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RESEARCH ARTICLE

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E74-like ETS transcription factor 3 expression and regulation in human intervertebral disc

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Abstract

Background: Intervertebral disc degeneration (IVDD) is one of the main causes of chronic low back pain. The degenerative process is often initiated by an imbalance between catabolic and anabolic pathways. Despite the large socio-economic impact, the initiation and progress of disc degeneration are poorly understood. Although intervertebral disc (IVD) and articular joint are not identical, their degenerative roads are remarkably similar. We, and another authors, previously demonstrated that E-74-like factor 3 (ELF3), a transcription factor induced by inflammatory mediators in various cell types including chondrocytes, is a central contributing factor for cartilage degradation. Thus, we aim to explore, for the first time, the expression, modulation, and the role of ELF3 in human IVD cells.

Methods: The presence of ELF3 in healthy and degenerated IVD tissues was initially determined by immunohistochemistry in annulus fibrosus (AF) and nucleus pulposus (NP). mRNA and protein expression were measured, respectively, by RT-qPCR and

Abbreviations: ADAMTS-5, A disintegrin and metalloproteinase with thrombospondin motifs 5; AF, annulus fibrosus; CCL3, C-C Motif Chemokine Ligand 3; COX-2, cyclooxygenase-2; COL2A1, collagen type II alpha 1 chain; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; ELF3, E74-like ETS transcription factor 3; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFNγ, interferon gamma; IL-1α, interleukin 1α; IL-1β, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; IVDD, intervertebral disc degeneration; LBP, lower back pain; LEPR, leptin receptor; LPS, lipopolysaccharides; MMP-3, matrix metalloproteinase 3; MMP-9, matrix metalloproteinase 9; MMP13, matrix metalloproteinase 13; NOS2, nitric oxide synthase 2; NP, nucleus pulposus; OA, osteoarthritis; PMSF, phenylmethylsulfonyl fluoride; PTGS2, prostaglandin-endoperoxide synthase 2; RA, rheumatoid arthritis; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TNFα, tumor necrosis factor alpha.

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Western blot in AF and NP IVD cells harvested from healthy individuals and IVDD patients. Overexpression of ELF3 was performed by transfection of AF IVDD cells with pESE-1: ELF3 expression vector or pCI: empty vector.

Results: Our results unveiled, for the first time, the expression of ELF3 in IVD tissues. ELF3 is notably upregulated in degenerated tissues compared to those from healthy patients. In addition, the stimulation of IVDD AF cells with various proinflammatory stimuli, showed marked increase in both mRNA and protein expression of ELF3. ELF3 overexpression in AF IVDD cells resulted in the upregulation of proinflammatory and catabolic genes such as PTGS2, NOS2, LCN2, IL-6, MMP13, and ADAMTS-5; whereas, ELF3 silencing resulted in the opposite results.

Conclusions: Our results support a novel role for ELF3 as a pro-inflammatory and pro-catabolic transcriptional mediator, whose targeting in IVD tissues might be of potential therapeutic relevance in disc degeneration.

KEYWORDS

catabolism, E74-like ETS transcription factor 3, ETS transcription factors, human intervertebral disc, inflammation, IVDD

1 | INTRODUCTION

Intervertebral disc degeneration (IVDD) is a multifactorial spine disorder driven by inflammatory and catabolic processes leading to a structural degenerative cascade of the intervertebral disc (IVD).[1] It causes low back pain (LBP), a prevalent cause of disability on a global scale.[1,2] IVDD is characterized by chronic low-grade inflammation that sustains a metabolic shift toward catabolic processes.[3] This shift leads to progressive extracellular matrix degeneration, fibrosis, loss of mechanical stability, diminished shock absorbent function of the IVD, and subsequent damage of the intervertebral tissues.[4,5] The degenerative and inflammatory processes in IVDD share many cellular and molecular similarities with osteoarthritis (OA).[6] Unveiling these similarities might enable more rapid advancement in identifying potential therapeutic targets for both conditions. The current therapeutic strategies for both pathologies focus on symptoms management, with very limited clinical success in reversing the degenerative process.[6,7]

In chondrocytes, the extracellular matrix remodeling, in both physiological and pathological conditions, was reported to be also regulated by ETS transcription factors.[8] The E-74-like factor 3 (ELF3) is typically expressed in epithelial tissues under normal physiological conditions.[8] However, it is induced by inflammatory triggers in various tissues and cell types, including cartilage, synovium, and in certain rheumatic degenerative diseases such as rheumatoid arthritis (RA) and OA.[9] Our group previously demonstrated that in response to inflammatory stimuli, ELF3 drives in chondrocytes a transcriptional control over the expression of inflammatory genes like NOS2, COX2, and LCN2.[10–12] In addition, ELF3 was shown to promote cartilage catabolism, to inhibit the expression of anabolic genes in chondrocytes,[13] and also to interrupt the interaction between Sox9-CBP and p300.[14]

Our laboratory also previously highlighted the existence of a loop between obesity and inflammation in cartilage, demonstrating how the adipokine leptin synergizes with IL-1β to aggravate ELF3 induction in chondrocytes.[15] Obesity is undoubtedly one of the most relevant risk factors for the onset and progression of IVDD.[16] Besides the abnormal and excessive mechanical loading, the production of adipokines by white adipose tissue has been proposed as a metabolic link between obesity and IVDD.[3,16] Adipokines were reported to contribute to the pathophysiology of degenerative, inflammatory, and autoimmune diseases.[17–20] Leptin itself was reported to have a catabolic role in articular cartilage.[21,22]

As described, although previous studies have established ELF3 involvement in inflammatory processes in conditions like OA, its specific implication in IVDD remains unexplored. To address this gap, our research aims to uncover the specific role of ELF3 in IVDD and identify new therapeutic targets for this condition. First, we studied the ELF3 expression levels in healthy and degenerated IVD tissues. Next, we assessed the effect of ELF3 overexpression and silencing on the mRNA expression of key catabolic and proinflammatory factors. We finally investigated whether the leptin-ELF3 loop, previously described in OA cartilage, is conserved also in IVD AF cell.[15] Here, we demonstrate, for the first time, the potential role of the transcription factor ELF3 as an inflammatory mediator in IVDD AF cells.

2 | MATERIALS AND METHODS

2.1 | Reagents

Fetal bovine serum (FBS), lipopolysaccharides from *Escherichia coli* (LPS), recombinant human interleukin 1α (IL-1α), leptin, tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and dexamethasone were obtained from MilliporeSigma (USA). Dulbecco's Modified Eagle's Medium (DMEM) and α modified minimum essential medium MEMα were purchased from Gibco (USA). Trypsin-ethylenediaminetetraacetic acid and penicillin–streptomycin mixture

were obtained from Lonza Group (Switzerland). Pronase and collagenase P were obtained from Roche Diagnostics (Germany).

2.2 | Tissue and cell isolation and culture

Healthy IVDs were obtained from deceased multiorgan donors with no clinical records of lumbar magnetic resonance imaging studies. IVDD samples were harvested from patients undergoing discectomy due to conditions such as spinal stenosis, adult degenerative scoliosis, disc herniation, and disc degeneration (Pfirrmann grades 4 and 5). Patients with infections or prior spinal malignancies were excluded from the study. All IVD samples were obtained under permission from the local ethics committee, according to the Declaration of Helsinki (Code 2017/279 from Galician Ethical Committee). We have used AF cells from healthy individuals (five males, two females, mean age: 49.7 ± 13.6) and from IVDD patients (four males, 10 females, mean age: 60.2 ± 12.5).

The isolation of disc cells followed a previously established protocol.[23] In brief, aseptically dissected disc tissue was finely diced and thoroughly rinsed with phosphate-buffered saline (PBS). After removing excess PBS, the tissue underwent a 20-min incubation with pronase at a final concentration of 1 mg/mL in DMEM/Ham's F12 at 37°C. Following this step, pronase was carefully removed, and the tissue was washed three times with PBS. The tissue was then digested with collagenase P at a final concentration of 1 mg/mL in DMEM/Ham's F12 supplemented with 10% FBS for 4 to 6 h at 37°C with gentle agitation. The resulting cell suspension was then filtered through a 40 μm nylon cell strainer (BD Biosciences Europe, Belgium) to remove any debris. Subsequently, the cells were centrifuged, washed with PBS, and resuspended in a mixture of DMEM and MEMα (in a 6:4 ratio) supplemented with 20% FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin. The cells were counted and plated into six-well culture plates, at a density of 2×10^5 cells per well, reaching a confluence of 75%–90%, and cultured for a maximum of 10 days for expansion. It is important to note that all cells utilized in these experiments were at passage number 2. The concentrations of used treatments were as follows: LPS at 100 ng/mL, IL-1α at 0.1 ng/mL, leptin at 800 nM, TNFα at 10 ng/mL, IFNγ at 10 ng/mL, and dexamethasone at 1 μM.

2.3 | Immunocytochemical assays

Immunohistochemistry was carried out automatically utilizing an Autostainer Link 48 immunostainer manufactured by Dako-Agilent (located in Santa Clara, CA, USA), as it was as previously described.[24] In brief, the slides underwent the following steps at room temperature: Heat-induced epitope retrieval was performed using a high pH solution (Dako-Agilent) for 20 min at 97°C. After that, a human polyclonal antibody targeting Anti-ESE1 antibody (ab97310) from Abcam (Cambridge, UK) was applied at a dilution of 1:100 for 30 min. The EnVision® + Dual Link System HRP, consisting of a dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-mouse and goat anti-rabbit immunoglobulins (Dako Agilent, K4065), was incubated for 20 min. The DAB + substrate-chromogen solution, composed of 1 mL of substrate buffer solution containing hydrogen peroxide and 20 μL of 3,3′-diaminobenzidine tetrahydrochloride chromogen solution, was applied for 10 min. Finally, EnVision FLEX hematoxylin was used for counterstaining, with a 15-min incubation period.

2.4 | Isolation of Total RNA and RT-qPCR Analysis

Total RNA was extracted using NZYol (NZYTech, Portugal) and the E.Z.N.A. Total RNA Kit I (Omega Bio-tek, USA), following the manufacturer's instructions. Subsequently, reverse transcription was carried out using the NZY First-Strand cDNA Synthesis Kit (NZYTech).

A SYBR-green-based quantitative real-time polymerase chain reaction (RT-qPCR) was realized in Agilent AriaMX Real-time PCR system (Agilent Technologies, Reference code. G8830A) following the standard protocol for RT2 SYBR Green qPCR Mastermix (Qiagen, Germany). Specific PCR primers were obtained from Qiagen for the target genes, including human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) PPH00150E, human nitric oxide synthase 2 (NOS2) PPH00173E, human matrix metalloproteinase 13 (MMP13) PPH00121B, human matrix metalloproteinase 3 (MMP-3), human matrix metalloproteinase 9 (MMP-9) PPH00152E, human ADAMTS-5 (A disintegrin and metalloproteinase with thrombospondin motifs 5) PPH09588A, human LCN2 (lipocalin-2) PPH00446E, human Interleukin-6 (IL-6) PPH00560B, and human prostaglandin-endoperoxide synthase 2 (PTGS2) PPH01136F. No-template controls were included in the analysis to ensure specificity. Additionally, melting curves were generated to confirm the presence of a single gene-specific peak.

The quantification of gene expression changes was determined using the comparative ΔΔCt method within Agilent Aria 2.0 (Agilent Technologies). The results were expressed as relative fold changes compared to a control (C-) and were normalized to the housekeeping gene GAPDH.

2.5 | Western Blot Analysis

Primary human IVD cells were seeded into six-well plates and then stimulated with the treatment depending on the experiment. After the designated treatment period, the cells were quickly washed with cold PBS and gently detached using a lysis solution (10 mM Tris–HCl [pH 7.5], 5 mM ethylenediaminetetraacetic acid [EDTA], 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride [PMSF], and a freshly prepared protease inhibitor mixture [Thermo Fisher Scientific, USA]).

The resulting cell lysates were then subjected to centrifugation at 14000*g* for 20 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotting procedures were performed as previously described.[25] Immunoblots were probed with specific

human antibodies against ELF3 (1:1000, GeneTex), MMP13 (1:500, Abcam, UK), and IL-6 (1:1000, Cell Signaling). Detection of immune complexes was achieved using secondary antibodies conjugated with horseradish peroxidase, specifically anti-rabbit antibodies (1:5000, GE Healthcare, USA).

Visualization of protein bands was accomplished using the Immobilon Western Detection kit (Millipore, USA). To confirm equal protein loading, all membranes were also incubated with anti-GAPDH antibodies (1:1000, MilliporeSigma). Image acquisition and analysis were conducted using the ChemiDoc MP Imaging System and Image Lab 6.0.1 Software, both from Bio-Rad Laboratories (USA).

2.6 | Transfection assay

The plasmid cDNA encoding ELF3 gene and the control pCI vector were provided by Dr. Mary Goldring, (Hospital for Special Surgery, New York).[13] For each transfection, 2×10^5 cells of healthy and degenerated AF disc cells were seeded in a six-well plate and allowed to adhere overnight until reaching approximately 70%–80% confluence. A transfection mixture was prepared using Opti-MEM I medium, 100 ng of plasmid cDNA (pESE-1: ELF3 expression vector or pCI: empty vector), and TransFectin™ Lipid Reagent (BioRad) at the manufacturer's recommended ratio for overexpression. For silencing, 20 nM of siRNA targeting the ELF3 gene from the TriFECTa® RNAi kit (Integrated DNA Technologies) was combined with the siLentFect reagent (BioRad) following manufacturer's recommendation. The mix was incubated at room temperature for 15 min to allow complex formation. The transfection mix was then added dropwise to the cells, which were then incubated at 37°C for 24 h to allow for efficient transfection. After 24 h of transfection, the transfection medium was replaced by DMEM and αMEM (6:4) supplemented with 20% FBS. Subsequently, at 48 h post-transfection, cells were stimulated with IL-1α at a concentration of 0.1 ng/mL for 24 h, followed by protein and mRNA extraction.

2.7 | Statistical analysis

Data are reported as the mean ± SEM of at least three independent experiments. Statistical analysis was performed using either one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test, paired students' t test, or ratio paired using the Prism computerized package (Graph Pad Software). A p value less than 0.05 was considered statistically significant.

3 | RESULTS

3.1 | ELF3 is expressed and regulated in IVDD

ELF3 expression was analyzed in both annulus fibrosus (AF) and nucleus pulposus (NP) tissues from healthy individuals, as well as in IVDD tissue. First, immunohistochemical assay revealed a positive immunostaining for ELF3 in both in healthy and IVDD tissues. Intriguingly, ELF3 immunostaining is likely more intense in AF degenerated tissue than in healthy. In addition, ELF3 immunostaining was predominantly localized into the perinuclear region but also evident in the cytoplasm (Figure 1A–C).

To corroborate these results, we performed RT-qPCR and Western blot analyses on harvested healthy and degenerated AF IVDD cells. As showed in Figure 1D, mRNA expression of ELF3 was found significantly higher in degenerated AF cells compared to AF healthy cells. This finding was further supported by Western blot analysis, where the ELF3 protein expression in AF degenerated cells was found significantly higher than that observed in healthy AF cells (Figure 1E,F).

3.2 | Effect of pro-inflammatory stimulation on ELF3 expression in healthy and IVDD AF cells

To investigate whether ELF3 mRNA expression might be influenced under inflammatory conditions, we exposed healthy and IVDD AF cells to various proinflammatory stimuli: LPS 100 ng/mL, IL1α 0.1 ng/mL, TNFα 10 ng/mL, and IFNγ 10 ng/mL.

Our results indicated a significant upregulation of ELF3 mRNA expression in healthy AF cells exposed to IL-1α (Figure 2A), as well as in IVDD AF cells exposed to LPS and IL-1α (Figure 2D). Intriguingly, neither TNFα nor IFNϒ were able to modulate ELF3 both in healthy and IVDD AF cells. This effect was also assessed by western blot analysis in healthy AF cells (Figure 2B,C), as well as in IVDD AF cells (Figure 2E,F). However, no significant variation was observed in terms of protein expression.

3.3 | Dexamethasone modulates ELF3 expression in IVDD AF cells

To evaluate the effect of anti-inflammatory treatment on the expression of ELF3 in degenerated AF cells, we treated with dexamethasone at a concentration of 1 μM in the presence or not of IL-1α at 0.1 ng/mL. Dexamethasone was selected due to its well-documented efficacy as a potent anti-inflammatory and immunosuppressive agent, commonly used in clinical settings. The concentration of 1 μM was chosen based on previous studies demonstrating its effectiveness in modulating inflammatory responses in similar cellular models.[15] This concentration has been shown to sufficiently reduce inflammatory mediator thereby providing a reliable measure of its anti-inflammatory capabilities in our experimental setup.

mRNA expression and Western blot analysis revealed a significant decrease in ELF3 protein expression in the dexamethasone pretreated IVDD cells (Figure 3A–C). The anti-inflammatory action of dexamethasone was demonstrated also by the significant reduction of the mRNA expression of the inflammatory and catabolic mediators IL-6 and MMP13 (Figure 3D,E). Furthermore, we performed the same

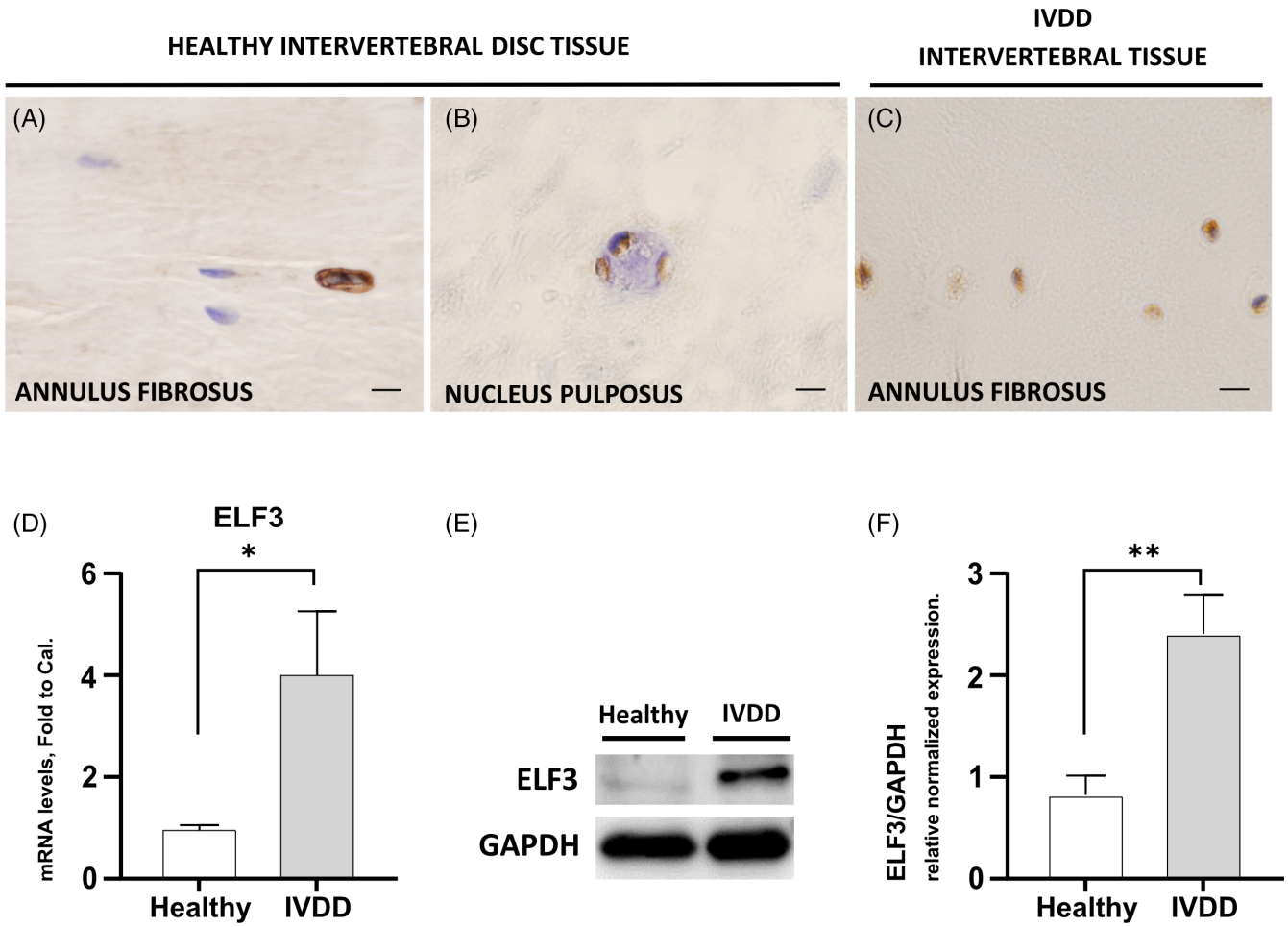

FIGURE 1 ELF3 localization in healthy (AF and NP) and human AF IVDD tissues. Immunohistochemical analysis showing the ELF3 protein presence (brown staining) in healthy AF (A), healthy NP (B), and IVDD AF (C) tissues. Images taken at 60× magnification. Scale bar = 10 μm D: MRNA expression of ELF3 in healthy versus IVDD AF cells. E-F: ELF3 protein expression in healthy versus IVDD AF cells. Comparisons referenced to healthy AF cells. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$ vs. control).

experiments in healthy AF cells, confirming that the treatment of the pre-stimulated healthy AF cells with dexamethasone significantly reduced ELF3 mRNA expression (Figure 3F).

3.4 | ELF3 mediates inflammation and ECM degradation and potentiates the expression of IL-1α-driven proinflammatory and catabolic factors in healthy and IVDD AF cells

To gain further insights into the potential pro-inflammatory role of ELF3 in disc degeneration, we transiently transfected healthy and degenerated AF cells with pCI-ELF3 vector or pCI expression empty vector. Overexpression of ELF3 in AF healthy cells resulted in a significantly upregulation of the pivotal catabolic extracellular matrix gene MMP13, along with the elevation of proinflammatory genes such as IL-6, LCN2, and PTGS2 (Figure 4B,E,G,H), with a tendency to increase of MMP3, MMP9, and ADAMTS5 (Figure 4C,D,F). To further explore the inflammatory and catabolic role of ELF3 in IVD, we stimulated transfected AF IVD cells with IL-1α. We observed a statistically significant upregulation of MMP13, MMP3, ADAMTS5, and IL-6 (Figure 4B,C,E,F), accompanied by a noticeable trend in LCN2 expression (Figure 4G). However, MMP9 and PTGS2 levels did not increase significantly when we stimulated transfected AF IVD cells with IL-1α (Figure 4D,H).

Furthermore, we performed these experiments on IVDD cells. The overexpression of the ELF3 in IVDD cells led to significant upregulation of key catabolic extracellular matrix genes, including MMP3 and MMP13, as well as proinflammatory genes such as IL-6 and PTGS2 (Figure 5B,C,E,H). Additionally, there was a tendency to increase, although not statistically significant, in the mRNA expression levels of MMP9, ADAMTS5, and lipocalin-2 (LCN2) (Figure 5D,F,G).

In IVDD cells, our findings showcased a statistically significant elevation in MMP13 (Figure 5B) and IL-6 (Figure 5E) mRNA expression, coupled with a noticeable upregulation trend in MMP3, MMP9, ADAMTS-5, LCN2, and PTGS2 expression (Figure 5C,D,F,G,H). We corroborated these results through Western blot analysis of the MMP13 and IL-6 genes in IVDD cells, revealing a statistically significant increase in both cases (Figure 6A,B).

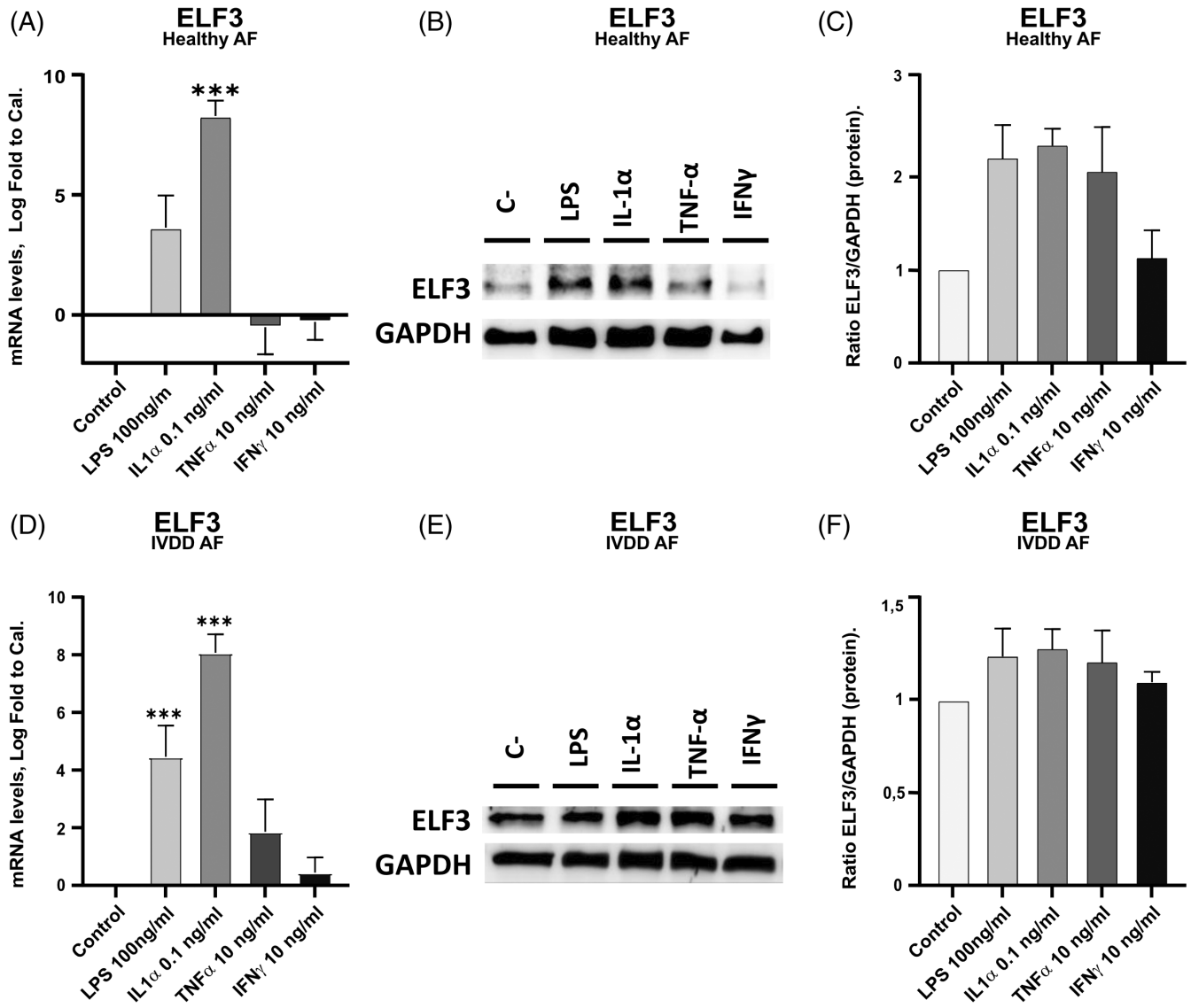

FIGURE 2 ELF3 mRNA and protein expression in healthy and IVDD AF cells stimulated with proinflammatory stimuli for 24 h. (A–C) Healthy AF cells: (A) mRNA expression, (B,C) Protein expression measured by Western Blot. (D–F) IVDD AF cells: (D) mRNA expression, (E,F) protein expression measured by Western Blot. Values are the mean ± SEM of at least three independent experiments (***$p < 0.001$ vs. control).

3.5 | ELF3 siRNA knockdown downregulates IL-1α driven MMP13 expression in IVDD

Based on the obtained results showing that ELF3 mediates inflammatory response in IVDD, we investigated the effect of silencing ELF3 in these cells using siRNA technology (Figure 7A). Knocked-down cells were stimulated in the presence or not of IL-1α. As shown in Figure 7B, a statistically significant decrease in MMP13 gene expression was observed, along with reductions ($p > 0.05$) in the expression of other matrix degradation genes such as MMP-3, MMP-9, ADAMTS5 (Figure 7C,D,F), and inflammatory genes like IL-6, LCN2, and PTGS2 (Figure 7E,G). We also performed Western Blot experiments, showing a significant decrease in MMP13 protein expression (Figure 8).

4 | SUPPLEMENTARY RESULT

4.1 | Does leptin modulate ELF3 expression?

We previously demonstrated that leptin synergizes with IL-1 inducing ELF3 in cartilage.[15] To investigate the potential role of leptin in the regulation of ELF3 in IVD cells, we initially analyzed the expression of leptin and its receptor (LEPR) in both healthy and IVDD AF cells and then stimulated these cells with human recombinant leptin alone or in combination with IL-1α. As shown in Figure S1A, both healthy and IVDD AF cells expressed leptin mRNA. Leptin mRNA was significantly reduced in AF degenerated (Figure S1A). To note, both healthy and IVDD AF cells expressed similar levels of mRNA encoding the functional leptin receptor (Figure S1B). Finally, leptin was unable to

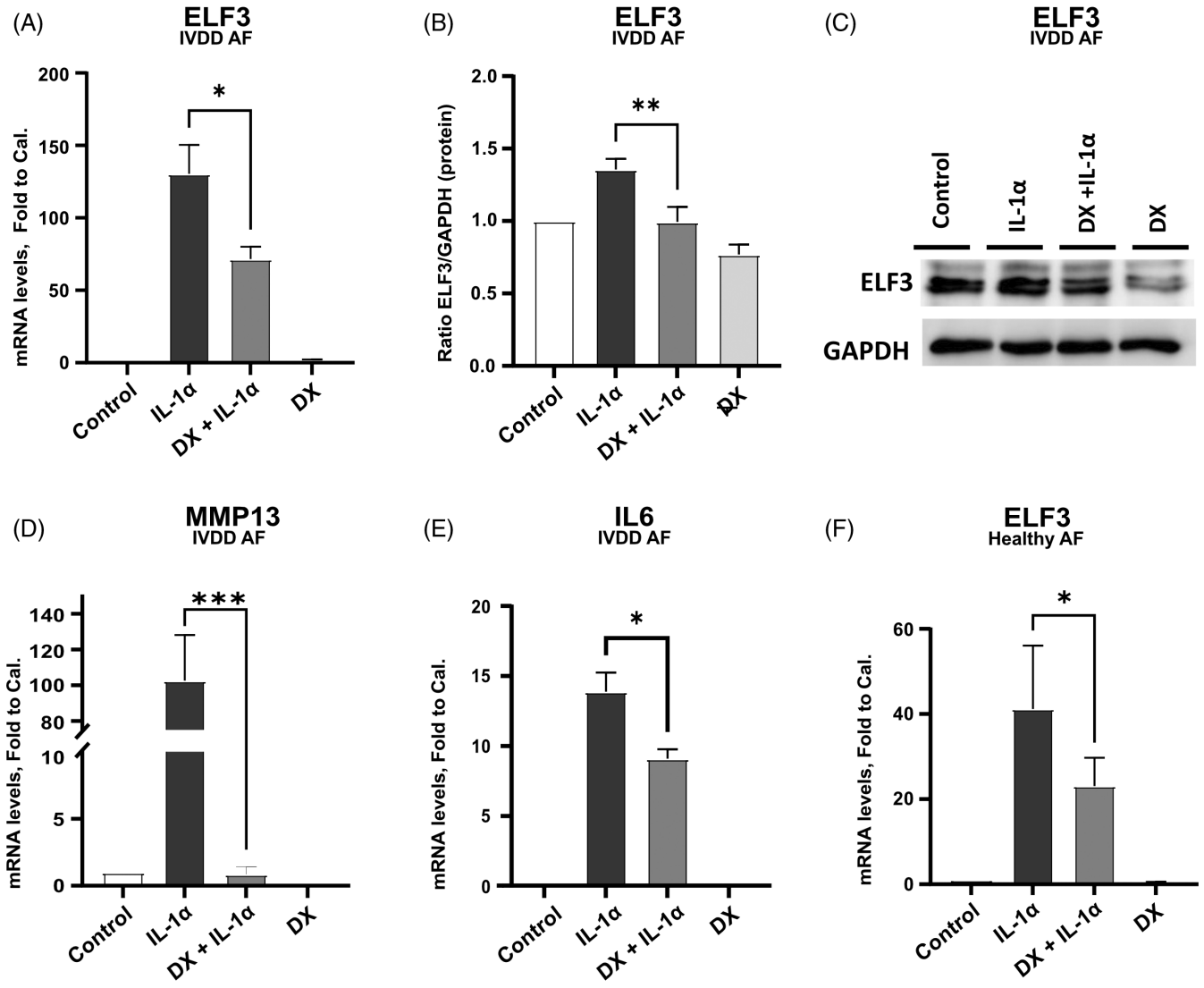

FIGURE 3 Dexamethasone downregulates the ELF3 expression. IVDD AF cells were pre-treated with dexamethasone at 1 μM and then stimulated for 24 h with IL-1α 0.1 ng/mL. (A) ELF3, (D) MMP13, (E) IL-6 mRNA expression in IVDD AF cells. (B,C) ELF3 protein expression in IVDD AF cells measured by Western Blot. (F) ELF3 mRNA expression in healthy AF cells. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$ vs. IL-1α).

modulate ELF3 expression in both healthy and IVDD AF cells even in the presence of IL-1α.

5 | DISCUSSION

Our current research demonstrated, for the first time, as far as we are aware, that ELF3 is expressed in healthy and IVDD tissue and cells, being upregulated in IVDD or by pro-inflammatory stimuli such as LPS or IL-1α in in vitro experiments with human AF cultured cells. One of the most relevant findings arising from our study is that ELF3 overexpression leads to the upregulation of critical pro-catabolic factors. In particular, those related with extracellular matrix turnover (MMPs and disintegrins) and those related to relevant pro-inflammatory pathways (IL-6, LCN2, and PTGS2). Taken together, these results suggest that ELF3 exerts a lasting catabolic effect by increasing the expression of inflammatory mediators and proteolytic enzymes, being able to promote IVD matrix breakdown and trigger inflammatory responses in healthy and degenerative AF cells. Altogether, these molecules can cooperate, resulting in the enhancement and perpetuation of the ECM-degrading processes at IVD level. In addition, ELF3, by itself, can trigger a severe increase in the expression of inflammatory mediators in healthy disc cells, suggesting that ELF3 has a clear role in the onset of disc degeneration. These data are in line with the previous published results regarding the role of ELF3 in cartilage degeneration.[15]

ELF3, a member of the ETS transcription factor family, is mainly expressed in epithelial cells and plays a crucial role in various cellular processes, including differentiation and inflammatory response.[26] Previous studies have demonstrated elevated ELF3 expression in human osteoarthritic cartilage as well as its significant involvement in the

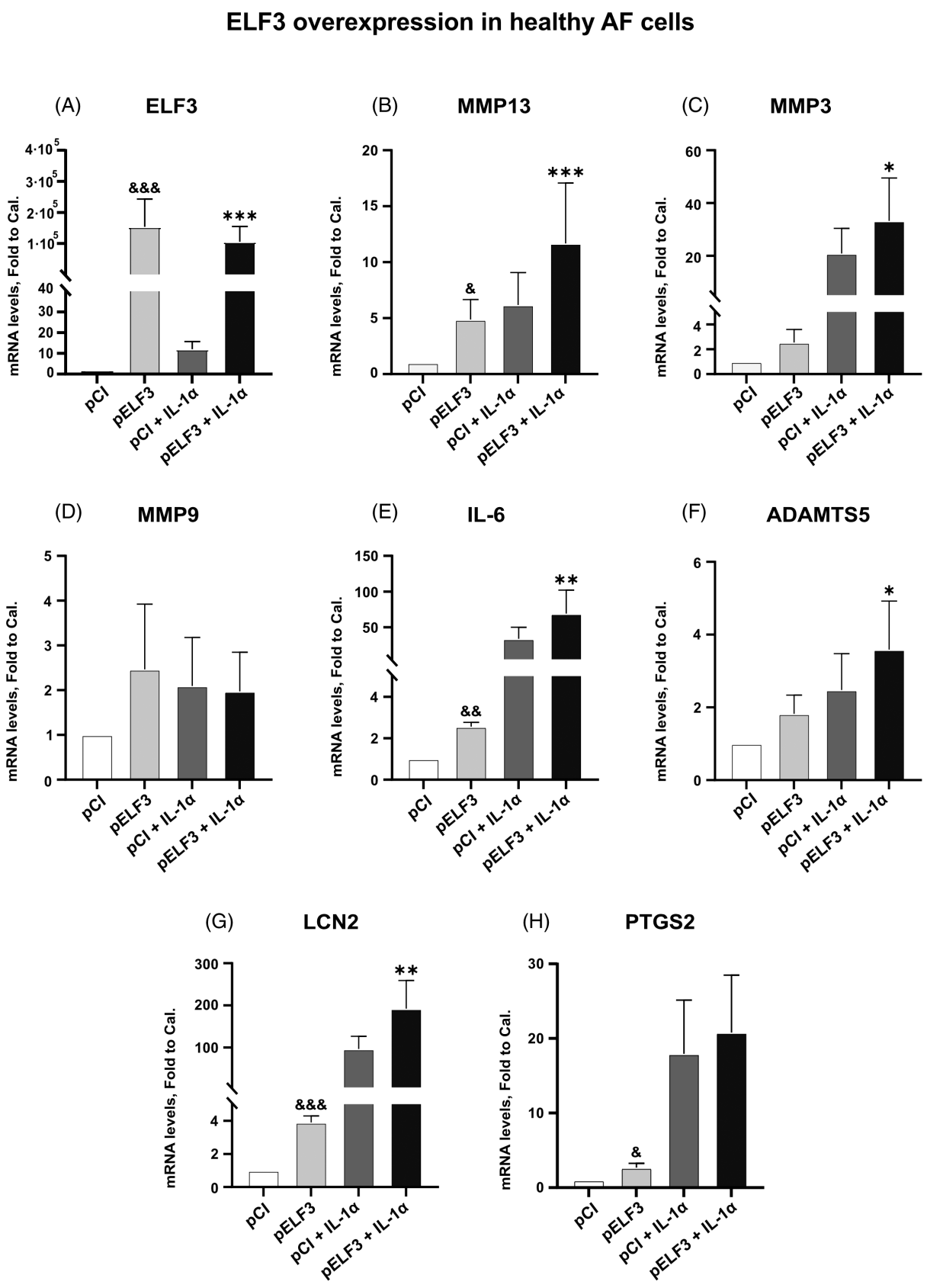

FIGURE 4 Overexpression of the ELF3 gene in combination with IL-1α 0.1 ng/mL in healthy AF cells. mRNA expression levels in healthy AF cells of ELF3 (A), MMP13 (B), MMP3 (C), MMP9 (D), IL-6 (E), ADAMTS5 (F), LCN2 (G), PTGS2 (H) in cells transfected with the empty plasmid (pCI) or the ELF3 gene overexpression plasmid (pELF3) alone and in combination with IL-1α 0.1 ng/mL treatment for 24 h. Data represent the mean ± SEM of at least three independent experiments. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, pELF3 compared to pCI and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, pELF3 + IL-1α compared to pCI + IL-1α.

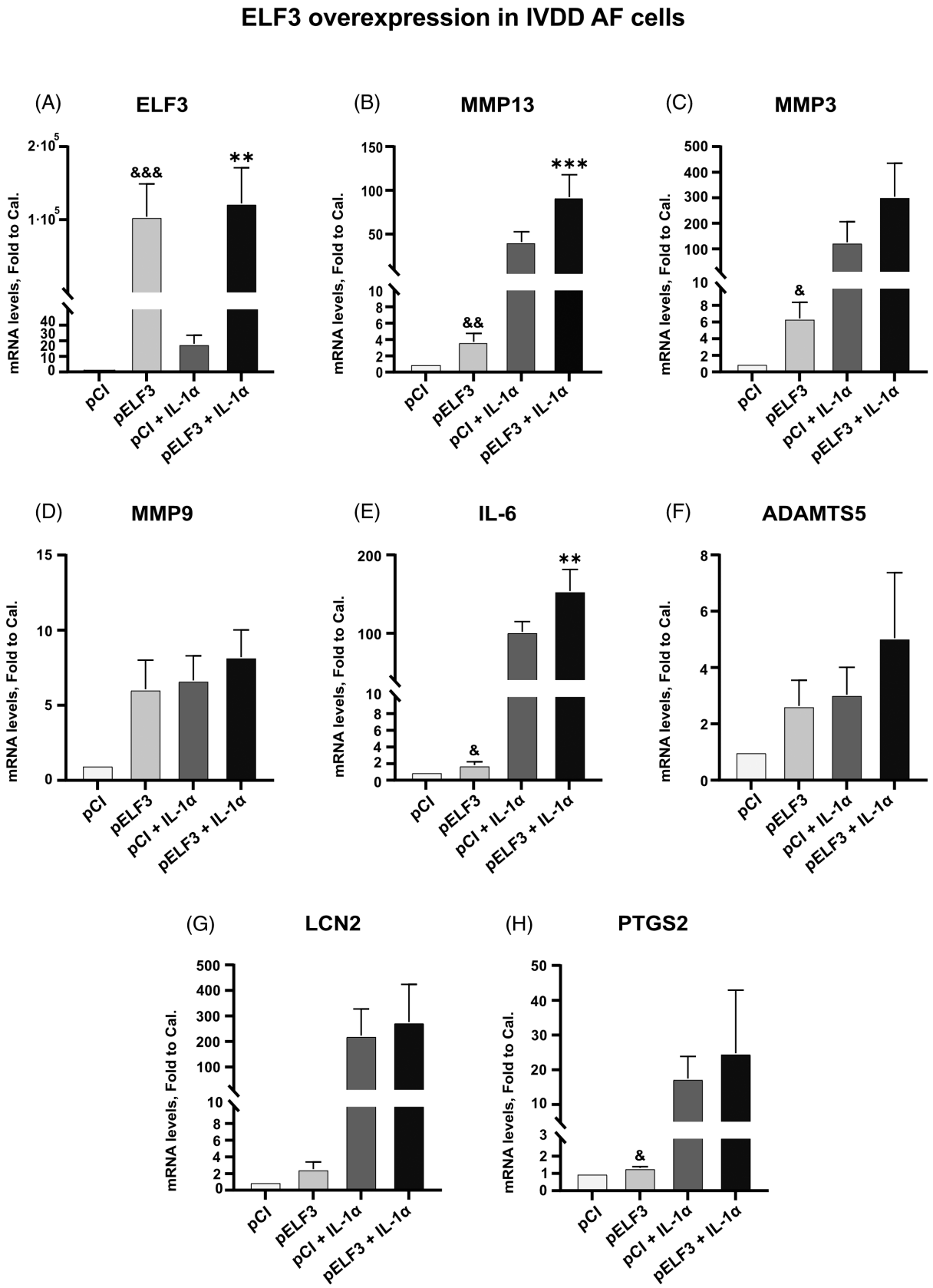

FIGURE 5 Overexpression of the ELF3 gene in combination with IL-1α 0.1 ng/mL in IVDD AF cells. mRNA expression levels in IVDD AF cells of ELF3 (A), MMP13 (B), MMP3 (C), MMP9 (D), IL-6 (E), ADAMTS5 (F), LCN2 (G), PTGS2 (H) in cells transfected with the empty plasmid (pCI) or the ELF3 gene overexpression plasmid (pELF3) alone and in combination with IL-1α 0.1 ng/mL for 24 h. Data represent the mean ± SEM of at least three independent experiments. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, pELF3 compared to pCI and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, pELF3 + IL-1α compared to pCI + IL-1α.

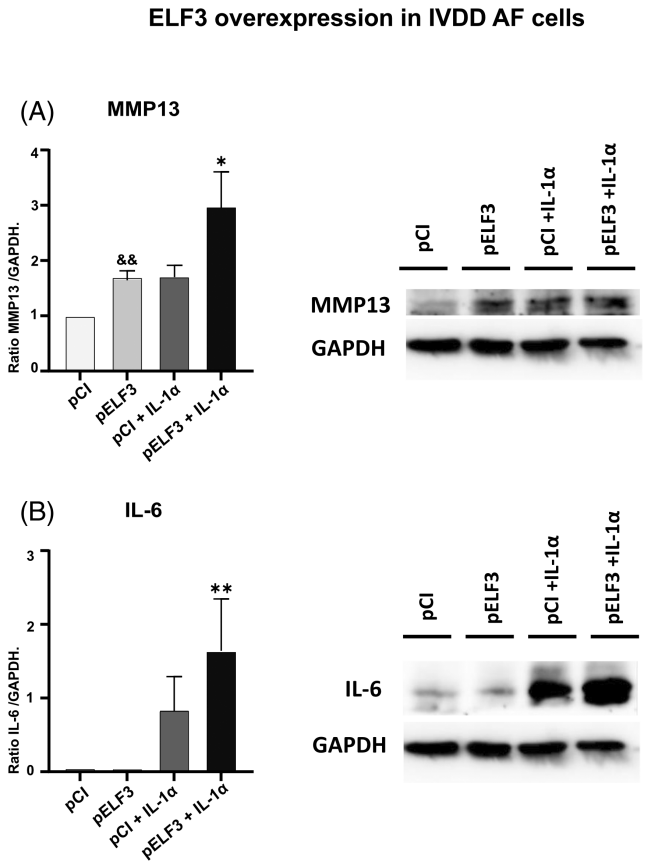

FIGURE 6 Overexpression of the ELF3 gene in combination with IL-1α in IVDD AF cells increases the protein expression of inflammatory and matrix degeneration genes. Protein expression levels in IVDD AF cells for MMP13 (A) and IL-6 (B) in cells transfected with the empty plasmid (pCI) or the ELF3 gene overexpression plasmid (pELF3) alone and in combination with IL-1α 0.1 ng/mL for 24 h. Data represent the mean ± SEM of at least three independent experiments. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, pELF3 compared to pCI and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, pELF3 + IL-1α compared to pCI + IL-1α.

expression of pro-inflammatory cytokines.[12,14,27–29] Our research group has also elucidated the interplay between ELF3 and the transcription factor NF-κβ in the induction of the proinflammatory adipokine lipocalin-2 expression in human and murine chondrocytes.[10] Furthermore, we also revealed the synergistic induction of ELF3 through leptin and IL-1β in OA in vitro models.[15]

IVDD shares many pathological similarities with OA, particularly in its advanced stages.[6] The molecular signature of IVDD and OA reveals a considerable overlap, including the expression of inflammatory mediators like COX-2, IL-6, IL-8, CCL3, IL-1β, and TNF-α and LCN2.[6,30,31] Additionally, the expression of catabolic factors, specifically MMP3, MMP13, and ADAMTS5, also shows significant similarity in both conditions.[6] In both IVDD and OA, cells within the IVD and articular joint, as well as M1 macrophages, sustain a state of low-grade inflammation producing proinflammatory cytokines such as IL-1β and TNF by.[32,33]

Another aspect arising from our results is that ELF3 expression, induced by a classic pro-inflammatory cytokine such as IL-1α, is down-modulated by a glucocorticoid drug with potent anti-inflammatory and immunosuppressive activity such as dexamethasone. Glucocorticoids are well known for their use in the clinical practice, suppressing inflammation and regulating the expression of inflammatory mediators.[34] To this regard, in our experimental set, dexamethasone was able to reduce, together with ELF3, also MMP13 and IL-6 expression. These data are in agreement with other previous published results obtained in human chondrocytes.[15]

Actually, ELF3 was able to control the promoter activities and gene expression of NOS2, COX2, and MMP13.[14] ELF3 activates MMP13, and represses COL2A1 transcription in cartilage, having an important role in cartilage catabolism.[35,36] In our prior work, we already reported an elevation of LCN2 expression in ELF3-overexpressed chondrocytes treated with IL-1α compared to cells treated alone with IL-1α. Conversely, ELF3 gene silencing led to a decrease in LCN2 expression.[10]

Matrix metalloproteinases (MMPs) and the ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) are the major mediators of the ECM degradation in IVDD. Many of these proteinases were reported to be upregulated in IVDD as well as in OA.[6,37,38] We also investigated the role of ELF3 in regulating the transcription of the collagenase (MMP13), the gelatinase (MMP9), the stromelysin (MMP3), and the aggrecanase (ADAMTS5), finding all of them all to be upregulated by the overexpression of ELF3. These results indicate an evident pro-catabolic activity of ELF3 that might potentially contribute to the ECM remodeling, and degradation. ELF3 was previously described to activate MMP13 transcription by binding to a conserved ETS site in its proximal promoter region.[29]

MMP9 is known to have a role in the pathogenesis of IVDD cells.[39,40] Zigouris et al. reported a significant correlation between MMP9 expression and histological degenerative scores in IVD tissues, supporting the fact that MMP9 plays a role in IVDD.[40] ELF3, as a pro-inflammatory transcription factor, has been linked to the regulation of MMP9 expression. In human chondrocytes, the overexpression of ELF3 had shown to increase the levels of LCN2, an adipokine known to form a covalent complex with MMP9.[41,42] Our previous research has highlighted the induction of LCN2 through a collaborative mechanism involving ELF3 and NFκB in chondrocytes.[10] In addition, LCN2 has been found to contribute to the stabilization of MMP9 in chondrocytes.[43] Remarkably, our current results in AF IVDD cells indicated that both LCN2 and MMP9 are upregulated by ELF3 overexpression and IL-1α. Further supporting this relationship, studies on pancreatic cell lines have reported a significant decrease in MMP9 expression following the silencing of the ELF3 gene, providing clear evidence of their interdependence.[44]

MMP3, which has been associated with the progression of IVDD, has also shown a link with the upregulation of its expression when NP cells are stimulated with IL-1β, consistent with our results following IL-1α stimulation.[45] In an in vivo surgical model of posttraumatic OA, it was reported that MMP3 tended to decrease when ELF3 was

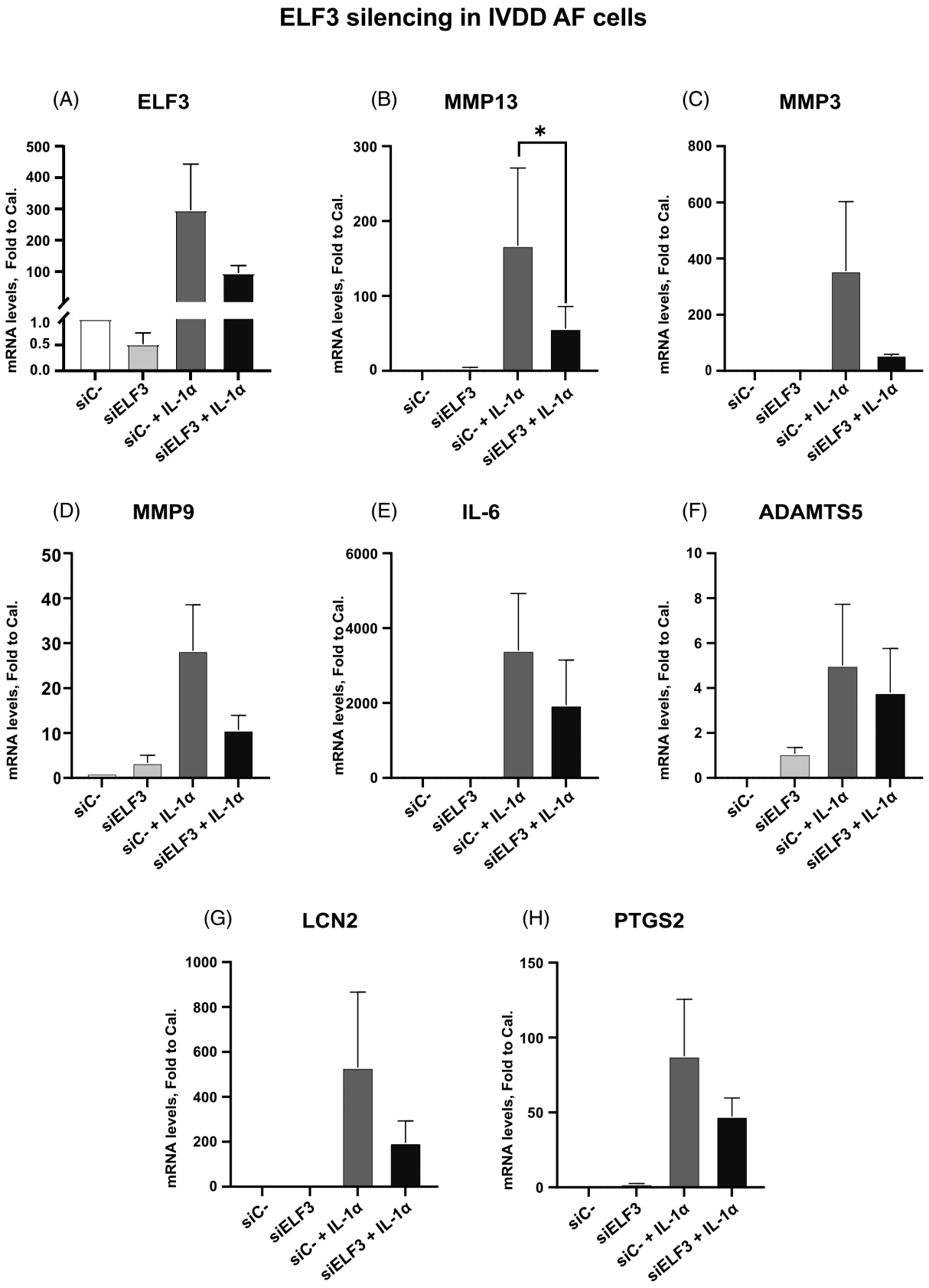

FIGURE 7 Silencing of the ELF3 gene in IVDD AF cells. mRNA expression levels in IVDD AF cells for ELF3 (A), MMP13 (B), MMP3 (C), MMP9 (D), IL-6 (E), ADAMTS5 (F), LCN2 (G), PTGS2 (H) transfected with the indicated siRNAs alone and in combination with IL-1α 0.1 ng/mL during 24 h. Data represent the mean ± SEM of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, siELF3 + IL-1α compared to siC- + IL-1α.

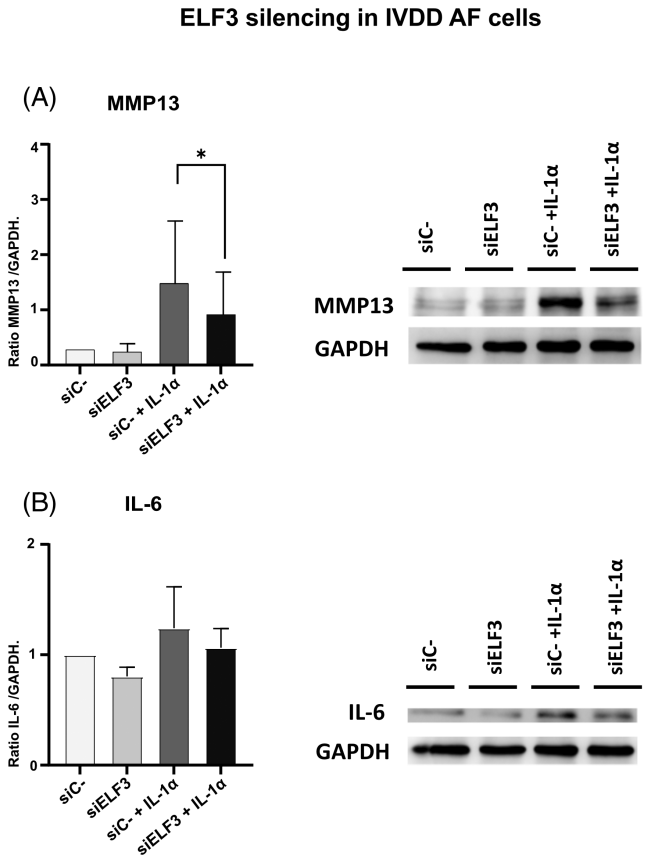

FIGURE 8 Effect of silencing of the ELF3 gene in IVDD AF cells on the protein expression levels of MMP13 (A) and IL-6 (B) transfected with the indicated siRNAs alone and in combination with IL-1α 0.1 ng/mL for 24 h. Data represent the mean ± SEM of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, siELF3 + IL-1α compared to siC- + IL-1α.

silenced.[27] Furthermore, this study indicated a significant decrease in the levels of NOS2 and MMP13 upon ELF3 silencing.[27]

As long as ELF3 had a direct relation with a collagenase (MMP13), we studied the aggrecanase ADAMTS-5, described to have a role in degrading ECM components in IVD, potentially undermining its structural stability.[46] In an in vivo rabbit annular puncture model of IVDD, suppressing ADAMTS-5 effectively mitigated disc degeneration, highlighting ADAMTS-5 as a potential therapeutic target for IVDD.[47] The increased expression of ADAMTS-5 in human herniated IVDs also suggests its involvement in the degenerative process of IVDD. Notably, ADAMTS5 is also reported to be upregulated in disc cells stimulated by IL-1β, which is in line with the findings presented in our paper.[46] As far as we are aware, this is the first evidence showing that overexpression of ELF3 tends to increase ADAMTS-5 expression in IVDD cells.

Obesity is recognized as a major risk factor to IVDD, not only for the mechanical stress, but also for the elevated levels of cytokines and adipokines secreted by white adipose tissue, exacerbating cellular inflammatory status.[3,31] The significant decrease in leptin mRNA expression in degenerative discs compared to healthy discs suggests a disruption in the homeostatic balance of adipokines within the disc environment. Adipokines, including leptin, play critical roles in maintaining cellular homeostasis, modulating inflammatory responses, and regulating extracellular matrix composition. In healthy discs, higher leptin levels likely might contribute to these regulatory processes, supporting the structural and functional integrity of the disc. Previously, our research group established that leptin, a master proinflammatory adipokine, can synergize with IL-1β to induce ELF3 expression in human chondrocytes.[15] Surprisingly, we did not observe this synergistic response in either healthy or IVDD AF cells. Similarly, our group has demonstrated that leptin did not synergize with IL-1β to potentiate the expression of several pro-inflammatory cytokines (such as NOS2, COX2, and IL-6) and adipokines (such as LCN2).[5] This synergism between leptin and IL-1β relies on the presence of the leptin receptor.[15] We found no difference in mRNA expression of leptin receptor between IVDD and healthy AF cells. However, we found significantly higher leptin expression in healthy AF cells compared to IVDD AF cells. The lower leptin expression in IVDD cells could be attributed to an impaired cellular functionality during the degenerative process that characterizes IVDD.

It is worth noting that circulant or local levels of leptin can influence the homeostasis of the IVD, either through its receptors or indirectly through cytokines induced by leptin.[48,49] A direct correlation between leptin levels and the levels of MMP-1 and MMP-3 has been observed in the synovial fluid of patients with OA. In these patients, stimulation with leptin alone and in combination with the inflammatory stimulus IL-1β increased the levels of MMP-1, MMP-3, and MMP-13.[50]

Very recently it has been demonstrated that ELF3 plays a significant role also in the synergy between IL-17 and TNF in synovial fibroblasts.[51] In these cells, it has been clear that some of the genes regulated by Iκβ are dependent on ELF3. Thus, the induction of ELF3 might be a collaborative activating route, since ELF3 is able to cooperate with AP-1 and NFκβ in prolonging the production of inflammatory mediators.

While our research provides significant insights into the role of ELF3 in IVDD, it is important to acknowledge certain limitations. First, we have to consider that our findings, suggesting a pro-catabolic role for ELF3, have been obtained primarily in in vitro experiments. Thus, the extrapolation of these findings to in vivo conditions will require further investigations to confirm the relevance of ELF3 as a therapeutic target. In addition, the current paper did not analyze specific signal transduction pathways that might be involved in the activity of ELF3 and this will be explorated in future research.

6 | CONCLUSION

Collectively, these findings deepen our understanding of ELF3's pivotal role in IVDD, highlighting its involvement in the inflammatory and

degenerative processes. Targeting ELF3 may offer a promising avenue for therapeutic interventions aimed at modulating these pathological processes in IVDD. Further investigations are needed to evaluate the therapeutic potential of targeting ELF3.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest9.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article and further inquiries can be directed to the corresponding author.

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