM regulators
Secreted factors

Fig. 2 Comparison of AF, NP and EP profiles. Venn diagram showing number of shared and exclusive proteins expressed by AF, NP and EP cells (**A**). Number of proteins expressed in AF, NP, EP cells (**B**-**D**) and media (**E**-**H**) with or without GDF6 treatment. Abundance of non-matrisome (grey) and matrisome (coloured) proteins in AF, NP and EP samples (**I**). Abundance of matrisome proteins in AF, NP and EP cells (**J**)

We also performed geneset analysis on the genes downregulated by GDF6 treatment in NP, AF and EP cells (Supplementary Fig. 1). In NP cells, GDF6 downregulates some genes associated with Myc targets associated with translation (such as EIF4E, EIF3J, EEF1B2, RPL6, RPL34, RPLP0, SNRPA1, and RPL14, In AF cells, GDF6 reduced the expression of genes associated with Myc targets associated with translation and RNA processing (such as NOLC1, EEF1B2, SNRPA, and RPL14). In EP cells, GDF6 downregulated genes associated with Mtorc1 (PNP, HMBS) and apoptosis (LGALS3, SQSTM1).

Whilst it was important to understand the effects of GDF6 on the intracellular proteins (which represent their function) of the different cell types, we were also interested in determining if GDF6 could affect the secretion of ECM proteins by the cells into the media (secretome), as ECM plays a major role in the integrity of IVD tissue that is maintained by the localised cells (Fig. 4A). In AF media, there was increase in the secretion of MFGE8, IGFBP2, FBN1, HSPG2, CST3, TIMP2, MMP2, SERPINF1/D1, POSTN, which was accompanied with a downregulation of COL2A1 (also observed in all GDF6-treated media samples), SPOCK1, and FSTL1. In NP media, treatment with GDF6 resulted in increased COL6A3, HTRA1, MFGE8,CTGF, EFEMP1, FBN2, CBLN4 and IGF1 which was accompanied by a downregulation of ECM1, CLEC3B, PLOD1, MMP2, LOX, IGFBP4, BGN, and FSTL1. Treatment of EP resulted an increase in HTRA1 and reduction of MMP2, F2, MFGE8, and FBN2. Geneset enrichment analysis of the media (Fig. 4B) showed enrichment of common pathways in AF, NP, and EP (EMT, coagulation, angiogenesis, apoptosis, hypoxia).

Discussion

Impact on matrisome and non-matrisome protein synthesis

This study elucidates the comprehensive effects of GDF6 on both matrisome (structural) and non-matrisome (cellular) protein synthesis within degenerate IVDs. We employed SILAC to analyze compartment-specific responses in NP, AF, and EP cells isolated from degenerated IVDs. The results revealed significant alterations in protein synthesis following GDF6 treatment across these three disc compartments.

Across all the compartments, GDF6 treatment led to synthesis of crucial matrisomal proteins, mostly in the form of proteoglycans and glycoproteins. Synthesis of specific proteins, particularly MFGE8 (milk fat globule-EGF factor 8), IGFBP2 (insulin-like growth factor binding protein 2), POSTN (periostin), THBS4 (thrombospondin 4), and IGFBP4 (insulin-like growth factor binding protein 4), suggests a multifaceted role of GDF6 in enhancing cellular responses in the context of intervertebral disc (IVD) health and regeneration. MFGE8 has been implicated in promoting cell survival and modulating inflammatory responses [19, 20]. Its upregulation in response to GDF6 treatment indicates a potential mechanism by which GDF6 enhances cellular viability in the IVD, especially under degenerative conditions. MFGE8 facilitates the phagocytosis of apoptotic cells, thus maintaining a healthy cellular environment and preventing chronic inflammation, which can exacerbate degeneration [21, 22]. IGFBP2 plays a crucial role in regulating the bioavailability of insulin-like growth factors (IGFs), which are vital for cellular growth, proliferation, and survival [23, 24]. The upregulation of IGFBP2 upon GDF6 treatment suggests that GDF6 not only stimulates growth factor signaling but also fine-tunes it through binding and modulating IGF activity. This balance may enhance cell proliferation and support tissue repair processes within the IVD, contributing to the overall regenerative capacity of the disc. By promoting POSTN expression, GDF6 may aid in the stabilization of the ECM [25], fostering a supportive niche for NP and AF cells, which is crucial for maintaining hydration and mechanical properties of the disc. Enhanced THBS4 expression could facilitate the formation of a more stable and integrated ECM, promoting cellular interactions that are critical for sustaining IVD health [26]. Similar to IGFBP2, IGFBP4 also modulates the actions of IGFs, albeit with a different regulatory mechanism [27]. The upregulation of IGFBP4 suggests that GDF6 treatment may create a more complex network of IGF regulation within the IVD. This could help fine-tune cellular responses to growth factors, promoting a balanced proliferation and differentiation process.

The upregulation of non-core matrisome proteins such as CLEC3B (C-type lectin domain family 3 member B), CST3 (cystatin C), TIMP2 (tissue inhibitor of metalloproteinases 2), MMP2 (matrix metalloproteinase 2), SERPINF1 (serpin peptidase inhibitor, clade F, member 1), SERPIND1 (serpin peptidase inhibitor, clade D, member 1), SERPINA7 (serpin peptidase inhibitor, clade A, member 7), SPOCK1 (sparc/osteonectin, cwcv, and kazal-like domains proteoglycan 1), and FSTL1 (follistatin-like 1) in response to GDF6 treatment indicates a complex interplay of signaling pathways that contribute to extracellular matrix (ECM) remodeling, cellular protection, and modulation of inflammatory

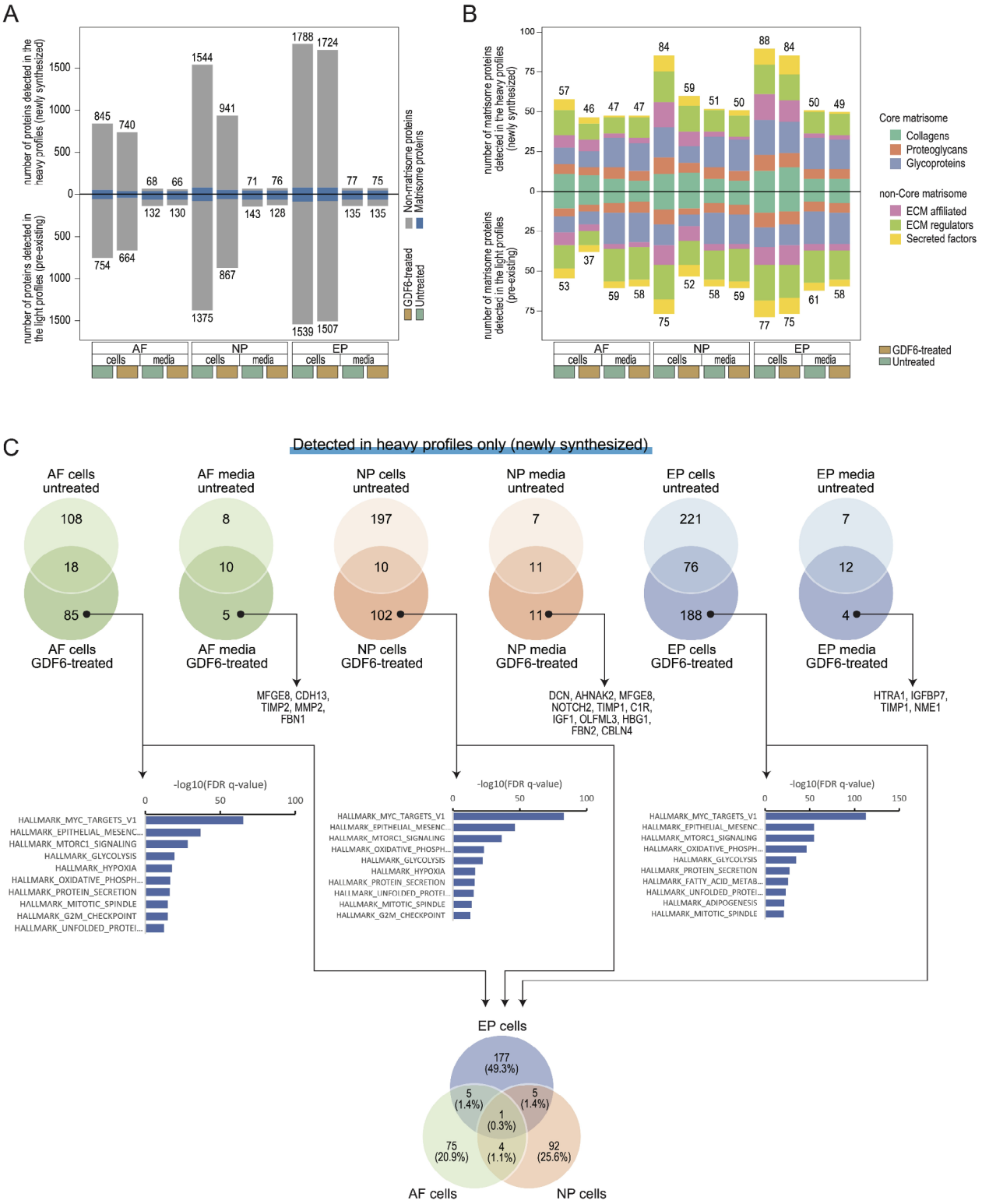

Fig. 3 Quantification of 'light' and 'heavy' non-matrisome, matrisome proteins (**A**) and the different categories of matrisome proteins (**B**) in AF, NP and EP samples. Newly-synthesised proteins (heavy) detected in GDF6-treated and untreated samples and related pathways assessed by gene-set enrichment analysis of newly synthesized cellular proteins (**C**)

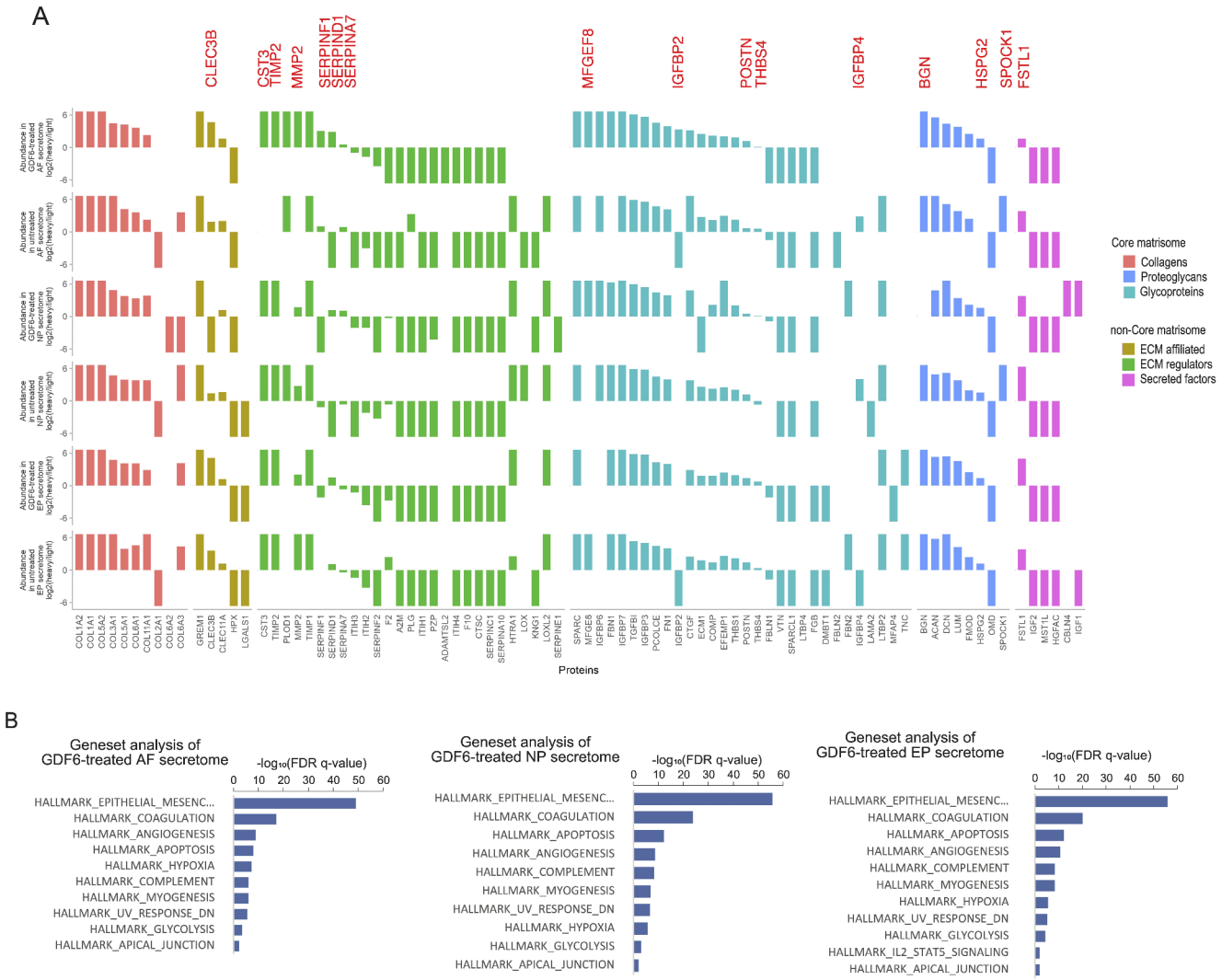

Fig. 4 Abundance of secreted matrisome proteins with/without treatment with GDF6, expressed as the log2(heavy/light) ratio (**A**). Genset analysis of secreted matrisome proteins after GDF6 treatment (**B**)

responses in the intervertebral disc (IVD) [28–32]. These proteins collectively contribute to maintaining disc health, promoting regeneration, and potentially mitigating the effects of degeneration.

Differential effects across disc compartments

Variability in cell response

GDF6 influences protein synthesis in cells from different intervertebral disc (IVD) compartments—annulus fibrosus (AF), nucleus pulposus (NP), and endplate (EP)—showing varied responses that highlight their unique regenerative roles. GDF6 treatment reduced protein synthesis across all cell types, with NP cells experiencing the most significant decrease (from 1,332 to 825 proteins), followed by AF cells (714 to 636). EP cells showed minimal change (1,484 to 1,478). Specific protein responses differed by compartment. AF cells upregulated proteins such as LOX and OGN, suggesting a role in maintaining structural integrity, while untreated AF cells expressed proteins linked to matrix degradation. NP-treated cells increased PCOLCE, OGN, ACAN, and HAPLN1/3 synthesis which are necessary for structural integrity and hydration [33]. EP cells treated with GDF6 had enhanced EMILIN1 and OGN synthesis, both of which are important for structural integrity of the tissue, while untreated cells expressed proteins associated with inflammation [34].

Our findings reveal that GDF6 induces smaller changes in the endplate EP compared to the AF and NP, likely due

to the EP's lower metabolic activity and transitional tissue composition. As a member of the BMP family (GDF5, 6, and 7), GDF6 is known for its chondrogenic and anti-osteogenic properties, inhibiting both early and late markers of osteogenic differentiation while promoting cartilaginous phenotypes [35]. This characteristic aligns with the limited changes observed in EP cells, which naturally exhibit a gradient toward osteogenic traits in degenerated states, and suggests that GDF6 primarily supports cartilage stability in the EP rather than extensive ECM remodelling [36]. Overall, these results underscore the compartment-specific effects of GDF6 and highlight the NP as a key target for therapies aimed at ECM turnover and hydration restoration in IVD regeneration.

Variability in secretomes

The secretome is vital for the dynamic regulation of the ECM by the resident cells, ensuring that it can adapt to damage repair, localised environmental changes, and maintain tissue integrity [37]. Secretome analysis of AF, NP, and EP cells highlights the compartment-specific effects of GDF6 treatment. Fewer secreted proteins were identified compared to cellular proteins: 55 in AF media, 59 in NP media, and 62 in EP media. GDF6 influenced the extracellular environment differently in each compartment. In the AF secretome, GDF6 upregulated matrix proteins including fibrillin-1 (FBN1), periostin (POSTN) and perlecan (HSPG2), while downregulating FSTL1, suggesting a shift toward a stable matrix environment [38]. Untreated AF media showed higher levels of inflammatory proteins, indicating an active degeneration response. In NP media, GDF6 promoted the secretion of proteins Connective Tissue Growth Factor (CTGF), Fibulin 3 (EFEMP1), Fibrillin-2 (FBN2) and Insulin-Like Growth Factor 1 (IGF1), while reducing matrix metalloproteinase-2 (MMP2), suggesting a focus on matrix preservation and reduced inflammation [39, 40]. Untreated NP secretomes had higher levels of fibrotic proteins, indicating a response to degeneration [41]. EP media showed fewer differences, but GDF6 increased cartilage HTRA1 levels and decreased MMP2 and F2. Overall, GDF6 appears to promote ECM stabilization and limit inflammation and degradation across IVD compartments, indicating its potential as a therapeutic agent for disc regeneration and maintenance.

Another noteworthy observation was the enrichment of genes associated with epithelial-to-mesenchymal transition (EMT) following GDF6 treatment in NP, AF, and EP cells. The induction of EMT-associated genes suggests that GDF6 may facilitate a shift in cellular phenotype that enhances the resilience of IVDs against degradation. EMT, characterized by the loss of epithelial traits and acquisition of migratory and invasive properties, can contribute to tissue repair and regeneration [42, 43]. In the context of IVDs, GDF6 may promote structural integrity and functionality by fostering cellular adaptations that counteract degenerative processes.

Implications for clinical & therapeutic strategies

The compartment-specific effects of GDF6 highlight its potential as a therapeutic agent for IVD degeneration. GDF6 modulates matrisome and non-matrisome protein synthesis to enhance ECM stability, reduce fibrosis, and maintain hydration, especially in the NP. Its effects can be tailored to target specific disc compartments depending on the stage of degeneration. In early-stage NP degeneration, GDF6 may restore proteoglycan levels and improve water retention, while in later stages, it could reduce AF stiffening and enhance ECM flexibility [12].

Our findings show that GDF6 treatment decreases the total number of proteins in NP cells but boosts the expression of critical ECM proteins. GDF6 also enriches genes associated with epithelial-to-mesenchymal transition (EMT), suggesting its role in slowing disc degeneration by replenishing matrisomal proteins. The selective downregulation of fibrotic proteins, while promoting protective non-matrisome proteins, further underscores GDF6's potential to restore hydration and mechanical properties across NP, AF, and endplate (EP) compartments. However, GDF6 therapies must be carefully tailored, as reduced levels of key structural proteins like collagen II and aggrecan may affect disc biomechanics [44]. Future studies should focus on optimizing GDF6 dosage and delivery methods.

Additionally, GDF6's modulation of the secretome suggests it may enhance the disc's self-repair capabilities by creating a regenerative microenvironment conducive to tissue repair and limiting further degeneration. This dual intracellular and extracellular modulation enhances the therapeutic versatility of GDF6, making it a compelling candidate for both direct intradiscal injection and broader regenerative strategies.

Limitations and future directions

While this study provides valuable insights into the proteomic changes induced by GDF6 in degenerated IVD cells, several limitations should be noted. The use of a single patient-derived degenerated disc sample limits the generalizability of the findings. Future research should include a larger cohort of patient samples to validate the effects of GDF6 across different stages of degeneration and in individuals with varying etiologies of LBP.

IVD degeneration is characterized by significant hydration loss, leading to reduced disc height and an increased risk of further degeneration. GDF6 plays a crucial role in

maintaining IVD hydration and mechanical properties. This study demonstrates that GDF6 enhances the synthesis of ACAN in NP cells, a key proteoglycan associated with the water retention capacity of the NP. While other proteins, such as FSTL1, were synthesised by NP and AF cells in response to GDF6, the newly synthesised proteins identified in this study did not fully align with the hydration matrisome reported by Tam et al. [34]. This discrepancy is likely due to the use of isolated cells, as opposed to cells in situ.

Additionally, while SILAC offers precise quantification of newly synthesized proteins, it does not capture the full complexity of ECM remodeling, including post-translational modifications and protein degradation, which are crucial for understanding disc biology. Future studies combining SILAC with other proteomic approaches, such as degradomics and transcriptomics, will be critical for elucidating the complete molecular pathways influenced by GDF6.

Conclusion

This study highlights GDF6's role in modulating protein synthesis in both matrisome and non-matrisome proteins within degenerated IVD compartments. Specifically, GDF6 enhances ECM stability and reduces fibrosis in NP and AF cells, demonstrating its potential to support ECM integrity and hydration. While these findings suggest that GDF6 could be a promising therapeutic candidate for disc degeneration by promoting cell survival and matrix production, this study provides preliminary evidence that must be further validated through randomized controlled trials and clinical studies to fully establish its efficacy and safety as a treatment strategy for degenerative disc disease.

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Data availability The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE repository with the following dataset identifier PXD029192.

Declarations

Ethics approval and consent to participate Ethics approval was obtained from Institutional Review Board (reference UW 13–576) and with informed consent by the patient to participate in accordance with the Helsinki Declaration of 1975 (revision 1983).

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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