Inhibiting IL-1 signaling pathways to inhibit catabolic processes in disc degeneration

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Title: Inhibiting IL-1 signalling pathways to inhibit catabolic processes in disc degeneration.

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Abstract:

Intervertebral disc degeneration is characterised by an imbalance between catabolic and anabolic signalling, with an increase in catabolic cytokines particularly IL-1β, a key regulator of IVD degeneration. This study aimed to investigate intracellular signalling pathways activated by IL-1β, and GDF-5 in the degenerate IVD to identify potential new therapeutic targets.

Human NP cells were cultured in alginate beads to regain *in vivo* phenotype prior to stimulation with IL-1β or GDF-5 for 30 minutes, a proteasome profiler array was initially utilised to screen activation status of 46 signalling proteins. Immunoflourescence was used to investigate activation of the NFκB pathway. Cell based ELISAs were then deployed to confirm results for ERK1/2, p38 MAPK, c-jun and IκB signalling. IHC was utilised to investigate native activation status within human IVD tissue between grades of degeneration. Finally cells were stimulated with IL-1β in the absence or presence of p38 MAPK, c-jun, JNK and NFκB inhibitors to investigate effects on MMP3, MMP13, IL-1β, IL-6 and IL-8 mRNA expression.

This study demonstrated three key signalling pathways which were differentially activated by IL-1β but not GDF-5; namely p38 MAPK, c-jun and NFκB. Whilst ERK 1/2 was activated by both GDF-5 and IL-1**.** Immunohistochemistry demonstrated p38 MAPK, c-jun and NFκB were activated during human IVD degeneration and inhibition of these pathways reduced or abrogated the catabolic effects of IL-1β, with inhibition of NFκB signalling demonstrating most widespread inhibition of IL-1β catabolic effects.

Introduction:

The intervertebral disc (IVD) is an important component of the spinal column enabling bending, flexion and torsion of the spine, composed of the cartilaginous end plates (CEP), the annulus fibrosus (AF) and the central gelatinous nucleus pulposus (NP). The major components are water, collagens, proteoglycans, non-collagenous proteins and elastins. The collagen fibres of the IVD provide a strong durable framework supporting the cells and confine the highly hydrated proteoglycan aggregates[1]. The normal human IVD contains chondrocyte-like cells within the NP and inner AF termed NP and AF cells, and fibroblast-like cells in the outer AF. These cells are responsible for the synthesis and regulation of the matrix in the disc and control the homeostasis between extracellular matrix (ECM) synthesis and ECM degradation[2]. Within the normal adult IVD the anabolic and catabolic processes which regulate the ECM are thought to be kept in balance by a number of growth factors (e.g. TGF, FGF, IGF, GDF) and cytokines (e.g. IL-1, IL-6, IL-8, TNFα) [3-5]. If this balance is disrupted in favour of catabolic processes, the IVD begins to degrade, resulting in disc degeneration which can lead to low back pain (LBP)[3]. An improved understanding of the homeostatic mechanisms and those seen during degeneration of the disc are crucial to understanding the biology of this important tissue.

Increasing data suggests that inflammatory cytokines produced by NP cells within the IVD during degeneration are responsible for matrix degradation and induction of painful stimuli[6-12], with a particular role for IL-1β hypothesized[6, 7, 9-11]. IL-1β has been shown to be increased in degenerate IVDs without a concordant increase in its antagonist (IL-1Ra)[6]. IL-1β has also been shown to regulate a pleathora of events linked to IVD degeneration such as increased matrix degradation, decreased matrix synthesis, increased pro inflammatory cytokine production and increased production of neurotrophic and angiogenic factors[6-10]. Spontaneous degeneration was seen in a mouse model where the natural inhibitor of IL-1β (IL-1Ra) was knocked out[11], which together with increased risks of LBP in patients with IL-1β polymorphisms[13-16], suggests a major role for IL-1β in the pathogenesis of disc degeneration.

A number of methods to inhibit the catabolic processes (e.g. matrix degradation via TIMPs) have been proposed, however whilst these have shown some promise, many target individual catabolic cytokines (e.g. IL-1, TNF), which carries a major shortcoming as many cytokines and other catabolic factors (e.g. NO, PGEs) are produced within a degenerate disc and other cytokines may replace the degenerate effects if one such as IL-1β is removed[3, 5].

One alternative would be to inhibit the intracellular signalling mechanisms common to catabolic processes, however many of these mechanisms are also deployed by the anabolic factors including growth factors within the disc. Anabolic factors have been proposed as alternative therapeutic strategies to adjust the balance towards matrix synthesis and of these GDF-5/6 (CDMP-1/2) appear to be an attractive growth factor[17-20] as its receptors are expressed by NP cells even in degenerate discs however unlike the majority of the growth factors which have been investigated are not expressed by the blood vessels which are found in degenerate discs[21]. It is important to target anabolic factors which do not have off target effects, such as potential detrimental angiogenesis and accompanied nerve ingrowth which could result from the use of growth factors whose receptors are also expressed by the blood vessels in the IVD.

Thus identification of intracellular signalling pathways activated by catabolic factors but not anabolic factors is essential to identify potential targets to inhibit degeneration without inhibiting anabolic processes. Recently p38 MAPK (Mitogen activated protein kinase)[22, 23] and NFκB (Nuclear factor kappa beta)[24-27] have been identified within IVD cells, as signal transduction pathways following IL-1β stimulation and inhibition of these pathways have shown partial inhibition of the catabolic mediators induced in response to IL-1β[22, 23]. However importantly these studies were investigated using IVD cells cultured in monolayer culture which do not represent the phenotype of NP cells *in vivo*, indeed very limited studies have investigated signalling molecule activation in chondrocyte like cells in a 3D culture system which more closely mimics the *in vivo* phenotype.

This study aimed to identify intracellular signalling pathways differentially expressed between catabolic (IL-1β) and anabolic (GDF-5) factors in human IVDs and determined the activation status of these pathways in native IVD tissues highlighting potential pathways for new therapies.

Materials and Methods:

Human IVD samples:

Human IVD tissue was obtained from surgery for micro discectomy or post mortem examination (processed within 72 hours after death) with informed consent of the patient or relatives. Ethical approval was obtained from Sheffield Research Ethics Committee (09/H1308/70).

Histological Grading:

Representative tissue samples were fixed in 10% v/v neutral buffered formalin (Leica, Milton Keynes, UK) and processed to paraffin wax. Haematoxylin and eosin stained sections were evaluated independently by two researchers (AB & CLM) to determine the extent of degenerative tissue changes. Sections were scored numerically between 0 and 12 based on the presence of cell clusters, fissures, loss of demarcation and haematoxophilia; a score of 0 to 3 indicates histologically non-degenerate IVDs; ≥4 indicates evidence of degeneration, as described previously[6].

NP cell isolation and culture:

Surgical NP tissue samples were finely minced and digested with 2U/ml protease (Sigma, Poole, UK) in DMEM (Gibco, Paisley, UK) for 30 minutes at 37°C, followed by 2mg/mL collagenase type I (Sigma) in DMEM for 4 hours at 37°C. Cells were maintained in DMEM supplemented with 10% heat inactivated foetal bovine serum (Gibco), 200U/mL penicillin/200µg/mL streptomycin (Gibco), 500ng/mL amphotericin B (Sigma), 2mM L-glutamine (Gibco) and 50µg/mL ascorbic acid (Sigma) (complete media). NP cultures were used for experimental purposes upto and including passage 2, note no NP samples which displayed histological evidence of infiltration were used for culture experiments, all cultures were completed with NP cells from individual patients no pooling of patient samples was performed. Cultures were maintained at all times at 37°C and 5% CO_2 in a humidified environment.

Alginate Culture and stimulation regimes

Following expansion in monolayer, NP cells were re-suspended in 1.2%w/v medium viscosity alginic acid (Sigma) in 0.15M sodium chloride (Fisher Scientific, Loughborough, UK) at a density of 4.0 x 10^6 cells/ml (which mimics native NP cell density). Alginate beads were formed via extrusion through a 19 gauge needle into 200mM $CaCl_2$ (Fisher Scientific), washed in 0.15M NaCl and media and maintained in complete media for 14 days prior to IL-1β or GDF-5 stimulation with culture media changed twice weekly. To investigate signalling molecule activation all treatments were performed with either 10ng/ml recombinant IL-1β (Peprotech, London, UK), 10ng/ml recombinant GDF-5 (Peprotech) or left untreated as controls. Stimulations were performed for 30 minutes prior to downstream analysis (30 minutes was selected from a preliminary time course experiment where 10,20,30,60 and 180 mins were investigated for NFkB activation (data not shown for time course)). Following stimulation alginate beads were dissolved in alginate dissolving buffer (55mM $C_6H_6Na_2O_7$ (Sigma); 30mM EDTA (Fisher); 0.15M NaCl; pH 6.0) for 10 minutes at 37°C to release cells for downstream analysis with arrays, immunofluorescence or in cell ELISAs.

R&D Proteome Array for intracellular signalling molecules:

NP samples derived from 2 patients (Grade 6, age 21 and 36) were used for this component of the study (Table S-1). Forty alginate beads were used for each treatment in duplicate. Following treatment and release from beads, cells were lysed with cell lysis solution containing protease inhibitors, and protein quantified, activation of 46 signalling molecules investigated using the Human Phospho-Kinase Proteome profiler array as per manufactures instructions (Cat: ARY003; R&D systems, Abingdon, UK).

NFκB Immunocytochemistry

NP samples derived from 3 patients (Age 29,32,36; Grade 6,7,10) were used for this study (Table S-1) were treated for 30 mins with 10ng/ml IL-1β, 10ng/ml GDF-5 or left untreated as controls. Following treatment and release from beads cell pellets were fixed in 4% w/v paraformaldehyde/PBS and cytospins formed spinning 300,000 cells per slide via 1500rpm 30 min cytospin (Shandon cytospin). Cells were washed in tris buffered saline (TBS) for 5 minutes. Non-specific binding sites blocked with 25% v/v goat serum (Abcam) in 1% w/v BSA in TBS for 1 hour at room temperature. Cells were incubated with rabbit polyclonal antibody against p65 NFκB (1/50) (Abcam) overnight at 4°C. Cells were washed 3 times for 5 minutes each in 0.1% v/v Tween 20/TBS for 5 minutes, cells were then incubated with FITC conjugated goat anti-rabbit IgG (1/250) (Abcam) at room temperature for 30 minutes. Cells were washed 3x5 minutes in 0.1% v/v Tween 20/TBS, mounted in 90% v/v glycerol/TBS and coverslips sealed with nail varnish. Cells were visualised and images captured on the Zeiss laser scanning confocal microscope at a magnification of 630X magnification using the Zen 2009 operating system.

Cell Based ELISAs: ERK1/2, p38 MAPK, IkB, c-Jun:

To confirm results from R&D proteome array on additional patients on key signalling molecules identified to be upregulated by IL-1β cell based ELISAs were utilised to investigate quantitative expression of the signalling molecules identified from the array (ERK 1/2, p38 MAPK, c-jun) together with investigating activation of the NFκB signalling pathway which is not included in the array, but demonstrated to be potentially differentially activated via immunofluorescence. This was investigated by determining levels of phosphorylated IκB which is the first stage in the activation of this signalling pathway. The cell based ELISAs enable quantification of total and phosphorylated proteins in cells *in situ* without the need to perform protein extraction. A further 3 patients were utilised for this component of the study (Age 29,32,36; Grade 6,7,10) (Table S-1), Alginate beads were transferred 1 bead per well of four black 96 well microplates (R&D Systems) in complete media (separate plates were used for detection of each signalling molecule). Following stimulations beads were solubilised in alginate dissolving buffer and plates centrifuged for 10 mins 400g to deposit cells on the base of the culture wells, and fixed in 4% v/v formalin/PBS. The amount of phosphorylated ERK1/2, p38 MAPK, c-Jun or IκB was measured using cell based ELISA as per manufactures instructions (R&D systems, UK). The plate was read fluorometrically using the Tecan Infinite 200 Pro with excitation at 540 nm and emission at 600 nm, then with excitation at 360 nm and emission at 450 nm. Expression of phosphorylated protein was then assessed between treatment groups.

Immunohistochemical identification of activation status *in vivo*

Immunohistochemistry (IHC) was utilised to identify expression levels of phosphorylated-p38 MAPK, phosphorylated-c-jun and phosphorylated-NFκB in native NP cells from different grades of degeneration to investigate activation status *in vivo* and whether this altered during degeneration. IHC was utilised rather than western blotting to ensure expression was quantified only within the native NP cells not any infiltrating cells, in addition IHC has the advantage that multiple patient samples can be investigated. Expression of each phosphorylated signalling molecule was investigated in 30 IVD tissue samples (Age: 45.5±14.4) (Table S-1), divided between three study groups: non-degenerate; histologically graded as intermediate degeneration and histologically graded as severe degeneration. Briefly, 4μm paraffin sections were dewaxed, rehydrated and endogenous peroxidase blocked using hydrogen peroxide. After washing in TBS, sections were subjected to heat induced antigen retrieval (10 minute microwave irradiation in 0.05M tris buffer, pH 9.5). Following TBS washing, non-specific binding sites were blocked at room temperature (RT) for 2hrs with 10%v/v normal goat serum in 1%w/v BSA/TBS. Sections were incubated overnight at 4°C with rabbit polyclonal primary antibodies against human phosphorylated-p38 MAPK (1:800;Abcam:ab4822), phosphorylated-c-jun (1:400;Abcam:ab32385) and phosphorylated-NFκB (1:100;Abcam:ab31481). Negative controls in which rabbit IgGs (Abcam) replaced the primary antibody at an equal protein concentration were used. Following TBS washes, sections were incubated in biotinylated goat anti-rabbit antiserum (1:300;Abcam) for 30 minutes at RT. Disclosure of secondary antibody binding was by HRP-streptavidin-biotin complex (Vector Laboratories, Peterborough, UK) with 0.08% v/v hydrogen peroxide in 0.65 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in TBS. Sections were counterstained with Mayer's Haematoxylin (Leica), dehydrated, cleared and mounted in Pertex (Leica).

All slides were visualised using an Olympus BX60 microscope and images captured using a digital camera and software program QCapture Pro v8.0 (MediaCybernetics, Marlow, UK). Evaluation of IHC was performed by counting 200 NP cells, with immunopositive cells expressed as a percentage of total count.

Stimulation of NP cells in alginate with IL-1β in presence of signalling inhibitors:

NP samples derived from 3 patients (Age 29,32,36; Grade 6,7,10) (Table S-1), 2 alginate beads per well were treated with DMSO at equivalent concentrations as those used for signalling inhibitors or with signalling inhibitors (p38MAPK inhibitor: 40µM SB203580[28] (Sigma, Poole, UK); c-jun inhibitor: 800µM c-jun peptide[29] (Tocris cat: 1989); JNK inhibitor: 20µm SP600125[30] (Sigma); or NFκB inhibitor: 10µM Helenalin[31] (ENZO, Exeter, UK)) for 1 hr prior to stimulation with 10ng/ml IL-1β for 48hrs or left unstimulated to act as signalling inhibitor only controls. All treatments were performed in triplicate on each patient independently.

RNA extraction, cDNA synthesis and Real time PCR:

Following stimulation NP cells were recovered from alginate culture and re-suspended in 0.06%w/v type I collagenase and incubated for 10 minutes at 37°C and cells recovered by centrifugation. RNA was extracted using Qiagen RNeasy Mini kit (Qiagen, Crawley, UK) as per manufacturers' protocol. cDNA was reverse transcribed using Moloney Murine Leukaemia Virus reverse transcriptase (Bioline) and random hexamers (Applied Biosystems, Warrington, UK). cDNA samples were interrogated by qRT-PCR for the expression of MMP3, MMP13, IL-1β, IL-6, IL-8 and aggrecan (Pre-designed primer/probe mixes Applied Biosystems). Plates were ran on a StepOnePlus real-time PCR machine for 50 cycles (Applied Biosystems). Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalised against two internal reference genes (GAPDH and 18S) and un-stimulated control samples. Stable expression of internal reference genes was confirmed by geNorm algorithm.

Statistical Analysis

All data from this study was shown to be non-parametric and as such statistical analysis for assays was performed using the Kruskal-Wallis test with Conover-Ingman post hoc tests used to investigate significant differences between treatment groups or grades of degeneration. All Statistical analysis was performed using statistical software: Stats-Direct.

Results:

Identifying differential signalling pathways between anabolic and catabolic factors:

Proteome profiler arrays were deployed to enable screening of the phosphorylation status of 46 intracellular signalling proteins following stimulation of human NP cells with either IL-1β or GDF-5 for 30 minutes which was shown from preliminary experiments to be the optimum time frame. Significant increases in phosphorylated forms of 3 signalling proteins were seen in cells following stimulation with IL-1β (c-jun, ERK1/2 and p38)(Figure 1, Table 1) and 2 following stimulation with GDF-5 (STAT 1 and 4)($P<0.05$)(Table 1). Interestingly IL-1β stimulation resulted in the significant down regulation of 12 signalling proteins: including STATs (STAT 3, 5A/B and 6); src family members (src, YES, Fyn, Hck, Lck); and other signalling proteins involved in focal adhesions (FAK, Paxillin)(Table 1). In addition decreased activation of MEK1/2 and mTOR was observed ($P<0.05$)(Table 1). Whilst GDF-5 only significantly inhibited 1 signalling protein (Lck) ($P<0.05$)(Table 1). Of particular interest pERK1/2 was increased following GDF-5 (6 fold) and IL-1β (30 fold) stimulation although only significantly so following IL-1β ($P<0.05$) (Table 1, Figure 1), whilst FAK was down regulated by both GDF-5 and IL-1β although again only significantly so following IL-1β stimulation ($P<0.05$) (Table 1, Figure 1). Surprisingly from the 46 signalling proteins investigated only three were shown to be differentially activated by IL-1β but not GDF-5, these were p38 MAPK, JNK and its downstream target c-jun ($P<0.05$) (Table 1, Figure 1). Caution should be taken when utilising these results as they were completed on 2 patients each in quadruplet, thus the biological significance cannot be concluded from this component of the study. Hence this component of the study was utilised to identify potential signalling pathways for further investigation in additional patient samples.

As the proteome profiler array did not include NFκB signalling this was investigated initially using immunofluorescence using a phosphorylated antibody which would only detect activated NFκB. NP cells cultured in alginate to maintain NP cell phenotype were stimulated with IL-1β or GDF-5. Cells which were unstimulated or stimulated with GDF-5 showed punctate staining only, whilst cells stimulated with IL-1β showed increased staining across the cell, including nuclear staining, suggesting activation of the NFκB pathway was more pronounced with IL-1 β stimulation (Figure 2).

Cell based ELISAs were then utilised to confirm the results from profiler and immunofluorescence on further patient samples. Phosphorylated ERK 1/2 was significantly increased following GDF-5 and IL-1β stimulation although induction was significantly more following IL-1β stimulation ($P<0.05$)(Figure 3A). GDF-5 stimulation had no stimulatory effect on the phosphorylation status of p38 MAPK, c-jun or IκB, although a significant decrease was seen in phosphorylated c-jun ($P<0.05$)(Figure 3), however IL-1β stimulation induced phosphorylation of p38 MAPK, c-jun and IκB, which was significant for p38 MAPK and c-jun ($P<0.05$)(Figure 3B,C,D).

Activation status of IL-1β induced intracellular signalling pathways *in vivo.*

To determine whether IL-1β induced signalling pathways: p38 MAPK, c-jun and NFκB are active within human IVDs and whether these are differentially activated within degenerate discs, human IVDs from differing grades of histological degeneration were investigated for IHC staining with phosphor specific antibodies. Cytoplasmic staining for phospho-p38 MAPK, c-jun and NFκB were identified in NP cells, particularly those cells in clusters (Figure 4). Percentage immunopositive cells for phosphorylated p38 MAPK and phosphorylated NFκB were significantly increased in discs with intermediate histological degeneration compared to histologically non-degenerate discs ($P<0.05$)(Figure 4). Whilst percentage immunopositive cells for phosphorylated c-jun was significantly higher in both high grade and intermediate grades of histological degeneration compared to histologically non degenerate discs ($P<0.05$)(Figure 4).

Effects of inhibitors of intracellular signalling on native mRNA expression in NP cells

Inhibition of p38 MAPK signalling decreased mRNA expression of MMP3, MMP13, IL-1β and IL-8 but not IL-6 in cells not stimulated with IL-1β although this only reached significance for MMP3 and IL-1β ($P<0.05$)(Figure 5). Inhibition of JNK or its downstream target c-jun in the absence of IL-1β resulted in significant decrease in native mRNA expression of IL-1β ($P<0.05$)(Figure 5), whilst only c-jun inhibition decreased native expression of MMP3, and JNK inhibition decreased native expression of IL-8 ($P<0.05$)(Figure 5), conversely native IL-6 mRNA expression was increased by inhibition of JNK or c-jun ($P<0.05$)(Figure 5). Inhibition of NFκB signalling significantly decreased mRNA expression of MMP13 ($P<0.05$)(Figure 5) in the absence of IL-1.

Inhibition of IL-1β actions via inhibition of intracellular signalling.

Real time PCR was utilised to investigate effects of signalling inhibitors on IL-1β actions. IL-1β stimulation of NP cells significantly increased mRNA expression of MMP3, MMP13, IL-1β, IL-6 and IL-8 (P<0.05)(Figure 5).

IL-1β stimulation of NP cells in the presence of a p38 MAPK inhibitor induced MMP3, IL-6 and IL-8 mRNA although to a lesser extent than IL-1β alone (P<0.05)(Figure 5), whilst p38 MAPK inhibition prevented stimulation of MMP13 mRNA by IL-1β but had no effect on the induction of IL-1β mRNA by IL-1β (Figure 5).

IL-1β stimulation of NP cells in the presence of a c-jun inhibitor induced MMP3, MMP13, and IL-1β mRNA although to a lesser extent than IL-1β alone (P<0.05)(Figure 5), whilst c-jun inhibition had no effect on IL-6 or IL-8 mRNA induction by IL-1β (Figure 5). IL-1β stimulation of NP cells in the presence of a JNK inhibitor induced MMP3, IL-1β and IL-8 mRNA although to a lesser extent than IL-1β alone (P<0.05)(Figure 5), whilst JNK inhibition had no effect on MMP13 or IL-6 mRNA induction by IL-1β (Figure 5).

IL-1β stimulation of NP cells in the presence of an inhibitor of NFκB signalling inhibited the IL-1β induced mRNA expression of MMP3, MMP13, IL-1β, IL-6 and IL-8 mRNA expression (P<0.05)(Figure 5).

Effect of signalling inhibitors on aggrecan mRNA expression.

In order to determine the effect of signalling inhibitors on normal anabolic mRNA expression the expression of aggrecan was investigated following inhibition of signalling factors. Inhibition of JNK or c-jun had no effect on mRNA expression of aggrecan, however inhibition of p38 MAPK or NFκB resulted in down regulation of mRNA for aggrecan although this failed to reach significance (P>0.05)(Figure S-1).

Discussion

This study aimed to investigate the intracellular signalling pathways activated by IL-1β in the degenerate IVD which were not activated by the anabolic factor GDF-5 to identify potential targets for new therapeutic strategies. It is important to identify signalling pathways which are not activated by anabolic factors such as GDF-5 to ensure native growth factors are not inhibited, whilst inhibiting catabolic processes.

Initial studies utilised the proteasome arrays to screen 46 signalling molecules to investigate activation of a wide range of signalling molecules and determine if these signalling molecules were differentially induced by IL-1β and not by the anabolic factor GDF-5. Interestingly, only a limited number of signalling molecules were activated by IL-1β in these cells and of these ERK1/2 was also induced by GDF-5, whilst p38, JNK and c-jun were induced significantly more by IL-1β than GDF-5, whilst this component of the study was completed on two patients. These results were further confirmed in additional patients using cell based ELISAs. IL-1β has been previously shown to induce expression of phosphorylated-ERK, p38 and JNK in human IVD cells in monolayer culture[32], and together with NFκB signalling in rat NP cells[33]. Furthermore as NFκB signalling was not included within the array, but previous studies have demonstrated this is a key signalling pathway activated by IL-1β in IVD cells[24-27], this was investigated within human NP cells cultured in alginate to maintain phenotype which demonstrated activation by IL-1β but not GDF-5, which was demonstrated using immunofluorescence and confirmed by cell based ELISAs. Unfortunately performing these experimental procedures in alginate beads to maintain the phenotype of the NP cells, alginate was shown to have high auto-fluorescence within immunofluorescence experiments which prevented quantification of this data, however the use of cell based ELISAs confirmed activation of the NFκB signalling pathway.

GDF-5 stimulation resulted in activation of STAT 1 and 4 which has not been previously demonstrated, however STAT 1 has been shown to be involved in the signalling of other growth factors in chondrocytes, and has been shown to be a key signalling molecule in FGF signalling[34], and IGFBP-3 signalling[35].

Interestingly IL-1β stimulation resulted in the down regulation of 12 signalling proteins: including STATs (STAT 3, 5A/B and 6), STAT3 has been shown to be expressed by human NP cells previously[36], STAT3 is associated with IL-6 signalling in chondrocytes where it has been shown to induce expression of cartilage matrix genes[37]. These anabolic effects of IL-6 were inhibited by STAT3 knockdown[37]. Thus the down regulation of STAT3 by IL-1β seen in the current study could be linked to the effects of IL-1 on matrix gene expression, however its role in the IVD is poorly understood and requires further investigation.

The combined down regulation of the src family proteins, FAK and Paxillin demonstrate a potential role for IL-1β in the disruption of focal adhesion signalling, src family protein kinases phosphorylate FAK and if these are inhibited integrin regulated adhesion to the extracellular matrix is disrupted[38]. Thus the inhibition of src family, FAK and paxillin signalling could explain the altered integrin signalling seen during disc degeneration[39].

mTOR is a key signalling pathway involved in autophagy, inhibition of the mTOR complex has been demonstrated to induce autophagy[40], thus the inhibition of mTOR shown here by IL-1β could provide a role for IL-1β induced autophagy in the IVD[40]. Additionally mTOR is involved in regulation of cellular senescence and inhibitors of mTOR have been shown to induce cellular senescence[40], which is also a key feature of disc degeneration[41]. As such the modulation of these signalling pathways by IL-1β requires further investigation particularly whether abrogating the inhibition can be targeted by potential therapies.

It should be noted that activation of these signalling molecules reported within this study were completed following 30 minutes, which was shown to be optimal from initial preliminary studies, it was not possible to complete all studies at multiple time points but it should be considered that as arrays investigated a wide range of signalling molecules further factors may be activated by these factors at alternative times, however the majority of studies investigating signalling factors do so over short time periods such as 30 mins.

Following the identification of p38, c-jun and NFκB signalling as potentially differentially induced by IL-1β, expression of phosphorylated forms of these proteins was determined in native human IVDs, which demonstrated all three signalling proteins were active within native NP cells with increased expression of phosphorylated-p38, c-jun and NFκB in discs with intermediate grades of histological degeneration compared to those discs graded as non-degenerate, whilst c-jun was also increased in discs with severe histological degeneration. Interestingly the discs with highest levels of expression are also those which have previously been shown to express highest levels of IL-1β i.e. discs with intermediate grades of degeneration[6]. Tolonen *et al.,* previously investigated protein expression of c-jun in human IVD samples and demonstrated expression in 74% of patient samples investigated[42], unfortunately it is not clear whether they utilised a phospho-specific antibody and they did not investigate whether expression altered during degeneration, however in agreement with the current study expression was seen within NP cell clusters[42]. Interestingly in stab induced degeneration in rat IVDs an induction of p-ERK and p-p38 was observed associated with increased immunopositivitiy for IL-1β and IL-6, in contrast no increase in p-JNK was observed[43]. Nerlich *et al.,* also demonstrated increased activation of NFκB signalling in human IVDs which increased with age and grade of degeneration[44]. Increased NFκB signalling with degeneration has been further confirmed recently[45]. Together this data supports the potential role of inhibitors of these signalling pathways in IVD degeneration.

Thus, this study went on to investigate the potential use of inhibitors of these signalling pathways within human NP cells derived from degenerate discs on a number of key targets which have been shown previously to be regulated by IL-1β. Interestingly inhibitors of p38 signalling and the JNK/c-jun pathway only partially abrogated effects of IL-1β suggesting these factors alone may not be sufficient to inhibit the plethora of effects induced by IL-1β (Figure S-2). Klawitter *et al.,* demonstrated partial inhibition of IL-1β effects via curcuma and curcumin with inhibition of IL-1β induced expression of MMPs and IL-6 but failed to inhibit effects on IL-1β, IL-8 and TNF expression, these effects were shown to be a result of JNK inhibition[32], whilst other studies have suggested curcumins actions are via inhibition of NFκB signalling[25, 46, 47]. Yu *et al.,* demonstrated abrogation of IL-1 induced inhibition of Sox9 and collagen type II expression by curcumin[46]. Further to this, Ma *et al.,* recently demonstrated curcumin could reduce degenerative changes in a rat degeneration model[25], both studies associating actions to inhibition of the NFκB pathway. Inhibition of p38 signalling in rabbit NP cells abrogated the effects of IL-1 on inhibition of matrix synthesis and partly reduced the effects on expression of Cox-2, MMP3, IL-1 and IL-6[23] and PGE_2[22] which agrees with the current study.

However inhibition of the NFκB signalling pathway demonstrated the most potential in the current study with abrogation of all the effects of IL-1β investigated. Wang *et al.,* also found that NFκB inhibition could completely abrogate IL-1β induced MMP3, whilst inhibition of p38/ERK/JNK only partially abrogated the effects of IL-1β[28]. In contrast IL-1β induced expression of ADAMTS4 could be blocked by inhibitors of NFκB, p38, JNK and ERK1 signalling[33], recently Sun *et al.,* further demonstrated the important role for NFκB signalling in the IL-1β induced expression of ADAMTS[24, 45]. Inhibition of NFκB signalling has been shown to abrogate a wide range of IL-1 induced effects, demonstrating the potential of this signalling target for inhibiting the plethora of IL-1 effects in the IVD[26]. The potential role of NFκB as a target for treating disc degeneration is further supported by the finding that NFκB signalling was increased in an accelerated aging mouse model, and catabolic effects could be blocked by inhibiting NFκB[48]. However, the current study demonstrated inhibition of the NFκB signalling did induce a baseline decrease in the expression of aggrecan mRNA expression, which although failed to reach significance should be treated with caution.

Conclusion

This study has highlighted three key pathways were identified which were differentially activated by IL-1β but not GDF-5; namely p38, c-jun and NFκB. All pathways were shown to be activated during human IVD degeneration and inhibition of these pathways reduced or abrogated the catabolic effects of IL-1β, with inhibition of NFκB signalling demonstrating more widespread inhibition of IL-1 effects and thus potentially holds most promise for future investigations (Figure S-2).

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References

1. Mwale F, Roughley P, Antoniou J. 2004. Distinction between the extracellular matrix of the nucleus pulposus and hyaline cartilage: A requisite for tissue engineering of intervertebral disc. Eur.Cell Mater. 8: 58-64.

2. Roughley PJ. 2004. Biology of intervertebral disc aging and degeneration: Involvement of the extracellular matrix. Spine 29: 2691-2699.

3. Le Maitre CL, Binch AL, Thorpe AA, Hughes SP. 2015. Degeneration of the intervertebral disc with new approaches for treating low back pain. J Neurosurg Sci 59: 47-61.

4. Le Maitre CL, Pockert A, Buttle DJ, Freemont AJ, Hoyland JA. 2007. Matrix synthesis and degradation in human intervertebral disc degeneration. Biochem Soc Trans 35: 652-655.

5. Risbud MV, Shapiro IM. 2014. Role of cytokines in intervertebral disc degeneration: Pain and disc content. Nat Rev Rheumatol 10: 44-56.

6. Le Maitre CL, Freemont AJ, Hoyland JA. 2005. The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. Arthritis Res Ther. 7: R732-R745.

7. Hoyland JA, Le MC, Freemont AJ. 2008. Investigation of the role of IL-1 and TNF in matrix degradation in the intervertebral disc. Rheumatology (Oxford) 47: 809-814.

8. Binch A, Cole AA, Breakwell LM, et al. 2014. Expression and regulation of neurotrophic and angiogenic factors during human intervertebral disc degeneration. Arthritis Res Ther 16: 416.

9. Phillips KL, Cullen K, Chiverton N, et al. 2015. Potential roles of cytokines and chemokines in human intervertebral disc degeneration: Interleukin-1 is a master regulator of catabolic processes. Osteoarthritis Cartilage .

10. Phillips KL, Chiverton N, Michael AL, et al. 2013. The cytokine and chemokine expression profile of nucleus pulposus cells: Implications for degeneration and regeneration of the intervertebral disc. Arthritis Res Ther 15: R213.

11. Phillips KL, Jordan-Mahy N, Nicklin MJ, Le Maitre CL. 2013. Interleukin-1 receptor antagonist deficient mice provide insights into pathogenesis of human intervertebral disc degeneration. Ann Rheum Dis 72: 1860-1867.

12. Bachmeier BE, Nerlich AG, Weiler C, et al. 2007. Analysis of tissue distribution of TNF-alpha, TNF-alpha-receptors, and the activating TNF-alpha-converting enzyme suggests activation of the TNF-alpha system in the aging intervertebral disc. Ann N Y Acad Sci 1096: 44-54.

13. Solovieva S, Lohiniva J, Leino-Arjas P, et al. 2005. Intervertebral disc degeneration in relation to the COL9A3 and the IL-1ss gene polymorphisms. Eur Spine J .

14. Karppinen J, Solovieva S, Luoma K, et al. 2009. Modic changes and interleukin 1 gene locus polymorphisms in occupational cohort of middle-aged men. Eur Spine J 18: 1963-1970.

15. Solovieva S, Kouhia S, Leino-Arjas P, et al. 2004. Interleukin 1 polymorphisms and intervertebral disc degeneration. Epidemiology 15: 626-633.

16. Paz Aparicio J, Fernandez Bances I, Lopez-Anglada Fernandez E, et al. 2011. The IL-1beta (+3953 T/C) gene polymorphism associates to symptomatic lumbar disc herniation. Eur Spine J 20 Suppl 3: 383-389.

17. Chujo T, An HS, Akeda K, et al. 2006. Effects of growth differentiation factor-5 on the intervertebral disc--in vitro bovine study and in vivo rabbit disc degeneration model study. Spine 31: 2909-2917.

18. Liang H, Ma SY, Feng G, Shen FH, Joshua Li X. 2010. Therapeutic effects of adenovirus-mediated growth and differentiation factor-5 in a mice disc degeneration model induced by annulus needle puncture. Spine J 10: 32-41.

19. Le Maitre CL, Freemont AJ, Hoyland JA. 2009. Expression of cartilage-derived morphogenetic protein in human intervertebral discs and its effect on matrix synthesis in degenerate human nucleus pulposus cells. Arthritis Res.Ther. 11: R137.

20. Clarke LE, McConnell JC, Sherratt MJ, et al. 2014. Growth differentiation factor 6 and transforming growth factor-beta differentially mediate mesenchymal stem cell differentiation, composition, and micromechanical properties of nucleus pulposus constructs. Arthritis Res Ther 16: R67.

21. Le Maitre CL, Richardson SM, Baird P, Freemont AJ, Hoyland JA. 2005. Expression of receptors for putative anabolic growth factors in human intervertebral disc: Implications for repair and regeneration of the disc. J Pathol 207: 445-452.

22. Studer RK, Aboka AM, Gilbertson LG, et al. 2007. p38 MAPK inhibition in nucleus pulposus cells: A potential target for treating intervertebral disc degeneration. Spine 32: 2827-2833.

23. Studer RK, Gilbertson LG, Georgescu H, et al. 2008. p38 MAPK inhibition modulates rabbit nucleus pulposus cell response to IL-1. J Orthop Res 26: 991-998.

24. Sun Z, Yin Z, Liu C, et al. 2015. IL-1beta promotes ADAMTS enzyme-mediated aggrecan degradation through NF-kappaB in human intervertebral disc. J Orthop Surg Res 10: 159-015-0296-3.

25. Ma T, Guo CJ, Zhao X, et al. 2015. The effect of curcumin on NF-kappaB expression in rat with lumbar intervertebral disc degeneration. Eur Rev Med Pharmacol Sci 19: 1305-1314.

26. Zhongyi S, Sai Z, Chao L, Jiwei T. 2015. Effects of nuclear factor kappa B signaling pathway in human intervertebral disc degeneration. Spine (Phila Pa 1976) 40: 224-232.

27. Wuertz K, Vo N, Kletsas D, Boos N. 2012. Inflammatory and catabolic signalling in intervertebral discs: The roles of NF-kappaB and MAP kinases. Eur Cell Mater 23: 103-19; discussion 119-20.

28. Wang X, Wang H, Yang H, et al. 2014. Tumor necrosis factor-alpha- and interleukin-1beta-dependent matrix metalloproteinase-3 expression in nucleus pulposus cells requires cooperative signaling via syndecan 4 and mitogen-activated protein kinase-NF-kappaB axis: Implications in inflammatory disc disease. Am J Pathol 184: 2560-2572.

29. Holzberg D, Knight CG, Dittrich-Breiholz O, et al. 2003. Disruption of the c-JUN-JNK complex by a cell-permeable peptide containing the c-JUN delta domain induces apoptosis and affects a distinct set of interleukin-1-induced inflammatory genes. J Biol Chem 278: 40213-40223.

30. Seguin CA, Bojarski M, Pilliar RM, Roughley PJ, Kandel RA. 2006. Differential regulation of matrix degrading enzymes in a TNFalpha-induced model of nucleus pulposus tissue degeneration. Matrix Biol 25: 409-418.

31. Lyss G, Knorre A, Schmidt TJ, Pahl HL, Merfort I. 1998. The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-kappaB by directly targeting p65. J Biol Chem 273: 33508-33516.

32. Klawitter M, Quero L, Klasen J, et al. 2012. Curcuma DMSO extracts and curcumin exhibit an anti-inflammatory and anti-catabolic effect on human intervertebral disc cells, possibly by influencing TLR2 expression and JNK activity. J Inflamm (Lond) 9: 29-9255-9-29.

33. Tian Y, Yuan W, Fujita N, et al. 2013. Inflammatory cytokines associated with degenerative disc disease control aggrecanase-1 (ADAMTS-4) expression in nucleus pulposus cells through MAPK and NF-kappaB. Am J Pathol 182: 2310-2321.

34. Krejci P, Prochazkova J, Bryja V, et al. 2009. Fibroblast growth factor inhibits interferon gamma-STAT1 and interleukin 6-STAT3 signaling in chondrocytes. Cell Signal 21: 151-160.

35. Spagnoli A, Torello M, Nagalla SR, et al. 2002. Identification of STAT-1 as a molecular target of IGFBP-3 in the process of chondrogenesis. J Biol Chem 277: 18860-18867.

36. Osuka K, Usuda N, Aoyama M, et al. 2014. Expression of the JAK/STAT3/SOCS3 signaling pathway in herniated lumbar discs. Neurosci Lett 569: 55-58.

37. Kondo M, Yamaoka K, Sakata K, et al. 2015. Contribution of the interleukin-6/STAT-3 signaling pathway to chondrogenic differentiation of human mesenchymal stem cells. Arthritis Rheumatol 67: 1250-1260.

38. Li L, Okura M, Imamoto A. 2002. Focal adhesions require catalytic activity of src family kinases to mediate integrin-matrix adhesion. Mol Cell Biol 22: 1203-1217.

39. Le Maitre CL, Frain J, Millward-Sadler J, et al. 2009. Altered integrin mechanotransduction in human nucleus pulposus cells derived from degenerated discs. Arthritis Rheum 60: 460-469.

40. Jiang L, Jin Y, Wang H, Jiang Y, Dong J. 2014. Glucosamine protects nucleus pulposus cells and induces autophagy via the mTOR-dependent pathway. J Orthop Res 32: 1532-1542.

41. Le Maitre C,L, Freemont A,J, Hoyland J,A. 2007. Accelerated cellular senescence in degenerate intervertebral discs: A possible role in the pathogenesis of intervertebral disc degeneration. Arthritis Res Ther. 9: R45.

42. Tolonen J, Gronblad M, Virri J, et al. 2002. Oncoprotein c-fos and c-jun immunopositive cells and cell clusters in herniated intervertebral disc tissue. Eur Spine J 11: 452-458.

43. Ulrich JA, Liebenberg EC, Thuillier DU, Lotz JC. 2007. ISSLS prize winner: Repeated disc injury causes persistent inflammation. Spine (Phila Pa 1976) 32: 2812-2819.

44. Nerlich AG, Bachmeier BE, Schleicher E, et al. 2007. Immunomorphological analysis of RAGE receptor expression and NF-kappaB activation in tissue samples from normal and degenerated intervertebral discs of various ages. Ann N Y Acad Sci 1096: 239-248.

45. Sun Z, Yin Z, Liu C, Tian J. 2014. The changes in the expression of NF-KB in a degenerative human intervertebral disc model. Cell Biochem Biophys .

46. Yu ZG, Xu N, Wang WB, et al. 2009. Interleukin-1 inhibits Sox9 and collagen type II expression via nuclear factor-kappaB in the cultured human intervertebral disc cells. Chin Med J 122: 2483-2488.

47. Jobin C, Bradham CA, Russo MP, et al. 1999. Curcumin blocks cytokine-mediated NF-kappa B activation and proinflammatory gene expression by inhibiting inhibitory factor I-kappa B kinase activity. J Immunol 163: 3474-3483.

48. Nasto LA, Seo HY, Robinson AR, et al. 2012. ISSLS prize winner: Inhibition of NF-kappaB activity ameliorates age-associated disc degeneration in a mouse model of accelerated aging. Spine (Phila Pa 1976) 37: 1819-1825.

Figure legends:

Figure 1: Proteasome kinase array for 46 intracellular signalling molecules. Representative arrays shown for control (A), IL-1β (B) and GDF-5 (C) stimulated human NP cells. D: Box and whisker plots shown for key kinases/transcription factor (ERK1/2 (Red); FAK (Blue); p38 MAPK (Green); JNK (Purple) and c-jun (Orange)) indicated on the representative images with matching colour boxes, colours indicated on legend for graph. Box and whisker plots show relative protein expression normalised to untreated controls in 2 patients each stimulated in duplicate and tested on arrays in duplicate. Significant differences between treatment groups shown with line above box and whiskers ($P<0.05$).

Figure 2: Representative images for p65-NFκB immunofluorescence from human NP cells cultured in alginate and stimulated with IL-1β (B) and GDF-5 (C) or unstimulated controls (A). Human NP cells from 3 patients cultured in alginate and treated in triplicate with IL-1β and GDF-5 or unstimulated controls.

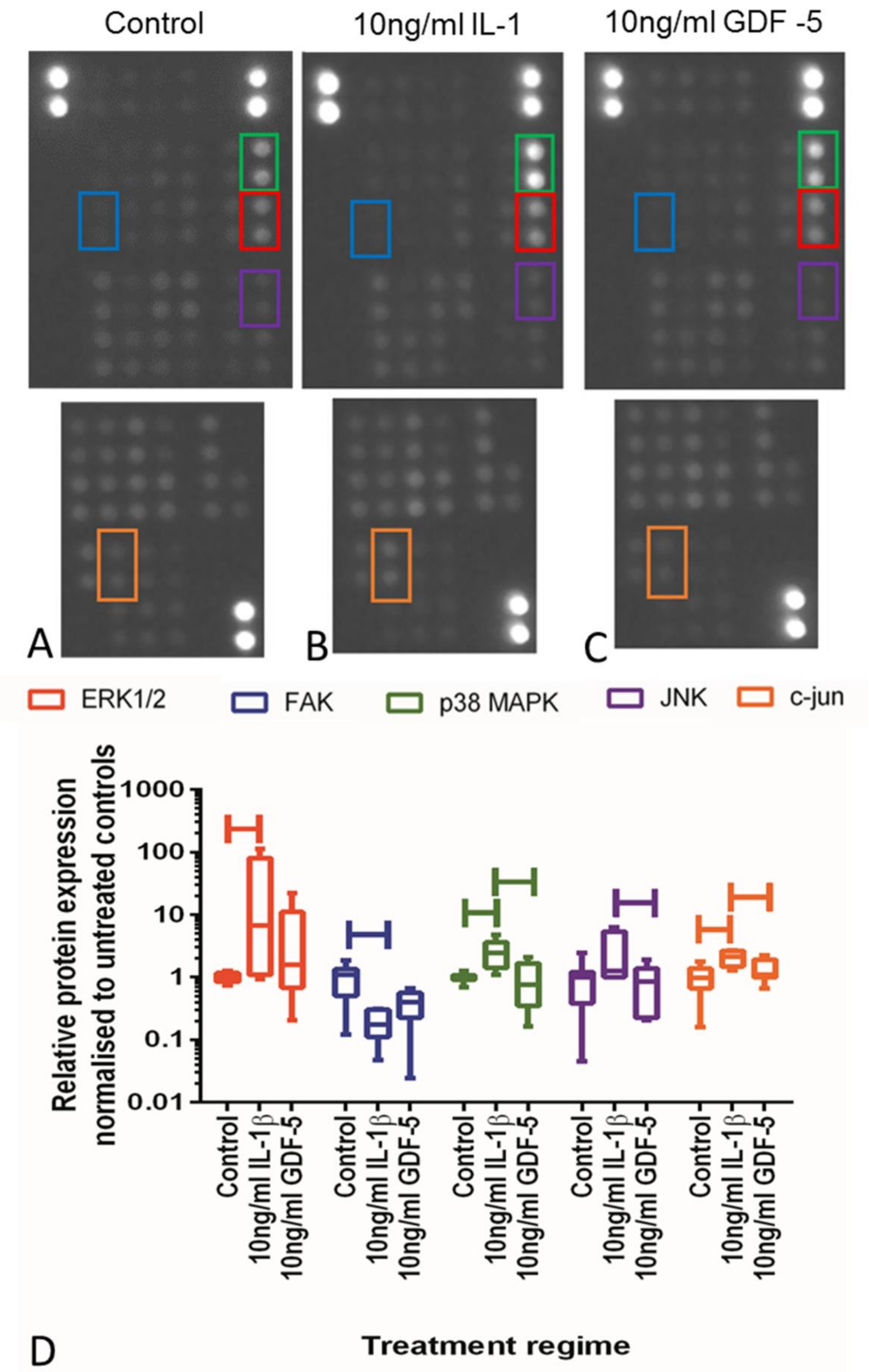
Figure 3: Cell based ELISAs for demonstrating relative phosphorylated ERK1/2 (A), p38 MAPK (B), c-jun (C) and IκB (D) protein expression normalised to untreated controls. Human NP cells from 3 patients cultured in alginate and treated in triplicate with IL-1β and GDF-5 or unstimulated controls. Statistical differences between groups demonstrated by bars and *=$P<0.05$.

Figure 4: Immunohistochemical analysis for phosphorylated p38 MAPK (A-D); c-jun (E-H) and NFκB (I-L) in human IVD discs. Representative images of immunohistochemical staining demonstrated for low grade degenerate discs (A,E,I); Intermediate grade discs (B,F,J); high grade discs (C,G,K). Percentage immunopositivty within NP cells with differing grades of degeneration (D,H,L). Statistical differences between immunopositivity for phosphorylated signalling molecules in intermediate grades of degeneration (4-6) or severe degeneration (≥7), compared to non degenerate discs (≤3) shown with *=$P<0.05$. (n=30).

Figure 5: Relative mRNA expression for MMP3 (A), MMP13 (B), IL-1β (C), IL-6 (D) and IL-8 (E) normalised to housekeeping genes (GAPDH and 18s) and untreated control cells. Data shown generated from NP cells sourced from three independent patients cultured in alginate and treated with or without IL-1β, with or without prior treatment with inhibitors of signalling molecules (p38 MAPK; c-jun, JNK and NFκB), all treatments performed in triplicate within each patient sample. * = significant difference between treated cells compared to untreated controls ($P<0.05$); ^ = significant difference between IL-1β stimulated cells following prior treatment with signalling inhibitors compared to stimulation with IL-1β alone $P<0.05$.

Figure S - 1: Relative mRNA expression for aggrecan normalised to housekeeping genes (GAPDH and 18s) and untreated control cells. Data shown generated from NP cells sourced from three independent patients cultured in alginate and treated with or without signalling inhibitors. All treatments performed in triplicate within each patient sample.

Figure S - 2: Schematic representing key findings.


Control
10ng/ml IL-1
10ng/ml GDF -5
A
B
C
ERK1/2
FAK
p38 MAPK
JNK
c-jun
Relative protein expression normalised to untreated controls
1000
100
10
1
0.1
0.01
Control
10ng/ml IL-1β
10ng/ml GDF-5
Treatment regime
D

1 Figure

1

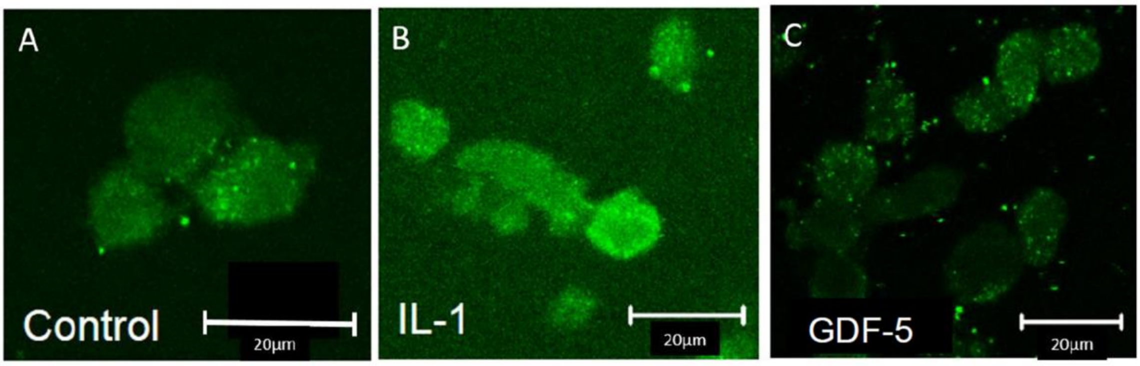

A
Control
20µm
B
IL-1
20µm
C
GDF-5
20µm

Figure 2

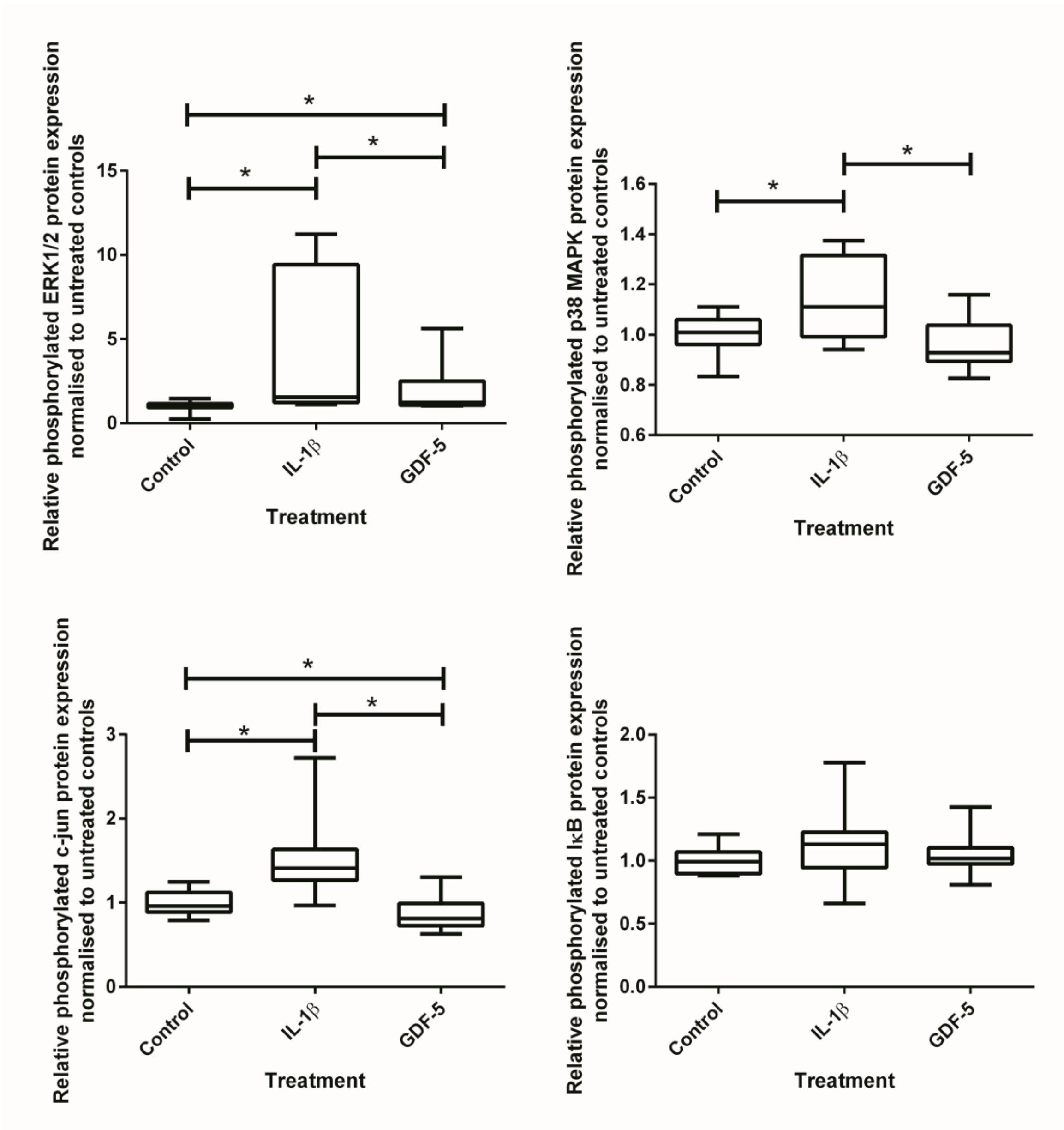

Relative phosphorylated ERK1/2 protein expression normalised to untreated controls
Relative phosphorylated p38 MAPK protein expression normalised to untreated controls
Relative phosphorylated c-jun protein expression normalised to untreated controls
Relative phosphorylated IκB protein expression normalised to untreated controls
Control
IL-1β
GDF-5
Treatment
*

Figure 3

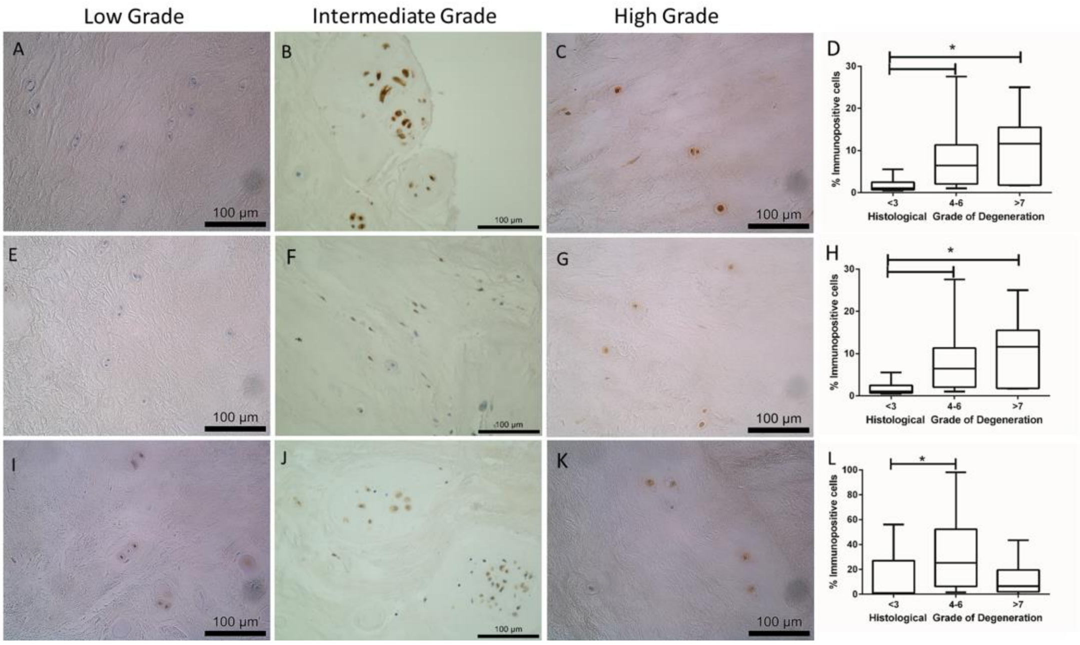


Figure 4

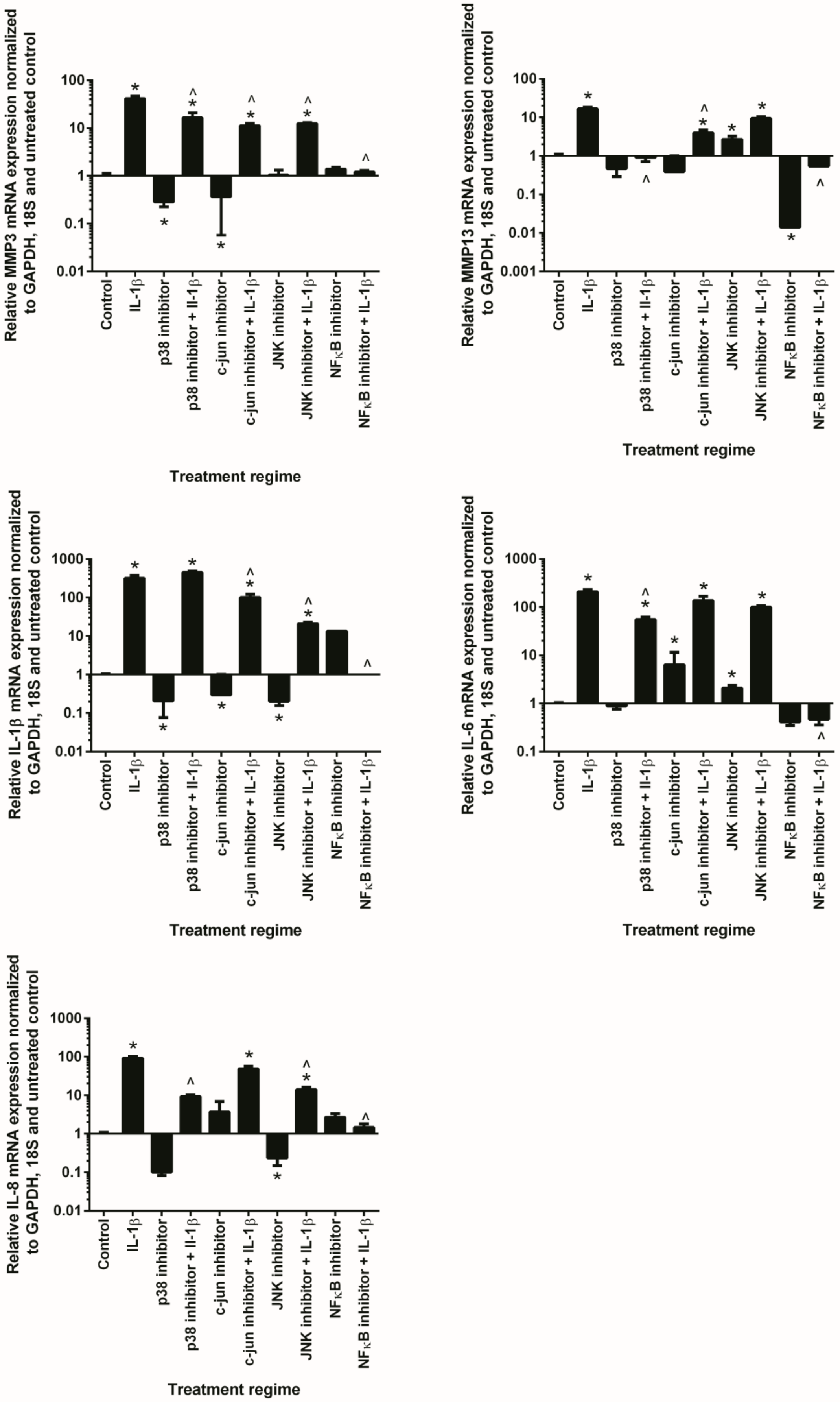
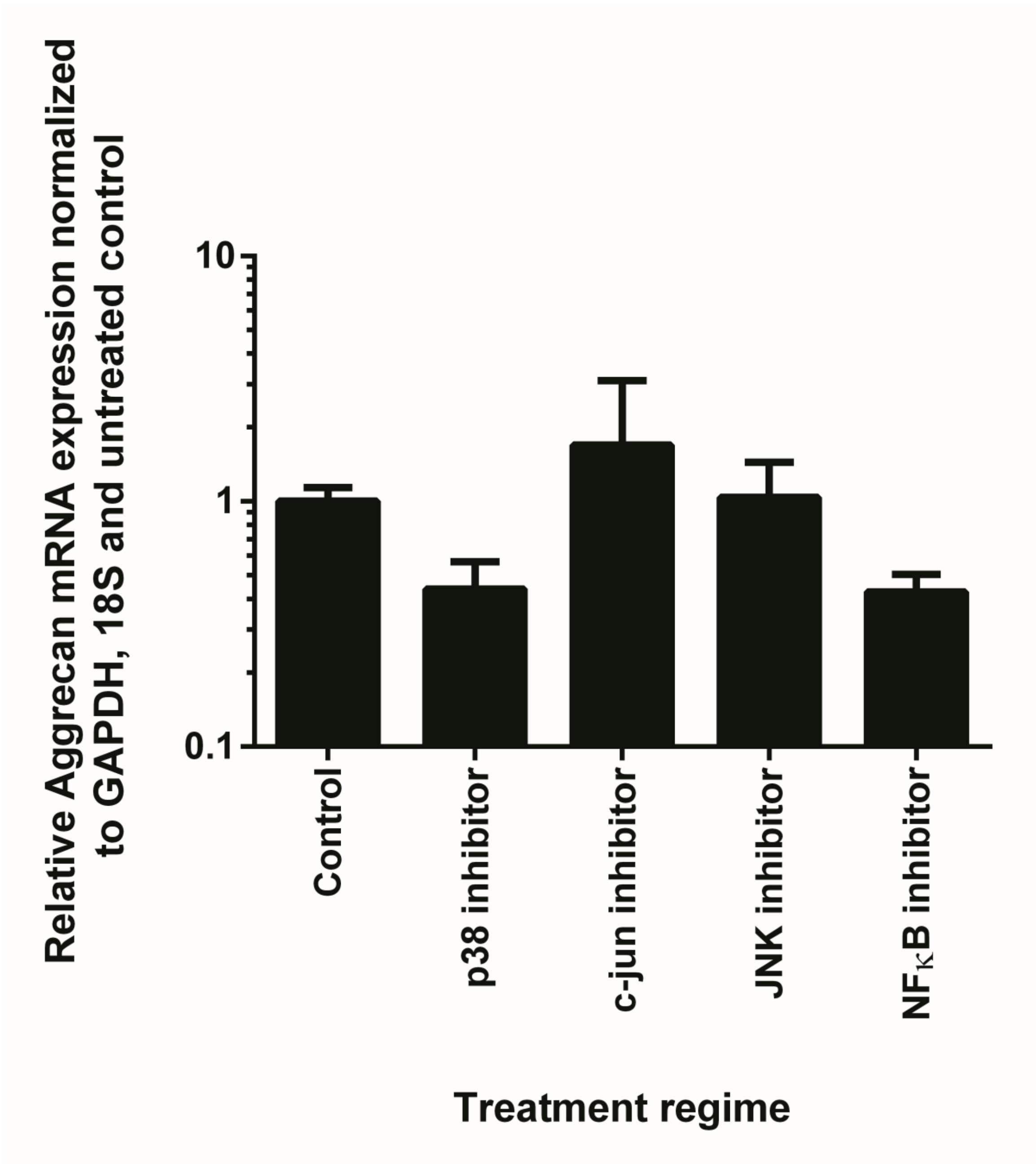
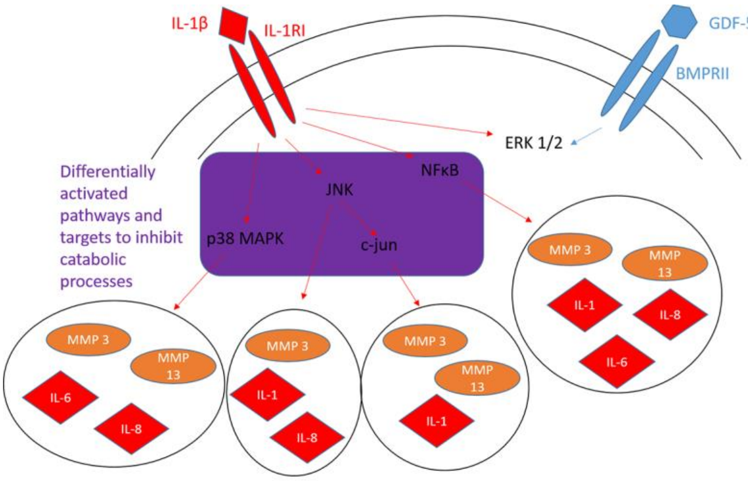

Relative MMP3 mRNA expression normalized to GAPDH, 18S and untreated control
Relative MMP13 mRNA expression normalized to GAPDH, 18S and untreated control
Relative IL-1β mRNA expression normalized to GAPDH, 18S and untreated control
Relative IL-6 mRNA expression normalized to GAPDH, 18S and untreated control
Relative IL-8 mRNA expression normalized to GAPDH, 18S and untreated control
Control
IL-1β
p38 inhibitor
p38 inhibitor + Il-1β
c-jun inhibitor
c-jun inhibitor + IL-1β
JNK inhibitor
JNK inhibitor + IL-1β
NFκB inhibitor
NFκB inhibitor + IL-1β
Treatment regime

Figure 5



Sup Fig 1


IL-1β
IL-1RI
GDF-5
BMPRII
ERK 1/2
NFκB
JNK
p38 MAPK
c-jun
Differentially activated pathways and targets to inhibit catabolic processes
MMP 3
MMP 13
IL-1
IL-6
IL-8

Sup fig 2