Importance of Matrix Cues on Intervertebral Disc Development, Degeneration, and Regeneration

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Abstract: Back pain is one of the leading causes of disability worldwide and is frequently caused by degeneration of the intervertebral discs. The discs’ development, homeostasis, and degeneration are driven by a complex series of biochemical and physical extracellular matrix cues produced by and transmitted to native cells. Thus, understanding the roles of different cues is essential for designing effective cellular and regenerative therapies. Omics technologies have helped identify many new matrix cues; however, comparatively few matrix molecules have thus far been incorporated into tissue engineered models. These include collagen type I and type II, laminins, glycosaminoglycans, and their biomimetic analogues. Modern biofabrication techniques, such as 3D bioprinting, are also enabling the spatial patterning of matrix molecules and growth factors to direct regional effects. These techniques should now be applied to biochemically, physically, and structurally relevant disc models incorporating disc and stem cells to investigate the drivers of healthy cell phenotype and differentiation. Such research will inform the development of efficacious regenerative therapies and improved clinical outcomes.

Keywords: intervertebral; matrix; cues; biomimetic; laminin; bioprinting; regeneration

1. Introduction

The extracellular matrix (ECM) is a non-cellular three-dimensional network of biomolecules and minerals that provides biochemical and physical support to the cells that produce it. The ECM of different tissues varies enormously but can generally be considered as a mixture of fibrous components, including collagenous and elastic fibres, plus a gel-like ground substance, containing proteoglycans, glycoproteins, enzymes, ions, and other non-fibrous elements. The ECM is able to transmit biochemical and physical cues to cells, affecting cellular morphology and phenotype. Changes to cellular activity/function result in linked changes to the ECM, meaning that matrix cues are important drivers of tissue development, homeostasis, ageing, and disease [1–5].

Matrix cues are received by cells via a range of biochemical and physical mechanisms, both directly and indirectly. Biochemical cues, for example, are sensed via cell-surface receptors such as integrins and N-cadherins [6]. Physical cues, such as matrix stiffness, are transmitted most notably through the intracellular actin cytoskeleton. Stiffness, or Young’s modulus, is determined by a range of factors including the relative abundance of matrix proteins, their orientation, the degree of fibre crosslinking, and the mechanical properties of individual fibres [7]. Structural and topographical cues, including fibre size, texture, regional alignment, and orientation are additionally important factors that influence cell morphology, phenotype, and matrix synthesis and degradation. A variety of cues are also sensed by cells indirectly, through changes in nutrient transport regulation, tissue
diffusion profile, and variations in hydrostatic pressure or mechanical loading. Such factors intrinsically link to other microenvironmental cues, including tissue pH, hydration, and oxygen concentration.

Understanding the impact of these various cues is of vital importance both for understanding tissue homeostasis and disease processes, and in regenerative medicine, where matrix cues may be exploited in cellular therapies to help enhance clinical outcomes. This review will therefore focus on the importance of changes to the intervertebral disc (IVD) ECM during development, homeostasis, ageing, and disease and will outline current attempts to integrate biochemical and physical matrix cues into cell-based experimental systems and regenerative therapies.

2. IVD Physiology, Structure, and Function

2.1. The IVD

The human spine contains 23 IVDs which separate the vertebral bones and whose primary functions are to absorb biomechanical forces and permit a range of motions in three dimensions [8]. The IVDs vary in size depending on their position within the spine, but the largest of the lumbar discs, found in the lower back, are approximately 4 cm in diameter and 7–10 mm thick. The IVDs retain a similar composition between disc levels [9], are generally axially symmetrical, and collectively contribute one third of total spinal height [10]. They are hydrated fibrocartilaginous structures consisting of two main regions, each contributing approximately 50% of tissue volume: first, a central gelatinous, proteoglycan-rich nucleus pulposus (NP) and second, an outer fibrous ring-like annulus fibrosus (AF). Each IVD is enclosed by two semi-rigid cartilaginous endplates (CEPs) that connect via the bony endplates (BEPs) to the vertebrae. The CEPs are thin (<1 mm) and difficult to isolate [11], so are frequently excluded from discussions surrounding the IVD; however, the CEPs are essential for nutrient transport and are believed to play a key role in maintaining IVD health [12–15]. Overall, the complex organisation of the IVD makes the organ a viscoelastic, non-linear, anisotropic structure with compressive, tensile, and shear strength in the axial and radial directions—properties highly suited to the IVDs’ primary structural function [8,16].

2.1.1. The NP

The central NP is an amorphous gelatinous tissue composed primarily of water (80% wet weight) interspersed with a loose matrix of collagen type II fibrils and aggregating proteoglycans, particularly aggrecan. Other collagens (types VI, IX, and XI), proteoglycans (biglycan, versican, decorin, lumican, fibromodulin, and perlecan), laminins, fibronectin, and elastin are also prevalent [17]. The NP’s main function is to distribute compressive strain. This is principally enabled by the large quantity of proteoglycans relative to collagens (the GAG/hydroxyproline mass ratio is 27:1) [18] and a correspondingly high level of hydration. The healthy young and adult NP has a Young’s modulus under unconfined compression of 0.3–5 kPa [8,16,19–24], is viscoelastic, and has been described as a ‘tethered fluid’ [25,26]. NP tissue is maintained by a sparse population of NP cells (~4 \times 10^3 cells/mm^3), which display a rounded, chondrocyte-like morphology [27,28] and express a range of phenotypic and functional markers, including Shh, Brachyury, KRT18/19, CA12, CD24, GLUT1, and stabilized expression of HIF1α protein [29].

2.1.2. The AF

The surrounding AF tissue, in contrast, is a highly organised structure comprising 25–30 discrete concentric rings, called lamellae, which are composed principally of fibrillar bundles of collagen type I, collagen type III, and elastin [30]. The AF’s main function is to distribute tensile and shear forces whilst constraining NP swelling [31]. The thickness of the lamellae increases with distance away from the NP, ranging between 0.05 mm in the inner AF (IAF) to 0.5 mm in the outer AF (OAF) [32], whilst lamellae are also organised in an angle-ply formation, with adjacent rings orientated obliquely at 30–60° to the spine. This
angle alternates between adjacent rings [33,34]. Fibre angle is elevated in the IAF compared to the OAF [35], and these structural features combined reflect the variations in mechanical loading experienced across different AF regions. Throughout the tissue, lamellae are furthermore connected by trans-lamellar cross bridges consisting of proteoglycans, most significantly aggrecan and versican, plus collagen type VI [36]. The impact these have on AF integrity and cellular behaviour has not been comprehensively studied (Table 1) [37,38], although micro-computed tomography (µCT) and other advanced imaging techniques are increasingly being applied to better understand the AF microstructure [33,39–41]. Overall, the complex structure of the AF confers it with excellent shear and tensile properties, with tensile Young’s modulus in the order of ~100 kPa.

### Table 1. Composition and biological function of key matrix components within healthy adult IVDs [8,28,42–47].

| Tissue | Matrix Component                          | Relative Composition (Dry Weight) | Structural/Biological Effects                                                                 |
|--------|------------------------------------------|-----------------------------------|------------------------------------------------------------------------------------------------|
| NP     | Collagen (esp. type II)                  | 5–20%                             | Confine hydrating proteoglycans within NP matrix. Modulation of cellular metabolism and extracellular signalling. |
|        | Proteoglycans (esp. aggrecan, versican, biglycan) | 35–65%                           | Generate swelling pressure; influence expression of NP cytoskeletal elements and matrix anabolism/catabolism during compression, particularly collagen fibrillogenesis. |
|        | Non-collagenous proteins (esp. laminin)  | 15–60%                            | Play a key role in matrix organisation through interaction with integrins. |
| AF     | Collagen (esp. type I)                   | 50–70%                            | Fibre orientation and composition ratio influences biomechanics and matrix production within IAF/OAF. Generate swelling pressure; influence expression of AF cytoskeletal elements and matrix anabolism/catabolism during tension and shear; regulate matrix assembly and repair following damage. |
|        | Proteoglycans (esp. aggrecan, versican, decorin) | 10–20%                           | Support development of elastic matrix and enable collagen fibre recovery after deformation. |
|        | Non-collagenous proteins (esp. elastin)  | 10–40%                            |                                                                                             |

The AF is maintained and populated by AF cells (~9 × 10^3 cells/mm^3), which display an elongated fibroblast-like morphology and express the phenotypic markers COL1A2, COL5A1, COL12A1, CD146, SFRP2, LAM1, THY1, and MKX [48,49]. The consensus regarding healthy adult AF cell phenotype remains fragile at present, although a recent study identified 1161 genes showing higher expression in AF than in NP cells [50]. Notably, there is no clear interface between NP and AF tissues; however, there is a discernible transition between IAF and OAF tissue types. Compared to OAF tissue, the IAF is richer in aggrecan, whilst collagen type I and type II fibrils are present in more equal quantities [34,51]. IAF tissue is also comparatively enriched in COL3A1, COL5A1, COL11A2, and proteins relating to matrix synthesis (PCOLCE) and remodelling (MXRA5), as well as those relating to WNT and BMP inhibition [9]. In contrast, the OAF is comparatively enriched in COL6A1/2/3, COL12A1, COL14A1, basement membrane and anchoring proteins, plus ligamentous, tendinous, and cartilaginous components, including tenomodulin and thrombospondins [9]. The presence of these particular ECM components likely reflects the OAF’s proximity and integration with the CEPs, BEPs, and ligamentous structures around the spine [14,15].

#### 2.1.3. The CEPs

The CEPs are semi-rigid layers of hyaline cartilage, approximately 0.6 mm in thickness that perform a range of functions. They have a 60% water content and an ECM rich in aggrecan and collagen type II at a ratio of 2:1 [32]. They therefore deform during mechanical loading, dissipating stress across the IVD [8], although their biochemical and physical properties are regionally dependent [53]. The CEPs also reduce the rate of water expulsion from the NP during compression, making them important regulators of
diffusion; nutrients, oxygen, metabolic by-products, and other small molecules are known to diffuse through the CEPs bidirectionally [54]. The CEPs are penetrated by a thin capillary network [55] and the diffusion profile of the CEPs during mechanical loading, age-related calcification, and IVD degeneration are active areas of study, both in basic research and in tissue engineering [14,27,54,56–60], because of the important role the CEPs play in IVD nutritional balance. The CEPs are populated by chondrocytes (1.5 \times 10^4 \text{ cells/mm}^3) [27,28] and whilst knowledge concerning phenotype is limited, COL10 has been identified as a potential CEP cell marker in adult IVDs [61]. CEP cells have also been shown to express high levels of the NP markers Brachyury and KRT19, plus the AF cell marker MKX [62].

Given the tissue’s similarities with both NP and AF tissues, the CEPs and role of CEP-related matrix cues deserve greater inclusion in future IVD discussions and regenerative approaches; CEP matrix cues and other instructive factors are almost certainly linked to NP and AF cell behaviour and phenotype, plus overall IVD development, homeostasis, ageing, and degeneration.

3. Changes to the ECM during IVD Development and Degeneration

3.1. IVD Development and Maturation

The NP develops from the precursor foetal notochord, a soft rod-like structure composed of clusters of vacuolated notochordal cells and an ECM rich in collagens (types II, VIII, XI, XV, and XXVII), glycoproteins (laminins, fibronectin, and fibrillins), and chondroitin sulphate (CS)-rich proteoglycans, such as aggrecan. Many of these have been found to vary regionally, indicating their diverse functional roles [63]. The notochord supports musculoskeletal development and provides a series of dynamic biochemical and physical matrix cues crucial for directing IVD development [64–67]. Over time, identifiable notochordal cells are lost from the NP, disappearing almost entirely by adolescence and skeletal maturity. The mechanisms driving the loss of notochordal cells in humans remain unclear and whether the notochordal cells differentiate into, or are replaced by, NP cells continues to be a source of major contention. Recent progress in identifying phenotypic markers for notochordal [68] and NP [29] cells has revealed that NP cells are derived directly from the notochordal cell population [69]. However, subpopulations of NP [70,71] and AF [72,73] cells with potentially differing ontogenies have been identified in adult IVDs, including those of non-notochordal origin. It is therefore speculated that a small notochordal cell population persists into adulthood, producing ECM that encourages the development of a healthy NP cell phenotype and guards against the onset of early IVD degeneration [74–77].

At all stages of NP development, cell–matrix interactions are important; however, notochordal ECM is especially rich in laminin isoforms. Laminins generally act as structural proteins in both basement membrane and the notochordal sheath [66,78], but are also known to encourage NP cell clustering behaviour in developing NP tissue [7]. Notochordal and young NP cells strongly express specific laminin isoforms (LM-511, LM-521, and LM-332) and receptors (integrins \( \alpha_6, \beta_1, \beta_4, \) and CD239) [78–80], whilst laminin–cell interactions are believed to encourage cell–cell interaction, healthy cell morphology, and the proteoglycan production required for notochordal and IVD development [78]. Developmental changes within the notochord and early NP have additionally been associated with the upregulation of molecular functions linked to laminin and BMP receptor binding, strengthening the view that laminins are particularly important biochemical matrix cues relevant to developmental research and regenerative therapies.

Notochordal ECM has a lower Young’s modulus (<1 kPa) than adult NP, another factor known to encourage notochordal cell clustering behaviour and NP tissue development [7,66,80,81]. From the third to tenth weeks of embryonic development, the notochord stiffens and eventually segments, influenced by a variety of biomolecules including the intracellular transcription factors Brachyury, SOX5/6, and PAX1, plus secreted morphogens and signalling molecules BMP 2/7 [82], Shh, and Noggin [83]. The 23 NP regions eventually result from this variety of interconnected cues and further research is required to
understand the full relevance of the spatial and temporal changes inherent to such cues as they drive development.

In contrast, the adult AF and CEP regions are of mesenchymal origin, developing from sclerotomal tissue surrounding the foetal notochord. From around the fifth week post-conception, this sclerotomal tissue condenses and expands, encompassing the notochord, whereupon elastin, fibrillin, and perlecan influence the formation of collagenous attachments between nascent AF lamellae and the cartilage and vertebral bodies [83,84]. Knowledge of the impact of cell–cell and cell–matrix interactions on AF development lags significantly behind that of NP and the notochord; however, it is suspected that the organisation of the AF is specified by physical cues present during early development. For example, the number of lamellae is fixed during early foetal development, with fibres subsequently thickening and increasing in volume according to the physical and geometrical stresses induced by the stiffening notochord and surrounding ECM [85]. It is furthermore proposed that early fibre organisation informs the development of intracellular actin stress fibres within developing AF cells [86], which in turn may guide the production of aligned ECM and help redistribute mechanical loading as the IVD develops. Intriguingly, the orientation and integration of AF fibres into the BEPs, CEPs, and surrounding tissues appears to be driven by physical constraints caused by the emergence of the endplates and vertebral bodies [34,85,87], implying that physical matrix cues are central to AF development.

3.2. IVD Degeneration

Degeneration is a broad term that encompasses the myriad changes taking place within the IVD, which ultimately result in a loss of structural integrity, pain, and an overall reduction in quality of life. Many of the changes that occur in the IVD due to age and degeneration are indistinguishable from one another, and as a result, the root causes of degeneration remain only partially understood. It is widely accepted, however, that a combination of mechanical loading, genetic factors, and reduced cellular activity are all responsible for the changes in matrix production that lead to the loss of tissue homeostasis and increased matrix degradation typically observed with degeneration [75,88–95].

The healthy IVD is avascular, with the possible exception of the extreme OAF, and so cells must maintain tissue health by balancing matrix anabolism and catabolism within each disc region. A variety of anabolic and anti-catabolic factors are therefore important for NP and AF health, including those from the TGFβ superfamily, matrix metalloproteinases (MMPs), aggrecanases/a disintegrin metalloproteinase with thrombospondin motifs (ADAMTS), and tissue inhibitors of metalloproteinases (TIMPs), along with a number of genes relating to structural proteins correlated with IVD degeneration. Damage-associated molecular patterns (DAMPs), including hyaluronic acid and fibronectin fragments, are also believed to be primary contributing factors to the inflammation and pain experienced by most sufferers of IVD degeneration [96,97].

Degeneration typically begins within the NP region, where an upregulation of inflammatory cytokine production leads to the downregulation of matrix synthesis, especially aggrecan and collagen type II. This shift coincides with an increase in matrix degradation via upregulation of matrix degrading enzymes (MMPs/ADAMTS), which results in proteoglycan fragmentation [98] and a corresponding loss in the ability of the tissue to retain water [99,100]. The linked loss in hydrostatic pressure leads to an overall reduction in disc height, which impairs the IVD’s overall mechanical integrity and encourages further degeneration, the start of a vicious cycle [101]. The NP also experiences a shift in the production of collagens, from predominantly collagen type II to the more fibrous type I, resulting in increased ECM stiffness, a key driver of NP degeneration [102,103] (Figure 1).
tissue to retain water [99,100]. The linked loss in hydrostatic pressure leads to an overall reduction in disc height, which impairs the IVD’s overall mechanical integrity and encourages further degeneration, the start of a vicious cycle [101]. The NP also experiences a shift in the production of collagens, from predominantly collagen type II to the more fibrous type I, resulting in increased ECM stiffness, a key driver of NP degeneration [102,103] (Figure 1).

Figure 1. Comparison of healthy and degenerate IVD ECM. Created with Biorender.com.

There is a growing body of work documenting the negative impact of increased stiffness on both NP and notochordal cell phenotype [7,66,104,105]. Interestingly, degeneration of the NP coincides with the loss of identifiable notochordal cells, a phenomenon observed spontaneously in many species, including humans and chondrodystrophic dogs, but not in pigs, rabbits, or non-chondrodystrophic dogs. Species which retain their notochordal populations do not generally suffer from significant IVD degeneration [103], reinforcing the view that notochordal cells are a promising target for regenerative therapies. Work is ongoing to determine the roles of master notochordal cell regulators and to identify how best to use these for the detection of early degeneration and to retard disease progression [105–109] using novel regenerative therapies.

Overall, such observations suggest physical matrix cues are important contributors to the initiation or progression of degeneration. Such a view is reinforced by changes occurring in the AF during degeneration, where the impacts appear mainly mechanical. For example, additional mechanical stress due to NP degeneration can lead to AF microfissuring and a widening of the interlamellar spaces, meaning lamellae lose their organisation and orientation due to load redistribution [102]. Stiffening of AF tissue is another contributing factor to this structural deterioration; however, it is the loss of aggrecan which particularly impacts AF health, since aggrecan is anti-angiogenic and its absence leads to neovascularisation [110]. AF vascularisation results in increased oxygen concentration and disrupted signalling pathways, and can be responsible for driving AF and NP cell senescence [111], further impairing tissue homeostasis and overall mechanical integrity. In the most serious instances of AF degeneration, herniation of the NP through the AF may even occur, permanently fissuring the already weakened AF structure [112,113].

Degenerative changes also take place in the CEPs and adjacent vertebral structures, although the significance of many of these remains unclear. Degeneration-associated CEP calcification is known to be important [13,14,56,60], as a loss of CEP permeability results in reduced nutrient transport and altered CEP mechanical profile [114], plus knock-on
effects for NP and AF cells. These include cellular quiescence [115,116], deregulation of matrix anabolism and catabolism [117], and a reduction in the release of exosomes, recently discovered to inhibit IVD degeneration and NP cell apoptosis [118,119]. Nerve ingrowth and neovascularisation through the CEPs into the AF are additional disease pathologies directly linked to pain [120], highlighting the links between the condition of the CEPs and other tissue regions, as well as the importance of understanding the range of matrix cues spanning all three regions as these change over time.

Transcriptomic and proteomic analyses have begun to reveal many of the IVD-wide and more subtle spatiotemporal changes in genes and proteins that may result in downstream regulation of matrix anabolism and catabolism or influence cell phenotype and matrix production, both in non-humans [73,121–127] and in humans (Table 2). Collectively, they have additionally identified age-related compositional differences and regionally specific variations in matrix proteins [128], indicating that some ageing and degenerative mechanisms act only locally within NP and AF tissues, as opposed to at the organ-wide level. Such novel approaches open up vast new avenues of study and will transform our understanding of the importance of different transcription factors influencing matrix cues during degeneration. In turn, this will allow the accelerated development of degenerative models and novel therapies incorporating a variety of spatiotemporally defined matrix cues.

Table 2. Important matrix genes and proteins identified during IVD omics studies in humans.

| Source | Method | Matrix-Related Genes/Proteins Investigated | References |
|--------|--------|------------------------------------------|------------|
| Transcriptomic | Microarray analysis | Identified CD24, IGF1 and eight other notochord-specific markers, plus molecules involved in inhibiting vascularisation (WISP2, Noggin, and EDN2) and inflammation (IL1-RN). | [105] |
| Foetal/notochord | scRNA-seq | Identified eight drivers of notochordal differentiation: PAX6, GDF3, FOXD3, TGFβ1, SOX5, LMX1A, LEFTY1, and LEFTY2. | [109] |
| Young/healthy NP | Microarray analysis | Identified marker genes including PAX1, FOXL1, HBB, CA12, and OVOs2 for NP; GDF10, CYTL1, IGBP, and FBN1 for articular chondrocytes. | [129] |
| Young/healthy NP and AF | scRNA-seq | Higher expression of COL2A1, COL9A3, and COL11A1 in NP. Signature transcription factor for NP (KDM4E) and AF (FOXM1) identified. | [130] |
| Young/healthy NP, AF, and CEP | scRNA-seq | Identified progenitor IVD cell markers plus PDGF and TGFβ cascades important for regulating NP microenvironment. | [131] |
| Young/healthy and degenerate NP, IAF, and OAF | Microarray analysis | Overall, showed relative enrichment of COL5A1, SERPINA5, and MXRA5 in IAF; LAMB2, THBS1, and CTSD in OAF. Identified that clearest transition in proteomic signature is between ECM of OAF and IAF, not between IAF and NP. Used integrin- and syndecan-binding laminin-mimetic peptides to upregulate 148 genes, including NP markers (Noggin and ITGAv6), and downregulate 277 genes, including known fibroblastic markers linked to matrix catabolism (CTGF). | [9], [132] |
| Degenerate NP | RNA-seq | High expression of genes encoding proteoglycan-rich ECM and TGF superfamly signalling pathways in NP (GpC6, INHBA, INHA); fibrous, vascular matrix and WNT/NOTCH signalling in AF (COL1/4/6, VEGFC, and JAG1/2). | [133] |
| Degenerate NP and AF | RNA-seq | Identified membrane-associated genes for cellular subtypes, including CLDN11, TMEF2, EFNA1 and NETO2 for NP; COLEC12, LPAR1, and CHIC1 for AF. Indicated that regulation of WNT signalling separates AF from NP cells. | [50] |
Table 2. Cont.

| Source | Method | Matrix-Related Genes/Proteins Investigated | References |
|--------|--------|-------------------------------------------|------------|
| Foetal NP | LC-MS/MS | Identified 1316 proteins, 1096 of them unique, and 10 significant protein clusters including collagens, SLRPs, and matrilins. | [134] |
| Foetal/notochord and degenerate NP | LC-MS/MS | Highly expressed COL14A1 identified for the first time. Identified 12 degeneration-linked proteins including interleukin-11, LTA, ECM1, and matrilin-3. | [135] |
| Foetal/notochord, young/healthy, and degenerate NP | ESI–LC–MS/MS | Identified ten ECM regulators and ECM affiliated proteins of interest, including four (P4HA1, PLOD1/2, and SERPINH1) involved in collagen biosynthesis. | [136] |
| Young/healthy and degenerate AF | MS and silver-stained 2-D electrophoresis gels | Degeneration led to decreases in three proteins and increases in seven, suggesting possible degenerative biomarkers and loss of cell adhesion ability. | [137] |
| Young/healthy NP and AF | LC-MS/MS and iTRAQ analysis | High level of lubricin and low levels of biglycan compared to seven other cartilaginous tissue types. | [138] |
| Young/healthy and degenerate NP and AF | LC-MS/MS and iTRAQ analysis | Increased levels of CILP and CILP2 in NP, HTRA, COMP, and CILP in AF with degeneration. | [139] |
| Young/healthy IVDs | Peptide location fingering | Indicated age-related structural differences in over one hundred ECM-associated proteins including COMP, CILP, CILP2, and LR1P. Regionally specific variations in collagen type II and type V, and aggrecan across ages. | [128] |
| Young/healthy and degenerate IVDs | LC-MS/MS | Observed reduction in structural and other matrix proteins including COL2A1, KRT, BGN, VCAN, and DCN with degeneration. | [140] |
| Degenerate NP and AF | FTMS/ITMSMS and iTRAQ analysis | Fifty-four and seventy-three proteins differentially regulated in NP and AF, including integrin-mediated cell adhesion pathways. | [141] |
| Bone marrow-derived stem cells (BMSCs) exposed to young/healthy and degenerate IVD environment | LC-MS/MS | Altered regulation of 224 and 223 proteins following exposure to healthy or degenerate IVD microenvironments compared to baseline secretome. Following trauma, MMP and IL production observed; however, CTGF, LTBP2, and TIMP1 were also recorded, indicating attempted inhibition of matrix degradation and inducement of NP cell growth and matrix production. | [142] |

**Metabolomic**

| Source | Method | Matrix-Related Genes/Proteins Investigated | References |
|--------|--------|-------------------------------------------|------------|
| DegeneraH HR MAS NMR to NP and AF | $^1$H HR MAS NMR spectroscopy | Correlation between degree of degeneration and metabolites including glycine and hydroxyproline, associated with significant collagen breakdown. Reduced abundance of CS observed in highly degenerate specimens. | [143] |

4. Incorporation of Matrix Cues into Experimental Models Using Biomaterials

4.1. The Need for Regenerative Therapies

Back pain is one of the leading causes of disability worldwide. In the UK alone, it has an estimated socio-economic cost of £12 billion [144], whilst in the USA the total impact may be as high as $100 billion [145]. It is predicted that 85% of individuals from Western societies will experience back pain at some point in their lives [146], and an estimated 632 million people are suffering from the condition at any one time [147]. However, treatments focus principally on pain management and non-invasive interventions such as physiotherapy. Intradiscal injections and the removal of severely degenerate discs are also common [148], whilst grafting and novel prosthetic implants are increasingly yielding superior clinical outcomes [149]. However, such innovations, whilst welcome, ultimately fail to address IVD degeneration and are reported in many instances to accelerate the breakdown of adjacent IVDs post-treatment [150–153]. The long-term prognosis for surgical procedures is thus unsatisfactory [154,155]. Efforts to physically repair degenerate IVDs, particularly through the use of AF sealants and fibrous composites, are progressing [156–158], but it is novel biological approaches that hold the most promise, including platelet-rich plasma (PRP) therapy [159–163], gene therapy, and cell-based therapies. Gene therapy, a broad class of treatments involving the genetic modification of cells for therapeutic effect, is proposed as the key to long-term inhibition of IVD degeneration [164,165]. Recent advances involving gene silencing via RNAi, gene editing using CRISPR, and the delivery of non-viral vectors to cells have hinted at the feasibility of targeting the inflammatory receptors of degenerate
IVD cells to inhibit degeneration by limiting matrix catabolism [166]. However, the field is still in its early phases, and many basic questions remain regarding clinical efficacy, cost-effectiveness, and long-term ethical implications.

4.2. Cell-Based Regenerative Approaches

Cell therapy, the implanting of cells into diseased tissue, offers another exciting regenerative strategy with real potential for IVD regeneration. Autologous and allogenic notochordal [167], NP [168], AF [169], chondrocyte [170], and pluripotent/multipotent stem cells [171–178] have all been trialled in cell-based therapies [17], with experimental objectives including to limit production of inflammatory cytokines, encourage matrix anabolism, and repopulate or stimulate native IVD cells [179]. All these cell types appear to have properties suitable for IVD regeneration. Whilst in vitro evidence is strong and there is compelling evidence from in vivo studies, current clinical trial data do not yet provide high quality evidence of efficacy [180], highlighting the need to promote appropriate cell function or, in the case of stem cells, lineage commitment. Short-acting growth factors are occasionally employed to stimulate cell function [181], but their high cost and the risk of off-target effects means that most cell therapies do not use growth factors, and cells are instead injected in isolation. Cells therefore lack many of the supporting biochemical and physical cues known to drive healthy phenotypes or discogenic differentiation and may instead receive abnormal cues linked to degeneration, limiting their regenerative potential. Cells sourced from or delivered to degenerate tissue show a strong tendency to de-differentiate or produce degenerative phenotypes, either due to a lack of cell function or because of cell death in the harsh microenvironment of the degenerate IVD [182–184]. This highlights the need to identify the most appropriate matrix cues to deliver to cells, in order to better control and direct their behaviour and health, along with ECM restoration.

If cell-based therapies are to improve, understanding how best to deliver these cues and minimise the impact of the degenerate IVD microenvironment is of vital importance. Comparatively little work has focused on how different cell types respond to the matrix cues presented by the degenerate IVD environment. These cues are substantially different from those in healthy IVD tissue, which are themselves different from those of the developmental IVD and foetal notochord, and whether the incorporation of 'healthy' matrix cues can be used to promote a healthy phenotype or drive more appropriate stem cell differentiation and matrix formation is an open question. There is therefore a real need to provide the cells used in cell therapies with matrix cues through the use of biomaterials in experimental models and in clinical regenerative therapies. The designing of systems that can deliver regionally specific instructive cues to cells is one of the priorities facing IVD regenerative research moving forward. If achieved, models can be developed that mimic IVD development, health, and degeneration, allowing the study of the role of specific matrix cues and the inclusion of cues into regenerative therapies, with the aim of restoring appropriately functioning ECM and reducing or eliminating pain.

4.3. Development of Biomimetic Systems for the Delivery of Matrix Cues

Research is ongoing to develop biomaterials and systems that can deliver biochemical and physical cues to cells and yield experimental insights regarding the roles of specific instructive cues within the IVD. The most notable body of work in this regard is the development of a library of laminin-mimetic peptide hydrogels for directing the NP cell phenotype in 3D. At least six integrin- and syndecan-binding peptide sequences have been developed which successfully drive healthier NP cell phenotypes [132]. The use of such systems provides the clearest indication yet that matrix cues can be used to directly modulate cell behaviours [185] and potentially mimic a combination of cues present in development, health, and degeneration. Peptide-conjugated alginate hydrogels have similarly been exploited, with cell-adhesive and syndecan-binding domains encouraging the production of NP-specific phenotypes within alginate cultures, in some instances regardless of presented cell-adhesive domains [186]. Such systems can in the future be used
to determine differences in transduction experienced by young/healthy and degenerate cell types and the importance of matrix cues and receptor interactions [186].

A number of separate biomaterials systems incorporating collagen-based instructive cues are also in development and have been shown to modulate cell phenotype and differentiation. For NP, collagen type II crosslinked with genipin has been used to promote the differentiation of adipose-derived stem cells (ADSCs) into NP-like cells via the Shh pathway [187], whilst genipin has been employed to stabilise a collagen type II and chondroitin sulphate gel capable of encouraging healthy NP-like expression in ADSCs and the partial restoration of NP [188]. A denatured form of collagen, gelatine, was also combined with hyaluronic acid and methacrylate in a photo-crosslinkable hydrogel to achieve NP-like differentiation of ADSCs and the reversal of degeneration in vivo [189], whilst, in one instance, type II collagen was combined with both hyaluronic acid and chondroitin sulphate in a rabbit degeneration model to encourage disc cell repopulation and matrix production [190]. For AF, scaffold materials containing collagen type I are common, increasingly within stiffness-tuneable materials [191–193] and with the addition of cells and growth factors [194]. An MSC-laden collagen type I gel was recently applied to reverse AF degeneration, albeit in sheep [195], whilst AF-derived stem cells have been trialled in a collagen type I-containing decellularised ECM (dECM) [196]. Many relevant collagen-based systems exist that have not yet been applied to IVD bioengineering; for example, combined collagen type I and type II blended hydrogels [197] with chondroitin sulphate [198] for MSC differentiation and articular cartilage repair, hinting at the use of combinations of matrix molecules simultaneously in NP, AF, and CEP research in future. This growing body of work is important, as it indicates collagen-based biomaterial culture systems can be designed to specifically deliver type I and type II-based instructive cues for the directing of cell phenotype and healthy matrix production, potentially in combination with glycosaminoglycans (GAGs) and other collagens [199,200].

Aggrecan, hyaluronic acid, and other GAG-based hydrogel systems and their biomimetic equivalents are needed moving forward. There is significant research in this regard applied to cartilage engineering [201–210], including systems that can be localised within ECM tissue through the incorporation of HA-specific binding peptides [201,205,206] and via steric interactions with collagen type VI and perlecan [211]. Some of these systems have been applied to the IVD [212], most notably in the case of a cytocompatible large aggrecan mimic, which has been chemically, structurally, and mechanically characterised and injected ex vivo into bovine NP tissue [213]. Proteoglycan-like systems principally mimic or incorporate heparan sulphate [214] and the chondroitin sulphate chains of aggrecan in order to confer a hydrating function [214,215]. However, they have also been used to aid differentiation [216] and deliver growth factors to mesenchymal stem cells (MSCs) [217], plus encourage collagen and GAG production in MSCs for NP regeneration [218]. Collectively, these studies demonstrate new opportunities for macromolecular matrix engineering that have potential to alter the degenerate IVD microenvironment and augment cellular therapies with growth factors, tissue engineered scaffolds, and the delivery of instructive matrix cues [211] (Table 3).

dECM-based alternatives have also advanced in recent years, although the bulk of research has focused on cartilage and bone tissue engineering applications, not IVD. Tissue-specific dECM is regarded as a promising alternative to other biomimetic matrix systems as they may enable a more accurate reflection of the native tissue environment [219,220], delivering collagens, proteoglycans, laminins, and other important matrix components [221]. In the case of IVD, dECM biomaterials have been used to provide scaffold materials and growth factors to AF-derived stem cells [196] and MSCs co-cultured with degenerate NP cells [222]. dECM biomaterials are increasingly being developed as scaffold coatings, within hydrogels, and for bioinks in 3D bioprinting [223–228].
| Matrix Cues(s) | Study Outcome | References |
|---------------|---------------|------------|
| **Type I collagen** | Injectable collagen gel upregulates aggrecan and collagen type I production for in vitro AF repair. Alginate-collagen porous scaffolds supported MSC proliferation and collagen type I production. Injectable high-density collagen gel partially repaired AF defect and remodelled by host fibroblasts into a fibrous cap. Injectable high-density collagen gel seeded with MSCs resulted in increased disc height, reduced Pfirrmann grade, and increased NP area. Injectable TGFβ1-supplemented collagen hydrogel resulted in production of collagen type I, CD146, MKX, and SM22α. | [192] [193] [191] [195] |
| **Type I collagen and HA** | Injectable collagen-HA hydrogel enabled growth factor delivery and supported growth and chondrogenic differentiation potential of MSCs and nasal chondrocytes. | [229] |
| **Type II collagen** | Injectable, crosslinked collagen hydrogel enriched with HA preserved NP cell morphology. Collagen microspheres aided pre-differentiation of ADSCs in degenerate IVD-like conditions. Collagen scaffold activated the Shh pathway in ADSCs, promoting NP-like differentiation. | [230] [189] [187] |
| **Type II collagen and HA** | Microgels influenced ADSCs to express high levels of collagen type II, aggrecan, and SOX9, and low levels of collagen type I. | [231] |
| **Type II collagen and CS** | CS incorporation resulted in increased production of NP-like ECM, including sulphated GAGs. | [232] [233] |
| **Type II collagen and HA and CS** | Cell-seeded scaffolds supported NP cell viability and resulted in maintenance of disc height. | [190] |
| **Laminin** | Injectable laminin-111 functionalized poly(ethylene glycol) (PEG) hydrogel resulted in significantly higher cell retention within NP. | [234] |
| **Laminin mimic** | Laminin-mimetic peptides resulted in cell signalling downstream of integrin and syndecan binding, promoted cell migration, and modulated NP behaviour similarly to full-length laminins. Integrin- and syndecan-binding peptide-conjugated alginate hydrogel elicited NP-specific phenotype and re-expression of more juvenile-like phenotype in NP cells. Integrin- and syndecan-binding peptide-conjugated alginate hydrogel used to identify novel mechanosensitive targets in NP cells, including several G Protein-Coupled Receptor genes. | [185] [186] [132] |
| **HA** | HA-pNIPAM hydrogel induced greater disc-like differentiation of MSCs compared to pre-differentiation, including collagen type II, SOX9, KR19, and CD24. Eight different HA-PEG hydrogel formulations used to identify key parameters influencing IVD cell–material interactions. Treatment with HA-based hydrogel resulted in downregulation of NGF and BDNF, plus suppression of IL1R1 in an in vitro inflammation model of NP. HA and PRP hydrogel blended with batroxobin gelling agent resulted in heightened sulphated GAG production in MSCs and chondrocyte-like differentiation. Assessed the efficacy of HA hydrogel pain alleviation, demonstrating altered glycosylation plus modulation of inflammatory and regulatory signalling pathways. HA oligosaccharides shown to stimulate MMPs, ADAMTs, and anabolic matrix repair genes. Gelatine-HA hydrogel promoted NP-like differentiation of ADSCs. | [235] [236] [237] [218] [238] [239] [188] |
| **HA and GAG mimic** | Used HA-PEG hydrogels to demonstrate the chondro-inductive potential of pentosan polysulphate, a sulphated semi-synthetic polysaccharide. | [240] |
### Table 3. Cont.

| Matrix Cues(s)     | Study Outcome                                                                                                                                 | References |
|--------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|------------|
| GAG mimic          | Cytocompatible biomimetic aggrecan analogue comprising a polymeric core conjugated to CS ‘bristles’ was shown to have comparable osmotic pressure to natural aggrecan and increase intradiscal pressure upon injection. | [212]      |
|                    | Pentosan polysulphate embedded within a gelatine-fibrin scaffold with MSCs resulted in restored disc height, morphology, and NP proteoglycan content. | [241]      |
|                    | Cytocompatible, large biomimetic aggrecan analogue comprising a polymeric core conjugated to CS was chemically, structurally, and mechanically characterised. Injection into ex vivo bovine NP showed localisation in the pericellular matrix. | [211]      |
|                    | Co-polymerised naAMPS and KSPA GAG mimic provided intrinsic swelling pressure and restoration of stiffness within ex vivo NP. | [214]      |
| Elastin            | Bioprinted silk fibronin hydrogel combined with elastin shown to support ADSC culture and enable creation of scaffolds with structural and mechanical properties similar to AF. | [242]      |
| Matrilin-3         | Gelatine microparticles loaded with TGFβ3 and matrilin-3 promoted chondrogenic differentiation of ADSC spheroids while preventing hypertrophy and terminal differentiation of cells. | [243]      |
| Decellularised AF ECM | Decellularised ECM and chitosan hydrogels increased production of collagen types I and II, and aggrecan, in AF-derived cells. | [196]      |

The development of biomaterials capable of delivering variations of combined cues to guide cell behaviour is a research bottleneck, and there are unfortunately no systems that can yet deliver a comprehensive range of the many physiologically relevant biochemical cues so far identified, including those that are laminin-, collagen-, aggrecan-, and GAG-based. Nor are there many that combine the use of these matrix molecules with stiffness-tuneable biomaterials for mechanical cue incorporation. This has been achieved in a small number of instances for IVD, using in situ crosslinkable peptide delivery systems [244,245] and laminin-functionalised pegylated hydrogels of varying stiffnesses [185]. Such approaches are generating novel data regarding mechanotransduction, notochord morphogenesis, intracellular signalling, cell differentiation, and matrix synthesis, which are of major relevance to IVD regeneration strategies moving forward [81,132].

As the range of biomaterials systems expands, the inclusion of comparatively unexplored molecules known to have matrix regulatory functions is an important step. For example, perlecan is speculated to directly impact gene regulation and matrix stabilisation within IVD cells [246,247]; however, its role in regulating healthy and degenerate phenotypes and stem cell differentiation remains unclear [248,249]. The impact of early protein and proteoglycan deposition on cellular behaviour within hydrogels has also largely been overlooked, with recent studies showing that the interface between newly produced pericellular matrix and biodegradable/dynamic hyaluronic acid-based hydrogels modulates MSC differentiation with some correlation to hydrogel crosslinking density [250,251]. This implies the effect of matrix cues may vary over time, with short-term impact heightened and long-term impact reduced, although such aspects are not well understood. It may be that spatial variations are important, with cues normally localised in the pericellular matrix having a more profound effect than cues from the inter-territorial matrix. The importance of laminin cues may be a clear example of this since cells interact more closely with laminins than collagen type II or aggrecan. Experimental systems must be designed that can determine exactly which biomaterial cues are masked in the short- and long-term and how these cues can be exploited through spatial and temporal patterning to direct regional effects.

One of the main inhibitors of progress in this regard is the difficulty of achieving spatiotemporal control over stiffness and viscoelasticity in existing hydrogel systems, along with spatiotemporal presentation of biochemical cues. Graphene-based delivery systems have been used to provide sustained local delivery of growth factors and have additionally been incorporated within collagen hydrogels for cartilage engineering and
MSC differentiation \[252,253\]. Work could focus in the short-term on the development of small molecule delivery systems such as graphene-based and BMP2/7-, GDF5/6-, and TGFβ-laden hydrogels \[253\] to help understand cell modulation and which cues are masked. In the longer-term, once comprehensive matrix instructive systems exist, work would be needed to replace growth factors entirely using a combined selection of more stable matrix cues.

4.4. Spatially Controlled Patterning of Matrix Cues Using Biofabrication Strategies

Advanced manufacturing techniques are increasingly being applied to model complex tissues and pattern matrix molecules with the aim of achieving spatiotemporal control over matrix cues, including electrospinning and 3D bioprinting. Electrospinning uses high-voltage electric fields to project polymer fibre melts and solutions onto charged surfaces at varying length scales and can produce highly aligned multi-layer fibre scaffolds \[254\]. The technique is therefore a popular tool for replicating AF fibre organisation, plus scaffolds for AF tissue engineering, both acellularly and in combination with AF and stem cells \[255–259\]. The technology has also been applied to model the AF-CEP interface \[260\], an intriguing approach that exploits electrospinning’s capabilities particularly well. Notable attempts have been made to investigate and maintain the phenotype of AF and stem cells in electrospun multi-lamellated IAF and OAF constructs by varying fibre diameter, orientation, and other physical cues \[261–263\], revealing much about the possible structure–function relationship of AF tissue and its component cells. For instance, AF-derived stem cells seeded on aligned polyurethane (PU) scaffolds have been shown to become elongated and better aligned, exhibiting heightened production of collagen type I and aggrecan compared to cells cultured on non-aligned scaffold \[264\]. AF-derived stem cells have additionally been shown to respond to fibre size, with AF-like morphology and phenotype promoted by the presence of larger fibres \[265,266\]. Electrospun scaffolds have also helped shed light on the stiffness- and topographical-dependency of Yes-associated protein (YAP) in AF-derived stem cells, hinting at electrospinning’s ability to modulate stem cell behaviour using structural and physical matrix cues \[267,268\].

It is notable how few attempts there have been to use electrospun systems incorporating biochemical or physical matrix cues within an IVD context \[268\]. Electrospun fibres containing dECM have been shown to be beneficial for the creation of scaffolds for cartilage tissue engineering; however, such outcomes have yet to be comprehensively demonstrated for IVD \[269\]. One of the only clear examples of biochemical cue inclusion using this method was achieved by blending TGFβ into a polymer scaffold solution before electrospinning. Seeded AF cells subsequently produced greater amounts of GAGs and collagens than when scaffolds lacking TGFβ were used \[270\]. More of this is needed moving forward, preferably with core matrix components as opposed to growth factors, although this is a technical challenge. It must be considered, however, that whilst electrospinning is a valuable tool, the technology is ultimately inappropriate for designing whole tissue engineered IVD models in a research or clinical setting. The creation of electrospun IVD models traditionally relies on the manual rolling of the AF and injection of an NP-like hydrogel, a labour-intensive process which introduces discontinuities between regions \[271–273\], whilst the stiffness of electrospun materials has generally been an order of magnitude higher (~MPa) than native ECM (~kPa). Any mechanical cues transmitted to cells are therefore not representative of those present in the native environment, even if the models are structurally useful. Nevertheless, several electrospun constructs have been implanted into small and large animal models \[192,274,275\], and electrospinning is likely to remain a core technique, both for basic IVD research and for the creation of total disc replacement devices and other tissue-engineered approaches.

An alternative scalable approach used to model NP, AF, and whole IVDs is 3D bioprinting. Bioprinting is an umbrella term for a powerful set of 21st century techniques including vat photopolymerisation, material jetting, and extrusion-based bioprinting, which allow the precise spatial delivery of cells, matrix components, growth factors, and
biomaterials [276–279]. This establishes bioprinting as an invaluable tool for the creation of multi-material ex vivo culture systems for basic research. The printing of low-viscosity materials such as hydrogels, whilst beneficial for cell culture, remains a challenge, and the shape fidelity of printed constructs is often compromised. To ensure fidelity and to main high cell viability, strategies such as Freeform Reversible Embedding of Suspended Hydrogels (FRESH) and Suspended Layer Additive Manufacturing (SLAM) have been developed to restrict fluid flow and improve printability [280–284]. These technologies could in theory allow the patterning of matrix cues in specific locations and concentrations within bioprintable hydrogel systems. IVD cells have been bioprinted to model NP and AF tissue using shear-thinning hydrogels; however, the development of biochemically and physically relevant printable biomaterials for IVD is a major bottleneck [285], particularly when modelling the stiffer AF region. To address this, hydrogels, including gelatine- [286] and gellan gum-based [287], have been co-printed with PCL scaffolds, increasing the stiffness of the bioprinted constructs. In one instance, CTGF and TGF-β were included [286], demonstrating that growth factors could be delivered in a spatially controlled manner and used to induce bone marrow stromal cell differentiation towards NP and AF cell phenotypes in different disc regions. Several attempts have also been made to bioprint hydrogel scaffolds that mimic the AF’s lamellar structure and the changing biochemical and physical properties across the OAF, IAF, and NP interfaces [269,288–293]. It is therefore possible to foresee the creation of whole IVD models for basic and clinical research, with IVD constructs being designed with the aid of patient MRI data [294,295].

It seems likely that bioprinting will be applied in combination with other advanced manufacturing technologies such as melt electrospinning direct writing (MEDW), the layer-by-layer assembly of melted fibres. This combined approach, sometimes termed hybrid bioprinting [296], has the potential to deliver matrix-reinforced cell-seeded hydrogels with tailored mechanical properties [297,298]. Whilst much of this research is in its early stages, the design and fabrication of hybrid MEDW-hydrogel systems for cartilage engineering is of particular relevance to IVD researchers, as there are several examples of chondrocyte- and ADSC-based systems [299–302] that could serve as inspiration for IVD models incorporating matrix cues. Since the importance of many matrix cues for IVD health and discogenic differentiation has been established (Table 1), the application of these cues within multiphasic biofabricated IVD and stem-cell-based systems seems a logical next step. The results of these experiments could pave the way for the creation of a series of developmental, healthy, and degenerate IVD models where the controlled use of relevant matrix cues leads to the design of regenerative therapies with better clinical outcomes.

5. Conclusions

The developing, healthy, and degenerate IVDs are primarily composed of ECM. Understanding the influence of biochemical and physical matrix cues on cellular activity and matrix production is therefore vital for the development of better clinical therapies targeting back pain. Lately, progress has been made in the design of experimental systems capable of delivering key matrix cues. The development of laminin- and proteoglycan-like mimics is a particularly encouraging step, although the development of biomaterials able to deliver spatially and temporally controlled cues remains a major challenge moving forward. Tissue-engineered systems, created using electrospinning and 3D bioprinting, are increasingly being used to create structurally relevant NP, AF, and CEP models incorporating a range of disc- and stem-cell types, and this enables the investigation of regionally specific matrix cues in vitro and in vivo within whole IVD systems. In the future, efforts should focus on the development of bioprintable materials capable of delivering physiologically relevant biochemical and physical matrix cues in combination with other advanced manufacturing techniques such as MEDW. If this is achieved, the effect of individual matrix cues can be better understood and employed in the design of efficacious regenerative therapies.
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