Towards an optimal microenvironment for nucleus pulposus regeneration: a glycobiology approach

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Chapter 5

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I certify that the thesis is all my own work and I have not obtained a degree in this University, or elsewhere, on the basis of this work.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4S-StarPEG</td>
<td>Poly(ethylene glycol) Ether Tetrasuccinimidyl Glutarate</td>
</tr>
<tr>
<td>AAA</td>
<td><em>Anguilla anguilla</em> agglutinin</td>
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<td>AAV</td>
<td>Associated-AdenoVirus</td>
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<tr>
<td>ADAMTS</td>
<td>A Distintegrin and Metalloproteinase with Thrombospondin Motifs</td>
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<td>ADSC</td>
<td>Adipose tissue Derived Stromal Cells</td>
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<td>AIA</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>APS</td>
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<td>BCA</td>
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<td>Brain-Derived Neurotrophic Factor</td>
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<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
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<td>Chondroitinase ABC</td>
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<td>CXCL</td>
<td>Chemokine CXC Motif Ligand</td>
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<td>DAPI</td>
<td>4′,6′-diamidino-2-phénylindole</td>
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<tr>
<td>DDD</td>
<td>Disc Degeneration Diseases</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>DIDS</td>
<td>4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid</td>
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<td>DMAEMA</td>
<td>N,N-DiMethylAminoEthyl MethAcrylate</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>ECM</td>
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<td>EDC</td>
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<td>HIF</td>
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<td>HMW</td>
<td>High Molecular Weight</td>
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<td>HNF-3β</td>
<td>Hepatocyte Nuclear Factor 3-Beta</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>HS</td>
<td>Heparan Sulfate</td>
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<td>Inner Annulus Fibrosus</td>
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<td>IGF</td>
<td>Insulin-like Growth Factor</td>
</tr>
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<td>IFN</td>
<td>Interferon</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>ITS</td>
<td>Insulin Transferrin Selenium</td>
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<td>IVD</td>
<td>Intervertebral Disc</td>
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<td>KS</td>
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<td>LMW</td>
<td>Low Molecular Weight</td>
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<td>Paraformaldehyde</td>
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<tr>
<td>WFA</td>
<td><em>Wisteria Floribunda</em> Agglutinin</td>
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<tr>
<td>Xyl</td>
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<td>XT-I</td>
<td>Xylosyltransferase I</td>
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Abstract

Neck and low back pain are the two highest causes of job-related disability in the UK and the USA. These pathologies are strongly related to intervertebral disc degeneration (IVD). Disc degeneration diseases (DDD) are characterised by changes in extracellular matrix (ECM) composition which lead to an important disorganisation of IVD tissue. Better knowledge of IVD biology and DDD in the last decades has promoted the development of new tissue engineering approaches to restore the disc function from a biological viewpoint. The ultimate objective of this thesis was to develop an optimal functionalized cell delivery system using an ECM-mimicking injectable hydrogel that enhances the production and the deposition of newly synthesised ECM to aid the regeneration of NP tissue. It was hypothesised that the modulation of the glycoenvironment of NP cells will promote the maintenance of their phenotype.

In the first phase of this thesis, an injectable type II collagen hydrogel stabilised with poly(ethylene glycol)ether tetrasuccinimidyl glutarate and supplemented with hyaluronic acid was successfully developed. The hydrogel system was stable in culture and had the capability to support cell growth. In addition, NP cells maintained a low type I collagen expression and the cell morphology after culture in the hydrogel. These characteristics, in addition to its injectable properties, make this hydrogel a promising candidate as a carrier of cells for future translation in vivo. The results obtained in this study highlighted the importance of ECM composition on NP cell behaviour. Highly glycosylated, the ECM of IVD tissue plays a crucial role on cell behaviour and IVD biology. Therefore, as a step forward, the glycoenvironment of the IVD was mapped in an effort to understand IVD glycoenvironment and its impact on IVD biology. A subset of specific and selective histological markers to distinguish the cell and ECM phenotypes of NP, AF and cartilage tissue and their stage of maturation was identified. The detailed CS composition and quantity of chondroitin sulfates (CS) revealed a change in sulfation pattern of CS with maturity. The depletion of CS has been shown to greatly affect IVD biology of the intervertebral disc and CS were chosen for the investigations conducted in the last part of this thesis. Therefore, the behaviour of GAGs, specifically of CS, and xylosyltransferase I (XT-I) and glucuronyltransferase I (GT-I), two key enzymes involved at crucial points of CS synthesis, was evaluated in a
bovine ageing IVD model. Important changes in GAGs composition during disc ageing were highlighted in this study. CS, specifically, were affected at a structural and quantitative levels with important changes in sulfated disaccharide composition upon ageing. A correlation between the expressions of XT-I and GT-I and CS content was shown in this study. The delivery via electroporation restored the expression of both enzymes at a protein level. A trend, although not significant, towards the increase of CS production after delivery of XT-I and GT-I was seen. In accord with the results of this study, the best therapeutic approach to modulate the expression of GAGs might be a dual delivery of XT-I and GT-I or in combination with aggrecan protein core up-regulation.

Glycans were shown in this thesis to be essential to IVD biology. A better understanding of their effects on cell behaviour will promote the development of new biological tissue engineering approaches for IVD regeneration.
Introduction

Sections of this chapter have been published and are under submission:

1.1. Introduction

Neck and low back pain are the two highest causes of job-related disability with significant associated social and economic costs [1], accounting for total annual healthcare costs estimated at £11 billion in the UK in 2000 [2], and between $50 to $90 billion each year in the USA [3]. Every year, 6.3 to 15.4% of the population experience low back pain for the first time with a recurrence rate of 24 to 80% [2]. The overall prevalence of these pathologies occurs from early twenties with a constant increase with age until 60-65 year-old [3]. Neck and low back pains are multifactorial disorders with many possible aetiologies from genetic to occupational factors [4]. Strongly associated with intervertebral disc (IVD) degeneration [5], these pains are defined epidemiologically as Òa process related to normal ageing as well as changes related to physical loading over the lifetimeÓ [6].

Disc degeneration diseases (DDD) are defined as Òan aberrant cell mediated response to progressive failureÓ [7]. The onset of the degeneration process results in the IVD being inherently incapable of adequate self-repair [5]. Currently, the most prevalent treatment modalities for spinal problems associated with DDD involve conservative methods including physical therapy [3], drugs [8] and interbody fusion surgery [1, 3, 9]. Interbody fusion is one of the gold standard procedures used for the treatment of DDD. However, this technique has many disadvantages; the main being the loss of a range of motion [9]. Tissue engineering strategies offer many potential advantages in the treatment of DDD by aiming to recover the mechanical properties of the disc. These approaches designed to restore the disc function from a biological viewpoint will have a significant impact in the near future on medical practices and will significantly reduce the associated social and economic costs [10].

1.2. Intervertebral Disc (IVD)

1.2.1. Structure and Function of the IVD

The spine is the central part of the skeleton which provides stiffness, support and mobility to the body, and protection to the spinal cord. It is composed of 33 bony vertebrae classified in five different categories according to their location: cervical (7), thoracic (12), lumbar (5), sacral (5) and coccygeal (4) vertebrae. These vertebrae are considered as load transmitting units which rest on the IVD [11, 12]. This structure is the major load bearing element especially during lateral and posterior shear, axial compression and flexion [12] (Figure 1.1).
The IVD lies between the vertebral bodies. These vertebrae are covered on both sides by a thin layer of hyaline cartilage, the cartilage end-plates, which links the IVD tissue to the vertebral bodies [5, 12, 13]. In the centre is found the nucleus pulposus (NP), a highly hydrophilic tissue. This tissue exhibits significant viscoelastic properties allowing the deformation of the IVD under loads [14, 15]. This deformation is confined by the annulus fibrosus (AF), a fibrous tissue firmly attached to the vertebral bodies and cartilage end-plates [12, 13]. This anatomic arrangement permits the AF to limit the vertebral movements, to transmit the loads from vertebrae to vertebrae and to maintain the pressure on NP while the NP provides flexibility to the structure [15]. In addition, the AF is reinforced by the presence of the anterior and posterior longitudinal ligaments [12, 13] (Figure 1.1).

1.2.2. Development of the IVD

During embryonic development, cells derived from the endodermal layer separate to form the notochord, which is a tissue precursor of the axial skeleton. The notochord is then surrounded by a thick three-layer of collagenous fibres with a large diameter containing mesenchymal stem cells [16]. This thick connective tissue sheath condenses around the notochord [17, 18]. The larges vacuoles present in the notochordal cells provide stiffness to the combined structure. Subject to compression, the structure of the developing embryo is then elongated and splits to form the vertebral bodies and the IVD structures [16, 19-22]. This phenomenon is under tight regulation by numerous signalling molecules and transcription factors such as Foxa2/HNF-3β, the secreted patterning factor Shh, brachyury, T-Box and Noto [20, 23].

1.2.3. Tissue Composition

The IVD tissue is a largely avascular and aneural tissue with a lack of nerves and vessels in its inner part and few in the outer superficial region of the AF [24-26]. The NP tissue, isolated during development from the vascular system, does not acquire an immunological tolerance. By this lack of vascularisation, the IVD is a relatively immuno-privileged zone which can, by consequent, trigger an autoimmune response if exposed to the immune system [27, 28].
Figure 1.1. Representation of the anatomical structure of the IVD. The nucleus pulposus (NP), a gel-like structure, is surrounded by a concentric fibrous ring: the annulus fibrosus (AF). The fibres of this tissue are attached to the vertebral bodies and the cartilage end-plates linking both vertebrae. The anterior and posterior longitudinal ligaments reinforce the function of the AF [12, 13].
Composed of two predominant tissue-types, the NP and AF, IVD tissue has a characteristic volume fraction of cells lower than that of other tissues [29], and which greatly decreases upon maturity [30]. The number of cells and the composition of the IVD vary according to the presence of notochordal cells, the age and the species [29]. The main metabolic activity of the IVD cells occurs mainly by consumption of oxygen and glucose and results in the formation of metabolites (lactic acid essentially) [5, 22, 31, 32]. The fluid movement within the highly hydrated extracellular matrix (ECM) allows the diffusion of the metabolites from the cells through the matrix to the capillaries of the cartilage end-plate and the outer AF [31, 33, 34].

1.2.3.1 Nucleus Pulposus (NP)

The NP is composed of a loose network of type II collagen fibres strongly associated with a high content of proteoglycans (PGs) to form a highly hydrated gel-like structure [5, 13, 23, 35].

- Cells

Two main cell-types, notochordal and NP cells, are present in this tissue in an age and state dependent manner. Both cell-types together occupy less than 1% of the total tissue volume [13].

Notochordal cells are large cells with a size greater than 30µm. They present a prominent actin cytoskeleton containing large vacuoles-like structures [36] that occupy greater than 25% of the cell volume [16, 18, 21] and provide the mechanical properties of the notochord tissue (see section 1.2.2). Notochordal cells persist throughout most of the adult life of some species while in other species they gradually disappear. In humans, for example, these cells disappear around the age of ten years [18, 21, 37]. The nature and role of the notochordal cells are still not well defined. It has initially been hypothesised that these cells play a role of organiser cells from their notochordal origin by directing the migration of the chondrocytes from cartilage end-plate and by stimulating the matrix synthesis [38-40]. Their disappearance is explained, in this case, by their entrance in apoptosis or necrosis following the fulfilment of their role. The other theory more widely adopted by the scientific community is a role for the notochordal cells of NP progenitor cells which undergo terminal differentiation forming the NP cells [38, 40, 41]. Producing a large amount of hyaluronic acid (HA) and glycosaminoglycans (GAGs) during the
development [5, 38], notochordal cells were shown to promote the synthesis of GAGs and aggrecan protein core [42]. They also produce themselves large chondroitin sulfate-proteoglycans (CS-PG), which, though a product of the aggrecan gene, differs structurally from the aggrecan present in mature tissue by the absence of keratin sulfate attached on the aggrecan core [13]. Brachyury [16] and galectin-3 were described as markers for notochordal cells. However, galectin 3 has recently been reconsidered as a specific marker due to the evidence of its expression in adult tissue and on the surface of NP cells [16, 43].

With a round morphology and a diameter of 10µm [18], NP cells were historically described as chondrocytes or chondrocytes-like cells by exhibiting a similar morphology and producing a similar ECM to those of chondrocytes [5, 44, 45]. However, in the recent years, variances between NP cells and chondrocytes have been identified. These differences explain the differential response of both tissues to their environment [45-47]. Substantial evidence in the recent years favours a notochordal origin to the NP cells [16, 38, 44] as opposed to a chondrogenic origin [39]. However, it remains unknown if NP cells are derived from the large vacuolated notochordal cells or if they are the result of aberrantly transformed notochordal cells [41]. As for the chondrocytes, NP cells are scattered and surrounded by a capsule in the tissue [5]. These cells have a low cell density of 4,000 cells/cm² [48] and represent less than 0.25% of the total volume of the tissue [45]. NP cells differ morphologically from chondrocytes by the presence of cytoplasm-filled processes. These processes, which penetrate some distance into the matrix, are believed to be mechano-sensors which respond to the mechanical stresses of the NP [45]. So far, no on/off marker have been identified in order to distinguish the three cell-types: NP cells, AF cells and chondrocytes. Some markers have been identified as differently expressed by the different cell-types such as cytokeratin-19 [49], FOXF1 [50], Pax-1 [50], CA-12 [50, 51], MGP [49, 52], more highly expressed in NP cells than in articular chondrocytes and AF cells, or GDF10 and CYLT-1 more expressed in articular chondrocytes [50], or GPC-3 and COMP highly expressed in AF cells [46, 49]. A differential matrix metalloproteinase (MMP) profile has also been observed in chondrocytes and NP cells. Indeed, MMP12 and MMP27 are not produced by NP cells while both of these enzymes are secreted by chondrocytes [53]. It is worth noting that a high variability of marker expression has been described in the literature [49, 54]. This variability has to be taken into consideration for the translation of
preclinical studies to human. Articular chondrocytes and NP cells can also be distinguished by the ratio of glycosaminoglycans (GAG) and type II collagen that is produced which leads to the deposition of two different structural ECM. Mwale et al. have reported a ratio 27:1 of GAG:collagen content for NP tissue as opposed to a ratio of 2:1 for hyaline cartilage [47].

Recent studies suggest the presence of progenitor cells [55-57] and the maintenance of remaining notochordal cells [16] within the NP tissue. A specific progenitor cell population Tie2 and GD2 positive was identified by Sakai et al. as active progenitor cells which markedly decrease with age and degeneration [57]. Their presence suggests a potential self-healing process of the IVD tissue [16, 57]. Both cell-types were described as being able to form colonies, to proliferate and to differentiate towards mesenchymal and NP lineages [16, 55-57].

- ECM

The ECM of the NP is unique in its composition and provides specific mechanical and biological functions the tissue [7, 14, 58]. NP tissue is composed of collagen and PGs. Type I collagen and type II collagen together make up to 80% of the total collagen content [5]. Small amounts of type VI, IX, X and XI collagens are also present. A role in tissue remodelling and organisation is attributed to these collagens [5, 59, 60]. Type II collagen in NP [44] is associated with PGs of which aggrecan has the highest concentration [23, 37, 61-63]. Mwale et al. described a ratio of PGs to collagen as 27:1 in NP tissue [47] which is greater than that of cartilage or AF tissue. Aggrecan is a large PG which contains chondroitin sulfate (CS) chains and a lesser amount of keratan sulfate (KS) chains covalently bound to a protein core [64, 65]. It binds specifically to hyaluronan via the N-terminal globular domain of the protein core [66] to form high molecular weight aggregates [65, 67]. Link-proteins increase the stability of the formed structure by the formation of additional bonds between HA and aggrecan [66-68]. The aggregates are more resistant to dissociation after pH changes, elevated temperature, high concentrations of urea, and hyaluronate oligosaccharides. This aggregation immobilizes PGs within the network of type II collagen fibres, thus enhancing the ability of the tissue to resist compressive deformation and to allow a better load distribution [35, 67]. Other PGs such as versican and small leucin rich proteoglycans (SLRP) (decorin, luminican, biglycan and fibromodulin) are also expressed in the ECM of the NP tissue. The
SLRPs influence cellular ECM deposition and collagen assembly [20, 23, 69]. The high negatively charged GAGs enable the retention of water within the tissue and therefore its reversible deformation nature. Connective proteins, such as elastin which provides elasticity and flexibility to the tissue [11] and laminin which promotes cell adhesion [70], also contribute to the tissue deformation. As the PGs are one of the main components studied in this thesis, their structure, regulation and role are elucidated in section 1.3.

1.2.3.2 Annulus Fibrosus (AF)

The AF tissue is composed of highly organised concentric rings of collagen fibres which play a dual mechanical role of maintaining the pressurization of NP and of transmitting loads across adjacent vertebrae [15].

- **Cells**

AF cells, derived from the mesenchyme, vary in cell morphology according to their location in the tissue. The cells adopt different morphologies according to the degree of organisation of the AF. Therefore, cells from the inner AF (IAF) exhibit a spherical shape with cytoplasmic elongation similar to that of the nearby NP cells. Cells of the outer annulus adopt an elongated shape similar to that of fibroblast [69, 71]. Cells are then orientated on the collagen fibres under mechanical stresses applied physiologically to the tissue [72]. Their number is higher than that of NP tissue with $9 \times 10^6$ cells/cm$^3$ with a higher density towards the outer AF (OAF) [48]. As for NP cells, no distinctive markers to differentiate AF cells from NP cells and chondrocytes have been identified [44]. Nevertheless, relative expression of markers were shown as higher expressed by AF cells including type V [46, 52], type I and type III collagens, cadherin 13, decorin, versican [46], tenomodulin, TNFAIP6, FoxF1, FoxF2 and aquaporin [54] compared to NP cells and chondrocytes. However, as for the NP cells, a high interspecies variability is noted in the transcriptomic profiling of AF cells [73].

AF cells were shown to respond to chemokine secretion, especially CXCL10 which stimulate AF cell migration in the annular defect [74]. This observation suggests a small potential for self-healing of the AF tissue which has been described previously [73, 75]. This hypothesis is accentuated by the presence of progenitor cells within the AF. These cells were shown to differentiate towards the
mesenchymal lineage [55, 56]. Further investigations have shown the potential of AF cells to dedifferentiate towards an AF progenitor cells after drug treatment [76].

- ECM

The ECM of the AF is highly organised in 15 to 25 concentric lamella of collagen fibres oriented approximately 60° to the vertical axis with alternating right and left lamellae [5, 69]. The composition of the ECM varies from OAF to IAF providing different mechanical properties and cell morphology to these two regions of the AF. The IAF ECM has a composition closer to that of NP tissue with an accumulation of type II collagen and GAGs while the OAF is mainly composed of type I collagen fibres [5, 23, 69] providing strength to the tissue [14, 77]. Along with type I collagen, type II, III, V, VI, IX, X and XI collagens are found [46, 52, 78]. The high deformation properties of the tissue are provided by the elastin network present between the lamella [5, 11], and the high PG content in the form of aggregcan, versican and SLRP such as biglycan, decorin, fibromodulin and lumican [23, 69, 79, 80]. These PG also play a role in the sequestration of growth factors within the tissue [69] and in the organisation of collagen fibrillogenesis [69, 81]. The GAGs associated with these PGs are therefore at a lower ratio than that observed in NP tissue (1.6:1, GAGs:coll) which allows the distinction of AF cells from NP cells [47].

1.2.4. Disc Degeneration

The progressive degeneration process is a multifactorial process [82] involving nutritional [5, 34], mechanical [83], biochemical [84-86] and genetic [63, 87-89] factors (Figure 1.2). It is difficult to distinguish DDD from the general process of IVD ageing with an absence of morphological or biochemical markers which allow the distinction of both processes [13]. The disappearance of notochordal cells has been shown to precede the degeneration process. However, the link between both phenomena is still unclear [18, 38]. The loss of notochordal cells coincides with the loss of PGs, one of the main features of IVD degeneration and ageing. During DDD, the tissue changes in morphology with an important disorganisation of the ECM. Annular lamellae become irregular, bifurcating and interdigitating, thus creating tears within the tissue [13, 90]. Collagen and elastin networks of the AF become disorganised and the PG content of the NP decreases leading to the formation of a less gel-like structure and a more fibrous tissue [5, 21]. The tissue, initially avascular and aneural, is invaded by vessels and nerves [91].
At a healthy state, a balance between anabolic and catabolic activities is observed in the tissue. The tissue is continually remodelled and the expression of matrix degrading enzymes matches the deposition of ECM [11, 13, 35]. During ageing and degeneration process, calcification of the cartilage end-plate occurs and limits oxygen and nutrient supplies. Cells undergo an anaerobic metabolism with high production of lactic acid [33, 34, 90] resulting in a decrease of pH within the tissue. ECM proteins are modified in composition and/or degraded. A decrease of CS chain length and an increase of KS chains are observed due to the decrease in oxygen supply. Therefore, cells do not respond adequately to mechanical or chemical signals and enter in an apoptotic state [29].

IVD cells, during the degeneration process, have been shown to produce many cytokines, such as IL-8, IL-6, IL-1β, IL-17 and TNF-α [86, 92, 93], and inflammatory mediators such as PGE2 and nitric oxide [93]. These cytokines and proinflammatory factors activate signalling pathways inducing the production of inflammatory, neurotrophic and angiogenic factors. Although TNF-α has been suggested to be implicated in disc degeneration, IL-1β seems the key molecule involved in the process of disc degeneration [94-96]. A direct link between the production of neurotrophic factors such as BDNF, neurephin 3, neuropilin 2 and NGF [24, 97, 98] and angiogenic factors such as VEGF GF family [26, 97, 99], and the activation of NFκB and MAPK pathways by IL-1β and TNF-α has been made [100, 101]. Along with these factors, a high secretion of matrix degrading enzymes under the form of matrix metalloproteinases (MMPs) and aggrecanases is observed. The induction of their expression and the repression of tissue inhibitor of matrix metalloproteinases (TIMP-1 and -2) play a crucial role in the degeneration of the ECM [96]. This deregulation is induced by the cytokines secreted by the IVD cells themselves or by the recruited macrophages after injury. IL-1β and TNF-α are believed to be the main activators of matrix degrading enzymes and inhibitors of TIMP [84, 86, 96, 102]. A wide range of MMPs and aggrecanases are secreted during disc degeneration including MMP-1-3, 7-10, -13, -14, -19 and -28 [85, 88, 96], a distintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and -5 [86, 102]. Their degrading action on the ECM leads to a loss of mechanical properties of IVD tissue [58] due to the loss in hydration [90]. Type II collagen and
Figure 1.2. Degeneration process and window of treatment. The optimal treatment window is narrowed to early to middle degeneration stages. At a late stage, the process is considered as irreversible leading to a total disc replacement or vertebrae fusion. Figure adapted from [104, 105].
type I collagen turnover leads to the formation of a more fibrous tissue. MMPs permit the infiltration of nerves and vessels ultimately induces lumbar pains experienced by the patient [91, 103].

1.3. Glycans

Particularly rich in glycans [23], the ECM of IVD tissues differs between tissue-types according to their functions and their status [20]. Glycans are an important part of the research performed in this dissertation. Their structures and their roles are described subsequently with a specific focus on CS and HA, two GAGs essential to the biology of the IVD.

1.3.1 Glycan Structure, Synthesis and Motifs

Glycan structures play an essential role in cell-cell and cell-matrix communications [106]. These communications contribute to maintain / regenerate tissues at a healthy state [107-109]. Highly and tightly regulated, glycans regulate signals that control cell differentiation, normal physiology, and even neoplastic transformation [107-111]. Glycans are post-translational modifications, free or carried by secretory or membrane anchored lipids and proteins [110, 112-114]. Coded by over 500 genes, their synthesis starts within the rough endoplasmic reticulum with the linkage of the first sugar and continues in the Golgi apparatus where most of the structural variations are performed [110, 112]. The biosynthesis of glycans is not template driven and involves coordinated expression of multiple glycosyltransferases, some of which have additional tissue-specific isoforms [113]. The two possible anomeric configuration of sugars (α/β), their different linkages (1→ 1, 2, 3, 4, 6 etc...) and ring sizes lead to a multitude of glycan conformations. Their complexity is accentuated by a lack of proof-reading, the branching of multiple glycans and the additional site-specific substitutes such as acetylation, phosphorylation and sulfation [112]. Different topological recognitions and structures are obtained which lead to multiple functions [110, 113]. Six families of glycans have been identified to date (Figure 1.3):

- **N-glycans**, branched polymeric structure with an initial glycan N-acetylgalactosamine (GalNAc) residues linked to an asparagine (Asn) residue of the Asn-X-Ser/Thr peptidic motif (Ser and Thr indicate serine and threonine).
- *O*-glycans, branched polymeric structure with an initial glycan a N-acetylglactosamine (GalNAc) or N-acetylgulosamine (GlcNAc) or mannose (Man) or fucose (Fuc) or glucose (Glc) residue linked to a Ser/Thr residue.

- GAGs, linear polymers linked to a Ser residue by an initial xylose (Xyl). This family includes heparan sulfate (HS), CS and dermatan sulfate (DS).

- Glycolipids, linked on ceramides with an initial motif galactose (Gal) or Glc-β-(1→4)-Gal residues.

- GPI-anchors, on which are attached the glycan chain GalNAc(NH₂)-α-(1→4)-Man-α-(1→6)-Man-α-(1→2).

- Hyaluronic acid (HA), a linear polymeric structure of the family of GAGs. This glycan is considered differently as it is not linked to any protein or lipid core [110, 113].

Glycans are located in the environment of many proteins such as growth factors, cytokines, receptors and enzymes modulating their action [115, 116]. Present at a cellular level, they constitute a coat on the cell surface referred to as glycocalyx. The glycans modulate protein and cell activities at cellular and ECM levels by their interaction with lectin peptidic domains and by their influence on protein conformations [114, 116, 117]. Further fucosylation and sialylation cap many N- and O-linked glycans. These modifications contribute to the glycan diversity and their involvement in cell-cell and cell-ECM communication. The knockout of fucosyl and sialyltransferases have provided evidence of a specific glycosignature mediating the cell biology [113, 118].

1.3.2 Glycosaminoglycans (GAGs)

Four different families of GAGs are known to date: CS, DS, HS and HA. DS, HS and CS are attached to a protein core forming PGs [119] while HA is the only free and non sulfated glycosaminoglycan in the body. HA has an important structural role in the ECM [66] but also possesses many biological functions [120] (Section 1.3.2.2).
Figure 1.3. Chemical diversity of glycans. Different classes of glycans have been described: N- and O-linked glycans, ceramides and GAGs. Along with the typical glycan backbones, fucosylations and sialylations occur which contributes to the glycan diversity. HS, CS, DS and HA correspond to heparin sulfate, chondroitin sulfate, dermatan sulfate and hyaluronic acid, respectively [110, 113].
GAGs, such as CS [121, 122], HA [123, 124] and HS [125, 126], are often used in scaffold for IVD and cartilage research. Their role, even if known as essential, is often underestimated for tissue regeneration mainly due to a lack of knowledge of their biological properties. Furthermore, their inappropriate use in therapeutics can cause severe side effects [127]. Recent studies have shown that cell behaviour is not only affected by the structure of GAGs but also by their sulfation patterns and size [128-131]. Structure, synthesis, turn-over and biological function of CS and HA, GAGs highly abundant in the ECM of the IVD [47, 132], are described below.

1.3.2.1 Chondroitin Sulfate Family

CS are GAGs attached to serine residues of a protein core. This structure forms PGs providing structural and biological functions to these proteins. Sixteen cell surface and ECM-linked CS-PGs have been identified to date. Differentially expressed in the body, they are associated with diverse role in the tissues (Table 1.2).

- Structure and Synthesis

CS chains consist of a repeat disaccharide motif formed by glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) [(GlcA-β-(1→3)-GalNAc-β-(1→4)]n]. The synthesis of these O-linked glycans is initiated via a conserved tetrasaccharide Xyl-Gal-Gal-GlcA, where Xyl and Gal indicate xylose and galactose, respectively [133]. This first step consists of the attachment of a xylose group on the serine/threonine residues [134] by a link Xyl-β-Ser [133-135]. This reaction is catalysed within the endoplasmic reticulum (ER) by two enzymes, the xylosyltransferases I and II (XT-I and XT-II). The structure is then transferred within the golgi apparatus where further residues (GlcA and GalNAc residues) are attached through the successive activity of glycosyltransferases (GalNAc transferases, GlcA transferase, chondroitin synthases, CS N-acetylgalactosaminyltransferases I and II, CS glucuronyltransferases and CS polymerising factors) [135-137].

- Regulation of CS Synthesis

The control of CS synthesis remains unclear. The regulation of their synthesis occurs at gene, enzymatic and turnover levels. The enzymes involved in their synthesis are under the control of growth factors such as TGF-β which regulates the production of CS in many tissues [128, 138]. Other growth factors such as BMP-2 and transcripton factors such as TonEBP (Tonicity sensitive transcription factor) and
HIF-1/-2 (hypoxia sensitive transcription factor) take part in the regulation of the enzymes of synthesis of CS [139-141]. At an enzymatic level, the rate-limiting nature of XT-I and XT-II allows an auto-regulation of CS synthesis from an early stage [142]. The elongation of the CS chains is also controlled via successive phosphorylation and sulfation phenomena which either enhance or repress the synthesis [143, 144]. For example, the sulfation of galactose residues stimulates CS chain initiation by increasing the affinity substrate to enzyme [143]. The knock-down of the chondroitin 4-O-sulfotransferase-I suggests that this enzyme facilitates the elongation of CS chains by chondroitin polymerases by affixing a sulfate group in position 4 of the GalNAc [137, 145]. Conversely, an inhibition of the synthesis is obtained after a 3-O-sulfation of the GlcA residue on the initial linkage tetrasaccharide or on the glycan in final position of the chain [146, 147].

- **Modification of Sulfation**

CS structure is modified by epimerization and sulfation at various degrees of the glycan chain providing high specificity and various biological activities [148]. Non-sulfated to trisulfated forms of CS are found. Sulfation can occur on the carbons 2 and 3 of the GlcA and on the carbons 4 and 6 of the GalNAc. Four types of CS are present in the ECM: CS A, C, D and E known as chondroitin-4-sulfate (C4S), chondroitin-6-sulfate (C6S), chondroitin-2,6-sulfate (C2,6S) and chondroitin-4,6-sulfate (C4,6S), respectively [149].

Sulfation patterns are generated from the simultaneous orchestration of different sulfotransferases in the golgi apparatus [150]. Different sulfotransferases have been identified to date: chondroitin/dermatan 4-O-sulfotransferase (C4ST/D4ST), chondroitin 6-O-sulfotransferase (C6ST), uronosyl 2-O-sulfotransferase (UA2OST), and N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase (GalNAc4S6ST). Three C4ST isoforms, two C6ST isoforms, D4ST-1 UA2OST and GalNAc4S6ST have been identified in mammals [145, 150]. These golgi-associated transmembrane proteins are present in very low quantity. Highly specific, they recognize a specific monosaccharide, its anomeric configuration (α or β) and the type of glycosylation (N-link or O-link) of the acceptor sugar (Table 1.1) [134].

The sulfation of glycan occurs by the transfer reaction of a sulfate group from a 3 phosphoadenosine-5 phosphosulfate (PAPS) on a hydroxyl group.
Figure 1.4. Sulfation of chondroitin sulfate (CS) process. (1) The inorganic sulfate is up-taken by the cell through active sulfate transporters regulated via DIDS inhibition. (2) After entrance in the cytosol, the inorganic sulfate ($\text{SO}_4^{2-}$) molecule is grafted on a 3$\text{\textacute{A}}$denosine-5$\text{\textacute{A}}$diphosphate (ATP) by ATP sulfurylase resulting in the formation of 3$\text{\textacute{A}}$denosine-5$\text{\textacute{A}}$diphosphosulfate (APS) and subsequent release of a molecule of inorganic phosphate (PPI). (3) The APS molecule is then converted to an active high energy form, the 3$\text{\textacute{A}}$denosine-5$\text{\textacute{A}}$diphosphosulfate (PAPS), catalysed by APS kinase releasing a molecule of 3$\text{\textacute{A}}$denosine-5$\text{\textacute{A}}$diphosphate (ADP). (4) The PAPS molecule is then translocated within the golgi lumen via PAPS transporters (PAPST1 and PAPST2). (5) Finally, the sulfate group is grafted on the CS chain by sulfotransferases (summarized in Table 1.1) [145, 150, 151].
Table 1.1. Sulfotransferases involved in the sulfation of chondroitin sulfates in position 2, 3, 4 and 6.

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<th>Sulfation type</th>
<th>Name</th>
<th>Glycan acceptor</th>
<th>Abbreviations</th>
</tr>
</thead>
<tbody>
<tr>
<td>2S</td>
<td>Uronosyl 2-O-sulfotransferase</td>
<td>GlcA</td>
<td>UA2OST</td>
</tr>
<tr>
<td>4S</td>
<td>Chondroitin/dermatan 4-O-sulfotransferases</td>
<td>GalNAc</td>
<td>C4ST/D4ST</td>
</tr>
<tr>
<td>3S</td>
<td>HNK-1 sulfotransferase</td>
<td>GlcA</td>
<td>HNK-1ST</td>
</tr>
<tr>
<td>6S</td>
<td>Chondroitin 6-O-sulfotransferase 1</td>
<td>GalNAc</td>
<td>C6ST-1</td>
</tr>
<tr>
<td>4,6S</td>
<td>N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase</td>
<td>4S-GalNAc</td>
<td>GalNAc 4S-6ST</td>
</tr>
</tbody>
</table>
of the chondroitin in position 2, 3, 4 and 6. The first event of this process is the entrance of a sulfate molecule in its ionic form (SO$_4^{2-}$) to the cytoplasm via an active transport process through sulfate transporters regulated by phenomena such as 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) inhibition [151]. After entrance to the cytosol, the inorganic sulfate (SO$_4^{2-}$) molecule is grafted on a 3'-adenosine-5'-triphosphate (ATP) by ATP sulfurylase resulting in the formation of 3'-adenosine-5'-phosphosulfate (APS) and the release of an inorganic phosphate (PPi) molecule. The APS molecule is then converted to an "active" high energy form, the PAPS, catalysed by APS kinase which leads to the release of a molecule of 3'-adenosine-5'-diphosphate (ADP). The PAPS molecule is then translocated within the golgi lumen via PAPS transporters (PAPST1 and PAPST2). Finally, the sulfate group is grafted onto the CS chain by sulfotransferases (Figure 1.4) [145, 150, 151]. A rapid turnover of the sulfation pattern is obtained by the successive action of sulfotransferases and sulfatases [152]. This turnover allows fast changes in the sulfation pattern and consequently in cell-cell and cell-ECM communication [20, 128, 152].

- **CS Turnover**

CS degradation occurs at a cellular level predominantly in the lysosomes [153] but also by clearance through the vasculature and lymphatic systems [154]. Highly specific, the degradation is performed by endoglycosidases which cleave CS chains into fragments according to the glycan stereo-configuration, the glycosidic linkage, the substitution pattern of the backbone, the pH of the compartment and the glycan residues adjacent to the site of cleavage. Sulfatases remove the potential sulfations on the fragment [155] to allow the action of exoglycosidases which perform the final cleavage [156]. External factors such as nitric oxide produced during inflammation and angiogenesis have been shown to favour the cleavage of CS by breaking down the terminal part of their chains [157].

- **Roles of CS**

The sulfation and / or phosphorylation of the CS provide different functions of these molecules through changes in their conformation and their interactions with receptors / growth factors / ECM molecules [134]. Carried by a protein core, they provide biological activity to the PG not only by direct activity on cell signaling or GF interaction but also by modifying the PG conformation that allow for activation or repression activities (Table 1.2).
Table 1.2. Chondroitin sulfate proteoglycans (CS-PGs), their location, function and interaction.

<table>
<thead>
<tr>
<th>Proteoglycan name</th>
<th>Location</th>
<th>Function</th>
<th>Interaction GF/ECM</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RPTP β</strong></td>
<td>CNS</td>
<td>RPTP β is a proteoglycan (PG) receptor expressed by glial and neuronal cells which, after cleavage of its transmembrane domain, is released within the ECM under the form of phosphacan (see below). - Induces β-catenin phosphorylation by binding HB-GAM resulting in an increased level of cell-cell adhesion under its monomeric form in CNS. - Increases the cell migration under its oligomeric form after its glycosylation by N-acetylglicosaminyltransferase Vb and galectin 1 binding in CNS. A similar process in involved in other tissues where similar role of RPTP β has been observed. - Regulates neurite outgrowth. - Binds in a calcium dependent manner to both tenascins R and C and modifies the growth supportive substrate by interaction with cell adhesion molecules. - Plays a role in the maturation and maintenance of synaptic structure and function. - Is involved in the nerve fibres organisation during development and maturity, axonal growth, cell migration and myelination.</td>
<td>Galectin 1, HB-GAM, phosphacan, FGF-2, FGF-7</td>
<td>[109, 150, 158-160]</td>
</tr>
<tr>
<td>Proteoglycan name</td>
<td>Location</td>
<td>Function</td>
<td>Interaction GF/ECM</td>
<td>Ref.</td>
</tr>
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<td>------------------</td>
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</tr>
<tr>
<td>NG2 MW=248kDa CS chain=1</td>
<td>CNS, blood vessels, NP cells, thyroid, skin, muscle, blood cells</td>
<td>NG2 is a transmembrane PG mainly expressed in the CNS involved in the cell mobility. - Is expressed at the surface of oligodendrocyte progenitor cells and in a variety of cell types following injury of the CNS. - Contributes to the chemical barrier formed after brain or spinal cord injuries. - Promotes endothelial cell motility and angiogenesis by interaction with galectin 3 and $\alpha 3/1$ integrin (tumor development, wound healing). - Plays a pivotal role in the dissemination of cancerous cells. - Enhances several cancers (acute lymphoblastic leukaemia, acute myeloid leukaemia, glioblastoma, melanoma, sarcomas).</td>
<td>PDGF, galectin 3, $\alpha 3/1$ and $\alpha 4/1$ integrins, type V1 collagen, FAK/PI3Ka/ERK pathway.</td>
<td>[161-168]</td>
</tr>
<tr>
<td>CD44* MW=37kDa CS chain=1</td>
<td>All tissues</td>
<td>CD44 is a transmembrane glycoprotein with a wide variety of polymorphism and post-translational modification. - Is covalently modified by several GAGs (CS, DS, HS and KS). Both CD44s and CD44v variants are modified with CS chains. - Its glycosylation modifies binding and presentation of ECM molecules such as fibrinogen (enhanced by CS/DS modifications, or chemokines (mediated by HS and/or CS modifications. - Contains a globular head region containing lectin motifs that can bind to HA. This binding is regulated by post-transcriptional variations such as sulfation of CS enhancing the adhesion of the receptor to HA or sialylation down-regulating the</td>
<td>Interaction with HA, aggrecan</td>
<td>[116, 169-171]</td>
</tr>
<tr>
<td>Proteoglycan name</td>
<td>Location</td>
<td>Function</td>
<td>Interaction GF/ECM</td>
<td>Ref.</td>
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<tr>
<td>CD44* (following)</td>
<td>adhesion to HA.</td>
<td>- Is involved in stem cell homing when sialylated and CS by adhesion to E- and L-selectin. &lt;br&gt; - Activates cell migration by interaction with HA. &lt;br&gt; - Promotes cell proliferation by its interaction with HER-2. &lt;br&gt; Implicates cell homing (stem cells and lymphocytes homing).</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syndecans*</td>
<td>All tissues</td>
<td>Syndecans are transmembrane proteins which carry both HS and CS chains. Four different genes are present in vertebrae (Syndecan-1, -2, -3, -4). &lt;br&gt; - Play a role in development, and in the regulation of tumor cell growth and cell matrix interaction (adhesion and invasion). &lt;br&gt; - Are temporally and specifically expressed (Syndecan-1: epithelial and mesenchymal tissues; syndecans 2 and 3: neuronal and musculoskeletal tissue, syndecan-4: all tissues). &lt;br&gt; - Syndecan-1 regulates cell-cell and cell-matrix interaction. &lt;br&gt; - Syndecan-2, predominant syndecan expressed during embryogenic development, modulates TGF-β. &lt;br&gt; - Syndecan-3 interacts with FGF-2, HGF/scatter factor and TGF-β allowing the maintenance of skeletal muscle fibroblasts in an undifferentiated state. &lt;br&gt; - Syndecan-4 modulates FGF-2 signalling, regulates cell migration and controls cell adhesion. This syndecan interacts specifically with ADAMTS-5.</td>
<td>FGF-2, Fibronectin, Vimentin, laminin</td>
<td>[171-173]</td>
</tr>
<tr>
<td>Proteoglycan name</td>
<td>Location</td>
<td>Function</td>
<td>Interaction GF/ECM</td>
<td>Ref.</td>
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</tr>
<tr>
<td>Syndecans*</td>
<td>(following)</td>
<td>interaction induces the activation of ADAMTS-5 and subsequently the degradation of aggrecan.</td>
<td>TGF-β 1, 2, 3, Inhibin A, BMP-2, -4, -7, GDF-5, FGF-2, TRAF-6</td>
<td>[168, 171, 174-176]</td>
</tr>
<tr>
<td>Betaglycan*</td>
<td>All tissues</td>
<td>Betaglycan is described as part-time PG as it is expressed under glycosylated and non glycosylated form.</td>
<td>TGF-β 1, 2, 3, Inhibin A, BMP-2, -4, -7, GDF-5, FGF-2, TRAF-6</td>
<td>[168, 171, 174-176]</td>
</tr>
<tr>
<td></td>
<td>MW=250-350kDa</td>
<td></td>
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<tr>
<td></td>
<td>CS chain=1</td>
<td>- Inhibits or enhances the signalling of TGF-β superfamily as co-receptor by modulating the presentation of TGF-β to type II TGF-β receptor.</td>
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<td></td>
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<td>- Dictates the trafficking of the signalling receptors for its various ligands influencing receptor fate and signalling output.</td>
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<tr>
<td></td>
<td></td>
<td>- Plays a role in BMP receptor signalling and trafficking by binding BMPs and GDF-5.</td>
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<tr>
<td></td>
<td></td>
<td>- Forms stable complexes with inhibin and type II activin receptor reducing their availability to propagate activin signalling.</td>
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<tr>
<td></td>
<td></td>
<td>- Regulates reproduction and foetal development.</td>
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<tr>
<td></td>
<td></td>
<td>- Modulates cancerous cell growth and migration acting as a tumor suppressor by a loss of sensitivity to TGF-β.</td>
<td></td>
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</tr>
<tr>
<td>Thrombomodulin</td>
<td>Vascular</td>
<td>TM is a cell surface PG critical mediator in the coagulation process with a part-time PG activity <em>i.e.</em> activities at both glycans and protein levels.</td>
<td>Thrombin, Protein C, LPS, HMGB-1</td>
<td>[145, 146, 177]</td>
</tr>
<tr>
<td>(TM)</td>
<td>endotheil, smooth muscle,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>patelet, monocytes,</td>
<td>- Contains a lectin-like domain involved in the anti-inflammatory properties of TM, and a CS chain involved in the anticoagulant properties.</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>MW=56kDa</td>
<td>- Acts as an anti-coagulant molecule by its co-factor for thrombin dependent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CS chain=1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteoglycan name</td>
<td>Location</td>
<td>Function</td>
<td>Interaction GF/ECM</td>
<td>Ref.</td>
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</tr>
<tr>
<td>Vascular</td>
<td>endothelium, smooth muscle cells, platelet, monocytes, cardiomyocyte, cancerous cells</td>
<td>TM is a cell surface PG critical mediator in the coagulation process with a part-time PG activity <em>i.e.</em> activities at both glycans and protein levels. - Contains a lectin-like domain involved in the anti-inflammatory properties of TM, and a CS chain involved in the anticoagulant properties. - Acts as an anti-coagulant molecule by its co-factor for thrombin dependent activation of protein C, its direct inhibition of fibrinogen cleavage by thrombin, and its indirect enhancement of the formation of the complex anti-thrombin III/thrombin. - Modulates inflammatory process by its potent anti-inflammatory activity by producing activated protein C, and suppressing thrombin activity, the HMGB1-mediated inflammation, and the interaction of LPS with TLR-4. - May maintain the integrity of endothelial junction by keeping a quiescent state of blood vessels. - Influences the growth and metastasis of some cancers.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Extracellular matrix proteoglycans**

<p>| Versican* | IVD, cartilage, V-rod, bone, skin | Versican is a lectican expressed in many tissues. The protein core and attached glycans provide both the structural role and the strong biological activity to Versican. - Enhances mesenchymal condensation during embryonic development. - Modulates cell adhesion, migration, proliferation, differentiation and the | Selectins, integrins, EGF receptors, fibulin-1, -2, tenascin R, |
| MW=373kDa | | | [170, 176, 178-181] | |
| CS chain=1-23 | | | | |</p>
<table>
<thead>
<tr>
<th>Proteoglycan name</th>
<th>Location</th>
<th>Function</th>
<th>Interaction GF/ECM</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brevican</td>
<td>CNS</td>
<td>Bervican is specifically expressed in the brain in a developmental manner by glial cells, neurons and astrocytes.</td>
<td>HA, chemokines, growth factors</td>
<td>[109, 164, 168, 182-184]</td>
</tr>
<tr>
<td>MW=160kDa</td>
<td>CS chain=1-3</td>
<td>- Is incorporated into the ECM or directly bound at the cell surface to sulfated cell surface glycolipids or receptors such as HA receptor.</td>
<td>CD44</td>
<td></td>
</tr>
<tr>
<td>Decorin*</td>
<td>All tissues</td>
<td>Decorin is a PG expressed in all tissues which can carry either dermatan sulfate and CS chains.</td>
<td>GFR, IGF, HGFR, LRP 1, TGF-β1, FGF-2, TNF-α, PDGF, IGF-1</td>
<td>[185-189]</td>
</tr>
<tr>
<td>MW=8kDa</td>
<td>CS chain=8-40</td>
<td>- Regulates cell signalling such as TGF-β/Nodal/Smad-2, BMP/Smad-1, MAPK/FGF, EGF, IGF and Met pathway, influencing cell differentiation,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteoglycan name</td>
<td>Location</td>
<td>Function</td>
<td>Interaction GF/ECM</td>
<td>Ref.</td>
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</tr>
</tbody>
</table>
| Biglycan*        | Bone, skin, dentin, | - Proliferation, migration and apoptosis.  
| MW=42kDa         | tendon, brain, muscle, | - Plays an essential role in matrix organisation and homeostasis via the regulation of collagen fibrogenesis and degradation.  
| CS chain=2       | cartilage, IVD, heart, liver | - Modulates cell adhesion and migration by binding fibronectin and thrombospondin (reduction of tumor growth in cancerous condition).  
|                  |                     | - Inhibits the production of angiogenic factors such as HIF-1\(\alpha\) and VEGF-\(\alpha\) and induces the production of anti-angiogenic factors secretion such as thrombospondin.  
|                  |                     | Is involved in fibrosis and inflammation phenomena with anti-inflammatory properties under its non degraded form and pro-inflammatory under its degraded form (damage-associated molecular patterns (DAMPs)).  
|                  |                     | Biglycan is present in many tissues under both glycosylated and non glycosylated forms. Similar in structure to decorin, it does not exhibit the same functions.  
|                  |                     | - Plays a role in fibrillar collagen organisation.  
|                  |                     | - Affects cell signalling under its cleaved form forming DAMPs after cleavage by BMP-1, MMP-2, MMP-3 and MMP-13. However, it does not present biological activity in its sequestered form as a signalling molecule.  
|                  |                     | - Is a proinflammatory molecule in its cleaved form. This cleaved form interacts with TLR214 and promotes IL-1\(\beta\) secretion by macrophages.  
|                  |                     | - Regulates muscle regeneration, synapse stability in neuromuscular junction, and  
|                  |                     | Pathogen recognition [172, 185, 188, 190-192] receptors (PRR), BMP-2, BMP-4, TLR-2, TLR-4, HuSK  

<table>
<thead>
<tr>
<th>Proteoglycan name</th>
<th>Location</th>
<th>Function</th>
<th>Interaction GF/ECM</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurocan</td>
<td>CNS but also in heart, T-lymphocytes and sclerotome during development</td>
<td>Neurocan belongs to the lectican family with a hyaluronan binding domain and a C-lectin type domain which interacts with ECM molecules and tenascins C and R. - Highly expressed in the perineural network (PNN) by neurons and glia during embryonic development in the brain before cleavage into two polypeptides of 150 and 130kDa after birth suggesting a role of neurocan in early plasticity. - Inhibits NCAM and L1/NgCAM and other cell adhesion molecules influencing neurite outgrowth, fasciculation and navigation. - Is over expressed after injury and acts as an inhibitory matrix component.</td>
<td>Link N protein, HA, heparin, FGF-2, HB-GAM, amphoterin, Tenascin C and R, NCAM, axonin 1, L1/NgCAM, GalNAcPTase, NCAM</td>
<td>[109, 159, 162, 164, 165, 193]</td>
</tr>
<tr>
<td>Aggrecan*</td>
<td>All tissues but in large quantity in IVD, cartilage, CNS, tendon</td>
<td>Aggrecan is the largest PG of the ECM on which are attached both KS and CS in large quantity. Structural component of the ECM, its main function is to provide mechanical properties to the tissue. - Forms aggregates by association of the protein to HA backbone via N-link protein providing the high stability and mechanical properties of the structure. - Post-transcriptional modification of aggrecan protein core with KS and CS provides the osmotic properties allowing water recruitment that enables resistance of compressive loads in cartilage, disc and tendon.</td>
<td>HA, link N-protein, tenascins</td>
<td>[25, 26, 63, 86, 178, 183, 194-196]</td>
</tr>
</tbody>
</table>

- bone formation.
- Promotes bone metastasis and affects osteoblasts differentiation.
- Promote the differentiation of progenitor cells in tendon towards a tendocyte phenotype through the control of BMP-2.
<table>
<thead>
<tr>
<th>Proteoglycan name</th>
<th>Location</th>
<th>Function</th>
<th>Interaction GF/ECM</th>
<th>Ref.</th>
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</thead>
<tbody>
<tr>
<td><strong>Degraded by different MMPs (MMP-1 to 20 according to the protein region) and ADAMTS activated by IL-1β and TNF-α.</strong></td>
<td>- Gene polymorphism is involved in the degeneration of both cartilage and IVD involving a change in the KS and CS lengths.</td>
<td>- Plays a role in fibrillogenesis, fibre lubricant and aggregation in tendon.</td>
<td>- Inhibits nerves growth, cell adhesion and migration.</td>
<td></td>
</tr>
<tr>
<td><strong>Allows the aggregation of the component of PNN in the brain with an uniquely important role in the formation and function of this structure. The role of aggrecan is still unclear. However, different potential functions are hypothesised such as (1) securing high-rate synaptic transmission via the hydration properties of aggrecan, (2) mechanically stabilising the synaptic contacts which results in a non-permissive role for the structural and dynamic plasticity of the nervous system function, (3) protecting the neurons by reducing the oxidative stress through scavenging the redox-active action.</strong></td>
<td>- Type IX collagen, composed of three polypeptidic chains, is a part-time PG with a CS chain.</td>
<td>- Is located on the surface of heterotypic type II/XI collagen fibres and aggregates.</td>
<td>- Regulates collagen fibril diameter, the interaction in the collagen network and the PG of the ECM.</td>
<td>[197-201] [197-201]</td>
</tr>
<tr>
<td>Proteoglycan name</td>
<td>Location</td>
<td>Function</td>
<td>Interaction GF/ECM</td>
<td>Ref.</td>
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</tr>
<tr>
<td>Perlecan*</td>
<td>All tissues</td>
<td>Perlecan is a small leucine heparan sulfate PG which can also carry CS.</td>
<td>Fibrillin-1, tropoelastin, BMP-2, HS GF</td>
<td>[79, 168, 178, 202, 203]</td>
</tr>
<tr>
<td>MW=470kDa</td>
<td></td>
<td>- Induces cell aggregation, condensation and differentiation toward a chondrogenic pathway in the early development.</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>- Plays an essential role in ECM organisation and stabilisation by its interaction with ECM components.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>- Sequesters, stabilises and presents a number of GF to their cellular receptor resulting in intracellular activation.</td>
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<tr>
<td></td>
<td></td>
<td>- Participates in mechanosensory processes by promoting cell-cell and cell-ECM connections.</td>
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<tr>
<td></td>
<td></td>
<td>- Promotes the progression of cancer such as colorectal carcinoma, head and neck carcinomas, hepatocellular carcinoma and melanoma.</td>
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</tr>
<tr>
<td>Serglycin*</td>
<td>Immune system</td>
<td>Most studies attribute the biological activity of serglycin to the CS chains of this PG while the protein core is described as inactive to date.</td>
<td>CS interaction with heparin and other receptors, MMP-9</td>
<td>[204, 205]</td>
</tr>
<tr>
<td>MW=14.3-16.7kDa</td>
<td></td>
<td>- Mediates the storage of the compounds of the mastocyte secretory granules (histamine, bactericidal proteins) and the granulopoiesis.</td>
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<tr>
<td>CS number=7-15</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Proteoglycan name</td>
<td>Location</td>
<td>Function</td>
<td>Interaction GF/ECM</td>
<td>Ref.</td>
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<tr>
<td>Phosphaean</td>
<td>CNS</td>
<td>Phosphaean is the extracellular variant to RPTP (\zeta/\beta) expressed by glial and neuronal cells. CS and their patterns were shown to influence the biological functions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW=173-175kDa</td>
<td></td>
<td>- Interacts with CD44 suggesting an important role in cell-cell interaction in immune reaction.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS number=2-5</td>
<td></td>
<td>- Activates MMPs such as MMP-2.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Activates MMPs such as MMP-2.</td>
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<tr>
<td></td>
<td></td>
<td>- Binds in a calcium dependent manner to both tenascins R and C.</td>
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<tr>
<td></td>
<td></td>
<td>- May modulate the RPTP (\zeta/\beta) action and cell interactions.</td>
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<tr>
<td></td>
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<td>- Interacts with cellular adhesion molecules to modify the growth supportive substrate.</td>
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<tr>
<td></td>
<td></td>
<td>- Plays a role in the maturation and maintenance of synaptic structure and function via its interaction with the receptors NCAM and L1.</td>
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<tr>
<td></td>
<td></td>
<td>- Is involved in nerve fibres organisation during development and maturity, axonal growth, cell migration and myelination.</td>
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<tr>
<td></td>
<td></td>
<td>- Shows a lower expression at the lesion site after brain or SCI injury. Its expression increases on a long term at the level of the glial scar.</td>
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<tr>
<td></td>
<td></td>
<td>NCAM, NgCAM, axonin-1, extracellular portion of voltage-gated sodium channels</td>
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<td>[109, 162, 165, 206, 207]</td>
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</tbody>
</table>

* PGs expressed in the IVD tissue.

GalNAcPTase: N-acetylgalactosaminylphosphotransferase
The severity of pathologies associated with a deficiency of sulfation enzymes highlights the importance of the role of GAG sulfation in tissue function and organisation [134, 208-210]. PGs possess specific properties associated to their protein core and their glycan substitution chain (Table 1.2). CS possess specific biological activity in a structural manner [204, 205]. Through their interaction with ECM proteins, receptors, cytokines and growth factors (GF), they modulate a myriad of functions [211-213]. CS properties are marked by highly specific interactions through their sulfation pattern with the proteins which enhance or repress biological activities [214]. These sulfation signatures appear to evolve over development, ageing and pathology altering the CS functions [20, 152, 215].

The interactions of CS and GFs, often sulfate-dependent [212], enhance the GF activity [211, 212]. Different mechanisms of action are involved in this enhancement of which the maintenance of the GFs in the proximity to their receptor. This phenomenon facilitates the presentation of the molecules to their receptor and stabilises their interaction [211, 216]. The CS/GFs interaction can also result in the formation of a functional complex GF-oligosaccharide, a complex released from the chains by an enzymatic cleavage, which activate and/or amplify the action of the GF [211, 214, 217]. Although the functions of CS are still not clear, the interactions of GFs/CS have been shown to regulate many events in the development from neural stem cell differentiation to the development of cartilage and bone [150, 210]. For example, the interactions of CS and TGF-β family have been shown to promote the development of bone and cartilage by stimulating the differentiation of mesenchymal stem cells towards osteogenic and chondrogenic pathways [216, 218]. The sulfated structures on CS chains also interact with FGF having different affinities depending on the presence of CS-B, CS-D and CS-E units on the chains. These complexes promote FGF-mediated cell signalling [219] and the activation of Ihh in cartilage and bone in a gradient-dependent manner highlighting the importance of the sulfation in development [134, 210].

A repression of the activity also occurs by retaining and masking of the molecules within the ECM network, by masking the active site of the receptor [220, 221], and by inhibition of down-streamed activated pathways [222]. While it is known that they interact with many cytokines, their role and mechanisms of action nevertheless remain unclear [212]. A dynamic CS substitution of CD44, hyaluronan-binding receptor, has been described in inflammation. This phenomenon allows a
dynamic control of the HA-receptor which regulates the inflammatory response and the recruitment of macrophages on the inflamed site of an injured tissue [221]. CS have also been described as modulating the inflammatory responses [220] by affecting the action of IL-β and TNF-α [214, 222], two inflammatory cytokines implicated in pathologies such as osteoarthritis or DDD [86]. IL1-β effect is diminished in chondrocytes by inhibition of the p38-MAPK pathway and the nuclear translocation of NFκB. This action decreases the inflammatory action of IL1-β involved in cartilage and IVD degeneration [222]. Similarly, CS-E has been shown to interact strongly with TNF-α and thus to antagonise its interaction with TNF-R1 [214].

CS have been shown not only to affect the behaviour of GFs and cytokines but also the organisation of the ECM [223]. Both protein cores and CS play a crucial role in the formation and organisation of tissues such as cartilage and IVD [63, 79, 205]. A major property of CS is their ability to retain water. The water retaining capacity of these molecules is proportional to the degree of sulfation [224]. The ECM network is constrained and greatly influences the morphogenesis and the tissue organisation at maturity [20, 22]. CS also interact with different types of collagen with a strong binding ability to type II and VII collagen [223] and are an essential component of the aggregates [66, 197] or perineuronal nets [225] providing strength and flexibility to the tissues [21, 188, 189]. CS and their sulfation patterns also influence the motility of the cells by reinforcing integrin signalling [152]. They provide, by the modulation of their expression and sulfation patterns, guidance to the tissue growth, self-renewal [210, 226] and pathology [130, 227].

1.3.2.2 Hyaluronic Acid (HA)

Unlike CS, DS and HS, HA is the only free and non sulfated glycosaminoglycan [131]. It interacts with receptors and ECM proteins [66, 131]. Although used clinically and in tissue engineering research [228], its functions still are not fully understood.

- **Structure and Synthesis**

HA chains are composed of a repeat disaccharide motif \([\text{GlcA-β(1→3)-GlcNAc-β(1→4)}]_n\) where GlcA and GlcNAc correspond to glucuronic acid and N-acetylglucosamine, respectively [131, 229]. Not a post-transcriptional modification of a protein, HA synthesis does not occur in the golgi apparatus but at the surface of
the cytoplasmic membrane via the polymeric activity of hyaluronan synthases (HAS). Three different forms of these transmembrane proteins are found. Although the specific role of these enzymes remains unclear, they are activated via different growth factors and produce HA with various degrees of polymerization and stability [131, 230]. While HAS1 and HAS2 are thought to preferentially secrete high molecular weight (HMW) forms of HA (from 2x10^6 to 6x10^6 Da), HAS3 has been shown to synthesise low molecular weight (LMW) HA chains [230]. The regulation of HAS is controlled directly through miRNA regulation [231] and also through growth factor expression such as TGF-β [232]. HA is a simple polymer which does not undergo epimerization or sulfation as the other GAGs. The large size of HA leads to its extrusion through the plasma membrane during its synthesis directly onto the cell surface or in the ECM [131, 230].

**HA Turnover**

HA turnover occurs during tissue remodelling, injury and pathology through several mechanisms: (1) internalization and degradation, (2) clearance from tissue matrices through the vasculature and lymphatic systems, and (3) scission of HA via free radicals under oxidative conditions promoted by divalent cations [131]. The turnover by internalization often occurs when HA cannot be cleared through the vasculature and lymphatic systems [131, 230]. During this process, HA binds to HA-receptors (layilin, CD44, receptor of HA mediated motility (RHAMM)) before being cleaved by a glycophosphatidylinositol linked enzyme, hyaluronidase-2 (HYAL-2) [131, 230, 232]. This enzyme, attached to the external part of the membrane, makes an initial cleavage of HA molecules generating fragments of 20 to 100 disaccharides. These fragments are then internalized in early endosomes and lysosomes where they are degraded into disaccharides by the acid-activated hyaluronidase HYAL-1 [131, 230, 233]. Further digestion by N-acetylglicosaminidases and N-glucuronidases are required to fully degrade the glycan in monosaccharide [230].

**Roles of HA**

Studies suggest that the size of HA molecules is an essential element to its structural and biological activities [131, 233]. HA interacts in the tissue with hyaladherins, a family of proteins which include receptors such as CD44, RHAMM, and ECM molecules such as link N-protein, aggrecan, versican, bervican, nerucan.
and TSG-6 [131, 229, 234]. These interactions are essential for the function of HA in cell communication, motility and morphogenesis [229, 232].

The main role attributed to HA is structural when the molecule is under a HMW form. Highly charged, HA is able to hold 10 to 10,000 times its weight in water and is, therefore, considered as osmotically active [120, 228, 230, 231]. Its volume at a hydrated state fulfills the roles of space filling, shock absorber and also of lubricant [131, 235, 236]. From a biological viewpoint, anti-angiogenic, anti-inflammatory and immunosuppressive effects are attributed to HMW HA. These effects are attributed to its potential to exclude inflammatory molecules and cells from the tissue, and to its ability to create a voluminous pericellular matrix which prevents the action of inflammatory molecules. HA binds to specific receptors such as CD44 and RHAMM on the surface of the cells [235, 237]. A cross-linking reaction occurs between receptor and HA inhibiting the receptor activation [237]. By these properties, HMW HA impairs the phenomena of phagocytosis, macrophage activation and the production of inflammatory cytokines. A diminution of inflammatory responses and apoptosis through the down-regulation of many factors such as IL-8, iNOS, aggrecanase 2, TNF-α and MMPs has been observed after administration of HMW HA to chondrocytes [238, 239].

During pathologies, injury and remodelling, HMW HA is cleaved to form LMW HA molecules composed of few glycans. LMW HA fragments function as damage-associated molecular pattern molecules which provide an endogenous "danger signal" [230]. These fragments have been shown to be tissue and size specific [131], promoting the secretion of inflammatory cytokines, the stimulation of angiogenesis and tissue remodelling [131, 233]. Depending on their size, they can promote activation and maturation of dendritic cells, and release of proinflammatory cytokines such as IL-1β, TNF-α, IL-6 and IL-12 [230, 237].

1.4. Tissue Engineering for IVD

Spinal fusion [1, 3, 9, 35] and total artificial disc replacement [240, 241] are the most current treatments for DDD. Spinal fusion is achieved with or without material to eliminate painful motion following the collapse of IVD [242]. This process is the focus of many studies undertaken to enhance the fusion process and to reduce the morbidity of the donor site [242, 243]. However, fusion leads to a loss of the mobility of spine [10, 104] and potential degenerative changes in the adjacent
IVD [244]. Full or partial disc replacements present the advantage of decreasing painful motion and also of restoring the mobility of the spine. Different technologies for disc replacements are currently on the market to preserve flexion, extension, lateral bending motions and to restore disc space height [245]. The use of hydrogel implants in poly(vinyl)alcohol has been proved to be efficient in a short term, with a significant delay of the degeneration process after implantation into baboons and rabbits [246, 247]. However, this type of materials has limitations. The long-term behaviour of the material is unknown [248]. Furthermore, some movement of the implant (ejection of the implant outside of the disc due to mechanical forces) [247] is observed because of the absence of adherence of this material to the native tissue. Their long-term functionality is also unknown because of their inability to integrate into the host tissue.

New strategies, from delivery of biological cues [95, 249, 250] to full disc replacement [251], are currently under development. The ideal tissue engineering approach should be able to meet anatomical and degeneration state requirements of the patient profile [38, 104]. Depending on the stage of degeneration, the complexity of the therapy increases (Figure 1.2) from a simple biological treatment to a complex tissue engineering approach [104]. However, the main hurdle encountered to develop a therapy for the IVD treatment is the difficulty of DDD detection and diagnosis at an early stage for an optimal tissue response [38, 252]. It is essential to determine the window of treatment. Premature treatments may accelerate the process of degeneration by altering the AF integrity [253-255] whereas late treatments may result in the need for a total disc replacement due to the acellularity of the disc at this advanced stage of degeneration [240, 251]. Many approaches are envisaged to restore the disc using a variety of materials and biological cues. However, many questions remain unanswered concerning the design of the scaffolds, including which cell-type to deliver, which type of scaffold is required and which biological cues are needed (Figure 1.5).

1.4.1 Cells and scaffold

Various therapies are proposed to respond to the loss of disc function. Most of the new tissue engineering approaches are based on the regeneration or replacement of the IVD using biomaterials enriched with cells and/or bioactive macromolecules.
1.4.1.1 Cells

The delivery of cells appears promising because of its ability to restore a balance between matrix production and degradation, which maintains integrity and tissue function. The potential of different cell-types is tested in the literature. The ideal cell source, however, has not yet been identified.

The logical candidate for cell based therapy for NP regeneration is NP cells themselves. Indeed, it is most likely that these cells behave as the native cells after reimplantation. NP cells have been shown to recover their phenotype in a three dimensional environment [256, 257] and in vivo after expansion in monolayer [258-260]. However, because of their low proliferation rate, a large number of cells is required for their expansion. Furthermore, autologous transplantation of NP cells is problematic. Harvesting cells from a healthy donor disc would compromise the integrity of this disc and will induce degeneration. However, the transplantation of allogenic cells has been shown to enhance the deposition of ECM and to maintain the disc function [258-261].

Another cell source tested is cells from articular cartilage. Chondrocytes share many characteristics with NP cells including those of secreting high levels of PGs and type II collagen [47]. Chondrocytes were demonstrated to resist the drastic environment of the disc and to enhance the deposition of both type II collagen and PGs [262]. Nonetheless, this approach can induce morbidity at the donor site. It is also important to highlight the phenotypic differences between NP cells and chondrocytes. There are several studies that report that both cell-types produce a similar but distinct ECM [50-52, 54]. Potential differences in matrix deposition can then be envisaged after transplantation which can affect the tissue behaviour.

Mesenchymal stem cells (MSCs), derived from various sources (bone marrow, adipose tissue, etc...), are considered as a potential source of cells to repopulate and regenerate the IVD [249, 263-266]. Their availability, differentiation and biological properties make them a candidate of choice for NP regeneration [17]. MSCs have been described as feeder cells [263] which influence resident cells by cell-cell interaction and by the secretion of paracrine factors that decrease the inflammation of the tissue [267], stimulate the resident cells to produce new ECM molecules [265, 268] and preventing the resident cells from apoptosis [269]. Several studies speculated on the ability of MSCs to differentiate towards a NP cell-like
phenotype [270-272]. However, the phenotype adopted by the cells described as the NP cell phenotype has not yet been clearly identified [50, 51]. The microenvironment of the IVD needs to be also considered in such therapies, as this drastic environment has been shown to influence the behaviour of MSCs. The limited nutrition, high osmolarity, high acidity and low oxygen tension of the tissue impair the potential of MSCs to repair the disc by decreasing their viability and their biological activity [273, 274].

The recent understanding of progenitor cells and notochordal cells within the IVD [16, 57, 275] have opened new perspectives for cell therapy. Progenitor cells have been identified in both IVD tissues (NP and AF). These cells have been shown to exhibit the same characteristics as those of MSCs. Highly proliferative, these cells were able to differentiate into multiple mesenchymal lineages [57, 275]. Their isolation, expansion and reimplantation can lead to their differentiation toward a NP lineage. Notochondal cells are present in the disc at an early stage of development and their number decreases with ageing (Chapter 1, paragraph 1.1.). These cells influence NP cell behaviour and secretion profile [16, 42]. Their stimulation or reimplantation is likely to stimulate the resident cells and induce regeneration of the tissue [21]. Nevertheless, it is important to note that for NP cells, autologous delivery of notochordal and progenitor cells compromises the integrity of the donor disc.

Multiple studies delivering NP cells, chondrocytes or MSCs within the IVD in vivo have been reported (Table 1.5). Although promising results were obtained from these studies, different hurdles were nevertheless encountered including a low survival rate of the injected cells [276, 277] and a potential leakage of the cells outside the disc resulting in the formation of osteophytes [266, 278]. The importance of the use of a scaffold for cell delivery was highlighted by the study of Bertram et al. in which a significant improvement of cell retention and survival was obtained [278]. Therefore, a therapy which associates scaffold and cells for NP regeneration appears essential.

1.4.1.2 Scaffold

Cell implantation, generally associated with scaffolds, has received a lot of attention in recent years.
Figure 1.5. Challenges for tissue engineering of the intervertebral disc (IVD). Different strategies are envisaged for the treatment of IVD degeneration from simple cell based therapy to composite strategies mixing cells and functionalized scaffolds.
The use of a cell carrier presents the advantages of providing physical support for implantation of cell into the disc. Furthermore, a specific cellular environment can be created [279] to direct a desired cellular activity such as cell proliferation or production of ECM. These scaffolds can be functionalised by gene or drug carriers to influence signalling pathways [280] and/or the production of new ECM [281] (Figure 1.5). Many biodegradable scaffolds were developed for NP, most of which are implantable [122, 123, 282]. Given the gel-like structure of the NP, a hydrogel structure appears to be the most promising structure for NP regeneration [283]. The difficulty for the design of a hydrogel for this purpose is to match the mechanical properties of the native NP tissue. Hydrogels have been shown to restore the range of mobility of the IVD [284]. The use of an injectable system presents several advantages such as reduced invasion during surgery, minimal damage of the AF, filling of the defect, and incorporation of multiple cues from cells to drugs [10, 256, 283]. A small number of injectable systems for NP regeneration are available on the market (Table 1.3).

A promising pre-clinical trial of an injectable hydrogel “NuCore™” (Spine Wave, USA) has been performed. This commercial hydrogel is composed of an in situ cross-linked copolymer of elastin and silk which mimics the biomechanical properties of the native nucleus. Trends of 93% and 92% of disc height maintenance were obtained in the central and posterior part, respectively. Furthermore, no inflammatory response was observed over the 24 weeks of clinical trial [285]. However, no biological factors are delivered to slow down or induce the regeneration of the disc which limits the use of this material to the replacement of the damaged disc.

The importance of the NP tissue microenvironment on the stem cells differentiation [286-288] and NP cell phenotype [124, 289, 290] has been highlighted in many studies. The major challenge to design a hydrogel for NP regeneration is the identification and the development of a material which resembles the native microenvironment of the tissue, and is capable of promoting phenotype maintenance, proliferation and differentiation of NP cells and/or stem cells. Cell carriers such as alginate [291], bioactive composite scaffolds including chitosan-glycerophosphate [292, 293], hyaluronan [256] type II collagen-aggre can-hyaluronan [10], fibrin-hyaluronan scaffolds [294] provide advantages as therapeutic solutions in the
following areas: (1) biodegradability, (2) injectability (less invasive than the use of surgical techniques), (3) preservation of the native tissue, (4) non-preclusion of future spinal surgery and multilevel disc treatments [35]. Ideally, this tri-dimensional matrix should closely mimic the environment of the native tissue [17]. An ideal scaffold should be biodegradable at a rate equivalent to that of ECM deposition and should not degrade with any toxic or inhibitory products. In addition, the scaffold should also have a chemical make-up that allows the transport of nutrients, metabolites and regulatory molecules to and from the cells [295].

Based on this information, an ideal hydrogel needs to match the native composition of the NP. The combination of datasets suggests that an association of different ECM molecules helps the guidance of stem cells differentiation into a NP cells-like phenotype [10, 256, 282, 296-298] and the maintenance of NP cells phenotype [10]. O’dialloran et al. have shown a better NP cell phenotype maintenance using a composite hydrogel of HA and type II collagen [10]. The low antigenic character of the type II collagen, its mechanical proprieties and its dominance in the composition of the native tissue, makes it a candidate of choice for IVD engineering [299, 300]. Copolymers of type II collagen have been reported as promising scaffolds for cartilage [301-303] and disc regeneration [265, 304]. Furthermore, Lu et al. reported an enhancement of the chondrogenic differentiation of ADSCs in type II collagen. In this study, they described an important effect of the type II collagen on the cells shape, an important determinant of stem cell differentiation [305]. Hyaluronic acid (HA), a glycosaminoglycan richly present in NP tissue [66, 67] (Paragraph 1.2.3.1), is widely used for cartilage and IVD repair in either its natural structure or in modified versions. Acellular HA hydrogels (HYAFF®-120) and cells-loaded HA hydrogels (HYADD®-3) were shown to induce some repair of IVD following nucleotomy. The biological effect of the derived material was not studied [306]. However, these promising results and the positive effect of HA on ECM biosynthesis, cell proliferation, cell migration and maintainance of chondrocytes and NP cell phenotype [294, 301, 307-309] suggest that the addition of HA in the system is beneficial to the development of an ideal microenvironment for NP regeneration. CS [310, 311] and HA [312] microenvironments have also been shown to promote cell aggregation and chondrogenic differentiation along with anti-inflammatory properties [313]. Type II collagen and HA properties make them candidates of
Table 1.3. Injectable cell-seeded systems developed for NP tissue engineering.

<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>Cells</th>
<th>Method</th>
<th>Outcome</th>
<th>Ref.</th>
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</table>
| **Type II atelocollagen collagen hydrogel** | Rabbit autologous bone marrow MSCs 1x10^6 cells/mL | In vivo rabbit degenerative model obtained by puncture | - MSCs were labelled using a recombinant adenovirus vector expressing Lac Z.  
- A type II atelocollagen at 0.3% supplemented with cells was injected in an IVD degenerative rabbit model.  
- The model was induced by puncture two weeks before the injection of the hydrogel.  
- Histology on the disc was performed to determine the level of degeneration (Hematoxylin and eosin, safranin O staining).  
- A deceleration of the IVD degeneration was observed after injection of a type II atelocollagen supplemented with MSCs. The primary morphological features of the disc were maintained with minimal degeneration and cell depletion and an increase of PG production. The injected MSCs survived over the 12-week experiment and adopted a NP cell-like morphology in the tissue. A high intensity of staining for PG was obtained after MSCs were injected which suggested the deposition of new ECM by the delivered cells. | [265] |
<p>| Hyaluronan hydrogel                | Rat MSCs 1x10^7 cells/mL    | In vivo rat IVD caudal model                                            | MSCs were able to survive and to proliferate after injection within the discs. A decrease of cell number was observed after injection in a HA carried which made this carrier not an | [314] |</p>
<table>
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<tr>
<th>Hydrogel</th>
<th>Cells</th>
<th>Method</th>
<th>Outcome</th>
<th>Ref.</th>
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<tbody>
<tr>
<td><strong>Fibrin hydrogel</strong></td>
<td>HeLaX1.5 cells 5x10^6 cells/mL</td>
<td><em>In vitro</em> Explant culture <em>In vivo</em> rabbit degenerative model obtained by mechanical axial compression</td>
<td>• Disc height was measured by X-Ray after each time-point. Ideal candidate as a cell vehicle.</td>
<td>[278]</td>
</tr>
<tr>
<td><strong>Type I and type II atelocollagen hydrogels</strong></td>
<td>Human NP HNPSV-1 1x10^6 cells/mL</td>
<td><em>In vitro</em></td>
<td>• Three types of hydrogel were prepared: 0.3% type I atelocollagen, 0.3% type II atelocollagen and 3% type I atelocollagen.</td>
<td>[264]</td>
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<td></td>
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<td>• Cell proliferation, DNA synthesis and content, and PG synthesis and accumulation were evaluated over 14 days.</td>
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<td>• Histology of the constructs was performed after four-weeks in culture.</td>
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<td>The injection of cells resuspended in medium led to a leakage of the cells outside of the tissue. This phenomenon was explained by the authors as being the intradiscal swelling pressure working against an injected non compressible liquid suspension. The injection of cells through a hydrogel allowed the immobilisation of cells within the discs as well as the support of cell growth.</td>
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<td>A higher cell proliferation was obtained after culture of HNPSV-1 cells in type I atelocollagen than in type II atelocollagen hydrogel. Although a higher expression of type I collagen was obtained in the 3% atelocollagen hydrogel, this hydrogel showed the highest production of type II collagen and PG in comparison to the two other gel-type.</td>
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<td>Hydrogel</td>
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<tr>
<td>Hyaluronic acid</td>
<td>Human chondrocytes</td>
<td>in vitro</td>
<td>- Cell viability was significantly greater in the layer cultures than in the monolayer cultures.</td>
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<tr>
<td>Nucore™ hydrogel</td>
<td>Acellular</td>
<td>in vivo</td>
<td>- The polymers of silk and elastin were produced using recombinant DNA</td>
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<tr>
<td>Polyalcohol</td>
<td>Human chondrocytes</td>
<td>in vitro</td>
<td>- The cells formed a gel-like structure and maintained their phenotype.</td>
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<tr>
<td>Type II atelocollagen</td>
<td>Bovine caudal IVD cells</td>
<td>in vitro</td>
<td>- The gel-like structure was retained independently of the method used to generate the hydrogel.</td>
<td></td>
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<tr>
<td>Chitosan/glycerophosphate hydrogel</td>
<td>Bovine caudal IVD cells</td>
<td>in vitro</td>
<td>- The mixture of thermogelling chitosan was made with sodium β-glycerophosphate (GP) resulting in a mixture of 2 and 8% GP for a 1% final concentration of chitosan.</td>
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| Ref. | 293 | 315 |

Introduction
<table>
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<th>Hydrogel</th>
<th>Cells</th>
<th>Method</th>
<th>Outcome</th>
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<tr>
<td>silk/elastin</td>
<td></td>
<td>In vitro organ culture/ in vivo</td>
<td>polymerisation occurred after 30 minutes of incubation. The hydrogel was non-cytotoxic. No extrusion of the hydrogel was seen during the mechanical tests,</td>
<td></td>
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<tr>
<td>Hyaluronic acid based hydrogel</td>
<td>Acellular</td>
<td>Ex vivo Human non degenerative lumbar IVD</td>
<td>The different hydrogels of this study presented mechanical properties similar to those of native tissue. No toxicity and cellular interactions were investigated in this study.</td>
<td>[316]</td>
</tr>
<tr>
<td>Type II collagen/ HA/ Aggrecan hydrogel cross-linked with microbial</td>
<td>Bovine NP cells (3-4 months-old) 5x10⁷/30µl</td>
<td>In vitro</td>
<td>The addition of HA and aggrecan in the structure increased the mechanical properties of the cross-linked type II collagen hydrogel. A good cell distribution was observed after culture within the hydrogel. DNA was</td>
<td>[10]</td>
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<tr>
<td>Hydrogel</td>
<td>Cells</td>
<td>Method</td>
<td>Outcome</td>
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<tr>
<td>transglutaminase</td>
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<td>hydrogel compositions over seven days in DMEM + 10%FBS</td>
<td>maintained constant. The volume of the various hydrogels was reduced over seven days in culture. However, the composite coll/HA hydrogel showed the optimal mechanical properties, cell distribution, cell viability and GAGs production.</td>
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<tr>
<td>Puramatrix™ (Self-assembling peptide)</td>
<td>Human bone marrow MSCs Passage 5</td>
<td>In vitro organ culture/ in vivo</td>
<td>hMSCs were mixed within the hydrogel before injection within the IVD.</td>
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<tr>
<td>hydrogel having amino sequence Ac-(RADA)4-CONH₂</td>
<td>Mini pigs, 6 month-old Porcine degenerative model obtained by puncture</td>
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<td>MRI was performed post-mortem to visualize disc appearance six months post transplantation.</td>
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<td>Matrix deposition and cell localization were assessed by histology (Alcian Blue von Gieson, Von Kossa staining), immunohistochemistry (type I and type II collagen, aggrecan and human nuclei), and gene expression (sox-9, type II and type I collagens, aggrecan and versican).</td>
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<td>MRI signals obtained for the treated discs were similar to those of normal discs after injection of the mixture MSCs and hydrogel. MSCs expressed chondrogenic markers after transplantation which indicates their differentiation toward a NP cell-like phenotype. The three dimensional microenvironment enhanced the differentiation of MSCs.</td>
<td>[317]</td>
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<tr>
<td>Hydrogel</td>
<td>Cells</td>
<td>In vitro/organ culture/ in vivo</td>
<td>Method</td>
<td>Outcome</td>
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<tr>
<td>Polyurethane fibrin</td>
<td>Human IVD cells</td>
<td>In vitro</td>
<td>• Polyurethane scaffold was minced in order to obtain PU spheroids. These spheroids were then mixed with a fibrin hydrogel.</td>
<td>A homogenous cell distribution was obtained with a high cell survival. NP cells were able to recover their phenotypic markers expression (type II collagen, aggrecan, sox-9) and the production of sGAGs after 14 days in culture.</td>
</tr>
<tr>
<td>structure</td>
<td>1.2x10⁶ cells/mL</td>
<td></td>
<td>• Cells were seeded onto PU spheroids for three days in DMEM/F12 + 10%FBS before being embedded in the fibrin hydrogel and cultured for up to 14 days.</td>
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<td>• Biochemical, histological and gene expression analyses were performed on the composite structure.</td>
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<td>Fibrin hydrogel</td>
<td>Porcine bone marrow MSCs</td>
<td>In vivo</td>
<td>• Bone marrow MSCs were transduced with Rv-Luc.</td>
<td>The implant was maintained after injection with the IVD. However, a rapid loss of activity of the implanted cells was observed. The authors suggested a focus on AF repair and sealing would increase the percentage of success of NP regeneration therapies.</td>
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<td></td>
<td>1x10⁷ cells/mL</td>
<td>mini pigs 2-3 year-old.</td>
<td>• microCT based analysis of the IVD after sacrifice to follow up the injected hydrogel.</td>
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<td>Nucleotomy before injection of</td>
<td>• Luciferase assay was performed for following up the injected cells’ activity.</td>
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<td>the hydrogel.</td>
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<tr>
<td>Fibrin hydrogel</td>
<td>Rabbit bone marrow MSC</td>
<td>In vivo</td>
<td>• PGF-TGF/β1 and MSCs-PGF-TGF/β1 hydrogels were obtained by mixing a fibrin</td>
<td>MSCs showed anti apoptotic properties which slowed down the degeneration rate. Type II</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
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<tr>
<td>Hydrogel</td>
<td>Cells</td>
<td>Method</td>
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<tr>
<td><strong>Chondroitin sulfate based hydrogel</strong></td>
<td>Bone stromal MSCs</td>
<td>25x10⁶ cells/mL</td>
<td>Four year-old goat induced model using an incision of the AF.</td>
<td>Collagen expression was maintained in the disc after injection of MSC-PFG TGF/β1 hydrogel. The injection of hydrogel alone did not show any deceleration properties on the degenerative discs.</td>
</tr>
<tr>
<td></td>
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<td>2x10⁶ cells/mL</td>
<td>Degenerative model by puncture of the disc.</td>
<td>The level of degeneration observed in this model was not fully constant and this did not allow the observation of disc height changes. The injection of bone stromal MSCs allowed the accumulation of more PGs than that of control discs. The authors concluded that a more severe injury, a larger sample size and a longer observation period were required to test the system used in this study.</td>
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<tr>
<td></td>
<td></td>
<td>In vitro organ culture/ in vivo</td>
<td>In vitro</td>
<td>Solution with PGF, TGF/β1 and MSCs. MRI, X-Ray and histological analyses were performed after puncture at week 4, 8 and 12 to check disc height and hydration levels. TUNEL assay was performed to determine cell viability, and type II collagen immunostaining and H&amp;E staining were performed to assess the level of disc degeneration.</td>
</tr>
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</table>

Introduction
<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>Cells</th>
<th>Method</th>
<th>Outcome</th>
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</thead>
</table>
| **Fibrin hydrogel**      | Human bone marrow MSCs and bovine NP cells | *In vivo* Sprague-Dawley rats 6 months-old Nucleotomy of the cranial discs. 6.25x10⁷ cells/mL | * Hematoxylin and eosin staining were performed and boost score evaluated.  
* Different formulations were tested: fibrin alone, MSCs resuspended in fibrin, NP cells resuspended in fibrin, and MSCs surrounded by NP in a fibrin gel (BCP).  
* BCP was formed seven days before implantation (75% MSCs inside, 25% NP on the outside).  
* Disc height and grade were determined after X-Ray and histological analysis.  
* Inflammation response was evaluated by ELISA. Cell response was evaluated by sGAG quantification and gene expression analysis.  
  The addition of BCP and MSCs constructs allowed the maintenance of disc height while a significant decrease of disc height was seen for the other groups tested. BCP showed a higher disc height level compared to the control group. The fibrin hydrogel allowed the retention of the cells within the disc. | [268] |
| **Injectable oxidized HA/ adipic acid dihydrazide hydrogel (oxy-HA/ADH)** | Rabbit NP cells  Passage 6 | *In vitro* | * Oxi-HA was prepared by incubation of HA with a sodium periodate solution. This solution at 6% was then mixed with various concentration of adipic acid dihydrazide (2, 4  
No toxicity of the hydrogel tested was evident on NP cells. An increase of aggrecan and type II collagen expression along with a decrease of type I collagen expression was obtained after 14 days in culture compared to | [260] |
<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>Cells</th>
<th>Method</th>
<th>Outcome</th>
</tr>
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<tbody>
<tr>
<td>Methacrylated carbomethyl-cellulose (MC) hydrogel</td>
<td>Bovine caudal IVD cells Passage 2 30x10^6 cells/mL</td>
<td>In vitro Lyophilised methacrylated CMC + 0.5% (w/v) photoinitiator was mixed with NP cells for a final concentration of 2.5%. Hydrogels were then crosslinked by UV and cultured in DMEM+10%FBS.</td>
<td>Higher increases of type II collagen expression and CSPG deposition with CMD supplemented with TGF-β3 were observed while no type I collagen was shown after 28 days in culture. The mechanical properties of</td>
</tr>
</tbody>
</table>
| Albumin hydrogel             | Human chondrocytes 1x10^6/mL - IVD cells 5x10^6/mL | In vitro (chorioallantoic membrane (CAM)) In vivo (subcutaneous mice model) | • Activated maleimido-albumin cross-linked with HS-PEG-HS was supplemented with HA, gelatin, VEGF or chondrocytes.  
• A culture of HUVEC cells was performed on the hydrogel and cell invasion assays were performed.  
• A mixture of the hydrogel and IVD cells were injected subcutaneously. Histological and immunohistochemistry were performed. | Endothelial cells were hardly able to adhere onto the hydrogel and showed an absence of proliferation on the albumin gel. Both in the CAM assay and subcutaneous in vivo study, blood vessels were not able to invade the hydrogel. This observation led the authors to suggest an angiogenic barrier role of the hydrogel which contributes to the regeneration of IVD. |

Hydrogel and 8%; w/v). Hydrogel properties (mechanical stability and swelling were assessed.  
• Cell viability and phenotype (gene expression and morphology) were assessed.  
and an increase of matrix degrading enzymes was observed and explained by the authors as the potential result of an increase of HA fragment of small molecular weight.
<table>
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<tr>
<th>Hydrogel</th>
<th>Cells</th>
<th>Method</th>
<th>Outcome</th>
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<tbody>
<tr>
<td>Thiol modified HA and elastin-like polypeptides composite (TM-ELP)</td>
<td>Human NP cells 10⁶ cells/400µl</td>
<td>In vitro In vivo rabbit model</td>
<td>The addition of ELP within the thiol modified HA hydrogel resulted in obtaining a stiffer hydrogel. IVD cells exhibited a high viability and a round morphology. Type II collagen was expressed by IVD cells over three weeks. A higher staining intensity was obtained for the GAGs after injection of TM-HA hydrogel in the disc. The MRI analysis showed a</td>
</tr>
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</table>

After 24h, medium was changed for growth medium or chondrogenic medium (DMEM+%ITS+1mM sodium pyruvate, 50µg/mL ascorbic acid 2-phosphate and 100nM dexamethasone, both supplemented with TGF-β3.

- ECM deposition was evaluated by chemical assay (sGAG and collagen production), histology and immunohistochemistry. Mechanical testing of the seeded hydrogel was performed.
<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>Cells</th>
<th>In vitro/organ culture/ in vivo</th>
<th>Method</th>
<th>Outcome</th>
<th>Ref.</th>
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</thead>
<tbody>
<tr>
<td>Thermosensitive chitosan/ gelatin/ β-glycerophosphate (C/G/GP) hydrogel</td>
<td>Rabbit NP cells (4 month-old)</td>
<td>In vitro</td>
<td>n=4. Efficacy of the system was assessed by MRI and alcian blue staining.</td>
<td>relative maintenance of disc height after injection of the hydrogel.</td>
<td>[320]</td>
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<td></td>
<td>5x10⁴ cells/well</td>
<td>in 24 well-plate</td>
<td>• Cells were seeded in monolayer for 18h before treatment with H₂O₂ to activate an oxidative stress.</td>
<td>C/G/GP hydrogel can be used as a sustained release system. FA was released over time from the hydrogel. FA-GP hydrogel allowed the retention of aggrecan expression and induced the production of ECM molecules (type II collagen). A role of FA in the regulation of H₂O₂ induced oxidative stress was suggested by the authors.</td>
<td></td>
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<tr>
<td>Fibrin hydrogel</td>
<td>Joint chondrocytes and MSCs</td>
<td>In vitro</td>
<td>Fibrinogen was obtained from the plasma of a mini-pig.</td>
<td>Joint chondrocytes were shown to survive up to 12 months after injection within the IVD. However, no viable MSCs were detected within the tissue. The production of ECM molecules by implanted chondrocytes was detected. A predominance of type II collagen expression was observed in the tissue after each time-point while no type I collagen was</td>
<td>[262]</td>
</tr>
<tr>
<td></td>
<td>Miniature swine</td>
<td>In vivo</td>
<td>• Groups tested in this study: joint chondrocytes in a fibrin gel and MSCs in a fibrin hydrogel. Viability of chondrocytes and MSCs was assessed in vitro by growing cells for two</td>
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<td>Hydrogel</td>
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<td><strong>Hydrogel</strong></td>
<td><strong>Cells</strong></td>
<td><strong>In vitro organ culture/ in vivo</strong></td>
<td><strong>weeks in presence of fibrin.</strong></td>
<td><strong>Ref.</strong></td>
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<td>• NP tissue was harvested three, six and 12 months after surgery. DNA content, sGAG and protein contents were assessed. Histology staining (H&amp;E, safranin O/fast green) and immunohistochemistry (type I collagen, type II collagen, link-N and chondromodulin I) were performed on tissue.</td>
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<td>• Joint chondrocytes allowed the production of ChM-I over 12 months. The authors hypothesised that the loss of expression of this protein along with the destruction of aggrecan with senescence of NP cells is linked to the inhibition of nerves and to vessels invasion.</td>
<td>[284]</td>
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<tr>
<td><strong>Oxidized HA (oHA)-gelatin hydrogel</strong></td>
<td>Acellular</td>
<td><em>Ex vivo</em></td>
<td>• oHA was obtained by addition of a sodium periodate solution. The mixture was then incubated with PEG to finish the reaction.</td>
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<td>• The hydrogel consisted of a mixture of gelatin and oHA at a weight ratio 7:3.</td>
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<td>• Mechanical tests were performed on <em>ex vivo</em> porcine disc.</td>
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<td>It was shown that an injectable system serves as a proxy for the mechanical properties of the discs and restores the range of mobility of the IVD.</td>
<td>[284]</td>
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<tr>
<td><strong>Poly N-acetylglucosamine (pGlcNAc) hydrogel</strong></td>
<td>Human NP cells $2 \times 10^4$ cells/mL</td>
<td><em>In vitro</em></td>
<td>• pGlcNAc (chitosan) nanofibers and gels were manufactured by the fermentation of a marine diatom and which was then mixed with dried DEAC.</td>
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<td>Higher expression of both aggrecan and type II collagen expression and GAGs production were observed after culture in gel 1, sulfated deacetylated derivate of the pGlcNAc</td>
<td>[321]</td>
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<tr>
<td>Hydrogel</td>
<td>Cells</td>
<td>In vitro organ culture</td>
<td>Method</td>
<td>Outcome</td>
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<tr>
<td>Glucidyl metachylate-HA (GM-HA) and HA-collagen based free matrix</td>
<td>Acellular</td>
<td>In vivo porcine lumbar spine</td>
<td>Hydrogel was added onto IVD cells and cultured for 28 days. • Cell viability, proliferation and phenotype (gene and protein expression) were evaluated.</td>
<td>nanofibers, compared to monolayer culture and the other hydrogel groups. The hydrogel showed a lower storage modulus than that of observed for the native tissue.</td>
<td>[322]</td>
</tr>
<tr>
<td>Thermoreversible hyaluronan grafted poly(N-isopropylacrylamide) (HA-pNIPAM)</td>
<td>Bovine NP cells (4-6 months-old) caudal discs 3x10⁶ cell/mL, passage 1</td>
<td>In vitro Ex vivo for seven days</td>
<td>Cells were seeded in the thermoresponsive hydrogel and cultured for one week in DMEM supplemented with 10% FCS • Cell viability and proliferation and gene</td>
<td>A high cell viability (~90%) after encapsulation and culture in vitro and in organ culture system was obtained. Cell cultured in the gel under the two different conditions retained markers specific of NP cell</td>
<td>[256]</td>
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<tr>
<td>Hydrogel</td>
<td>Cells</td>
<td>Method</td>
<td>Outcome</td>
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</table>
| Injectable silk fibroin/polyurethane composite hydrogel | Rabbit MSCs 3x10⁵ cells | *In vitro* Implantation in a porcine *ex vivo* model. | - Silk fibroin solution (5 wt%) was mixed with polyurethane (PU) at a ratio 1:1 and incubated for 5 to 30 min.  
- BMSCs were seeded in the material and toxicity assessed after seven days in culture.  
- The injectability and the use of hydrogel was tested in practice in a porcine *ex vivo* IVD.  
A low toxicity of the material was observed after culture of BMSCs onto the hydrogel.  
Cell growth and proliferation were obtained onto the materials. | [323] |
| Oxidized HA and oxidized HA-gelatin | Rabbit 2x10⁶ cells/mL, Passage 6 | *In vitro* | - Cells were encapsulated in the hydrogel up to seven days in DMEM/F12 + 10%FBS.  
- Cell viability and gene expression were assessed after seven days in culture. | High cell viability and proliferation were obtained after culture within the two different hydrogels developed in this study. At a phenotypic level, a decrease of type I collagen along with an increase of chondrogenic markers, type II collagen, aggrecan, sox-9, biglycan and decorin, were observed | [324] |
<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>Cells</th>
<th>In vitro/ organ culture/ in vivo</th>
<th>Method</th>
<th>Outcome</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>EVICEL® (fibrinogen and thrombin) hydrogel</td>
<td>Human umbilical tissue derived stem cells 6.6x10^6/mL</td>
<td>In vivo</td>
<td>• Disc puncture was performed three weeks before the actual treatment. • Umbilical tissue derived stem cells were extracted from human umbilical cord, characterised and expended before implantation. • Treatment groups studied: cells + PBS, cells + hydrogel, hydrogel alone. • Disc hydration was monitored by MRI for 12 weeks. Histology and biochemical analyses were performed after 12 weeks.</td>
<td>The range of measurement performed in this study suggested a response of the IVD tissue to the injection of cells via the fibrin carrier. However, no significant difference between the different treatment groups and the untreated group was seen. A higher cellularity in the cell treated disc was observed. However, no evidence resulted from this study to demonstrate a full return to the non degenerative state.</td>
<td>325</td>
</tr>
</tbody>
</table>

*Note: NZW rabbit puncture model*
choice for NP tissue engineering and they were therefore chosen as main components of the hydrogel developed in chapter 2.

The ideal treatment of the IVD tends towards being a complex association of cells and biologically active molecules incorporated into a carrier for delivery. The association of the macro-scale (carrier of cells) and micro-scale (carrier of gene or/and proteins) carriers for tissue engineering may be conducive to slow down the process of degeneration of IVD and consequently to regenerate the IVD tissue (Figure 1.5).

1.4.2 Gene Therapy

Many factors from GFs to cytokine inhibitors have been identified as having a biological influence on the IVD [35, 95, 326]. The delivery of GFs such as TGF-β1, OP-1 and GDF-5 has been reported to stimulate the production of PGs in vitro and in vivo [35, 327]. These results suggest a therapeutic significance of the delivery of GFs. However, the short life of these proteins reduces the potential of these therapies for DDD [328, 329]. The long process of degeneration and the low potential of healing of this tissue require a prolonged exposure to stimulating factors to restore its biochemical composition and function.

- Viral Therapy vs. Non-Viral Therapy

Gene therapy presents the advantage of a sustained exposure of resident IVD cells to the therapeutical molecules (growth factors [280], transcription factors [326], and RNA interference [281]) by using the secretory machinery of cells. Two groups of gene delivery systems are commonly used: viral and non-viral [249, 330, 331]. Viruses present natural properties of cell infection which leads to a high number of transfected cells and therefore to a high production of the proteins of interest. To date, this approach is the most efficient system for gene delivery [330]. However, numerous disadvantages can be stated for the use of viruses of which the integration within an oncogenic coding sequence of the genome represents a significant risk [331, 332]. Non-viral gene delivery has received a great deal of attention in recent years to overcome the problems associated with viral gene delivery [330]. The ease of engineering allows the integration of a large transgene and of regulating sequences for a better control of expression [332-334]. However, a significant disadvantage of non-viral systems is the low transfection efficiency of these vectors compared to that of viral system [335]. The recent progress for improving gene transfer via
electroporation [336] and polymeric or lipidic carriers [335, 337-339] shows promising results with a significant improvement in transfection efficiency.

- **Gene Therapy of the IVD**

The first gene delivery approach for DDD treatment has been reported by Nishida *et al.* in 1999. In this study, the authors reported the delivery of TGF-β1 via an adenoviral vector for promoting PG production in a degenerated rabbit disc [340]. Although the results of this study were promising, only a few studies using gene therapy since then have been reported (Table 1.4). Most of these studies are based on the use of a viral vector [94, 95, 103, 340-351]. However, the use of such a technology is limited by many issues [335, 352]. The main issue is the concern on the dislodgement of the injected vector [331, 350, 352]. The misplaced injection, leakage or incorrect dosage of the therapy can lead to serious side effects such as the ossification of the external tissue [331, 352, 353]. Different approaches have been developed in the recent years to address these issues by non-viral gene therapy approaches [335, 351, 354] by the delivery of drug-activated adenoviral or associated-adenoviral vectors such as AAV-Rheoswitch and Ad/FasL-GFPTET [350, 352].

To date, most of the studies developed have the aim of restoring the balance between matrix catabolism and anabolism lost during disc degeneration. For this purpose, many studies have focused their attention on the delivery of GFs such as TGF-β1, sox-9, BMP-2, GDF-5, IGF-1, BMP-7 (Table 1.4) which promote the synthesis of ECM molecules (type II collagen, PGs). However, many of these GFs are induced in other phenomena in addition to up-regulating type II collagen and PGs. BMPs, for example, regulate bone formation. It is therefore essential to take account of the potential side effects associated with the delivery of such factors following extradiscal leakage or over dosage of this therapy. Targeting the catabolism of the ECM has been envisaged with promising results after the delivery of IL-1 Receptor antagonist *via* an adenoviral vector [94, 95]. Recent investigations of siRNA delivery *in vivo* [281, 355] have demonstrated the potential of gene down-regulation which opens a new perspective on the control of ECM catabolism. These approaches, however, while promising, only slow-down the process of degeneration of the IVD but do not restore matrix anabolism. A more specific targeted gene therapy approach can reduce the secondary effect of GF delivery. The loss of CS during disc degeneration leads to a loss of water content and therefore a loss of the
### Table 1.4. Studies using gene therapy approaches for IVD regeneration

<table>
<thead>
<tr>
<th>Cell type</th>
<th>In vitro / ex vivo / in vivo</th>
<th>Vector</th>
<th>Gene of interest</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondrocytes from cartilage end-plate</td>
<td><em>In vitro</em> bovine chondrocyte in monolayer passage 0</td>
<td>Retrovirus (MFG)</td>
<td>IL-1Ra</td>
<td>MAG retrovirus transduced a low percentage of chondrocytes (~1%). This low level was explained by the non-dividing nature of the IVD cells. Nevertheless, IL-1Ra production was obtained after transduction.</td>
<td>[356]</td>
</tr>
<tr>
<td>IVD cells</td>
<td><em>In vivo</em> rabbit skeletally mature model</td>
<td>Type five adenovirus</td>
<td>TGF-β1</td>
<td>TGF-β1 gene was successfully transferred within NP cells via the adenoviral vector. An increase of TGF-β1 protein and PG expression was observed seven days after injection compared to the untreated disc.</td>
<td>[340]</td>
</tr>
</tbody>
</table>
| IVD cells | *In vitro* human IVD cells monolayer passage 1  
*In vivo* rabbit degenerative model obtained by puncture | Adenovirus | Sox-9 | The adenovirus encoding for sox-9 efficiently transduced human IVD cells inducing a significant increase of type II collagen. In the *in vivo* rabbit model, rabbit NP cells maintained a chondrocytic morphology over a period of five weeks compared to the punctured disc. | [342] |
<p>| IVD cells | <em>In vitro</em> human cells in monolayer | Type five adenovirus | TIMP-1 and BMP-2 | BMP-2 and TIMP-1 were successfully delivered to IVD cells and promoted the synthesis of PG <em>in vitro</em>. The delivery of TIMP-1 is another approach to promote the up-regulation of PG synthesis within the disc. | [343] |</p>
<table>
<thead>
<tr>
<th>Cell type</th>
<th>In vitro / ex vivo / in vivo</th>
<th>Vector</th>
<th>Gene of interest</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVD cells</td>
<td>In vitro rat AF cells in monolayer and in three dimension (alginate beads)</td>
<td>Type five adenovirus</td>
<td>Lim Mineralisation Protein-1 (LIMP-1)</td>
<td>LIMP-1 over-expression via viral delivery increased the production of PG by IVD cells in monolayer, in a three dimensional system and in vivo via the activation of BMPs expression (in particular BMP-2 and BMP-7).</td>
<td>[344]</td>
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<tr>
<td></td>
<td>In vivo rabbit skeletally mature model</td>
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<tr>
<td>NP and inner AF cells</td>
<td>In vitro human cells passage 2 or earlier in monolayer, in three dimension (agarose culture)</td>
<td>Adenovirus</td>
<td>IL-1 receptor antagonist (IL-1Ra)</td>
<td>A significant increase of IL1Ra in monolayer and in the three dimensional system was obtained after transduction with the adenovirus. The expression of the transgene was constant up to two weeks in ex vivo conditions. This expression was correlated with a significant decrease of MMPs expression over time.</td>
<td>[94]</td>
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<td>Ex vivo degenerated human IVD</td>
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<tr>
<td>NP tissue</td>
<td>Ex vivo degenerated human IVD</td>
<td>Adenovirus</td>
<td>IL-1Ra</td>
<td>The delivery of IL-1Ra diminished the expression of matrix degrading proteinases over a sustained period.</td>
<td>[95]</td>
</tr>
<tr>
<td>NP cells</td>
<td>In vitro ovine in monolayer</td>
<td>Lipofectamine™ and adenovirus</td>
<td>Human telomerase reverse transcriptase (hTRT)</td>
<td>NP cells were successfully transfectected using a non-viral cationic liposomal system allowing the expression of hTRT. This enzyme prolonged the matrix production and the cellular lifespan.</td>
<td>[345]</td>
</tr>
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Introduction
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<thead>
<tr>
<th>Cell type</th>
<th>In vitro / ex vivo / in vivo</th>
<th>Vector</th>
<th>Gene of interest</th>
<th>Results</th>
<th>Ref.</th>
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<tbody>
<tr>
<td><strong>NP cells</strong></td>
<td>In vitro</td>
<td>Electroporation</td>
<td>GDF-5</td>
<td>GDF-5 plasmid was successfully delivered by electroporation to NP cells, promoting the cell proliferation, an increase of GAG/DNA ratio, and type II collagen and aggrecan expressions. The inhibitory action of GDF-5 on matrix degrading enzymes allowed the accumulation of matrix within the tissue.</td>
<td>[351]</td>
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<td></td>
<td>balb/c mice NP cells passage 1 in alginate bead</td>
<td>of pCMV-IRES-hrGFP-GDF-5</td>
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<tr>
<td><strong>NP and AF cells</strong></td>
<td>In vitro</td>
<td>Adenovirus</td>
<td>Cocktails of TGF-β1, IGF-1 and BMP-2</td>
<td>The therapeutic gene transfer of TGF-β1, IGF-1 and BMP-2 genes allowed the up-regulation of PG in human IVD cells. Their simultaneous delivery demonstrated a higher enhancement of PG synthesis compared to the delivery of a single therapeutic gene.</td>
<td>[341]</td>
</tr>
<tr>
<td></td>
<td>human IVD cells passage 1 in alginate beads</td>
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<tr>
<td><strong>AF and NP cells</strong></td>
<td>In vitro</td>
<td>Adenovirus Ad-BMP12 Retrovirus RbBMP-2 and RbBMP-12</td>
<td>BMP-2 and BMP-12</td>
<td>The delivery of BMP-2 and BMP-12 through viral vectors induced the stimulation of higher rates of matrix protein synthesis and did not cause any osteogenic changes in NP and AF cells. However, this technique needs to be taken carefully as the potential exposure of extradiscal tissues to BMP-2 can induce ossification of this tissue.</td>
<td>[353]</td>
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<td>human IVD cells passage 0 in monolayer for transduction before culture in pellet</td>
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<tr>
<td><strong>IVD cells</strong></td>
<td>In vivo</td>
<td>Adenovirus</td>
<td>GDF-5</td>
<td>The delivery of GDF-5 via a viral vector allowed the compensation of cell loss after injury associated with an</td>
<td>[346]</td>
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<td>balb/c mouse degenerative</td>
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</table>
model obtained by puncture

**NP cells**  
*In vitro* cells from beagle dog in monolayer  
Recombinant adeno-associated virus 2 (AAV-2)  
**BMP-7**  
NP cells were successfully transduced via AAV vector allowing the up-regulation of BMP-7. BMP-7 overexpression promoted the accumulation of PG and collagens over 14 days. [357]

**NP cells**  
*In vitro* human NP cells passage 2 in monolayer  
AAV  
hTRT  
The delivery of hTRT induced the decrease of the telomerase activity, and, therefore a decrease of cell senescence. [358]

**IVD**  
*In vivo* rabbit degenerative model obtained by puncture  
AAV  
BMP-2 and TIMP-1  
The simultaneous delivery of BMP-2 and TIMP-1 promoted the production of PG after degeneration resulting in a higher hydration level and the partial recovery of the mechanical properties of the control IVD. [349]
mechanical properties of the tissue [63, 90]. Targeting the up-regulation of their synthesis will allow a better control the anabolism regulation without encountering the side effects observed following the use of GFs. The dose and time effects and delivery site of the therapy can also be controlled by different strategies such as a drug or environment-dependent activation or a targeted delivery via plasmid design, electroporation or the use of carriers for gene delivery. An ex vivo gene transfer can overcome the low rate of transfection of NP cells via non-viral gene transfer [335]. Although this technique is not ideal, it can be combined with the cell delivery approach described previously. It presents then the double advantage of repopulating the disc and of delivering the therapeutic molecule with a better control.

1.5 Objectives and Hypotheses

The ultimate goal of this study was to develop an optimal functionalised cell delivery system using an ECM-mimicking injectable hydrogel that enhances the production and the deposition of newly synthesised ECM to aid the regeneration of NP tissue. It was hypothesised that the modulation of the glycoenvironment of NP cells will promote the maintenance of their phenotype and function.

This thesis involved three different research topics: cell therapy, glycobiology and gene therapy (Figure 1.5). These three areas were explored with the aim of developing an injectable hydrogel system with a tunable microenvironment for NP regeneration. To achieve this goal, the project was divided into three different phases: (1) development of an injectable hydrogel system (Phase I), (2) identification of the cellular glyco-microenvironment of the IVD (Phase II), and (3) development of a gene therapy strategy for the enhancement of the glyco-microenvironment (Phase III) (Figure 1.6). The specific hypotheses and objectives developed in these different phases are listed below.

1.5.1 Phase I (Chapter Two)

Overall aim: To develop an injectable system which mimics the ECM composition of NP tissue and promotes the maintenance of NP cell phenotype.

Hypotheses:

- An injectable type II collagen hydrogel stabilised with poly(ethylene glycol)ether tetrasuccinimidyl glutarate (4S-StarPEG) is a suitable carrier for cells allowing their encapsulation.
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Figure 1.6. Thesis overview. The project developed here involved three different research topics: cell therapy, glycobiology and gene therapy. Three different main objectives were explored in an effort to identify the optimal microenvironment for NP regeneration: (1) Development of an injectable hydrogel system (Chapter 2), (2) Identification of the cellular glyco-microenvironment of the IVD (Chapter 3), and (3) Development of a gene therapy strategy for the enhancement of the glyco-microenvironment (Chapter 4).
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- To assess mechanical and injectable properties of the fabricated hydrogel.
- The supplementation of the hydrogel with HA will provide an environment more conducive to the maintenance of the NP cell phenotype.

Objectives:
- To evaluate the potential of 4S-StarPEG to cross-link type II atelocollagen.
- To assess the cellular toxicity of the injectable system after encapsulation of ADSCs and NP cells.
- To evaluate the NP cell behaviour after enrichment of the type II atelocollagen hydrogel with varying HA concentrations.

1.5.2 Phase II (Chapter Three)

Overall aim: To map the glyco-environment of the IVD in an effort to understand cell-cell and cell-ECM cross-talk.

Hypotheses:
- Cartilage, NP and AF tissues present different glycosylation profiles at a cellular and extracellular level.
- A differential expression of glycans is observed in cartilage, NP and AF tissues upon maturation at a cellular and extracellular level
- NP, AF and cartilage tissues differ in CS composition from immaturity to maturity.

Objectives:
- To assess the glycan expression in chondrocytes, NP and AF cells and in the ECM of cartilage, NP and AF tissues using lectin technology.
- To identify the glyco-signature of chondrocytes, NP and AF cells and their respective ECM.
- To compare the glyco-environment of the different tissue-types upon maturation at a cellular and ECM level.
- To quantify and to compare the sulfation pattern of CS from immature to mature of NP, AF and cartilage tissues.

1.5.3 Phase III (Chapter Four)

Overall aim: To enhance the production of CS by NP cells by up-regulating via a non-viral gene therapy approach xylosyltransferase I (XT-I) and
glucuronyltransferase I (GT-I), the key enzymes involved at crucial points of CS synthesis.

**Hypotheses:**
- GAGs composition changes in an ageing bovine model are correlated to a decrease of XT-I and GT-I expression.
- The up-regulation of both XT-I and GT-I enzymes promote CS synthesis in NP cells.

**Objectives:**
- To assess the behaviour of GAGs and two of the enzymes involved in their synthesis (XT-I and GT-I) with the ageing.
- To fabricate and produce plasmids encoding XT-I and GT-I.
- To deliver these plasmids to IVD cells by electroporation.
- To evaluate the expression of CS after delivery of XT-I and GT-I plasmids to IVD cells.

**1.6. Reference**


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[201] Jackson G, Marcus-Soekarman D, Stolte-Dijkstra I, Verrips A, Taylor J, Briggs M. Type IX collagen gene mutations can result in multiple epiphyseal
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Chapter 2

Development of a type II collagen/hyaluronic acid hydrogel as a cell vehicle for NP regeneration

Sections of this chapter have been published:

E. Collin, S. Grad, D. Zeugolis, P. Weiss, J. Guicheux, M. Alini, A. Pandit,
Injectable Hydrogel as a Reservoir System for Nucleus Pulposus Regeneration.
Biomaterials. 2011: 32(11): 2862-2870
2.1 Introduction

Back and neck problems are the second leading cause of disability with global prevalence of 60 to 80% amongst individuals between 20 and 50 years old [1]. The pathophysiologies of degenerative intervertebral discs (IVD) have a remarkable socio-economic impact and associated healthcare expenditure of over 90 billion dollars annually [2]. The aetiology of disc degenerative diseases is associated with an abnormal response at a cellular and molecular level due to genetic malignancies and/or environmental factors [3]. At the cellular level, the degeneration process is characterised by a decline in the cell population caused by a decrease in pH, nutrient and oxygen supply [4, 5]. At a molecular level, a change of the extracellular matrix (ECM) composition is noted with a decrease in type II collagen and aggrecan content. This phenomenon leads to a loss of water content that compromises the swelling properties of the disc [6]. The structure of the disc becomes more fibrotic with an increase in type I collagen promoting the vascularisation and innervation of the disc [7, 8]. A structural disorganisation of the nucleus pulposus (NP) and the annulus fibrosus (AF) is induced leading to a loss of disc height [9]. This leads to an overall effect on the biomechanics of the spinal column [10]. Behavioural changes of the ligaments, muscles and other associated structures lead to painful pathophysiologies [8, 11]. The onset of the degeneration process ultimately results in the intervertebral disc being inherently incapable of adequate self-repair [8].

Tissue engineering strategies offer many potential advantages in the treatment of disc degeneration diseases (DDD). Therapeutic strategies involve the use of stem cells [12, 13], chondrocytes and disc cells [14], molecules (growth factors, enzymes) [5, 15] and/or matrix (scaffolds) [16-18]. An ideal cell carrier should provide advantages as therapeutic solutions in the following areas: (1) biodegradability; (2) injectability; (3) preservation of the native tissue; and (4) non-preclusion of future spinal surgery and multilevel disc treatments [11, 18]. The three-dimensional matrix should closely mimic the tissue environment with a degradation rate matching that of ECM regeneration/deposition and without any toxic or inhibitory products. In addition to being highly biocompatible, the scaffold should have a structural make-up that permits the transport of nutrients, metabolites and regulatory molecules to and from the cells [14, 19]. The major challenge is the identification and the development of a matrix that is similar to the native tissue along with being capable
of promoting phenotype maintenance, proliferation and differentiation of NP cells and/or stem cells.

The low antigenic character of collagen and its mechanical properties make it a candidate of choice for intervertebral disc engineering [20, 21]. Type II collagen is a major and uniformly distributed component of the ECM of the NP tissue. Hydrogels based on this molecule enhance chondrogenesis of adipose tissue-derived stem cells and possess higher chondrogenic properties than those of type I collagen [22]. However, non-cross-linked type II collagen presents poor mechanical properties and low resistance to enzymatic degradation [23-25]. An injectable system allows minimal invasion via the annulus fibrosus as well as the material to adopt the irregular shape of the defect [11]. In order to improve stability and mechanical properties, cross-linkers such as glutaraldehyde and carbodiimide have been used. However, these molecules have been shown to be highly toxic, which limits their use as cell reservoir implantable structures [24, 26]. Consequently, different approaches using non-toxic chemical cross-linkers have been developed [27, 28] and in particular the use of poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG) has been advocated. Taguchi et al. have shown the properties of this molecule to cross-link type I collagen in the absence of toxicity for the encapsulation of chondrocytes [28].

Hyaluronic acid (HA), a glycosaminoglycan richly present in NP tissue, has been reported to have a positive effect in stimulating ECM biosynthesis, cell proliferation, cell migration and phenotype maintenance of chondrocytes and NP cells [29-32]. Halloran et al. have shown improved NP cells phenotype maintenance using a composite hydrogel of HA and type II collagen [16]. Furthermore, the use of high molecular weight HA has been advocated for prevention of arthritis because of its notable anti-inflammatory and anti-vascular effects [33, 34]. It has also been reported that HA-enriched type II collagen hydrogels possess the biological benefits of HA without the mechanical limitations [35]. Therefore, in this study an injectable cell-carrier system comprised of type II collagen hydrogel optimally stabilised with 4S-StarPEG, enriched with varying hyaluronic acid concentrations, was designed. It was hypothesised that such a system will be a suitable carrier for cells allowing for their encapsulation and injection and that the presence of HA will provide an environment more conducive to the maintenance of NP cell phenotype.
Figure 2.1. Reaction of type II atelocollagen and 4S-StarPEG. The succinimidyl groups react with the amine groups present on the molecules of type II collagen at 37°C.
2.2 Material and Methods

2.2.1 Materials and Reagents

Type II porcine cartilage atelocollagen was purchased from Symatese Biomateriaux (France). High molecular weight hyaluronic acid (HA) was purchased from Contipro group, CPN (Czech Republic). 4-arm polyethylene glycol succinimidyglutarate Mw 10,000 Da (4S-StarPEG) was purchased from JenKem Technology USA (Allen, TX). Trinitrobenzene sulfonic acid (TNBSA) was purchased from Pierce Protein Research Products, Thermo Scientific (Ireland). Quant-iT™ PicoGreen® dsDNA Reagent was purchased from Invitrogen (Bio Sciences Ltd., Dun Laoghaire, Ireland). All other materials and reagents were purchased from Sigma-Aldrich (Ireland) unless otherwise stated.

2.2.2 Type II Atelocollagen Hydrogel Preparation and Stabilisation

Type II atelocollagen was dissolved in 0.05M acetic acid at a final concentration of 5mg/mL and filtered (0.22µm, Corning, Germany). The pH of the solution was adjusted to 7.4 using 1M NaOH and 5X Phosphate Buffer Saline (PBS). 4S-StarPEG was then added to final concentrations of 0.5mM, 1mM and 2mM. 0.625% glutaraldehyde (GTA) was used as positive control. The solutions were incubated for 1h at 37°C in a humidified atmosphere of 5% CO₂ to induce gelation (Figure 2.1).

2.2.3 Hydrogel Stability

2.2.3.1 Quantification of Free Amines using TNBSA Assay

Residual primary amine groups of the type II atelocollagen hydrogels were determined using TNBSA assay as previously described [26]. Briefly, hydrogels were incubated in 0.1M sodium bicarbonate pH 8.5. 0.01% of TNBSA was added to the samples and incubated for 2h at 37°C. The reaction was stopped using 10% sodium dodecyl sulphate and 1M HCl. The samples were then incubated at 120°C for 15 minutes. Absorbance of each sample was read at 335nm and the free amine groups quantified by interpolating values from a linear standard curve of known concentrations of glycine.
2.2.3.2 Enzymatic Stability (Collagenase Assay)

Resistance of the hydrogels to enzymatic digestion was evaluated using collagenase assay, as described previously [36]. Briefly, hydrogels were incubated for 1h in 0.1M Tris-HCl (pH 7.4), containing 50mM CaCl\(_2\) at 37°C. Subsequently, bacterial collagenase type IV (770 units/mg, extracted from *Clostridium histolyticum*, reconstituted in 0.1M Tris-HCl at a concentration of 10U) was added. After incubation for 48h at 37°C, the enzymatic reaction was stopped by the addition of 0.25M EDTA. After vacuum dehydration, the remaining mass was weighed and normalised to the remaining mass of GTA cross-linked hydrogels.

2.2.3.3 Gel Point and Mechanical Properties (Rheology)

In order to identify the gel time of the hydrogel as a function of cross-linking, rheological measurements were performed at 37°C using a Haake Modular Advanced Rheometer System (MARS) rheometer (Thermo Haakes, Germany) as described previously [37]. Briefly, type II atelocollagen, 5X PBS and 1M NaOH alone, with 0.625% GTA, or with different concentrations of 4S-StarPEG (0.5mM, 1mM and 2mM), were added to the plate at 37°C. The rheometer was equipped with a circulating water bath to accurately control the temperature. To minimise the influence of water loss on mechanical behaviour, samples were coated with paraffin oil. Dynamic frequency sweep experiments were carried out to determine the storage (\(G'\)) and loss (\(G''\)) moduli as a function of time at 37°C. The measurements of the storage (\(G'\)) and loss (\(G''\)) moduli during the gelation were recorded as a function of time for five different frequencies (a, b, c, d and e rad/s) using multi-wave facilities. The gel point was defined as the time that \(G'\) equalled \(G''\). The impact of HA on the storage *modulus* of the hydrogel was investigated.

2.2.3.4 Cytotoxicity of 4S-StarPEG on Adipose tissue-Derived Stem Cells (ADSCs)

ADSCs were extracted from rabbit adipose tissue. The tissue was digested by collagenase type I at 0.025% for 1h under agitation at 37°C. The enzymatic reaction was stopped by addition of complete medium (DMEM, 10% FBS, 1% P/S). The stromal fraction was collected by centrifugation of five minutes at 1,200rpm, suspended and filtered on a cell strainer 70\(\mu\)m (BD Sciences, Switzerland). After 24h of incubation at 37°C in a humidified atmosphere of 5% CO\(_2\), cells were washed in
order to eliminate the contaminant cells (blood cells and adipocytes). Media was changed every 2-3 days and cells were maintained sub-confluent. Adipogenic, chondrogenic and osteogenic differentiation assays were conducted to confirm the nature of the extracted cells.

- Characterisation of Rabbit ADSCs

**Osteogenic differentiation** Cells were cultured in a 6-well plate at a density of 10,000 cells/cm². After 24h of incubation at 37°C under 5% CO₂, the medium was changed for a differentiation medium consisting of complete DMEM supplemented with 10mM β-glycerophosphate, 10nM of vitamin D₃ and 0.05mM ascorbic acid. The medium was changed every 2/3 days for 28 days. Non-treated cells were used as a control. After 28 days, cells were fixed in formalin for 1h and stained with alizarin red S (Figure 2.2.C)

**Adipogenic differentiation** Cells were cultured in a 6-well plate at a density of 30,000 cells/cm². After 24h of incubation at 37°C under 5% CO₂, the medium was changed for a differentiation medium consisting of complete DMEM supplemented with 10µg/mL insulin, 0.5mM isobutylmethylxanthamine, 1µM dexamethasone and 200µM indomethacine. The medium was changed every 2/3 days for 14 days. Non-treated cells were used as a control. After 14 days, cells were fixed in formalin for 1h and stained with Oil Red O solution (Figure 2.2.B).

**Chondrogenic differentiation** Cells were cultured in pellet (1,000,000 cells per pellet) using a differentiation medium composed of DMEM 1% P/S, ITS (6.25 µg/mL insulin; 6.25µg/mL transferrin; 6.25ng/mL sodium selenite), ascorbic acid (50nM) and TGF-β1 (10ng/mL) for 21 days. Pellets were fixed in formalin for 1h. Histological sections were prepared and stained for GAGs production using toluidine blue (Figure 2.2.A).

- 4S-StarPEG Toxicity Evaluation

After the third passage, 20,000 cells were mixed in the 4S-PEG at different concentrations: 0.1mM, 0.5mM, 1mM and 2mM. Cell viability and proliferation were measured using respectively AlamarBlue™ cell metabolic assay and PicoGreen® assay after 24h according to the supplier (Figure 2.3).

The hydrogels were seeded with ADSCs at the third passage. Briefly, type II atelocollagen and 5X PBS solutions (see section 2.2) were mixed and the pH
adjusted to 7.4 by addition of NaOH. 150,000 cells/mL were then added to the mixture. 4S-StarPEG was subsequently added to a final concentration of 0.5mM, 1mM and 2mM. 300µl of the mixture was poured into a cell culture insert (BD Falcon, BD Biosciences, 24 well plate 0.4µm pores) and was maintained at 37°C in a humidified atmosphere of 5% CO₂ for 1h before the addition of complete medium (DMEM, 10% FBS, 1% P/S) onto the hydrogels formed. The medium was routinely changed every two days. Cells were allowed to grow for 2, 7 and 14 days at 37°C in a humidified atmosphere of 5% CO₂. Cell viability was determined using the Trypan Blue exclusion test.

2.2.4 Enrichment of the Optimal 4S-StarPEG Type II Atelocollagen Hydrogel with HA

After identification of the optimal 4S-StarPEG concentration (1mM), the hydrogel was supplemented with high molecular weight HA. The influence of HA incorporation was evaluated biomechanically (Figure 2.4) and biologically (nucleus pulposus cell studies, gene expression assays, cell morphology and distribution studies). HA was dissolved in 5X PBS and 0.4M NaCl [38] and subsequently mixed with type II atelocollagen at molar ratios of 9:0, 9:1, 9:4.5 and 9:9. Neutralisation with 1M NaOH and stabilisation with 1mM 4S-StarPEG followed.

2.2.4.1 Isolation of Nucleus Pulposus Cells

Five-month-old calf bovine fresh tails were collected directly after sacrifice from a local slaughter house. Soft tissues surrounding IVDs (muscles and ligaments) were manually removed. Each IVD was sectioned transversally through the center and NP tissue was harvested from both halves. Tissues were washed twice with Tyrode’s Balanced Salt Solution (TBSS). Following this wash, NP tissues were digested with 0.19% of pronase (Roche, Switzerland) for 1h at 37°C under agitation. After pronase inactivation by successive washes with TBSS, DMEM supplemented with 10% FBS and 1% P/S containing 32IU/mL of collagenase type II (327IU/mg, Worthington Biochemical Corporation, Germany) was added. The mixture was incubated under orbital agitation overnight at 37°C in a humidified atmosphere of 5% CO₂ and was subsequently filtered through a 70µm cell strainer. Cells obtained were centrifuged for five minutes at room temperature at 1,200rpm and counted.
2.2.4.2 Hydrogel Seeding with Nucleus Pulposus Cells

The hydrogels were seeded with NP cells directly after cell extraction. Briefly, type II atelocollagen and hyaluronic acid solutions (see section 2.4) were mixed and the pH adjusted to 7.4 by addition of NaOH. 800,000 cells/mL were then added to the mixture. 1mM 4S-StarPEG was subsequently added. The mixture was maintained at 37°C in a humidified atmosphere of 5% CO₂ for 1h before the addition of complete medium (DMEM, 10% FBS, 1% P/S) onto the formed hydrogels. The medium was routinely changed every two days. Cells were allowed to grow for 2, 7 and 14 days at 37°C in a humidified atmosphere of 5% CO₂.

2.2.4.3 NP Cell Viability in the Hydrogel

After 2, 7 and 14 days, cell viability was assessed using Live/Dead assay. Briefly, hydrogels were incubated in serum free medium supplemented with 10µM calcein AM green (Fluka, Germany) and 1µM ethidium homodimer-1 (Sigma Aldrich, Switzerland) for 30 minutes. Stained samples were visualised on an inverted Confocal Laser Scanning Microscope (CLSM) (LSM510, Zeiss, Jena, Germany). CLSM stacks were imaged at 5µm image intervals. Three fields per hydrogel, with three hydrogels per condition, were imaged for each experiment. Viable and dead cells were counted using the custom-made macro23 ImageJ software as described before [39]. The cell viability was then estimated on a subset of 40 consecutive images in the stack. The first image was chosen as 10 slides from the surface of the hydrogel to prevent the variation caused by the surface.

2.2.4.4 Proliferation of NP Cells

Hydrogels were digested overnight at 56°C by 0.5mg/mL of proteinase K. PicoGreen® Assay was used to assess NP cell proliferation as per manufacturer’s guidelines. A standard curve based on known concentrations of DNA was used to determine the DNA content. The sample fluorescence was measured using a microplate reader (VICTOR3 VÊ Multilabel Counter, PerkinElmer BioSignal Inc, USA) at 480nm excitation and 520nm emission.

2.2.4.5 Distribution of NP Cells

The dissector stereological method was used to determine the variation in cell distribution pattern into the hydrogel. CSLM stacks of 200µm were analysed to determine the distribution pattern (i.e. percentage of covariance) of cells seeded
within the scaffold. For each field previously imaged (see section 2.2.4.b), sections from positions separated by a distance of 20µm were analysed. Cells within the dissector square were counted, whilst cells touching the inclusion lines (bold lines) were excluded. Cells were counted when they appeared for the first time in any subsequent section (Figure 2.2.8.B1).

2.2.4.6 Gene Expression

After 7 and 14 days, total RNA was extracted using a variant of Trizol isolation. Briefly, TriReagent® (Invitrogen, Ireland) was added to the hydrogels. Hydrogels were mechanically disrupted using Tissue Lyser System (Qiagen, Germany). Phase separation was performed using bromo-chloro-propane and total RNA was purified using Nucleospin® RNA II kit (Macherey-Nagel, Germany), according to the supplier’s protocol. Total RNA quantity and purity were determined using an ultraviolet spectrometer (NanoDrop® ND-1000 Spectrophotometer). Reverse transcription (RT) was performed using the TaqMan® RT system according to the manufacturer’s protocol (Applied Biosystems, Switzerland). Gene transcription was examined using real-time polymerase chain reaction (PCR). Reactions were performed and monitored using an ABI™ 7500 sequence detection system (Applied Biosystems) using the TaqMan® gene expression Master Mix (Applied Biosystems) and specific primer sequences and probes (5`FAM-3`TAMRA) for procollagen 2α1, procollagen 1α2 and aggrecan (see Appendix X). Gene transcription was normalised in relation to transcription of the housekeeping human 18S. The 2^ΔΔCt method was used to calculate relative gene expression for each target gene.

2.2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism®, Version 5 (USA). Data were compared using one-way analysis of variance (ANOVA) followed by a Tukey comparison test. Values were considered as significantly different with a p<0.05.
Figure 2.2. Differentiation capability of rabbit ADSCs. (A) Toluidine blue staining of histological sections of pellet after three weeks in chondrogenic differentiation conditions culture. (B) Differentiation to an adipogenic lineage (Oil red O staining) after two weeks in culture in presence (B1) or absence (B2) of differentiation medium. (C) Differentiation to an osteogenic lineage (Alizarin red staining) after four weeks in culture in presence (C1) and absence (C2) of differentiation medium.
Figure 2.3. Rabbit ADSCs activity (percentage of reduction of AlamarBlue™) and proliferation (DNA content) measured 24h after having been mixed with 4S-StarPEG used as a cross-linker. Non-treated cells were used as a control. Data represents mean ± standard deviation (n=3) (t-test 0.05%).
2.3 Results

2.3.1 4S-StarPEG Cross-Linking Efficiency

2.3.1.1 Free Amine Groups Content after Cross-Linking

TNBSA assay was used to evaluate the amine content of the scaffolds produced in this study (Figure 2.4.A). A significant decrease in free amine groups was observed for all concentrations of 4S-StarPEG used ($p<0.05$). At 1mM of 4S-StarPEG, a plateau was reached and no statistical difference noted between 1mM and 2mM cross-linked hydrogel ($p<0.05$).

2.3.1.2 Resistance to Enzymatic Degradation and Stability in Culture

Bacterial collagenase digestion assay was used to evaluate the enzymatic stability of the produced hydrogels (Figure 2.4.B). Non-cross-linked samples were completely degraded within the experimental period (48h). A higher stability compared to the other concentrations used was obtained for 1mM cross-linked hydrogel (10.97 ± 1.68% - 0.5mM, 5.89 ± 2.33% - 1mM, 12.75 ± 2.04% - 2mM) ($p<0.05$). GTA fixed hydrogels and 1mM cross-linked hydrogel exhibited a non-significantly different stability after 48h degradation.

2.3.1.3 Gel Point and Mechanical Properties

Rheological studies were carried out to estimate the gel time of the hydrogel, i.e. the time that $G'$ equalled $G''$. No statistical difference ($p<0.05$) of gel time was observed as a function of 4S-StarPEG concentration, with a gel point of 8.49±1.81 minutes for the 1mM cross-linked hydrogel (Figure 2.5). GTA induced almost instantaneous gelation of the hydrogel, whilst the non-cross-linked samples were not gelled for the duration of the experiment (Figure 2.4.C).

Self-hardness of the hydrogel was evaluated by measurement of storage modulus after 24h from the beginning of the cross-linking process. No statistical differences in storage moduli ($p<0.05$) were obtained (Figure 2.4.C). Equivalent storage moduli were obtained after 1h and 24h of incubation (Figure 2.6).

2.3.1.4 Cytotoxicity of 4S-StarPEG on Adipose Derived Stem Cells

No statistical difference ($p<0.05$) was noted between the viability of encapsulated ADSCs in the non-cross-linked hydrogels (84.98±15.4%) and encapsulated cells in the most highly cross-linked hydrogel (2mM) (77.23±19.92%).
after one day in culture. Over 80% ADSCs viability was observed after encapsulation into the cross-linked type II collagen hydrogel independently of the concentration of cross-linker used after seven and 14 days in culture (Figure 2.4.D). GTA cross-linked hydrogels led to the death of all encapsulated cells (data not shown).

2.3.2 HA Incorporation within the Optimal Stabilised Type II Atelocollagen Hydrogel

2.3.2.1 Hydrogels Stability

Good stability of the hydrogels in culture was observed during this study. Complementary visual inspection of the hydrogels up to 14 days in culture revealed no shrinkage (Figure 2.7). Furthermore, the mass of the hydrogels remained constant for the experimental period (14 days in culture) (Figure 2.8).

2.3.2.2 NP Cells Viability over 14 Days

NP cell viability was found to range from 89.33±5.84 to 98.08±0.99% independently of the HA concentration and the number of days in culture (p<0.05) (Figure 2.9.A).

2.3.2.3 Homogenous NP Cells Distribution

The coefficient of variation between the different planes of the hydrogels ranged from 18.10±2.89% to 31.15±6.58%, which indicates a low variation of cell distribution into the hydrogels and between the different groups (Figure 2.9.B). No statistically significant difference between the different groups was noticed (p<0.05).

2.3.2.4 NP Cells Proliferation and Morphology

After seven days in culture, no cell proliferation was seen independently of the concentration of HA used. After 14 days, a significant increase in DNA content was noted in the absence of HA (6.33±0.95ng of DNA/mg of hydrogel) and consequently an associated increase in cell numbers, whilst in the presence of HA, the cell number remained constant (Figure 10.A).

This phenomenon was confirmed microscopically with cell proliferation onto the surface of the hydrogel in the absence of HA (Figure 2.11.A). This increase in cell numbers was associated with a change in cell morphology. Cells at the surface of the hydrogel lost their round morphology to adopt a more elongated fibroblast-like morphology with numerous short extensions with randomly directed processes.
(Figure 2.11.6). Under this layer, clusters of cells with a round morphology and multiple cytoplasmic elongations were observed in a transient layer. Deeper within the hydrogel, a chondrocyte-like round shape was noted. These changes occurred independently of the concentration of HA used. However, this phenomenon was more pronounced in the absence of HA.

2.3.2.5 NP Cells Gene Expression

After seven days, no difference in gene expression between the different groups of hydrogels tested was observed for the three genes tested ($p<0.05$). A decrease in type II collagen and aggrecan was noted when compared to day 0 (0.03±0.01 to 0.02±0.01-fold and 0.11±0.10 to 0.32±0.14-fold respectively; $p<0.05$). However, the type I collagen expression remained stable independently of the concentration of HA used (between 1.69±0.88-fold increase and 0.59±0.51-fold decrease) (Figure 2.10.B).

2.4 Discussion

The idea of using injectable biomaterials following discectomy for nucleus augmentation was introduced in the early 1960s [11]. Since then, numerous synthetic [40] or natural [16, 41-43] hydrogels have been developed for nucleus pulposus regeneration [40, 44, 45]. The repopulation of the disc using adult mesenchymal stem cells (MSCs) or NP cells has been extensively investigated in the recent years with promising in vivo results [13, 44]. The driven hypothesis of these studies is that an increase in viable cells within the IVD will result in an increase in the deposition of newly synthesised ECM [46] which will potentially restore the disc function. Although the capability of stem cells to form new tissue has been illustrated [13], the association scaffold with cells appears to be essential to protect the cells from mechanical loads, to provide a template for the newly synthesised ECM [46] and to guide the differentiation of stem cells toward a NP cell-like phenotype [47, 48]. A scaffold that mimics the natural environment of NP and can act as a reservoir system for cells has been hypothesised to be the ideal system for NP regeneration [18, 20, 22]. Consequently, a type II atelocollagen/hyaluronan/4S-StarPEG injectable cell carrier system was investigated in this study.

4S-StarPEG molecule is a pegylated structure presenting four terminal N-hydroxysuccinimidyl (NHS) reactive groups. NHS-terminal groups react with the
Figure 2.4. (A) Quantification of free amine group after cross-linkage with 4S-StarPEG. * and # denote significant differences between the different groups (n=3, one-way ANOVA, p<0.05). (B) Degradation by collagenase. Glutaraldehyde (GTA) was used as a control. * denotes a significant difference between the different groups (n=3, one-way ANOVA, p<0.05). (C) Gel point and storage modulus of the different groups of acellular cross-linked hydrogels. GTA was used as a control. No statistical differences were noted for the gel point between the different groups (n=3, one-way ANOVA, p<0.05). (D) ADSCs viability within the hydrogels after cross-linking with variable concentrations of 4S-StarPEG. No statistical differences were noted for the viability between the different groups (n=9, one-way ANOVA, p<0.05).
Figure 2.5. Gel point measurement. Storage *modulus* and loss *modulus* were measured for one hour at 37°C under different frequencies. The storage *modulus* obtained after one hour is equivalent to the storage *modulus* obtained after 24h incubation at 37°C (Figure 2.4.C).
Figure 2.6. Storage modulus of the different groups of acellular cross-linked hydrogels. GTA was used as a control. No statistical differences were noted relative to the enrichment of hyaluronic acid (n=3, one-way ANOVA, p<0.05).
**Figure 2.7. NP cell-seeded scaffold after one, seven and 14 days in culture.** The different molar ratio hydrogels are presented in this figure (type II collagen:hyaluronan).
Figure 2.8. Weight in mg of cell-seeded type II atelocollagen/HA hydrogels after one, two, seven and 14 days in culture. No mass loss was observed after 14 days in culture (n=9, one-way ANOVA, p<0.05).
Figure 2.9. NP cell viability and distribution within the type II atelocollagen/HA hydrogels. (A) Cell viability within the hydrogel. * No significant difference between the different groups (n=9, one-way ANOVA, Tukey test $p<0.05$). (B) NP cell distribution into the hydrogels. (B1) Live dead image from stack used for the quantification. (B2) Percentage of covariance into the different groups of hydrogels. Data were represented as mean ± standard deviation. No significant differences between the different groups at $p<0.05$ were noted (n=9; one-way ANOVA).
Figure 2.10. (A) NP cell proliferation within the hydrogels. DNA content within the hydrogels after one, two, seven and 14 days was evaluated with varying hyaluronic acid concentrations (n=9; one-way ANOVA, p<0.05). (B) Gene expression of the NP cells within the different types of hydrogels after seven days. Type II collagen, type I collagen and aggrecan expressions were quantified after seven days in culture with the type II collagen hydrogels with varying ratios of HA (n=9; one-way ANOVA, p<0.05).
Figure 2.11. Cell morphology of NP cells within type II atelocollagen hydrogels containing different ratios of HA stained by Live/Dead assay staining after 14 days in culture. (A) Type II collagen/HA hydrogel ratio 9:0. (B) Type II collagen/HA ratio 9:1. (C) Type II collagen/HA hydrogel ratio 9:4.5. (D) Type II collagen/HA hydrogel ratio 9:9. Viable cells appear in green (calcein staining) and dead cells in red (ethidium bromide staining).
amine groups of the type II collagen molecules inducing their cross-link (Figure 2.1) and consequently stabilising the structure. The use of 4S-StarPEG as an in situ cross-linking agent for type II collagen was evaluated in this study. At a biochemical level, 1mM 4S-StarPEG decreased the percentage of free amine groups to 30.7±16.3%. This value did not decrease further after cross-linking with 2mM 4S-StarPEG showing that a maximum amount of cross-linking had been reached (Figure 2.4.A). Degradation by collagenase showed an efficient cross-linking reaction leading to the formation of a stable hydrogel (Figure 2.4.B). 1mM cross-linked hydrogel presented a stability equivalent to GTA cross-linked hydrogel (Figure 2.4.B). However, a reduction in stability after degradation by collagenase was observed when the concentration of cross-linker increased to 2mM, showing that high levels of cross-linker have a detrimental effect on the scaffold stability.

Injectability, implant mobility following implantation and stability are important considerations for the design of a NP treatment [18, 40, 46]. An injectable hydrogel system is advantageous since it sets on the site, is potentially bonded with the adjacent native tissue and prevents the movement of the implant [11]. Furthermore, this less invasive system prevents damage to the AF leading to alteration of the tissue, its mechanical properties and consequently its function [11, 18, 45]. GTA stabilisation induced instant gelation that prohibits injectability, whilst non-cross-linked scaffolds failed to self-assemble within the experimental period (1h). On the other hand, the 4S-StarPEG stabilised the injectable system after approximately eight minutes at 37°C independently of the concentration of 4S-StarPEG used. The highest storage modulus was obtained after less than 1h incubation equivalent to the storage moduli obtained after 24h (Figure 2.7). This setting time is acceptable, since it not only allows time for the surgeon to inject the hydrogel, but also allows the accurate placement of the hydrogel at the site and prohibits any potential leakage of the gel after injection [11]. The cross-linker concentration did not modify the time of gelation of the hydrogel (Figure 2.4.C). It did however affect its storage modulus after 24h (0.5mM-160.350.49Pa; 1mM-633.07±131.04Pa). For this reason, this property can be used to vary the stiffness of the hydrogel without changing its injectability properties.

Better understanding of IVD degeneration at a cellular and molecular level and high interest in stem cells potential in regenerative medicine arouse major interest in cell delivery as a therapeutic approach. The high toxicity of chemical
cross-linkers like EDC/NHS and GTA, commonly used for stabilisation, does not allow an \textit{in situ} cross-link [26]. No toxicity of the 4S-StarPEG to the NP cells and ADSCs was observed with viability over 80\% (Figures 2.4.D and 2.9.A). GTA cross-linked hydrogels were used as control for this experiment (data not shown). Cells into the hydrogels were dead because of the toxicity of this molecule [26]. This absence of toxicity makes the 4S-StarPEG a candidate of choice for future use as an \textit{in situ} cross-linker.

HA has been shown to enhance stem cell differentiation and NP cell phenotype maintenance [49]. Furthermore, this molecule, an unsulfated glycosaminoglycan richly present in the native tissue, binds to aggrecan molecules to create aggregates [18]. These aggregates are involved in the retention of water into the IVD and consequently in its mechanical function. Mechanical properties of the hydrogel were not affected after enrichment with HA ($p<0.05$) (Figure 4S, Supplementary information). At a cellular level, NP cells viability was not compromised in the presence of the different HA concentrations used.

A homogenous distribution of the NP cells into the hydrogel was noted after seven days in culture without a significant HA effect (CV\% 30\%) (Figure 2.9.B2). A homogenous distribution of the cells into the hydrogels conducts a maximum ECM-cells interaction and a best cellular response to the permissive micro-environment. The incorporation and the uniform dispersion of cells within the hydrogel allow the anticipation of a homogenous repopulation of the IVD. Moreover, this uniform distribution could be used for the functionalisation of the hydrogel using potential therapeutic agents such as TGF-\beta or BMPs [11].

After 14 days in culture, an increase in DNA content was observed in the NP cells cultured in the absence of HA in the type II collagen hydrogels (Figure 2.10.A). This increase reports a cell proliferation correlated to a modification of cell morphology accentuated in the absence of HA. Three layers could be observed in this hydrogel as in the disc: a fibroblastic cell morphology on the surface, then clusters of cells with a round morphology and multiple cytoplasmic elongations and deeper a chondrocyte-like round shape (Figure 2.11). One of the limitations of \textit{in vitro} studies using hydrogels is the low solubility and diffusion of oxygen and nutrients within the hydrogel-creating gradients [50]. NP cells were maintained viable within the hydrogel during this study without being impacted by these potential gradients. However, they adopted different morphologies within the
hydrogels depending on their location. Discs cells and chondrocytes can adopt a fibroblastic shape in a high oxygen and glucose environment \([51, 52]\) similar to the shape adopted by the cells on the surface of the hydrogel. Deeper in the hydrogel, with a lower level of oxygen and nutrients, cells maintained a round morphology. These observations, \textit{i.e.} high cell survival and the maintenance of NP cells morphology deep within the hydrogel, are due to the natural environment of the disc cells with low oxygen and nutrient supplies \([4, 5]\). Nevertheless, further experiments would need to be conducted to confirm the presence of this gradient.

The presence of HA seemed to slow down the process of dedifferentiation at a morphological level. However, the concentration of this molecule did not seem to influence this phenomenon. Furthermore, it did not induce any change at a gene expression level after seven days in culture (Figure 2.10.B). Gene expression level at 14 days could not have been determined due to the mixed cell populations observed microscopically. A modification of NP cells phenotype was noted compared to cells after extraction with a decrease of type II collagen and aggrecan expression. No significant difference was observed for the level of type I collagen, a major marker for NP cells phenotype loss, after seven days in culture. As type I collagen is one of the major markers for the loss of NP cell phenotypic loss, this result suggests that the hydrogel had some positive effect on the maintenance of the cell phenotype.

### 2.5 Conclusions

- Type II atelocollagen hydrogel was successfully stabilized with 4S-PEG with better stability after degradation by collagenase, a decrease of free amine groups in the structure and suitable mechanical properties.
- The system possesses injectable properties with a gelling time of eight minutes.
- The mechanical properties of the hydrogel were not altered by the addition of HA. A good cell viability and distribution were obtained after encapsulation of NP cells and ADSCs.
- HA promotes the maintenance of NP cell phenotype. An absence of NP cell proliferation was observed in presence of HA. A round morphology of NP cells was maintained with an increased concentration of HA.
- After seven days in culture, a low level of type I collagen expression was maintained in NP cells. However, a decrease of type II collagen and aggrecan expression was observed.
A potential correlation between the conditions of culture and the loss of phenotype can therefore be drawn, which highlights the importance of maintaining cell-seeded scaffolds in an \textit{in vivo}-like culture conditions. Consequently, these observations need to be taken into consideration for future studies, including trials for stem cell differentiation toward a NP cell phenotype. Additional biochemical and functional analyses would be required for further characterisation of the hydrogel developed in this study.

2.6 Future Studies

A pilot study to test the hydrogel developed herein in an \textit{in vivo} environment was performed in an ageing rabbit model with spontaneous degeneration. Promising results were obtained in this study (see Appendix X). However, multiple limitations to the model did not give conclusive evidence. In order to overcome these limitations, an organ culture system was developed to test this hydrogel in an \textit{in vivo}-like environment in a cost-effective manner (Appendix X). Further investigations in this system and later in a large animal model would be required to validate the system developed in this chapter.

The results obtained in chapter 2 highlighted the importance of the ECM composition on NP cell behavior. The highly specialised ECM composition of NP tissue plays a crucial role on cell behavior. However, the effects of this ECM on the cell behavior are not yet well understood. The identification of this ECM composition can help in understanding the cell-ECM communication and consequently in the design of the optimal tissue engineering approach. For this reason, the subject of chapter 3 was the identification of the glycoenvironment of the IVD tissue.

2.7 References


Chapter 3

Glycoenvironment of the Intervertebral Disc: From Immaturity to Maturity

Sections of this chapter are under submission:

E. Collin, M. Kilcoyne, S. Grad, S. White, M. Alini, L. Joshi, A. Pandit, Unique glycophenotypes and sulfation patterns for intervertebral disc and articular cartilage cells and tissues upon maturation, submitted to
3.1 Introduction

Glycans play an essential role in the modulation of many biological processes from development to pathology. Associated with cell membrane, intracellular proteins or lipids, and extracellular matrix (ECM), glycans mediate cell-cell and cell-ECM communication [1] by regulating growth factor interaction and distribution [2], receptor activation and interaction [3], and ECM protein adhesion [4]. Their structure and composition, both tissue and cell specific [5, 6], vary from development to maturity [7, 8] and from healthy to pathological states [9]. It is generally understood that the glycan environment of the intervertebral disc (IVD) influences its biology and mechanical properties [10]. However, not much is known on the glyco-signature of cells and ECM of this tissue or of the influence of this signature on disc degeneration diseases (DDD).

Particularly rich in glycans [11], the ECM of the IVD differs between tissue-types according to their functions and their maturity (Figure 3.1). The nucleus pulposus (NP) contains a large quantity of type II collagen, aggrecan and hyaluronic acid (HA) while the annulus fibrosus (AF) is mainly composed of type I collagen and fibronectin [12, 13]. The association of aggrecan with HA molecules via link N proteins allows the formation of aggregates within the type II collagen network that accumulates a large amount of water molecules. This structure confers NP a resilience to mechanical stresses [11, 14, 15]. The highly organized collagen fibers of the AF form a ring around the hydrated NP maintaining the pressure on this tissue. AF also allows the transmission of loads to the adjacent vertebral bodies [16, 17]. Aside from these molecules, collagens type III, V, VI, IX and XI [11, 18] and connective proteins such as laminin and elastin [11] are expressed in both tissues at minor concentrations. The ECM of both NP and AF tissues also contains other large PGs such as versican and small proteoglycans (PG) such as decorin, biglycan, lumican and fibromodulin at different concentrations. These molecules are differentially expressed from embryonic development to degeneration of the IVD in relation to their function in disc biology [11, 18].

During embryonic development, the mesenchyme condenses around the notochord to form the vertebral bodies and IVD [19, 20]. Derived from the notochord, the NP tissue contains two main cell types: the notochordal cells and the NP cells [19, 20]. Recent findings tend to favour a notochordal origin of the NP cells [21, 22] while the cells of the AF are derived from the mesenchyme [13].
Notochordal cells are generally described as being lost with age although they are found to persist in many species after birth [19, 23]. These cells greatly influence the production of ECM molecules, especially proteoglycan (PG). The loss of notochordal cells is described as to precede the ageing and degeneration processes associated with a depletion of PG and a significant decrease in cell number [24]. Therefore, the phenomena which occur in IVD from immaturity to maturity are crucial to its structure and function. However, little is known on the progression between these two states. During ageing and disc degeneration processes, a tissue imbalance is induced by the secretion of many cytokines (mainly IL-1β and TNF-α) and matrix degrading enzymes (MMP-1, -2, -3, -7, -8, -9, -13, ADAMTS-5, etc....) [12, 25]. The tissue becomes more solid, less flexible and more fibrotic. The cell population decreases greatly and the ECM composition is severely affected, the most notable being at the PG level [12]. The depletion of PG encourages vascularisation and nerve ingrowth leading to pain [26, 27].

The changes occurring upon the maturity of the IVD appear to be critical to the maintenance of the tissue in a healthy stage. A better understanding of these changes allows the development of new therapeutic approaches that aim to restore the IVD functionality. These approaches tend to replenish the ECM with a scaffold that is functionalised with cells and different biological molecules (ECM molecules, growth factors, and inhibitory molecules) which would stimulate the regeneration of the tissue [18, 28]. One of the strategies envisaged is the delivery of stem cells for the repopulation of the IVD tissue [28]. So far, studies ensure their differentiation into a chondrogenic lineage by analysing the expression of a small panel of known markers including type II collagen, aggrecan and sox 9 [29, 30]. NP cells are often referred as "chondrocytes-like" cells. Chondrocytes and NP cells exhibit different morphologies and secrete a comparable ECM composition. ECM from both cartilage and NP tissue differ at a quantitative level providing different mechanical properties and functions to cartilage and IVD tissues [31]. It is therefore essential to know the exact cell phenotype and ECM composition of these tissues to verify the differentiation of stem cells toward the right phenotype, and to obtain the appropriate secretion of newly synthesised ECM. Numerous studies were performed to underline the differences between chondrocytes, NP and AF cells [32-35]. However, so far, no clear "on/off" markers have been identified which distinguish chondrocytes, NP and AF cells. A few markers were identified as being specific to the cell-types as
cytokeratin-19 [34], FOXF1 [31], Pax-1 [31], CA-12 [31, 32], MGP [34, 36], more highly expressed in NP cells than in articular chondrocytes and AF cells, or GDF10 and CYLT-1 more expressed in articular chondrocytes [31], or GPC-3 and COMP highly expressed in AF cells [33, 34]. Some negative markers such as MMP-12 and MMP-27 expressed uniquely by chondrocytes are used to differentiate NP cells from chondrocytes [37].

Cell-cell and cell-ECM interactions play crucial roles during the development of the IVD by profoundly influencing cell behaviour and ECM deposition. Numerous glycoproteins and PG expressed within the IVD tissue regulate its behaviour by influencing many biological functions [20, 38]. Glycoproteins are expressed in the extracellular matrix, intracellularly and on the surface of the cells. They are formed by the attachment of oligosaccharides chains to asparagin (N-linked) or serine/threonin (O-linked) residues resulting of post-translational modifications [1, 6]. Glycan expressions were shown to reflect the phenotypic status of chondrocytes in vitro with specific lectin binding profiles associated with their immortalisation and dedifferentiation [6]. The terminal structure of glycans (fucosylated, sialylated, mannosylated structures) contributes to the glycan diversity and their involvement in cell communication and tissue remodelling [39-41] (Figure 3.2). The knockout of specific enzymes such as fucosyl and sialyltransferases has provided evidence of the importance of terminal modifications of glycans in development, pathology and ageing [42, 43]. Large linear polymeric glycans attached a protein core, the glycosaminoglycans (GAG), play an important structural role in the ECM but also possesses many biological functions [44].

Different groups of GAGs are described: chondroitin/dermatan sulfate, keratan sulfate, heparan sulfate and HA [1]. Chondroitins sulfate (CS) (Figure 3.1), repeat unit of [\( \beta 4\)]GlcA-\( \beta -(1 \beta 3)\)-GalNAc-\( \beta -(1 \beta 4)\) motif attached on a protein core such as aggrecan or versican, are the most abundant GAGs present within the IVD [20]. Their sulfation patterns greatly influence the biology of the disc not only by allowing the retention of water within the disc but also by guiding the behaviour of the IVD cells [20]. These patterns are also shown to dictate the fate of other tissues such as cartilage [45], nerves [7, 46] and of immune cells [47] through interaction with growth factors, receptors and other ECM molecules [7, 20]. CS signature was shown to be modified under pathological conditions in cartilage [45] and can also be used as putative biomarkers for progenitor cells in this tissue [48]. It can therefore be
believed that the access of the glycoalx and ECM signature of cartilage, NP and AF tissues will not only allow the differentiation of the three different cell-types but also provide a glyco-phenotype signature for each cell type and ECM at different stages of development and later for degeneration.

Lectin histochemistry, immuno-histochemistry and high performance liquid chromatography (HPLC) analysis were used to identify the glyco-environment of IVD and cartilage tissues. Lectins are proteins naturally present in plants, invertebrate and vertebrate organisms regulating multiple biological processes from development to pathology [4]. Extracted from plants and invertebrates, their high affinity for glycans is often used as a tool to detect and to determine the glycan structures of glycoconjugates including glycoproteins, glycolipids and glycosaminoglycans [4, 49]. Lectin histochemistry allows the identification and the localization of glycan moieties within the tissue and provides information on the glycodynamics of a tissue over development, ageing and/or pathology [49]. CS quantity and structure, shown as essential in cartilage and IVD tissues phenotype maintenance [5, 20], were further investigated by HPLC in order to reveal both their quantity and composition in the ECM upon maturity.

Glycans expressed at the cell surface and in the ECM of cartilage, NP and AF tissues were screened using lectin technology in order to identify the glycosignature of chondrocytes, NP and AF cells and their respective ECM across different maturation states (Figure 3.2). The hypothesis of this study was that IVD and cartilage tissues could be selectively distinguished based on glyco-biomarkers at cell and ECM levels and also that change in glycan expression and structure occur on the cell surfaces and in the ECM with maturation of the IVD and cartilage tissues which influence their fate. The glycoenvironment of immature (three month-old) and mature (eleven month-old) cartilage and IVD tissues were therefore compared at a cellular and ECM level.

3.2 Material and Methods

3.2.1 Material and Reagents

Tetramethylrhodamine isothiocyanate (TRITC)- and fluorescein isothiocyanate (FITC)-labelled lectins were sourced from EY Laboratories (San Mateo, CA, USA). Bovine serum albumin (BSA), proteinase K, chondroitinase ABC
Figure 3.1. Cartoon representations of IVD, cartilage and harvested tissues for analysis. (A) The IVD is composed of the nucleus pulposus (NP) surrounded by the annulus fibrosus (AF). On both side of the vertebral body is found the cartilage end-plate (CEP). (B) The articular cartilage (AC) from the knee, which covers the end of the femur, the top of the tibia and the back of the patella, rests on the meniscus (M). For both tissues, IVD and AC, half was used for histology and the other half for biochemical analyses.
Figure 3.2. Symbolic representation of the main glycan motifs detected during this study. The behaviour upon maturity of fucosylated, sialylated, high glycans, terminal N-acetylgalactosamines (GalNAc) and T-antigens motifs in terminal position of glycans were assessed by immuno- and lectin histochemistry. Chondroitin sulfate quantity and structure were evaluated by HPLC analysis. Glycans were represented with the international symbol nomenclature [50].
Glycoenvironment of the intervertebral disc

(ChABC) from *Proteus vulgaris*, essentially protease-free, 4Nj-diamidino-2-phenylindole dihydrochloride (DAPI) and the CS-56 antibody were procured from Sigma-Aldrich Co. (Dublin, Ireland). The BSA was periodate-treated [51] and used for all histochemistry. The bicinchoninic acid (BCA)™ Protein Assay Kit was from Pierce® Biotechnology (Thermo Fisher Scientific Inc., Dublin, Ireland). Amicon® Ultra molecular weight cut off (MWCO) centrifugal filters were obtained from Millipore (Dublin, Ireland). The anti-chondroitin 6-sulfate (C6S), and anti-blood group Lewisb antibodies were sourced from Abcam® (Cambridge, UK) ProLong® Gold antifade and Alexa Fluor® 488-conjugated donkey anti-mouse secondary antibody were sourced from Life Technologies™ (Dublin, Ireland) and unsaturated chondroitin sulfate disaccharide standards were procured from Dextra Labs Ltd. (Reading, UK). All other reagents used were sourced from Sigma Aldrich Co. unless otherwise stated and were of the highest grade available.

3.2.2 Tissue Collection and Digestion

Three and 11 month-old ovine spine and articular cartilage were collected from a local abattoir directly after sacrifice. Soft tissues surrounding IVDs (L4-L5) and cartilage (muscles and ligaments) were removed, and tissues were harvested. For each disc, half of the tissue was fixed for histochemistry with 4% paraformaldehyde (Figure 3.1). After three washes with 1X phosphate buffered saline (PBS; pH 7), tissues were infiltrated overnight with 20% sucrose. Specimens were flash-frozen in liquid nitrogen-cooled isopentane and 5µm frozen sections were cut on a Leica™ CM 1850 cryostat (Laboratory Instruments & Supplies Ltd., Ireland). Tissue sections were collected on Superfrost® Plus slides (Fisher Scientific Inc., Dublin, Ireland) and stored at -20°C until use. NP and AF tissues were separated from the remaining IVD tissue. Both tissues and cartilage were digested with proteinase K (0.5mg/mL) overnight at 56°C and stored at -20°C before further analyses.

3.2.3 Expression of Glycan

3.2.3.1 Histochemistry of Lectin

The slides were washed three times for five minutes each with buffered saline supplemented with Ca²⁺ and Mg²⁺ ions (TBS; 20 mM Tris-HCl, 100 mM NaCl, 1 mM each of CaCl₂ and MgCl₂, pH 7.2) with 0.05% Triton X-100 (TBS-T) and then blocked with 2% periodate-treated BSA in TBS for one hour. Sections were washed
again three times for five minutes each in TBS and incubated with the fluorescently-labelled lectin diluted in TBS for one hour (Table 3.1). Inhibitory controls were carried out in parallel to verify that the lectin binding was glycan-mediated [52, 53]. The lectins were pre-incubated in 100mM of the appropriate haptenic sugar (see Table 1 for inhibitor specificity and concentration) for one hour at room temperature (RT) beforehand. The sections were washed five times with TBS with 0.05% Tween 20 and counterstained with DAPI (1/2,500; diluted in TBS) for 20 minutes. The slides were washed again in TBS with 0.05% Tween 20 before mounting with ProLong® Gold antifade (Life Science™, Dublin, Ireland) (Appendix DD). The slides were kept at 4°C in the dark for one day before imaging. Imaging was performed using an inverted epifluorescent microscope (Olympus IX81, Mason Technologies, Dublin, Ireland). Five random images per slide for each tissue were taken and analysed.

3.2.3.2 Immunohistochemistry of Lewis^b (Le^b) and H1 Antigen

Slides were washed three times in 1X PBS. For all immunohistochemistry, sections were blocked with 5% goat serum in 1X PBS for one hour before overnight incubation at 4°C with the primary antibody anti-blood group Le^b antibody (Abcam®, Cambridge, UK) or H1 antigen (anti-blood group H1 (O) antigen antibody (Abcam®, Cambridge, UK)) (Table 3.1). Negative control sections were incubated without primary antibody. After five washes with 1X PBS with 0.05% Tween 20 for five minutes each, the sections were incubated at RT for one hour with the secondary antibody (AlexaFluor 488® donkey anti-mouse) diluted at 1/1,000. The slides were then washed five times with 1X PBS with 0.05% Tween 20 for five minutes each before being counterstained with DAPI diluted at 1/2,500 in 1X PBS for ten minutes. The sections were washed three times with 1X PBS with 0.05% Tween 20 before mounting with ProLong® Gold antifade (Life Science™, Dublin, Ireland). The slides were kept at 4°C in the dark for one day before imaging (Appendix DD). Imaging was performed using an inverted epifluorescent microscope (Olympus® IX81, Mason Technologies, Dublin, Ireland). Five images per slide for each tissue-type were taken and analysed as described in section 3.2.5.
3.2.4 Quantification of Chondroitin Sulfate

3.2.4.1 Colorimetric Quantification of sGAG by DMMB assay

sGAG content was quantified by the dimethylmethylene blue (DMMB) method as previously described [54]. Chondroitin 4-sulfate from bovine trachea was used as a standard (Appendix N). The absorbance was measured at 535nm on a Varioskan® Flash Spectral Scanning Multimode Reader. DNA quantification for sGAGs content normalisation was performed using the Quanti-IT™ Picogreen assay (Invitrogen, Dublin, Ireland) according to the supplier's instruction (Appendix G). Protein concentration was determined by the microBCA method [55] using BSA as standard.

3.2.4.2 Quantification of CS by HPLC

100µL of the proteinase K-digested NP, AF or cartilage tissues (113.5 to 584.1 µg of protein) was filtered through a 3kDa molecular weight cut off (MWCO) centrifugal filter with 100µL HPLC-grade water according to manufacturer's instructions. Water used throughout the procedure was HPLC-grade. Briefly, all centrifugal filters were washed with water prior to use. The retentate was eluted from the filter with 50µL of water, and 390µL of digestion buffer (50mM Tris-HCl, 60mM sodium acetate, pH 8.0) and 10µL of chondroitinase ABC (ChABC, 100mU). Eluate was then digested at 37 °C for three hours with gentle agitation (300rpm). The digested mixture was immediately filtered using a 3kDa MWCO spin filter as above and the lower molecular weight filtrate was dried in a vacuum centrifuge (~ two hours). The dried digested samples were stored at -20°C until analysis.

Following this, digested samples were dissolved in 200µL water. 10µL was injected on an Alliance™ 2695 HPLC instrument (Waters, Dublin, Ireland). The content of unsaturated disaccharides ADi-0S, ADi-4S and ADi-6S was determined by a method described by Ji et al. [56] with minor modifications indicating the presence of C0S, C4S and C6S in the undigested tissue sample.

Briefly, chromatographic separations were carried out on an Ultratech® 5ODS C18 (250 x 4.6mm) (HPLC Technology, Inc., Cheshire, UK) or a Synergi™ column (250 x 4.6mm, 4µm, 80 Å) (Phenomenex Inc., Torrance, CA, USA) at 25°C at a flow rate of 1.1mL/minutes. The eluate was monitored at an absorbance of 232nm on a Waters™ 2489 UV-Vis detector. The mobile phase consisted of (A) 1 or
2mM aqueous tetrabutylammonium bisulfate (TBAB) and (B) 1 or 2mM TBAB in 2:1 mixture of acetonitrile and water, with starting conditions of 80% A and 20% B. The gradient of 20-65% B was applied over seven minutes, held at 65% B for five minutes and returned to 20% B by 12.5 minutes. The system was re-equilibrated at 20% B for ten minutes before the next sample injection. The disaccharide content of each sample was identified by comparison with appropriate chromatographed standards under the same HPLC conditions as the sample and quantified by comparison with the appropriate standard curve generated by injection of known concentrations of the standards. Data were normalized by the quantity of DNA and represented as $\mu$g of disaccharide per $\mu$g of DNA.

### 3.2.4.2 Immunohistochemistry of CS

Immunohistochemistry was performed for two different antigens: C6S (anti-chondroitin 6-sulfate antibody (Abcam®, Cambridge, UK)), and C6S and C4S (CS-56 antibody (Sigma-Aldrich, Dublin, Ireland)) (Table 3.1). For the detection of C6S, an antigen retrieval by tissue digestion with 0.25U/mL of ChABC diluted in digestion buffer (50mM Tris-HCl, 60mM sodium acetate, pH 8.0) for 30 minutes was performed. Slides were then washed three times in 1X PBS. For all immunohistochemistry staining, sections were blocked with 5% goat serum in 1X PBS for one hour before overnight incubation at 4°C with the primary antibodies (see Table 3.1 for primary antibodies dilution). Negative control sections were incubated without primary antibody. After five washes with 1X PBS with 0.05% Tween 20 for five minutes each, the sections were incubated with the secondary antibody (AlexaFluor 488® labelled-donkey anti-mouse (Life Science™, Dublin, Ireland)) diluted in 0.1% goat serum at 1/1,000 for one hour at RT. The slides were then washed five times with 1X PBS with 0.05% Tween 20 for five minutes each before being counterstained with DAPI diluted at 1/2,500 in 1X PBS for ten minutes at RT. The sections were washed three times with 1X PBS with 0.05% Tween 20 before mounting with ProLong® Gold antifade (Life Science™, Dublin, Ireland). The slides were kept at 4°C in the dark for one day before imaging. Imaging was performed using an inverted epifluorescent microscope (Olympus® IX81, Mason Technologies, Dublin, Ireland). Five random images per slide were taken and analysed as described in section 3.2.5.
3.2.5 Image Analysis and Fluorescence Intensity Quantification

Quantification of fluorescence intensity of the digital images was done using Image J software (National Institutes of Health, USA). From each image, five random cells and five random ECM areas were digitally marked and the intensity of fluorescence measured [57]. The intensity of fluorescence values were then normalised by the surface area. An average of the five values was taken as the quantification of cells and ECM for one image. The average of quantification from the five images was taken for the quantification of one tissue per animal. The average fluorescence intensity quantification from five animals with standard error of the mean was represented (Appendix EE).

3.2.6 Clustering Analysis

For clustering analysis, data were normalised to maximal individual lectin intensity (100%). Clustering analysis was then performed in Hierarchical Clustering Explorer 3.0 (National Institutes of Health, USA) without normalisation across lectins, for clustering parameters complete linkage and Euclidean distance for clustering linkage method and similarity measure, respectively.

3.2.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism®, Version 5 (USA). Data were compared using ANOVA followed by Tukey comparison or the non parametric Kruskal-Wallis test followed by Dunns comparison test when the population followed a normal and a non normal distribution (D’Agostino and Pearson omnibus normality test), respectively. Values were considered as significantly different with a \( p < 0.05 \).

3.3 Results

An inhibition by the inhibitory glycans was observed for all lectins used in this study (data not shown).

3.3.1 Fucosylated Motifs

The binding observed for the three fucose-specific lectins (Table 3.1), UEA-I, AAA and LTA, was unique for each lectin which indicated an interaction of each lectin with different glycan motifs.
Table 3.1. Lectins and antibodies used for tissue histochemistry, their specificities, concentrations and inhibitory glycans [58].

<table>
<thead>
<tr>
<th>Lectin/antibody</th>
<th>Abbreviation</th>
<th>Binding specificity</th>
<th>Concentration (mg/mL)</th>
<th>Inhibitory glycan</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lotus tetragonolobus</em> agglutinin</td>
<td>LTA</td>
<td>Fuc-α-(1Y 3), Fuc-α-(1Y 6), Fuc-α-(1Y 2)</td>
<td>15</td>
<td>100mM Fuc</td>
</tr>
<tr>
<td><em>Ulex europaeus</em> agglutinin I</td>
<td>UEA-I</td>
<td>Fuc-α-(1Y 2)</td>
<td>10</td>
<td>100mM Fuc</td>
</tr>
<tr>
<td><em>Aleuria aurantia</em> agglutinin</td>
<td>AAA</td>
<td>Fuc-α-(1Y 6), Fuc-α-(1Y 3)</td>
<td>20</td>
<td>100mM Fuc</td>
</tr>
<tr>
<td>Anti-Lewis(^b) antibody</td>
<td>Le(^b)</td>
<td>Fuc-α-(1Y 2)-Gal-β-(1Y 3)-[Fuc-α-(1Y 4)GlcNAc]</td>
<td>1.25x10^{-3}</td>
<td>---</td>
</tr>
<tr>
<td><em>Sambucus nigra</em> agglutinin I</td>
<td>SNA-I</td>
<td>Neu-α-(2Y 6)-GalNAc &gt; Lac, GalNAc &gt; Gal</td>
<td>20</td>
<td>100mM Lac</td>
</tr>
<tr>
<td><em>Maackia amurensis</em> agglutinin</td>
<td>MAA</td>
<td>Neu-α-(2Y 3)-GalNAc, Gal-3S &gt; Lac</td>
<td>20</td>
<td>100mM Lac</td>
</tr>
<tr>
<td><em>Griffonia simplicifolia</em> isolectin</td>
<td>GS-I-B(_4)</td>
<td>Terminal α-Gal</td>
<td>10</td>
<td>100mM Gal</td>
</tr>
<tr>
<td><em>Concanavalin A</em></td>
<td>Con A</td>
<td>α-Man &gt; α-Glc &gt; α-GlcNAc, complex biantennary structures</td>
<td>20</td>
<td>100mM Man</td>
</tr>
<tr>
<td><em>Peanut agglutinin</em></td>
<td>PNA</td>
<td>Gal(Gal-β-(1Y 3)-GalNAc(T-antigen)) &gt; GalNAc &gt; Lac &gt; Gal, terminal β-Gal</td>
<td>10</td>
<td>100mM Gal</td>
</tr>
<tr>
<td><em>Artocarpus integrifolia</em> agglutinin</td>
<td>Jacalin (AIA)</td>
<td>Gal, Gal-β-(1Y 3)-GalNAc (T-antigen), Gal-α-(1Y 6), sialylation independent</td>
<td>10</td>
<td>100mM Gal</td>
</tr>
<tr>
<td><em>Soya bean</em> agglutinin</td>
<td>SBA</td>
<td>GalNAc &gt; Gal</td>
<td>10</td>
<td>100mM GalNAc</td>
</tr>
<tr>
<td><em>Wisteria floribunda</em> agglutinin</td>
<td>WFA</td>
<td>GalNAc, GalNAc-α-(1Y 6)-Gal &gt; GalNAc-α-(1Y 3)-GalNAc (Forsmann antigen) &gt; GalNAc &gt;&gt; Lac &gt; Gal, GlcA-α-(1Y 3)-GalNAc</td>
<td>10</td>
<td>100mM GalNAc</td>
</tr>
<tr>
<td>Anti-chondroitin-4- and -6-sulfate antibody (CS-56 clone)</td>
<td>CS-56</td>
<td>GlcA-α-(1Y 3)-GalNAc-4S, GlcA-α-(1Y 3)-GalNAc-6S</td>
<td>1/200 dilution</td>
<td>---</td>
</tr>
<tr>
<td>Anti-chondroitin-6-sulfate antibody</td>
<td>C6S</td>
<td>GlcA-α-(1Y 3)-GalNAc-6S</td>
<td>1/200 dilution</td>
<td>---</td>
</tr>
</tbody>
</table>
Glycoenvironment of the intervertebral disc

UEA-I lectin is known to bind to the α-(1→2)-linked fucose group of the H-antigen, antigen part of the O, B and A blood group antigenic determinants (Table 3.1, Figure 3.4.A) [53]. Differences in binding of this lectin were noted between tissue-types and age-groups. A significant decrease of UEA-I lectin binding on NP and AF cells was observed (9.10% and 22.73%, respectively) upon maturity while the binding intensity was significantly higher (p<0.05) on chondrocytes in 11 month-old tissues (Figure 3.4.C). Both NP and AF cells presented a higher binding intensity than chondrocytes in three month-old tissue (p<0.05). A similar pattern of UEA-I binding to that of cell binding was observed for the ECM of both AF and cartilage tissue although no significant difference of binding was noted for the NP tissue (p<0.05) (Figure 3.4.C). ECM of AF in the immature tissues contained more α-(1→2)-linked fucose and antigen H motifs than other tissues with a binding intensity 14.69% and 20.59% higher than that seen in NP and cartilage tissues, respectively.

α-(1→6)-, α-(1→3)- and α-(1→2)-linked fucosylated motifs are recognised by LTA lectin (Figure 3.5.A) with a higher affinity for α-(1→6)-linked fucosylated motifs (Table 3.1). No binding of LTA occurred on the NP and AF cells although this lectin bound greatly chondrocytes independently of the age-group. A higher binding intensity was seen with the maturity for this cell-type (22.1% higher in 11 month-old tissue). This absence of binding indicates the absence of expression of terminal α-(1→6)-linked fucose and/or Le^x motifs [23, 24] on the surface of the IVD cells. These motifs were, however, present within the ECM of the three tissue-types independently of the age-group. As with chondrocytes, the cartilage ECM showed a greater binding with maturity (15.6% higher for 11 month-old tissue than for three month-old tissue) while no significant differences in binding intensity were observed for NP and AF tissues with maturity (p<0.05) (Figure 3.5.C). Overall, cartilage tissue showed a higher binding intensity of LTA lectin to motifs in the ECM independently of the age.

The lectin AAA recognises specifically both H-antigen and Le^b epitope [25] (Table 1, Figure 3.4.A). No significant difference in binding intensity of AAA onto AF cells was observed with maturity (p<0.05) while the motifs detected on NP cells decreased (11.9%). Unlike NP cells which showed a lower binding intensity upon maturity, chondrocytes membranes showed a significantly higher binding intensity (12.29%) in 11 month-old tissues (Figure 3.6.C). Interestingly, no significant
difference in the expression of the motifs recognised by AAA ($p<0.05$) was observed in ECM of NP and AF between the both tissues. The expression of the motifs recognised by AAA was also not modified in ECM of both tissues upon maturity. However, cartilage ECM showed a slight but significant ($p<0.05$) decrease, in the binding intensity with maturity (6.83%). It is also noticeable that the ECM of cartilage had a lower content of the motifs recognised by AAA compared to both NP and AF ECM independently of the age-groups. As $\text{Le}^b$ motif was not detected in the ECM after immunostaining (Figure 3.7.B), the binding intensity observed in the ECM after AAA staining can be attributed to the presence of H antigen (Figure 3.7.C). The type I H antigen was not detected by immunohistochemistry (data not shown). This absence of detection shows that the H antigen detected by UEA-I and AAA was type II, III or IV H antigen (Figures 3.4 and 3.6).

A similar profile of binding of those of AAA and UEA-I lectins was observed for the detection of $\text{Le}^b$ motif by immunohistochemistry (Figure 3.7.C). A significant decrease in the detection of $\text{Le}^b$ motif was noted between the different age-groups for both NP and AF cells (25.62% and 38.73%, respectively). As for chondrocytes, the binding intensity increased by 32.18% with the maturity. The overall binding intensity was higher onto the NP cells than it was AF cells and chondrocytes independently of the age. The $\text{Le}^b$ motif was not detected within the ECM of the three tissues with an absence of staining regardless of the age.

### 3.3.2 Sialylated Motifs

Sialic acids are negatively charged, nine carbon sugars that are widely expressed not only at the surface of cells but also in the ECM of all tissues. A switch of linkage of the sialic groups has been shown to be critical for biological events such as cell inflammation [9, 59] or cell proliferation [60]. The lectins SNA-I and MAA distinguish between $\alpha$-(2,6)- or $\alpha$-(2,3)-linked sialic acid, respectively [17].

Apart from AF cells which, remarkably, did not show any staining, SNA-I lectin bound to NP cells and chondrocytes for the two age-groups (Figure 3.8). The binding intensity was significantly lower for NP cells in the mature tissue (51.87% decrease) while the profile was reversed for chondrocytes where SNA-I binding intensity was 47.04% more intense for the 11 month-old chondrocytes. SNA-I lectin bound motifs present in all the tissues types at the ECM level. However, the binding intensity differed between tissue-type and maturation. In both NP and AF tissues,
binding intensities were significantly higher in the three month-old compared to the 11 month-old tissue (42.64% and 28.21% greater, respectively). By contrast, a significant decrease of binding was noted in the cartilage between the two different age-groups. In addition, for both age-groups, AF ECM expressed more \( \alpha-(2,6) \)-linked sialic acid than the ECM of the NP. However, the difference was greater in the mature animals (11.83% and 29.56% greater intensity in three month-old and 11 month-old AF ECM, respectively) (Figure 3.8.C).

As well as having an affinity for \( \alpha-(2,3) \)-linked sialic acid, MAA is also known to bind to Gal-3-SO\(_4\) [61] (Table 1 and Figure 3.9.A). MAA lectin bound to NP and chondrocytes for each age-group but, as for SNA-I lectin, it did not bind to the AF cells. The difference in MAA binding intensity for cells between age groups and tissue types was not as pronounced after staining with MAA lectin (Figure 3.9) as SNA-I lectin (Figure 3.8). NP cell binding intensity decreased upon maturation (12.27%) while the chondrocyte binding intensity remained constant \((p<0.05)\) for both age-groups. Overall, the binding intensity of MAA on chondrocytes was lower than that of NP cells (29.91% and 19.18% lower in three and 11 month-old tissues, respectively). In the ECM, the binding intensity of MAA lectin in the AF tissue was greater than NP tissue in immature animals (24.59%). This difference was not observed upon maturity where the intensity levels were not significantly different \((p<0.05)\). The intensities of binding to the ECM remained constant with maturation in the NP tissue \((p<0.05)\). As for the AF tissue, a greater binding intensity was observed in the immature ECM of cartilage tissue than mature tissue (17.83% higher). More marked differences in \( \alpha-(2,3) \)-sialylation between age-groups and tissue-types similar to that revealed by SNA-I lectin for \( \alpha-(2,6) \)-sialylation might be present. However, these differences could be masked by an increase of sulfated galactose motifs masking the clear-cut differences (Figure 3.9.C).

### 3.3.3 High Mannose Type Motifs

Con A lectin is often employed as a general recognition molecule for \( N \)-linked glycosylation because of its ability to recognise \( \alpha \)-linked mannose, high-mannose-type and complex type biantennary structures (Table 3.1 and Figure 3.10.A) [16, 17].

A sequentially decreasing binding intensity trend was observed between cells of the different immature tissue-types (47.12% and 61.38% for AF cells and
chondrocytes, respectively, compared to NP cells) (Figure 3.10.C). No significant difference in binding intensity onto NP cells was observed upon maturation \( (p<0.05) \). Remarkably, Con A lectin did not bind onto AF cells in the 11 month-old tissue. For this age-group, Con A lectin demonstrated a greater binding onto the chondrocytes than that of three month-old tissue \( (41.99\%) \). However, the binding intensity on chondrocytes in 11 month-old tissue was still lower than the binding intensity on NP cells \( (35.39\%) \). In the immature ECM, as the trend of binding followed onto the cells, an iterative decrease in binding intensity of Con A lectin was observed from NP to AF to cartilage tissue \( (lower \text{ compared to NP ECM of 26.88\% and 42.91\% for AF and cartilage ECM, respectively}) \). The lectin bound as much to the ECM of the NP tissue as it did to the ECM of the AF tissues for the 11 month-old tissue-group \( (p<0.05) \). Upon maturation, binding intensity increased for the three tissue-types. However, the increment observed for the NP ECM was not statistically significant \( (p<0.05) \) whereas the binding intensity for AF and cartilage ECM increased of 26.92 and 37.98 \%, respectively.

### 3.3.4 Galactose Motifs

PNA and Jacalin lectins both recognise the Gal-\( \beta-(1\rightarrow 3) \)-GalNAc motifs also known as T-antigen \( (\text{Table 3.1, Figure 3.11.A, Figure 3.12.A}) \). Although Jacalin lectin detects the T-antigen motif independently of the sialylation, PNA lectin binds only to the non sialylated motif. The presence of terminal \( \alpha \)-Galactose present on some form of T-antigens was detected using GS-I-B4 lectin \[62\].

PNA lectin bound only to the chondrocytes independently of the age-group which indicated an absence of the T-antigen motif on both NP and AF cells \( (\text{Figure 3.11.C}) \). An increase of the binding intensity was observed upon maturity onto the chondrocytes \( (41.72\%) \). The T-antigen was detected in the ECM of three tissue-types with differences with maturity and tissue-types. Similar to the chondrocytes, cartilage ECM showed an increase of binding intensity with ageing \( (32.68\%) \). A similar trend was followed for the NP ECM with a 28.11\% increase of the binding intensity in 11 month-old tissue compared to three month-old tissue. A slight decrease, but not significantly different \( (p<0.05) \), was noted between the binding intensities of AF ECM of three and 11 month-old tissues. Overall, the binding intensities were higher in both NP and cartilage ECM than that of AF ECM \( (p<0.05) \).
whereas no differences were observed between NP and cartilage ECM for either age-groups.

While PNA lectin bound only chondrocytes, Jacalin lectin bound to all three cell-types (Figure 3.12). NP cells were the only cell-type to show a decrease in the binding intensity upon maturity (13.6%) whereas no statistically significant differences were observed for chondrocytes and AF cells ($p<0.05$). The binding intensity was lower on chondrocytes than on AF and NP cells irrespectively of the age-group. An increment of the binding intensity was noted for the cartilage ECM whereas the T-antigen expression remained constant upon maturation in NP and AF ECM. Jacalin lectin bound greater the ECM of AF tissue than that of cartilage and NP ECM independently of the age-group ($p<0.05$).

Terminal $\alpha$-Galactose motifs were detected on the surface of all cell-types for both age-groups (Figure 3.13). An increase of binding intensity of GS-I-B$_4$ was noted for chondrocytes and NP cells upon maturity (38.09% and 62.64% increase, respectively) while the binding intensity decreased onto AF cells (33.04%). NP cells showed the lowest binding intensity (28.63% and 63.88% lower than chondrocytes and AF cells, respectively). However, the binding intensity increased greatly with maturity at which the NP cells presented the highest binding intensity (15.47 and 30.76% higher than chondrocytes and AF cells, respectively). A comparable binding intensity pattern to the one observed for cells was noted at an ECM level. Indeed, the recognition of the terminal $\alpha$-galactose motif by GS-I-B$_4$ increased upon maturity for both cartilage and NP ECM (11.54% and 44.04% increase, respectively) while a decrease of binding intensity was noted for AF ECM (28.78%). At three month-old, AF tissue showed the highest binding intensity of GS-I-B$_4$ compared to that of cartilage and NP ECM. At 11 month-old, although the binding intensity did not differ between NP and AF tissues, cartilage ECM presented a binding intensity lower than that of NP and AF ECM ($p<0.05$).

3.3.5 Terminal GalNAc Motifs

SBA and WFA lectins both have affinities for terminal GalNAc residues on oligosaccharides while WFA is known to bind chondroitin sulfate independently of the sulfation pattern (Table 3.1, Figures 3.12.A and 3.13.A).

SBA lectin bound to motifs present on both NP cells and chondrocytes whereas no binding of the lectin was observed onto AF cells (Figure 3.14.C). The
binding intensity was 42.04% greater on chondrocytes upon maturity while no significant variation of the binding intensities between three and 11 month-old NP cells was noted ($p<0.05$). At an ECM level, the terminal motifs detected by SBA were expressed in the three tissue-types. Although the binding intensity increased with age on chondrocytes, a drastic decrease of the binding intensity was observed for the ECM of this tissue (92.22%). By contrast, NP ECM was greater bound by the SBA lectin in 11 month-old tissue compared to three month-old tissue (66.48%). No significant differences in binding intensity between the different age-groups were observed for the AF ECM ($p<0.05$). Overall, the cartilage ECM showed the highest binding intensities for both age-groups ($p<0.05$).

WFA lectin binding showed a similar profile to that of SBA lectin binding (Figure 3.15.C). Indeed, as for SBA lectin, WFA lectin did not recognise any motif onto AF cells. For both chondrocytes and NP cells, an increase of binding intensities was noted upon maturity with greater bindings of 42.03 and 31.64%, respectively. The binding intensities were higher for NP cells than for chondrocytes independently of the age. Likewise, a higher binding intensity was observed for the NP ECM than for the cartilage ECM. Similar profiles of binding were obtained for the ECM of the NP and AF tissues with an increase of binding intensities with maturity of 21.46 and 21.39%, respectively. A similar trend was noted for the cartilage with a greater binding intensity in 11 month-old ECM of 25.04% while the overall binding intensity was lower than that of the two other tissues ($p<0.05$).

### 3.3.6 Chondroitin Sulfate Composition

GAGs can densely substitute a protein core, forming PG. PG are important components of articular cartilage and IVD tissues. Their structure and quantity are essential to the maintenance of tissue function [5, 12, 63].

CS-56 antibody recognises specifically both sulfated chondroitin 4- and 6-sulfates (C4S and C6S). None of these disaccharides were detected by CS-56 on the cell surface of the three cell-types (Figure 3.16). However, their motifs were recognised within the ECM of the three tissues.

Although the overall quantity of CS disaccharides stayed constant, the structure of compositional disaccharides may change with maturity. For this reason, a staining of the specific C6S disaccharide was performed to discern more detailed differences between the different groups. As for the staining with CS-56 antibody,
the C6S antibody did not detect any pattern on the cell surface of the three tissue-types (Figure 3.17). A significant decrease in the binding intensity was observed upon maturity for AF and cartilage tissue. Indeed, the binding intensity was decreased by 49.59% in AF ECM, while in the cartilage ECM a decrease of 92.93% was found. Although no statistically significant differences in the binding intensity for C6S motif was noted in the ECM of the three tissue-types in three month-old tissues, an iterative decrease of the binding intensity was seen between the three tissue-types in 11 month-old ECM.

In order to assess the exact composition of these CS motifs, the quantifications of sGAGs and CS disaccharide were performed by DMMB assay (Figure 3.18.A) and HPLC analysis, respectively (Figure 3.18.B). Although NP tissue showed a higher sGAG content for the both age-groups than that of the AF tissue, no statistically significant differences were observed between the different age-groups ($p<0.05$) (Figure 3.18.A). Remarkably, the content of sGAG in cartilage tissue for both age-groups was lower than those of NP and AF tissues ($p<0.05$).

The CS disaccharide content, obtained by HPLC analysis, showed the same trend as that of total sGAG content (Figure 3.18.B). However, no statistically significant differences were observed between the different age-groups and tissue-type ($p<0.05$). Nevertheless, the percentage of the specific disaccharides C0S, C4S and C6S varied upon maturity with an inversion of the sulfation profile for the AF tissue (Figure 3.19.B). Indeed, for this tissue, a low quantity of C4S was measured in the three month-old tissue ($0.10\pm0.01 \mu g$ of CS / $\mu g$ of DNA) in parallel with a high quantity of C6S ($0.24\pm0.09 \mu g$ of CS / $\mu g$ of DNA). Upon maturity, the quantity of C4S increased to reach $0.21\pm0.07 \mu g$ of CS / $\mu g$ of DNA while, inversely, the quantity of C6S dropped to $0.03\pm0.01 \mu g$ of CS / $\mu g$ of DNA (Table 3.2). A similar trend was seen for NP tissue. However, the variations of disaccharide composition between different ages were not statistically significant ($p<0.05$) (Figure 3.19.A) for this tissue. Remarkably, the composition of CS of the cartilage tissue did not change in profile with similar percentages of C0S, C4S and C6S upon maturation ($p<0.05$) (Figure 3.19.C).

3.3.7 Comparison of Cell and ECM Glycoprofile

The averages of binding intensities for each lectin were clustered in an effort to identify similarities in the cells and ECM glyco-profiles between the different
tissue-types and age-groups. Although a high similarity was observed between the individual cell-types upon maturity, they exhibited a distinct glycosylation profile according to their maturity (Figure 3.20.A). The analysis revealed strong similarities between the cells from the same tissue-type upon maturity with a first segregation by tissue-type. The second cluster obtained shows that NP cells exhibited a glycophenotype closer to that of chondrocytes unlike AF cells which were more distinct from these two cell-types (similarity score of 0.22 from the two other cell types). In addition, the glycosylation of the ECM showed a completely different segregation profile (Figure 3.20.B). In contrast to cells, similarities were seen to be age-dependent for the matrix composition and were not tissue-type dependent. A high similarity between NP and AF ECM was observed for both age-groups (similarity scores of 0.70 and 0.79 for three and 11 month-old ECM, respectively). While a segregation between the glyco-profile of 11 month-old tissues was obtained, cartilage ECM of three month-old tissue exhibited a clearly distinct profile from the other tissue-types (similarity score of 0.19).

3.4 Discussion

Cytokines, growth factors, hormones and morphogens control development, maturation, ageing and diseases of a tissue [64] by highly regulated mechanisms in which glycans of the glycocalyx and the ECM play important roles [7, 8, 20, 64, 65] through binding, sequestration, activation and bioactive signalling of molecules [59, 64, 65]. Cellular glycosylation and glycoproteins present a highly transient signature, tightly regulated by biologically active molecules and the cell micro-environment [66]. Their glycodynamics can dictate the fate of a tissue during development, ageing and pathology [49]. Maturity of the IVD is often associated with the loss of notochordal cells. This event is believed to precede the degeneration process involving a profound change in PG content [12, 17]. The glyco-dynamics of ovine NP, AF and cartilage tissues upon maturity were examined and compared in an effort to understand the phenomena that occur at this essential stage of IVD development.

The Bovidae family have been described in the literature as one of the most suitable animal model for the study of the IVD behaviour because of their similarities to human IVDs in their mechanical properties [67-69], biochemical composition [24], loss of notochordal cells with maturity [19, 70], and gene expression [35].
Figure 3.3. Representative images of hematoxylin and eosin stained ovine NP (1-2), AF (3-4) and cartilage tissue (5-6) at three and 11 month-old. Extracellular matrix proteins and nuclei are stained in pink/red and purple/blue, respectively. Scale bar = 100µm.
Figure 3.4. (A) Representation of the fucosylated structures detected by FITC-labelled UEA-I lectin. UEA-I lectin binds to $\alpha$-(1\(\rightarrow\)2)- and $\alpha$-(1\(\rightarrow\)6)-linked fucose and Le$^s$ motifs. (B) Representative fluorescent images of the stained fucosylated motifs detected by UEA-I in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. Fucosylated motifs and nuclei are stained in green and blue, respectively. Scale bar = 100$\mu$m. (C) Quantification of lectin binding intensity. Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at $p<0.05$. 
Figure 3.5. (A) Representation of the fucose linkages detected by FITC-labelled LTA lectin. LTA lectin binds to $\alpha$-(1→2), $\alpha$-(1→3)- and $\alpha$-(1→6)-linked fucose motifs. (B) Representative fluorescent images of the stained fucosylated motifs detected by LTA in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. Fucosylated motifs and nuclei are stained in green and blue, respectively. Scale bar = 100µm. (C) Quantification of lectin binding intensity. Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at $p<0.05$. 
Figure 3.6. (A) Representation of the fucosylated structures detected by FITC-labelled AAA lectin. AAA lectin binds to α-(1→3)- and α-(1→6)-fucosylated N-acetylglucosamine motifs. (B) Representative fluorescent images of the stained fucosylated motifs detected by AAA in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. Fucosylated motifs and nuclei are stained in green and blue, respectively. Scale bar = 100μm. (C) Quantification of lectin binding intensity. Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at p<0.05.
Figure 3.7. (A) Representation of the structures of the Le<sub>b</sub> motif. The motif Le<sub>b</sub> consists on Fuc-α-(1→2)-Gal-β-(1→3)-[Fuc-α-(1→4)-]GlcNAc motif derived from the H antigen type I. (B) Representative fluorescent images of Le<sub>b</sub> motifs in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. Fucosylated motifs and nuclei are stained in green and blue, respectively. Scale bar = 100µm. (C) Quantification of lectin binding intensity. The motif Le<sub>b</sub> was not detected within the ECM. Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at p<0.05.
Figure 3.8. (A) Representation of the structures of $\alpha$-(2→6)- sialylated N-acetylgalactosamine detected by FITC-labelled SNA-I lectin. (B) Representative fluorescent images of the sialylated motifs detected by SNA-I in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. Sialylated motifs and nuclei are stained in green and blue, respectively. Scale bar = 100 $\mu$m. (C) Quantification of lectin binding intensity. Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at $p<0.05$. 

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Figure 3.9. (A) Representation of the $\alpha(2\rightarrow3)$-sialylated galactose motifs structures detected by FITC-labelled MAA lectin. (B) Representative fluorescent images of stained sialylated motifs detected by MAA in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. Sialylated motifs and nuclei are stained in green and blue, respectively. Scale bar = 100$\mu$m. (C) **Quantification of lectin binding intensity.** Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at $p<0.05$. 
Figure 3.10. (A) Representation of the structures detected by TRITC-labelled ConA lectin. ConA lectin binds to α-Mannose, α-Glucose and α-N-acetylglucosamine. (B) Representative fluorescent images of the N-glycans motifs detected by ConA in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. N-Glycans motifs and nuclei are stained in red and blue, respectively. Scale bar = 100µm. (C) Quantification of lectin binding intensity. Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at p<0.05.
Figure 3.11. (A) Representation of non sialylated T-antigen structures detected by FITC-labelled PNA lectin. PNA lectin binds to non sialylated Gal-β-(1→3)-GalNAc (T-antigen) motifs. (B) Representative fluorescent images of the stained non sialylated T-antigen motifs detected by PNA in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. T-antigen motifs and nuclei are stained in green and blue, respectively. Scale bar = 100 µm. (C) Quantification of lectin binding intensity. Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at p<0.05.
Figure 3.12. (A) Representation of the T-antigen structures detected by FITC-labelled Jacalin lectin. Jacalin lectin binds to sialylated and non sialylated Gal-β-(1→3)-GalNAc (T-antigen) motifs. (B) Representative fluorescent images of stained sialylated and non-sialylated T-antigen motifs detected by Jac in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. T-antigen motifs and nuclei are stained in green and blue, respectively. Scale bar = 100μm. (C) Quantification of lectin binding intensity. Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at p<0.05.
Figure 3.13. (A) Representation of the terminal $\alpha$-galactose detected by GS-I-B$_4$ lectin. (B) Representative fluorescent images of stained motifs in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. Terminal $\alpha$-galactose motifs and nuclei are stained in green and blue, respectively. Scale bar = 100$\mu$m. (C) Quantification of lectin binding intensity. Data were normalized to surface area and represented as mean $\pm$ standard error of the mean (n=5). * denotes significant differences between the different groups at $p<0.05$. 
Figure 3.14. (A) Representation of the terminal galactose and N-acetylgalactosamine structures detected by FITC-labelled SBA lectin. (B) Representative fluorescent images of stained motifs detected by SBA lectin in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. Terminal motifs and nuclei are stained in green and blue, respectively. Scale bar = 100µm. (C) Quantification of lectin binding intensity. Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at $p<0.05$. 
Figure 3.15. (A) Representation of the terminal GalNAc-Gal and chondroitin disaccharides GlcA-GalNAc motifs detected by FITC-labelled WFA lectin. (B) Representative fluorescent images of the stained motifs detected in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. Motifs and nuclei are stained in green and blue, respectively. Scale bar = 100µm. (C) Quantification of lectin binding intensity. Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at p<0.05.
Figure 3.16. Representation of the structures of C4S and C6S motifs detected by CS-56 antibody. The C4S and C6S are composed of a glucuronic acid coupled to a sulfated N-acetylgalactosamine in position 4 and 6, respectively. (B) Representative fluorescent images of C4S and C6S motifs staining in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. C4S and C6S motifs and nuclei are stained in green and blue, respectively. Scale bar = 100µm. (C) Quantification of antibody binding intensity. Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at p<0.05.
Figure 3.17. **Representation of the structure of C6S motif.** The C6S is composed of a glucuronic acid coupled to a sulfated N-acetylgalactosamine in position 6. **(B)** Representative fluorescent images of C6S motifs staining in ovine NP (1-2), AF (3-4) and cartilage tissues (5-6) at three and 11 month-old. C6S motifs and nuclei are stained in green and blue, respectively. Scale bar = 100 µm. **(C) Quantification of antibody binding intensity.** Data were normalized to surface area and represented as mean ± standard error of the mean (n=5). * denotes significant differences between the different groups at p<0.05.
Figure 3.18. Age and tissue-related changes of (A) total sGAG content of ovine IVD quantified by DMMB assay and (B) CS content quantified by HPLC. Data were normalized to DNA content and represented as mean ± standard error of the mean (n=5). * represents significant differences at $p<0.05$. 
Table 3.2. Quantity of C0S, C4S and C6S disaccharides (µg of disaccharide / µg of DNA) in three and 11 month-old ovine NP, AF and cartilage tissues. Data were normalized to DNA content and represented as mean ± standard error of the mean (n=5). No significant difference was seen between the different groups (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>C0S</th>
<th>C4S</th>
<th>C6S</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 month-old NP</td>
<td>0.14±0.03</td>
<td>0.19±0.03</td>
<td>0.16±0.15</td>
<td>0.47±0.11</td>
</tr>
<tr>
<td>11 month-old NP</td>
<td>0.25±0.08</td>
<td>0.43±0.14</td>
<td>0.04±0.01</td>
<td>0.85±0.23</td>
</tr>
<tr>
<td>3 month-old AF</td>
<td>0.13±0.02</td>
<td>0.10±0.01</td>
<td>0.24±0.09</td>
<td>0.47±0.09</td>
</tr>
<tr>
<td>11 month-old AF</td>
<td>0.10±0.03</td>
<td>0.21±0.07</td>
<td>0.03±0.01</td>
<td>0.37±0.10</td>
</tr>
<tr>
<td>3 month-old cartilage</td>
<td>0.10±0.04</td>
<td>0.15±0.06</td>
<td>0.05±0.02</td>
<td>0.30±0.12</td>
</tr>
<tr>
<td>11 month-old cartilage</td>
<td>0.10±0.04</td>
<td>0.14±0.05</td>
<td>0.04±0.02</td>
<td>0.29±0.10</td>
</tr>
</tbody>
</table>
Figure 3.19. Percentage of disaccharides C0S, C4S and C6S in three month-old and 11 month-old ovine (A) NP, (B) AF and (C) cartilage tissue. Data were normalized to DNA content and represented as mean ± standard error of the mean (n=5). * represents significant differences at $p<0.05$. 
Figure 3.20. Clustering analysis of (A) the average cell glycosylation profile and (B) the average ECM glycosylation profile. Data were normalized to maximal individual lectin intensity (100%) (n=5). Rows and columns denote age / tissue-group and lectins, respectively. The color scale for glycan staining is presented as follows: red represents the highest expression across all samples, black represents mean expression, and green represents lowest expression across the samples.
Therefore, ovine lumbar IVDs (L4-L5) were studied here to evaluate the glyco-dynamics. Bovine and ovine IVDs resemble human IVDs by the occurrence of notochordal cells with a few cells at birth which decrease rapidly with maturity [19, 70, 71]. Notochordal cells seemed to be absent or at a low number for both age-groups as no cells with notochordal cell morphology were observed in NP tissues (Figure 3.3).

Fucosylated glycans play an important role in a variety of biological and pathological processes such as tissue development [8], angiogenesis, inflammation, cell adhesion, cancer and cell homing [3]. Present on cell surface glycoconjugates and also on ECM proteins (including fibronectin, aggrecan and AGP), fucose residues regulate adhesion and cell-cell interactions via adhesion proteins such as selectins [72]. Similar patterns of binding intensity with slight differences were observed after staining with Le\(^b\) antibody, AAA and UEA-I lectins suggesting the detection of different fucosylated patterns at both cellular and extracellular level. At a cellular level, there was a decrease of the fucosylated motif binding intensity on both NP and AF cells. Minimal differences in the binding intensity of these motifs were noted in the ECM of either tissue which may indicate a similar trend of this glyco-modification in NP and AF tissues. LTA lectin did not bind the glycocalyx of AF and NP cells; however, AAL lectin did recognise fucosylated motifs on both cell-types. LTA lectin binds with a higher affinity to the motif Fuc-\(\alpha-\)(1\(\rightarrow\)6) whereas AAA has a higher affinity for the motif Fuc-\(\alpha-\)(1\(\rightarrow\)3) (Table 3.1). By consequence, it is believed that the motif Fuc-\(\alpha-\)(1\(\rightarrow\)6) is not present onto NP and AF cells. However, chondrocytes distinctively expressed Fuc-\(\alpha-\)(1\(\rightarrow\)6) with an increase of this motif upon maturity (Figure 3.5). Similarly, an increase of Fuc-\(\alpha-\)(1\(\rightarrow\)2), Fuc-\(\alpha-\)(1\(\rightarrow\)3) and Le\(^b\) motifs was observed upon maturity (Figures 3.4, 3.6, 3.7). Fucosylated motifs are up-regulated during rheumatoid arthritis and osteoarthritis suggesting their role in the homeostasis of cartilage [73].

Fucosylated motifs are closely related to the presence of sialylated motifs in the regulation of protein binding, conformation and activation. The simultaneous increase of \(\alpha-\)(2\(\rightarrow\)3) sialylation, \(\alpha-\)(1\(\rightarrow\)3) and \(\alpha-\)(1\(\rightarrow\)4) fucosylation enhances the adhesion, migration and invasion [39], differentiation and the maintenance of cell phenotype [40]. This is attained by modulation of growth factor interactions with their receptors [74], and cell interactions via C-lectin domain proteins such as selectins [72] or CD44 receptor [3]. These sialylation and fucosylation motifs were
detected at a cellular and extracellular level in the three tissues. Their decrease on NP and AF cells upon maturity suggests a decrease of the metabolic activities related to differentiation, migration and maturation of the IVD tissues.

Keratan sulfate (KS) chains are glycosaminoglycans formed by a succession of Gal-β-(1→4)-GlcNAc motifs which can be sialylated in α-(2→3) and α-(2→6), and fucosylated in α-(1→3) linkage in a tissue and age-dependent manner [75, 76]. KS are attached onto fibronectin and aggrecan protoglycans present in the ECM of both cartilage and IVD [12, 13, 76]. KS fucosylation in α-(1→3) and sialylation in α-(1→6) are described as modulating the formation of the fibrillar network by controlling the fibril diameter and interfibrillar spacing of collagen molecules for the matrix formation and rebuilding [75-78]. Terminal Fuc-α-(1→3) was reported to increase in human cartilage from birth to adolescence to maturation along with an increase of galactose sulfation and the appearance of Neu-α-(2→6) expressed independently of the age [75, 76]. A similar trend for these motifs was observed in this study (Figures 3.8, 3.9 and 3.6). The presence of both terminal Neu-α-(2→3) and Neu-α-(2→6) motifs is likely to be related to the organisation of the AF tissues over development. A higher expression of sialylated motifs was observed in the immature tissue which points to a potential role of these molecules in the organization and remodelling of the AF tissue.

Sialylation motifs of the cell surface and ECM and their alterations influence cell behaviour by modifying not only cell adhesion, proliferation and differentiation [8, 79, 80] but also the organisation of the ECM [75]. Many glycoproteins and GAGs involved in development, homing and inflammation are substituted with sialic acid.

At a cellular level, α-(2→3)- and α-(2→6)-linked sialic acid motifs, recognised by MAA and SNA-I lectin, respectively, were highly expressed at the cell surface of NP cells compared to chondrocytes while no expression of these motifs was found on AF cells (Figure 3.9.C). Both motifs are notably recognised by both galectin 1 and galectin 3, β-galactoside binding proteins with high affinity for poly-N-acetyl-lactosamine. Involved in multiple biological roles from cell adhesion to cell signalling regulation [4, 81, 82], the roles of galectins are dictated by their affinity to specific glycan motifs. Galectins 1 and 3 were described to be expressed within both IVD [35, 83-85] and cartilage tissues [86, 87] onto the cell surface and within the ECM. Galectin 1 acts as a homofunctional cross-linker binding to ECM glycoproteins such as laminin, fibronectin or elastin [81, 88]. Its role is yet not fully
known but it has been reported to be involved in specific roles such as cell adhesion to ECM, collagen network organisation, mRNA splicing, cell growth, apoptosis and cell cycle regulation [81, 88]. In cartilage, this lectin mediates chondrocytes maturation and differentiation, and the progression of tissue calcification [81]. The absence of expression of α-(2→3)- and α-(2→6)-linked sialic acid motifs on AF cells (Figures 3.8, 3.9) suggests a role of the galectin 1 in the adhesion of cells to the ECM and not in cell-to-cell communication for this tissue. Galectin 1 might play a role in the organisation of type I collagen by binding to α-(2→3)-sialic acid motif within the ECM. The higher expression of α-(2→3)-sialic acid observed in the AF ECM compared to those of NP and cartilage tissues might be related to the higher organization of AF collagen fibers required for the mechanical strength of this tissue [89, 90]. At a cellular level, Minogue et al. reported a higher expression of galectin 1 by NP cells compared to chondrocytes which follows the trend observed for Neu-α-(2→3)-Gal expression (Figure 3.9). A role of this motif in cell-to-cell communication through galectin-1 can therefore be envisaged. Galectin 3 is expressed within the notochordal cells, NP and AF cells [83-85]. Its expression was shown to induce degradation of the matrix in cartilage and also in the degeneration of the IVD by recognition of AGES and activation of MMPs. The presence of motifs recognized by galectin 3 suggests its role in the ECM alteration of the IVD upon maturation. By controlling the matrix degrading enzymes [86, 87], this galectin might modulate the remodelling of IVD tissues via its interactions with Neu-α-(2→3)-Gal and Neu-α-(2→6)-Gal.

It is also important to note the expression of numerous markers on IVD cells carrying α-2,3-linked sialylation such as TrkA [91, 92] or both α-2,3- and α-2,6-sialylation such as neural cell adhesion molecule (NCAM) [8, 33], β1-integrin, CD44 [3] and CD24 [34, 60, 93] modulating cell communication, cell adhesion, tissue maturation [3, 33, 34, 60, 93]. Glycosylation regulates their activation and binding activity [94]. A modulation of the expression of these markers in IVD will consequently influence the patterns of sialylated motifs.

T-antigen (β-Gal-(1→3)-GalNAc) motifs are commonly used as a marker for pre-chondrogenic condensation during the development [41]. This marker was not detected on the glyocalyx of NP or AF cells (Figure 3.11). Baffi et al. reported an absence of binding of PNA in IVD tissue which is indicated by the absence of staining of IVD tissue during the embryonic development with a clear demarcation
between the positively stained cartilage end-plate and the intervertebral tissue [41]. The notochordal origin of the NP cells may be confirmed by the absence of staining for this marker suggesting a different lineage of both cell types. On a functional aspect, the T-antigen plays a role in the activation of TGFBR-2, a transcription factor controlling chondrogenesis [41]. The AF and NP cells do not express β-Gal-(1\(\rightarrow\)3)-GalNAc motifs detected by PNA which points towards their differentiation through a different pathway than that of chondrogenesis. Although β-Gal-(1\(\rightarrow\)3)-GalNAc was present on NP and AF cells, this motif was detected after staining with Jacalin lectin which indicates a potential modification of the T-antigen (sialylation, sulfation or addition of α-galactose) [95]. α-(2,6)- or α-(2,3)-linked sialylations were detected on NP cells which indicate a potential modification of the T-antigen by these motifs (Figures 3.10, 3.11). The absence of sialic acid motifs from the surface of AF cells suggests the presence of a different modification of the T-antigen. These differences of expression suggest a specific role of the T-antigen and its structure in the maintenance of the cell communication of the IVD.

The staining of terminal α-galactose motif revealed the presence of two cell subpopulations within the AF tissue (Figure 3.13). One of these subpopulations may correspond to the AF progenitor cells found in AF tissue [96] which exhibit a different glycosylation profile than that of AF cells. Further investigations will be necessary to confirm this observation. However, it is worth noting that progenitor cells were found in both NP and AF tissues [97, 98] although cells present in the NP tissue did not display a difference in glycosylation profile for the set of lectins used in this study. Stem cells express high mannose type oligosaccharide on the cell surface [99]. The presence of high mannose type N-glycans on the overall cell population suggests the presence of these motifs not only on the surface of progenitor cells but also on the surface of NP and AF cells and chondrocytes (Figure 3.10). These motifs were not detected onto 11 month-old AF cells. High mannose type N-glycans were shown to play a crucial role in the initiation and progression of osteoarthritis by regulating MMP13 and ADAMTS-5 in response to IL-1α regulation in osteoarthritis [100]. However, their roles remain unclear. Their action on matrix degrading enzymes during skin remodelling [101] suggests a similar function to the high mannose type N-glycans over the development of cartilage and IVD tissues. In accord with the observation performed for the fucosylated and sialylated motifs (Figures 3.4 to 3.9), a decrease of the remodelling activity might be envisaged for the
AF tissue at 11 months with the absence of staining of AF cells. In the ECM, Con A lectin was more intense upon maturity for both cartilage and AF tissue whereas the quantity in the ECM of NP tissue did not change (Figure 3.10).

During disc and cartilage degeneration, CS chains decrease in number and size [102-104]. No significant differences in sGAGs and CS overall content were observed in this study with maturity (Figure 3.18). Nevertheless, it is important to note that the sGAG quantity was lower in cartilage tissue (Figure 3.18.A) than both NP and AF tissues. This difference in quantity explains the difference in mechanical properties of both tissues [105]. sGAGs are related to the hydration of the tissue and hence plays an essential role in the organisation of the ECM and its water content [40, 105]. Although small changes in CS content were observed (Table 3.2, Figure 3.8.C), the profile of sulfation of the AF and NP tissues differed not only from tissue-types but also age-groups (Figure 3.19). The sulfation profile of AF tissue changes considerably in profile with the maturity with an inversion of the sulfation pattern from a dominance of C6S at 3 months, consistent with the study of Hayes et al. in embryonic IVD [11], to a dominance of C4S at 11 months. This switch might be related to a change of the mechanical loads on the tissue upon maturity, shown to affect the sulfation patterns of CS in cartilage [45]. Furthermore, from a biological point of view, a change in the sulfation patterns dictates the inhibition of nerve growth and vascularisation within a tissue [46, 106]. In immature tissues, vessels were observed (data not shown) in the outer AF at three months while no vessels were noted in 11 month-old tissue which suggests a higher vascularisation of the AF at immaturity compared to mature AF. At maturity, the percentage of C4S is higher compared to the percentage of C6S which may be related to a greater ability of the tissue to inhibit nerve and vessels growth in the IVD tissue, known as avascular and aneural at a healthy stage [12]. A similar trend was observed for the NP although no significant difference was seen. As described in previous reports, the composition in disaccharide in cartilage did not change with maturity [107]. However, a significant decrease of C6S was noticed in this tissue over time suggesting a significant decrease of the overall sulfated CS (Figure 3.17). The absence of CS motif at the surface of chondrocytes and NP cells suggests that the motif recognised by WFA in the glycocalyx of these two cell-types is the terminal GalNAc-Gal motif (Figure 3.15).

Hierarchical clustering was used to identify the differences in glycosylation profiles between the different tissue-types and age-groups (Figure 3.20). Although
Power *et al.* showed a clear separation of the chondrocytes gene expression profiles from AF and NP cells [32], chondrocytes and NP cells present a closer glycosylation profile than that of AF cells for the 13 motifs screened in this study. The profiling by fluorescently-labelled lectins and antibodies demonstrates that chondrocytes, NP and AF cells are phenotypically distinct by glycosylation profiles, independent of the maturity with a distinct glyco-phenotype of AF cells (Figure 3.20.A). This difference correlates to several gene analysis studies which report similarities between chondrocytes and NP cells [31, 33, 34]. However, remarkable differences between the different cell-types were noticed with the detection of on-off markers such as the T-antigen motif (PNA staining) and Fuc-α-(1→6)- (LTA staining) expressed only on chondrocytes, or the α-(2→3-) and α-(2→6)-linked sialylation absent on AF cells. Studies have been reported that illustrate differences between the IVD cells and chondrocytes at a transcriptomic level [32, 33, 35]. Although differences of gene/protein expression levels were shown between cartilage, NP and AF tissues, no specific on/off marker have been identified. CD24 was described as only expressed on NP cells [93]. However, its validity as specific marker has been debated as it was not found in other studies [32, 34]. It is essential to point out that the validation of the markers highlighted requires validation in human tissue as a high variability between species can be observed for the disc [24, 32]. Differences in ECM composition were also shown in this study in addition to the well known differences in type II collagen and PG contents (Figure 3.20.B). Cell-ECM interactions strongly affect the behaviour of the IVD and cartilage tissues and contribute to the different cellular responses to mechanical loadings between the various anatomic zones [108, 109]. The different anatomical and mechanical functions of both tissues may explain the structural changes in glycans composition or these functions may be a product of changes caused by the changes in glycan expression. The ECM seems to adopt a different glyco-signature in an age-dependent manner responding to the anatomical and mechanical demands in respect of the development of the tissues.

### 3.5 Conclusions

- Specific spatial and temporal glycans patterns throughout maturity were highlighted in this study.
- The differences in glycan patterns are related to remodelling activities.
• Specific glyco-markers were identified which allows distinction between NP and AF tissues and cells.
• An on/off switch of markers was shown that may have ramifications in the differentiations of the cells.
• A modification of the CS content was observed upon maturity. This observation alongwith the significant decrease of GAGs during the degeneration of the IVD was the focus of further investigations.

   It is important to underline differences observed from species to species in gene expression [31, 32, 34, 35] and in glycosylation profiles [59], and their changes in pathological conditions for these three tissues. Therefore, it is essential to specifically characterise the glycosylation profile in humans to validate the specific marker characteristics of NP, AF and cartilage tissues observed in this study. This study presents promising results with findings which allow researchers in the IVD field to isolate NP and AF cells in a tissue, identify potential cell-cell and cell-ECM phenomena crucial in the degeneration process and eventually to design new regenerative approaches for IVD and cartilage therapies.

3.6 References


Glycoenvironment of the intervertebral disc


Chapter 4

Xylosyltransferase I and glucuronyltransferase I: potential targets for chondroitin sulfate up-regulation in NP cells?

Sections of this chapter are under submission:

E. Collin, O. Carroll, M. Peroglio, E. See, D. Hendig, M. Kilcoyne, M. Alini, S. Grad, A. Pandit, *Xylosyltransferase I and glucuronyltransferase I: two potential targets for chondroitin sulfates up-regulation in NP cells*, submitted to
4.1 Introduction

The distinction between disc degeneration and normal ageing process of the intervertebral disc (IVD) remains unclear. Nevertheless, IVD degeneration occurs irrespective of the ageing process because of external factors (e.g. environmental and genetic factors). IVD degeneration is also associated with an exacerbated pain [1, 2]. Both degeneration and ageing of the disc are characterised by an important catabolism of the IVD extracellular matrix (ECM) resulting in the loss of mechanical functions [1, 3, 4].

The degradation of the ECM is induced by many proinflammatory factors such as IL-1β and TNF-α [5, 6]. These two cytokines have been suggested to activate the production of matrix-degrading enzymes such as MMP-7, MMP-13 and ADAMTS-4 inducing the degradation of collagens and proteoglycans [5, 7]. Different therapeutic strategies have been developed either to promote ECM production by the delivery of growth factors (GFs: TGF-β1, BMP-2, sox-9, TIMP-1, GDF-5) which stimulate anabolic activities [8-12], or to inhibit catabolic activities via the inhibition of proinflammatory cytokines [13, 14]. Although the stimulation of anabolic activities showed promising results, several drawbacks to these studies were emphasised including the side effect of BMP-2 or BMP-9 delivery, both growth factors playing a role in bone formation [15]. Targeting the catabolism of the ECM has shown promising results [14]. However, this approach only slows down the degeneration process but it does not restore matrix anabolism.

Proteoglycans (PGs) are glycoproteins on which glycosaminoglycan (GAG) chains are attached. The family of GAGs include: heparan sulfate, chondroitin sulfate (CS), dermatan sulfate and keratan sulfate (KS) (Chapter 1, section 1.3.2) [16]. PGs possess different structural and biological properties according to their substitution chains [17-22]. Their polyanionic nature contributes to their biological functions by interaction with many cytokines, receptors, growth factors and extracellular molecules [23, 24]. Aggrecan is the predominant PG present in the ECM of IVD tissue (15-20% of the annulus fibrosus (AF) and 65% of the nucleus pulposus (NP) dry weight) [1, 25, 26]. This large PG can carry KS chains and a large number of CS chains [25, 27, 28]. The functional properties of aggrecan reside in the high content of chondroitin sulfates on the molecule, and in its ability to create aggregates with the molecules of HA which provide mechanical strength and high hydrodynamic
XT-I and GT-I delivery for sGAGs upregulation

capabilities to the IVD tissue [1, 4, 27, 29-31]. The aggregation immobilizes PGs within a network of collagen fibres, thus enhancing the ability of the tissue to resist compressive deformation and to provide better load distribution [32, 33]. During IVD degeneration and ageing processes, a change in the structure of GAG chains occurs. This phenomenon leads to the formation of an aggrecan molecule with less and shorter CS chains and more KS [31, 34]. The depletion of CS chains results in a decrease of tissue hydration [1], a loss of fluid movement [30, 35, 36], cell apoptosis [3, 36, 37], a loss of nerve growth inhibition [38, 39], and, ultimately, the loss of disc function [3, 25, 40]. Cell behaviour is not only affected by the structure of CS but also their sulfation pattern [24]. The chondroitin 4-sulfate (C4S), chondroitin 6-sulfate (C6S), chondroitin 2,6-sulfate and chondroitin 4,6-sulfate disaccharides provide biological activities to CS chains by direct activity on cell signalling or growth factor interaction and by modifying PG conformation [24, 41]. Although many studies have highlighted the importance of CS in tissue development and pathologies, little is known on the structure and content of CS. Important variations in sulfation pattern of CS have been reported during embryonic development [42] and maturation (Chapter 3). However, no study has reported CS composition upon ageing.

The synthesis of CS chains is initiated by a tetrasaccharide Xyl-Gal-Gal-GlcA (Xyl, Gal and GlcA indicate xylose, galactose and glucuronic acid, respectively) attached to the Asn-X-Ser/Thr peptidic sequences of the protein core. The attachment of the initial glycan is catalysed in the endoplasmic reticulum by xylosyltransferase I (XT-I) and xylosyltransferase II (XT-II) enzymes [43]. After transfer of the protein in the golgi apparatus, a succession of glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) [(GlcA-β-(1→3)-GalNAc-β-(1→4))n] (Chapter 1, 1.3.2.1) glycans [44] is polymerised on the previously attached tetrasaccharide through the successive activity of glycosyltransferases (Figure 4.1) [45, 46].

XT-I, a rate-limiting enzyme [47], is considered as a key regulatory factor of GAGs synthesis because of its role as a primer for chain elongation and its tight regulation of the biosynthesis pathway [43, 48, 49]. The expression of XT-I is, as with aggrecan, induced by TGF-β1 via ERK1/2 and p38 MAPK [50]. A decrease of XT-I has been noted during osteoarthritis. This decrease is directly mediated by IL-1β which inhibits XT-I promoter by promoting Sp3 transcription factor production [48]. XT-I expression and its effects have not yet been reported in the IVD tissue.
β-1,3-glucuronyltransferase-I (GT-I) also plays a key role in GAGs synthesis as the branching point of various GAG chains. This enzyme catalyzes the transfer of a glucuronyl moiety from a UDP-gluconic acid onto the galactose of the initial tetrasaccharide [51]. Responsive to TGF-β, BMP-2 and TonEBP [52, 53], the expression of GT-I, like XT-I, is down-regulated by IL-1β leading to a decrease of CS synthesis [51]. This enzyme has been involved in CS synthesis in cartilage and IVD tissues [51, 52].

Therefore, the behaviour of GAGs, specifically CS, and both XT-I and GT-I enzymes were evaluated in a bovine ageing IVD model. It was hypothesised that the expression of both enzymes will correlate with the behaviour of GAGs within AF and NP tissues upon ageing. In the second phase of the project, plasmids encoding for XT-I and GT-I were delivered to NP cells by electroporation. The effect of the non-viral delivery of both XT-I and GT-I plasmid on the production of CS was investigated. It was hypothesised that the up-regulation of XT-I and GT-I within NP cells will increase the production of CS.

4.2. Material and Methods

4.2.1 Materials and Reagents

Anti-XT-I antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Anti-GT-I antibody (anti-B3GAT1) was purchased from Abnova (Heidelberg, Germany). Prolong® Gold antifade, secondary antibodies AlexaFluor® 555 mouse anti-goat and AlexaFluor® 555 donkey anti-mouse), 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) and Quant-iT™ PicoGreen dsDNA Reagent were purchased from Life Technologies™ (Dublin, Ireland). Human GT-I cDNA clone (NM_018644) was purchased from Origene (Rockville, USA). All other materials and reagents were purchased from Sigma-Aldrich® (Dublin, Ireland) unless otherwise stated.

4.2.2 GAGs, XT-I and GT-I Behaviour

4.2.2.1 Tissue Collection and Preparation

Bovine caudal IVD from six month-old, two and eight year-old animals were collected directly after sacrifice from the local slaughter house (n=5 for each age-group). Soft tissues surrounding IVDs (muscles and ligaments) were removed. Five
Figure 4.1. Schematic of CS synthesis adapted from [54]. The CS result from the polymerisation of a succession of glycans on the serine residue of Asn-X-Ser/Thr peptidic motif via different enzymatic reactions. The enzymes are represented in green while the protein core is represented in orange.
discs of each age-group were then fixed in 4% neutral buffered PFA overnight at 4°C for histology (see section 4.2.2) while five other discs were digested for GAGs biochemical analysis (see section 4.2.2).

4.2.2.2 Histology

After fixation, discs were washed three times with 1X PBS and infiltrated with 20% sucrose overnight at 4°C. Specimens were flash-frozen in liquid nitrogen-cooled isopentane and 5µm frozen sections were cut on a Leica CM 1850 cryostat (Laboratory Instruments & Supplies Ltd., Ireland). Sections were collected on Superfrost® Plus slides (Fisher Scientific Inc., Dublin, Ireland) and stored at -20°C until use.

- **Safranin O/Fast Green Staining**

Sections were washed three times with 1X PBS before staining. The sections were then stained with Safranin O (proteoglycans staining) and fast green FCF (collagen staining). After dehydration through a series of ethanol and xylene baths, sections were mounted with DPX mounting solution. After drying, slides were viewed under a light microscope (Olympus™ Fluorescence Microscope, Middlesex, UK) (Appendix Y).

- **Immunohistochemistry**

Immunohistochemistry was performed for three different antigens: XT-I detected by goat anti-XT-I (anti-XYLT1) antibody, GT-I detected by mouse anti-GT-I (anti-B3GAT-1) antibody and chondroitin-6-sulfate (C6S) and chondroitin-4-sulfate (C4S) detected by the CS-56 antibody. For the detection of XT-I and GT-I, an antigen retrieval was required by digestion with proteinase K at 20µg/mL (30U/mg) for five minutes at 37°C. After digestion, slides were washed three times in 1X PBS. For all stainings, sections were blocked with 5% goat serum in 1X PBS for one hour before overnight incubation at 4°C with the primary antibodies diluted at 1/200. Negative control sections were incubated without primary antibody. After five washes with 1X PBS with 0.05% Tween 20 of five minutes each, the secondary antibody (AlexaFluor 555® mouse anti-goat for anti-XT-I antibody, and AlexaFluor 555® donkey anti-mouse for anti-GT-I and CS-56 antibodies) diluted at 1/1,000 was incubated for one hour at room temperature (RT) onto the sections. The slides were then washed five times with 1X PBS with 0.05% Tween 20 for five minutes each before being counterstained with DAPI diluted at 1/2,500 in 1X PBS for 10 min at
RT. The sections were washed three times with 1X PBS with 0.05% Tween 20 before mounting with Prolong® Gold antifade. The slides were cured at 4°C in the dark for one day before imaging. Imaging was performed using an inverted epifluorescent microscope (Olympus IX81, Mason Technologies, Dublin, Ireland). Five images per slide were taken and analysed as described in 4.2.4.

4.2.2.3 GAGs Quantification

- Biochemical Quantification of Total sGAG by DMMB

  sGAG content was quantified by the dimethylmethylene blue (DMMB) method. Chondroitin 4-sulfate from bovine trachea was used as a standard, and absorbance was measured at 535 nm. DNA quantification for sGAGs content normalisation was performed using the Quanti-iT™ Picogreen assay (Invitrogen, Dublin, Ireland) according to the supplier’s instruction.

- Biochemical Quantification of CS by HPLC

  100 µL of the proteinase K-digested NP, AF or cartilage tissues was filtered through a 3 kDa molecular weight cut-off (MWCO) centrifugal filter with 100µL HPLC-grade water according to manufacturer’s instructions. All centrifugal filters were washed with HPLC-grade water prior to use and water used throughout the procedure was HPLC-grade. The retentate was eluted from the filter with 50µL of water, and 390µL of digestion buffer (50mM Tris-HCl, 60mM sodium acetate, pH 8.0) and 10µL of ChABC (100 mU) were added. The mixture was then digested at 37 °C for 3h with gentle agitation (300rpm). The digested mixture was then immediately filtered as above (3 kDa MWCO) and the lower molecular weight filtrate was dried in a vacuum centrifuge (approximately 2h). The dried, digested samples were then stored at -20°C until analysed. For analysis, digested samples were dissolved in 200µL water and 10µL was injected on an Alliance 2695 instrument (Waters, Dublin, Ireland) and the content of unsaturated disaccharides Δdi-0S, Δdi-4S and Δdi-6S was determined according to Ji, et al. [55] with minor modification. In brief, chromatographic separations were carried out on an Ultratech 5ODS C18 (250 x 4.6 mm) (HPLC Technology, Inc., Cheshire, U.K.) or a Synergi column (250 x 4.6mm, 4µm, 80 Å) (Phenomenex Inc., Torrance, CA, USA) at 25°C at a flow rate of 1.1mL/min and the eluate was monitored at 232nm absorbance on a Waters 2489 UV-Vis detector. The mobile phase consisted of (A) 1 or 2mM aqueous tetrabutylammonium bisulfate (TBAB) and (B) 1 or 2mM TBAB in 2:1 mixture of
acetonitrile and water, with starting conditions of 80% A and 20% B. The gradient of 20-65% B was applied over seven minutes, held at 65% B for five minutes and returned to 20% B by 12.5 min. The system was re-equilibrated at 20% B for ten minutes before the next sample injection. The disaccharide content of each sample was identified by comparison to appropriate standards chromatographed under the same HPLC conditions as the sample and quantified by comparison to the appropriate standard curve generated by injection of known concentrations of the standards. Quantification data is presented in Table 4.1 as µg of disaccharide per µg of DNA for normalization.

4.2.3 Up-regulation of XT-I and GT-I via Electroporation

4.2.3.1 Plasmid Preparation

Human XT-I mRNA and protein sequences were homologous to those of bovine at 90 and 94%, respectively. Human GT-I mRNA and protein were shown to be homologous to those of bovine at 88 and 96%, respectively. Human XT-I cDNA was carried on a pcDNA3.1/CT-GFP plasmid [49]. This plasmid was generously provided by Dr Hendig from Rurh-Universität-Bochum, Bad Oeynhousen, Germany. A human GT-I cDNA was cloned from human cDNA clone (NM_018644, Origene, Rockville, USA). Briefly, cDNA clone was digested using Not-I (New England Lab, USA) before amplification of the gene of interest by long proof-read long amplification (Extensor Long Range PCR polymerase ReadyMix kit, Thermo Scientific - Fisher, Ireland) according to the supplier’s indications. PCR products were then run on an 1% agarose gel. PCR products were extracted and purified using the MiniElute® kit (Qiagen, Manchester, UK). After purification and A overhangs, PCR product was cloned in a pCR™-2.1-TOPO® vector (Invitrogen, Ireland) following the manufacturer’s instructions. The size of positive clones was checked by electrophoresis on an 1% agarose gel after digestion using Nco I (Promega, Ireland) and their sequence by sequencing (MWG-Operon, Germany).

After validation by sequencing, both plasmids were amplified and purified using the Qiagen Plasmid Mega® Purification Kit (Qiagen, Manchester, UK) (Appendix Q).
4.2.3.2 NP and AF Cells Extraction

Two year-old bovine fresh tails were collected directly after sacrifice from a local slaughter house. Soft tissues surrounding IVDs (muscles and ligaments) were manually removed. Each IVD was sectioned transversally through the center and NP tissue was harvested from both halves. Tissues were washed three times with Hank’s Balanced Salt Solution (HBSS). Following this wash, NP and AF tissues were digested with 0.19% of pronase for 1h at 37°C under agitation. After pronase inactivation by successive washes with HBSS, DMEM supplemented with 10% FBS and 1% P/S containing 32IU/mL of collagenase type II was added. The mixture was incubated under orbital agitation overnight at 37°C in a humidified atmosphere of 5% CO₂ and was subsequently filtered through a 70µm cell strainer. Cells obtained were centrifuged for eight minutes at 1,200rpm and counted. Cells were then cultured up to 80% confluency before trypsinisation (1X Trypsin/EDTA) and electroporation.

4.2.3.3 Cell Electroporation

The procedure followed for cell electroporation was performed according to the method described in the Amaza™ Nucleofector™ for human chondrocyte kit (Lonza, Ireland). Briefly, cells were resuspended in electroporation solution at a density of 50,000 cells per 100µl. 2µg of the plasmid DNA of interest was added to the cells. The mixture was then transferred into an electroporation cuvette and electroporated using the program U-028 of Amaza™ Nucleofector™. Immediately after electroporation, cells were seeded onto a tissue culture plate at a density of 50,000 cells per cm² or in a 8-well chamber slide (LabTek™, Chamber Slide™ System, Thermo-Fisher™, Dublin, Ireland) at a density of 25,000 cells per well.

Different treatment groups were prepared as follows: (1) cells were electroporated with XT-I plasmid (XT-I); (2) cells were electroporated with GT-I plasmid (GT-I); (3) cells were mock transfected with green fluorescent protein-encoding plasmid used as control (EV); (4) cells were electroporated without plasmid. Two groups resulted from this last group: cells treated with IL-1β (CA) and untreated cells (CA-IL1). After electroporation, cells were cultured for four days at 37°C under 5% CO₂ with a medium change at day 2. Medium was then removed and cells were treated by chondroitinase ABC (Ams Biotechnology®, Abingdon, UK) at 0.2U/mL to digest the remaining CS. Briefly, cells were washed three times with
serum-free DMEM before addition of 0.2U/mL of chondroitinase ABC diluted in digestion buffer (pH8; 100mM Tris, 100mM sodium acetate, 0.02% BSA). Digestion reaction was allowed for three hours at 37°C. Cells were then washed three times in DMEM supplemented with 10% FBS and 1% P/S. DMEM supplemented with 10% FBS and 1% P/S and 10ng/mL of IL-1β was then added onto XT-I-electroporated cells (XT-I), GT-I-electroporated cells (GT-I), EV-electroporated cells (EV) and cells alone (CA). Cells of the CA-IL group were cultured within the same medium not supplemented with IL-1β. Cells were then cultured for two days before analysis.

4.2.3.4 Gene Expression

After two days, total RNA was extracted using a variant of Trizol isolation. Briefly, TriReagent® (Life Technologies™, Dublin, Ireland) was added to the cells. Phase separation was performed using chloroform, and total RNA from this step was purified using RNeasy mini kit (Qiagen, Manchester, UK) according to the supplier’s protocol (Appendix H). Total RNA quantity and purity were determined using an ultraviolet spectrometer (NanoDrop 2000 Spectrophotometer, ThermoScientific Inc., USA). Reverse transcription (RT) was performed using the reverse transcription system (Promega, UK) in accordance with the manufacturer’s protocol (Appendix I). Gene transcription was examined using real-time polymerase chain reaction (PCR). Reactions were performed and monitored by StepOne Plus™ detection system (Applied Biosystems, USA) using the QuantiFast SYBR Green gene expression PCR kit (Qiagen, UK) (Appendix L) for the targeted genes (Appendix M). Gene transcription was normalised in relation to transcription of the housekeeping bovine 18S. The 2^\text{ΔΔCt} method was used to calculate relative gene expression for each target gene according to Pfaffl method [56].

4.2.3.5 Immunocytochemistry and Lectin Cytochemistry

Immunohistochemistry was performed for three different antigens: XT-I detected by goat anti-XT-I antibody (Santa Cruz, USA), GT-I detected by mouse anti-B3GAT-1 antibody (Abnova, GmbH, Heidelberg, Germany) and C6S and C4S detected by the CS-56 antibody (Sigma-Aldrich®, Dublin, Ireland). Two days post-treatment, cells were blocked with 4% neutral buffered PFA for one hour at RT. Cells were then washed three times in 1X PBS. Samples were incubated in a
permeabilising solution consisting of 1X PBS supplemented with 0.2% Triton-X, 24mM NaCl, 1.6mM MgCl₂ and 160mM of sucrose for five minutes at 0°C.

For all staining, a blocking step with 5% goat serum in 1X PBS for one hour was performed before overnight incubation at 4°C with the primary antibodies diluted at 1/200. Negative control sections were incubated without primary antibody. After five washes with 1X PBS with 0.05% Tween 20 of five minutes each, the secondary antibody (AlexaFluor® 488 mouse anti-goat for anti-XT-I antibodies, and AlexaFluor® 488 donkey anti-mouse for anti-GT-I and CS-56 antibodies) diluted at 1/1,000 was incubated for one hour at room temperature (RT) onto the sections. The cells were then washed five times with 1X PBS with 0.05% Tween 20 for five minutes each before being counterstained with DAPI diluted at 1/2,500 in 1X PBS (Life Technologies™, Ireland) for 10 minutes at RT. The sections were washed three times with 1X PBS with 0.05% Tween 20 before mounting with ProLong® Gold antifade. The slides were stored at 4°C in the dark for one day before imaging. Imaging was performed using an inverted epifluorescent microscope (Olympus IX81, Mason Technologies, Dublin, Ireland). Five images per slide were taken and analysed as described in 4.2.4.

A WGA lectin staining was also performed to reveal the golgi apparatus. After permeabilisation, a blocking step with 2% periodate-treated BSA in TBS was performed for one hour. Slides were washed again three times for five minutes each in TBS and incubated with the fluorescently-labelled lectin diluted in TBS for one hour. An inhibitory control was carried out in parallel to verify that the lectin binding was glycan-mediated [57, 58]. The lectin was pre-incubated in 100mM of the appropriate haptenic sugar (see Table 1 for inhibitor specificity and concentration) for one hour at room temperature (RT) beforehand. The sections were washed five times with TBS with 0.05% Tween 20 and counterstained with DAPI (1/2,500) for 20 minutes. The cells were washed again in TBS with 0.05% Tween 20 before mounting with ProLong® Gold antifade. Slides were cured at 4°C in the dark for one day before imaging. Imaging was performed using an inverted epifluorescent microscope (Olympus IX81, Mason Technologies, Dublin, Ireland). Five images per slide were taken and analysed for each tissue.
4.2.3.6 GAG Quantification

2mL of medium for each sample was freeze-dried for concentration. The lyophilized medium was resuspended in 250µl water. 100µL of the medium was filtered through a 3 kDa MWCO centrifugal filter with 100µL HPLC-grade water according to manufacturer's instructions. All spin filters were washed with HPLC-grade water prior to use and water used throughout the procedure was HPLC-grade. The retentate was eluted from the filter with 50µL of water, and 390µL of digestion buffer (50 mM Tris-HCl, 60 mM sodium acetate, pH 8.0) and 10µL of ChABC (100mU) was added. The mixture was then digested at 37°C for 3h with gentle agitation (300rpm). The digested mixture was then immediately filtered as above (3 kDa MWCO) and the lower molecular weight filtrate was dried in a vacuum centrifuge (approximately 2h). The dried, digested samples were then derivatized according to the protocol from Skidmore et al. [59] with 10µL of BODIPY® stock solution (Invitrogen, Life Technologies™, Ireland). The methanol contained in the BODIPY® solution was evaporated by vacuum-speed centrifugation for 30 min before the addition of 5µL of the fluorescence labeling solution to each of the reaction mixtures. After resuspension, the samples were incubated for 4h at room temperature. 5µL of sodium borohydride reducing solution was added to reduce the imine bound in a more stable amine bound. The reduced reaction mixture was then incubated for 30 minutes at RT protected from the light before being flash-frozen and lyophilized. Samples were kept at -80°C until further analysis.

CS analysis was performed in accordance with a modified protocol of Skidmore et al. [59]. In brief, chromatographic separations were carried out on a strong anion exchange column ProPac PA 1.4mmx250mm (Thermo Scientific, Dublin, Ireland). The column was conditioned by washing in isocratic HCl followed by a wash in 1mM NaOH and a final wash with HPLC grade water. All washes were performed in a flow of 1mL/min at 50°C for 30 minutes. The eluates were monitored on a Varian™ 920-LC analytical HPLC equipped with a FP-2020 Plus™ Intelligent Fluorescence Detector (Jasco, UK). After column conditioning, 20-50ul of standard disaccharides or digested samples were injected onto the column followed by 100% Solvent A (5mM NaOH) for 20 minutes to allow the elution of free tag. The fluorescently-labelled standards and samples were eluted in a linear gradient of Solvent B (2M NaCl, 5mM NaOH) from 0-50% (final concentration 1M NaCl) over 60 minutes at a flow rate of 1mL/min. Following elution, the column was washed in
isocratic Solvent B for five minutes at 1mL/min before regeneration in Solvent A for 20 minutes at 1mL/min. The eluted fluorescence was monitored at wavelengths Ex=488nm and Em=520nm. Four reference peaks were identified for chondroitin-0-sulfate (C0S), chondroitin-4-sulfate (C4S), chondroitin-6-sulfate (C6S) and hyaluronic acid (HA). The disaccharide content of each sample was identified by comparing to appropriate standards chromatographed under the same HPLC conditions as the sample and quantified by comparing to the appropriate standard curve generated by injection of known concentrations of the standards.

4.2.4 Image Analysis and Fluorescence Intensity Quantification

Quantification of fluorescence intensity of the digital images was done using Image J software (National Institutes of Health, USA). The intensity of fluorescence was measured from five different images for each set of cells (n=3) [60]. The intensity of fluorescence values were then normalised by the cell number. An average of the five values was taken as the quantification of CS deposition. The average of quantification from the five images was taken for the quantification of one cell-group (n=3). The average fluorescence intensity quantification from the three sets of cells with standard error of the mean was represented (Appendix EE).

4.2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism®, Version 5 (USA). Data were compared using one-way analysis of variance (ANOVA) followed by a Tukey comparison test. Values were considered as significantly different with a $p<0.05$.

4.3 Results

4.3.1 GAGs, XT-I and GT-I Expression

Bovine IVDs exhibited different gross morphology upon ageing (Figure 4.2). With ageing, the tissue appeared less hydrated and less flexible. The quantity of NP tissue decreased greatly with increasing ratios AF surface / NP surface (2.08, 11.64 and 14.3, respectively). Along with these gross morphological differences, the ECM displayed distinct morphological features with ageing. An iterative decrease of GAG staining intensity (Safranin O – Red) was observed in both NP and AF tissues (Figure 4.4). This decrease was associated with a change in the ECM organisation profoundly marked in eight year-old tissue (Figure 4.3, Figure 4.4). For this age-
group, an increase of cellularity was seen with characteristic clusters formed of multiple chondrocyte-like cells (Figure 4.3E, Figure 4.4E). These clusters were delimited by a rim of matrix with a higher intensity of staining. An irregular GAG deposition in NP tissue along with a disorganisation of the collagen fibres in the AF tissue was seen. Cell distribution became sparse. Annular tears were observed in the AF (Figure 4.3.F, Figure 4.4.F).

4.3.1.1 GAGs and CS Quantification

Similarly, an iterative decrease of sGAG content was revealed by DMMB assay in both NP and AF tissues upon ageing (Figure 4.5A). sGAG content was higher in NP tissue than in AF tissue for each age-group ($p<0.05$). ECM of NP of six month-old tissue contained six and three times more sGAG than two and eight year-old NP ECM, respectively. A comparable, but less pronounced, trend was observed for the AF ECM.

The sulfated CS disaccharide content, obtained by HPLC analysis, showed a similar profile to that observed for the total sGAG content with an age-related decrease of CS content. However, this trend was not statistically significant (Figure 4.5). The quantity of specific disaccharides C0S, C4S and C6S varied with ageing. The total CS disaccharide content decreased in NP tissue from 0.19±0.08 µg of CS / µg of DNA in six month-old tissue dropping to 0.066±0.01 µg of CS / µg of DNA in eight year-old tissue (Table 4.1). In AF tissue, the total CS disaccharide content dropped from 0.20±0.07 µg of CS / µg of DNA in six month-old tissue to 0.02±0.01 µg of CS / µg of DNA in eight year-old tissue. NP and AF tissues do not display the same changes in CS composition upon ageing (Figure 4.6). Although the percentage of C0S in the tissues remained constant with ageing for both tissue-types, significant differences were noted in C4S and C6S disaccharide profiles. At six month-old and eight year-old, NP tissues showed a comparable profile with similar percentages of C0S, C4S and C6S disaccharides (Figure 4.6A). NP ECM in two year-old tissue exhibited an inverse profile with a higher percentage of C6S disaccharides and a lower percentage of C4S disaccharides. A progressive switch of sulfation profile was noted in the AF from six months to eight years. Eight year-old AF ECM contained more C6S disaccharides than C4S disaccharides. However, for this tissue, the variations in percentage of disaccharide between the different age-groups were not significantly different (Figure 4.6B).
4.3.1.2 Decrease of XT-I and GT-I Expression

XT-I and GT-I, two enzymes involved in the synthesis of CS, were stained by immunohistochemistry in order to study their expression over ageing. XT-I was detected in both NP and AF tissues at a cellular level. No expression of the enzyme was detected in the ECM. XT-I expression decreased significantly in two year-old tissues while the enzyme was no longer detected in eight year-old IVD tissues (Figure 4.7).

Likewise, GT-I was detected in both AF and NP tissues at a cellular level. No expression of the enzyme was detected within the ECM. A significant drop of GT-I expression was observed in IVD tissue with ageing to reach a non-detectable point in eight year-old tissue (Figure 4.8).

4.3.2 XT-I and GT-I Up-regulation via Non-viral Therapy in NP cells

After identifying a correlation between the quantity of GAGs and the expression of XT-I and GT-I, the up-regulation of both enzymes via non-viral gene delivery approach to enhance the production of CS by NP cells was evaluated. Plasmid encoding for GT-I was prepared and characterised as described in Appendix O. Both XT-I and GT-I plasmids were delivered by electroporation. This technique was preferred to chemical non-viral delivery because of its higher efficiency of transfection of NP cells (Appendix II). Cells were treated with IL-1β to stimulate an inflammation profile of NP cells [14, 61]. The expression of XT-I and GT-I proteins and their biological activity were assessed by gene expression, immunocytochemistry and HPLC analysis.

4.3.2.1 Increase of XT-I and GT-I mRNA Level after Electroporation

A significant decrease of both XT-I and GT-I mRNA expression were observed after treatment with IL-1β (2/3 and ½-fold, respectively) (Figure 4.9A-B). The cells electroporated with XT-I plasmid exhibited a high expression of XT-I (Figure 4.9A) (p<0.05). The level of expression of this enzyme was also greater after electroporation with GT-I plasmid (83-fold) while the expression of XT-I after electroporation with AV was similar to that of CA. GT-I plasmid was highly expressed in NP cells after electroporation (Figure 4.9B) (p<0.05). The introduction of XT-I and EV-plasmids induced a higher expression of GT-I (2 and 6-fold, respectively). The level of mRNA encoding for aggrecan was not changed after
treatment with IL-1β. However, an increase of aggrecan expression was noted after the introduction of exogenous plasmid DNA within the cells. A higher expression was observed in XT-I-treated cells (9-fold) (Figure 4.9C).

4.3.2.2 Increase of XT-I and GT-I Protein Expression

At a protein level, a 60% decrease of binding intensity of the antibody detecting XT-I was observed after IL-1β treatment. A higher binding intensity was obtained after delivery of XT-I plasmid to NP cells compared to empty vector (EV)-treated cells and cells alone (CA) (85 and 73%, respectively). No significant difference between the binding intensities was observed between XT-I treated cells and cells without IL-1β treatment (Figure 4.10).

Likewise, 89% decrease of binding intensity of the antibody detecting GT-I was observed after IL-1β treatment of NP cells. A higher binding intensity was noted after delivery of GT-I plasmid compared to EV-treated cells and CA (91 and 81%, respectively). No significant difference was noted between the binding intensities of GT-I treated cells and cells without IL-1β treatment (Figure 4.11).

4.3.2.3 CS Expression

Deposited and cell surface CS were quantified by image analysis of CS-56. No significant difference of binding intensity was observed between the different treatment-groups (Figure 12A).

The quantification of total CS secreted in the medium by HPLC revealed a decrease of CS production after treatment with IL-1β (p<0.05). A slight increase of CS content after treatment with XT-I and GT-I plasmids was noted compared to CA and EV-treated cells. A high inter-individual variability was noted during the quantification of CS by HPLC. Although the same trend toward an increase of CS synthesis was observed, no significant differences between the different treatment-groups were shown (p>0.05) (Figure 12B). Based on power analysis calculations, a sample size of cell lots extracted from different animals would be required to obtain significant differences between the treatment groups.

4.4 Discussion

During degeneration and ageing of the IVD, PG depletion induced by IL-1β occurs [14, 52, 61]. Aggrecan, main PG of IVD tissue, substituted with many GAGs of which CS is the most abundant [52]. During the degeneration and ageing, the size
Figure 4.2. Gross morphology of six month-old (A), two year-old (B), and eight year-old (C) bovine caudal intervertebral discs. A decrease of the NP/AF ratio is observed upon ageing.
Figure 4.3. Representative images of hematoxylin and eosin stained bovine NP (A, C, E) and AF (B, D, F) tissues at six month-old (A-B), two year-old (C-D), and eight year-old (E-F). Extracellular matrix proteins and nuclei are stained in pink/red and purple/blue, respectively (n=5, scale bar = 100µm).
Figure 4.4. Representative images of Safranin O Fast Green stained NP (A, C, E) and AF (B, D, F) tissues at six month-old (A-B), two year-old (C-D), and eight year-old (E-F). Collagens (green) and GAGs (red) are stained in green and red, respectively. Black arrows indicate clusters of chondrocytes-like cells in E and tears within the AF tissue in F (n=5; Scale bar = 400μm).
Figure 4.5. Age and tissue-related changes of (A) total sGAG content of bovine IVD quantified by DMMB assay and (B) sulfated CS content quantified by HPLC. Data were normalised to DNA content and represented as mean ± standard error of the mean (n=5). * represents significant differences at p<0.05.
Table 4.1. Quantity of C0S, C4S and C6S disaccharides (µg/µg of DNA) in six months, two and eight years bovine IVD tissues. Data were normalized to DNA content and represented as mean ± standard error of the mean (n=5). * represents significant differences at p<0.05.

<table>
<thead>
<tr>
<th></th>
<th>C0S</th>
<th>C4S</th>
<th>C6S</th>
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<td><strong>NP</strong></td>
<td></td>
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<tr>
<td>6 month-old</td>
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<td>2 year-old</td>
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<td>0.11±0.02</td>
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<td>8 year-old</td>
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<td>0.01±0.00</td>
<td>0.07±0.01</td>
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<tr>
<td><strong>AF</strong></td>
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<tr>
<td>6 month-old</td>
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<td>0.03±0.03</td>
<td>0.20±0.07</td>
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<tr>
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<td>0.06±0.02</td>
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<td>8 year-old</td>
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Figure 4.6. Percentage of disaccharides C0S, C4S and C6S in six month-old, two and eight year-old bovine (A) NP and (B) AF tissues. Data were normalised to DNA content and represented as mean ± standard error of the mean (n=5). * represents significant differences at $p<0.05$. 
Figure 4.7. Representative fluorescent images of XT-I expression in bovine NP (A, C, E) and AF (B, D, F) at six month-old (A-B), two year-old (C-D) and eight year-old (E-F). Nuclei are stained in blue (DAPI) and XT-I in green (FITC-coupled secondary) (n=5; Scale bar = 100\(\mu\)m).
Figure 4.8. Representative fluorescent images of GT-I expression in bovine NP (A, C, E) and AF (B, D, F) at six month-old (A-B), two year-old (C-D) and eight year-old (E-F). Nuclei are stained in blue (DAPI) and GT-I in green (FITC-coupled secondary) (n=5; Scale bar = 100µm).
Figure 4.9. mRNA expression of XT-I (A), GT-I (B) and aggrecan (C) after electroporation of NP cells. XT-I, GT-I and aggrecan expression were quantified two days after treatment with IL-1β. * denotes significant differences between the different treatment-groups at $p<0.05$ (n=3).
Figure 4.10. (A) Representative fluorescent image of XT-I expression within NP cells after electroporation. XT-I, nuclei and golgi apparatus are stained in red, blue and green, respectively. (B) Quantification of antibody binding intensity to XT-I protein. Data were normalised to the cell number and represented as mean ± standard deviation. * denotes significant differences between the different groups at \( p<0.05 \) (n=3; Scale bar = 10µm).
Figure 4.11. (A) Representative fluorescent image of GT-I expression within NP cells after electroporation. GT-I, nuclei and golgi apparatus are stained in red, blue and green, respectively. (B) Quantification of antibody binding intensity to GT-I protein. Data were normalised to the cell number and represented as mean ± standard deviation. * denotes significant differences between the different groups at $p<0.05$ (n=3; Scale bar = 10µm).
Figure 4.12. (A) Quantification of CS-56 antibody binding intensity to deposited and cell surface CS. Data were normalised to cell number. (B) Total CS content in the culture medium quantified by HPLC. Data were normalised to the quantity of CS secreted by non-treated cells. No significant difference was found between the different groups at $p<0.05$ for A and B.
and number of CS carried by aggrecan decrease influencing IVD at both structural and biological levels [25, 42, 62]. In this study, the behaviour of GAGs, specifically CS, was investigated in an ageing bovine model. This model was chosen because of its similarity to human IVDs due to its mechanical properties, loss of notochordal cells [63], gene expression [64], and GAGs behaviour [63].

Miyazaki et al. attributed the similarity between human and bovine GAGs behaviour to the absence of notochordal cells within the tissue [63]. These cells were shown to greatly influence the production of GAGs. Notochordal cells were not observed in six month-old tissue (Figure 4.3). As a consequence of notochordal cell disappearance, the quantity of sGAG produced decreases and an increase of sGAGs occurs resulting in an imbalance in anabolic and catabolic activities [63]. This phenomenon results in the formation of a less hydrated and flexible tissue with ageing [3]. These characteristics were observed in the ageing bovine model evaluated here (Figure 4.2). At a histological level, a decrease of GAGs staining was noted between six month-old and two year-old tissues which suggests a decrease of anabolic activities by IVD cells with ageing. Tissues in eight year-old IVD exhibited characteristic features of degeneration [65] i.e. increase of cellularity, presence of clusters formed of multiple chondrocytes-like cells delimited with a higher stained rim of matrix and an irregular GAG deposition in NP tissue as well as a disorganisation of the ECM with tear formation in AF tissue (Figure 4.4).

Close observation of total sGAGs including CS, HS, KS and DS revealed an iterative decrease of their quantity upon ageing (Figure 4.5A). CS, the most abundant sGAGs within the IVD tissue, were shown to play essential structural and biological roles in IVD [52]. Their polyanionic nature allows retention of water within the tissue [66] and interaction with GFs and cytokines [62]. These properties confer to CS specific roles in tissue development, remodelling and pathology [67-69]. The CS composition was specifically assessed by HPLC. As for the total sGAG content, an iterative decrease of sulfated CS content was observed upon ageing (Figure 4.5B). However, this decrease was less marked than that of total sGAG content. This observation suggests that the overall sGAG content including CS, HS, KS and DS was affected by the phenomenon of ageing. As for the ovine model (Chapter 3), changes in CS disaccharide composition were noted upon ageing. The same trend in CS composition was observed in ovine and bovine IVD tissues. An inversion of sulfation profile occurs with maturity (from six month-old to two year-old) with a
higher level of C4S in six month-old tissue and a higher level of C6S in two year-old tissue (Figure 4.6A). Eight years NP ECM displayed a similar trend to that of six months NP ECM. These variations in CS disaccharide composition can be attributed to changes in mechanical stresses on the discs (e.g. bending, flexion) shown to modify the CS sulfation pattern [70]. GFs and cytokines interact with specific sulfated disaccharide in a time and gradient manner [22, 24]. The modification of CS disaccharides also suggests that there is a role of these molecules in the regulation of GF and cytokines during the development and ageing processes of IVD.

AF ECM of ovine and bovine IVD did not display the same profile (Figure 4.6B). Indeed, ovine AF ECM changed upon maturity from a predominant composition of C4S disaccharide to a predominant composition of C6S disaccharide similarly to NP ECM (Chapter 3, Figure 3.18). However, bovine IVD displayed the same trend of composition upon maturity. Eight year-old AF ECM contained less C4S disaccharides and more C6S disaccharides than did the two other age-groups (Figure 4.6B). It is possible that the switch in sulfation pattern observed in ovine disc occurred at an earlier stage of IVD life-time. It is important to highlight the differences in mechanical stresses applied on caudal and spinal IVDs and also the considerable interspecies differences [71] which may be at the root of any differences in CS disaccharide composition.

The results of this study highlighted the variations in CS composition and content which occur during IVD ageing. A significant decrease of the overall CS content was observed in this study (Table 4.1). The biosynthesis of CS is performed by the polymerisation of GlcA and GalNAc catalysed by different enzymes [44, 72]. XT-I and GT-I are considered to play key roles in CS synthesis [48, 50, 51]. The behaviour of both enzymes was evaluated by immunohistochemistry upon ageing. XT-I and GT-I were detected in both AF and NP tissues (Figure 4.7 and Figure 4.8). A decrease of XT-I and GT-I occurred upon ageing to reach an undetectable level in eight year-old tissue. Therefore, a correlation between CS behaviour and their enzymes of synthesis can be made.

Better understanding of disc biology has enabled the development of different therapeutic approaches for disc regeneration and repair [33]. Most of these strategies are based on the implantation or injection in the disc of a scaffold which can be carrier of cells and therapeutic molecules [33, 72]. Different therapeutic molecules were investigated, such as GFs (sox-9, BMPs, TGF-β) and cytokine anagnost
(IL1Ra, anakinra) [14, 73], in an attempt to up-regulate anabolism or to down-regulate catabolism [33]. Although these approaches have shown promising effects, the limited short-life of GFs, enzymes and other effective molecules limits their use for long term therapeutic strategies [51, 74, 75]. A nucleic acid transfer strategy which specifically aimed to stimulate CS synthesis was developed in this study.

Cell treatment with IL-1β affected the expression of both XT-I and GT-I at mRNA and protein levels (Figures 4.9-4.11) in accordance with previous reports in chondrocytes [48, 7]. This phenomenon is due to the repression of the XT-I promoter activity by IL-1β [48]. The level of aggrecan was not affected by IL-1β between treated and non-treated cells was observed. An increase of aggrecan mRNA expression was noted after introduction of exogenous plasmid DNA by electroporation within NP cells. Exogenous plasmid DNA was shown to induce some level of inflammatory responses [78]. Khair et al. has reported an early induction of aggrecan expression in response to IL-1β treatment [48]. It can be hypothesised that a compensatory phenomenon occurred in response to electroporation and treatment with IL-1β which induced the maintenance or increase of aggrecan expression.

The over-expression of XT-I and GT-I enzymes by plasmid DNA delivery was shown to restore the level of both enzymes at a protein level after treatment with IL-1β (Figures 4.9-4.11). The next objective of this study was to investigate whether the delivery of XT-I or GT-I to NP cells have a beneficial effect on the CS production. The potential of XT-I and GT-I over-expression to restore the production of CS production was assessed at a cellular level by immunocytochemistry. No significant difference in CS deposition between the treatment groups was noted. The CS released in the medium were quantified by HPLC. A slight increase of CS content was noted after treatment with both XT-I and GT-I. However, this increase was not statistically significant. A high inter-individual variability was noted during the quantification although the same trend was observed for the different sets of cells extracted from three different animals used in this study. A rate-limiting nature of XT-I and GT-I has been suggested [48, 51, 79]. This property can be at the origin of the slight increase of CS synthesis observed in this study. Bai et al. have reported the formation of various intermediate variants along with normal GAG chains after up-regulation via non-viral delivery of GT-I plasmid in deficient Chinese hamster ovary cells. In this study, the authors have shown a two-fold increase of GAG synthesis compared with that observed in wild-type cells which suggest a rate-limiting nature
of GT-I [79]. The rate-limiting properties of GT-I have, however, been reconsidered by Venkatesan et al. who have reported an increase of GAG synthesis after up-regulation of GT-I in cartilage explants [51]. The results obtained in this chapter are in accordance with the results obtained by Bai et al. Nevertheless, different factors also have to be taken into consideration such as the direct inhibition of XT-I and GT-I activities by IL-1β which may influence their activity after up-regulation [48, 77]. Finally, a monolayer culture model was used in this study. GAG production has been reported as optimal in a three dimensional environment [80]. A higher up-regulation of CS via non-viral delivery of GT-I has been reported after treatment in a cartilage organ culture system than seen after treatment in a chondrocyte monolayer culture [51]. It is likely that an up-regulation in NP tissue of both enzymes can induce a higher GAG synthesis.

4.5 Conclusion

- Significant changes in GAGs composition during disc ageing were observed upon ageing for both NP and AF tissues with a higher expression of chondroitin 6-sulfate in two year-old tissues.
- A correlation between the expressions of XT-I and GT-I was shown in this study.
- IL-1β affected the expression of XT-I and GT-I in vitro but also the expression of CS.
- XT-I and GT-I were successfully up-regulated in NP cells after electroporation with evidence at gene and protein expression levels.
- No significant increase of CS synthesis was observed after treatment.

The changes observed in GAGs and their enzymes of synthesis give an appreciation of the importance of CS composition on disc biology. Results indicate that the best therapeutic approach to modulate the expression of GAGs might be a dual delivery of XT-I and GT-I or in combination with aggrecan protein core up-regulation.

4.6 References


Chapter 5

Summary and Future Directions
5.1 Introduction

Neck and low back pain is often associated with disc degeneration diseases (DDD). These diseases represent one of the major causes of disability with significant socio-economic consequences. Current treatments for DDD often lead to loss of spine mobility. Different tissue engineering approaches are being developed in order to treat IVD degeneration by promoting tissue regeneration and therefore maintaining IVD mechanical properties. Disc degeneration is characterized by significant changes in glycan composition. Glycans influence the IVD biology by their biological influence on cellular phenotypes [1, 2]. However, their signature and composition are not yet well known and understood. A better knowledge and understanding of the disc glycobiology is therefore essential for the design of an optimal microenvironment for NP regeneration. It is believed that such an environment will promote the deposition of new ECM to aid the regeneration of the tissue. The overall goal of this project was to develop a new tissue engineering paradigm for disc regeneration that is informed by identifying and promoting the maintenance of the glycobiology environment of the IVD.

5.2 Summary

5.2.1 Phase I – Type II Collagen/HA Hydrogel

The objective of phase I (Chapter 2) was to develop an injectable system which mimics the ECM composition of NP and which promotes the maintenance of the NP cell phenotype. Thus, the potential of a stabilised type II collagen hydrogel using poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG) cross-linker, enriched with various concentration of hyaluronic acid (HA), was investigated. The stability of type II collagen hydrogel was determined by assessing free amine groups, resistance to enzymatic degradation, and gel point. The potential toxicity of the cross-linker was initially tested by exposure to adipose stromal cells (ADSCs). After addition of HA (molar ratio type II collagen:HA 9:0, 9:1, 9:4.5, 9:9) within the hydrogel, the behaviour of the encapsulated NP cells was evaluated using cell viability, cell proliferation assay, gene expression analysis, cell distribution and cell morphology. A significant decrease in the free amine groups of collagen was observed, confirming successful cross-linking. The concentration of 4S-StarPEG did not influence the gelling time of the hydrogel developed (eight minutes at 37°C).
1mM cross-linked hydrogel yielded the most stable hydrogel after enzymatic degradation and this hydrogel was therefore used for the subsequent experiments. No toxicity of the 4S-StarPEG was noted for the ADSCs or NP cells regardless of the concentration of 4S-StarPEG used. NP cell viability was higher than 80% after each time-point, two, seven and 14 days in culture independently of the concentration of HA used. Interestingly, after 14 days cells did not proliferate in presence of HA while a proliferation was observed in its absence. At a gene expression level, HA did not influence NP cells phenotype after seven days in culture. Type I collagen mRNA expression was maintained similar to that of native NP cells. The optimally stabilised and functionalised type II collagen/HA hydrogel system developed in this study shows promise as an injectable reservoir system for intervertebral disc regeneration.

The results obtained in Chapter 2 highlight the importance of the ECM composition on NP cell behaviour. The highly specialized ECM composition has a significant role on cell behaviour. However, the effects of this ECM on the cell behaviour are yet not well understood. The identification of this ECM composition can help the understanding in cell-ECM communication and therefore to design the optimal therapy for regenerative approach.

5.2.2 Phase II – Glycoenvironment of the IVD

Glycans play an essential role in cell-cell and cell-extracellular matrix (ECM) communication during development, ageing and pathology. Specifically, sulfated glycosaminoglycan (sGAG) content dramatically influences cellular signalling and differentiation. At present, little is known of the glycosylation pattern of the intervertebral disc (IVD) cells and tissues and their alteration during development. The difficulty in distinguishing IVD cells and ECM phenotypes makes the identification of selective biomarkers for determining cell phenotype critical to assess the state of tissue for basic cell biology research and translational work in regenerative therapies. Currently, the phenotypes of the IVD cells and their ECM, the AF and NP can only be distinguished by gene expression analysis with relative ratio of expression of cell markers or ECM macromolecules. Furthermore, questions still remain about the relationship of NP cells to articular chondrocytes known to have a similar cell phenotype.

Consequently, the second phase of this thesis (Chapter 3) was to investigate the glycosignature of the glycocalyx and the ECM of the IVD tissue. Based on lectin
and immuno-histochemistry with statistical and clustering analyses of three and eleven month-old ovine IVD and cartilage tissues (n=5), a subset of specific and selective histological markers to distinguish the cell and ECM phenotypes (NP, AF and cartilage tissues) and their stage of maturation was identified. Clustering analysis revealed a closer relationship between NP cells and chondrocytes, in agreement with previous observations of a common phenotype. Interestingly, the relationship between glycoprofiles of the different extracellular matrices was age and tissue-dependent. In this case, a higher similarity between NP and AF tissues was observed. The detailed chondroitin sulfates (CS) composition and quantity of each tissue type (AF, NP and cartilage) at each stage was determined by enzymatic digestion and HPLC analyses. While the quantity of CS was not altered with age, the CS sulfation pattern differed in the AF and NP tissues with disc maturation. An important change in CS composition in IVD biology has been shown in this study. These glycans are essential structurally because of their properties of water retention and also in cell signalling. Modulating their expression will enhance the production of functional ECM by NP cells.

5.2.3 Phase III – Up-regulation of GAG Production

The depletion of CS within the IVD during DDD results in a decrease of tissue hydration, a loss of fluid movement, cell apoptosis, a loss of nerve growth inhibition and ultimately, the loss of disc function. Although many studies have highlighted the importance of CS in tissue development and pathology, little is known of their structure and content during IVD ageing. Therefore, the behaviour of GAGs, specifically of CS, and xylosyltransferase I (XT-I) and glucuronyltransferase I (GT-I), two key enzymes involved at crucial points of CS synthesis, was evaluated in a bovine ageing IVD model. It was hypothesised that the behaviour of both enzymes correlates with the behaviour of GAGs upon ageing. Important changes in GAGs composition during disc ageing were highlighted in this study. CS were affected at a structural and quantitative levels with important changes in sulfated disaccharide composition upon ageing. A decrease of GAGs and CS was seen on maturity which correlated with the decrease of the expressions of both XT-I and GT-I.

Therefore, it was hypothesised in Chapter 4 that the up-regulation of XT-I and GT-I within NP cells increases the production of CS. Both XT-I and GT-I
expression was affected by IL-1β. The delivery via electroporation restored the expression of both enzymes at a protein level. A decrease of CS was noted after treatment with IL-1β. A trend towards the increase of CS production after delivery of XT-I and GT-I was seen. However, no significant difference was noted. In accord with the results of this study, the best therapeutic approach to modulate the expression of GAGs might be a dual delivery of XT-I and GT-I or in combination with aggrecan protein core up-regulation. GAGs, especially CS, need to be taken into consideration for a better understanding of IVD biology.

5.3 Limitations

5.3.1 Phase I (Chapter 2)
In the course of this study, the quantification of GAGs was required to assess the phenotypic changes of NP cells. This quantification is usually performed by DMMB assay [3-5], a test based on the interaction of the cationic 1,9-dimethylene blue dye with the negatively charged sulfated GAGs. Due to the nature of this test, any highly charged molecules can interact with the dye. As HA is a negatively charged glycan, it interacts with DMMB at high concentrations. The hydrogel developed in Chapter 2 has a high HA content. The interaction of this molecule with DMMB makes the quantification of sulfated GAGs difficult. For this reason, this quantification was not done. Therefore, HPLC techniques, developed in the later part of this thesis, are a potential alternative to quantify GAGs.

A mixture of cells with different morphologies, expressing various cell phenotypes, was observed within the hydrogel after 14 days in culture. Elongated cells were present on the surface of the hydrogel whereas rounded cells were present in the centre. Consequently, for this time-point, it was not possible to obtain gene expression of type II collagen, aggrecan or type I collagen. Laser dissection of the hydrogel will allow the isolation of the different cell populations and permit the analysis of gene expression of the different cell-types.

5.3.2 Phase II (Chapter 2)
A rabbit ageing model was used in the study conducted following the study performed in Chapter 2 (Appendix GG). The degeneration/ageing process of the lumbar discs from different levels of the rabbit spine was observed to be inconsistent. This variation in degeneration results in high variability in the data.
Figure 5.1. Schematic representation of the main milestones achieved during the project. The different phases are inherently linked toward the development of an optimal NP cell microenvironment.
Furthermore, leakage of the hydrogel due to the limited disc space was observed in these animals. Small animal models, rodents and rabbits, retain notochordal cells over their lifespan. Consequently, NP maintains a more gel-like structure even at maturity unlike the NP of other larger mammalian species such as bovine or human which lose their notochordal cells at an early age. For these reasons, a large animal model is better-suited for the study of IVD tissue engineering approaches. However, the access to large animal models is limited due to the cost involved and restricted access. Indeed, such a model requires monitoring disc height and hydration in addition to the need for long term care. Another major limitation for in vivo studies of disc tissue engineering approaches is the absence of an in vivo model with a similar posture to that of the human spine.

Alternative strategies for therapy screening are envisaged by using an ex vivo and bioreactor systems. The organ-culture allows the application of mechanical forces on the discs to mimic those in the human environment. However, the results of such systems are not fully comparable to in vivo models as they do not possess the mechanical support of the surrounding soft tissues, does not initiate an inflammatory responses, and, additionally, the cells have a different metabolic activity to that of the in vivo IVD.

In both in vivo and ex vivo models, a leakage of the hydrogel was observed. It is noteworthy that this leakage is due to the high pressure and a lack of space within the disc. An essential step for the potential use of these strategies is the development of an approach that allows the creation of a cavity large enough for injection of the hydrogel, while avoiding the collapse of the disc throughout the hydrogel injection procedure.

5.3.3 Phase III (Chapter 3)

The model used for identification of the glyco-microenvironment of IVD in this thesis was the ovine lumbar intervertebral disc. Although two age-groups were studied, the original plan was to extend the study to a third group of five year-old animals. However, sourcing of animals in this higher age-group was not possible in Ireland. The study was therefore limited to two age-groups and extended to the comparison of IVD tissues with articular cartilage tissue.

The use of human samples is more relevant for the investigation of the glycomic profile of the IVD. Indeed, a high interspecies variability is observed in
IVD biology. Differences of gene and protein expressions are underlined between the animal models used currently for disc biology studies and those of human discs. However, access to human samples is highly regulated. Human discs used in most of the studies reported in the literature have been harvested from cadavers [6, 7]. Thus, discs from different degeneration levels can be collected, although a high inter-variability between degeneration groups is observed. Furthermore, access to young healthy patients is limited. Regardless of these limitations, a bank of human discs will be beneficial to this study. The access to human discs from cadavers is not authorized in Ireland, which limits the access to only pathological or post-traumatic discs obtained after surgery. However, the use of these discs shows some disadvantages as they are pathological in nature (mostly herniated discs). Furthermore, to date, the classification of pathological disc is difficult and is not well established.

5.3.4 Phase IV (Chapter 4)

The original plan in Chapter 4 was designed to transfect ADSCs, NP and AF cells in a three dimensional environment. Different trials were performed using several transfecting agents (HA-PEI nanoparticles [8], PEI, SuperFect® [9], Xfect™ [10], hyperbranched 2-(dimethylamino)ethyl methylacrylate (DMAEMA)/ ethylene glycol dimethacrylate [11]), a range of DNA concentrations (from 1µg to 10µg per 100,000 cells), and different techniques of transfection (polyplexes added on and within the hydrogel) (Figure 6.2). None of these trials were successful. These unsuccessful transfection results can be explained by availability of the polyplexes to the cells. Also, cell-polyplexes interactions, mechanical properties of the scaffolds, scaffold-polyplexes interaction have been shown to be essential parameters influencing the transfection in three-dimension [12]. Functionalisation of the polyplexes via antibodies or lectins (see paragraph 6.3.3b) will be an eventual strategy to explore in order to improve cell/polyplexes interactions in a three dimensional environment.

5.4 Future Directions

With the outcomes and limitations resulting from this project, it is possible to envisage various directions this research can take. Some of these are elucidated in the following sections.
Figure 5.2. HA-PEI nanoparticles, SuperFect® and PEI transfection capabilities of ADSCs in two and three dimensional environments. Polyplexes (10µg DNA per 100µl of hydrogel) were mixed within type II collagen/HA hydrogel seeded at 1,000,000 cells per mL. DNA alone and monolayer cultures were used as control. Data were represented as mean ± SD. * represents significant differences at $p<0.05$ ($n=3$; one-way ANOVA).
5.4.1 Large Animal Model and Organ Culture

One of the limitations of this project was the *in vivo* model used (Chapter 1, Appendix GG). Small animal models are relevant for disc research and allow the screening of numerous conditions within a reasonable cost. However, these models are not ideal because of their significant differences to those of human spine and the difficulties encountered in the use of such models (Chapter 5 section 5.2.2).

An organ-culture system is an alternative because of its low cost, its availability, and the control of the disc environment (confined culture, possibility to apply mechanical stresses similar to those of human, *etc*). Several studies have been reported on the use of this *tool* to elucidate disc biology [13], to identify the effects of the environmental phenomena such as nutrient deprivation [14] or mechanical loads [15, 16], and to assess potential therapies [17]. The use of an organ culture system can mimic an *in vivo*-like environment to illustrate the ability of the therapeutic strategy to slow down the degeneration of the IVD. Testing the stem cell loaded hydrogel within this system will allow for the determination of (1) its integration within the tissue, (2) the potential differentiation of stem cells towards a NP cell phenotype, (3) the behaviour of the hydrogel and delivered cells under mechanical stresses, and (4) the potential of the hydrogel to induce some level of regeneration.

However, this system will not provide all the responses required to evaluate the potential of the hydrogel for a therapeutic application. Thus, the translation of the hydrogel developed in Chapter 2 to a large animal model is required to determine its behaviour at a more physiological level and its potential application in human therapeutics. Previously used for testing IVD therapeutic strategies [18], the ovine model is reported as a good model for studying intervertebral disc interventions [19]. Even if differences between human and ovine discs are observed (vertebral end-plate size, shape, range of motion, mechanical properties, ECM composition and cells [19, 20]), this model is more appropriate than a small animal model because of its similarity in structure and mechanics to those of human and ovine IVD [20-23]. Furthermore, on a technical note, the space for injection within the discs relative to their size will be greater, thus allowing a controlled nucleotomy and a higher volume of injected hydrogel. As a result, more explants tissue will be available for analysis;
disc height and hydration, gene expression, ECM proteins composition and histology analysis can therefore be assessed.

Some concerns remain as to the use of induced vs. spontaneous models to evaluate IVD regeneration strategies. The induced models such as degeneration by stab wound [24, 25] or enzymatic digestion [26] provide a quick and controlled degeneration of discs. However, such models do not induce a "real" degeneration process and are, potentially, reversible [20]. A spontaneous model of degeneration will therefore be more relevant to the research question posed as it is closer to pathological conditions.

5.4.2 Glycan Composition

The delivery of stem cells through an injectable hydrogel supplemented with an optimal concentration of hyaluronic acid (HA) has been explored in Chapter 2. A potential future approach based on this project is the design of a cell microenvironment similar to that of progenitor cells / stem cells in IVD. Matrix components of the extracellular matrix can provide stimulatory or inhibitory effects leading stem cells / progenitor cells toward different roles (repopulation of the tissue, secretion of paracrine factors, etc.) [27]. In this thesis, the microenvironment of the immature and mature IVD was investigated. The importance of glyco-signature and glyco-microenvironment changes in cell communication has been highlighted by the identification of changes in glyco-cell surface markers and glyco-composition of the ECM during the maturation of the disc (Chapter 3). Glycans have been shown to modulate and to mediate cell-cell, cell-matrix and cell-molecule interactions. These properties provide glycans with critical functions in the development and the maintenance of tissues at a healthy stage [28] as well as in the maintenance of the cell microenvironment [29]. A specific glyco-environment directed with specific sulfations patterns has been shown to modulate the neuronal stem cells response to the tissue [30, 31]. Engineering the cell microenvironment will maintain the stem cells at an undifferentiated state promoting the production of paracrine factors. These factors have been shown to stimulate the synthesis and the deposition of ECM by the resident cells [32, 33]. They are also known to have immunosuppressive properties [32]. Indeed, the conditioned medium of pretreated MSCs has been shown to modulate the inflammatory environment of osteoarthritis cartilage by decreasing the expression of IL-1β and matrix degrading enzymes [34] crucial in disc degeneration.
Summary and future directions

diseases [35]. Sugars are optimally positioned to help cell-cell and cell-ECM communication. Stem cell / progenitor cell interactions within their microenvironment promote the maintenance of the cells at a multipotent and self-renewing state and the secretion of paracrine factors [32, 36] (Figure 5.3).

Such a stem cell microenvironment system can also be used as a production factory for relevant biomolecules. Stem cells can be engineered genetically in order to produce specific molecules such as IL-1 receptor antagonist [37]. The incorporation of such engineered cells within the hydrogel will enable a constant production of molecules of interest. It can be hypothesized that this engineered environment will slow down the degeneration process by constantly stimulating the resident cells which will restore the equilibrium matrix degradation - matrix production (Figure 5.3).

5.4.3 Improvement of the Gene Therapy Approach

Two hurdles that are critical for the success of potential gene therapy approaches have been highlighted in this project.

The first hurdle was a leakage of the hydrogel observed during the pilot in vivo and organ culture studies performed during this project (Chapter 2, Appendices GG and HHI). Such leakage, also reported in the literature by Vadala et al. [38], can lead to a displacement of the therapy toward the dura, spinal cord, vertebral body and ligaments associated with the spine [39, 40]. This, in turn, can lead to dramatic effects on the surrounding tissues. For example, a displacement of transformed cells which up-regulates GAGs (Chapter 4) will compromise the function of the spinal cord and dura by increasing of CS-PG production [41, 42].

The second hurdle observed during this project was a steady decrease in the activity of CMV-promoter plasmids over a period of one to two weeks (Appendix X). This decrease of activity may be attributable to an inhibition of the promoter activity by cytokines, by the binding of a repressor protein, by its methylation or by the loss of a positive activator [43]. Most of the strategies for intervertebral disc regeneration are conducted over a long period and consequently require a long expression of the transgene to be efficient.

Different approaches can be envisaged to encounter the use of virus and to maintain a local and long expression of the transgene. Two complementary approaches are presented below:
Plasmid Engineering

Plasmid sequences can be manipulated to be specifically expressed in the target tissue under defined conditions for obtaining a controlled activation of the exogenous plasmid DNA. Different strategies have been reported to promote local, controlled and long term expression of plasmid DNA (see Table 6.1 for the listing of different plasmids used currently in gene therapy approaches).

The controlled transcriptional activation of the transgene expression can be obtained by using specifically controlled promoters which allow for a longer and/or targeted expression of the transgene [43, 44]. Different promoters such as β-actin, elongation factor 1-α (EF1-α), or ubiquitin confer a sustained expression of the transgene. Their activity has been described as being lower than cytomegalovirus promoters; but, when coupled with viral or cellular enhancer elements, an activity superior to that of the native cellular promoters is observed. However, despite this improvement of the maintenance of expression, no control of the location of expression is obtained with this system. Controlled expression can be obtained via the use of drug controlled promoter such as the system TET which allows transgene expression after treatment with tetracycline. Vadala et al. managed to successfully deliver this system to NP cells via a viral vector [40]. These drug activated vectors are still under development and further work will be needed to envisage their application in the form of plasmid. Another approach that can be conceptualized is the use of tissue-specific [43, 45] or environmental-specific promoters such as HIF-1α induced promoters [43, 46]. Different approaches using environment-specific promoters will potentially be efficient to respond to the drastic environmental conditions of IVD (low oxygen, low glucose, and acidic pH environment) [47, 48]. The production of ECM molecules in IVD is regulated by HIF-1α protein [49, 50]. The avADSCular nature of IVD induces a strong constitutive expression of HIF-1α [49] which makes HIF-1α induced promoter a promoter of choice for IVD gene therapy.

Another important aspect of the plasmid design is the inflammatory response to the transfection of exogenous DNA to the cells. Plasmid DNA vectors are prokaryotic in origin, and as such are foreign entities to mammalian cells. The innate immune system is able to recognize pDNA vectors principally by their higher frequency of unmethylated CG dinucleotides (CpG), ligands of toll-like receptor 9,
compared to those of mammalian DNA [51]. Furthermore, an inactivation of the promoter can be observed by methylation of these sites. An elimination of these CpG from the vector will by consequent decrease inflammatory response by reducing the immune cell activation and will maintain a longer expression of the transgene [44, 51-53].

These observations highlight the potential of specific plasmid design as an approach for gene therapy for the IVD. Other approaches can be envisaged based on the development of functionalised vectors which promote specific and sustained delivery of the transgene.

- **Functionalisation of particles**

Sustained plasmid DNA delivery to NP and AF cells has been shown to be successfully obtained using HA particles without compromising their viability [8]. It is believed that their functionalisation with antibody/antibody fragments and/or lectins directed towards specific cell surface proteins and glycans, respectively, will improve their up-take and their specificity towards the targeted cells *i.e.* NP or AF cells. Previous studies have demonstrated the improvement of the up-take of polymeric systems after attachment of antibodies/antibody fragments at their surface [54]. Furthermore, the tethering of lectins on carbon nanotubes has been shown to improve their binding to cell surface of MCF-7 cells [55]. Thus, functionalisation of HA nanoparticles with such molecules is a promising approach for improving the up-take and for targeting a specific cell population (Figure 5.4).

In order to target and deliver these particles to a specific cell-type, the identification of cell surface markers for NP and AF cells is required. Power *et al.* identified the carbonic anhydrase XII as specific marker of NP cells [6]. However, the expression of this molecule has been shown to change with age and degeneration. Such a marker can be used for targeting a specific stage of degeneration. Nevertheless, a stable marker with a switch on/off expression will be more suited to targeting specifically NP, AF cells or chondrocytes. Though these markers are not yet recognised at a protein level, such a marker has been identified at a glycan level (*Chapter 3*). Future work can be focused on the functionalisation of the particles with SBA, WFA, SNA or MAA lectins for a specific target of the NP cells.
5.4.4 Modulating the Inflammation in the Disc: a Way to Reduce ECM Degradation

Degeneration and injury of the IVD are characterised by an increase of inflammatory cytokines [56]. They are secreted by the IVD cells themselves and by macrophages that infiltrate the tissue after injury [57]. These cytokines promote imbalance in anabolic and catabolic phenomena in the degeneration process [37, 58]. In Chapter 4, an approach to up-regulate the GAGs anabolism within the disc was developed. Further strategies focusing on the catabolic phenomena can be investigated in order to slow down the degeneration process. The degradation of the ECM is induced by the secretion of numerous cytokines [35, 59] which leads to innervation and vascularisation of the disc. IL-1β plays an essential role in this process. A significant increase of this cytokine along with a decrease of IL-1 receptor antagonist was shown in the progression of disc degeneration diseases [37]. IL-1β induces the secretion of other inflammatory factors such as IL-6, IL-8, COX-2 [35, 60], growth factors such as VEGF, NGF or BDNF [59], and numerous matrix metalloproteinases such as MMP-3, ADAMTS-5 [35] via the activation of p38-MAPK and NFκB (Figure 6.5). IL-1β is also involved in the decrease of ECM synthesis by having a direct role in the decrease of GT-I and XT-I expression [49, 61-63], and in aggrecan expression [35, 37, 58].

To date, different strategies have been envisaged to down-regulate IL-1β and its effects. Most of the strategies are based on the inhibition of the interaction of the cytokine and its receptor (IL-1R). Le Maitre et al. showed a significant decrease of matrix degrading proteases after the viral delivery of IL-1R antagonist [37]. In another study, this antagonist, under the form of anakinra, a recombinant non glycosylated form of IL-1R, was delivered via PLGA microspheres for sustained delivery to NP cells, demonstrating an attenuation of the IL-1β effects for seven days [64]. Additionally, the delivery of antibodies directed against IL-1β and TNF-α at the level of herniation allows the reduction of the NP-induced effects on nerve conduction velocity and, consequently, on pain [65]. The inhibition of p38-MAPK by Sb203190 inhibitor in rabbit NP tissue was shown to restore the production of ECM molecules (type II collagen and proteoglycans), anabolic factors (Sox-9, TGFβ), and a decrease of the production of catabolic factors (MMPs, COX-2, IL-6) [58]. Thus, a strategy to reduce the impact of such a cytokine will reduce the matrix
degrading proteases secretion, restore ECM synthesis, and will inhibit innervation and vascularisation of the disc.

Different non-viral gene delivery approaches can be envisaged to decrease the effect of IL-1β. The delivery of siRNA targeted against IL-1β would efficiently reduce the effect of the cytokine and restore the catabolic/anabolic balance in the IVD tissue. Non-direct targets against this cytokine can also be envisaged to regulate IL-1β cell signalling pathway (Figure 6.5). The delivery of siRNA against p38-MAPK is an approach to inhibit this pathway and to slow down not only the disc degeneration process by the inhibition of IL-1β but also of numerous matrix degrading enzymes and inflammatory cytokines (Figure 5.5). In vitro and ex vivo assays performed by Ellman et al. showed that the inhibition of MyD88 by MyD88 inhibitor peptide (MyD88i) allows an increase of proteoglycan production and a decrease of MMPs secretion [66]. The inhibition of this protein up-stream of p38-MAPK and NFκB allows a direct intracellular target against the activation of the IL-1β receptor (Figure 6.5). Furthermore, a better control of the transcriptome can be obtained by targeting microRNA (miRNA). These molecules, single stranded endogenous RNA of 16 to 35 nucleotides, are believed to constitute a complex regulatory network for the transcriptome [67]. Their expressions were shown to be deregulated in numerous pathologies, notably in osteoarthritis. miRNA-140 was shown to be down-regulated along with a decrease of Sox-9 expression and the up-regulation of ADAMTS-5 and IL-1β expressions in this pathology [68, 69]. ADAMTS-5, the major enzyme for aggrecan degradation, was shown to be tightly regulated by this miRNA [68, 70]. Its behaviour within the disc has not yet been reported.

These results highlight the potential of targeting IL-1β signalling pathway to slow down disc degeneration. The sustained and targeted delivery of siRNA against p38-MAPK, NFκB or MyD88 using a reservoir system such as type II collagen or HA spheres will allow a sustained and targeted effect not provided by inhibitors.
Figure 5.3. Engineered glyco-microenvironment for progenitor / stem cells as a secretory factory for IVD regeneration. This concept is based on the type II collagen hydrogel developed in Chapter 2. This hydrogel will be supplemented with a mixture of glycans previously identified as a component of the cell microenvironment. These glycans will allow the maintenance of stem cell properties which supplement the system. Paracrine factors secreted by the cells influence the resident cells and promote the deposition of ECM.
### Table 5.1 List of different promoters used for gene therapy approaches.

<table>
<thead>
<tr>
<th>Promoter type</th>
<th>Examples</th>
<th>Expression profile</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transient expression promoter</td>
<td>CMV, SV-40, RSV-LTR</td>
<td>Strong promoter leading to a high expression with a steady decrease of activity after one to two weeks.</td>
<td>[43, 71]</td>
</tr>
<tr>
<td>Sustained expression promoter</td>
<td>EF-1α, β-actin, CAG, Ubiquitin</td>
<td>Long term expression with transgene detection from eight to 24 weeks.</td>
<td>[72, 73]</td>
</tr>
<tr>
<td>Synthetic promoters</td>
<td>CMV-UBB, CMV-EF-1</td>
<td>Promoter designed as one or two enhancer elements are fused to a heterologous promoter sequence allowing a strong and long expression over time.</td>
<td>[72, 73]</td>
</tr>
<tr>
<td>Regulated promoters</td>
<td>GRP78-GRP94, HIF-1α</td>
<td>Tissue, environment or drug-specific promoter which is induced only in the specific tissue, environment or in presence of a specific drug.</td>
<td>[40, 43, 44, 74]</td>
</tr>
</tbody>
</table>
Figure 5.4. Functionalised HA nanoparticles system. HA nanoparticles developed [8] are loaded with plasmid DNA or siRNA/miRNA. Antibodies (1) and/or lectins (2) that recognise cell surface markers (transmembrane proteins and glycans) are grafted on the surface of these particles.
Figure 5.5. Potential strategies for inhibition of the effect of IL-1β. The signalling pathway was adapted from [60]. Different potential strategies can be envisaged: (1) inhibition of the receptor via IL1 receptor antagonist for example, (2) inhibition of the IL-1β pathway activation by MyD88 inhibitors, (3) inhibition of p39-MAPK or (4) NFκB pathways. Both pathways, p39-MAPK and NFκB are downstream of IL1 receptor activation.
5.5 Conclusions

The conclusions from the research studies described in this thesis can be summarized as follows:

5.5.1 Phase I

The principal objective of this phase was to develop an injectable system which mimics the ECM composition and which promotes the maintenance of NP cell phenotype.

Conclusions:

- Type II atelocollagen hydrogel was successfully stabilized with 4S-StarPEG with better stability after degradation by collagenase, a decrease of free amine groups in the structure and suitable mechanical properties.
- The system possesses injectable properties with a gelling time of eight minutes.
- The mechanical properties of the hydrogel were not altered by the addition of HA. A good cell viability and distribution were obtained after encapsulation of NP cells and ADSCs.
- HA promotes the maintenance of NP cell phenotype. An absence of NP cell proliferation was observed in presence of HA. A round morphology of NP cells was maintained with an increased concentration of HA.
- After seven days in culture, a low level of type I collagen expression was maintained in NP cells. However, a decrease of type II collagen and aggregan expression was observed.

5.5.2 Phase II

The principal objective of this phase was to identify the glyco-environment of the IVD in an effort to understand cell-cell and cell-ECM cross-talk.

Conclusions:

- Specific spatial and temporal glycans were highlighted in this project.
- The differences in glycan patterns are related to remodelling activities.
- Specific glycomarkers were identified which allows distinction between NP, AF and chondrocytes.
A modification of the chondroitin sulfates (CS) content and composition was observed upon maturity. This observation along with the significant decrease of GAGs during the degeneration of the IVD was the focus of further investigation.

5.5.3 Phase III

The principal objective of this last phase was to enhance the production of CS by IVD cells via a non-viral gene therapy approach by delivery of XT-I and GT-I, key enzymes involved at crucial points of CS synthesis.

Conclusions:
- A significant decrease of CS was observed with ageing in bovine caudal IVD.
- The composition of CS changed upon ageing for both NP and AF tissues with a higher expression of chondroitin 6-sulfate in a two-year-old tissue.
- Decreases of XT-I and GT-I expression were shown with ageing.
- XT-I and GT-I were successfully upregulated in NP cells after electroporation with evidence at gene and protein expression levels.

5.6 References


Summary and future directions


Summary and future directions


Summary and future directions


Summary and future directions


Appendices
I. Protocols
A. List of components and reagents

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type II collagen</td>
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<tr>
<td>Poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG)</td>
<td>JenKem Technology, Allen, TX, USA</td>
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<td>Hyaluronic acid</td>
<td>Contipro Group, Dolni Dobrouc, Czech Republic</td>
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<td>NaCl</td>
<td>Sigma Aldrich\textsuperscript{®}, Dublin, Ireland</td>
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</tr>
<tr>
<td>Na\textsubscript{2}HPO\textsubscript{4}</td>
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<tr>
<td>KH\textsubscript{2}PO\textsubscript{4}</td>
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<td></td>
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<tr>
<td>Acetic acid</td>
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<td>Collagenase type II C-5138</td>
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<tr>
<td>(\text{d}\text{b})Glycerophosphate</td>
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<td>Monoclonal anti-chondroitin sulfate clone CS-56</td>
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<td>Proteinase K</td>
<td>Roche Applied Science, West Sussex, UK</td>
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<td>1,9-dimethyl methyleneblue</td>
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<td>Sodium borohydrate</td>
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<td>Pronase</td>
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<td>TNBSA (2,4,6-Trinitrobenzene sulfonic acid)</td>
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<td>Extensor long range PCR polymerase kit</td>
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<td>Goat anti-XT-I antibody</td>
<td>Santa Cruz Biotechnology®, Inc., Santa Cruz, CA, USA</td>
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<td>Mouse anti-GT-I antibody</td>
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<td>BODIPY®</td>
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<td>Bioscience, Dun Laoghaire, Ireland</td>
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<td>Glucuronyltransferase I plasmid sc110387</td>
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<td>Not I restriction enzyme</td>
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<td>MiniElute® kit</td>
<td>Qiagen, West Sussex, UK</td>
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<td>Rneasy® Kit</td>
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<td>Ireland</td>
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<td>Lonza, ISIS Ltd., Bray, Ireland</td>
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<td>Anti-blood group Le&lt;sup&gt;a&lt;/sup&gt; antibody</td>
<td>Abcam®, Cambridge, UK</td>
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<tr>
<td>Anti-chondroitin 6-sulfate antibody</td>
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<td>Primers</td>
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<td>TUNEL Assay</td>
<td>GenScript&lt;sup&gt;®&lt;/sup&gt;</td>
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<tr>
<td>Chrondoitinase ABC</td>
<td>Amsbio&lt;sup&gt;®&lt;/sup&gt;</td>
</tr>
<tr>
<td>Δ Disaccharides (HA, C0S, C4S, C6S)</td>
<td></td>
</tr>
</tbody>
</table>
B. Hydrogels composition and preparation

Note: All procedures were performed in an ice bath unless otherwise stated.

Fabrication of type II collagen hydrogels

Porcine type II collagen was reconstituted in 0.05M acetic acid at a concentration of 5mg/mL, and stored at 4°C with gentle rocking. A concentration of 5mg/mL was chosen for the investigation as per the previous protocol of Dr Damien O’Halloran [1]. Fabrication of the hydrogel is based on the reaction of the NHS groups carried by the 4S-StarPEG with the amine groups of the type II collagen molecules (Figure B1). After eight minutes at 37°C, the hydrogel is formed. To ensure the complete gelation of the hydrogels, they were kept for one hour at 37°C before use.

![Figure B1. Type II collagen and Poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG).](image)

The succinimidyl groups react with the amine groups present on the molecules of type II collagen at 37°C.

Procedure

a) Prepare a solution of 5X Phosphate Buffer Saline (PBS) from a 10X stock solution (NaCl 1.368M, KCl 0.0268M, Na₂HPO₄ 0.101M, KH₂PO₄ 0.0176).
b) Mix 5X PBS and type II collagen together, and bring the pH to 7.4 by addition of NaOH 1M. Each component of the hydrogel needs to be added in the order indicated in Table B1. The addition of 5X PBS in collagen will induce the formation of fibrils.
c) Mix the suspension and check the pH by pH strips.
d) Add 20µl of 10mM 4S-StarPEG solution to the hydrogel. The 4S-StarPEG solution is to be resuspended just before use.
e) Mix well before dispensing in the well.
f) Incubate for one hour at 37°C before analysis.
Table B1. Acellular hydrogel preparation.

<table>
<thead>
<tr>
<th>Component</th>
<th>Order</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS5X</td>
<td>1</td>
<td></td>
<td>70µl</td>
</tr>
<tr>
<td>NaOH</td>
<td>2</td>
<td>1M</td>
<td>9-12µl</td>
</tr>
<tr>
<td>Type II Collagen</td>
<td>3</td>
<td>5mg/mL</td>
<td>200µl</td>
</tr>
<tr>
<td>4S-StarPEG</td>
<td>4</td>
<td>2mM, 1mM, 0.5mM</td>
<td>40µl, 20µl, 10µl</td>
</tr>
</tbody>
</table>

Fabrication of composite hydrogel

As previously described, type II collagen was reconstituted in 0.05M acetic acid at a concentration of 5mg/mL and stored at 4°C overnight with gentle rocking. Hyaluronic acid (HA) (750,000 to 1,000,000 Da) was dissolved at 4°C overnight under agitation in 5X PBS supplemented with 0.4M NaCl to prevent the formation of polyionic complexes [2] at concentrations of 1.57mg/mL, 7.14mg/mL and 14.3mg/mL. 70µl of these solutions was used for the hydrogel preparation in order to obtain a weight ratio collagen:hyaluronan of 9:0, 9:1, 9:4.5 and 9:9 respectively. HA was sterilised by filtration on filter 0.22µm before use.

Procedure

1. Mix HA solution and type II collagen together according to the weight ratio (Table 2), and bring the pH to 7.4 by addition of NaOH 1M. Each component of the hydrogel needs to be added in the order indicated in Table 3. The addition of 5X PBS onto the collagen induces the formation of fibrils.
2. Mix the suspension and check the pH by pH strips.
3. Add 20µl of 10mM 4S-StarPEG solution resuspended just before use to the hydrogel.
4. Mix well before to dispense within the well.
5. Incubate for one hour at 37°C before analysis.
6. Add cells at a concentration of 1,000,000 cells/mL into the hydrogel suspension (see appendix D for cell preparation). Cells have to be washed before addition within the hydrogel to remove any trace of phenol red.
7. Resuspend just before use the 4S-StarPEG in 1X PBS at a concentration of 10mM, and add 20µl to the hydrogel.
8. Mix the suspension and incubate for one hour at 37°C before addition of medium.
Table B2. Preparation of composite acellular type II collagen/hyaluronan hydrogel.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg/mL)</th>
<th>Volume (µL)</th>
<th>Quantity for one hydrogel (mg)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type II Collagen</td>
<td>5</td>
<td>200</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>1.57</td>
<td>70</td>
<td>0.1</td>
<td>9:1</td>
</tr>
<tr>
<td>HA</td>
<td>7.14</td>
<td>70</td>
<td>0.5</td>
<td>9:4.5</td>
</tr>
<tr>
<td>HA</td>
<td>14.3</td>
<td>70</td>
<td>1</td>
<td>9:9</td>
</tr>
</tbody>
</table>

Table B3. Preparation of composite cell-loaded type II collagen/hyaluronan hydrogel.

<table>
<thead>
<tr>
<th>Component</th>
<th>Order</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>1</td>
<td></td>
<td>70µl</td>
</tr>
<tr>
<td>NaOH</td>
<td>2</td>
<td>1M</td>
<td>9-12µl</td>
</tr>
<tr>
<td>Type II Collagen</td>
<td>3</td>
<td>5mg/mL</td>
<td>200µl</td>
</tr>
<tr>
<td>Cells</td>
<td>4</td>
<td>200,000 cells/hydrogel</td>
<td>100µl</td>
</tr>
<tr>
<td>4S-StarPEG</td>
<td>5</td>
<td>1mM</td>
<td>20µl</td>
</tr>
</tbody>
</table>

C. Isolation of adipose stem cells from rabbit adipose tissue

Tissue isolation

1. Sacrifice a three to four month-old rabbit of (four to five kg) using pentobarbitone injection (140mg/kg).
2. Add a sterile field on a table.
3. Wash the lower midline of the rabbit abdomen with alcohol to disinfect it (alcohol at 70%).
4. Cut the first skin layer of this area with scissors. The adipose tissue is just below this skin at the level of the groins.
5. Harvest the tissue and collect it in transport media. During the process of isolation, take care to eliminate the blood vessels to avoid contamination of the cell population with vascular cells.

Cells isolation (under hood)

1. Wash the tissue several times with Hank's Balanced Salt Solution (HBSS) to eliminate any blood potentially present (two to three times).
2. Incubate the tissue with collagenase type I at 0.025% for 1h at 37°C under agitation.
3. Add one volume of complete medium to stop the reaction.
4. Allow the digested tissue to settle for several minutes under the hood. Two layers are obtained:
   - Top: fat and adipocytes
   - Bottom: stromal fraction
5. Centrifuge for five minutes at 300g (1,200rpm).
6. Discard the top layer (by pipetting).
7. Resuspend the last phase and filter on cell trainer 70µm.
8. Centrifuge for eight minutes at 300g.
9. Wash the cells with complete medium.
10. Count cells. For this, dilute the cells to 1/32 to count them. Seed cells at 10,000,000 cells/mL (a lot of cells will be eliminated after 24h).

After 24h, the media is changed to eliminate vascular cells and adipocytes (these cells adhere after 72h). The media is then changed every two days and cells are kept subconfluent to avoid a spontaneous differentiation.

**Media**

**Transport medium**: -HBSS/DMEM (V:V)
-10% FBS
-1% P/S

**Complete media**: -DMEM
-10% FBS
-1% P/S

**D. Isolation of nucleus pulposus (NP) and annulus fibrosus (AF) cells**

Cells were isolated from six month-old and two year-old bovine tails.

**Tissue isolation:**
1. Dissect the tails (Figure D1A).
2. Eliminate muscles and ligaments. Isolate intervertebral discs (IVD) with a scalpel (Be careful not to damage the discs during this process).
3. Cut across the disc and collect NP and AF from each part. This tissue is then added in HBSS until cell isolation. It is important to avoid contamination of cells from the intermediate zones (cartilage endplate and bone tissue) (Figure D1B).
Figure D1. Isolation of cells of interest from designated areas from bovine tails.

(A) Arrows indicate the IVD. NP and AF tissues are harvested from upper (B) and lower (C) caudal disc.

**Cells isolation: under hoods**

1. Wash the tissues with HBSS three times.
2. Incubate for 1h at 37°C with 0.19% pronase (40mL) under agitation.
3. Remove the supernatant.
4. Wash three times with complete medium to inhibit the enzyme.
5. Resuspend in 40 mL of DMEM containing 32IU/mL of collagenase type II.
6. Incubate at 37°C under agitation overnight.
7. Filter the suspension on a cell strainer 70µm.
8. Inhibit the collagenase type II by addition of complete medium.
9. Centrifuge the cells for eight minutes at 1,200rpm.
10. Wash the pellet with complete media (three times).
11. Centrifuge the cells for eight minutes at 1,200rpm.
12. Resuspend in 10mL of complete media.
13. Count the cells (dilution 1/2, 1/4 and 1/8).
14. Seed 10,000 cells/cm² if they are seeded in monolayer. Seed the cells within the hydrogel for cell-seeded hydrogel.
E. **Rheology experiment**

Instrumentation:

Rheological measurements were performed at 37°C using a Haake MARS (Modular Advanced Rheometer System) rheometer (ThermoHaakes, Germany). The rheometer was equipped with a circulating water bath to control the temperature. To minimize the water loss influencing the mechanical behaviour, samples were coated with paraffin oil.

**Determination of gel point**

1. Prepare the hydrogels as described in Appendix B.
2. Place the mixture on the sensor (transducer 2) as soon as the cross-linker is added (Figure E1).
3. Start measurement for the five frequencies applied: 1, 1.8, 3.2, 5.6 and 10Hz at a fixed total shear stress (1Pa) of the storage modulus ($G'$), loss modulus ($G''$) and the damping factor ($\tan(\delta)$).
4. Plot $G'$, $G''$ and $\tan(\delta)$ in ordinate and the time (seconds) in axis. The gel point is the time corresponding at $G'$=$G''$ so $\tan(\delta)$=1.

![Figure E1. Schematic representation of the dynamic mechanical tester.](image)

**Self hardening study**

Dynamic frequency sweep experiments were carried out to determine the storage ($G'$) and loss ($G''$) moduli as a function of time at 37°C. The evolution of the storage ($G'$) and loss ($G''$) moduli during the gelation was recorded as a function of time for five different frequencies (a, b, c, d and e rad.s$^{-1}$) using multiwave facilities [3].

1. Prepare the hydrogels as described in Appendix B.
2. Pour the hydrogels into a 12 well-plate.
3. Incubate for one hour at 37°C before addition of medium.
4. Place a sand paper (waterproof silicon carbide paper FEPA P220 (Stuers)) on the sensor to avoid any sliding.
5. Immerse the sensor in the medium before measurement (Figure E2).
6. Search the hydrogel with the transducer 1. The hydrogel is reached when the transducer detects a force of 0.10N.
7. Apply mechanical stresses from 0.5Pa to 80Pa on the hydrogel.
8. Plot the values obtained as follows:
   a) $G' - G''$ (Pa) vs. frequency (rad.s$^{-1}$) (log scale)
   b) Normalised moduli (Pa) $G' - G''$ vs. normalised frequency
   c) Multiwave test $\rightarrow$ time dependence of the storage $G'$ and loss moduli
   d) $G' - G''$ (Pa) vs. time (minutes) (different frequencies)
   e) $G' - G''$ (Pa) vs. frequency (rad.s$^{-1}$).

Figure E2. Haake MARS (Modular Advanced Rheometer System) rheometer set up for self hardening study. The system is linked to a water-bath in order to maintain the system at 37°C.
F. Quantification of amine group by TNBSA assay

![Chemical reaction occurring during TNBSA reaction](image)

**Figure F1. Chemical reaction occurring during TNBSA reaction.** TNBSA reacts with the primary amine group of collagen to produce a chromogenic derivative.

**Protocol**
1. Prepare a standard curve with glycine in sodium bicarbonate pH 8.5 (0.1M) (H$_2$N-CH$_2$-COOH) (100nmol, 50nmol, 25nmol, 10nmol, 5nmol and 0nmol).
2. Add 250µL of 0.01% TNBSA in 0.5mL of each sample. Mix well. The dilution of the stock solution concentrated at 5% in the sodium bicarbonate buffer.
3. Incubate at 37°C for two hours to allow the reaction (Figure F1).
4. Add 250µl of 10% SDS plus 125µl of 1M HCl.
5. Measure the absorbance at 335nm.

In order to hydrolyse the hydrogel, the samples are incubated at 120°C for 15 minutes or can be autoclaved after the reaction step *i.e.* after the addition of HCl. The absorbance is measured at 335nm as for the standard curve.

G. Cell proliferation study: PicoGreen® Assay

1. Wash tissues/cells/scaffold with HBSS.

For cells:
   a) Remove the media and gently rinse the cells with HBSS.
   b) Add 250µl of DNase free water.
   c) Freeze-thaw cells three times (freeze at -80°C for 15 minutes minimum and defreeze at room temperature until it is completely defrosted).

For hydrogel or tissue:
   a) Wash the constructs with HBSS.
   b) Transfer the construct in an eppendorf, and digest it by 1mL of proteinase K at a concentration of 1mg/mL at 56°C overnight (O/N).
   c) Use the digestion product directly or freeze it at -20°C.
2. Prepare a standard curve in a 96 well plate in accordance with table G1:
   a) Make up 1X TE buffer (initial solution at 20X).
   b) Make up 2µg/mL DNA stock solution (100µg/mL DNA standard) and 50ng/mL DNA stock solution (2µg/mL DNA stock solution).

Note: Prepare the standard curve with DNase free water.

Table G1. Protocol for the preparation of a standard curve.

<table>
<thead>
<tr>
<th>Final DNA concentration (ng/mL)</th>
<th>Volume (µl)</th>
<th>Volume of 2µg/mL stock solution (µl)</th>
<th>Volume of 50ng/mL stock solution (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>50</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>100</td>
<td>90</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>95</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

4. Transfer 100µl of each sample in the 96 well plate.
5. Make up the diluted PicoGreen® solution: 5.376mL 1X TE + 27µL concentrated PicoGreen® (enough for the standard curve in triplicate and 24 samples).
6. Add 100µl of diluted PicoGreen® to each well.
7. Incubate at room temperature for two to five minutes in the dark.
8. Read the plate for fluorescence (excitation: ~480nm; emission: ~520nm).
9. Plot a graph concentration vs. the fluorescence values. Determine the concentration of DNA as a function of the standard curve.

H. Total RNA extraction using trizol technique combined with RNeasy® kit

Extraction
1. Homogenize the cells by 1mL Trizol onto the hydrogel/tissue by a tissue ruptor or by pipetting for cells onto tissue culture plastic (TCP).
2. Store homogenate for five minutes at room temperature (RT) (complete dissociation of nucleoprotein complexes)
3. Heat at 37°C for ten minutes.
4. Vortex for 15 seconds.
Separation phase

5. Add 0.2mL of chloroform per 1mL of Trizol.
7. Incubate for 15 minutes at RT.
8. Centrifuge at 12,000g max (tr/min) for 15 minutes at 4°C.

Following the centrifugation, three different phases:
- a lower red phenol-chloroform phase
- an interphase
- an aqueous phase (translucent).

Collect the clear upper aqueous phase (~ 650 µl) and add in a fresh tube.

9. Slowly, add one volume of 70% ethanol (in three equal aliquots) mixing by inversion.
10. Apply 700 µl sample from 9. to RNeasy column; centrifuge for 15 seconds at 8,000g and discard flow-through. Repeat for remaining sample.
12. Add 350 µl of RW1 buffer to the centre of column, centrifuge for 15 seconds at 8,000g and discard flow-through.
13. Add 10 µl DNase stock solution to 70 µl Buffer RDD and add the DNase incubation mix directly onto the RNeasy column. Incubate at RT for 15 minutes.
14. Add 350 µl of RW1 buffer to center of column, centrifuge for 15 seconds at 8,000g and discard flow-through.
15. Transfer column to a new 2mL collection tube. Add 500 µl RPE to the centre of column, centrifuge for 15 seconds at 8,000g and discard flow-through.
16. Add 500 µl of RPE buffer to the centre of column, centrifuge for 15 seconds at 8,000g, discard flow-through and centrifuge for a further two minutes at 8,000g.
17. Transfer the column to new 1.5mL tube, add 30 µl RNase-free water onto the column, incubate at RT for one minute and centrifuge for one minute at 8,000g.
19. Take back the 30 µl of eluate and add it again onto the column, incubate at RT for one minute and centrifuge for one minute at 8,000g. Adjust the volume of water according to the quantity of RNA to extract.
20. Measure the concentration using the nanodrop and freeze at -80°C.

RNA quantification and purity determination

1. Calibrate the spectrometer with RNase free water.
2. Measure the absorbance of the RNA at 230nm, 260nm and 280nm.

¬ 1 unit of $A_{260}$ = 40 µg/mL of RNA
Concentration of RNA sample = 40 x A260 x dilution factor = x µg/mL
Quantity of RNA = concentration x volume of sample in mL = x µg

Purity

If the ratios A260/A280 and A230/260 are superior at 1.8-1.9, a pure RNA is obtained (max of the ratio 2.2).

Note: The ratio of reading at A260/A280 provides an estimation of the purity of RNA with respect to contaminants that absorb in the UV such as protein; and the ratio at A230/A260 provides an estimation of the purity of RNA with respect to organic contaminant.

I. Protocol for reverse transcription

RNA target and primers preparation

Note 1: For the entire procedure, use sterile, nuclease-free tubes pre-chilled on ice

1. For 20µl reverse transcription reaction, mix:
   RNA Template up to 1µg
   Primers Oligo (dT)15 Primer and Random primer 0.5µg
   Nuclease free water to a final volume of 5µl

2. Incubate at 70°C for five minutes (denaturation the RNA template and the primers).

3. Quick-chill at 4°C for five minutes and hold on ice (or direct in the bath of ice).

Note 2: Keep on ice during the preparation of the reverse transcription mix.

Preparation of the reverse transcription (RT) mix

1. Prepare the RT mix as indicated in Table I1.

Table I1. Reverse transcription master mix composition.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per 1 RT (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-Free Water</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>ImProm-II™ 5X Reaction Buffer</td>
<td>4</td>
<td>1X</td>
</tr>
<tr>
<td>MgCl2, 25mM</td>
<td>2.4</td>
<td>3mM</td>
</tr>
<tr>
<td>dNTP mix (10mM)</td>
<td>1</td>
<td>0.5mM</td>
</tr>
<tr>
<td>Recombinant RNasin Ribonuclease Inhibitor (20/40U/µl)</td>
<td>1</td>
<td>1u/µl</td>
</tr>
<tr>
<td>ImProm-II™ Reverse Transcriptase</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
2. Dispense 15µl of the mix into the reaction tubes onto the template/primers mix.
3. Mix gently and run the RT program after addition of the tube onto a DNA engine according to the program described in Table I2.

**Table I2. Reverse transcription program.**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing</td>
<td>25°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Extension</td>
<td>42°C</td>
<td>60 minutes</td>
</tr>
<tr>
<td>Heat-Inactivation Reverse Transcriptase</td>
<td>70°C</td>
<td>15 minutes</td>
</tr>
</tbody>
</table>

After this step, proceed with the PCR or store at -20°C.

**J. Endpoint PCR**

1. Prepare the master mix according to the supplier directions (see Table J1)

**Table J1. PCR master mix.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/PCR (µl)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl2, 25mM</td>
<td>3</td>
<td>1.5mM</td>
</tr>
<tr>
<td>Buffer 5X</td>
<td>5</td>
<td>1X</td>
</tr>
<tr>
<td>Nucleotide Mix, 10mM</td>
<td>1</td>
<td>200µM</td>
</tr>
<tr>
<td>Forward Primer, 100pmol</td>
<td>1</td>
<td>1µM</td>
</tr>
<tr>
<td>Reverse Primer, 100pmol</td>
<td>1</td>
<td>1µM</td>
</tr>
<tr>
<td>Taq Polymerase (5u/µL)</td>
<td>0.25</td>
<td>1.25/50µL</td>
</tr>
<tr>
<td>Template DNA</td>
<td>x</td>
<td>≤0.5µg/50µL</td>
</tr>
<tr>
<td>Nuclease Free Water, Up to</td>
<td>x</td>
<td>Final volume of 50µL</td>
</tr>
</tbody>
</table>

1. Add the cDNA template in individual RNase free tubes in triplicate and add a negative control (replacement).
2. Mix well the Master Mix by pipeting and add into each well a volume to obtain a final volume of 50µL.
3. Place the tubes on the PCR thermocycler.
4. Start the run according to the program described in Table J2.

Note: The PCR program is adapted according to the primers properties.
Table J2. PCR Program for XT-I and GT-I primers.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>2 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>1 minute</td>
<td>25 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>56°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 minute 30</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>5 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Soak</td>
<td>4°C</td>
<td>Indefinite</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

5. Run the PCR products on an agarose gel 2% (see appendix K for preparation)

K. Agarose gel

For a gel at 1%,

1. Mix 1 g of agarose in 100 ml of Tris-Acetate-EDTA buffer (TAE) 1X.
2. Boil the mixture until full dissolution of the agarose.
3. Add SybrSafe (1/10,000 dilution).
4. Pour gel within a rack and wait until full polymerisation.
5. Remove the comb and load the samples. (Comb 20-wells: 13-15 µl, Comb 12-wells 1.5 mm: 20 µl)
6. Run at 90 V for 1 h and read under UV using the transilluminator.

L. Real-time PCR protocol (Sybr Green technique)

1. Prepare a dilution of cDNA template to obtain a concentration of 10 ng per well.
   The cDNA concentration should not exceed 100 ng per well for 25 µl final volume
   and the volume added 10% of the final volume.
2. Prepare the master mix according to the supplier (see Table L1)

Table L1. Real-time PCR master mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/PCR (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Quantifast SYBR</td>
<td>12.5</td>
<td>1.5mM</td>
</tr>
<tr>
<td>Green PCR Master Mix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward Primer, pmol</td>
<td>0.25</td>
<td>1 µM</td>
</tr>
<tr>
<td>Reverse Primer, pmol</td>
<td>0.25</td>
<td>1 µM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.49</td>
<td>0.00 ng/reaction</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>10.51</td>
<td>Final volume of 25 µl</td>
</tr>
</tbody>
</table>
3. Add the cDNA template in each well in triplicate and add a negative control (replace the cDNA template by nuclease free water).
4. Mix the Master Mix well by pipetting and add in each well a volume to obtain a final volume of 25µL.
5. Add an optical plastic cover on the plate.
6. Centrifuge one minute at 1,400rpm.
7. Place the plate in the machine.
8. Start the run according to the program described in Table L2.

**Table L2. Real-time PCR Program.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp. (°C)</th>
<th>Time</th>
<th>Ramp Rate</th>
<th>N° of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Step</td>
<td>50</td>
<td>2 minutes</td>
<td>Maximal/Fast Mode</td>
<td>1</td>
</tr>
<tr>
<td>Initial Activation</td>
<td>95</td>
<td>5 minutes</td>
<td>Maximal/Fast Mode</td>
<td>1</td>
</tr>
</tbody>
</table>

**Two-Step Cycling**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp. (°C)</th>
<th>Time</th>
<th>Ramp Rate</th>
<th>N° of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 seconds</td>
<td>Maximal/Fast Mode</td>
<td>40</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60</td>
<td>30 seconds</td>
<td>Mode</td>
<td></td>
</tr>
</tbody>
</table>

**Final Step**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp. (°C)</th>
<th>Time</th>
<th>Ramp Rate</th>
<th>N° of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 seconds</td>
<td>Maximal/Fast Mode</td>
<td>1</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60</td>
<td>20 seconds</td>
<td>Mode</td>
<td></td>
</tr>
<tr>
<td>Final Denaturation</td>
<td>95</td>
<td>15 seconds</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For a proper analysis, it is preferable to have a Tm around 60°C and a sequence amplified of about 100bp.

**M. Primers sequences and probes**

**Table M1. Bovine primers and probes for TaqMan real-time PCR assay (Chapter 2).**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Forward (5’-3’)</th>
<th>Primer Reverse (5’-3’)</th>
<th>Probe (5’FAM-3’TAMARA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan</td>
<td>CCA ACG AAA CCT ATG ACG TGT ACT</td>
<td>GAC CTC GTT GGC TGC CTC</td>
<td>ATG TIG CAT AGA AGA CCT CGC CCT CCA T</td>
</tr>
<tr>
<td>Procollagen 1A2</td>
<td>TGC AGT AAC TTC GTG CCT AGC A</td>
<td>CGC GTG GTC CTC TAT CTC CA</td>
<td>CAT GCC AAT CCT TAC AAG AGG CAA CTG C</td>
</tr>
<tr>
<td>Procollagen 2A1</td>
<td>AAG AAA CAC ATC TGG TTT GGA GAA A</td>
<td>TGG GAG CCA GGT TGT CAT C</td>
<td>CAA CGG TGG CTT CCA CTT CAG CTA TGG</td>
</tr>
<tr>
<td>HAS-1</td>
<td>GGC ACC CAC TGT ACC TTT</td>
<td>CGA GGT GTA CTT GGT GGC ATA G</td>
<td>CGT CAC CTC ACC AAC CGC ATG CT</td>
</tr>
</tbody>
</table>
Table M2. Bovine primers for SybrGreen real-time PCR assay (Chapter 5).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Forward (5’-3’)</th>
<th>Primer Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan</td>
<td>TCT ACC TCT ACC CCA ACC AG</td>
<td>GCA CTA CCC TCC TCT TCT TC</td>
</tr>
<tr>
<td>18 S</td>
<td>TCA ACA CGG GAA ACC TCA C</td>
<td>CGC TCC ACC AAC TAA GAA C</td>
</tr>
<tr>
<td>XT-I</td>
<td>TCC CCC AAT GAC TTC AAG CC</td>
<td>CCC GAT GAT TTC CTG ATT CAC C</td>
</tr>
<tr>
<td>GT-I</td>
<td>TTG ATG CTA CTG CTC CTC G</td>
<td>GCT CCT CCT GCT TCA TCT TG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGT TGT CTG ATC CAT GCC C</td>
<td>TGA GTG AGT AGA AGT GAG AGA G</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>ATA GCA ACA ATT CCT GGC G</td>
<td>AAG CCC TCT ATT TCC TCT CTG</td>
</tr>
</tbody>
</table>

Table M3. Human primers for SybrGreen real-time PCR assay (Chapter 5).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Forward (5’-3’)</th>
<th>Primer Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XT-I</td>
<td>CAC ACC CAA GTC CGC TCA TC</td>
<td>GCT GGT GCT TCT TCT TC</td>
</tr>
<tr>
<td>GT-I</td>
<td>TTA CTG CCT CAC AGA AGC C</td>
<td>TCA AAG TCC TGA AAA TGA ACC</td>
</tr>
</tbody>
</table>

N. Sulfated glycosaminoglycan (sGAG) detection assay

1. Prepare a stock solution of 1mg/mL chondroitin 4-sulfate in dH₂O. Store aliquots at −20°C.
2. Prepare DMMB reagent (1,9-Dimethyl-methylene blue) as follows:
   - Dissolve 16mg DMMB powder in 1L dH₂O containing 3.04g glycine and 2.37g NaCl (aluminium covered).
   - Stir overnight (room temperature) and adjust pH to 3.0 with 1M HCl.
3. Wash cell-seeded scaffolds with HBSS.
4. Add 1mL proteinase K (0.5 mg/mL) and digest overnight at 56°C.
5. Prepare standards in eppendorfs from the stock solution (step 1) to obtain concentrations of 2.5 / 1.25 / 0.625 / 0.3125 / 0.156 / 0.078µg/20µl so 125, 62.5, 31.25, 15.625, 7.8, 3.9µg/mL. Prepare standards in duplicate. The two blanks consist of PBS or complete DMEM (Dulbecco’s Modified Eagle’s Medium).

Note:
- Dilute standards with 1X PBS when measuring sGAG scaffold content.
Appendices

- Dilute standards with complete DMEM when measuring sGAG content in medium. A loss of linearity in the standard curve is then observed at concentrations above 1.25 µg/well.

6. Dispense 20 µl of standards and 20-50 µl of samples into wells of a 96-well assay plate (transparent). Samples are measured in duplicate.

7. Add 200 µl DMMB colour reagent to each well.

8. Tap the plate gently and immediately read the absorbance in a plate reader at 535nm.

9. Prepare a linear standard curve A<sub>535</sub> vs. sGAG content (µg/well) and extrapolate sGAG content of samples from their A<sub>535</sub> values.

O. Plasmid cloning: PCR amplification, A overhangs addition and TOPO TA cloning

A) Resuspension and amplification of the plasmid from Origene

1. Thaw chemically competent bacteria on ice for approximately 30 minutes.

2. Add 1-5 ng of expression plasmid per 10 µl of competent cells. Mix gently by flicking tube with the finger or by pipetting.

3. Incubate on ice for approximately 30 minutes.


5. Place on ice immediately for three to five minutes.

6. Add 250 µl of recovery medium (SOC medium), mix gently by inverting, and incubate at 37°C for 60 minutes.

7. Mix and spread 50-100 µL on Lysogeny Broth agar-Ampicillin (LB agar-AMP) plates (100 µg/mL) (according to the resistance gene carried by the plasmid) at different dilution factors (dilution at 10 and 50% and non diluted).

8. Incubate O/N at 37°C and store the remaining solution at 4°C.

9. Perform a MiniPrep isolation as described in appendix Q.

10. Check the plasmid's size on an agarose gel at 1%.

B) PCR amplification of the gene of interest

1. Digest the plasmid previously isolated with the restriction enzyme Not I as follows:
   a) Prepare the digestion mix (Table O1).
Table O1. Composition of the digestion mix by Not I.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume for 1 RT (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Template</td>
<td>1 µg</td>
</tr>
<tr>
<td>Not I Restriction Enzyme</td>
<td>0.5</td>
</tr>
<tr>
<td>Acetylated BSA</td>
<td>0.2</td>
</tr>
<tr>
<td>Buffer</td>
<td>2</td>
</tr>
<tr>
<td>Water, up to</td>
<td>20</td>
</tr>
</tbody>
</table>

b) Incubate for 1h at 37°C.

c) Run the digestion product on an agarose gel of 1%. Two bands at 4kb would be observed.

2. Extract the bands from the agarose gel and purify as follows:
   a) Add five volumes of buffer PB1 to one volume of gel.
   b) Centrifuge for one minute at 17,000g to bind the DNA to the column.
   c) Add 0.75mL of buffer PE to the column to wash.
   d) Centrifuge for one minute at 17,000g. Discard the flow-through.
   e) Centrifuge for an additional minute to remove the ethanol traces.
   f) Elute DNA with 20µL of water.

3. Amplify the gene of interest i.e. glucuronyltransferase I by PCR.
   a) Prepare the PCR mix as indicated in Table O2.

Table O2. Long amplification PCR master mix composition.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume for 1 RT (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extensor PCR Master Mix</td>
<td>25</td>
</tr>
<tr>
<td>Primer Forward, Xba-I</td>
<td>1</td>
</tr>
<tr>
<td>Primer Reverse, Xba-I</td>
<td>1</td>
</tr>
<tr>
<td>Water</td>
<td>20.8</td>
</tr>
<tr>
<td>DNA Template</td>
<td>2.2</td>
</tr>
</tbody>
</table>

a) Prepare a negative control without cDNA (replace the volume of cDNA by nuclease free water in that case).

b) Distribute the mix in tube.

c) Add the cDNA template and start the program as described in Table O3.
Appendices

Table O3. PCR program for long amplification of GT-I.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial denaturation</strong></td>
<td>94°C</td>
<td>2 minutes</td>
<td>1</td>
</tr>
<tr>
<td><strong>Initial amplification step</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>10 seconds</td>
<td>10</td>
</tr>
<tr>
<td>Annealing</td>
<td>68°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>68°C</td>
<td>4 minutes</td>
<td></td>
</tr>
<tr>
<td><strong>Long amplification step</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>10 seconds</td>
<td>15 cycles +</td>
</tr>
<tr>
<td>Annealing</td>
<td>68°C</td>
<td>30 seconds</td>
<td>10s/cycle</td>
</tr>
<tr>
<td>Extension</td>
<td>68°C</td>
<td>4 minutes</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>68°C</td>
<td>7 minutes</td>
<td>1</td>
</tr>
</tbody>
</table>

C) A overhangs addition
1. After extraction from gel, place the vials on ice.
2. Add 5µl of 5X GoTaq PCR buffer, 1µl of dNTP and a 0.5µl of GoTaq polymerase per tube.
3. Mix well.
4. Incubate at 72°C for eight to ten minutes.
5. Place the vials on ice.

The DNA amplification product is now ready for ligation into pcDNA™ 3.3-TOPO®.

D) TOPO TA cloning
1. Mix the reaction gently and incubate for five minutes at room temperature (22-23°C) (Table O4).

Note: For most applications, five minutes will yield a sufficient number of colonies for analysis. The length of the TOPO TA cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For larger PCR products (>1kb), increasing the reaction time may yield more colonies.

Table O4. TOPO TA cloning reaction mix for a final volume of 6µl.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume for reaction (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product/digestion product</td>
<td>2</td>
</tr>
<tr>
<td>Salt solution</td>
<td>1</td>
</tr>
<tr>
<td>Sterile water</td>
<td>2</td>
</tr>
<tr>
<td>TOPO TA vector</td>
<td>1</td>
</tr>
</tbody>
</table>
2. Prepare a negative control without insert (replace the volume of cDNA by nuclease free water in that case), and a positive control with the insert provided by the supplier.

3. Place the reaction on ice and proceed to transforming using heat shock competent *Escherichia coli*.

4. Add 1-5ng of expression plasmid 10µl of competent cells. Mix by flicking the tube with the finger or by pipetting gently.

5. Incubate on ice for approximately 30 minutes.


7. Place on ice immediately for three to five minutes.

8. Add 250µl of recovery medium (SOC medium), mix gently by inverting, and incubate at 37°C for 60 minutes.

9. Mix and spread 50-100µL on Lysogeny Broth agar-Ampicillin (LB agar-AMP) plates (100µg/mL) (according to the resistance gene carried by the plasmid) at different dilution factors (dilution at 10 and 50% and non diluted).

10. Incubate O/N at 37°C and store the remaining solution at 4°C.

**Screening of the bacteria colonies**

After step 10, colonies are screened before amplification in order to determine which colonies contain the insert of interest.

1. Pick ten colonies from the culture obtained in step 10.

2. Add one tip in 10µL of nuclease free water for PCR analysis and one tip in SOC medium.

3. Incubate in SOC medium for one hour at 37°C under agitation and keep the suspension at 4°C until further analysis.

4. Perform a PCR using human GT-I primers (Table M1 Appendix M) according to Appendix J and run the product on an agarose gel at 1% for one hour at 80V (Figure O1).

![Figure O1. Electrophoresis of GT-I amplicons for screening of GT-I positive clones.](image)

The positive clones were annotated from C1 to C5. Each of the positive
clones was digested with Nco-I to check plasmid size and sent for sequencing to validate the insert.

5. Perform a digestion of the plasmid with NcoI restriction enzyme on the GT-I positive clones. Four bands were obtained: 3,500bp, 2,500bp, 2,000bp and 1,000bp (Figure O2).

6. Send to sequencing the positive clones and blast the results against the human genomic plus transcript database.

Figure O2. Electrophoresis of the digested products of the positive GT-I clones by NcoI. (P) Non digested plasmid (C) Digested plasmid.

P. Homology of XT-I and GT-I in human and bovine.

mRNA and protein sequences from *Bos Taurus* and *Homo sapiens* (Tables P1 and P2) were aligned against each other using the basic local alignment tool (BLAST®, NCBI, Bethesda, USA). For the mRNA homologies, the two sequences were aligned with nucleotide blast (blastn) for the alignment of two sequences with the highly similar sequences algorithm (Megablast). For the protein homologies, the two sequences were aligned using protein blast (blastp) with alignment if two sequences with blastp algorithm (protein-protein blast).

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Homo sapiens NM_022166.3</th>
<th>Bos taurus XM_002698037.1</th>
<th>% Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>NP_071449.1</td>
<td>DAA154492.1</td>
<td>94%</td>
</tr>
</tbody>
</table>
Table P2. Percentage of homology between human and bovine GT-I.

<table>
<thead>
<tr>
<th></th>
<th>Homo sapiens</th>
<th>Bos taurus</th>
<th>% Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA</td>
<td>NM_012200.3</td>
<td>NM_205805.3</td>
<td>88%</td>
</tr>
<tr>
<td>Protein</td>
<td>NP_036332.2</td>
<td>NP_991374.2</td>
<td>96%</td>
</tr>
</tbody>
</table>

Q. **Plasmid amplification**

A) **Plasmid recovery**

1. Add 100\(\mu\)l of nuclease free water to obtain a final concentration of 100ng/\(\mu\)l.
2. Close the tube and allow to settle O/N at 4°C (or allow to settle for 10min at RT).
3. Mix well and quick spin to concentrate the liquid in the bottom. Store at -20°C.

B) **Amplification plasmid into E. Coli (competent bacteria)**

Transformation

1. Thaw chemically competent bacteria on ice for approximately 30min.
2. Add 1-5ng of expression plasmid to 10\(\mu\)l of competent cells. Mix by flicking tube with the finger or by pipetting gently.
3. Incubate on ice for approximately 30 minutes.
5. Place on ice immediately for three to five minutes.
6. Add 250\(\mu\)l of recovery medium (SOC medium), mix gently by inverting, and incubate at 37°C for 60 minutes.
7. Mix and spread 50-100\(\mu\)L on Lysogeny Broth agar-Ampicillin (LB agar-AMP) plates (100\(\mu\)g/mL) (according to the resistance gene carried by the plasmid) at different dilution factors (dilution at 10 and 50% and non diluted).
8. Incubate O/N at 37°C and store the remaining solution at 4°C.

MiniPrep culture after eight hours

1. Make a starter culture by picking up one colony per 5-10mL of LB-AMP (AMP at 200\(\mu\)g/mL).
2. Let the culture grow for 16h at 37°C under agitation.

C) **Purification of the plasmid by miniPrep Qiagen**

Principle of Qiagen miniPrep
The process used for the plasmid extraction and preparation is based on an alkaline lysis of bacterial cells followed by the adsorption of DNA onto the silica column in the presence of a high concentration of salts. Three basic steps are followed: (1) preparation and clearing of a bacterial lysate, (2) adsorption of DNA onto the QIAprep membrane, (3) washing step and elution of plasmid DNA.

1. Prepare the different buffers.
   - Add the provided RNase A solution to buffer P1, mix and store at 2-8°C.
   - Add ethanol (96-100%) to the buffer PE (volume indicated on the bottle).
   - Check buffers P2 and N3 for the presence of salt precipitation. If it is present, redissolve the salts at 37°C.

2. Grow bacterial cultures in tubes or flasks according to the volume of the culture.

3. Harvest the bacterial cells by centrifugation at 8,000rpm for 3 minutes at RT in eppendorf or at 5,400rpm for 10 minutes at 4°C in 15mL tubes.

4. Resuspend pelleted bacterial cells in 250µl of buffer P1 and transfer to an eppendorf. No cell clumps should be visible after resuspension of the pellet.

5. Add 250µl buffer P2 and mix thoroughly by inverting the tube 4-6 times. Do not leave the solution more than five minutes in contact with the DNA. Do not vortex.

6. Add 350µl buffer N3 and mix immediately and thoroughly by inverting the tube four to six times.

7. Centrifuge for ten minutes at 13,000rpm. A compact pellet will be obtained.

8. Apply the supernatant to the QIAprep centrifugal column.

9. Centrifuge for 30-60 seconds and discard flow-through.

10. Wash the QIAprep centrifugal column by adding 0.5mL buffer PB and centrifuge for 30-60 seconds. Discard the flow-through. This step allows the elimination of the traces of nuclease activity into the pellet.

11. Wash QIAprep centrifugal column by adding 0.75mL buffer PE and centrifuging for 30-60 seconds.

12. Discard the flow-through and centrifuge for an additional minute to remove residual wash buffer.

13. Place the QIAprep column in a clean 1.5mL eppendorf. To elute DNA, add 50µl of buffer EB (10mM Tris-HCl, pH8.5) or water to the center of each QIAprep®
centrifugal column, allow it to stand for one minute and centrifuge for one minute.

R. **Chondrogenic differentiation of ADSCs**

1. After extraction, culture rabbit ADSCs with complete medium *i.e.* DMEM supplemented with 10% FBS and 1% P/S.
2. At passage three, cells are trypsinised and counted.
3. Wash cells with DMEM.
4. Centrifuge for five minutes at 1,200rpm. Resuspend the cells in the differentiation medium.
5. 1,000,000 cells were added in 15mL tube and centrifuged for five minutes at 1,200rpm.
6. Leave the lid of the tubes loose and incubate the pellets at 37°C for 21 days. Change the medium every two to three days.

**Chondrogenic differentiation medium**

Stock solution

- DMEM, 1% P/S; 1% L-Glut
- Insulin-Transferrin-Selenite (ITS) 6.25µg/mL

This stock solution was kept at 4°C for the duration of the differentiation assay after preparation. Before each medium change, supplement the medium with TGF-β at 10ng/mL and 50nM ascorbic acid, final concentration.

Note: prepare the ascorbic acid solution from a solution at 1M to a solution of 1µM just before use as described below:

- Solution at 1M
  0.99g ascorbic acid is resuspended in 5mL of DMEM, 1% P/S.
- Solution at 1mM
  Add 5µl of the solution at 1M in 5mL of DMEM, 1% P/S.
- Solution at 1µM
  Add 5µl of 1mM solution in 5mL of DMEM, 1% P/S supplemented with ITS.

S. **Adipogenic differentiation**

1. Seed rabbit ADSCs at a density of 3x10⁴ cells/cm² in a six-well plate. The surface of a well is of 9.6cm² so 288,000 cells were seeded per well.
2. Incubate in complete medium \textit{i.e.} DMEM supplemented with 10\%FBS and 1\% P/S for 24 hours.

3. After 24 hours, change the medium for the differentiation medium (see below for the composition).

4. Change medium twice a week for 14 days, alternate day two induction, day four resting medium.

\textbf{Composition of adipogenic induction differentiation medium}

- DMEM
- 10\%FBS
- 1\%P/S
- Dissolve insulin 10\(\mu\text{g/mL} \) at pH2-3. Aliquot and frozen at -20\(^\circ\text{C}\).
- Dissolve the isobutylmethylxanthine (IBMX) at 0.5mM by heating in boiling water. Aliquot and freeze at -20\(^\circ\text{C}\).
- Dissolve dexamethasone 1\(\mu\text{M} \) in ethanol. Aliquot and freeze at -20\(^\circ\text{C}\).
- Indomethacine 200\(\mu\text{M} \)

\textbf{Composition of adipogenic resting differentiation medium}

The resting medium consists of complete medium supplemented with insulin (no IBMX, indomethacin and dexamethasone).

- DMEM
- 10\% FBS
- 1\% P/S
- Insulin 10\(\mu\text{g/mL} \)

\textbf{T. Osteogenic differentiation}

1. Seed rabbit ADSCs at a density of 1x10\(^4\) cells/cm\(^2\) in a six well-plate.
2. Incubate in complete medium \textit{i.e.} DMEM supplemented with 10\%FBS and 1\%P/S for 24 hours.
3. After 24 hours, change the medium for the differentiation medium (see below for the composition).
4. Change medium twice a week for 28 days.

\textbf{Composition of osteogenic induction differentiation medium}

- DMEM
- 10\% FBS
- 1\%P/S
- β-Glycerophosphate 10mM
- Vitamin D₃ 10nM. Dissolution in ethanol. Aliquot and freeze at -20°C.
- Ascorbic acid 0.05mM

U. **Hematoxylin and Eosin staining**

For cryosections:

1. Place the slides in a bath of tap water Dip
2. Haematoxylin 8 minutes
3. Transfer the slides under running water 8 minutes
4. Bath of 50% ethanol Dip
5. Bath of 70% ethanol Dip
6. Bath of 80% ethanol Dip
7. Bath of 96% ethanol Dip
8. Eosin bath 2 minutes
9. Bath of 96% ethanol Dip
10. Bath of 96% ethanol Dip
11. Bath of 100% ethanol Dip
12. Bath of 100% ethanol Dip
13. Bath of 100% ethanol Dip
14. Bath of 100% ethanol Dip

From this step onwards, all steps are performed under fumehood:

15. Bath of 50% ethanol/50% xylene 4 minutes
16. Bath of Xylene 4 minutes
17. Bath of Xylene 4 minutes
18. Clean the coverslip with xylene
19. Add mountain solution (DPX) on the slide
20. Cover with coverslip
21. Allow to dry overnight

V. **Adipose tissue collection and hydrogel injection in an in vivo rabbit model.**

A) **Adipose tissue collection for ADSCs isolation**

During the time of surgery, capnograph, EtCO₂, heart rate, breath rate and SpO₂ are monitored.
1. Inject intramuscularly 200µg/kg of medetomidine and 20mg/kg of ketamine.
2. After 10-15 minutes, perform an endotracheal intubation of the animal.
3. After anesthesia, shave the surgical site to remove the hair.
4. Wash the surgical area with betadine and place the surgical cloth (Figure V1 (A)).
5. Maintain the animal under perfusion with 0.9% NaCl (10mL/kg/h) and inhalation of 1 to 2.5% isoflurane during the surgery according to the reaction of the rabbit.
6. Inject 0.1mg/kg of morphine intravenously five minutes before incision and repeat the injection during the surgery depending on the reaction of the rabbit.
7. Make an incision on the lower midline area through the outer dermal layers.
8. Harvest the adipose tissue present on both side of this line and collect the tissue in transport medium (Figure V1 (C)).
9. Suture the incision.

**Figure V1. Adipose tissue collection** (A) Field of surgery in the lower midline area (B) Adipose tissue sampling (C) Adipose tissue collection in transport medium (D) Suture of the incision.

10. Inject metacam subcutaneously for the recovery of the animal. Fentanyl patches (25µg/h) were placed on the sides of the incision at the end of the surgery as painkiller.
11. Proceed to the ADSCs isolation as described Appendix C.
Appendices

B) Hydrogel injection in rabbit IVDs.

During the time of surgery, capnograph, EtCO$_2$, heart rate, breath rate and SpO$_2$ were monitored.

1. Inject in intramuscular 200µg/kg of medetomidine and 20mg/kg of ketamine.
2. After 10-15 minutes, perform an endotracheal intubation of the animal.
3. After anesthesia, shave the surgical site to remove the hair.
4. Wash the surgical area with betadine and put the surgical cloth in place (Figure V2 (A)).
5. Maintain the animal under perfusion with 0.9% NaCl (10mL/kg/h) and inhalation of 1 to 2.5% isoflurane during the surgery according to the reaction of the rabbit.
6. Inject 0.1mg/kg of morphine intravenously five minutes before incision and repeat the injection during the surgery depending on the reaction of the rabbit.
7. Make an incision on the midline area of the abdominal cavity through the outer dermal and muscles layers of the abdominal cavity (Figure V2 (A)).
8. Empty the abdominal cavity on cloth pads soaked in saline solution.
9. Place the needle holder at the level of the IVD of interest.
10. Prepare the hydrogel as described appendix A.
11. Inject the hydrogel using a luer lock Hamilton syringe to secure the needle and the injection (Figure V2 (C)). According to the position, different mixtures were injected i.e. sham, cells alone, hydrogel alone and hydrogel plus cells (Figure V3).
12. Wait for one minute before removing the needle from the disc.
13. Replace the organs within the abdominal cavity.
14. Suture the incision.
15. Keep the animal under anaesthesia for a further 30 minutes before recovery to allow the hydrogel to settle (avoid any movement from the animal).
16. Inject metacam subcutaneously for the recovery of the animal. Place fentanyl patches (25µg/h) on the sides of the incision at the end of the surgery as painkiller.
17. Monitor the animals for three months before sacrifice. Measure the disc height and hydration by X-Ray and MRI each month as described in appendices W and X.
Figure V2. Injection within rabbit IVDs (A) Midline incision of the abdominal cavity (B) X-Ray for the location of the injection site (C) Injection (D) Suture of the incision.

Figure V3. Schematic representation of rabbit lumbar spine and the injection points of the experiment groups within the IVD. L1-L2 IVD: control, L2-L3: sham, L3-L4: cell alone, L4-L5: hydrogel + cells, L5-L6: hydrogel alone.

W. Measurement of disc height

X-rays were performed at the veterinary school at Nantes, France. The software used for analysis is the OsiriX 3.9.2 Imaging Software.

After X-Ray, check the spine anatomy on the front snapshot before analysis:
1. Check sacralisation of L6-L7 and L7-S1.
2. Check for any lumbosacrum formation.
3. Check if the iliac crests are properly located at the level of L6-L7 IVD. On the profile snapshot, the edge of the iliac crests should be at the level of L6-L7 IVD.

After having checked these different points, the disc height is measured (Figure W1).
1. Measure of the diameter of the disc.
4. Draw a line on each edge of the vertebrae.
5. Draw a line between these two lines. The length of this line gives the diameter of the disc.
6. Draw with the pencil tool a circle at the level of the cartilage endplates (white signal). If there are two signals, place the circle between the both signals to avoid any bias.

![Figure W1. X-Ray for disc height determination.](image)

Disc height calculation:

\[
\text{Disc height} = \frac{\text{Disc surface}}{\text{Diameter}}
\]

X. Disc hydration measurement

MRI were performed at the veterinary school ONIRIS at Nantes, France. The software used for analysis is the OsiriX 3.9.2 Imaging Software.

After analysis of the X-ray to check for any potential deformation, the signal of MRI is determined (Figure X1).
1. Select the images in MRI T2 sagittals.
2. Start to count the vertebrae from S1 and skip the evaluation of the disc L7-S1.
3. Amplify the image at the level of the disc.
4. Choose the pencil tool and surround the disc without including the end-plate in hyper signal which would interfere with the real signal (surround in the black space).
5. Note the mean value of the signal.
6. Balance the signal with the medullar signal. For this, select a square of 0.20 to 0.25cm² in front of the disc. Be careful not to select this square in the cerebrospinal fluid. The medullar signal should be constant on the image. Take two images if it is not constant.

7. **Figure X1. MRI for determination of hydration.** (A) Transversal MRI of rabbit spine. (B) Enlargement of the transversal MRI with measurements of the disc signal and medullar signal.

Calculation of hydration level

\[
\text{Hydration level} = \frac{\text{Disc signal}}{\text{Medullar signal}}
\]

Y. **Safranin O / fast green FCF staining procedure**

This staining is performed to detect GAGs within the tissue. The safranin O stains the proteoglycans in red. The Fast Green FCF stains collagen in green. Nuclei, stained with the hematoxyclin, appear in purple.

1. Prepare the following solutions:
   - Weigert's Iron Hematoxylin Solution
Stock Solution A:
Hematoxylin 1g
95% Ethanol 100mL

Stock Solution B:
29% Ferric chloride in water 4mL
Distilled H₂O 95mL
Hydrochloric acid 1mL

Mix equal parts of stock solution A and B (stable for four weeks at room temperature).

Safranin O Stain (0.1% w/v)
Safranin O 0.1g
Distilled H₂O 100mL

Fast Green FCF Stain (0.001% w/v)
Fast green FCF 0.01g
Distilled H₂O 1000mL

2. Wash samples three times with 1X PBS.

**Table Y1. Dehydration steps**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>96% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>96% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>2 minutes</td>
</tr>
</tbody>
</table>

3. Stain with Weigert’s iron hematoxylin solution for 12 minutes.

4. Dip five times in acid alcohol (1mL 37% hydrochloric acid / 100mL 70% ethanol).

5. Place in running tap water for eight minutes.

6. Stain with Fast Green FCF solution for five minutes.

7. Dip ten times in 1% acetic acid solution.

8. Stain with Safranin O solution for five minutes.

9. Dehydrate the samples in the following series of solutions (Table Y1).
10. Keep samples in xylene bath and one-by-one, place DPX solution on slides and press coverslips on top.

11. Let the samples dry overnight to allow DPX to cure. Before viewing the samples under microscope, clean the slides.

Z. Oil Red O assay

Oil Red O staining is performed to detect the lipid production by adipogenic cells. After induction of ADSCs in adipogenic pathway, lipids are stained and semi-quantified. Lipids appear red and nuclei appear blue.

Note 1: All procedures involving formalin must be performed under a fume hood. Make sure not to leave the cells dry for more than 30 seconds throughout this assay.

Note 2: Gently add and remove all reagents indirectly to the monolayer to avoid cell detachment. For example, drip the reagent down the side of the culture plate.

A) Fixation of adipogenic cultures
1. Remove cultures from incubator and place in a fume hood.
2. Remove media from the wells, always first from the control.
3. Gently rinse the plate with 2mL 1X PBS.
4. Remove the PBS and add 2mL 10% formalin. Incubate for 30-60 minutes at RT.

B) Preparation of Oil Red O staining solution
1. Prepare a stock solution by weighing out 300mg of Oil Red O powder and adding this to 100mL 99% isopropanol. This stock solution is stable one year from its date of manufacture.
2. In the fume hood, mix three parts of Oil Red O stock solution with two parts distilled water. Incubate for ten minutes at RT. This working solution is only stable for two hours.
3. Place a piece of Whatman filter paper in a funnel above a vessel.
4. Filter the Oil Red O working solution completely through the filter funnel.

C) Staining of adipogenic differentiated cultures
1. Remove the formalin from each well and discard it according to the chemical waste disposal procedure.
2. Gently rinse each well with 2mL distilled water. Remove and discard as formalin waste.
3. Add 2mL 60% isopropanol to each well and allow it to sit for five minutes.
4. Remove isopropanol and add 2mL Oil Red O working solution to each well, be sure to cover the entire monolayer. Incubate for five minutes at RT.
5. Remove Oil Red O and rinse cultures with RT tap water until the water rinses off clear.
6. Add 2mL hematoxylin stain into each well. Make sure to cover the entire monolayer. Incubate one minute at RT.
7. Remove the hematoxylin and rinse the cultures with RT tap water until the water rinses off clear.
8. Add 2mL tap water to each well and view on a phase contrast microscope.

**D) Semi-quantification of lipids**
1. Add 1mL of isopropanol (100%).
2. Maintain under agitation for five minutes.
3. Transfer the dye in eppendorfs.
4. Centrifuge two minutes at 8,000rpm (elimination of debris).
5. Read at 490nm on the plate reader.

**AA. Set up for organ culture experiment**
From two year-old bovine tails:
1. Cut the discs at both sides of the vertebrae.
2. Remove the bony part from the cartilage endplate by scraping as much as possible.
3. Wash disc with 0.9% NaCl (Buffer 1) for five minutes.
4. Wash with betadine for five minutes.
5. Wash with HBSS containing 55mM trisodium citrate and 50µg/mL gentamicin (Buffer 2) for 10-15 minutes.
6. Wash overnight disc with DMEM supplemented with 5% FBS and 50µg/mL gentamicin and 20mM trisodium citrate at 37°C (Medium 1).
7. Culture the disc in DMEM supplemented with 10% FBS and 25µg/mL of ascorbic acid and 1%P/S in a six well-plate.

**Media composition:**
- NaCl buffer: 9g NaCl in 1L dH2O
- HBSS buffer: HBSS with 55mM trisodium citrate and 50µg/mL gentamicin
- Medium 1: DMEM with 5%FBS and 50µg/mL gentamicin and 20mM trisodium sulfate
BB. Terminal Deoxynucleotidyl Transferase dUTP nick end labeling (TUNEL) assay

For cryo-sectioned cut,

1. Allow the slides to dry for 15 minutes at RT.
2. Wash them three times for five minutes with 1X PBS.
3. Incubate for ten minutes with protease K at 20µg/mL (30U/mg) (Sigma-Aldrich, Dublin, Ireland) at 37°C.
4. Rinse the slides three times with 1X PBS.
5. Block the slides with BSA 2% for one hour at RT.
6. Rinse three times with 1X PBS.
7. Incubate with the TUNEL reaction mixture (50µl) (See below for reaction mixture composition).
8. Incubate for 1h at 37°C.
9. Wash the slides three times with 1X PBS for five minutes.
10. Incubate with Extravidin®-TRITC conjugated diluted at 1/1,000 for 1h at RT.
11. Rinse slides with PBS 1X three times, for five minutes each.
12. Counterstain with DAPI (1/2,000) for ten minutes.
13. Mount and cure the slides overnight before reading on an inverted epifluorescent microscope (Olympus IX81, Mason Technologies, Dublin, Ireland) (excitation: 541, emission: 572).

Controls for the assay:

Negative control: Mixture without TdT.

Positive control: Digest the tissue with DNase I solution at 30,000 units for 30 minutes at 37°C. Wash three times with 1X PBS, five minutes each after step 5. Then, proceed as described from step 6.

Negative control secondary: Incubate the sample with Extravidin®-TRITC conjugated alone.

Note: The controls are essential in this test for its validation.
CC. Lectin staining

1. Dry the slides for 15 minutes at room temperature (RT) before washing.
2. Wash the tissues three times for five minutes each with Tris buffer saline, pH 7.2 (TBS; 20 mM Tris-HCl, 100 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$).
3. Block the tissue with 2% periodate-treated BSA in TBS for 1 hour at RT.
4. Wash the tissues three times for five minutes each with TBS.
5. Incubate with the fluorescently-labelled lectin in TBS for one hour (Table 3.1 Chapter 3 for lectins and concentrations). Inhibitory controls were carried out in parallel to verify that the lectin binding was glycan-mediated [4, 5]. The lectins were pre-incubated in 100mM of the appropriate haptenic sugar at RT for one hour (Table 3.1 chapter 3 for haptenic sugar).
6. Wash the tissues three times for five minutes each with TBS with 0.05% Triton X-100 (TBS-T).
7. Incubate with DAPI (1 in 2,500 dilution in PBS) for 20 minutes.
8. Wash the tissues three times for five minutes each with TBS with 0.05% Triton X-100 (TBS-T).
9. Mount with ProLong Gold® antifade and cure the slide at 4°C 24h before reading.

DD. Immunohistochemistry

Immunohistochemistry was performed for different antigens: C6S (anti-chondroitin 6-sulfate antibody), C6S and C4S (anti-CS-56 antibody), Le$^b$ (anti-Le$^b$ antibody) (Table 3.1 chapter 3), XT-I and GT-I.

A) Detection of chondroitin-6-sulfate

1. Dry the slides for 15 minutes at room temperature (RT) before washing.
2. Wash the slides three times with 1X PBS.
3. Digest the tissues with 0.25U/mL of chondroitinase ABC in PBS for 30 minutes.
4. Wash the slides three times with 1X PBS.
5. Block the slides with 5% goat serum for one hour.
6. Incubate the primary antibody diluted in 0.1% goat serum at 1/200 overnight at 4°C. Negative control sections were incubated without primary antibody.
7. Wash five times with 1X PBS with 0.05% Tween 20 for five minutes each.
8. Incubate for 1h with the secondary antibody (AlexaFluor 488 donkey anti-
mouse, Invitrogen, Ireland) diluted at 1/1,000 at RT.
9. Wash five times with 1X PBS with 0.05% Tween 20 for five minutes each.
10. Counter stain with DAPI diluted at 1/2,500 in 1X PBS (Invitrogen, Ireland)
    for ten minutes at RT.
11. Wash three times with 1X PBS with 0.05% Tween 20 for five minutes each.
12. Mount with ProLong Gold antifade and cure the slide at 4°C 24h before
    reading.

B) Detection of Le$^b$, chondroitin-6-sulfate and chondroitin-4-sulfate by
immunohistochemistry
1. Dry the slides for 15 minutes at RT before washing.
2. Wash the slides three times with 1X PBS.
3. Block the slides with 5% goat serum for one hour.
4. Incubate the primary antibody diluted in 0.1% goat serum at 1/200 overnight
   at 4°C. Incubate the negative control sections without primary antibody.
5. Wash five times with 1X PBS with 0.05% Tween 20 for five minutes each.
6. Incubate for 1h with the secondary antibody (AlexaFluor® 488 donkey anti-
mouse, Invitrogen, Ireland) diluted at 1/1,000 at RT.
7. Wash five times with 1X PBS with 0.05% Tween 20 for five minutes each.
8. Counter stain with DAPI diluted at 1/2,500 in 1X PBS (Invitrogen, Ireland)
    for ten minutes at RT.
9. Wash three times with 1X PBS with 0.05% Tween 20 for five minutes each.
10. Mount with ProLong Gold® antifade and cure the slide at 4°C 24h before
    reading.

C) XT-I and GT-I detection
1. Dry the slides for 15 minutes at room temperature (RT) before washing.
2. Wash the slides three times with 1X PBS.
3. Digest with proteinase K at 20µg/mL (30U/mg) (Sigma-Aldrich, Dublin,
   Ireland) for five minutes at 37°C.
4. Wash the slides three times with 1X PBS.
5. Block the slides with 5% goat serum for one hour.
6. Incubate the primary antibody diluted in 0.1% goat serum at 1/200 overnight
   at 4°C. Incubate the negative control sections without primary antibody.
7. Wash five times with 1X PBS with 0.05% Tween 20 for five minutes each.
8. Incubate for 1h with the secondary antibody (AlexaFluor® 488 donkey anti-
mouse, Invitrogen, Ireland) diluted at 1/1,000 at RT.
9. Wash five times with 1X PBS with 0.05% Tween 20 for five minutes each.
10. Counter stain with DAPI diluted at 1/2,500 in 1X PBS (Invitrogen, Ireland) for ten minutes at RT.
11. Wash three times with 1X PBS with 0.05% Tween 20 for five minutes each.
12. Mount with ProLong® Gold antifade and cure the slide at 4°C 24h before reading.

EE. Quantification of lectin and antibody binding using image analysis
1. Open image in Image J.
2. Surround the cells with circle tool of ImageJ or select an area around 150 for the measurement of the extracellular matrix.
3. Go to plugins and select analysis then measure and set label
4. Press m to perform the measurement.
5. Select in the measurement window: area/mean/Stddev/Min/Max/Median. For this, go to results, select measurements and select Area/Mean/Stddev/Min/Max/Median.

The measurement is performed on five independent cells and five independent extracellular matrix areas. All data were reported to the area so the size of the area can vary between the different images. However, a constant area was preferred (around 150).
6. Save the measurement table.

FF. Preparation and derivatisation of disaccharide for HPLC analysis

Disaccharide preparation
1. Thaw required samples and chondroitinase ABC (ChABC) on ice.
2. Wash 3kDa MWCO centrifugal filter by 500µl HPLC grade water. Centrifuge at 15,000rpm to get >400µL of water filtered.
3. Remove the excess of water.
4. Place the tube in a fresh eppendorf tube.
5. Place 100µL of thawed samples and 100µL of HPLC grade water onto the column.
6. Filter samples by centrifugation for 15 minutes at 4°C and 15,000g to obtain a solution of 50µL.
7. Use a 10µL or 200µL pipette tip to gently aspirate retentate from the sample chamber.
8. Add 390µL of 1X digestion buffer (Tris-HCl 50mM; sodium acetate 60mM) and 10µL of ChABC to each sample.
9. Place the samples at 37°C for 3h under shaking at 300rpm.
10. Wash another set of filters as previously described (Steps 2-3).
11. Centrifuge the remaining liquid into the bottom of the tube (maximum speed at 4°C for one minute).
12. Immediately transfer the digested sample into the clean filter.
13. Filter samples by centrifugation for 15 minutes at 4°C 15,000rpm.
14. Place tubes in the vacuum centrifuge to dry for approximately 2h or freeze dry them.
15. Store samples until HPLC analysis.

Disaccharide derivatisation
1. Add the sample disaccharide or Δ disaccharide reference standard (~1nmol each) to individual 0.5mL centrifuge tubes.
2. Freeze dry or vacuum concentrate the samples.
3. Transfer 10µL BODIPY stock solution to the bottom of each tube and briefly spin the tube at 10,000rpm in a microcentrifuge.
4. Evaporate the methanol using a speed vacuum concentrator at 36°C for 30min. *Protect the tubes from light with foil. Depending on the samples, the volume of fluorescence labeling solution and BODIPY can be adjusted if the ratios are kept constant.*
5. Add 5µL of fluorescence labeling solution to each of the reaction mixtures and resolubilise the sample and fluorophore. Briefly spin the tubes at 10,000rpm. *It is really important to properly resuspend at the bottom of the tube and through solubilisation by repeated up and down pipetting before centrifugation.*
6. Incubate mixture at RT for 4h (make sure they are protected from the light).
7. Carefully add 5µL sodium borohydride reducing solution onto the side of each tube to prevent contact with the mixture.
8. Cap the tubes and then gently tab the tubes onto a hard surface to allow mixing. The reaction mixture will bubble vigorously. In this step, the imine bound (between saccharide and fluorophore) will be reduced to a more stable amine.

9. Incubate the reduced reaction mixture at RT for 30 minutes. Make sure that it is protected from the light.

10. Flash freeze samples in liquid nitrogen.

11. Lyophilise all samples (Samples can be kept for 3 months at -80°C after this step).

12. Resuspend the samples in 10µL of fluorescent sample reconstitution buffer (Samples can be kept at -80°C for one month after this step). The volume changes according to the samples.

**Bodipy**
- Dissolve 5mg BODIPY in 1mL methanol.
- Transfer to a suitable storage container to prevent evaporation.
- Pre chill tubes on ice and conduct everything on ice.
- Store at -20°C.

**Fluorescence labelling solution:** Prepare just before use to prevent water absorption 17:3 DMSO:ethanoic acid; 300µl of glacial acetic acid + 1.7mL of anhydrous DMSO

**Sodium borohydride reducing solution:** 1M NaBH₄
- 38mg in a 1.5mL centrifuge tube.
- Resuspend in 1mL of HPLC water.

**Fluorescent sample reconstitution buffer**
vol:vol DMSO:water
750µl of DMSO and 750µl of water. Stock at -20°C prior to use.
II. Supplementary information
Investigating the Performance of the Type II Collagen Hyaluronic Acid in an In Vivo Rabbit Model: a Pilot Study

Introduction

In chapter 2, a type II collagen/HA hydrogel was developed as a carrier of ADSCs for NP regeneration. However, its effectiveness as a cell carrier in vivo was not tested in this chapter. Rabbit models, widely used in the literature, are described as valuable because of their cost-effectiveness and their degeneration/ageing process which follows an alteration of PG metabolism similar to that of human discs [6]. Here, a spontaneous model was preferred to an induced degenerated model because of its similarity to that of human degenerative disc diseases.

Several in vivo studies have reported the evidence of the differentiation of stem cells towards a NP cell-like phenotype after injection within the intervertebral disc and the capabilities of these cells to deposit a newly formed ECM [7, 8]. Stem cells were shown not only to differentiate toward a NP cell-like phenotype but also to stimulate the endogenous cells promoting the repopulation of the NP, and the increase of matrix proteins production such as aggrecan [9]. However, the delivery of stem cells within the IVD has been shown to induce the differentiation of stem cells toward a NP cell-like phenotype and the deposition of newly synthesised ECM. The number of cells delivered to the disc appeared to be crucial to positively impact the behavior of NP tissue. Indeed, Serigano et al. reported that an insufficient number of delivered cells did not induce the regeneration and that, conversely, an excessive number of cells induced apoptosis of the delivered and resident cells due to an imbalance between nutrients and cell numbers [10]. An average cell number of 1,000,000 cells/mL relative to their optimal cell number was tested in this study.

Several studies reported the beneficial effect of stem cells on IVD regeneration [10-12]. However, the results of these studies, although promising, still suffer from effectiveness of cell transfer. An increase in cell viability in the carrier after injection within the disc was obtained [8]. For this reason, an injectable functionalized hydrogel system appears to be a promising approach for disc regeneration. Therefore, it was hypothesised that the maintenance and/or regeneration of the intervertebral discs can be achieved through delivery of an injectable type II collagen/HA reservoir containing ADSCs within the NP.
Materials and Methods

Animal experiments were carried out according to the European Community Guidelines for the care and use of laboratory animals (DE 86/609/CEE). This study was approved by the ethics committee of the National Veterinary School of Nantes. 30 month-old New Zealand white rabbits (Charles River, France) were used in this study (n=4).

- Anaesthesia and Surgery

Each rabbit was initially anesthetised by intramuscular injection of medetomidine (200µg/kg) and ketamine (20mg/kg). Five to ten minutes after injection, rabbits were intubated endotracheally. The hair was shaved across the midline and the skin was desinfected. 1mg/kg of morphine was injected subcutaneously before surgery. Rabbits were maintained under perfusion with 0.9% NaCl (10mL/kg/h) during the procedure. The anaesthesia was maintained using inhalation of 1 to 2.5% isoflurane by monitoring the reaction of the rabbit. The field was prepared with betadine, and draped in a sterile manner. 0.1mg/kg of morphine was injected intravenously five minutes before incision and injections were repeated during the surgery by observing the reaction of the rabbit. Metacam (AIMS) was injected subcutaneously on recovery. At the end of the surgery, fentanyl patches (25µg/h) were placed. During surgery, capnograph, EtCO₂, heart rate, breath rate and SpO₂ were monitored.

- Adipose Tissue Collection (Appendix C)

ADSCs were extracted from each rabbit and autologously reimplanted. The tissue was digested by collagenase type I at 0.025% for 1h under agitation at 37°C. The enzymatic reaction was stopped by addition of complete medium (DMEM, 10% FBS, 1% P/S). The stromal fraction was collected by centrifugation of five minutes at 1,200rpm, suspended and filtered on a cell strainer 70µm (BD Sciences, Switzerland). After 24h of incubation at 37°C in a humidified atmosphere of 5% CO₂, cells were washed in order to eliminate the contaminant cells (blood cells and adipocytes). Cells were cultured for another 24h before reinjection within the IVD. A part of the cells was kept for differentiation analysis in order to confirm the nature of the extracted cells as described previously [13].

- Preparation and Injection of the Type II Collagen Hydrogel

The hydrogels were seeded with NP cells directly after cell extraction. Briefly, type II atelocollagen and hyaluronic acid solutions (see appendix B) were
mixed and the pH adjusted to 7.4 by addition of NaOH. 1,000,000 cells/mL were then added to the mixture. 1mM 4S-StarPEG was subsequently added just prior to injection. The hydrogel was filled in a 500µl luer-lock Hamilton-syringe.

- **Injection Procedure and Treatment Groups**

Five groups were implanted on each rabbit spine. After locating the discs of interest, treatments were applied as follows: L1-L2 control group, L2-L3 cell alone, L4-L5 hydrogel plus cells and L5-L6 hydrogel alone). A puncture of the disc with the syringe was considered as the sham group. 25µl of hydrogel was injected in the discs. L1-L2 disc was used as control disc.

- **Monitoring of Disc Height and Hydration (Appendices W and X)**

Before monitoring disc height and hydration by MRI and X-Ray, the animals were anesthetized by intramuscular injection of xylazine (Bayer, France) and ketamine (Merial, France). Disc hydration was assessed by MRI scans 0, 30 and 60 days after surgery using a Tesla clinical magnet (Siemens Magnetom Harmony/Syngo). A 2.5mm midsagittal section image was obtained using a T2-weighted imaging sequence (T2ws) (TR, 5,000ms; TE, 111ms). In parallel with MRI scans, X-ray radiographies were performed using a radiograph machine (Convix 80 generator and Univesix 120 table) from Picker International (Uniontown, USA). Coronal and sagittal planes radiographs were taken with a collimator-to-film distance of 100cm, an exposure of 100mA per s and a penetration power of 48kVp. Images obtained after MRI and X-Ray were analysed with Osirix software (Osirix Foundation, Switzerland) according to the protocols described in appendices W and X.

- **Histology**

After 60 days, animals were sacrificed by intramuscular injection of ketamine (25mg/kg) followed by intravenous injection of sodium pentobarbital (1.2g/kg). The IVDs were then isolated with a part of the vertebral bodies on both sides and fixed for four days in 4% PFA. After decalcification for 24h in a Decalcifer® solution (Surgipath, USA), the samples were dehydrated and incubated with Histosol® (Shendon, Belgium). Specimens were then embedded in paraffin and cut into 3µm sections. After deparaffinisation, tissues were stained with Hematoxylin and Eosin, Alcian Blue, Periodic Acid Shiff-Alcian Blue, Masson staining and type II and type I collagen immunostainings. Images were taken on an upright fluorescent microscope (Olympus Microscope BX51, Mason Technologies, Ireland).
Appendices

Results

- Disc Height and Hydration Levels

No significant difference in percentage of hydration was observed between the different treatment groups. However, a trend showing the maintenance of disc hydration over 90 days was seen after the injection of the cell-loaded hydrogel. The other treatment groups (injection of cell alone and hydrogel alone) as the non-treated and sham groups showed a decrease of disc hydration up to 20% at day 90. However, no significant difference was observed between day 0 and day 90 (Figure GG1). For this measurement, based on power analysis calculations, a sample size of eight animals will be required to obtain significant differences between the different treatment-groups.

Similar trends were observed for disc height and disc hydration. A relatively stable height index was observed over the period of 90 days after injection of the cell-loaded hydrogel. The other treatment and control groups showed a decrease of disc height up to 20% at day 90 (Figure GG2). For this measurement, based on power analysis calculations, a sample size of 13 animals will be required to obtain significant differences between the different treatment-groups.

Gross Observations

At day 90, animals were sacrificed and lumbar spines were harvested. Outgrowths on the treated discs were noted. A higher vascularisation of the outgrowths was seen on the discs injected with cell alone. The cell-loaded hydrogel presented lower vascularisation of the outgrowth tissue. These outgrowths were not detected on the X-ray images which suggests an absence of mineralisation.

- Histological Observations

At a histological level, higher expression of GAGs (Figure GG3) and type II collagen (Figure GG4) were observed in the outgrowth of the cell-loaded hydrogel. The injection point was noticeable on images with failure of AF. An increase of type I collagen expression was observed after injection at the level of the outer AF compared to that of control discs (Figure GG6). At a higher magnification, the presence of tissue with the same characteristic as that of NP was seen in the injection point at the level of the AF. No inflammatory cells in the tissue were observed while the cells exhibited a chondrocytic morphology (Figure GG7).
Appendices

Figure GG1. Hydration level 30, 60 and 90 days after treatment relative to day 0. L1-L2 IVD: control, L2-L3: sham, L3-L4: cell alone, L4-L5: hydrogel + cells, L5-L6: hydrogel alone, L6-L7: control.

Figure GG2. Disc height level 30, 60 and 90 days after treatment relative to day 0. L1-L2 IVD: control, L2-L3: sham, L3-L4: cell alone, L4-L5: hydrogel + cells, L5-L6: hydrogel alone, L6-L7: control.
Figure GG3. Gross morphology of rabbit spine 60-days post-treatment. Outgrowths on the top of the disc were observed after extraction of the spine (yellow arrows). L1-L2 IVD: control, L2-L3: sham, L3-L4: cell alone, L4-L5: hydrogel + cells, L5-L6: hydrogel alone.
Figure GG4. Representative images of IVD sections from (1) control group (L1-L2), (2) sham group (L2-L3), injection of (3) cells alone (L3-L4), (4) hydrogel and cells (L4-L5) and (5) hydrogel alone (L5-L6) after staining with (A) hematoxylin and eosin, (B) alcian blue and (C) Periodic Acid Schiff-Alcian Blue. The outgrowths observed on the disc are indicated by the red arrows. Scale bar = 200µm.
Figure GG5. Representative images of IVD sections from (1) control group (L1-L2), (2) sham group (L2-L3), injection of (3) cells alone (L3-L4), (4) hydrogel and cells (L4-L5) and (5) hydrogel alone (L5-L6) after staining with (A) masson trichrome, and immunohistochemistry of (B) type II collagen and (C) type I collagen. The outgrowths observed on the discs are indicated by the red arrows.
Figure GG6. Representative image of the AF rupture present at the injection site. Red arrows indicate the injury site. AF, NP and IAF represent annulus fibrosus, nucleus pulposus and inner annulus fibrosus, respectively.
Figure GG7. PAS-BA staining of the outgrowth present on the IVD after the injection of hydrogel and cells (L4-L5). AF and O represent annulus fibrosus and outgrowth, respectively.
Discussion

A type II collagen hydrogel supplemented with HA and stabilized with 4S-StarPEG was developed previously (Chapter 2) [13]. Promising results were obtained after in vitro characterization with good stability after degradation by collagenase. Good cell viability and distribution with maintenance of NP cells phenotype (Chapter 2) was also seen [13]. At a mechanical level, in addition to a good stability, promising rheological properties and a short gelation time (~8min) was seen. The logical following step of this project was to test this hydrogel in vivo, in order to validate the hypothesis that the delivery of an injectable type II collagen/HA hydrogel carrier of ADSCs will induce the regeneration of the IVD tissue and restore/maintain disc height and hydration level of the IVD. A rabbit model presenting a spontaneous degeneration process over ageing was used in this study. This type of model follows a similar pattern of degeneration to that observed in humans [6, 14]. Each rabbit showed IVD from L1-L2 to L6-L7 with a Pfirrmann score between 2 and 3 on a scale of 4 indicating some levels of degeneration allowing the use of most of the discs for the study (data not shown).

The disc height index and disc hydration level were monitored over three months. No significant differences between the different group treatments were observed for either analyses (Figures GG1 and GG2). However, a trend toward the maintenance of disc height and hydration level was noted after the injection of cell loaded hydrogel while a decrease of both disc height and hydration level was noted after injection of cells alone, hydrogel alone and the sham group. For the sham group, only a puncture of the disc with a needle was performed. Largely discussed in the literature, the puncture of the disc during the injection process has been reported to greatly influence the degeneration of the IVD by damaging the AF [15, 16]. Some studies report that the size of the needle used during the injection is important [16] while others report that any injury of the AF induces/promotes the degeneration of the disc [15, 17]. In this study, no differences in hydration level were observed between treated groups sham, cell alone, hydrogel alone and control disc with a similar trend for all groups over 90 days. It is likely that this decrease of both disc height and hydration is due to the natural ageing phenomenon of the disc [6] and not to the effect of the disc puncture. This aspect was confirmed histologically where no difference in staining intensities and tissue morphology was observed (Figure GG4).
However, the injection site was noticeable on the discs with injection. Interestingly, NP tissue was observed in the injury site suggesting that the leakage of the NP is due to the pressure applied during the injection (Figure GG6).

At a macroscopic level, outgrowths were observed at the surface of the left anterolateral AF after the injection of a treatment group within the IVD (Figure GG3). These outgrowths suggest a leakage of the treatment outside the disc. A similar phenomenon was reported by Vadala et al. after injection of MSCs and Olmarker et al. after puncture and leakage of NP onto the discs surfaces [18]. In this study, the authors described the outgrowths as being osteophytes. These structures are formed of a mineralized tissue in the centre surrounded by a layer of cartilage [19, 20]. Gross inspection of the outgrowths presented on the disc revealed their vascularisation. The vascularisation observed for the cell alone exhibited a higher degree of vascularisation than the discs treated with hydrogel alone and hydrogel supplemented with cells (Figure GG3). The outgrowths for these treatment-groups presented a cartilaginous aspect (Figure GG7). Further histological analyses of these structures revealed a high expression of type II collagen (Figure GG5) and GAGs (Figure GG4) within the tissue indicating a cartilaginous nature of these nodules after injection of the cell supplemented hydrogel and the hydrogel alone. The expression of type I collagen suggests the formation of a fibrotic cartilaginous tissue. No mineralized tissues were observed within the outgrowths suggesting that the outgrowths were not osteophytes. Cells in these nodules presented a chondrogenic morphology (Figure GG6). These outgrowths may be the result of the secretion of bioactive substances present in the NP tissue [18] or secreted factors by the injected stem cells, or the leakage of hydrogel [19]. Further investigations are required in order to identify the nature of the cells present in the outgrowths.

Several studies have shown a therapeutic effect of the injection of stem cells within the IVD [10, 21-24]. A relative maintenance of disc height and hydration level was observed after injecting cells alone or using a scaffold as cell carrier to the disc [21, 22]. The level of degeneration of the discs in this model is not sufficient to observe an impact of the therapy on the disc. The use of aspiration, broadly described in the literature, would lead to a similar level of degeneration [8, 10, 21, 22]. This nucleotomy also provides an augmentation of the injection site.

Cell carriers are promising tools for disc regeneration as they increase the efficiency of cell retention within the IVD [25]. The results of this study suggest that
the use of a cell carrier, although promising, will need to be associated with a strategy to seal AF in order to promote the retention of the cell carrier within the IVD. The IVD is a highly pressurized tissue [26] which limits the space of injection. The formation of a space by nucleotomy prior to injection will allow the release of pressure within the disc, the injection of a greater volume of cell carrier and consequently a lower risk of hydrogel leakage. The multiple limitations of this pilot study do not lead to conclusive results. Further studies are required to determine cellular retention and the assessment of the degeneration level. In addition, the sample size prevented a thorough analysis of this study.
HH. Development of an Organ Culture System: a Pilot Study

Introduction

Organ culture systems have been envisaged for the last ten years as an alternative model for the screening of therapeutic strategies [27, 28] and the study of the mechanisms inducing the disc degeneration [29-31]. Their use is greatly valued as they address multiple disadvantages of animal models such as high cost, absence of a model with similar mechanical properties to human discs as well as ethical issues. Cell behaviour in such a system is also considered as more relevant than in vitro cell models by maintaining the cells in the highly specialised ECM of the IVD. The hypothesis under this project was that IVDs can be cultured ex vivo for 21 days with maintenance of cell viability for their use as a cost-effective alternative model for hydrogel screening (Figure HH1).

Materials and Methods

- Disc Extraction

Two year-old bovine fresh tails were collected directly after sacrifice from a local slaughter house. Soft tissues surrounding IVDs (muscles and ligaments) were manually removed. IVDs were extracted by sectioning discs on both sides of the cartilage endplate (Appendix AA). Discs were washed three times for five minutes with 0.9% NaCl and once with betadine for five minutes before being immersed in a HBSS solution supplemented with 55mM trisodium citrate and 50µg/mL gentamicin for 10 to 15 minutes. Discs were then incubated overnight in DMEM supplemented with 5% FBS, 50µg/mL and 20mM of trisodium citrate at 37°C. The IVDs were then used for injection trials or cultured for two, seven, 14 and 21 days in culture for the viability study.

- Evaluation of Cell Viability by TUNEL Assay

After 2, 7, 14 and 21 days, IVDs (n=5) were fixed in a solution of 4% paraformaldehyde in 1X PBS overnight. After three washes with 1X PBS, tissues were infiltrated overnight with 20% sucrose. Specimens were flash-frozen in liquid nitrogen-cooled isopentane and 5µm frozen sections were cut on a Leica™ CM 1850 cryostat (Laboratory Instruments & Supplies Ltd., Ireland). Sections were collected on Superfrost® Plus slides (Fisher Scientific Inc., Dublin, Ireland) and stored at -20°C until use. Sections were then washed three times for five minutes each.
were sectioned at three different levels as described in figure HH2. Five slides of 5µm were collected at each level. A gap of 200µm was observed between each level.

After a cure of 15 minutes at room temperature and three washes with 1X PBS, an antigen retrieval was performed by digestion of the tissue with proteinase K at 20µg/mL (30U/mg) for ten minutes at 37°C. After three washes with 1X PBS, slides were blocked with 2% BSA for one hour at 37°C. Tissues were then incubated with the TUNEL reaction mixture (GenScript, USA) for one hour. Slides were then washed three times for five minutes with 1X PBS + 0.05% Tween 20 and incubated for one hour with Extravidin®-TRITC conjugated (Sigma Aldrich®, Ireland) diluted at 1/1,000 for one hour at room temperature. After three washes of five minutes each, slides were counterstained with DAPI (1/2,000) for ten minutes. Sections were then mounted with Prolong® Gold antifade and cured overnight at 4°C before reading. A mixture without TdT and a digested tissue with DNase I solution at 30,000 units for 30 minutes were used as negative and positive controls, respectively. A staining with the Extravidin®-TRITC conjugated alone was also performed as negative control.

- **Tissue Imaging and Cell Quantification**
  Quantification of cell number from the digital images was done using Image J software (National Institutes of Health, USA, http://rsbweb.nih.gov/ij/). For each image, the total cell number and the positively stained cells were counted. For each section, five images were taken. Total cell number and positively stained cell number were calculated to obtain the percentage of dead cells per section. This percentage was measured for each section from five different discs (one disc per animal with n=5 animals).

- **Optimisation of the Hydrogel Injection**
  After extraction, a cavity in the disc was formed to allow hydrogel injection. Agarose supplemented with blue coomassie stain was melted and injected within the cavity. After 10 minutes, the disc was sectioned to localise the agarose gel within the tissue (Figure HH4).
Figure HH1. Schematic representation of the IVD organ extraction and preparation. After dissection of the tail, the discs were cut on both sides of the cartilage end-plates. The discs were then incubated overnight with sodium citrate to remove blood clots present in the cartilage end-plates. After three washes, a puncture was performed in the discs and the hydrogel injected.
Figure HH2. Illustration of IVD sampling observed during the organ culture study. The tissue was cut into three different sections with 200µm between sections. Five sections were collected for each section.
Figure HH3. Cell viability within the bovine IVD accessed by TUNEL assay after extraction (1), and 2 (2), 7 (3), 14 (4) and 21 (5) days in culture. Negative control as no TdT and positive CT (tissue digested by DNase before staining) were used. White arrows indicate positively stained cells. (Scale bar = 100µm) (n=5).
Figure HH4. Representations of bovine IVD after injection. (A) An incision of 2-3mm was performed on the side of the organ. (B) Representation of a cavity. (C) Location of the injection site after two days in culture.
Results

- **Disc Extraction**

  The discs were successfully cut on both sides of the vertebral bodies at the level of the cartilage end-plates. Dissections were performed in such a way that these structures were preserved in order to prevent swelling and to maintain the disc integrity. The bone remaining on the cartilage end-plates was removed. Blood clots within the cartilage end-plates vessels were dissolved by overnight treatment with sodium citrate [32]. By this treatment, the pores of the cartilage end-plates were cleaned which allow the diffusion of nutrients. Discs were then cultured for 21 days. No fungal or bacterial infection was observed over this period of culture.

- **Cell Viability**

  Three zones were scanned to assess the cell viability within the IVD tissue as described in Figure HH2. Over 90% of viability was obtained after 2, 7, 14 and 21 days in culture (Figure HH3).

- **Optimisation of the Hydrogel Injection**

  Different techniques were tested to inject hydrogels within the IVD: (1) no cavity, (2) punch biopsy, (3) midline incision associated with a small nucleotomy, and (4) L incision associated with a nucleotomy. The injection of the hydrogel within the IVD without prior nucleotomy was not possible due to the high pressure present in the tissue. The L incision technique allowed the formation of a large cavity which permitted the injection of a large volume of hydrogel and a better control of this volume. However, this technique profoundly damaged the AF. A cavity of reasonable size which allowed the injection of up to 50µl of hydrogel was obtained after punch biopsy or midline incision (Figure HH4). The midline incision followed by nucleotomy did not compromise the integrity of the AF but induced more damage of the surrounding tissues. A clear and clean cavity was obtained after the formation of a cavity by punch biopsy.

**Discussion**

The objective of this study was to develop an organ culture system which can be used for hydrogel screening. The preservation of the disc structure permits the study of NP cell behaviour in an *in vivo*-like environment as well as preliminary screening of the effect of therapies for IVD treatments. Over 90% of cell viability
was observed within the hydrogel after 21 days in culture which indicates the disc survival over this time period.

Different techniques of hydrogel injection were tested. The high pressure within the IVD [33] did not allow the injection of the hydrogel without nucleotomy. A nucleotomy, partial or complete, therefore seems essential to the hydrogel injection. Several techniques were envisaged in this study. Most of these techniques led to the different levels of damages of the AF. This damage can induce the full failure of the AF [15] which can lead to the extrusion of the hydrogel. Sealing of the AF is important to maintain the scaffold within the tissue [34].

Organ culture has been used in the recent years to study cell behaviour in the ECM environment, the process of disc degeneration, and the effect of external factors and therapies [28, 29, 35]. This system allows the maintenance of the disc cells in an *in vivo* like environment and the study of external factors such as mechanical forces reproduce closely the native environment.
II. Evaluation of Transfection Techniques for Non-Viral Delivery of Plasmid DNA to ADSCs and IVD Cells

Introduction

Many concerns with the use of non-viral gene therapy approaches have been raised because of toxicity, immunogenicity and oncogenesis associated with viral vectors [36, 37]. Alternative non-viral methods have showed promising results over the last few years [36]. Two types of non-viral methods are reported: physical and chemical techniques [38, 39]. These techniques, easier to engineer, allow a better controlled and targeted expression of the transgene [37, 40]. Physical and chemical gene transfer techniques were evaluated to determine which technique most efficiently delivers plasmid to ADSCs, NP and AF cells.

Materials and Methods

- Cell Extraction and Seeding
  
  ADSCs were extracted from adipose rabbit tissue as described in appendix C. Briefly, the tissue was digested by collagenase type I at 0.025% for 1h under agitation at 37°C. The enzymatic reaction was stopped by addition of complete medium (DMEM, 10% FBS, 1% P/S). The stromal fraction was collected by centrifugation of five minutes at 1,200rpm, suspended and filtered on a cell strainer 70µm (BD Sciences, Switzerland). After 24h of incubation at 37°C in a humidified atmosphere of 5% CO₂, cells were washed in order to eliminate the contaminant cells (blood cells and adipocytes). Cells were cultured DMEM supplemented with 10%FBS and 1%P/S at 37°C under 5%CO₂ and passaged twice before use.

  Two year-old bovine fresh tails were collected directly after sacrifice from a local slaughter house. Soft tissues surrounding IVDs (muscles and ligaments) were manually removed. Each IVD was sectioned transversally through the centre and NP tissue was harvested from both halves. Tissues were washed twice with Tyrode’s Balanced Salt Solution (TBSS). Following this wash, NP tissues were digested with 0.19% of pronase (Roche, Switzerland) for 1h at 37°C under agitation. After pronase inactivation by successive washes with TBSS, DMEM supplemented with 10% FBS and 1% P/S containing 32IU/mL of collagenase type II (327IU/mg, Worthington Biochemical Corporation, Germany) was added. The mixture was incubated under orbital agitation overnight at 37°C in a humidified atmosphere of 5% CO₂ and was
subsequently filtered through a 70μm cell strainer. Cells obtained were centrifuged for five minutes at room temperature at 1,200rpm and counted (Appendix D). Cells were cultured in DMEM supplemented with 10%FBS and 1%P/S at 37°C under 5% CO₂ until they reached 80% confluency. Cells were then passaged and seeded for the transfection studies in glass chamber slides or tissue culture plate.

- Transfection Studies

For the polymeric transfection techniques, cells (ADSCs, NP and AF cells) were seeded in monolayer at a density of 50,000 cells in DMEM supplemented with 10% FBS and 1% P/S for 24h before treatment. Transfection procedures were performed with 2μg of DNA per 50,000 cells for all polymeric agents. PEI was used at a polymer:DNA ratio of 2:1. Superfect® was used at a ratio of 8:1 as per the manufacturer’s instructions. X Fect™ was used at a ratio 0.3μl of polymer per μg of DNA as per manufacturer’s instructions. All mixtures were adjusted to a volume of 50μl of serum free medium and incubated for 30 minutes to allow complexation before addition onto the cells.

The procedure followed for cell electroporation was performed according to the method described in the Amaxa™ Nucleofector™ for human chondrocyte kit (Lonza, Ireland). Briefly, cells were resuspended in electroporation solution at a density of 50,000 cells per 100μl. 2μg of plasmid DNA of interest was added to the cells. The mixture was then transferred into an electroporation cuvette and electroporated using the programs U-028 for NP and AF cells and U-23 for ADSCs of Amaxa™ Nucleofector™. Immediately after electroporation, cells were seeded onto a tissue culture plate or chamber slides at a density of 50,000 cells per cm².

- Evaluation of Transfection Efficiency

Gaussian Princeps luciferase plasmid (New England Labs®, USA) and Green Fluorescence Protein plasmid were delivered to the three cell-types.

Secreted luciferase activity was measured in the medium using BioLux® Gaussian Luciferase Assay kit (New England Labs®, USA) as per manufacturer’s instruction. GFP transfected cells were fixed 48h after transfection with 4% paraformaldehyde in 1X PBS. Slides were mounted with ProLong® Gold antifade (Life Technologies™, Ireland). Slides were kept at 4°C in the dark for one day before imaging. Imaging was performed using an inverted epifluorescent microscope (Olympus IX81, Mason Technologies, Ireland). Ten images per slide were taken and analysed for cells extracted from three different animals (n=3). Quantification of cell
number from the digital images was done using Image J software (National Institutes of Health, USA, http://rsbweb.nih.gov/ij/). For each image, the total cell number and the positively stained cells were counted.

Results

The use of chemical gene carriers (Xfect™, Superfect® and PEI) increased the transfection efficiency of the plasmid DNA ($p<0.05$). While Xfect™ showed capabilities of transfection of ADSCs, the relative light units obtained for this transfecting agent is lower than that of Superfect® and polyethylenimine (PEI) (Figure II1).

PEI showed a higher toxicity level as reported in many studies [39, 41]. Because of the lower toxicity of Superfect®, this transfecting agent was used for further investigations. Although this agent showed a high capability of transfection of ADSCs, NP and AF cells with a high luminescence activity, a low number of transfected cells was counted after transfection (Figure II2.B). Electroporation was evaluated as an alternative method to improve the efficiency of transfection. A higher gene transfer in ADSCs, NP and AF cells compared to that of Superfect® was obtained with 66.53±7.88% of GFP-positive cells (Figure II2.A). A high efficiency of gene transfer was noted for NP and AF cells after electroporation with 70.16±15.4 and 70.76±13.1% of transfected cells, respectively (Table III1, Figure II3).
Figure III1. X Fect™, SuperFect® and PEI transfection capabilities of ADSCs. Data plotted represents mean ± standard deviation (n=3). * shows significant differences at $p<0.05$. 

$\text{Relative Light Units (Arbitrary Unit)}$

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<th>Transfecting agent</th>
<th>$1E+00$</th>
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</table>

* shows significant differences at $p<0.05$. 

Appendices
Figure II2. Transfection efficiency of ADSCs by (A) electroporation and (B) Superfect®. An efficiency of 66.53±7.88% and 3.84±1.65% was observed for electroporated cells and transfected cells with Superfect®, respectively. Nuclei are stained blue and GFP in green (Scale bar = 100μm).
Figure II3. GFP positive cells after electroporation of (A) ADSCs, (B) NP and (C) AF cells. Nuclei are stained blue and GFP in green (Scale bar = 100µm).
Table III. Percentage of electroporated cells. Results are expressed in percentage ± standard deviation (n=5).

<table>
<thead>
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<th>Cell-type</th>
<th>Percentage of transfected cells by electroporation (%)</th>
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<tbody>
<tr>
<td>Rabbit ADSCs</td>
<td>66.53±7.88</td>
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<tr>
<td>Bovine NP cells</td>
<td>70.16±15.4</td>
</tr>
<tr>
<td>Bovine AF cells</td>
<td>70.76±13.1</td>
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</table>
Discussion

Significant progress for non-viral gene therapy using chemical vectors has been observed recently [36, 39, 41]. The capabilities of transfection of different chemical agents (Xfect™, Superfect® and PEI) were evaluated in this study. The different polymeric agents used in this study showed various capabilities of transfecting ADSCs, NP and AF cells (Figure II1). The highest luciferase activities observed were obtained after transfection with PEI and Superfect®. A high toxicity of PEI was seen (data not shown) [42]. By consequent, Superfect® was used for further investigations. Although a high luminescence activity was obtained after transfection using this agent (Figure II1), a low number of transfected ADSCs was observed (Figure II2B). Similar results were obtained after transfection of NP and AF cells using Superfect® (data not shown). Primary cells, progenitor cells and stem cells have been shown to be more difficult to transfect through chemical vectors [36]. Physical non-viral gene delivery showed promise as an alternative method for transfecting difficult-to-transfect cells. This technique has been used to transfect IVD cells in vivo [38, 43]. Used as an alternative method in this study, a high number of transfected cells were obtained after electroporation of the three cell-types (Table II1). This technique was consequently chosen for the investigations conducted in Chapter 4.
Appendices

JJ. Journal publication and conference proceedings

Published Manuscripts (8)


Manuscripts to be submitted (3)


Book Chapter (2)


Invention Disclosures Filed (1)


Conference Presentations (11)


9. E. Collin, S. Grad, D. Zeugolis, P. Weiss, J. Guicheux, M. Alini, A. Pandit, Injectable Hydrogel as a Reservoir System for Nucleus Pulposus Regeneration,
Appendices

Poster Presentation at eCM XI: Cartilage & Disc Repair and Regeneration, Davos, Switzerland, 28th -30th June 2010
KK. References


Appendices


