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ORIGINAL ARTICLE

Expression and activity of hyaluronidases HYAL-1, HYAL-2 and HYAL-3 in the human intervertebral disc

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Abstract

Purpose Hyaluronic acid plays an essential role in water retention of the intervertebral disc (IVD) and thus provides flexibility and shock absorbance in the spine. Hyaluronic acid gets degraded by hyaluronidases (HYALs), and some of the resulting fragments were previously shown to induce an inflammatory and catabolic response in human IVD cells. However, no data currently exist on the expression and activity of HYALs in IVD health and disease.

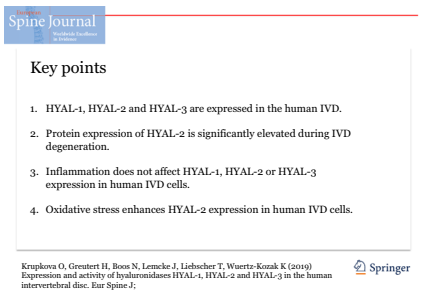
Methods Gene expression, protein expression and activity of HYALs were determined in human IVD biopsies with different degrees of degeneration ($n = 50$ total). Furthermore, freshly isolated human IVD cells ($n = 23$ total) were stimulated with IL-1β, TNF-α or H_2O_2, followed by analysis of HYAL-1, HYAL-2 and HYAL-3 gene expression.

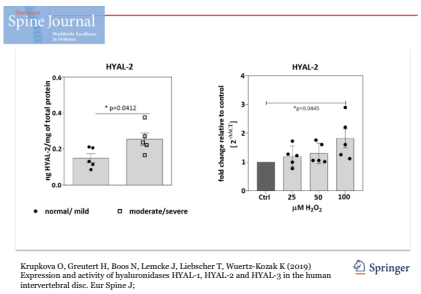
Results Gene expression of HYAL-1 and protein expression of HYAL-2 significantly increased in moderate/severe disc samples when compared to samples with no or low IVD degeneration. HYAL activity was not significantly increased due to high donor–donor variation, but seemed overall higher in the moderate/severe group. An inflammatory environment, as seen during IVD disease, did not affect HYAL-1, HYAL-2 or HYAL-3 expression, whereas exposure to oxidative stress (100 µM H_2O_2) upregulated HYAL-2 expression relative to untreated controls.

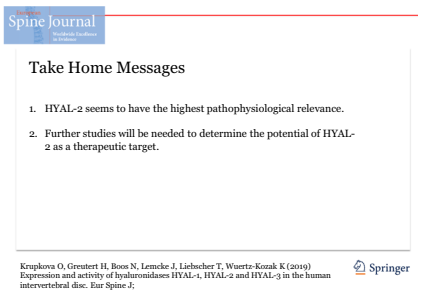
Conclusion Although HYAL-1, HYAL-2 and HYAL-3 are all expressed in the IVD, HYAL-2 seems to have the highest pathophysiological relevance. Nonetheless, further studies will be needed to comprehensively elucidate its significance and to determine its potential as a therapeutic target.

Graphic abstract

These slides can be retrieved under Electronic Supplementary Material.







Olga Krupkova and Helen Greutert have contributed equally to this work.

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Keywords Degenerative disc disease · Extracellular matrix · Hyaluronic acid · Glycosaminoglycan · Oxidative stress · Inflammation

Introduction

Glycosaminoglycans (GAGs), which are major components of the extracellular matrix (ECM) of numerous connective tissues, including the intervertebral disc (IVD), are a family of negatively charged heteropolysaccharides. Chondroitin sulfate and keratan sulfate as the IVD-typical GAGs bind to an extended protein core, resulting in the brush-like proteoglycan (PG) named aggrecan. The G1 domain at the N-terminal end of aggrecan interacts with hyaluronic acid (HA) and link protein and thus provides the hydrated gel structure seen in healthy IVDs, specifically in the nucleus pulposus (NP) [1, 2]. Hyaluronic acid (HA) itself is composed of repeated disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine with a molecular weight of 10^3–10^4 kDa (high molecular weight, HMW-HA) [3, 4].

Ample evidence has been provided over the past decades for the degradation of aggrecan with IVD degeneration. In fact, enzymatic action by matrix metalloproteinases (MMPs) and aggrecanases can target the interglobular domain (between the G1 and G2 domains), with MMP degradation typically happening earlier than aggrecanase degradation [5, 6]. Interestingly, mechanical overloading as a contributor to degeneration was shown to enhance MMP-associated cleavage of aggrecan [7]. Furthermore, changes in spatial distribution and disaccharide sulphation patterns of GAGs are commonly observed during IVD aging and degeneration [8–10].

HA degradation can occur via prokaryotic enzymes, including bacterial β-endoglycosidases and bacterial β-exoglycosidases [11]. However, in the context of ECM maintenance and degeneration in human health and disease, the eukaryotic HA-degrading endo-β-*n*-acetylhexosaminidases—also termed hyaluronidases (HYALs)—are of highest relevance, albeit other eukaryotic enzymes (e.g., *β*-glucuronidase) also possess HA degrading activity [11]. HYALs preferentially degrade HA through cleavage at the β-(1,4)-linkage [12], but can also affect the integrity of chondroitin sulfate at reduced activity [13].

Six HYAL-like gene sequences have been identified in humans so far, sharing about 40% of their identity with one another: HYAL-1, HYAL-2, HYAL-3, HYAL-4, PH20 (= SPAM1) as well as a pseudo gene HYAL-Phyal1 that is transcribed in humans, but is not translated [13]. Of these, HYAL-1 and HYAL-2 have been investigated in most detail. While both of these enzymes are located on chromosome 3p21.3 [14], they show differences with regard to substrates, location within the cellular compartment and cleavage products [11, 15].

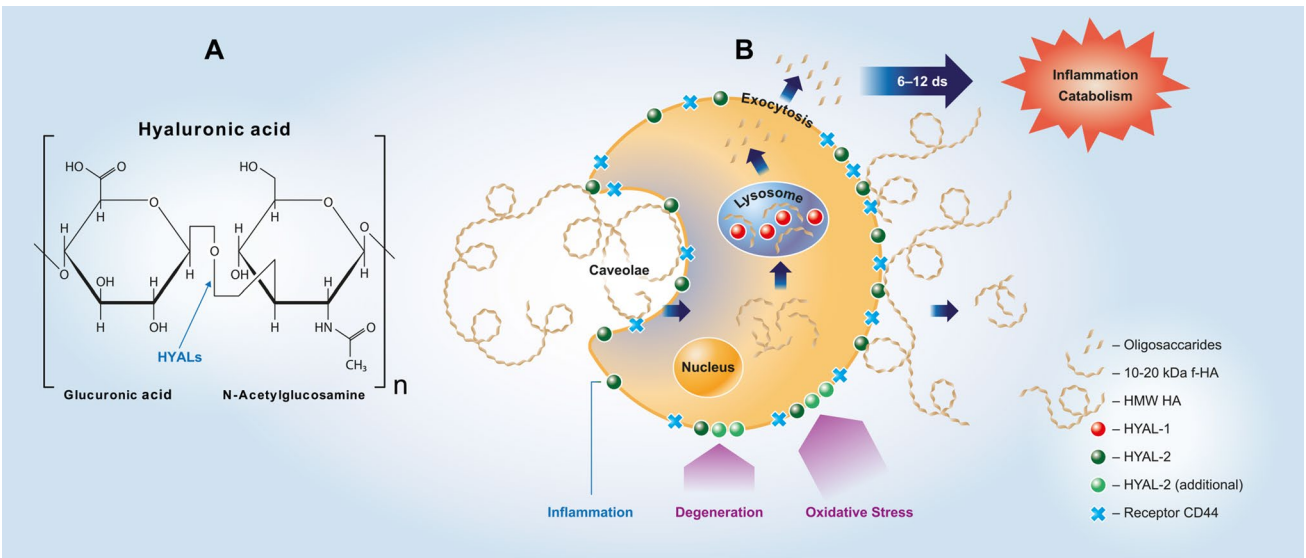

Fig. 1 Mechanism of HA degradation. **a** HA is cleaved by HYALs at hexosaminidic β (1–4) linkages between glucuronic acid and *N*-acetylglucosamine. **b** Membrane-anchored HYAL-2 cleaves HMW HA to small MW HA, which is then internalized into lysozomes and further cleaved to oligosaccharides by HYAL-1. In the IVD, oligosaccharides have been shown to exhibit biological (e.g., pro-inflammatory) activity. In the IVD, the expression of HYAL-2 is increased with oxidative stress as well as with degeneration (but in a subset of samples), yet not in a simulated inflammatory environment

The process of HA cleavage (Fig. 1) is initiated by HYAL-2, a cell surface glycosylphosphatidylinositol-anchored protein that acts in cooperation with CD44 [16, 17], by degrading HMW-HA into intermediate sized fragments of approximately 10–20 kDa [14]. These fragments are partially taken up by the cell upon interaction with surface HA receptors (endocytosis) [14, 15] and partially released into the extracellular space [17]. Upon endocytosis, HYAL-1 further degrades these fragments into oligosaccharides (predominantly tetrasaccharides) within endosomal-lysosomal structures in the cell. Fragments are then released by exocytosis or further cleaved by the lysosomal enzymes β-D-glucuronidase and β-*N*-acetyl-D-hexosaminidase into individual sugars [13, 15, 18]. Aside from enzymatic reactions, in vivo degradation of HA can also occur through reactive oxygen species (ROS), as described in detail by Stern et al. [11].

Larger HYAL-2-induced fragments (oligomers) and smaller HYAL-1-induced fragments (e.g., tetramers) have both been described to possess biological activity, thereby affecting a variety of cellular processes in a cell-type and size-specific manner [19–23]. More specifically, various types of HA fragments were shown to be able to promote inflammation in, e.g., immune cells (10–400 kDa) [19] (35kDA) [24], chondrocytes (6-mer oligosaccharides) [25] and also IVD cells (6–12 disaccharides) [26] (Fig. 1), although not all studies show an effect (4 mer as well as range between 4 and 200 kDa) [27, 28]. Interestingly, inflammatory conditions have been described to further induce the expression and activity of HYALs in certain cell and tissue types [29, 30], although it is currently unclear whether cells in degenerated IVDs (which are characterized by inflammation [31, 32]) retain a similar feedback mechanism.

Aside from inflammation, the degenerating IVD possesses various other microenvironmental characteristics that might affect HYAL metabolism, including reduced pH [33, 34] and enhanced oxidative stress [35]. It has previously been shown that the activity of HYALs is pH-dependent. While human HYAL-1 as a lysosomal enzyme is most active around pH 4.0 [36], the pH optimum for the membrane-associated HYAL-2 is around 6.0 [17]. Such a reduced pH is typically achieved through the activation of the Na^+/H^+ exchanger 1 (NHE1) upon interaction with CD44 [16]. As degeneration-associated accumulation of lactic acid in IVDs leads to a drop in pH as low as 5.7 in severely affected tissues [33, 34], the pH microenvironment of the IVD may further modulate HYAL-2 activity. Concomitant with a degeneration-associated reduction in pH, enhanced generation of ROS has been described in the IVD [35, 37]. While ROS have previously been shown to induce post-translational oxidative modification of collagens such as crosslinking and unfolding [38, 39], their role in modulating the HYAL metabolism have not yet been investigated. However, bronchial epithelial cells demonstrated increased HYAL-2 expression and activity when exposed to ROS [40] and similar response patterns may also exist for IVD cells.

Despite the functional importance of HA (and PGs) in the IVD, the role and regulation of HYALs in IVD health and disease are currently unknown. Therefore, the aim of this study was to identify the expression and activity of HYAL-1, HYAL-2 and HYAL-3 in human IVD tissue with different degrees of degeneration and to test whether their expression is regulated under inflammatory or oxidative stress conditions in vitro.

Materials and methods

Human IVD tissue

The collection of human IVD tissue from patients undergoing spinal surgery was approved by the Cantonal ethics Committee Zurich, Switzerland (#EK-16/05) as well as by the Ethics Committee of the Charité Berlin, Germany (#EA2/087/11). Informed consent was obtained from all patients.

Samples used for direct analysis of HYAL expression/activity ($n = 34$, mixture of NP and AF) were immediately cooled after intraoperative excision and thereafter shock frozen at − 80 °C. Using preoperative MRIs, the degree of IVD degeneration in these samples was determined according to Pfirrmann et al. [41], using an adopted 4-grade classification scale as previously described [42, 43]. Specifically, discs were graded as non-degenerated (grade 1), mildly degenerated (grade 2), moderately degenerated (grade 3) and severely degenerated (grade 4) (Table 1).

Samples used for cell isolation (Pfirmann grade 3–4, disc herniation or DDD) were transferred into DMEM/F12 (D8437, Sigma-Aldrich, USA) with 3% anti–anti (15240062, Gibco, USA) upon intraoperative excision and immediately transported to the laboratory for further processing ($n = 15$). Due to the degeneration status of the specimens as well as the posterior surgical approach used in most cases, no separation of nucleus pulposus and annulus fibrosus was performed.

Primary cell culture

Tissue samples of mixed degeneration grades were cut into pieces and incubated in a sterile solution of 0.2% collagenase NB4 (17454, Serva, Heidelberg, Germany) and 0.3% dispase II (04942078001, Roche, Basel, Switzerland) in phosphate-buffered saline (PBS) for 4–8 h. Then, the cell suspension was filtered using a 70-µm cell strainer (352350, BD Bioscience, Switzerland) and expanded up to passage 3 in a 2D monolayer culture containing DMEM/F12 media with 10%

Table 1 Donor information

Nos.	Degeneration grade	Age	Sex	Pathology	Level
1	Normal	41.4	M	T	L1/2
2	Normal	25.3	M	T	L2/3
3	Normal	45.3	M	T	Th11/12
4	Normal	58	M	T	L4/5
5	Normal	24.5	M	T	L5/S1
6	Normal	46.6	M	T	TH 11/12
7	Normal	44.3	F	F	Th12/L1
8	Normal	36.4	F	F	Th5/6
9	Normal	39.4	M	F	Th6/7
10	Normal	41.4	M	F	Th12/L1
11	Normal	30.0	M	F	Th12/L1
12	Mild	41.3	F	DH	L4/5
13	Mild	63.7	M	DDD	L4/5
14	Moderate	26.2	M	DH	L5/S1
15	Moderate	58.1	M	DH	L3/4
16	Moderate	70.2	F	DH	L4/5
17	Moderate	52.7	F	DDD	L3/4
18	Moderate	61.7	M	DDD	L4/5
19	Moderate	68.0	F	DDD	L4/5
20	Moderate	71.1	F	DDD	L4/5
21	Moderate	62.4	F	DDD	L5/S1
22	Moderate	25.6	M	R	L5/S1
23	Severe	66.1	F	DDD	L4/5
24	Severe	48.4	M	DDD	L5/S1
25	Severe	74.8	F	DDD	L3/4
26	Severe	55.9	F	DDD	L3/4
27	Severe	77.4	F	DDD	L4/5
28	Severe	74.8	F	DDD	L3/4
29	Severe	56	F	DDD	L2/3
30	Severe	58.2	F	DDD	L4/5
31	Severe	54.1	M	R	L3/4
32	Severe	52.0	M	R	L3/4
33	Severe	62.8	M	R	L4/5
34	Severe	68.3	F	DH	L4/5

Donors used for tissue analyses

DH, herniation; DDD, degenerative disc disease; T, trauma; R, radiculopathy; F, fracture; uk, unknown

FCS (F7524, Sigma-Aldrich, USA) and 1% anti–anti, with medium changes twice a week.

Cell stimulation with IL-1β or TNF-α

For cytokine stimulation experiments, IVD cells ($n=5$) were seeded into 6-well plates at a density of 33,000 cells/cm^2. After 24 h, cells were rendered serum free (DMEM/F12 + 1% anti–anti) for 2 h. To determine the best cytokine stimulatory response, cells were incubated with 0.1, 1, 5 or 10 ng/mL recombinant IL-1β (211-11, Peprotech/LuBioScience, Switzerland) or 0.1, 1, 10 or 100 ng/mL recombinant TNF-α (315-01A, Peprotech/ LuBioScience, Switzerland) in serum-free medium for 18 h. All concentrations were controlled for cytotoxicity (data not shown). In addition, a time course experiment with stimulation times of 2, 6 and 18 h for 5 ng/ml IL-1β or 10 ng/ml TNF-α was conducted.

Table 2 Primer information

Target gene	Assay identification number
HYAL 1	Hs00201046_m1
HYAL 2	Hs01117343_g1
HYAL 3	Hs00185910_m1
GAPDH	Hs02786624_g1
TBP	Hs00427620_m1

Target genes and assay identification (ID) numbers of corresponding TaqMan primers (TaqMan Gene Expression Assays; Thermo Fisher Scientific)

Cell stimulation with H_2O_2

For H_2O_2 stimulation experiments, IVD cells ($n=5$) were seeded at a density of only 11,500 cells/cm^2 into T25 flasks or 6-well plates and allowed to settle and adapt to culturing conditions for further 48 h prior to the oxidant treatment. Thereafter, cells were rendered serum free for 2 h and consequently exposed for an additional 2 h to H_2O_2 (H1009, Sigma-Aldrich, Switzerland) at concentrations of 25, 50 and 100 µM to ascertain the strongest physiological ROS effect on the cells. After the treatment, the oxidative medium was immediately replaced by fresh DMEM/F12 supplemented with 10% FCS and 1% anti–anti and cells allowed to recover from oxidative stress for 24 h [44]. All concentrations were controlled for cytotoxicity (data not shown).

HYAL gene expression in IVD tissue and treated IVD cells

To isolate RNA from IVD biopsies with normal/mild ($n=8$) and moderate/severe ($n=16$) degeneration, shock-frozen tissues were pulverized in liquid nitrogen using a custom-made device, immersed in Trizol (15596-018, Invitrogen, USA), exposed to phase separation with chloroform and subsequently subjected to column-based purification (12183025, PureLink RNA Mini Kit, ThermoFisher, Switzerland) as previously described [45]. To isolate RNA from cultured cells ($n=5$/treatment), the PureLink RNA Mini Kit or the Trizol/choloroform method was used.

RNA from tissues or cells was reverse transcribed to cDNA using the Reverse Transcription Reagents (4374966, ThermoFisher, Switzerland), and real-time

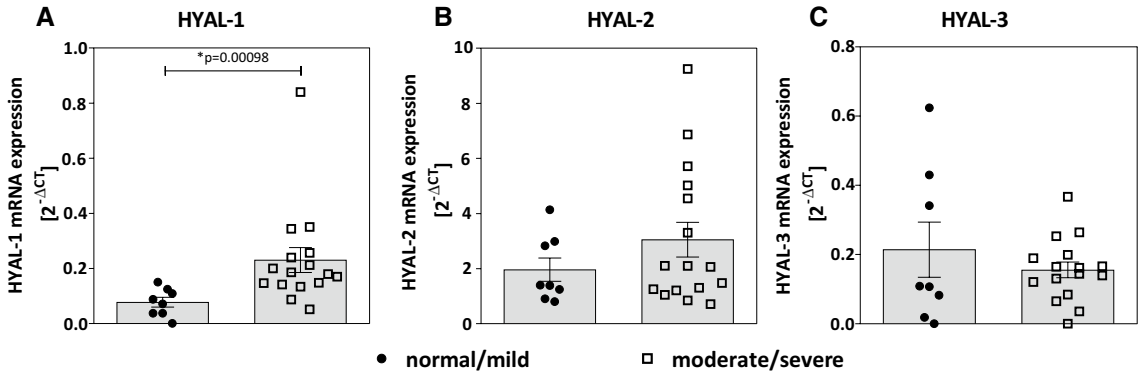

Fig. 2 Gene expression of HYALs in IVD tissue. Disc tissues were collected from patients undergoing elective spinal surgeries. **a** Gene expression of HYAL-1 was upregulated in moderate/severe disc degeneration group, when compared with the normal/mild group. **b** Gene expression of HYAL-2 was not changed; however, some degenerated samples showed high gene expression. **c** Gene expression of HYAL-3 was unchanged. Results were calculated by $2^{-\Delta Ct}$ method relative to TBP and analyzed by Mann–Whitney *U* test due to non-normality of data ($n=8$ in normal/mild group, $n=16$ in moderate/severe group)

PCR analysis was performed by TaqMan Gene Expression assays (ThermoFisher, Switzerland), using primers/probes for HYAL-1, HYAL-2 and HYAL-3 (Table 2). Additionally, HO-1 mRNA expression was measured to verify induction of oxidative stress in H_2O_2 treated cells. TBP was used as the housekeeping gene for all experiments except for H_2O_2 treatments, in which GAPDH was more stably expressed than TBP. Gene expression in the tissue was normalized to the housekeeping gene and shown as 2^{-dCt} values. Gene expression of stimulated cells was normalized to the housekeeping gene and to the untreated control and shown as 2^{-ddCt}.

HYAL protein expression

To isolate protein from IVD biopsies with normal/mild ($n=5$) and moderate/severe ($n=5$) degeneration, samples were pulverized as described above and then immersed in PBS supplemented with 100x protease inhibitors (78425, Pierce, USA) with three freeze-thaw cycles at − 72 °C on dry ice, as recommended by the ELISA's manufacturer.

Total protein concentration was determined by Bradford assay as described by the manufacturer (500-0006, Bio-Rad, Switzerland). Protein expression of HYAL-1 (tissue/cells), HYAL-2 (tissue/cells) and HYAL-3 (tissue) was detected on total protein samples (20–40 µg for tissue, 10–20 µg for cells, 100 µl for supernatants) by ELISA (MBS703230, My BioSource, USA; E1126 h, Lubio Science, Switzerland). Results are expressed as ng HYAL per mg of total protein in the IVD tissue or as the ng per ml in cultured cells, based on the sum of cell lysates and supernatants.

Total HYAL activity

Total HYAL activity in IVD biopsies with normal/mild ($n=5$) and moderate/severe ($n=5$) degeneration was analyzed with a commercial HYAL activity kit (Ra003-01-HAK, Amsbio, UK), according to the manufacturers instruction. Briefly, defrosted and washed tissue biopsies were immersed in base buffer defined by the manufacturer (10–50 mg tissue/ml) and then homogenized by Polytron mixer. The homogenized sample were centrifuged, the supernatant collected and total protein assessed by Bradford assay. The specific HYAL activity was determined on 50 µl supernatant and expressed as ng HA removed per minute and mg total protein applied.

Statistical analysis

Comparisons between treatment groups and control group were conducted using unpaired *t* test if data were normally distributed, or Mann–Whitney test if data were not normally distributed. Comparison between different groups was analyzed using one-way ANOVA for normally distributed data, with the Tukey's multiple comparisons test. All analyses were done using the Graph Pad Prism statistical program, with a significance level of $p < 0.05$.

Results

The expression of hyaluronidases in normal and degenerated discs

Gene expression of HYAL-1, HYAL-2 and HYAL-3 in human disc tissue was analyzed in relation to the degeneration grade (grades 1 and 2 = normal/mild, grade

3–5 = moderate/severe, as defined by Pfirrmann et al. [41] (Fig. 2). The expression of HYAL-1 significantly increased in moderate/severe disc samples when compared to the normal/mild group ($p = 0.0098$). The expression of HYAL-2 was not significantly affected by degeneration ($p = 0.4167$), although selected donors showed exceptionally high expression levels. Gene expression of HYAL-3 was not significantly different between the grades ($p = 0.834$). Protein expression of HYAL-1 and HYAL-2 in human disc tissue was analyzed as well (Fig. 3a, b). HYAL-2 protein significantly increased in moderate/severe disc samples, when compared to the normal/mild samples ($p = 0.0412$), while HYAL-1 protein was unchanged ($p = 0.769$). Overall HYAL activity tended to increase in the moderate/severe group ($p = 0.1886$) (Fig. 3c).

The effects of inflammation and oxidative stress on the expression of hyaluronidases in disc cells

As shown above, HYAL expression/activity was partially upregulated in discs with higher degeneration grade. Therefore, the influence of main hallmarks of DDD (inflammation and oxidative stress) on the expression of HYALs was tested next. Gene expression of HYAL-1, HYAL-2 and HYAL-3 was analyzed in primary human disc cells treated with increasing concentrations of pro-inflammatory cytokines IL-1β (0.1–10 ng/mL) (Fig. 4a–c) and TNF-α (0.1–100 ng/mL) (Fig. 4d–f). Pro-inflammatory cytokines did not influence gene expression of HYAL-1, HYAL-2 and HYAL-3. Gene expression of HYAL-1, HYAL-2 and HYAL-3 was also tested in primary human disc cells treated with increasing concentrations of oxidative stress inducer H_2O_2 (25–100 µM) (Fig. 5a–c). H_2O_2 at 100 µM dose-dependently upregulated gene expression of HYAL-2 ($p = 0.0445$ to control) and HYAL-3 ($p < 0.05$–25 and 50 µM), while the expression of HYAL-1 only tended to increase ($p = 0.1276$).

Discussion

This study showed for the first time that HYAL-1, HYAL-2 and HYAL-3, the three major HA-degrading enzymes, are expressed in IVD issue and isolated cells. Interestingly, HYAL-1 expression was increased with increasing degeneration, although this was only observed on the mRNA level, but not on the protein level. This may possibly be explained by negative feedback loops related to HA metabolism and/or alternative splicing. It has been shown that under certain conditions (e.g., hypoxia), the HYAL-1 mRNA can undergo alternative splicing (previously identified, e.g., in kidney cells and tumors), generating multiple mRNA species, only one of which is translated into protein [13, 46, 47]. While HYAL-2 gene expression was not significantly increased with degeneration, several severely degenerated samples demonstrated explicitly high HYAL-2 mRNA levels that may have contributed to the significant increase in the protein level with progressing degeneration, as shown in Fig. 3. However, the hyaluronidase activity of HYAL-2 remains controversial and has been described as limited, especially compared to HYAL-1 [18], possibly explaining why its significant protein enhancement with degeneration did not coincide with a significant (but merely tendential) increase in HYAL activity. The majority of our control samples came from patients with traumatic IVD injuries or vertebral fractures. Although our tissue donors are usually operated soon after the traumatic incident, we cannot exclude the possibility that the expression and/or activity of HYALs were altered in some control samples. Nevertheless, our data

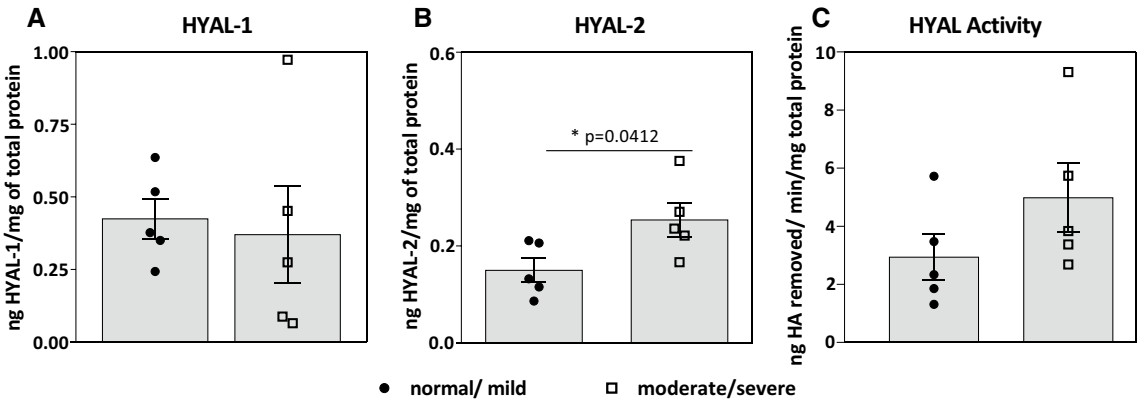

Fig. 3 Protein expression and activity of HYALs in IVD tissue. Disc tissues were collected from patients undergoing elective spinal surgeries. **a** Protein expression of HYAL-1 was not significantly different between the normal/mild disc degeneration group and the moderate/severe group. **b** Protein expression of HYAL-2 was upregulated in moderate/severe samples. **c** Total activity of HYALs tended to increase in the moderate/severe group. Results were calculated as ng of HYAL present (**a**, **b**) or removed (**c**) per mg of total protein and analyzed by unpaired t test due to normality of data ($n = 5$ in each group)

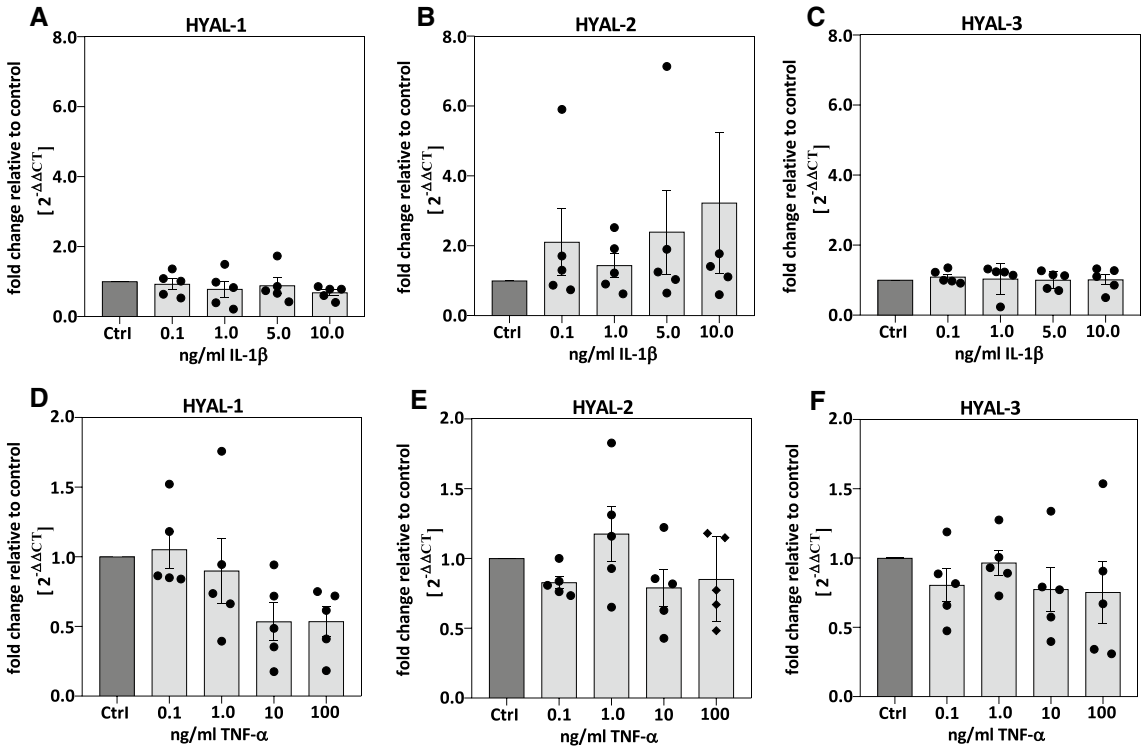

Fig. 4 Gene expression of HYALs in primary IVD cells treated with pro-inflammatory cytokines. Primary cell cultures were prepared from disc tissues collected during spinal surgeries and treated with increasing concentration of IL-1β and TNF-α for 18 h. The effects of IL-1β (0.1–10 ng/mL) on gene expression of **a** HYAL1, **b** HYAL2 and **c** HYAL3. The effects of TNF-α (0.1–100 ng/mL) on gene expression of **d** HYAL1, **e** HYAL2 and **f** HYAL3. Results were calculated by $2^{-\Delta\Delta Ct}$ method relative to the untreated control, with TBP as housekeeping gene and analyzed by one-way ANOVA with Tukey post hoc test due to normality of data ($n=5$)

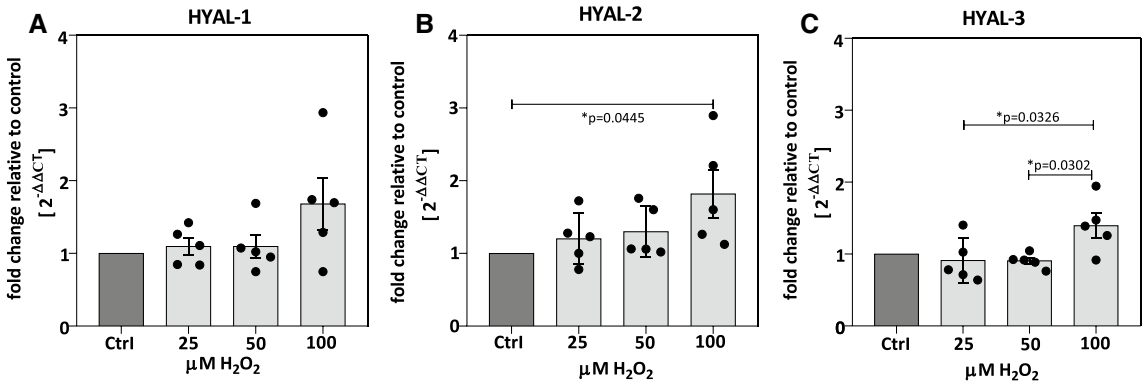

Fig. 5 Gene expression of HYALs in primary disc cells treated with reactive oxygen species. Primary cell cultures were prepared from disc tissues collected during spinal surgeries and treated with increasing concentration of H_2O_2 for 2 h. The effects of H_2O_2 (25–100 µM) on gene expression of **a** HYAL-1, **b** HYAL-2 and **c** HYAL-3. Results were calculated by $2^{-\Delta\Delta Ct}$ method relative to the untreated control, with GAPDH as housekeeping gene and analyzed by one-way ANOVA with Tukey post hoc test due to normality of data ($n=5$)

grouped as traumatic IVDs vs. other samples did not show any pattern that would indicate an effect of the trauma itself.

It should furthermore be noted that the high data variability within the two degeneration groups for all targets indicates that HYAL expression and activity is not only affected by the grade of degeneration, but also by other factors that were not controlled for due to the small sample size. Possible examples include, but are not limited to, diabetic conditions [48], *P. acnes* infection [49] and tissue loading patterns [50, 51]. Higher number of samples will be needed to better identify trends and statistically significant differences in future studies. Furthermore,

future studies would ideally investigate NP and AF cells separately, although our own preliminary data on bovine NP and AF cells indicate marginal zonal differences in HYAL expression.

Increased HYAL expression and/or activity have been described in numerous pathologies, such as during asthma (within the epithelium) [30], cartilage hypertrophy [52] or osteoarthritis and rheumatoid arthritis (within the knee synovium/synoviocytes) [53, 54]. HYAL deficiency or knockdown/knockout have also been described to promote certain pathologies, leading, e.g., to swelling of the periarticular masses [55] as well as to progression of osteoarthritis [56, 57]. These studies demonstrate that HYALs seem to play a crucial role in cartilage homeostasis, which not only shows similar composition [58], but also comparable degenerative processes and pathophysiological conditions [59] as the IVD. As in other tissues, HA in the IVD is cleaved by HYALs at hexosaminidic β (1–4) linkages between glucuronic acid and N-acetylglucosamine. Membrane-anchored HYAL-2 cleaves HMW HA to small MW HA, which is then internalized into lysozomes and further cleaved by HYAL-1 to oligosaccharides, which possess biological (e.g., pro-inflammatory) activity (Fig. 1).

Importantly, pathological degeneration of the IVD and cartilage are both characterized by increased level of inflammatory mediators [31, 32, 60–62] and reactive oxygen species (ROS) [35, 63, 64] within the tissue. Our goal was thus to analyze whether HYAL expression is regulated by exposure to pro-inflammatory cytokines or ROS, as would occur during DDD. However, we could not observe any significant changes in HYAL-1, HYAL-2 or HYAL-3 expression upon stimulation with IL-1β or TNF-α, independent of the concentration (Fig. 4) or the analysis time point (data not shown). In contrast, articular cartilage chondrocytes were previously shown to respond to IL-1β and TNF-α, treatment with an upregulation of HYAL-1, HYAL-2 and HYAL-3 gene expression [29] and similar response patterns were observed in airway epithelial cells [30].

Although we did not observe an upregulation of HYALs upon IL-1β or TNF-α stimulation, IVD cells responded to higher concentrations of H_2O_2 (100 µM) with increased expression of HYAL-2 and HYAL-3 (Figs. 1, 5). Feng et al. [65] summarized that various disc cells derived from different species show mitochondrion-dependent ROS production and that ROS can induce p38, ERKs, JNKs, p65 and Akt in IVD cells, thus leading to increased expression of matrix degrading enzymes and pro-inflammatory cytokines. In this study, we used H_2O_2 for cell stimulation as H_2O_2 has previously been identified in human NP tissues, hence representing a physiological type of ROS [66]. Similar to IVD cells, exposure to ROS (by xanthine oxidase) increased HYAL-2 expression and activity via the p38MAPK signaling pathway in human bronchial epithelial cells [40].

Overall, our and other studies demonstrate that ROS is not only involved in HA metabolism via direct cleavage of HA [11], but also via induction of HYAL expression, which in turn will then contribute to HA degradation. This notion is supported by the fact that exposure of airway cells to ROS leads to sustained HA degradation that lasts for at least 24 h [40]. Furthermore, GAG degradation by HYALs is also associated with increased expression of various MMPs [67], a group of enzymes that is known for its crucial role in ECM degradation in various tissues, including the IVD [68, 69].

In conclusion, our data not only show expression of HYAL-1, HYAL-2 and HYAL-3 in the IVD, but furthermore points to HYAL-2 as the most relevant HYAL as it increased during degeneration and further upregulated by ROS, a hallmark of DDD. Further studies will be needed to confirm the relevance of HYAL-2 in IVD health and disease and to determine its potential as a therapeutic target.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. The authors have full control of all primary data.

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