MUSCULOSKELETAL PATHOLOGY

Hypoxia and Hypoxia-Inducible Factor-1 α Regulate Endoplasmic Reticulum Stress in Nucleus Pulposus Cells

Implications of Endoplasmic Reticulum Stress for Extracellular Matrix Secretion

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Endoplasmic reticulum (ER) stress is shown to promote nucleus pulposus (NP) cell apoptosis and intervertebral disc degeneration. However, little is known about ER stress regulation by the hypoxic disc microenvironment and its contribution to extracellular matrix homeostasis. NP cells were cultured under hypoxia (1% partial pressure of oxygen) to assess ER stress status, and gain-of-function and loss-of-function approaches were used to assess the role of hypoxia-inducible factor (HIF)-1 α in this pathway. In addition, the contribution of ER stress induction on the NP cell secretome was assessed by a nontargeted quantitative proteomic analysis by sequential windowed data independent acquisition of the total high-resolution mass spectra—mass spectrometry. NP cells exhibited a lower ER stress burden under hypoxia. Knockdown of HIF-1 α increased C/EBP homologous protein, protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) levels, whereas HIF-1 α stabilization decreased the expression of ER stress markers *Ddit3*, *Hsp5a*, *Atf6*, and *Eif2a*. Interestingly, ER stress inducers tunicamycin and thapsigargin induced HIF-1 α activity under hypoxia while promoting the unfolded protein response. NP cell secretome analysis demonstrated an impact of ER stress induction on extracellular matrix secretion, with decreases in collagens and cell adhesion—related proteins. Moreover, analysis of transcriptomic data of NP tissues from aged mice and degenerated human discs showed higher levels of unfolded protein response markers and decreased levels of matrix components. Our study shows, for the first time, that hypoxia and HIF-1 α attenuate ER stress responses in NP cells, and ER stress promotes inefficient extracellular matrix secretion under hypoxia. (*Am J Pathol* 2021, 191: 487–502; <https://doi.org/10.1016/j.ajpath.2020.11.012>)

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Low back pain is one of the leading causes of disability. Although the pathophysiology is not wholly understood, disc degeneration is considered one of the major factors contributing to the disease process.¹ The intervertebral disc is composed of three compartments: nucleus pulposus (NP), the central, aneural, and avascular tissue, rich in proteoglycans; annulus fibrosus, circumferential, collagen-rich tissue; and superior and inferior cartilaginous endplates. More importantly, because of the physiologically hypoxic environment of the NP, resident cells rely on hypoxia-inducible factor (HIF)-1 α signaling to function.² In contrast to other cell types, regulation of HIF-1 α is unique in these cells; it is robustly expressed under normoxia and shows only a minimal increase in its stability under hypoxia.³ Importantly, previous studies of FoxA2^{Cre};HIF-1 α ^{fl/fl} mice with targeted deletion of HIF-1 α in the notochord have unequivocally shown that the absence of this key transcription factor compromises NP compartment development and cell survival *in vivo*.⁴

The endoplasmic reticulum (ER) is a highly conserved organelle responsible for folding and maturing newly synthesized secretory and transmembrane proteins. ER homeostasis can be compromised by different stimuli, such as high protein demand, inflammatory processes, reactive oxygen species, or mutant proteins, resulting in the accumulation of misfolded and unfolded proteins in the ER lumen. This condition is termed ER stress and leads to an adaptive response, termed the unfolded protein response (UPR), known to be an essential mechanism for cell survival.⁵ The chaperone glucose-regulated protein 78/immunoglobulin heavy-chain-binding (GRP78/BiP), encoded by the *HSPA5* gene, is a master regulator of ER homeostasis. It directly promotes protein folding, contributes to proteasomal degradation of misfolded proteins through retrograde transport across ER membrane, and interacts with UPR transducers: inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6).⁶ Accumulation of unfolded proteins in the ER results in dissociation of BiP from these transducers, initiating their respective UPR pathways that involve molecular chaperones, protein processing enzymes, and autophagy to prevent or promote the clearance of unwanted proteins. Briefly, IRE1 senses ER stress and activates IRE1, which then splices X-box binding protein 1 mRNA, up-regulating UPR target genes responsible for ER-associated protein degradation and folding proteins such as protein disulfide isomerase.⁷ In the presence of high levels and unresolved ER stress, IRE1 can also activate tumor necrosis factor receptor-associated factor 2 and apoptosis-signaling kinase 1, leading to C-Jun N-terminal kinase (JNK) activation and apoptosis.⁸ Likewise, PERK is also an ER transmembrane kinase whose activation prevents the formation of ribosomal complexes, decreasing global mRNA translation and attenuating ER work loading.⁹ In parallel, PERK can also activate ATF4,

which up-regulates other UPR target genes involved in apoptosis, such as C/EBP homologous protein (CHOP).¹⁰ In addition, UPR activates ATF6, driving ATF6 to the Golgi, where it is cleaved and then translocated to the nucleus to promote the transcription of UPR genes involved in protein folding, processing, and degradation.¹¹

Several studies have shown the importance of the UPR in various disease contexts, including intervertebral disc degeneration.¹² Recent studies show that modulation of ER stress protects NP cells from apoptosis and ameliorates intervertebral disc degeneration.¹³ Interestingly, a cross talk between hypoxia, HIF-1 α , and UPR activation has been reported in other cell types.^{14,15} However, little is known regarding how ER stress is governed and regulated in the hypoxic niche of the NP compartment. Therefore, we asked the questions: Is ER stress regulated by hypoxia in NP cells, and does HIF-1 α play a role in this regulation? Our study shows, for the first time, that in NP cells, hypoxia reduces the ER stress burden, and HIF-1 α activity modulates the UPR. We also demonstrate that induction of ER stress results in an inefficient secretion of extracellular matrix, characterized by decreased secretion of a wide spectrum of collagens and cell adhesion-related proteins *in vitro* and *in vivo*.

Materials and Methods

Cell Culture and Treatments

NP cells were isolated from adult male Sprague Dawley rats (350 g) in accordance with the protocol (number 00959) approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University (Philadelphia, PA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Corning, Corning, NY; 10-013-CV) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO; F6178) supplemented with antibiotics in T25 flasks until confluent [passage 0 (P0)]. Cells were passaged into T75 flasks for expansion (P1) and used for experiments until P2/3. Cells were maintained in a Hypoxia Workstation (InvivoO₂ 300; Baker Ruskinn, Sanford, ME) with a mixture of 1% O₂, 5% CO₂, and 94% or 90% N₂ from 8 hours up to 5 days; cells were also treated with either tunicamycin (1 to 10 μ g/mL; Sigma, St. Louis, MO; T7765) or thapsigargin (20 to 80 nmol/L; Sigma; T9033) for 24 hours. Concentrations of these ER stress-inducing drugs were chosen on the basis of published literature and the absence of reduced cell viability during the treatment period.¹⁶

Transmission Electron Microscopy

Primary rat NP cells were plated in 10-cm plates and were cultured in either normoxia or hypoxia for 24 hours. Cells were trypsinized, collected as a pellet, and fixed overnight at 4°C with 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4. After

subsequent buffer washes, the samples were post-fixed in 2.0% osmium tetroxide for 1 hour at room temperature, and then washed again in cacodylate buffer, followed by distilled water (dH₂O). After dehydration through a graded ethanol series, the cell pellet was infiltrated and embedded in EMbed-812 (Electron Microscopy Sciences, Hatfield, PA; 14900). Thin sections were stained with lead citrate and examined with a JEOL 1010 electron microscope (Peabody, MA) fitted with a Hamamatsu digital camera (Bridgewater, NJ; C4742-95) and AMT Advantage image capture software (Woburn, MA). Assessment of ER morphology was performed by analyzing three randomly selected regions of interest at a magnification of $\times 26,000$ from 13 to 14 NP cells from normoxia and hypoxia groups, respectively.

Reagents and Plasmids

LV-shHIF-1 α (clone 232222) and control pLKO.1 lentiviral plasmids were purchased from Sigma-Aldrich. The following plasmids were obtained from the Addgene (Watertown, MA) repository: luciferase reporter vector containing a hypoxia response element (HRE-Luc; number 26731), developed by Navdeep Chandel; and psPAX2 (number 12260) and pMD2G (number 12259), developed by Didier Trono.¹⁷

Lentiviral Particle Production and Viral Transduction

HEK 293T cells (ATCC, Manassas, VA; CRL-3216) were plated in 10-cm plates (5×10^6 cells per plate) in complete DMEM 1 day before transfection. Cells were transfected with 9 μ g of short hairpin RNA plasmid control (ShCtr) or HIF1A knockdown (ShHIF1A) plasmids along with 6 μ g psPAX2 and 3 μ g pMD2.G. After 16 hours, transfection medium was removed and replaced with complete DMEM. Lentiviral medium was harvested at 48 to 60 hours after transfection, mixed with 7% PEG 6000 (Sigma-Aldrich; 81253) solution, and incubated overnight at 4°C to precipitate virus particles. The PEG solution was removed from the virus medium before transduction by centrifugation at $1500 \times g$ for 30 minutes to pellet the virus particles. NP cells were plated in complete DMEM 1 day before transduction. Cells in 10-cm plates were transduced with 8 mL of fresh DMEM with 10% heat-inactivated fetal bovine serum containing viral particles, along with 8 μ g/mL polybrene. Twenty-four hours later, the medium was removed and replaced with complete DMEM. Cells were harvested for protein extraction 4 days after transduction to ensure maximum knockdown efficiency without affecting cell viability.

Real-Time RT-PCR Analysis

Total RNA was extracted from NP cells using RNeasy mini columns (Qiagen, Hilden, Germany). Before elution from the column, RNA was treated with RNase-free DNase I (Qiagen). Purified RNA was converted to cDNA using EcoDry Premix

(Clontech, Mountain View, CA). Template cDNA and gene-specific primers were added to the SYBR Green master mixture (Applied Biosystems, Foster City, CA), and mRNA expression was quantified using the Step One Plus Real-Time PCR System (Applied Biosystems). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used to normalize gene expression.¹⁸ Melting curves were analyzed to verify the specificity of the real-time RT-PCR and the absence of primer dimer formation. Each sample was analyzed in duplicate and included a template-free control. All primers used were synthesized by IDT, Inc. (Coralville, IA).

Protein Extraction and Western Blot Analysis

Following treatment, cells were placed on ice and washed with ice-cold phosphate-buffered saline. All of the wash buffers and the final cell lysis/resuspension buffers included $1 \times$ Complete Mini Protease Inhibitor Cocktail (Roche, South San Francisco, CA; 11836153001), NaF (5 mmol/L; Sigma-Aldrich; 201154), and Na₃VO₄ (200 μ mol/L; Sigma-Aldrich; S6508). Total cell proteins were resolved by electrophoresis on 8% to 12% SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes (EMD Millipore, Burlington, MA; IPVH00010). The membranes were blocked with 5% nonfat dry milk in 1% Tween 20 (Bio-Rad, Hercules, CA; 161-0781) in tris-buffered saline and then incubated overnight at 4°C in 5% nonfat dry milk in 1% Tween 20 in tris-buffered saline with antibodies against glyceraldehyde-3-phosphate dehydrogenase (1:1000; number 3583S), CHOP (1:1000; number 2895), ATF4 (1:1000; number 11815), BiP (1:1000; number 3183), and PERK (1:1000; number 5683p), from Cell Signaling Technology (Danvers, MA), and ATF6 (1:1000; number NBP1-40256), from Novus Biologicals (Littleton, CO). The membrane was also incubated with primary antibodies against HIF-1 α (1:1000; BD Transduction Laboratories, San Jose, CA; 610958) or β -tubulin (1:10,000; Developmental Studies Hybridoma Bank, Iowa City, IA; E7). Immunolabeling was detected using the Amersham ECL Reagent (Thermo Fisher Scientific, Waltham, MA; 45-002-401). Tubulin and glyceraldehyde-3-phosphate dehydrogenase were used as loading controls and to normalize protein expression analysis for densitometry quantification in ImageJ software version 1.53e (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>).¹⁹

Secretome Collection and Concentration

The secretome of NP cells (P2) was assessed following the protocol by Fraga et al.²⁰ Briefly, cells were seeded at a density of 12,000 cells/cm² and maintained for 2 days in culture under hypoxia. Cells were then washed three times with phosphate-buffered saline and incubated with Opti-MEM medium (31985070; Thermo Fisher), with or without tunicamycin (5 mg/mL) or thapsigargin (40 nmol/L) for 24 hours. Following the treatment, the medium was collected

and centrifuged at $400 \times g$ for 10 minutes to remove any cell debris, concentrated ($100\times$) by centrifugation using a 5-kDa cutoff concentrator (Vivaspin; GE Healthcare, Chicago, IL), and frozen at -80°C until used for proteomic analysis.

Untargeted MS Proteomics and Data Analysis

To characterize the secretome, a nontargeted quantitative proteomic analysis by sequential windowed data independent acquisition of the total high-resolution mass spectra (SWATH)—mass spectrometry (MS) was performed.^{21,22} Dried secretome samples were solubilized in 30 μL Laemmli buffer containing 1 μg of a recombinant protein [maltose-binding protein (MBP)—green fluorescent protein] to be used as internal standard (IS) and retention time calibrator.²³ Each sample (25 μL) was used for SWATH-MS analysis, and 5 μL of each replicate was combined to generate representative pools of each experimental group to be used for protein identification/library generation [information-dependent acquisition (IDA) experiments]. All samples were digested with trypsin by Short-GeLC approach,^{21–24} with acrylamide as the alkylating agent. Samples were analyzed on a Triple TOF 5600 System (ABSciex, Framingham, MA) in two phases: IDA of the pooled samples and SWATH acquisition of each individual sample for protein quantification.

Differentially secreted proteins between control versus tunicamycin and control versus thapsigargin groups were identified using multiple *t*-test analyses ($P < 0.1$).^{25,26} Similarly, differentially secreted proteins between the three groups were identified using the Kruskal-Wallis test ($P < 0.1$ cutoff).^{25,26} Principal component analysis was performed using Z-score values calculated from the normalized protein signals of all identified secreted proteins. Differential secreted proteins were then used for hierarchical clustering analysis, using the Pearson correlation method. Graphical analysis was performed with the Multiple Experiment Viewer (MeV) software version 4.9.0 (Bioinformatics, Austin, TX).

Dynamic profiles of the proteins among the experimental conditions were determined by heat map and unsupervised clustering analyses using the R package Mfuzz,²⁷ which performs fuzzy c-means clustering. Unsupervised clustering was performed for the standardized levels of the 355 altered proteins, with a *P*-value cutoff of 10% using a Kruskal-Wallis test. The number of clusters was set to 4, and the fuzzifier coefficient was set to 4.08.²⁸ The optimal number of clusters was determined by first plotting the minimum centroid distance across a range of cluster values and then observing the cluster value at which this distance plateaued. Membership value represents how well the protein profile fits the average cluster profile. All of the proteins with a membership value < 0.5 were not associated with any cluster. The final list of proteins from clusters 1 and 3 was used for gene ontology (GO) process characterization of up-regulated and down-regulated common proteins. GO process characterization of protein lists was performed using Protein Analysis Through

Evolutionary Relationships (PANTHER) overrepresentation analysis version PANTHER15.0.²⁹ The MS proteomics data were deposited to the ProteomeXchange Consortium (<http://www.proteomexchange.org>) via Proteomics Identifications database (PRIDE) (<https://www.ebi.ac.uk/pride>, last accessed December 3, 2020), with the data set identifier PXD019151.^{30,31}

SWATH Acquisition

Samples were analyzed on a Triple TOF 5600 System in two phases: IDA of the pooled samples and SWATH acquisition of each individual sample. Peptides were resolved by liquid chromatography (nanoLC Ultra 2D; Eksigent, Dublin, CA) on a MicroLC column ChromXP C18CL (300- μm internal diameter $\times$ 15-cm length; 3- μm particles; 120- $\text{\AA}$ pore size; Eksigent) at 5 $\mu\text{L}/\text{minute}$ with the following multistep gradient: 0 to 2 minutes linear gradient from 2% to 5%, 2 to 45 minutes linear gradient from 5% to 30%, and 45 to 46 minutes at 35% of acetonitrile in 0.1% formamide (FA) and 5% dimethyl sulfoxide. Peptides were eluted into the mass spectrometer using an electrospray ionization source (DuoSpray Source; ABSciex) with a 50- μm internal diameter stainless steel emitter (NewObjective, Littleton, MA).

IDA experiments were performed for three fractions of the condition-specific pooled samples: the mass spectrometer set to scanning full spectra (350 to 1250 m/z) for 250 milliseconds, followed by up to 100 MS/MS scans (100 to 1500 m/z from a dynamic accumulation time, minimum 30 milliseconds for precursor above the intensity threshold of 1000, to maintain a cycle time of 3.3 seconds). Candidate ions with a charge state between 2 and 5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation, and one MS/MS spectrum was collected before adding those ions to the exclusion list for 25 seconds (mass spectrometer operated by Analyst TF 1.7; ABSciex). Rolling collision was used with a collision energy spread of 5.

For SWATH-MS-based experiments, the mass spectrometer was operated in a looped product ion mode,³² and the same chromatographic conditions were used as in the IDA run described above. A set of 60 windows (Supplemental Table S1) of variables (width containing 1 m/z for the window overlap) was constructed, covering the precursor mass range of 350 to 1250 m/z . A 250-millisecond survey scan (350 to 1500 m/z) was acquired at the beginning of each cycle for instrument calibration, and SWATH MS/MS spectra were collected from 100 to 1500 m/z for 50 milliseconds, resulting in a cycle time of 3.3 seconds from the precursors ranging from 350 to 1250 m/z . The collision energy for each window was determined according to the calculation for a charge 2 ion centered on the window with variable collision energy spread in accordance with the window.

A specific library of precursor masses and fragment ions was generated by combining all files from the IDA experiments and used for subsequent SWATH processing.

Libraries were obtained using ProteinPilot software version 5.0.1 (ABSciex), using the following parameters: i) searched against a database composed by the *Rattus norvegicus* database from SwissProt (release at May 2020; 8094 sequences) and the sequences of the recombinant proteins used as IS (MBP—green fluorescent protein—IS)²³; ii) used acrylamide alkylated cysteines as fixed modification; and iii) used trypsin as digestion type. An independent false discovery rate (FDR) analysis using the target-decoy approach provided with the ProteinPilot software was used to assess the quality of the identifications, and positive identifications were considered when identified proteins and peptides reached a 5% local FDR.^{33,34}

Data processing was performed using the SWATH processing plug-in for PeakView version 2.0.01 (ABSciex), and peptides and target fragment ions were selected automatically from the library using the criteria described by Lambert et al.³⁵ After retention time adjustment using the IS peptides, up to 20 peptides, with up to five fragments each, were chosen per protein, and quantitation was attempted for all proteins in the library file that were identified <5% local FDR.

The target fragment ions were automatically selected, and peak groups were scored following the peak group confidence threshold that was determined on the basis of an FDR analysis. Peptides that met the 1% FDR threshold in at least two of the three biological replicates were retained, and the peak areas of the target fragment ions of those peptides were extracted across the experiments using an extracted-ion chromatogram window of 3 minutes with 100 ppm extracted-ion chromatogram width.

The levels of the proteins were estimated by summing all of the transitions from the peptides for a given protein that met the criteria described above (an adaptation of Collins et al)³² and normalized to the IS.²³

Analysis of Differentially Expressed Genes of NP Tissue from Aged Mice and Degenerated Human NP Tissues

Differentially expressed gene analysis comparing 23-month-old ($n = 7$) versus 6-month-old C57BL/6 ($n = 7$) mice was performed using transcriptomic expression array data deposited by our group ($P < 0.05$; <https://www.ncbi.nlm.nih.gov/gds>; accession number GSE134955). Gene expression data for human NP samples were deposited by Kazezian et al³⁶ in Gene Expression Omnibus database (<https://www.ncbi.nlm.nih.gov/gds>; accession number GSE70362³⁷). Differentially expressed genes from the human data set were obtained from transcriptome-based principal component analysis and clustering analysis, as reported previously by Novais et al³⁸: cluster 1 (degenerated discs; $n = 3$) versus cluster A (healthy discs; $n = 5$; $P < 0.05$).

Statistical Analysis

All cell culture experiments were performed at least three independent times. Data are presented as the means $\pm$ SEM.

Differences between groups were analyzed by the *t*-test and one-way analysis of variance for normally distributed data or *U*-test or Kruskal-Wallis test for data with nonnormal distributions ($P < 0.05$). Statistical analysis was performed using GraphPad Prism version 7.0c (GraphPad Software, La Jolla, CA).

Results

Hypoxia Decreases ER Stress in NP Cells

ER stress has been linked to NP cell death and intervertebral disc degeneration.¹³ To understand the effect of hypoxia on ER stress modulation, NP cells were cultured in either normoxia (21% partial pressure of oxygen) or hypoxia (1% partial pressure of oxygen) for 24 hours before visualizing the endoplasmic reticulum with transmission electron microscopy (Figure 1A–D, and Supplemental Figure S1).² ER dilation, as observed by transmission electron microscopy, is one of the most widely used and highly specific hallmarks to evaluate ER stress activation in cells.⁵ Transmission electron microscopy analysis showed that, in contrast to NP cells cultured under hypoxia, the ER was enlarged in cells cultured under normoxia, suggesting ER stress. Moreover, quantitative RT-PCR analysis showed decreased mRNA expression levels of *Hspa5*, *Ddit3*, *Atf4*, *Atf6*, and *Ern1* under hypoxia in a time-dependent manner (Figure 1E). However, *Eif2a*, *Ero1a*, and *Pdia2* mRNA levels did not follow the same trend (Figure 1E). Western blot analysis of NP cells cultured under hypoxia from 8 hours to 5 days showed decreased levels of CHOP and PERK after 48 and 72 hours, respectively, of hypoxic culture (Figure 1F and G), whereas ATF4 and BiP protein levels did not change under hypoxia (Figure 1G). These results suggested that, although hypoxic conditions promote the homeostasis of the ER in the NP cells, exposure to supraphysiological oxygen levels leads to increased ER stress.

Tunicamycin and Thapsigargin Treatments Induce Canonical Unfolded Protein Response and HIF-1 α Activity by NP Cells

To study the effect of ER stress response under physiological niche condition of NP cells,³⁹ we cultured NP cells under hypoxia and treated them with ER stress-inducing compounds: tunicamycin (which blocks N-linked glycosylation), and thapsigargin (which is a noncompetitive inhibitor of the sarco/endoplasmic reticulum Ca²⁺ ATPase).⁵ Quantitative RT-PCR analysis showed a clear increase of *Hspa5*, *Ddit3*, *Eif2a*, *Atf4*, *Atf6*, and *Ire1* mRNA levels following tunicamycin and thapsigargin treatments (Figure 2A). However, *Ero1l* and *Pdia2* mRNA levels did not change with treatment. Furthermore, Western blot analysis of CHOP, phosphorylated PERK, ATF4, ATF6, and BiP proteins showed a similar increase in levels after tunicamycin and thapsigargin treatments (Figure 2B).

Figure 1 Hypoxia decreases endoplasmic reticulum (ER) stress markers in nucleus pulposus (NP) cells. **A–D:** Representative transmission electron microscopy images of primary NP cells cultured under normoxia [NX; 21% partial pressure of oxygen (pO_2)] and hypoxia (HX; 1% pO_2) for 24 hours. Cells under hypoxia evidence thinner ER compared with dilated ER seen in cells cultured under normoxia (arrow). ER morphologic assessment was based on observing three randomly selected high-magnification areas from 13 to 14 NP cells under normoxia and hypoxia, respectively, chosen from three independent experiments. **E:** Quantitative RT-PCR analysis demonstrates time-dependent decreases in levels of *Hspa5*, *Ddit3*, *Atf4*, *Atf6*, and *Ern1* mRNA expression under hypoxia. *Eif2a*, *Ero1a*, and *Pdia2* mRNA levels do not decrease under hypoxia. **F:** Western blot analysis of NP cells cultured under hypoxia for 8 hours to 5 days show decreases in C/EBP homologous protein (CHOP) and protein kinase R-like endoplasmic reticulum kinase (PERK) after 48 hours under hypoxia. **G:** Densitometric analysis of independent Western blot experiments confirms significant decreases in CHOP and PERK after 48 hours under hypoxia. Immunoglobulin heavy-chain-binding protein (BiP) and activating transcription factor 4 (ATF4) protein levels are not altered by hypoxia. Values are shown as means $\pm$ SEM (**E** and **G**). $n \geq 3$ independent experiments (**A–G**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus NX. Scale bars: 2 μ m (**A** and **B**); 500 nm (**C** and **D**). Ctr, control; TUBB, β -tubulin.

To explore the impact of ER stress on HIF-1 α activity, HRE reporter activity was measured after tunicamycin and thapsigargin treatments. The elevated HRE reporter activity suggested that increased HIF-1 α activity was prompted by tunicamycin and thapsigargin (Figure 2C).

HIF-1 α Regulates ER Stress Response in NP Cells

Few studies have shown a relationship between HIF-1 α activity and ER stress under hypoxic conditions.¹⁴ Because HIF-1 α is as an essential transcription factor for the survival

Figure 2 Endoplasmic reticulum (ER) stress markers and hypoxia-inducible factor (HIF)-1 α activity are up-regulated in response to tunicamycin (Tun) and thapsigargin (Thap) treatments in nucleus pulposus cells. **A:** Quantitative RT-PCR analysis shows dose-dependent increases in mRNA levels of ER stress markers (*Hspa5*, *Ddit3*, *Eif2a*, *Atf4*, *Atf6*, and *Ire1*) by tunicamycin and thapsigargin treatments. **B:** Western blot analyses of C/EBP homologous protein (CHOP), activating transcription factor 4 (ATF4), ATF6, immunoglobulin heavy-chain-binding protein (BiP), and phosphorylated protein kinase R-like endoplasmic reticulum kinase (pPERK) after tunicamycin and thapsigargin treatments show increased levels of these ER stress makers. **C:** Luciferase (luc) assay using an hypoxia response element (HRE) reporter shows that HIF-1 α activity is up-regulated by tunicamycin and thapsigargin treatments. Values are shown as means $\pm$ SEM (A and C). $n \geq 3$ independent experiments (A–C). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus Ctr. Ctr, control; TUBB, β -tubulin.

and function of NP cells,^{4,19,40} we asked the question, whether or not it regulates ER stress in NP cells. NP cells were transduced with a lentivirus encoding ShHIF1A to stably suppress HIF1A expression. The HIF-1A knockdown was confirmed by quantitative PCR (Figure 3A). Interestingly, although *Ddit3*

expression levels increased in the ShHIF1A cells, *Hspa5*, *Eif2a*, and *Atf6* levels were decreased in these cells (Figure 3A), and *Atf4*, *Ero1b*, and *Pdia6* levels were unchanged (Figure 3A). At the protein level, there was a trend toward increased abundance of CHOP, PERK, and ATF6

Figure 3 Hypoxia-inducible factor (HIF)-1 α activity modulates unfolded protein response (UPR) in nucleus pulposus (NP) cells. **A:** Quantitative RT-PCR analysis shows insignificant changes in levels of *Hif1a* and increased *Ddit3* levels, followed by decreases in *Hspa5*, *Eif2a*, and *Atf6* levels in the short hairpin RNA HIF1A plasmid knockdown (ShHIF1A) group. *Atf4*, *Ero1b*, and *Pdia6* mRNA levels do not change between ShHIF1A and ShCtrl (control plasmid). **B:** Western blot analysis of NP cells transduced with ShCtrl or ShHIF1A shows an increase in protein levels of C/EBP homologous protein (CHOP), activating transcription factor 6 (ATF6), and protein kinase R-like endoplasmic reticulum kinase (PERK) in the HIF-1 α silenced group. **C:** Densitometric analysis of Western blot data shown in **B**. **D:** Tunicamycin (TUN)-treated cells show a significant increase of *Ddit3*, *Hspa5*, *Atf6*, and *Eif2a* mRNA levels relative to untreated controls (CTs). DMOG-treated cells show no changes in UPR markers when administered alone, but present a reduction in levels of *Ddit3*, *Hspa5*, *Atf6*, and *Eif2a* expression when added with tunicamycin. Values shown are means $\pm$ SEM (**A**, **C**, and **D**). $n \geq 3$ independent experiments (**A–D**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus ShCtrl; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ versus TUN. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine guanine phosphoribosyltransferase. T+D, tunicamycin plus DMOG treatment.

levels after HIF-1 α silencing (Figure 3, B and C). Further exploring the potential contributions of HIF-1 α in regulating ER stress, dimethylxylglycine (DMOG), a pan HIF–prolyl hydroxylase inhibitor, was used to stabilize HIF-1 α and increase its activity.⁴¹ Accordingly, NP cells were pretreated with DMOG for 2 hours before inducing ER stress with tunicamycin. As expected, tunicamycin significantly increased *Hspa5*, *Ddit3*, *Eif2a*, and *Atf6* levels (Figure 3D), which were reduced by DMOG treatment. Altogether, these data suggest that HIF-1 α modulates ER stress responses in NP cells.

The NP Cell Secretome in Hypoxia is Enriched with Metabolite Interconversion Enzymes, Translation Proteins, Cytoskeletal-Related Proteins, and Collagens

The importance of matrix homeostasis and secretion in the maintenance of intervertebral disc function has been reported.⁴² To delineate the NP cell secretome under physiological conditions, we collected the conditioned media of cells cultured under hypoxia and performed proteomic analysis

(Figure 4A). A total of 767 secreted proteins were identified by the mass spectrometry (Figure 4A). To understand the secretome composition, gene ontology analysis was performed using PANTHER.²⁹ Regarding protein class, most of the secreted proteins aligned with metabolite interconversion enzymes (21.3%), translation proteins (15.6%), cytoskeletal proteins (14.8%), and protein-modifying enzymes (11.8%) (Figure 4B). Molecular function analysis implicated these proteins in binding (43%), catalytic (37.8%), and structural molecule activity (10.1%) (Figure 4C). Furthermore, biological process analysis showed an association between these differentially secreted proteins and cellular processes (30.8%), metabolic processes (19.5%), cellular components (12.8%), and biological regulation (10.4%) (Figure 4D). Detailed analysis of cellular processes revealed an association with cellular metabolic processes, cellular component organization, cellular response to stimuli, cell communication, actin filament–based processes, cell cycle, cell death, export from the cell, and processes utilizing autophagic mechanisms (Figure 4E). Notably, COL1A2, COL1A1, FN, ACTA,

Figure 4 Secretome profile of primary nucleus pulposus (NP) cells cultured under hypoxia. **A:** Schematic detailing crucial steps in NP cell secretome analysis and the total number of identified proteins. **B–D:** Gene ontology (GO) analyses of identified proteins were categorized on the basis of the most represented protein classes (**B**), molecular functions (**C**), and biological processes (**D**), using the PANTHER software version PANTHER15.0. **E:** Level 1 biological analysis was performed to further detail the cellular processes seen in **C**. Results are shown as the percentage of proteins that are part of each individual GO category. **F:** Top 50 secreted proteins by NP cells under hypoxia; normalized protein values are shown. Ctr, control.

CO3A1, ACTN1, VIME, and BiP were the most abundantly secreted proteins from NP cells (Figure 4F).

ER Stress Induction in NP Cells Compromises Cellular Secretory Pathways, Resulting in Diminished Matrix Secretion

To elucidate the influence of ER stress on the secretion profile of NP cells, we collected the conditioned media from

cells cultured under hypoxia, with or without tunicamycin or thapsigargin, and performed a proteomic analysis (Figure 5A). Although tunicamycin treatment resulted in 9% variance of secreted proteins (67 proteins), thapsigargin lead to a variance of 42% (325 proteins) (Figure 5A). More importantly, each treatment group distinctly clustered, as reflected by the principal component analysis (Figure 5B and C). A total of 87% (58 proteins) of differentially secreted proteins in the tunicamycin group were down-

Figure 5 Endoplasmic reticulum stress induction under hypoxia results in inefficient secretory pathways accompanied by lower matrix secretion in nucleus pulposus cells. **A**: Schematic of the total number of secreted proteins detected by mass spectrometry and those showing significant changes on tunicamycin (Tun) and thapsigargin (Thap) treatments ($P < 0.1$). **B**: Principal component analysis (PCA) of control (Ctr) and Tun. **C**: PCA of Ctr and Thap. **Dotted lines** represent y axis at the origin (**B** and **C**). All identified secreted proteins were used for PCA clustering. **D**: Up-regulated and down-regulated protein percentages from Tun versus Ctr. **E**: Heat map and hierarchical clustering analysis of differentially secreted proteins between control- and tunicamycin-treated individual biological samples. **F**: Gene ontology (GO) enrichment for biological processes of the down-regulated proteins from the Tun group. **G**: Up-regulated and down-regulated protein percentages from Thap group versus control. **H**: Heat map and hierarchical clustering analysis of differentially secreted proteins between control- and thapsigargin-treated individual biological samples. **I**: GO enrichment for biological processes of the down-regulated proteins from the Thap group. Binomial statistical test with false discovery rate (FDR) adjustment and a cutoff of $P < 0.05$ was used for enrichment analyses. **J** and **K**: Top down-regulated proteins from the comparison between tunicamycin and control and thapsigargin and control groups. Three independent biological samples were analyzed per group. SFM, serum-free media.

Figure 6 Endoplasmic reticulum stress induction by tunicamycin (Tun) and thapsigargin (Thap) treatments decreases secretion of collagens and adhesion-related proteins in nucleus pulposus cells. **A–C:** Dynamic profiles of secreted proteins were determined from unsupervised clustering of 355 proteins identified as differentially secreted across the three groups. **B and C:** Schematic representation of the 92 commonly up-regulated (cluster 1) and the 15 commonly down-regulated proteins (cluster 3) across treatment conditions. **D:** Cluster 3 was used to perform the gene ontology (GO) enrichment analysis for biological processes of the down-regulated proteins. **E:** Representation of commonly down-regulated proteins from cluster 3. **F:** Cluster 1 was used to perform the GO enrichment analysis for biological processes of the up-regulated proteins. **G:** Representation of commonly up-regulated proteins from cluster 1. Binomial statistical test with false discovery rate (FDR) adjustment and a cutoff of $P < 0.05$ was used for enrichment analysis. Ctrl, control; IRES, internal ribosome entry site.

Figure 7 Aged C57BL/6 mice (BL6; 23-month-old versus 6-month-old mice) and degenerated human discs (degenerated versus healthy) showed increased expression of endoplasmic reticulum (ER) stress markers along with decreased expression of several extracellular matrix components. **A** and **B**: Analysis of differentially expressed genes (DEGs) showed increases in ER stress makers, secretion of regulatory genes, and down-regulation of several matrix components in aged BL6 mice. **C** and **D**: DEG analysis showed an increase in ER stress makers, secretion of regulatory genes, as well as down-regulation of several matrix components in human degenerated nucleus pulposus tissue samples (cluster A, healthy; and cluster 1, degenerated). Representative DEGs from GSE134955 (C57BL/6) and GSE70362 (human) deposited data ($P < 0.05$). **Dotted lines** separate the genes accordingly to their biological class. **Block arrows** next to y axis show directionality of change. **E**: Schematic showing the relationship between hypoxia, hypoxia-inducible factor (HIF)-1 α , and ER stress regulation in nucleus pulposus (NP) cells. NP cells evidence lower levels of ER stress under hypoxia in an HIF-1—dependent manner. HIF-1 α modulated ER stress response by down-regulating *Ddit3* and modulating *Atf6* and *Eif2a* expression. Activation of the ER stress—unfolded protein (UP) response pathway in NP cells results in an inefficient secretion of collagens and cell adhesion—related proteins. $n = 7$ per group (**A** and **B**); $n = 5$ for cluster A (**C** and **D**); $n = 3$ for cluster 1 (**C** and **D**).

regulated, whereas 13% (9 proteins) were up-regulated (Figure 5D and E), relative to the untreated control group. GO enrichment for biological processes using the PANTHER overrepresentation analysis²⁹ was performed to

study the biological associations of these differentially secreted proteins. Accordingly, down-regulated proteins in the tunicamycin group showed significant enrichment for extracellular structure organization, collagen fibril

organization, response to oxygen-containing compound, response to stress, thyroid hormone, cytokines, and tendon development processes (Figure 5F). Moreover, the thapsigargin group showed a higher percentage of differentially secreted proteins to be up-regulated, 93% (302 proteins) (Figure 5G and H). Interestingly, similar to the tunicamycin group, GO enrichment analysis of down-regulated proteins in the thapsigargin group showed association with collagen fibril organization, extracellular structure organization, response to thyroid hormone, and tendon development processes (Figure 5I), and up-regulated proteins in the thapsigargin group were enriched for cytoskeleton organization, actin filament-based processes, and catabolic processes (Supplemental Figure S2, A and B). The top down-regulated proteins in the tunicamycin group were VPS45, MVD1, VLDLR, PCOC1, and FBLN5 (Figure 5J). However, FBLN5, PCOC1, CO1A1, CO3A1, CO1A2, and CO11A1 were the most significantly down-regulated proteins in the thapsigargin group (Figure 5K). The top up-regulated proteins secreted in the tunicamycin group were LAMP1, AIFM1, and PALLD (Supplemental Figure S2B); and TBA1C, SRC8, and HPBP1 in the thapsigargin group (Supplemental Figure S2C).

Subsequent comparison of the secretion profiles of all three groups identified 355 variant proteins (Figure 6A). More importantly, to account for possible off-target effects (not related to ER stress induction) of tunicamycin and thapsigargin treatments and to delineate the consequences of ER stress on NP cell secretion profiles, only the clusters of secreted proteins showing changes in the same direction across treatments were selected for further analysis (Figure 6B and C). Differentially secreted proteins were thus clustered on the basis of their dynamic profiles (Figure 6B and C). Accordingly, cluster 1 identified 92 common up-regulated proteins, and cluster 3 identified 15 common down-regulated proteins (Figure 6B and C). Cluster 3 was used to perform a GO enrichment analysis for biological processes and study the down-regulated proteins. From this analysis, the most enriched pathways were related to collagen fibril organization, extracellular structure organization, supramolecular fiber organization, and extracellular matrix organization (Figure 6D). The secretion of collagens was decreased by both tunicamycin and thapsigargin treatments. There was also a significant decrease in other important matrix modulators and cell adhesion proteins such as FBLN5, PCOC1, C1QT5, B2MG, CATH, NID1, and HA12 (Figure 6E). In addition, biological enrichment analysis of up-regulated proteins showed association with cytoskeleton organization, actin cytoskeleton organization, and actin filament-based processes (Figure 6F). Likewise, there was a significant increase in AIFM1, CPNS1, HPBP1, ARP5L, BAX, ARPC5, DCTN2, MLP3B, SEPT9, TWF1, and VINC (Figure 6G).

Taken together, these results suggest the importance of ER homeostasis in the maintenance of an efficient extracellular matrix secretion.

Increased UPR, Along with Decreased Expression of Matrix Components, is Seen in NP Tissues from Degenerated Human and Aging Mouse Discs

UPR is known to cause and contribute to a wide spectrum of pathologies found in humans, including disc degeneration.¹² To determine the *in vivo* relevance of our *in vitro* findings, recently deposited microarray data sets comparing NP transcriptional profiles from 23-month-old C57BL/6 (old) with 6-month-old C57BL/6 (young) mice (GSE134955)³⁸ and degenerated versus healthy human disc samples (GSE70362)³⁶ were analyzed. Differentially expressed genes determined by comparing old and young mice showed a clear increase in the expression of UPR markers (*ATF6*, *Ddit4*, *Ddit3*, *Ero1lb*, *Pdia2*, *Ern1*, *Bcl2*, and *Becn1*) and secretion regulatory genes (*Fbln2*, *Ddx19b*, *Ddx1*, *Hspb7*, *Lmod3*, *Lmod2*, *Pak1*, *Actn2*, and *Actn3*), among others (Figure 7A). Concomitantly, old mice showed significant down-regulation of several extracellular matrix components and regulatory genes concerned with matrix synthesis, including *Col6a4*, *Col4a2*, *Col17a1*, *Col23a1*, *Col4a3bp*, *Col16a1*, *Col19a1*, *Col25a1*, *Col22a1*, *Col20a1*, *Col8a2*, *Col5a1*, *Col5a2*, *Has1*, and *Has3* (Figure 7B). In line with our *in vitro* findings and analysis of aging mice, NP tissues from degenerated human discs presented increased levels of ER stress markers (*EIF2AK4*, *ATF6*, *DDIT4L*, *ERO1A*, *PDAI6*, *MAPK8IP2*, and *BCL2*) and secretory regulation genes (*FBLN5*, *SEPT11*, *TWF1*, and *SPTBN1*) (Figure 7C). Likewise, there was a down-regulation of several matrix components and enzymes involved in matrix synthesis and degradation, including *COL9A2*, *COL6A2*, *COL11A2*, *COL16A1*, *LOC101928841*, *COL11A2*, *COL27A1*, *HAS2*, *MGEA5*, *HYAL1*, and *ACAN* (Figure 7D). These results suggest a correlation between increased UPR, decreased matrix expression and/or secretion, and disc degeneration, underscoring the importance of understanding ER stress regulation in the context of the pathogenesis of intervertebral disc degeneration.

Discussion

ER stress is known to play an important role in cell fate and survival in pathologies underlined by hypoxic conditions.¹⁴ Notably, unlike in other cell types,^{15,43,44} ER stress markers in NP cells decreased in a time-dependent manner under hypoxia. This result was interesting, considering hypoxic conditions and HIF-1 α activity activate and promote UPR,^{45,46} leading to activation of the apoptotic ER stress pathway through CHOP.¹⁵ However, it is important to underline that NP cells are uniquely adapted to survive and function in their physiologically hypoxic microenvironment,² and they have been shown to noncanonically regulate well-described biological mechanisms associated with hypoxia. Previous studies have demonstrated unique HIF-1 α activation and regulation in NP cells^{3,47,48} and that

hypoxia induces a noncanonical autophagy in them, independent of HIF-1 α and mammalian target of rapamycin (MTOR) signaling.² This hypoxic, time-dependent decrease in ER stress highlights the importance of the relationship between ER stress regulation and the long-term survival and function of NP cells. Moreover, both HIF-1 α loss-of-function and gain-of-function studies using ShRNA and DMOG treatments demonstrated a clear role for HIF-1 α in down-regulating CHOP under ER stress conditions, further supporting its importance as a master regulator of NP cell metabolism,^{19,40} homeostasis,⁴ and modulation of UPR.^{12,41}

Because hypoxia reduced ER stress levels, we sought to determine whether these cells activated canonical UPR under hypoxia through the master regulators IRE1, PERK, and ATF6. Using a previously described pharmaceutical approach,⁵ we showed that tunicamycin and thapsigargin treatments activated a canonical UPR by NP cells under hypoxic conditions. This was interesting, because although ER stress regulation by oxygen tension seemed unique to NP cells, mechanistically speaking, UPR activation was controlled in the same manner as in other skeletal cell types, such as chondrocyte⁴⁹ and osteoblast.⁵⁰

ER stress has been correlated with intervertebral disc degeneration, and more specifically, with NP cell viability.¹³ In addition, disc degeneration is characterized by reduced cell viability and matrix production, as well as amplified inflammatory pathways and extracellular matrix (collagens and aggrecan) degradation.^{42,51–54} Relevant to matrix homeostasis, UPR is shown to play a role in collagen secretion and matrix remodeling in other cells types.⁵⁵ To investigate the consequences of increased ER stress under hypoxic conditions, we performed a proteomic analysis of the proteins secreted from NP cells. Interestingly, despite some qualitative differences, both tunicamycin and thapsigargin resulted in overall induction of UPR in NP cells, with some clear distinctions in the observed secretomic changes. A previous study of fibroblasts showed a significant increase in the secretion of several chaperones when treated by thapsigargin (which depletes ER calcium) but not with tunicamycin (which inhibits protein glycosylation).¹⁶ Interestingly, independent of the differences in up-regulated proteins between treatments, down-regulated proteins indicated that treatments affect similar biological processes. This was supported by collagens and other cell adhesion-related proteins being commonly underrepresented. Analyses of other ER stress models have implicated a role of ER stress in modulating collagen levels, further supporting the role of ER stress in regulating matrix production and secretion by NP cells.⁵⁵ Interestingly, proteomic analysis comparing degenerated and healthy human discs has shown an enrichment of pathways related to cytoskeletal (actin organization), immune response (T-cell receptor signaling), and cell adhesion pathways, similar to what was observed in this study.⁵⁶ Moreover, another study investigating the secretory signature of muscle cells showed that cytoskeleton and extracellular matrix pathways are

modulated under physiological and stressful conditions.⁵⁷ Altogether, these results suggest that ER stress promotes an inefficient secretory pathway in NP cells, which may help explain the loss of cell viability and correlation between ER stress and intervertebral disc degeneration.^{12,13,58}

Correlations between disc degeneration and ER stress are established,¹² with stress loading,⁵⁹ impaired calcium homeostasis,⁶⁰ and injury⁶¹ being shown to alter matrix expression and promote UPR in animal models of disc degeneration. Xu et al⁶¹ showed that administration of hydrogen sulfide ameliorates disc degeneration in conjunction with decreased levels of ER stress. A recent study of the annulus fibrosus, the outer compartment of the intervertebral disc, also demonstrated elevated ER stress markers with increasing levels of degeneration.¹² Likewise, analyses of NP tissues from naturally aged C57BL6 mice and human degenerated discs presented herein reflect a similar increase in UPR markers. This was not surprising, given the conservation of cellular and molecular mechanisms across species in the context of the disc.^{38,39,42} Further supporting our secretome data, we also document a decrease in the expression of several matrix components in these data sets, implying associations between ER stress, impaired secretion, and disc aging and degeneration. These results thus suggest that ER stress is present in degenerated NP and annulus fibrosus compartments and may contribute to disease pathogenesis.

In summary, our study, for the first time, links ER stress to inefficient matrix secretion in the NP and clearly demonstrates the importance of HIF-1 α and oxygen levels in modulating ER stress responses in NP cells (Figure 7E).

Author Contributions

E.J.N., H.C., V.M., M.V.R., I.M.S., A.J.S., and B.M. designed the study; E.J.N., H.C., V.M., and S.I.A. collected and analyzed data; E.J.N., M.V.R., I.M.S., A.J.S., and B.M. prepared the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.11.012>.

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