Development of a novel 3D human cartilage model system to investigate changes in cartilage associated with osteoarthritis

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„Wissen und Erkennen sind die Freude und Berechtigung der Menschheit“

„Science and knowledge are the joy and entitlement of humanity“

Alexander von Humboldt, german naturalist and explorer (1769-1859)
Abstract

Osteoarthritis (OA) is the most prevalent form of degenerative joint diseases and affects about 13% of the world’s population with patients over 65 years of age reflecting the largest group of patients (Matthews and Hunter 2011). OA is characterized by a progressive degeneration of joint cartilage and results in impaired function of affected joints, pain and negatively affects quality of life of patients. Several biomarkers for the detection of early OA were already described (Mobasher 2013), but none of them is reliable and quantifiable. Therefore, a need for novel biomarkers for OA exists to improve diagnosis when overt changes in cartilage are not yet detectable.

The aim of my thesis was to identify novel biomarkers for OA which can be used for early diagnosis. I focused on changes in the glycosylation of proteoglycans, especially on chondroitin sulphate (CS) glycosaminoglycan (GAG) chains. Using human chondrocyte progenitor cells, a novel 3D cartilage model was developed and characterized. The resulting cartilage constructs showed similar biochemical, histological and mechanical properties like native articular cartilage. To investigate the effects of inflammatory cytokines, which are also present in OA, on changes in expression of glycosylation-related genes, treated and untreated constructs were analyzed using microarrays. Analysis showed that although genes for GAG chain synthesis were down-regulated, the sulphotransferase GalNAc4S-6ST was significantly up-regulated. This enzyme catalyzes the formation of GalNAc4,6diS, which has high biological activity (Mikami and Kitagawa 2013). In addition, the expression of serglycin was strongly increased after inflammatory stimulation. These results show that novel epitopes containing GalNAc4,6diS or serglycin could be potential biomarkers for OA.

Besides the experiments with cartilage constructs, analysis of the CS chain of decorin isolated from human skin fibroblasts showed that inflammatory stimulation alters the length and composition of CS chains.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2-AB</td>
<td>2-aminobenzamide</td>
</tr>
<tr>
<td>ACI</td>
<td>Autologous chondrocyte transplantation</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>A disintegrin and metalloprotease with trompospondin motif</td>
</tr>
<tr>
<td>AMAC</td>
<td>2-aminoacridone</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C-4-S</td>
<td>Chondroitin-4-sulphate</td>
</tr>
<tr>
<td>chABC</td>
<td>Chondroitinase ABC</td>
</tr>
<tr>
<td>chAC-I</td>
<td>Chondroitinase AC-I</td>
</tr>
<tr>
<td>chB</td>
<td>Chondroitinase B</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CTX-II</td>
<td>collagen II C-telopeptide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMOADS</td>
<td>Disease-modifying osteoarthritis drug</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulphate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDS</td>
<td>Ehlers-Danlos syndrome</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>SLRP</td>
<td>Small leucine rich proteoglycan</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA buffer</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline containing 0.01% Tween 20</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor b</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>Xyl</td>
<td>Xylose</td>
</tr>
<tr>
<td>XylT</td>
<td>Xylosyltransferase</td>
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Chapter 1:

Introduction
1 Introduction

1.1 Arthritis

Arthritis is a collective term for degenerative joint diseases, which can be subdivided by their cause. The most common forms of arthritis are osteoarthritis, rheumatoid arthritis and gout, but also lupus and psoriasis. In contrast to osteoarthritis, which is mainly caused by progressive joint deterioration through use, rheumatoid arthritis is a major autoimmune disease, characterized by active destruction of cartilage and bone (Abdel-Nasser et al. 1997). The following paragraphs will focus on osteoarthritis and describe the disease in more detail.

1.2 Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis and characterized by the degeneration of articular cartilage in joints. In Europe, one OA-damaged joint is replaced every 1.5 minutes (WHO Scientific Group 2003) and an estimated 1 million joints are replaced each year (Wood et al. 2013). In the United States it is predicted that 19 million people will be suffering from arthritis between 1995 and 2020 (Iorio et al. 2008). Mostly the weight-bearing joints of knee, hip, and lower spine are affected by OA, and to a lower extent feet and hands. It occurs when the dynamic equilibrium of breakdown and regeneration of joint tissue becomes unbalanced by outside influences, for example when the load-bearing capacity of a joint is exceeded (Eyre 2004). During the progression of the disease, the articular cartilage becomes more and more eroded, and this ultimately leads to complete loss of cartilage in weight bearing areas of the joint. This results in loss of motility of the affected person and can be very painful.

In an early stage of OA, chondrocytes from the articular cartilage form clusters through clonal expansion at the damaged site and the concentration of angiogenic growth factors in the extracellular matrix increases (Goldring 2000; Aurich et al. 2005). Repair fails and leads to an imbalance in the matrix, which
results in degradation. Increasing concentrations of matrix destructive proteases like matrix-metalloproteases (e.g. MMP-13) and aggrecanases (ADAMTS-4 & -5) (Karsenty 2005; Burrage et al. 2006), increasing apoptosis of chondrocytes and altered synthesis of matrix components lead to the formation of a matrix that is unable to bear normal mechanical stresses. As a result, the tissue enters a vicious cycle where matrix breakdown outweighs synthesis and regeneration. Because of the aneural nature of cartilage, patients have no symptoms until surrounding neural tissue of the bone becomes affected (Bijlsma et al. 2011). However, recent evidence shows that OA is not only cartilage-driven, but that also synovial tissue and bone play an important role. Synovial inflammation is evident in OA (Sellam and Berenbaum 2010) and may lead to generation of neurotransmitters and ultimately pain.

In synovial inflammation, proinflammatory and catabolic mediators are produced by synovial macrophages and negatively alter the balance between degradation and generation of cartilage matrix, leading to an excessive production of proteolytic enzymes that degrade cartilage matrix. Due to this, synovial inflammation in turn is amplified (Bondeson et al., 2006). Synovial inflammation can occur in early and late stages of OA and contributes to the vicious cycle of progressive joint degeneration (Bijlsma et al. 2011).

The main factors for the development of OA are excessive loading of joints due to obesity, aberrant joint alignment, peak forces and joint injury in sport, as well as wear and tear of joints during a lifetime. Age is therefore considered to be a main factor. It has been shown that the prevalence of OA affects a large proportion of all people above the age of 65 (Badley and Wang 1998). However, injuries and the presence of a genetic predisposition can promote the development of OA (Balint and Szefeny 2000). Moreover, women are more often affected than men, which indicates a sex-specific predisposition.

OA is commonly associated with changes in the subchondral bone of the affected joint. This can be the formation of osteophytes, bone remodeling, subchondral sclerosis and attrition. These are all important features for the radiographic diagnosis of the disease. Due to the fact that some of these changes not only occur in the later stages of OA, but also even before cartilage
degradation, it is suggested that subchondral bone changes could be an initiating or major contributing factor for OA (Intema, Hazewinkel, et al. 2010; Intema, Sniekers, et al. 2010).

Usually patients see a doctor when they have symptoms like joint pain and loss of movement and function. These are typically experienced by patients with well-established disease and hence, substantial cartilage damage may already have occurred. Pain occurs occasionally, but is typically worst during and after a period of weight-bearing activities. Stiffness in the morning and evening is another symptom often experienced by patients suffering of OA. It occurs after longer inactivity of the joint, e.g. in the morning after sleep and in the evening and usually resolves in a couple of minutes. Additional features that can be associated with OA are psychological in nature like depression and unsound sleep, which worsen the quality of a patient’s life (Bijlsma et al. 2011). Differences between a healthy and osteoarthritic knee joint are illustrated in figure 1.1.

![Figure 1.1 Comparison of a healthy and osteoarthritic (OA) knee joint. The OA joint is characterized by inflamed synovium, degraded cartilage and formation of osteophytes. Reprinted by permission from Macmillan Publishers Ltd: [Nature reviews. Drug discovery.] (Wieland H, Michaelis M, Kirschbaum B, Rudolphi K “Osteoarthritis - an untreatable disease?” Volume 4, Issue 4, Pages 331-344), copyright (2005). Link to the journal.](image-url)
1.3 Diagnosis of OA

Diagnosis of OA must be carried out carefully to exclude pain and functional symptoms of other origins. Therefore, the site of joint pain has to be carefully evaluated. For example, patients with hip OA often feel pain in the corresponding knee. Based on this, spine and neurological examination is often required. To identify the source of pain, serological tests that screen for the presence of anti-cyclic citrullinated peptide antibodies or uric acid can be used to exclude rheumatoid arthritis and gout (Majithia and Geraci 2007). It is very difficult to correctly assess the severity of joint damage in OA without using imaging techniques. The use of biopsies for histological and biochemical analysis of cartilage, and bone is not feasible, since the tissue changes that occur are often focal and could be missed by taking random biopsies. Moreover, taking a biopsy would in itself cause irreversible damage to the joint cartilage. Imaging of the joint cartilage is rarely used to confirm the diagnosis of OA, but is a useful tool to establish the severity of the joint damage and monitor the progression of the disease (Bijlsma et al. 2011).

Imaging of OA currently relies on conventional radiography. This technique is cheap, fast and easy to perform with minimal risk for the patient. However, restrictions are that only calcified bone can be visualized, giving an indirect measurement of cartilage thickness, and without providing any information on the synovial tissue (Bijlsma et al. 2011). Several classification models based on radiography have been developed (Kellgren and Lawrence 1957; Altman and Gold 2007), which are used to grade the progression of OA in several joints. The grading systems are based on the formation of osteophytes, joint-space narrowing and bone sclerosis. Other than radiography, more advanced imaging techniques are available for a more detailed visualization of osteoarthritic joints. These are magnetic resonance imaging (MRI), computer tomography (CT) and ultrasound (Bijlsma et al. 2011). CT provides a three-dimensional image and uses contrast agents, which allows visualizing cartilage next to bone. Nevertheless, the high radiation exposure of the tissue is a major disadvantage. By using ultrasound, even soft tissues such as synovial tissue can be visualized in several planes without contrast agents.
However, limitations of this technique are the depth rapid loss of resolution with increasing depth of tissue penetration. MRI allows the quantitative assessment of morphology (volume, and thickness) and integrity of cartilage (Eckstein et al. 2005; Eckstein et al. 2006). By providing a large number of different imaging sequences and scoring systems, it is a very valuable technique to monitor the state of joint damage. Nonetheless, high costs and time consuming image acquisition and analysis of the data are limiting factors for its applicability. Various different specialized imaging modes for MRI have been developed which give a more complex view into cartilage quality. T2 MRI relaxation time for example allows determining the collagen orientation and density of cartilage (Dunn et al. 2004; Regatte et al. 2006). In addition, T1ρ MRI can be used to map proteoglycan distribution in cartilage (Menezes et al. 2004; Borthakur et al. 2006). The difference between T1 and T2 MRI is that different aspects of the proton spins are measured. Therefore, some tissues can be visualized with a higher or lower resolution, which is strongly influenced by the individual water content.

1.3.1 Biological markers for osteoarthritis

Biochemical markers might help to understand the pathophysiology of OA and help in diagnosing OA. They are generally molecules or catabolic products released by the joint into body fluids (blood, urine and synovial fluid): for example, remnants from extracellular matrix turnover (collagen and proteoglycan fragments) and changes in cellular metabolism (collagen synthesis byproducts, proteases, cytokines) derived from articular cartilage, synovial tissue and subchondral bone (Mobasher 2012). To date, MMP-3 in serum and collagen II C-telopeptide (CTX-II) in urine are the best characterized commercially available biomarkers (Kraus et al. 2011). They have been tested extensively and show expected relations to radiological and clinical findings in OA. Additional markers, which are still under investigation, include further collagen synthesis byproducts (CPII and PIICP in serum) or degradation products (C2C, Col2-1 and Col2-1NO2 in serum and urine), proteoglycans (cartilage oligomeric matrix protein in serum, aggrecan CS846...
epitope in serum), glycosaminoglycans (hyaluronic acid and keratan sulphate content in serum) and finally markers of bone resorption (CTX-1 and NTX-1). However, none of the so far available biomarkers is indicative on its own for the prognosis and diagnosis of OA. Especially in an aged population, changes in protein turnover can have many different reasons which leads to a relatively low specificity of a single marker. It is suggested that for a correct diagnosis of the stadium of disease progression, a combination of several biomarkers together with imaging techniques will be needed (Goldring and Goldring 2007). For all biomarkers, substantial variations in baseline levels exist between individuals. Furthermore, there is a lack of adequate knowledge about systemic origin, metabolism and kinetics for many markers (Bijlsma et al. 2011). Given the age profile of the population in many western countries, there is a pressing need for reliable, simple to attain and cost effective biomarkers for OA (Kraus et al. 2011), especially for the diagnosis and patient stratification in an early stage, when overt changes in the joint have not occurred, and therefore the disease may be modified through intervention.

1.4 Current treatment of osteoarthritis

There is no cure for OA. However, many different surgical procedures, and cell and tissue transplantation methods have been proposed to promote the regeneration or repair of the articular cartilage in situ (Buckwalter 1997; Dewan et al. 2014). For the management of OA, there are three treatment modalities available: non-pharmacological, pharmacological and surgical. For most patients, a combination of these options is used, tailored to the individual needs of the patient. Regarding non-pharmacological options, physiotherapy and exercise, joint protection, weight loss and other modalities for reducing the weight on the damaged joint have shown a positive effect (W. Zhang et al. 2007; Zhang et al. 2008). Paracetamol is the first choice analgesic to reduce joint pain. However, if this does not prove to be efficient, non-steroidal anti-inflammatory drugs (NSAIDs) are another option (Hunter 2011a). In recent years, a new drug type called DMOADS (disease-modifying OA drugs) has emerged (Hunter 2011b). DMOADS are modifying the progression of OA by
slowing down or preventing cartilage degradation or synovial inflammation. To give a few examples, inhibitors of IL-1, nitric oxide synthase or matrix metalloproteases (Hunter 2011b) belong to the group of DMOADS.

Surgical options of OA treatment are the intra-articular injection of bioactive compounds such as glucocorticoids, which are an effective tool for treating inflammatory flares of OA (Bellamy et al. 2006). Principal surgical treatments are osteotomy to correct the joint alignment, micro fracture, cartilage transplantation, and autologous chondrocyte transplantation (ACI). ACI describes the removal of a small amount of intact cartilage from a non-load-bearing region of articular cartilage for the extraction of intact chondrocytes. The cells are expanded *ex vivo* and transferred back into the patient once a sufficient number of cells was generated (Dewan et al. 2014). The expanded chondrocytes are then transferred into the cartilage lesion of the patient.

To date, severely damaged cartilage cannot be repaired using the above described techniques. The only possible way to restore mobility of the patient is total knee arthroplasty (Buckwalter 1997). The replacement of the affected joint has proved to be very effective, but can only be carried out beyond a certain age due to the limited life span of the joint prosthesis. Young patients will require a replacement prosthesis after several years, but this may be less successful because the prosthesis can be replaced only about two times in total for each joint due to changes in the underlying bone structure.

### 1.5 Chondrocytes and cartilage

Chondrocytes are the sole cell-type present in cartilage. As a consequence, cells are at a considerable distance to neighboring cells and the tissue lacks a vascular and neural connection. Therefore, the main nutrition/waste exchange is achieved by diffusion and fluid movement, when cartilage is compressed and expanded during joint movement. To overcome the resulting low oxygen tension inside cartilage (10% at the surface layer and <1% in deep layers), chondrocytes cover their energy requirements by glycolysis and therefore, they normally contain comparably small numbers of mitochondria (Archer and
Francis-West 2003). Chondrocytes are generally round cells, except at tissue boundaries where they have a discoid or flattened shape, like at the surface of articular cartilage. The main functions of chondrocytes is synthesis and maintenance of the extracellular matrix of supporting structures like articular, tracheal and nasal cartilage in order to resist mechanical strain (Archer and Francis-West 2003). During development, chondrocytes form cartilage and subsequently epiphyseal growth plates that allow for longitudinal growth of bones during postnatal development. This is achieved by chondrocytes in three different ways: through rapid proliferation and differentiation of cells, through secretion of matrix proteins, matrix mineralization and regulation of angiogenesis and through programmed cell death (Goldring 2012).

The extracellular matrix surrounding chondrocytes can be divided into three distinct areas (Fig. 1.2). Firstly, the pericellular zone that directly surrounds the cell, and contains molecules that directly interact with cell surface receptors, for example hyaluronic acid binding to CD44. It is also rich in proteoglycans (predominantly aggrecan, but also SLRPs like decorin, biglycan, lumican and fibromodulin to a lower extent) and non-collagenous proteins like the cell membrane associated molecule anchorin CII and microfibril-forming collagens like collagen type VI (Mollenhauer et al. 1984; Hagiwara et al. 1993). Moreover, it contains little to no fibrillar collagen. Next is the territorial zone, which surrounds the pericellular zone. Collagen fibers situated at various angles form a fibrillar basket around the chondrocytes, which may provide mechanical protection (Buckwalter and Mankin 1998). Finally, the interterritorial zone has the furthest distance to the cell. It fills out most of the space in cartilage and contains collagen fibrils of large diameter as well as large aggregates of aggrecan and hyaluronic acid (Heinegård and Saxne 2011).
Figure 1.2 Composition of the chondrocyte extracellular matrix (ECM). The ECM is divided into the pericellular, territorial and interterritorial zone. Each zone has a distinct protein composition which results in specific functions in the ECM. Proteins involved in cell attachment to ECM (fibronectin, syndecan and CD44) are found in the pericellular zone. Aggrecan and other proteoglycans (decorin, biglycan, fibromodulin) are present in all three zones. Matrilins (1 and 3), and collagen molecules (type II, VI, IX, XI) can be found predominantly in the territorial and interterritorial zone.

Cartilage found at different body sites is distinct, both due to differences in embryonic origin of the cells as well as environmental conditions. Articular cartilage covers the bone surface in synovial joints and is biochemically different from other cartilage. It has a low cell to matrix ratio: in humans cartilage contains approx. 1% cells (Stockwell 1967). It is responsible for the weight-bearing abilities of articular joints and therefore a painless movement. Articular cartilage is comprised of several zones (Fig. 1.3). Starting from the surface, there is the superficial zone which contains discoid chondrocytes and well-ordered collagen fibers. It is also the thinnest zone, consisting of two layers: a sheet of fine fibrils, which is also called lamina splendens, and the cell-containing layer. Relative to other layers, the matrix composition of the superficial layer shows high fibrillar content and little proteoglycan content. In addition, water and fibronectin concentrations are also highest in this layer. The collagen fibrils are arranged parallel to the joint surface, providing more tensile stiffness and strength than deeper zones. It has also been suggested that the superficial layer protects the cartilage matrix from the immune system due to its function as a barrier for large molecules from synovial fluid (Buckwalter and Mankin 1998). Below the superficial zone lies the middle zone. It has several times the volume of its overlying layer and contains chondrocytes of spheroidal shape, which produce a matrix rich in collagen fibrils with large diameters. Moreover, a higher concentration of proteoglycans and lower concentration of water than in the superficial zone can be found (Buckwalter and Mankin 1998). The zone below the middle zone is called deep zone and contains spherical chondrocytes, which accumulate together vertical to the joint axis. This zone contains the largest collagen fibrils and highest concentration of proteoglycans. This is followed by the zone of calcified matrix, which is directly on top of subchondral bone (Hayes et al. 2007). The chondrocytes of this zone have a smaller volume and appear to be completely surrounded by calcified cartilage (Buckwalter and Mankin 1998).
Figure 1.3 Cellular organization of articular cartilage. Articular cartilage is organized in four different zones, starting with the superficial zone at the top, followed by the middle zone, deep zone and finally the calcified zone. Each zone is characterized by a unique organization of collagen fibers and chondrocytes. The subchondral and cancellous bone provides a strong base for articular cartilage.

1.6 Chondrocyte progenitor cells

Chondrocyte progenitor cells are a population of adult mesenchymal stem cells, residing in the superficial zone of articular cartilage. They are capable of differentiating into a chondrogenic, osteogenic or adipogenic lineage, becoming chondrocytes, bone cells or adipocytes, respectively. This cell population was initially discovered and isolated by Dowthwaite et al. (2004), using differential fibronectin adhesion. The principle of this method is that the progenitor cells are more rapidly and strongly adhering to a fibronectin-coated surface due to high levels of expression of $\alpha_5\beta_1$ integrin (Jones and Watt 1993). In contrast to normal full-depth chondrocytes which lose their chondrogenic potential after 8 population doublings (PD) (Dell’Accio et al. 2001; Schnabel et al. 2002; Cournil-Henrionnet et al. 2008), chondrocyte progenitor cells keep their chondrogenicity even after extensive culture in monolayers and a high number of PD, without losing the chondrocyte specific transcription factor SOX9 (Williams et al. 2010).

1.7 Cartilage extracellular matrix

The extracellular matrix (ECM) found in cartilage is a complex composite of macromolecules, which are capable of self-assembly, mostly by non-covalent bonds. The major components are collagens, elastin, hyaluronic acid, non-collagenous glycoproteins and proteoglycans (Gentili and Cancedda 2009; Heinegård and Saxne 2011). The ECM surrounds the cells and serves as a scaffold. The organization of collagen fibrils into a fibrillar meshwork provides elasticity and mechanical strength and provides a substrate for cell migration and adhesion. In addition, it creates a barrier for cell penetration and filtration of macromolecules (Hocking et al. 1998; Kresse and Schönherr 2001). The ECM is also a reservoir of growth factors and modulates their activity. By interacting with matrix molecules, growth factors become sequestered from their signaling receptors, potentially activated by proteolytic processing and presented to the cells in a specific conformation or in the context of ECM components. There is evidence that ECM molecules can also exhibit a potent direct signaling function by interacting with matrix receptors like integrins or
1.8 Collagen
Collagens are an essential component of ECM in many tissues. They provide stability to the matrix and are important for the mechanical properties and stiffness of the tissue (Hulmes 2008). Collagen molecules are trimeric, being composed of three alpha chains. Roman numerals are used to classify collagens based on the order of discovery and structural homology (Birk and Bruckner 2011). The chains form a triple helical structure, which can be up to 300 nm long in the case of fibril forming collagens type I, II and III. The individual chains are characterized by a repeating \((\text{Gly-X-Y})_n\) motif, with \(X\) and \(Y\) being any amino acid but often proline and hydroxyproline (Birk and Bruckner 2011). In addition to hydroxyproline, hydroxylysine is also found in collagens. Both unique amino acids are important for triple helix stability. One turn of the alpha chain helix is composed by only three amino acids. It has the shape of a left handed helix, whereas the triple helix has a right handed twist.

Collagens are synthesized as procollagens, containing N- and C-terminal propeptides. This was assumed to prevent the formation of collagen fibrils inside the cell although recent data from Kadler and colleagues suggest that fibrillogenesis may occur in specialized intracellular compartments (Canty-Laird et al. 2012). After translation at the ribosomes, the procollagen alpha chains are post-translationally modified in the endoplasmic reticulum (Hulmes 2008). Prolyl- and lysyl-hydroxylases form hydroxyproline (mostly to proline at the Y position) and hydroxylysine (only to lysine in the Y position), respectively. Hydroxylysine can be modified through addition of monosaccharides. After these modifications, folding of the triple helix starts from the C-terminal propeptides to the N-terminal part of the molecules. By passing through the Golgi system, the triple helix is further modified through addition of oligosaccharides. Finally, N- and C-terminal propeptides are cleaved off by proteases (for example ADAMTS-2/-3/-14, BMP-1), and the collagen molecules assemble into fibrils. The resulting supramolecular structures are...
further cross-linked by lysyl oxidases, which provides additional mechanical stability. Interestingly, a recent paper by Bruckner and colleagues demonstrated transglutaminase crosslinks in mature collagen fibrils (Wang et al. 2014). The fibrils can contain many different types of collagen molecules. In cartilage, collagen type II is the predominant fibril forming collagen (Eyre 2002). These collagen fibrils also contain collagen type IX and XI (Mendler et al. 1989; Bruckner et al. 1985). Furthermore, several non-collagenous proteins like COMP and the SLRPs decorin, biglycan and fibromodulin are involved in regulation and organization of fibrillar growth (Birk and Bruckner 2011). An additional type of collagen only found in cartilage is type X collagen. It belongs to the group of network-forming collagens and is associated with chondrocyte hypertrophy and mineralization (Birk and Bruckner 2011).

1.9 Proteoglycans
Proteoglycans are proteins that contain one or more glycosaminoglycan chain attached to their core protein (Esko et al. 2009). Most proteoglycans also contain N-linked oligosaccharide branches. Cartilage contains two major classes of proteoglycans. On the one hand there are large aggregating proteoglycans with >100 GAG chains like aggrecan, on the other hand small leucine-rich proteoglycans (SLRPs) like decorin, biglycan, lumican and fibromodulin, which contain only a small number of GAG chains (Buckwalter and Mankin 1998). About 90 percent of total cartilage proteoglycans are made up by aggrecan, filling most of the interfibrillar space. Aggrecan contains GAG chains made from chondroitin sulphate and keratan sulphate, whereas SLRPs are variably glycanated in a tissue specific manner with either chondroitin sulphate or dermatan sulphate (decorin, biglycan) or keratan sulphate (lumican, fibromodulin).

1.9.1 Aggrecan
The predominant large proteoglycan found in cartilage is aggrecan. Next to collagen type II, it is one of the major components of articular cartilage ECM.
The core protein is composed of 3 globular domains (G1-G3), an interglobular domain (IGD) and attachment sites for chondroitin sulphate and keratan sulphate chains. The fully processed protein contains about 100 chondroitin sulphate chains and about 50 keratan sulphate chains (Wight et al. 2011). Its name relates to its ability to form large aggregates through binding to hyaluronic acid chains within the extracellular matrix. Aggrecan binds non-covalently to hyaluronic acid through its N-terminal G1 domain in combination with link protein, forming large proteoglycan aggregates (Buckwalter and Rosenberg 1982; Buckwalter and Rosenberg 1983). Link protein is an important factor involved in the binding of aggrecan to hyaluronic acid. Mouse studies have shown that knock-down of link protein results in deformed limbs and early postnatal death (Czipri et al. 2003), probably because of insufficient ECM organization. The large aggregates comprised of aggrecan and hyaluronic acid are compressed within the collagen type II network to about a third of their normal volume. This leads to an enormous charge density resulting from the sulphate esters and carboxyl groups within chondroitin sulphate and to a smaller extent keratan sulphate. The attracted water molecules together with the constrained GAG chains create a large swelling pressure on the collagen network (Wight et al. 2011). This leads to compressibility of the tissue and excellent resilience to load of the cartilage ECM, which is ultimately the major function of the tissue.

1.9.2 Small Leucine-Rich Proteoglycans
The group of small leucine rich proteoglycans (SLRP) is part of the LRR (leucine-rich repeat proteins) superfamily. In general they are defined by a degenerate consensus sequence which is characterized by leucins in conserved positions: \(x Lx x N \pm x a x x \pm x \pm x x \pm x\), where \(L\) is leucine or isoleucine, \(N\) is asparagine (but can also be substituted with threonine or cysteine), \(x\) is any amino acid, \(\pm\) indicates a loose requirement for an amino acid and “a” indicates an aliphatic amino acid (Kobe and Deisenhofer 1994). Repeats can have a length between 20-29 amino acids, but most common are 24 amino acids. SLRPs additionally possess a unique N-terminal
domain, which is flanked by cysteine clusters and contains four similarly spaced cysteine residues within a 20 amino acid stretch at its N-terminal end, forming disulphide bonds. At its C-terminal end, two cysteine residues are forming an intra-chain disulphide bond. Every member of the SLRP family has a terminal LRR domain, which runs through the C-terminus of the individual protein, and therefore does not satisfy the requirements of a true repeat. However, it has been suggested that the repeats that reside between the cysteine clusters are the ones that are functionally and structurally important (Hocking et al. 1998).

The SLRP gene family has expanded over the last decades and includes 17 proteins (Iozzo 1997; Hocking et al. 1998; Iozzo 1999). Even though not all of them contain at least one GAG chain, they all share a common functionality. They are classified into five distinct groups based on homology and conservation at the genomic and protein level and their specific N-terminal cysteine-rich clusters (Table 1.1) (Schaefer and Iozzo 2008).

An additional typical structural feature of SLRPs is the so-called “ear repeat” motif. Decorin, for instance, contains this structure, which is made of the 11th LRR that extends laterally from the main body at the C-terminal end, where a cysteine forms a disulphide bond with a second cysteine in the 12th LRR (McEwan et al. 2006). All class I-III SLRPs contain the ear-repeat, but not class IV and V. Therefore, it was suggested that this specific feature is an indicator for the “true” SLRP family. Nevertheless, this is true only on a structural, but not functional level. For example, tsukushi (class IV) is like biglycan (class I) a potent modulator of bone morphogenetic protein (BMP) (Chen et al. 2004; Ohta et al. 2004; Moreno et al. 2005). Podocan (class V) inhibits cell growth and binds to collagen type I like some of the class I-III proteins (Shimizu-Hirota et al. 2004).
Table 1.1 Overview of small leucine-rich proteoglycans (SLRP). Members of the same class share the cysteine-rich cluster consensus sequence. Adapted from Schaefer et al. (2008).

<table>
<thead>
<tr>
<th>SLRP class</th>
<th>Proteins</th>
<th>Cysteine-rich cluster consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Decorin, Biglycan, Asporin</td>
<td>Cx3Cx6C</td>
</tr>
<tr>
<td>II</td>
<td>Fibromodulin, Lumican, PRELP, Keratocan, Osteoadherin</td>
<td>Cx3Cx9C</td>
</tr>
<tr>
<td>III</td>
<td>Epiphythcan, Opticin, Osteoglycin</td>
<td>Cx3Cx9C</td>
</tr>
<tr>
<td>IV</td>
<td>Chondroadherin, Nyctalopin, Tsukushi</td>
<td>Cx3Cx8-17C</td>
</tr>
<tr>
<td>V</td>
<td>Podocan, Podocan-like protein 1</td>
<td>Cx3.4Cx9C</td>
</tr>
</tbody>
</table>

### 1.9.2.1 SLRP class I proteins

The group of class I SLRPs consist of decorin, biglycan and asporin. Their N-terminal cysteine cluster forms 2 disulphide bonds. Decorin and biglycan contain one and two chondroitin / dermatan sulphate chains, respectively, in contrast to asporin, which lacks the xylosyltransferase consensus sequence and therefore harbours no GAG chain. That is why it is not a proteoglycan. One general feature of all class I SLRPs is the similar exonic organization of the eight exons with conserved intron/exon junctions (Schaefer and Lozzo 2008). The amino acid sequence of asporin contains a stretch of asparagine residues, which forms a negatively charged acidic domain that can also be found in osteoadherin (class II), epiphythcan (class III) and podocan (class V). Decorin, biglycan, and asporin are commonly expressed in cartilage.

### 1.9.2.2 Decorin

Decorin is a biologically active component of the ECM and not just a structural protein (Merline et al. 2009). It is ubiquitously expressed throughout tissues but is post-translationally modified in a tissue specific manner and was first cloned from a human embryonic fibroblast cell-line (Oikarinen et al. 1989).
There are various names for it like PG40 (due to the mass of its core protein, ~40 kDa), PG-S2, bone proteoglycan-II, small leucine-rich-proteoglycan-1B, dermatan sulphate proteoglycan-II, but many of them are not used anymore (Chen and Birk 2011). Its molecular size is about 100 kDa, which includes the attached GAG chain and the core protein. Human decorin consists of 359 amino acids that fold into 12 tandem leucine-rich repeat (LRR) domains, which are flanked by cysteine-rich loops (Fig. 1.4, 1.5). The twelve LRRs are what give decorin a curved solenoid, horseshoe-like shape with a parallel β-sheet on the inner concave surface (Fig. 1.5). In addition, decorin contains a structural feature called “ear repeat”, as described above. This is thought to contribute to the folding of the 12\textsuperscript{th} LRR and may also be involved in ligand recognition (Chen and Birk 2011). Its pre-propeptide contains a 16 amino acid signal sequence, which leads the translating protein into the ER where it is cleaved co-translationally. Next, the propeptide, which is composed of 14 amino acids, regulates the attachment of the GAG chain. Both core protein and GAG chain contribute to the biological function of decorin (Seidler and Dreier 2008). Decorin can be substituted with two GAG chains in some species, but only one is found in decorin of mammals. One chain, either of chondroitin sulphate or dermatan sulphate type, depending on the tissue, is covalently attached to a serine-residue (Ser34) in the N-terminal part of the human protein (Seidler and Dreier 2008). Traditionally, dermatan sulphate is found in soft tissues (e.g. skin) and chondroitin sulphate in musculoskeletal tissue (e.g. bone and cartilage), although dermatan sulphate is also found during the early stages of bone and predentine formation (Waddington et al. 2003). In addition, three N-glycosylation sites are present (Glössl et al. 1984). It is thought that N-glycosylation contributes to folding and secretion of the protein (McEwan et al. 2006). In its function as a matrix proteoglycan, decorin regulates lateral growth and therefore diameter of collagen fibrils. It “decorates” the collagen I fibrils by binding to their “d” or “e” bands (Scott and Parry 1992). Decorin slows down assembly of collagen fibrils, resulting in a reduction in fibril diameter, at least \textit{in vitro} (Vogel et al. 1984). Decorin also binds to other collagens, e.g. type I, II, III, IV, VI, and XIV (Bidanset et al. 1992; Ehnis et al. 1997; Santra et al. 2002). \textit{In vivo}, decorin was detected in the
interterritorial region around chondrocytes in articular cartilage, being preferentially associated with thicker fibrils, whereas it was absent on the thinner fibrils in the territorial region (Hagg et al. 1998). Besides its regulatory function in fibril formation, decorin also acts as a bridging molecule between collagen types I and VI, where it interacts with both collagens via different binding sites (Nareyeck et al. 2004). Furthermore, it was shown that a complex of decorin and matrilin-1 can act as a bridge between collagen type II and VI fibrils, whereby decorin binds to the globular N-terminal domain of collagen type VI (Wiberg et al. 2001).

Decorin not only serves as a structural element in the ECM, but its core protein also serves as a signalling molecule. It is a ligand for the insulin-like growth factor receptor (ILGFR) (Schönherr et al. 2005) and the epidermal growth factor receptor (EGFR) (Seidler et al. 2006). While decorin activates the IGFR-Akt signalling axis, it has been reported to block the EGF-R signalling in cancer. Therefore it has been proposed as a potential therapeutic agent in cancer treatment (Seidler et al. 2006; Neill et al. 2012).

It has long been known that decorin regulates cellular growth (Yamaguchi et al. 1990). This is mediated by its ability to engage the above receptors, influencing regulation at certain checkpoints in the cell cycle (Merline et al. 2009). A TGF-β-dependent inhibition of Chinese hamster ovary (CHO) cells (Yamaguchi et al. 1990), arterial smooth muscle cells (Fischer et al. 2001), human hepatic stellate cells (Shi et al. 2006) and fibroblasts (Zhang et al. 2007) were also observed.

It was reported that some SLRPs dimerize with high affinity (Scott et al. 2004; McEwan et al. 2006), but this may be true only in vitro and not in vivo. They are more likely to be monomeric with regards to their biological function (Goldoni et al. 2004). For instance, the binding site for collagen type I in the concave shape of decorin’s LRR6 (Kalamajski et al. 2007) would not be accessible in a dimeric decorin. Moreover, in regards of the dimensions of the decorin core protein (Scott et al. 2004), a decorin dimer would not fit into the EGFR binding groove. However, a detailed understanding of monomer-dimer transition and its regulation is outstanding to fully answer this question. A
possible situation for SLRPs undergoing monomer-dimer transitions is the exposure of their specific binding sites. As a result, the functional activity of a SLRP would be regulated by the intrinsic affinity for its cognate receptor and this could contribute to specialization and functional differentiation (Santra et al. 2002; McEwan et al. 2006; Schaefer and Iozzo 2008).

In the decorin-knockout mouse, collagen type I fibrils are irregular, showing a cross-sectional contour presenting notches and frequent protuberances (Danielson et al. 1997; Corsi et al. 2002; McEwan et al. 2006; Schaefer and Iozzo 2008). As a consequence, the skin is fragile, has a markedly reduced tensile strength and the dermis is thinner compared to the wild type. This phenotype is similar to the fragile skin observed in humans suffering from Ehlers-Danlos syndrome (EDS) (Danielson et al. 1997). EDS is a heterogeneous syndrome, which involves skin hyperflexibility, joint hypermobility and tissue fragility of different severity depending on the underlying genetic defect / EDS type (Mao and Bristow 2001). The decorin-knockout mouse also shows a random orientation of collagen fibrils in the periodontal ligament (Hakkinen et al. 2000), which underlines the regulatory role of decorin in collagen fibril organization.
Figure 1.4 Schematic structure of the class I SLRP decorin. It contains a signal peptide (SP, cyan), propeptide (PP, pink), and two cysteine rich domains (yellow) flanking the 12 leucine-rich repeat domains (blue and yellow, respectively). Signal- and propeptide are removed prior to secretion. One GAG chain of the chondroitin / dermatan sulphate type is attached to a serine residue in the N-terminal part. In addition, three N-linked oligosaccharides can be found on leucine-rich repeat domains. Based on Chen et al. (2001).

Figure 1.5 Crystal structure of decorin (PDB #1XKU). Leucine-rich repeat domains (LRRs, green) are flanked by N- and C-terminal cysteine-rich domains (red). Decorin adapts a curved shape that has been described as horseshoe-like in some literature, but the actual structure is too rigid to undergo bending to such an extent. The figure was prepared using Pymol software package (Schrödinger 2010) and data set #1XKU from the PDB (www.pdb.org) created by Scott et al. (2004).
1.9.2.3 SLRP class II proteins
The class of type II SLRPs consist of 5 proteins: fibromodulin, lumican, PRELP, keratocan and osteoadherin. Apart from osteoadherin, all proteins are expressed in cartilage. One feature that distinguishes this class from others is the presence of sulphated tyrosine residues in their N-terminal domain, which could contribute to the polyanionic character of SLRPs (Ameye and Young 2002; Corsi et al. 2002; Goldberg et al. 2003). They mostly contain KS and polylactosamine, a non-sulphated variant of KS. The genes of class II SLRPs have a similar exonic organization of 3 exons, and a large exon that encodes most of the LRRs (Schaefer and Iozzo 2008).

1.9.2.4 SLRP class III proteins
Epiphycan, opticin and osteoglycin embody the group of class III SLRPs. These proteins are characterized by a genomic organization of seven exons and a number of six LRRs (Schaefer and Iozzo 2008). Like class II SLRPs, their N-terminal domain contains sulphated tyrosine residues (Ameye and Young 2002). All three proteins are expressed in cartilage.

1.9.2.5 SLRP class IV proteins
To the novel class of type IV SLRPs belong tsukushi, chondroadherin, and nyctalopin (Bech-Hansen et al. 2000; Pusch et al. 2000; Ohta et al. 2004). Nyctalopin is the first described glycosylphosphatidyl-anchored SLRP and the second that is linked to the X chromosome. Together with tsukishi it contains eleven homologous LRRs, which are flanked by N-terminal cysteine-rich regions. Like some class I SLRPs (Chen et al. 2004; Moreno et al. 2005), tsukushi is a BMP inhibitor, which forms a complex with chordin and BMP (Ohta et al. 2004; Ohta et al. 2006). Unlike tsukushi and nyctalopin, chondroadherin is expressed by chondrocytes.
1.9.2.6 SLRP class V proteins

Class V SLRPs are a new non-canonical class of SLRPs (Schaefer and Iozzo 2008). This class is composed of two genes: podocan, which is located on chromosome 1 (Ross et al. 2003) and podocan-like protein 1, which can be found on chromosome 19. Although the proteins contain different C-terminal cysteine-rich clusters compared to other SLRP classes, they have 20 LRRs, which are homologous to some class I and II SLRPs (Schaefer and Iozzo 2008). However, podocan is able to bind collagen type I and inhibits cell growth by inducing p21 expression (Shimizu-Hirota et al. 2004). None of the class V proteins is expressed in cartilage.

1.10 Glycosaminoglycans

Glycosaminoglycan (GAG) chains consist of a linear, unbranched chain of disaccharide units, which are made of a composition of a hexosamine (N-acetylgalactosamine or N-acetylglucosamine), and a hexuronic acid (HexA, glucuronic acid or iduronic acid) or hexose (galactose) (Esko et al. 2009). Due to the carboxyl-group of the hexuronic acid and the potential sulphate esters, which can be attached to the hexuronic acid and the amino sugar, GAG chains are strongly negatively charged. As a result, they have a very large binding capacity for water molecules, which imparts osmotic pressure and elasticity on the tissue, an important feature of cartilage as already discussed. Several different GAG chain types can be distinguished, depending on its composition: hyaluronic acid, heparin/heparan sulphate, chondroitin sulphate, dermatan sulphate, and keratan sulphate (Table 1.2).

Hyaluronic acid (HA) consists of GlcA-β-1,3-GlcNAc disaccharide units, which are β-1,4 linked to each other. The length can reach up to $1 \times 10^5$ units in one chain (Esko et al. 2009). The disaccharide units are not sulphated. Unlike all other GAG types, it is synthesized by enzymes, HA syntheses, at the cell surface. HA is an amphipathic helix and is not attached to a core protein.

Heparin and heparan sulphate (HS) consist of GlcA-β-1,4-glucosamine or IdoA-α-1,4-glucosamine disaccharide units, which are α-1,4 linked to each
other (Esko et al. 2009). Heparin is only produced in mast cells, whereas HS is produced throughout all tissues. Heparin differs from HS by the amount of modifications of the sugar residues. Its disaccharide units are more sulphated and contain a significantly higher amount of iduronic acid compared to HS. Moreover, heparin is also a strong anticoagulant because of its ability to bind to antithrombin-III due to its negative charge.

The disaccharide units of keratan sulphate (KS) consist of Gal-β-1,4-GlcNAc, which are β-1,3 linked to each other. Both sugars can be sulphated at their C-6 atom, but Gal is rarely found to be sulphated (A. Plaas et al. 2001). The two types of KS (KS I and KS II) can be distinguished by their linkage to the protein core. KS I is predominantly found in cornea and N-linked to an asparagine residue of the core protein. KS II in contrast is O-linked to a serine/threonine residue via GalNAc.

Chondroitin sulphate (CS) and dermatan sulphate (DS) consist of GlcA-β-1,3-GalNAc units and IdoA-β-1,3-GalNAc, respectively. The subunits are β-1,4 linked to each other (Esko et al. 2009). The hexuronic acids can be sulphated at the C-2 position and the GalNAc at the C-4 and C-6 position. DS is derived from CS. The enzyme C5-epimerase uses GlcA as a substrate to epimerize the carboxyl group at its C-5 atom, which then becomes IdoA. The reverse reaction that prevents the “flip-back” of the carboxyl group is blocked by the sulphation of the hydroxyl group at the C-4 atom of GalNAc (Kusche-Gullberg and Kjellen 2003). If the epimerization and sulphation reactions are coupled, the two involved enzymes may interact and form a complex with each other. Since not all GlcA-residues in CS are epimerized to IdoA, DS GAG-chains usually contain a mixture of CS and DS.

The biological function of GAG chains is strongly influenced by its composition and also post-translational modification. A large body of investigation was performed on the function of HS chains found attached on cell surface proteoglycans like syndecans or glypicans (Zako et al. 2003; Multhaupt and Couchman 2012; Gallagher 2006). The biological function of these HS-proteoglycans partly depends on the variety of posttranslational modifications of the HS chains, e.g. sulphation and epimerization. These modifications lead
to the ability of the HS chain to bind growth factors or regulate the activation of cell surface receptors, which was shown for FGFs, TGF-β superfamily, Wnt, and others (Zako et al. 2003). This example shows that the structural patterns generated during GAG chain synthesis and modification are very important in the overall biological context and may also be influenced in the pathogenesis of degenerative joint diseases like osteoarthritis.
Table 1.2 Overview of GAG chains. Each type consists of different disaccharide subunits, which are repeatedly connected with each other. They can also be specifically modified through the addition of sulphate groups at designated hydroxyl groups of the hexuronic acid and the hexosamine.

<table>
<thead>
<tr>
<th>GAG Type</th>
<th>Subunit Structure</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin sulphate</td>
<td>GlcA-β-1,3-GalNAc-β-1,4</td>
<td>Sulphation on C-4 and/or C-6 of GalNAc and on C-2 of GlcA</td>
</tr>
<tr>
<td>Dermatan sulphate</td>
<td>IdoA-α-1,3-GalNAc-β-1,4</td>
<td>Sulphation on C-4 and/or C-6 of GalNAc (must be present) and on C-2 of IdoA</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>GlcA-β-1,3-GlcNAc-β-1,4</td>
<td>No modifications</td>
</tr>
<tr>
<td>Keratan sulphate</td>
<td>Gal-β-1,4-GlcNAc-β-1,3</td>
<td>Sulphation on both C-6 of Gal and GlcNAc</td>
</tr>
<tr>
<td>Heparin / Heparan sulphate</td>
<td>GlcA-β-1,4-GlcNAc-α-1,4 (top)</td>
<td>Sulphation on C-2 and C-6 of Glucosamine and on C-2 of IdoA or GlcA</td>
</tr>
<tr>
<td></td>
<td>IdoA-α-1,4-GlcNAc-α-1,4 (bottom)</td>
<td></td>
</tr>
</tbody>
</table>
1.10.1 **Synthesis of chondroitin sulphate**

CS/DS chains consist like all other GAGs (except HA) of three distinct structural regions: a linkage region which connects the reducing end of the chain to the core protein, a large region between the reducing and non-reducing end which contains the repetitive disaccharide units, and a non-reducing end terminal region (Midura et al. 1994). CS/DS and heparin/HS share the same tetrasaccharide linkage sequence Xyl-Gal-Gal-GlcA. Xyl is connected by an O-glycosidic linkage to a serine residue in the core protein sequence. This reaction is catalyzed by a xylosyl transferase (XylT). No single consensus recognition pattern of XylT for setting a starting point for GAG chain synthesis onto the core protein sequence has been identified yet. However, in general a glycine follows a substituted serine, and the Gly-Ser pair is led by acidic amino acid residues (Kjellen and Lindahl 1991). Alignment of sequences of over 50 CS attachment sites from 19 proteoglycans identified a potential consensus sequence of a-a-a-a-Gly-Ser-Gly-a-b-a, where “a” is a Glu or Asp and “b” is a Gly, Glu or Asp. However, this does not explain why often potential Gly-Ser pairs are not substituted. Therefore, other factors like proximity to other substituted sites, downstream sequences, or secondary structures may be important. Other sequences and structural features may contribute to the specification of the type of GAG, for example CS/DS or heparin/HS chains on the mutual linkage region (Silbert and Sugumaran 2002).

The basic building blocks of GAGs are disaccharide units, which originate from sugar nucleotide precursors like UDP-Xyl, UDP-Gal, UDP-GlcA, UDP-GalNAc, and UDP-GlcNAc. The main precursor for their synthesis is glucose, whereas galactose derived from lactose can be the precursor for UDP-Gal, UDP-GlcA and UDP-Xyl. They serve as substrate for the various glycosyltransferases involved in chain formation (Table 1.3) (Silbert and Sugumaran 2002). The sugar nucleotides are directly added to the appropriate acceptor sites without the formation of any intermediates. The synthesis of the linkage region and the disaccharide polymer is segregated within the endoplasmic reticulum (ER), transfer vesicles and Golgi system. However, unlike N-glycosylation of glycoproteins which occurs co-translationally, GAG chain synthesis occurs on the folded core proteins.
The initial addition of xylose by XylT (Table 1.3) starts in the ER and continues in the early Golgi (Silbert and Sugumaran 2002). The following additions of the two galactoses are catalyzed by two different galactose transferases (GalT-1 and GalT-2) (Kearns et al. 1993). They reside in different parts of the cis/medial Golgi (Sugumaran et al. 1992). Next, the first GlcA residue is added by GlcA 1 transferase (GlcAT-1) in the medial/trans Golgi regions, where also CS polymerization takes place (Sugumaran et al. 1998). The attachment of the first GalNAc residue by GalNAc 1 transferase (GalNAcT-1) determines the fate of the forming GAG-chain in becoming a CS/DS polymer. This transferase is also responsible for further polymerization of the chain by adding GalNAc (Uyama et al. 2002). When the first added hexosamine is a GlcNAc, the GAG chain becomes heparin/HS. Despite all that, the GalNAcT-1 must be able to differentiate between the primers for CS and heparin/HS in some way. This might be regulated by the capping of undesired primers. The enzymes involved may also recognize structures or sequences of the core protein which appear as transient modifications of the linkage region (Kitagawa et al. 1999; Nandanaka et al. 1999). CS is the predominant GAG on cartilage proteoglycans.

Recent studies with mice deficient of GalNAcT-1 showed that their cartilage was smaller and contained only half of the CS amount of the respective wild type or heterozygous mice. In addition, CS in collagen type II fibres (collagen IX associated with fibre surface) were abnormally arranged. Despite the fact that GalNAcT-2 is also able to attach a GalNAc residue to the polymerizing CS chain and to the linkage tetrasaccharide, it was apparently not able to compensate for the loss of GalNAcT-1. This indicates that GalNAcT-1 plays an important role in CS synthesis in developing cartilage (Watanabe et al. 2010).

The polymerization of the GAG chain takes place in a highly organized manner with GlcAT-2 and GalNAcT-2 acting in concert adding sugar monomers to the non-reducing end of the forming chain, which can be as large as 70 kDa (Silbert and Sugumaran 2002). It is notable that each sugar unit is added independently and the addition of the following sugar is delayed until the
concentration of the activated binding block with the required sugar is high enough. As a reason for this, it is thought that the GalNAc transferase activity and GlcA transferase activity are on two different enzymes (Silbert and Reppucci 1976). However, a protein, which has both transferase activities was isolated and has been denoted as chondroitin synthase 1 (ChSy-1). This protein is unable to add a GalNAc to the GlcA residue in the linkage region, which separates it from the GalNAcT-1. However, ChSy-1 shows both β1,3-GlcA transferase and β1,4-GalNAc transferase activities (Kitagawa et al. 2001). Interestingly, the chondroitin polymerizing activity could not be demonstrated by recombinantly expressed ChSy-1, indicating a more complex mechanism for this enzyme. Kitagawa and colleagues discovered, that the presence of another protein denoted as chondroitin polymerizing factor (ChPF), was required for full ChSy-1 activity (Kitagawa et al. 2003). Furthermore, two more chondroitin polymerases (ChSy-2 and ChSy-3) were cloned (Yada, Gotoh, et al. 2003; Yada, Sato, et al. 2003). Remarkably, all chondroitin polymerases and ChPF on their own show only very little enzyme activity, but distinct chondroitin polymerization was achieved when any two of these enzymes were combined. Two additional enzymes with both GalNAcT-1 and GalNAcT-2 activity were more recently discovered. They are called ChGn-1 and ChGn-2 and are thought to be able to catalyse the initiation and also polymerization of the CS chain (Uyama et al. 2002; Gotoh et al. 2002; Sato et al. 2003; Uyama et al. 2003). A schematic representation of all the steps in the biosynthesis of CS chains is given in Fig. 1.6.
Table 1.3 Summary of enzymes involved in synthesis of CS/DS GAG chains with their corresponding abbreviation used in literature and respective substrate (underlined). Partially adapted from Fransson et al. (2000) and Mikami et al. (2013).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abbreviation</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>xylosyl-transferase</td>
<td>XylT</td>
<td>..D/E..SG..(D/E)</td>
</tr>
<tr>
<td>galactosyl-transferase 1</td>
<td>GalT-I</td>
<td>Xyl-Ser</td>
</tr>
<tr>
<td>galactosyl-transferase 2</td>
<td>GalT-II</td>
<td>Gal-Xyl-Ser</td>
</tr>
<tr>
<td>glucuronyl-transferase 1</td>
<td>GlcAT-I</td>
<td>Gal-Gal-Xyl-Ser</td>
</tr>
<tr>
<td>glucuronyl-transferase 2</td>
<td>GlcAT-II</td>
<td>GalNAC-β1,4-GlcA</td>
</tr>
<tr>
<td>chondroitin N-acetylgalactosaminyl-transferase 1</td>
<td>GalNAcT-I</td>
<td>GlcA-Gal-Gal-Xyl-Ser; GlcA-β1,3-GalNAc</td>
</tr>
<tr>
<td>chondroitin N-acetylgalactosaminyl-transferase 2</td>
<td>GalNAcT-II</td>
<td>GlcA-Gal-Gal-Xyl-Ser; GlcA-β1,3-GalNAc</td>
</tr>
<tr>
<td>chondroitin synthase 1</td>
<td>ChSy-1</td>
<td>GlcA-β1,3-GalNAc; GalNAc-β1,4-GlcA</td>
</tr>
<tr>
<td>chondroitin synthase 2</td>
<td>ChSy-2</td>
<td>GlcA-β1,3-GalNAc; GalNAc-β1,4-GlcA</td>
</tr>
<tr>
<td>chondroitin synthase 3</td>
<td>ChSy-3</td>
<td>GlcA-β1,3-GalNAc; GalNAc-β1,4-GlcA</td>
</tr>
<tr>
<td>chondroitin polymerizing factor</td>
<td>ChPF</td>
<td>GlcA-β1,3-GalNAc</td>
</tr>
<tr>
<td>chondroitin GalNAc transferase 1</td>
<td>ChGn 1</td>
<td>GlcA-Gal-Gal-Xyl-Ser; GlcA-β1,3-GalNAc</td>
</tr>
<tr>
<td>chondroitin GalNAc transferase 2</td>
<td>ChGn 2</td>
<td>GlcA-Gal-Gal-Xyl-Ser; GlcA-β1,3-GalNAc</td>
</tr>
<tr>
<td>dermatan sulphate/C-5 epimerase</td>
<td>DS-epi</td>
<td>GlcA-GalNAc</td>
</tr>
</tbody>
</table>
Figure 1.6 Schematic overview of the CS synthesis. Initially, the tetrasaccharide linkage region is synthesized, followed by the assembly of repeating disaccharide subunits forming the CS chain. It can be further modified by specific sulphotransferases and epimerases. XylT: xylosyl-transferase, GalT-I: galactosyl-transferase 1; GalT-II: galactosyl-transferase 2; GlcAT-I: glucuronyl-transferase 1; GlcAT-II: glucuronyl-transferase 2; GalNAcT-I: Chondroitin N-acetylgalactosaminyl-transferase 1; GalNAcT-II: Chondroitin N-acetylgalactosaminyl-transferase 2; ChSy: chondroitin synthase; ChPF: chondroitin polymerizing factor; ChGn: chondroitin GalNAc transferase; C4ST: chondroitin 4-O-sulphotransferase; C6ST: chondroitin 6-O-sulphotransferase; D4ST: dermatan 4-O-sulphotransferase; UST: uronyl 2-O-sulphotransferase; GalNAc4S-6ST: GalNAc 4-sulphate 6-O-sulphotransferase; DS-epi: GlcA C-5 epimerase (DS epimerase).

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### 1.10.2 Post-translational modification of chondroitin sulphate

CS can be modified by kinases, sulphotransferases and epimerases, which takes place in the *trans* Golgi compartments (Silbert and Sugumaran 2002). The sulphation of CS/DS chains can be considered as the most important type of posttranslational modification. Here, 3'-phosphoadenosine 5'-phosphosulphate (PAPS) serves as substrate for sulphotransferases. PAPS is derived from ATP by a single enzyme with both ATP sulphurylase and APS kinase activity (Lyle et al. 1994). The main proportion of sulphate groups is found within the repeating disaccharide subunits of the chain. However, one or two Gal residues in the linkage region were found to be sulphated as well. Interestingly, Gal 4-sulphate is only found in chondroitin 4-sulphate chains and Gal 6-sulphate is only found in chondroitin 6-sulphate chains. Furthermore, no Gal sulphotransferase specific for the linkage region was identified, which indicates that these modifications are catalyzed by the same transferases that sulphate GalNAc residues (Silbert and Sugumaran 2002).

In general, the sulphation of CS/DS is highly variable. Other than the possible sulphation of the linkage region, a diverse range of differently sulphated disaccharide units can be found. The possible CS chain permutations consist of GalNAc 4-sulphate and GalNAc 6-sulphate only, both 4- and 6- sulphated GalNAc in the same GAG chain, GalNAc which is 4,6-sulphated, and also non-sulphated. Moreover, the GlcA can be 2-sulphated and also rarely 3-sulphated. DS usually contains disaccharide units consisting of IdoA 2-sulphate and GalNAc 4-sulphate, but was also found containing GalNAc 6-sulphate as the hexosamine (Silbert and Sugumaran 2002). Additionally, GlcA 2-sulphate in CS is relatively rare. The different forms of CS are listed in Table 1.4.
Table 1.4 Different types of CS chains. Each type comprises a different modification of the basic disaccharide subunit GlcA-GalNAc

<table>
<thead>
<tr>
<th>Chondroitin-type</th>
<th>Systematic name</th>
<th>Chemical nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS A</td>
<td>chondroitin 4-sulphate</td>
<td>GlcA-GalNAc4S</td>
</tr>
<tr>
<td>CS B</td>
<td>dermatan sulphate</td>
<td>IdoA2S-GalNAc4S</td>
</tr>
<tr>
<td>CS C</td>
<td>chondroitin 6-sulphate</td>
<td>GlcA-GalNAc6S</td>
</tr>
<tr>
<td>CS D</td>
<td>chondroitin 2,6-sulphate</td>
<td>GlcA2S-GalNAc6S</td>
</tr>
<tr>
<td>CS E</td>
<td>chondroitin 4,6-sulphate</td>
<td>GlcA-GalNAc4,6diS</td>
</tr>
</tbody>
</table>

Enzymes known as sulphotransferases catalyze the addition of sulphate groups to the above mentioned positions on GlcA, IdoA, GalNAc and also Gal (Fig. 1.7, Table 1.5). Sulphation of GAG chains appears to occur during the polymerization of the chain rather than on the completed chain (Sugumaran and Silbert 1990). Sulphotransferases are type II transmembrane proteins with an N-terminal cytoplasmic domain, a transmembrane domain and a stem region followed by a catalytic domain. So far, little is known about their organization in the Golgi system, but they are likely to form complexes with other enzymes in the same biosynthetic pathway (Kusche-Gullberg and Kjellen 2003).

To date, three different GalNAc-4-O-sulphotransferases (CS4ST1-3) have been cloned and characterized (Yamauchi et al. 2000; Hiraoka et al. 2002; Kang et al. 2002). Moreover, a GalNAc-4-O-sulphotransferase that adds a sulphate group to a GalNAc which is next to an IdoA (D4ST1) has also been cloned and characterized (Evers et al. 2001). CS chains modified by these sulphotransferases are named CS-A and CS-B, respectively (Table 1.4). Pacheco and colleagues showed that there is a close connection between the 4-O-sulphation of GalNAc and epimerization of GlcA to IdoA by C-5-epimerase. Down regulation of D4ST-1 led to a substantial reduction of IdoA residues, which are located in block structures. However, C-5-epimerase activity was not affected (Pacheco et al. 2009). As already mentioned, this may
be due to the fact that the 4-O sulphation of GalNAc prevents the back-epimerization of IdoA to GlcA (Silbert and Sugumaran 2002).

C6ST is a sulphotransferase that catalyses 6-O sulphation of GalNAc (Yamauchi et al. 2000), leading to the generation of CS-C (Fig. 1.7, Table 1.4, Table 1.5). A higher degree of sulphation can be achieved by the sequential sulphation of C6ST followed by UST, a sulphotransferase specific for 2-O sulphation of GlcA/IdoA (Kobayashi et al. 1999). This leads to the formation of CS-B and CS-D (Fig. 1.7, Table 1.4, Table 1.5). Moreover, disulphated CS-E can be generated through 4-O sulphation by C4ST1 followed by 6-O sulphation of GalNAc4S-6ST (Ohtake et al. 2001). This sulphotransferase needs 4-O sulphated GalNAc as a substrate (Mikami and Kitagawa 2013).

Figure 1.7 Pathways of modifications of CS/DS chains. Based on the substrate specificities of CS sulphotransferases, two pathways based on 4-O and 6-O sulphation can be determined. DS synthesis is based on the initial formation of IdoA by DS-epi, followed by subsequent 4-O sulphation of the adjacent GalNAc.

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Table 1.5 Summary of mammalian CS/DS sulphotransferases and kinases described in literature, including the corresponding abbreviation and substrate (underlined). Adapted from Kusche-Gullberg et al. (2003)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abbreviation</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>chondroitin 4-O-sulphotransferase 1</td>
<td>C4ST1</td>
<td>GlcA-GalNAc-</td>
</tr>
<tr>
<td>chondroitin 4-O-sulphotransferase 2</td>
<td>C4ST2</td>
<td>GlcA-GalNAc-</td>
</tr>
<tr>
<td>chondroitin 4-O-sulphotransferase 3</td>
<td>C4ST3</td>
<td>GlcA-GalNAc-</td>
</tr>
<tr>
<td>dermatan 4-O-sulphotransferase 1</td>
<td>D4ST1</td>
<td>IdoA-GalNAc-</td>
</tr>
<tr>
<td>chondroitin 6-O-sulphotransferase</td>
<td>C6ST</td>
<td>GlcA-GalNAc-</td>
</tr>
<tr>
<td>chondroitin 4-sulphate 6-O-sulphotransferase</td>
<td>GalNAc4S-6ST</td>
<td>GlcA-GalNAc4S-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IdoA-GalNAc4S-</td>
</tr>
<tr>
<td>galactosaminyl uronyl 2-O-sulphotransferase</td>
<td>UST</td>
<td>GlcA-GalNAc6S-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IdoA-GalNAc±4S-</td>
</tr>
<tr>
<td>xylosyl-kinase</td>
<td>Fam20B</td>
<td>Gal-Gal-Xyl-Ser</td>
</tr>
</tbody>
</table>
It has recently been reported that C4ST-1 regulates GalNAc-4O-sulphation and chain length (Klüppel et al. 2005; Uyama et al. 2006). It was demonstrated that CS chain elongation is regulated by C4ST-1 in cooperation with ChGn 2 (Izumikawa et al. 2011). They proposed a model for CS chain polymerization as follows (Fig. 1.8 A): when GlcA is at the non-reducing terminus, ChGn 2 catalyzes the addition of a GalNAc. Next, C4ST-1 facilitates the 4-O-sulphation of this residue. Triggered by this, a GlcA is added by ChSy-1 and ChPF (polymerase complex) and the chain elongation continues (left column). In the case of a GalNAc presented at the non-reducing terminus (right column), it is subsequently 4-O-sulphated by C4ST-1. In the next step, a GlcA is added by the polymerase complex, and then a GalNAc by ChGn 2, which is again 4-O-sulphated by C4ST-1 and so on (Izumikawa et al. 2011). In addition to the above described conventional pathway of CS chain synthesis, a different model was described (Anggraeni et al. 2011). In contrast to the chain polymerization starting from the GlcA of the tetrasaccharide linker by the CS polymerase complex, the first GalNAc is attached to GlcA by ChGn 1 (Fig. 1.8 B). This serves as a substrate for C4ST-2, leading to 4-O sulphation of GalNAc. From then on, chain synthesis is facilitated by the CS polymerase complex (Mikami and Kitagawa 2013).

An early posttranslational event is the phosphorylation at the C-2 atom of xylose in the linker region by Fam20B (Fig. 1.8 C). The modification of xylose is stable in aggrecan from cartilage ECM, but only transient in decorin. Depending on the length of the forming linkage region, phosphorylation goes from 50 to 60% at the Ser-Xyl and Ser-Xyl-Gal stage up to 90% at the Ser-Xyl-Gal-Gal stage (Mikami and Kitagawa 2013). However, the phosphate group is removed during the addition of the first GlcA residue to the linkage region. Probably the phosphorylation serves as a signal for intracellular trafficking or in regulating other following modifications (Silbert and Sugumaran 2002). In a recent study, Wen and colleagues found that phosphorylation of Xyl strongly increases the activity of GalT-II, and that the knockout of Fam20B leads to a truncated, sialic acid-capped linkage tetrasaccharide that cannot be further elongated (Wen et al. 2014).
Two modifications that result in the termination of GAG chain synthesis are performed by human natural killer cell carbohydrate antigen 1 (HNK-1) sulphotransferase and EXTL2, respectively. HNK-1 catalyses the transfer of a sulphate group to the C-3 atom of a terminal GlcA in the tetrasaccharide linker region (Mikami and Kitagawa 2013), which leads to the inhibition of CS chain elongation (Fig. 1.8 D). However, this was only observed so far for the proteoglycan thrombomodulin (Nakagawa et al. 2011). EXTL2, a glycosyltransferase that catalyses the addition of GlcNAc to the terminal GlcA of the linkage region, was found to be able to add $\alpha$-GalNAc or $\alpha$-GlcNAc to GlcA, which in return inhibits the further synthesis of CS or HS (Fig. 1.8 D). Although this was so far only observed in vitro, it could indicate a possible regulatory mechanism for GAG chains (Mikami and Kitagawa 2013).

Overall, the polymerization of CS/DS chains is a highly sophisticated process, which is not yet completely understood. Even the findings from enzyme-knockouts can be controversial in the light of a possible compensatory effect of other CS polymerizing enzymes, which cannot be completely excluded (Professor Kazuyuki Sugahara, Hokkaido University, Hokkaido, Japan, personal communication).
Figure 1.8 Mechanisms of CS synthesis. A: Conventional model of CS chain polymerization by ChGN 2 and C4ST-1. ChGn 2 attaches a GalNAc to the terminal GlcA of the linkage region, which is then 4-O sulphated by C4ST-1. Followed by this, the chain elongation is taken forward by a polymerization complex composed of ChSy-1 and ChPF (left column). If GalNAc is present at the terminus, it is first sulphated by C4ST-1 and then serves as a substrate for the chain polymerization complex (right column). B: A different mechanism describes the addition of the first GalNAc by ChGn 1, followed by 4-O sulphation by C4ST-2. From then on, chain elongation is performed by a CS polymerization complex. C: The linkage region xylose can be phosphorylated at the C-2 atom by Fam20B. D: Addition of an α-GalNAc or α-GlcNAc to the linkage region GlcA prevents further synthesis of CS and HS. Similar, 3-O sulphation of GlcA by HNK-1 supresses CS formation. Reprinted from Biochimica et Biophysica Acta, Volume 1830, Issue 10, Pages 4719 – 4733, Authors: Mikami T., Kitagawa H.; "Biosynthesis and function of chondroitin sulfate." Copyright (2013), with permission from Elsevier.
1.10.3 Termination of chondroitin sulphate synthesis

Usually CS/DS chains terminate at their non-reducing end variably with nonsulphated GlcA, GalNAc4S, GalNAc6S, GalNAc4,6diS or nonsulphated GalNAc (Silbert 1978; Otsu et al. 1985). However, some degree of specificity was found showing that GalNAc4S at the non-reducing end (Silbert and Freilich 1980) or pre-terminal to GlcA at the non-reducing end (Cogburn and Silbert 1986) of a CS chain blocks the further attachment of GlcA or GalNAc. Analysis of swarm rat chondrosarcoma aggrecan showed that GalNAc4,6diS as the non-reducing terminal disaccharide was 60 times more abundant than other disaccharides (Midura et al. 1995; Mikami and Kitagawa 2013). Moreover, it was shown that bone marrow derived mast cells isolated from GalNAc4S-6ST knockout mice produced larger CS chains compared to normal mice (Ohtake-Niimi et al. 2010). Although the mechanism responsible for chain termination is not yet fully understood, GalNAc4,6diS seems to be involved in a chain termination mechanism.

1.10.4 Degradation of GAG chains

In eukaryotes, GAG chain degradation takes place endogenously mostly in lysosomes, where chains are broken down initially by endo-glycosidases. The resulting oligosaccharides are further degraded by exo-glycosidases, acting on the non-reducing end terminus of the sugar, and sulphatases, which liberate the individual monosaccharides (Prabhakar and Sasisekharan 2006). Two enzymes known to be involved in the degradation of CS chains are HYAL1 and HYAL4, which are both endo-β-N-acetylgalactosaminidases specific for CS and HA (Mikami and Kitagawa 2013).

For a detailed analysis of the GAG disaccharide composition, the long polysaccharide chains need to be degraded into their smallest component: the disaccharide subunit. This is accomplished by polysaccharide lyases and hydrolases of bacterial origin, which specifically attack the glycosidic linkages between the disaccharide subunits. Lyases display a degree of specificity for heparin, HS, CS and HA and are therefore classified as heparinases, chondroitinases and hyaluronidases, respectively (Linhardt et al. 1986).
Lyases cleave disaccharides by catalyzing an elimination reaction, where the C5-hydrogen of the hexuronic acid part of the disaccharide gets removed, forming an unsaturated double-bond between the C4 and C5 atom. Lyases can only cleave glycosidic bonds on the non-reducing end of hexuronic acids as its carboxyl group is involved in the catalytic mechanism (Ernst et al. 1995). Hydrolases are the major class of polysaccharide degrading enzymes in higher animals. They are specific for heparin, HS, CS/DS, HA and KS. Unlike lyases, these enzymes are catalyzing a hydrolytic cleavage of glycosidic bonds, where a proton is donated to the glycosidic bond resulting in forming an oxonium ion and therefore cleavage of the bond. Then, a proton donated from H₂O is neutralizing the oxonium ion. In contrast to lyases, hydrolases are not restricted to the glycosidic bond between individual disaccharides, but can also cleave within a disaccharide. This feature and also the absence of an unsaturated double bond in the hexuronic acid makes hydrolases not preferable enzymes for GAG disaccharide analysis. Without the double bond, disaccharides are not absorbing UV light (232 nm) or easily undergo labeling reactions (Ernst et al. 1995).

In the following sections, the three types of GAG chain degrading enzymes are described in more detail.

1.10.5 Chondroitinases

Chondroitinases are specific for GAG chains comprised of CS and DS. Within this enzyme family, several chondroitinases are specific for only a certain type of CS depending on the sulphation of GalNAc or the position of the carboxyl group of the hexuronic acid (Cs vs DS). The substrate specificity is part of the enzyme nomenclature. For example, chondroitinase ABC (ChABC) cleaves both 4-sulphated CS (CS-A) and 6-sulphated CS (CS-C) as well as DS (CS-B).

ChABC utilized for GAG analysis is commonly derived from the bacterium *Proteus vulgaris*, which is used for large scale production and purification (Yamagata et al. 1968; Hamai et al. 1997) of this enzyme. Alternatively, it is recombinantly expressed in *Escherichia coli* (Prabhakar et al. 2009; Prabhakar
et al. 2005). ChABC does not cleave heparin or HS, but shows some activity towards HA (Ernst et al. 1995). It depolymerizes CS/DS chains from the non-reducing end disaccharide towards the core protein and is therefore denoted as an exolytically cleaving chondroitinase.

ChAC is derived from *Arthrobacter aurensis* and *Flavobacterium heparinum*. It is specific for CS. In fact, DS inhibits ChAC activity in a competitive way with a $K_i$ of $10 – 60$ mM. Two different isoforms of ChAC exist depending on the expressing organism. ChAC-I (*F. heparinum*) is degrading CS chains in an endolytic fashion by randomly generating oligosaccharides initially and disaccharides in the later stages of the reaction. In contrast, ChAC-II (*A. aurensis*) degrades exolytically from the non-reducing end generating primarily disaccharides from the outer portions of the CS chain (Hiyama and Okada 1976).

ChB is another chondroitinase derived from *F. heparinum*. It is specific for DS chains only (Ernst et al. 1995). Additionally, it only cleaves disaccharides that comprise a 4-sulphated GalNAc (Michelacci and Dietrich 1974). In contrast to ChAC which is inhibited by DS, ChB is not inhibited by CS chains.

ChC has a higher catalytic activity towards 6-sulphated CS than towards 4-sulphated CS. Like ChAC-I and ChB, it is also expressed by *F. heparinum*. Unlike ChABC, it is also able to depolymerize HA (Ernst et al. 1995). However, due to its very selective substrate specificity, it is not commonly used for GAG disaccharide analysis.

1.10.6 Heparinases

Heparinases are lyases that specifically depolymerize heparin and HS. No cross-reactivity with other GAG types have been reported. Heparinases are divided into three classes. Class 1 is mainly specific for heparin, class 2 cleaves heparin as well as HS and class 3 can only cleave HS (McLean et al. 1985). All the isoforms can be found in *F. heparinum*, however heparinase 1 was also found to be expressed in other soil bacteria (Ernst et al. 1995). Heparinase 2 is also known as heparitinase 2 and heparinase 3 is also known as heparitinase 1.
1.10.7 **Hyaluronidases**

Hyaluronidases can be found in bacteria as well as animal tissues. Depending on the source, the hyaluronidases have different cross-reactivities towards other GAGs and from different depolymerization products. For instance, hyaluronidase from *Peptostreptococcus sp.* shows a low activity towards CS-A and CS-C, but not CS-B and heparin (Tam and Chan 1985). It completely depolymerizes HA into disaccharides, in contrast to the more commonly used bovine testicular hyaluronidase, whose cleavage products are mostly hexa- and tetrasaccharides (Yamagata et al. 1968).

1.11 **Changes in GAG chain structure identified in disease states of cartilage**

During the last decades, increasing evidence accumulated regarding the alteration of GAG chain fine structure in OA. Early work from Mankin and Lipiello (1971) showed that OA hip cartilage contained an increased amount of chondroitin-4-sulphate (C-4-S) and decreased amount of KS compared to normal cartilage (Mankin and Lipiello 1971), which was subsequently confirmed by others (Michelacci et al. 1979). As the ratio of C-4-S to C-6-S is higher in fetal and young cartilage than in adult cartilage, chondrocytes in OA cartilage seem to produce an immature type of extracellular matrix. Moreover, a reduced HA content (Sweet et al. 1977) as well as longer and more CS chains per protein (Cox et al. 1985; Carney et al. 1985) were observed in OA cartilage. Further studies using specific antibodies raised against GAG chain epitopes revealed the re-appearance of epitopes in OA cartilage which are normally absent in adult cartilage but present in fetal cartilage (Rizkalla et al. 1992; Visco et al. 1993; Slater et al. 1995; Cs-Szabó et al. 1995; Carlson et al. 1995). Taken together, the observed differences in GAG chain structure in OA cartilage indicate that changes in the expression of GAG chain synthesizing and modifying enzymes take place during OA pathology.
1.12 **Aims of the thesis**

The overarching aim of the project is to identify potential biomarkers that can aid in the diagnosis of joint disease. We speculate that disease processes lead to alterations in glycosylation of proteins and that specific carbohydrate epitopes constitute highly specific markers for underlying pathological processes.

The hypothesis to be tested is:
Glycosylation of secreted proteoglycans is altered by biochemical processes triggered in the joint in response to inflammatory processes in cartilage.

The specific aims to investigate the hypothesis are:

1. To analyze the composition and fine structure of GAG chains of secreted proteoglycans under different conditions, e.g. decorin
2. Development of a 3D *in vitro* model of “articular” cartilage
3. Using the cartilage model for the analysis of tissue-specific expression patterns of proteins involved in carbohydrate synthesis and modification under inflammatory conditions similar to osteoarthritis using microarrays
4. Investigate changes in carbohydrates on proteins predicted from changes in gene expression of enzymes involved in carbohydrate synthesis/modification
Chapter 2:

Establishing HPLC disaccharide analysis methods and analysis of decorin GAG chain composition
2 Establishing HPLC disaccharide analysis methods and analysis of decorin GAG chain composition

2.1 Introduction

The basic building blocks of GAG chains are disaccharide units. The gross composition of the disaccharides determines the GAG chain type, e.g. CS/DS (GlcA/IdoA-GalNAc), heparin / HS (GlcA-GlcNAc), KS (Gal-GlcNAc) or HA (GlcA-GlcNAc). Except for HA, all disaccharides can be modified by the attachment of sulphate groups on the hydroxyl groups attached to carbon atoms 2, 4, and 6 on the glucopyranose sugar ring. This modification is performed by sulphotransferases during GAG chain synthesis in the endoplasmic reticulum/Golgi complex (Mikami and Kitagawa 2013). To be able to analyze the composition of differentially sulphated disaccharide units, the GAG chain is first depolymerized, which is achieved by specific lyases or hydrolases (Ernst et al. 1995). For example, chondroitinases are lyases specific for CS, DS and also HA to a lesser extent. They catalyze the elimination of the β1,4-glycosidic bond between the hexosamine of one unit and the hexuronic acid of its adjacent unit, introducing a Δ4,5-unsaturated bond in the hexuronic acid. Heparinases, cleaving heparin and HS, belong to the same family of polysaccharide lyases. A different mechanism is used by hydrolases, which cleave KS and HA. The respective hydrolytic cleavage does not introduce an unsaturated bond in the hexuronic acid. In addition, they can also cleave within a disaccharide unit in contrast to lyases.

A variety of different methods have been developed to analyze the structure and composition of GAG chains, ranging from gel electrophoresis (Yamada et al. 1992), fluorescence-assisted carbohydrate electrophoresis (FACE) (Calabro et al. 2000; A H Plaas et al. 2001), NMR analysis (Yamada et al. 1992), mass spectrometry (Linhardt et al. 1992; Zamfir et al. 2009; Sisu et al. 2011), capillary zone electrophoresis (Kitagawa et al. 1995; Lamari et al. ...
HPLC allows for the analysis of the relative composition of GAG chains by comparison of the elution profile to a set of disaccharide standards, indicating the relative proportion of differentially sulphated disaccharide units of a particular type present in the chain. These can be separated due to differences in their negative charge and position of the negative sulphate groups on the disaccharide. Common methods for HPLC analysis are “normal phase” chromatography approaches, which are similar to ion exchange chromatography. Here, an increasing concentration of a high ionic strength buffer, such as 1 M NaCl, is applied onto the column and disaccharides elute in the order from weakly charged (non-sulphated) to highly charged (three sulphate groups). Disaccharides can be detected by measuring absorbance at 232 nm due to the $\Delta 4$, 5-unsaturated bond introduced by the lyase depolymerization reaction. Sensitivity for normal phase HPLC analysis can be increased by chemically labeling disaccharides with fluorescent dyes, e.g. 2-aminobenzamide (2-AB).

Alternatively, “reverse phase” chromatography approaches use differences in hydrophobicity of the molecules for separation. However, disaccharides are highly hydrophilic, which makes it necessary to chemically link them to a highly hydrophobic molecule. For example, 2-aminoacridone (AMAC) is a hydrophobic fluorescent dye which is used for disaccharide labeling (Plaas et al. 2001; Kitagawa et al. 1995; Jackson 1994). The advantage of the fluorescent label is a substantial increase in sensitivity. In reversed phase HPLC, highly charged disaccharides elute first and weakly charged elute last with increasing organic solvent, e.g. methanol.

In this chapter, several HPLC analysis methods for GAG chains were evaluated for disaccharide analysis of CS/DS to establish a method for characterization of changes in GAG composition of selected proteoglycans in the context of osteoarthritis. These represent the predominant GAG chains found conjugated to decorin, a small leucine-rich repeat proteoglycan which is found in cartilage and skin (Reed and Iozzo 2003). It is composed of a core protein and a single GAG chain which itself is composed of either CS or DS,
depending on the tissue. Decorin derived from articular cartilage has a GAG chain which consists mostly of CS (70-80 %) and a small amount of DS (20-30%), whereas skin decorin has a GAG chain consisting mostly of DS (~80%) (Tiedemann et al. 2001; Cheng et al. 1994). While decorin may not turn out to be suitable as a biomarker for OA, it is used here as a model protein to establish relevant methodology as it has been shown to be highly sensitive to changes in the biosynthesis machinery in cells subjected to different conditions (Jianyun Yan et al. 2011).

2.1.1 **Aims for this chapter:**

1. Establish a sensitive HPLC disaccharide analysis method for the determination of GAG chain disaccharide composition.

2. Purify decorin from human skin fibroblasts and human chondrocyte progenitor cells.

3. Compare the GAG chain composition of decorin purified from different sources.
2.2 Materials and Methods

2.2.1 HPLC analysis of disaccharides using CarboPac PA1 column

A protocol for the HPLC analysis of disaccharide subunits was adapted, which describes the separation of chondroitinase-ABC-digested CS/DS disaccharide units using a CarboPac PA1 matrix (Dionex, Idstein, Germany) (Midura et al., 1994). It is based on the principle of ion exchange chromatography. Buffer solutions were 0.1 M NaOH (buffer A) and 0.1 M NaOH, 1 M NaCl (buffer B). For their preparation, ultrapure H₂O (>18 MΩ), HPLC-grade NaCl and NaOH (Fisher Scientific, Loughborough, UK) were used. The solutions were filtered through a 0.22 μm nitrocellulose membrane (Millipore, Watford, UK) and degassed. Due to the use of alkaline buffers, the disaccharides had to be reduced prior to analysis, as otherwise, the disaccharides would experience degradation from “alkali peeling” reactions (Whistler and Bemiller 1958). For this, 1 M NaBH₄ in 0.1 mM NaOH was added to the sample to give a final concentration of 25 mM NaBH₄. The reaction mixtures were incubated at 38°C for 30 min and then acidified by adding 25 μl of 1 M acetic acid per 100 μl reaction mixture. Samples were subsequently incubated on ice for 10 min, filtered through a 0.45 μm syringe filter (Millex®-HV, Millipore, Watford, UK) and transferred to 1.5 ml glass vials for HPLC analysis. For the separation of reduced disaccharides, the following linear buffer gradient (buffer A (%): buffer B (%)) was used: 0 min (98:2), 12 min (98:2), 62 min (40:60), 70 min (0:100), 85 min (0:100). Due to the introduction of a Δ4,5-unsaturated double bond at the hexuronic acid unit of the disaccharides by chondroitinases, elution could be monitored at a wavelength of 232 nm. The column was regenerated with 100% buffer A. Disaccharide standards (Sigma Aldrich, Gillingham, UK and Dextra, Reading, UK) were used to determine the elution position of the individual disaccharides. Data analysis was performed using Dionex Chromeleon software (Dionex, Idstein, Germany).
2.2.2 **Labeling of chondroitin/dermatan sulphate disaccharides with 2-aminobenzamide (2-AB)**

For labeling with 2-AB, 10 µl 2-AB labeling mix (0.35 M 2-AB/1.0 M NaBH₃CN/30% acetic acid (v/v) in DMSO) were added to the dried sample and incubated for 2 h at 65°C (Kinoshita and Sugahara 1999). The labeling mix needs to be prepared fresh and in a defined order of steps. Firstly, a solution of 30% acetic acid (v/v) in DMSO is prepared (solution A). Secondly, crystalline 2-AB is added to solution A to give a final concentration of 0.35 M 2-AB (solution B). Subsequently, NaCNBH₄ can directly be dissolved in solution B to a final concentration of 1.0 M. Labeled disaccharides were analyzed immediately or stored at -20°C.

2.2.3 **Removal of excess 2-AB from the labeled disaccharide sample**

Since 2-AB was added in excess over the disaccharides in the labeling reaction, free 2-AB needed to be removed to reduce background signal to improve detection of small amounts of labeled disaccharides. Two methods are described for the removal of excess 2-AB in the literature: paper chromatography and chloroform extraction (Mizumoto and Sugahara 2012). Paper chromatography was performed by applying the labeling mixture (10 µl) on a Whatman 3MM filter paper strip. The running phase was butanol/ethanol/water, 4:1:1 (v/v). After separation, the strip was dried and the original position as well as areas from the middle of the strip harbouring the 2-AB labeled disaccharides were cut out under UV-light. The cut out paper slices were placed into Mini Bio-Spin™ chromatography columns (BioRad) and washed four times with 300 µl distilled H₂O to elute the disaccharides. The eluates were pooled and dried in a vacuum concentrator (Christ RVC 2-25 CD plus and Christ CT 02-50 SR, Osterode am Harz, Germany) for about 5 h at 30°C.

Chloroform extraction was performed by adding 500 µl distilled H₂O to a disaccharide sample, followed by an equal amount of chloroform. The solution was vigorously mixed and then centrifuged for 15 sec at 1000 x g to establish phase separation. The chloroform phase was carefully removed and discarded.
and this step repeated for 5 to 10 more times. The resulting aqueous solution was finally dried using the vacuum concentrator.

2.2.4 HPLC analysis of 2-AB labeled disaccharides using YMC Pack Polyamine II column

Analysis of 2-AB labeled disaccharides was performed using a Dionex ICS-3000 system in combination with a YMC Pack Polyamine II ion exchange column (5 µm particle size, 12 nm pore size, 250 x 4.6 mm, YMC Europe GmbH, Dinslaken, Germany) as described (Kinoshita and Sugahara 1999). Buffer solutions were 16 mM NaH$_2$PO$_4$ (buffer A) and 1 M NaH$_2$PO$_4$ (buffer B). The solutions were filtered through a 0.22 µm nitrocellulose membrane and degassed as before. Dried samples of 2-AB labeled disaccharides were dissolved in buffer A to a final volume of 500 µl. To remove any residual particles, samples were filtered (0.45 µm filter) before applying them into 1.5 ml glass vials for HPLC analysis. The column was heated to 30°C. For the separation of 2-AB-labeled disaccharides, 100% buffer A was applied for the first 10 min, followed by a linear gradient up to 40% buffer B / 60% buffer A over 60 min at a flow rate of 1 ml/min. Emission of labeled disaccharides at 420 nm was recorded by a RF 2000 fluorescence detector (Dionex, Idstein, Germany) while exciting at 330 nm. The column was regenerated with 2 column volumes of 100% buffer B. Disaccharide standards (Sigma Aldrich and Dextra, Reading, UK) were labeled with 2-AB prior to use to determine specific elution times of the differentially sulphated disaccharide units. Data analysis was performed using Chromeleon software package (Dionex) as described.

2.2.5 Labeling of chondroitin/dermatan sulphate disaccharides with 2-aminoacridone (AMAC)

Labeling of disaccharides was carried out essentially as described (Jackson 1994; Kitagawa et al. 1995; Deakin and Lyon 2008). All samples were freeze-dried prior to labeling. The dried sample was reconstituted in 10 µl of 0.1 M repurified AMAC (see below) in DMSO/acetic acid (85:15, v/v) and incubated for 15 min at room temperature. Subsequently, 10 µl of freshly prepared 1 M
NaBH₃CN were added and the labeling reaction was carried out at 37°C for 18 h. Samples were either subjected to HPLC analysis immediately or stored at -20°C.

2.2.6 Repurification of AMAC using hydrophobic interaction chromatography

Repurification of AMAC was carried out as described (Deakin and Lyon 2008). 10 mg of AMAC were dissolved in 1 ml DMSO/acetic acid (85:15, v/v) and made up to 5 ml with H₂O. The solution was applied to a column packed with Macro-prep tert-butyl HIC media (2 ml bed volume, Bio-Rad), which was equilibrated in H₂O. The resin was washed with 20 ml of 20% methanol to remove any contaminants. Pure AMAC was subsequently eluted with 100% methanol. Repurified AMAC appeared as a bright yellow solution. Methanol was removed by evaporation.

2.2.7 HPLC analysis of AMAC-labeled disaccharides using Kinetex C-18 column

For HPLC analysis, a Dionex ICS-3000 system was used in combination with a Kinetex C-18 column (2.6 μm particle size, 100 Å pore size, 100 x 4.6 mm, Phenomenex, Macclesfield, UK). Buffer solutions were 0.1 M ammonium acetate (buffer A) and methanol (buffer B). AMAC-labeled samples (20 μl) were diluted to a volume of 500 μl in buffer A, centrifuged at 18,000 x g for 5 min, filtered through a 0.45 μm syringe filter (Millex®-HV, Millipore, Watford, UK) and applied to 1.5 ml glass vials. The column was heated to 40°C. For the separation of AMAC-labeled disaccharides, the following buffer gradient (buffer A (%): buffer B (%)) at a flow rate of 1 ml/min was used: 0 min (100:0), 5 min (90:10), 20 min (75:25), 25 min (0:100). Emission of labeled disaccharides was recorded by the RF 2000 fluorescence detector at 520 nm while exciting at 427 nm. The column was regenerated with 2 column volumes of 100% buffer B. Disaccharide standards (Sigma Aldrich and Dextra) were AMAC-labeled and subsequently used to determine the elution times of the differentially sulphated disaccharide units. Data analysis was performed using
Chromeleon software package (Dionex) as described. Deakin et al. (2008) pointed out that some disaccharides are more readily labeled with AMAC than others. Therefore, correction factors were determined to compensate for the differential labeling rates (Table 2.1) (Deakin and Lyon 2008). Those were used in all calculations of AMAC-labeled disaccharides in this study.

Table 2.1 Correction factors for AMAC-labeled disaccharides (Deakin and Lyon 2008)

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>Correction factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ2,4,6triS</td>
<td>1.91</td>
</tr>
<tr>
<td>Δ2,4diS</td>
<td>2.28</td>
</tr>
<tr>
<td>Δ2,6diS</td>
<td>1.00</td>
</tr>
<tr>
<td>Δ4,6diS</td>
<td>1.37</td>
</tr>
<tr>
<td>Δ2S</td>
<td>1.05</td>
</tr>
<tr>
<td>Δ4S</td>
<td>1.47</td>
</tr>
<tr>
<td>Δ6S</td>
<td>1.26</td>
</tr>
<tr>
<td>Δ0S</td>
<td>1.52</td>
</tr>
</tbody>
</table>

2.2.8 Cell culture

Human skin fibroblasts (Bonacker cells) (Glössl et al. 1984) were cultured in minimal essential medium (MEM) containing 10% heat-inactivated fetal calf serum (FCS), non-essential amino acids (NEAA), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Paisley, UK). Human chondrocyte progenitor cells were cultured as described in chapter 3 (section 3.2.2). These cells originate from primary human skin fibroblasts obtained from a skin biopsy.

For passaging, the cells were washed with PBS before addition of trypsin/EDTA (0.05% v/v, Gibco, Cat. No. 25300-062), and incubation for 5 min at 37°C. Trypsination was stopped through addition of a 4-fold volume of serum-containing medium. Cells were collected by centrifugation at 1,500 x g for 5 min and resuspended in fresh medium. Cells were seeded at a density of
1.3 x 10^5 cells/cm^2 into a new tissue culture flask (T-75, Sarstedt, Leicester, UK).

For collection of conditioned medium, cells were cultured to approximately 70% confluency in medium containing 10% heat-inactivated FCS and then switched to low serum medium (4% FCS). The cells were cultured for 3 more days, and then the medium was collected and supplemented with a Tris-HCl pH 7.5 stock solution to a final concentration of 5 mM and also protease inhibitors (10 mM 6-aminohexanoic acid, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulphonylfluoride (PMSF), 0.5 mM benzamidine, from a 10x stock solution) and stored at -20°C. Decreasing the serum concentration in the medium reduces the contamination from serum proteins while maintaining sufficient growth factor activity not to cause changes in GAG structure (Fiedler, 2007). Just before anion exchange chromatography, Triton X-100 was added to a final concentration of 0.1%.

2.2.9 Purification of decorin by anion exchange chromatography

In order to partially purify the proteoglycan decorin from conditioned medium of human skin fibroblasts, 1 liter of conditioned medium was filtered through a Whatman filter paper (No. 4, Whatman, Maidstone, UK) and applied overnight to an anion-exchange column containing DEAE-Cellulose DE52 matrix (weak anion exchanger; column 1.6 cm x 11 cm, Whatman Biosystems, Maidstone, UK) at a flow rate of 1 ml/min. The column had been pre-equilibrated with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100 and protease inhibitor cocktail (see 2.2.8). After sample application, the column was washed with 12 column volumes using the above described buffer prior to step elution as follows: By applying 36 column volumes of the same buffer, but containing 400 mM NaCl, weakly bound proteins were eluted. Tightly bound proteins, including proteoglycans, were subsequently eluted with the same buffer containing 1 M NaCl (Fiedler 2007). During this step, 7 fractions of 14 ml were collected and subsequently protein containing fractions were pooled. To reduce the NaCl-concentration and to remove Triton X-100, the pooled sample was dialyzed (Spectra/Por MWCO: 12-14.000, Spectrum Laboratories,
Rancho Dominguez, USA) extensively against 20 mM Tris-HCl, pH 7.4, 400 mM NaCl at 4°C. To purify decorin further, the sample pool was applied to a column containing Source 15Q matrix (strong anion exchanger; column 1 cm x 9.5 cm, GE Healthcare, Buckinghamshire, UK), which had been pre-equilibrated in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl. The flow rate was 1 ml/min. After sample application, the column was washed with the same buffer containing 400 mM NaCl until a baseline of absorbance at 280 nm was reached. Then the column was eluted with a linear gradient of 400 mM to 600 mM NaCl over 2 column volumes. The NaCl concentration was then increased to 1 M and 14 fractions of 14 ml each were collected. Decorin containing fractions were pooled and stored at -20°C for further analysis.

2.2.10 Digestion of decorin with chondroitinase ABC, AC-I and B
To depolymerize CS/DS chains, samples were incubated with 5 mU chondroitinase ABC (chABC, protease free, derived from *Proteus vulgaris*, ICN Biomedicals, Aurora, UK) per 1 µg decorin in 50 mM Tris-acetate, 60 mM sodium acetate, pH 8.0, for 2 h at 37°C. To specifically depolymerize DS, samples were incubated with 5 mU chondroitinase B (chB, Iduron, Manchester, UK) per 1 µg of decorin in 50 mM Tris-HCl, pH 8.0, 2 mM CaCl$_2$ for 2 h at 37°C. Specific cleavage of CS was performed by addition of 5 mU chondroitinase AC-I (chAC-I, Sigma Aldrich) per 1 µg of decorin in 50 mM Tris-HCl, pH 7.3 and incubation for 2 h at 37°C. The specificity of lyases is outlined in Table 2.2.

Table 2.2 Chondroitinases used in this study and their substrate specificity

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Organism</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>chondroitinase ABC</td>
<td><em>P. vulgaris</em></td>
<td>CS, DS, HA (low)</td>
</tr>
<tr>
<td>chondroitinase AC-I</td>
<td><em>F. heparinum</em></td>
<td>CS, HA (low)</td>
</tr>
<tr>
<td>chondroitinase B</td>
<td><em>F. heparinum</em></td>
<td>DS</td>
</tr>
</tbody>
</table>
2.2.11 **Protein concentration determination**

In order to determine the protein concentration of a sample, the Pierce BCA Protein Assay (Thermo Scientific, USA) was used. This assay works on the basis of the quantitative reduction of Cu$^{2+}$ to Cu$^{+}$ by proteins and the chelation of Cu$^{+}$ with two molecules of bicinchoninic acid (BCA), of which the absorbance can be detected at 562nm. 25 µl of a sample of unknown concentration was mixed with 200 µl BCA working solution in a 96 well plate. At the same time, a dilution series of bovine serum albumin (BSA, 25 µg/ml to 2.0 mg/ml) was also mixed with working solution. The mixtures were incubated at 37°C for 30 min. The protein concentration of the unknown sample was calculated from the BSA calibration curve using non-linear regression using Microsoft Excel®.

2.2.12 **Ethanol precipitation of proteins**

The precipitation of proteins for SDS-PAGE analysis was achieved by adding 9 volumes of ice cold ethanol (Sigma Aldrich) to 1 volume of sample solution, followed by incubation for 2 hours at -20°C or overnight at 4°C. The precipitated protein was collected by centrifugation at 13,200 x g for 20 min at 4°C. The supernatant was carefully removed and the pellet dried to remove the remaining ethanol. Then, the pellet was resuspended in a 1:1 mixture of 8 M Urea and 2x SDS-sample buffer (see below). The samples were extensively mixed and boiled for 2 min prior to separation by SDS-PAGE.

2.2.13 **SDS-Polyacrylamide-gel electrophoresis**

For SDS-PAGE, Novex Tris-Glycine gels with a linear gradient of 4-20% polyacrylamide (Invitrogen) were used. After rinsing the gel with H$_2$O, it was placed into an XCell™ Surelock gel chamber (Invitrogen). Protein samples were mixed with an equal amount of 2x sample buffer (126 mM Tris-HCl, pH 6.8, 20% glycerol (v/v), 4% SDS (w/v), 0.02 % bromophenol blue; for reducing conditions, 2% 2-mercaptoethanol was added), boiled for 2 min, mixed and centrifuged for 1 min at 13,200 x g immediately before loading onto the gel to remove aggregates. The gel was run at 125V constant voltage (~ 35 mA) for 2 h in running buffer (25 mM Tris, pH 8.8, 192 mM glycine, 1% (w/v) SDS).
Gels were calibrated with low molecular weight standards (GE Healthcare Life Sciences, Little Chalfont, UK).

2.2.14 Silver staining of polyacrylamide gels

Silver staining of SDS-PAGE gels is a very sensitive method to visualize proteins. The level of sensitivity approaches ~5-30 ng per protein band (Blum et al. 1987). The method developed by Blum et al. (1987) uses Ag⁺-ions, which are complexing with Glu, Asp and Cys- residues in the polypeptide chain. After addition of formaldehyde, the Ag⁺-ions are reduced to Ag⁰ and precipitate as pure silver. The SDS-PAGE gel was fixed for at least 1 h in fixing solution (50% ethanol (v/v) and 12% acetic acid (v/v) in H₂O). After washing three times with 50% ethanol (v/v), the gel was incubated in 0.8 mM Na₂S₂O₅ pentahydrate for 1 min. Next, the gel was washed extensively in H₂O, incubated in staining solution (12 mM AgNO₃, 0.015% formaldehyde (v/v)) and again washed with H₂O. The bands were made visible through incubation in developing solution (380 mM Na₂CO₃, 0.015% formaldehyde (v/v)). The reaction was stopped by adding fixing solution.

2.2.15 Western blotting

Western blotting involves the transfer of proteins from a gel onto a membrane for detection of proteins by specific antibodies. After separating a protein sample in a SDS-PAGE gel, the gel was equilibrated in transfer buffer (12 mM Tris, 96 mM glycine, 20% methanol (v/v)). After that, a sandwich free of air was prepared that contained the components in the following order starting from the cathode-side of the blotting chamber: 3x sponges, 2x filter paper, gel, Protran nitrocellulose membrane (Whatman, Maidstone, UK), 2x filter paper, 3x sponges. The transfer was performed at a constant current of 125 mA (~25 V) for 2h in transfer buffer. After transfer, the membrane was washed in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl). To visualize proteins and mark the migration of non-prestained protein markers, the membrane was incubated in a Ponceau S staining solution (0.1% Ponceau S (w/v), 5% acetic acid (v/v), Sigma Aldrich) for 5 min. The molecular weight
marker bands and position of lanes were marked with a pencil and the membrane subsequently washed in TBS until the bands were fully destained. In order to block non-specific binding sites of the membrane, it was incubated in 5% skimmed milk (Sigma Aldrich) in TBS (blocking buffer) for 1 h. A primary antibody against a protein of interest (Table 2.3) was diluted to the desired concentration in blocking buffer. The incubation time of the membrane with primary antibody depended on the nature of the antibody used and varied between 1 h at room temperature and overnight at 4°C. The membrane was washed three times for 5 min with TBS-T (0.01% Tween 20 in TBS) while shaking. An appropriate secondary antibody conjugated to horseradish peroxidase (HRP) made up in blocking buffer was added and the membrane incubated for 1 h at room temperature. The membrane was again washed three times for 5 min with TBS-T, followed by a 5 min wash step in TBS. Bound antibodies were visualized by chemiluminescence using a solution containing ECL Plus (GE Healthcare) and subsequent exposure of the membrane to a photographic film (Hyperfilm ECL, GE Healthcare) for different times to optimize the signal.

**Table 2.3 Antibodies used in this chapter for Western blotting**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution factor</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal anti-decorin</td>
<td>1:500</td>
<td>Dr Elke Schönherr, Cardiff University</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-biglycan</td>
<td>1:500</td>
<td>Dr Elke Schönherr, Cardiff University</td>
</tr>
<tr>
<td>Swine anti-rabbit IgG/HRP conjugate</td>
<td>1:1000</td>
<td>P0399, Dako, Cambridgeshire, UK</td>
</tr>
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**2.2.16 Stripping of nitrocellulose membrane**

For re-probing, the membrane was transferred into a roller bottle and stripped in 10 ml stripping buffer (62.5 mM Tris-HCl, pH 6.8, 0.5% SDS (w/v), 90 mM 2-mercaptoethanol, all from Sigma Aldrich) at 50°C for 30 min while agitating in a PersonalHyb hybridization oven (Stratagene, La Jolla, USA). Afterwards,
the membrane was washed three times with TBS-T for 5 min. Finally, the membrane was blocked again with 5% skimmed milk in TBS for 1 h prior to incubation with antibodies as described above.
2.3 Results

2.3.1 HPLC disaccharide analysis using anion exchange chromatography of unlabeled disaccharides

For the qualitative and quantitative analysis of CS/DS chain composition, a protocol used by Midura et al. (1994) was adapted. A program for disaccharide analysis on the Dionex ICS-3000 HPLC machine equipped with a CarboPac PA1 column (Dionex) was developed. Although high sensitivity with a detection limit of 50 ng CS/DS disaccharides by UV detection was described by Midura et al. (1994), it had to be tested if this was achievable with our system. In addition, the exact retention times for the different expected CS/DS disaccharides needed to be determined. For this, 100 μl of 1 mg/ml solutions of ΔHexA-GalNAc (Δ0S), ΔHexA-GalNAc2S (Δ2S), ΔHexA-GalNAc4S (Δ4S), ΔHexA-GalNAc6S (Δ4S), ΔHexA-GalNAc2,4diS (Δ2,4diS), ΔHexA-GalNAc2,6diS (Δ2,6diS), and ΔHexA-GalNAc4,6diS (Δ4,6diS) (Δ indicates an unsaturated double bond between C4 and C5 of the hexuronic acid) were subjected to reduction with NaBH₄ as described in section 2.2.1. The standards were applied to the CarboPac PA1 column either individually or as a mixture and absorbance at 232 nm was measured. The reduced CS/DS disaccharides eluted in the following order: Δ0S (7.7 min), Δ4S (27.6 min), Δ2S (32.0 min), Δ6S (34.0 min), Δ4,6diS (51.6 min), Δ2,4diS (52.7 min), and Δ2,6diS (59.3 min) (Fig. 2.1). Peaks observed between 17 and 25 min were a result of buffer contaminants as shown previously (Midura et al. 1994). The elution times in the mixed sample were consistent with those of each standard run separately. For the determination of the detection limit, lower concentrations of disaccharide standards (1.1 μg and 0.11 μg of each standard) were separated. Analysis of the resulting chromatograms showed, that 1.1 μg of each disaccharide was still more than sufficient for identifying clear peaks (not shown). When analyzing 0.11 μg of each disaccharide, absorbance was markedly reduced to <10 mAU, although peaks could still be identified (Fig. 2.1 B). Therefore, the sensitivity of this method in combination with the available HPLC system was around 100 ng, which is in line with published data (Midura et al. 1994).
Figure 2.1 Disaccharide analysis using CarboPac PA1 column

A: Chromatogram of the HPLC analysis of reduced CS/DS disaccharide standards (~11 µg each) on a CarboPac PA1 column. Absorbance was detected at 232 nm and a buffer gradient with increasing NaCl-concentration applied to the column (dotted line). Disaccharides eluted in accordance of their apparent charge density due to attached sulphate groups. The blue spectrum shows the absorbance of disaccharide standards and the red spectrum the absorbance detected when only buffer was applied to the column.

B: Determination of the detection limit for disaccharide analysis. The amount of standard applied was reduced to ~0.11 µg each to confirm the expected sensitivity of the method. The small peak indicating the elution of Δ0S is marked with a circle.
2.3.2 HPLC disaccharide analysis using 2-aminobenzamide labeling and anion exchange chromatography

Due to very small GAG amounts expected in the present biological samples, a detection limit of about 100 ng per disaccharide achieved by detecting unlabeled disaccharides was considered to be not sensitive enough. Therefore, a modified method using fluorescence-labeled disaccharides was established. The fluorescent dye 2-aminobenzamide (2-AB) was used to label disaccharides (Kinoshita and Sugahara 1999). Labeling was performed by reductive amination of the sugar aldehyde (Fig. 2.2). Separation was achieved using an increasing concentration of NaH$_2$PO$_4$ on a Pack Polyamine II column (YMC) under normal phase HPLC conditions. The retention time of each disaccharide standard was individually determined using 1 ng per disaccharide. The labeled CS/DS disaccharides eluted in the following order (Fig. 2.3A): Δ0S (11.6 min), Δ2S (32.4 min), Δ4S (33.8 min), Δ6S (34.2 min), Δ2,6diS (49.6 min), Δ2,4diS (51.4 min), Δ4,6diS (55.7 min), Δ2,4,6triS (71.5 min). For the labeling reaction, 350 mM 2-AB were used, which was in large excess over disaccharide. The high concentration of 2-AB was nevertheless necessary to ensure complete labeling of all disaccharides in the sample. The remaining free 2-AB interferes with the HPLC disaccharide analysis (overlapping elution), hence removal is imperative. To remove free 2-AB from the labeled disaccharides, several methods have been described: e.g. chloroform extraction and paper chromatography (Mizumoto and Sugahara 2012). Both methods were assessed on their applicability and efficacy. Both chloroform extraction (Fig. 2.3B) and paper chromatography (Fig. 2.3C) were effective in removing free 2-AB, which eluted within the first 10 minutes of the HPLC analysis as indicated (red frame). However, paper chromatography was more effective than chloroform extraction, and was therefore adapted for further analysis. This difference could be particularly important for the detection of small amounts of Δ0S, which eluted around 11.6 min and therefore close to free 2-AB.

Using this approach, a detection limit of 100 pg was determined (not shown). However, the necessity of a post-labeling cleanup could be disadvantageous due to variations in the recovery of labeled disaccharides from filter paper.
Despite the high sensitivity, a major problem of this method was the elution times of Δ4S and Δ6S disaccharides. Those were eluting very close to each other (Fig. 2.3), and therefore may not be sufficiently separated to ensure the correct analysis of the integrated area from each peak. Better separation of the two peaks could have only been achieved by significantly decreasing the slope of the NaH$_2$PO$_4$ gradient, which would have resulted in a much longer overall running time per sample. Apart from this, labeling of disaccharides with 2-AB and the analysis using normal phase HPLC with a Polyamine II column in combination with increasing concentrations of NaH$_2$PO$_4$ is a very sensitive method to detect even very small quantities of CS/DS disaccharides in biological samples.
Figure 2.2 Labeling of disaccharides using fluorescent dyes.

To increase the sensitivity of detecting sulphated ΔHexA-GalNAc disaccharides generated as a product from chondroitin-lyase enzymes using HPLC, they were labeled with the fluorophore 2-aminobenzamide (2-AB) or 2-aminoacridone (AMAC). Sugar molecules in solution are in an equilibrium between a closed conformation (pyranose) and open form (aldehyde). By applying acidic conditions, the equilibrium shifts towards the open form. The NH₂-group of 2-AB and AMAC attacks the sugars’ aldehyde group via nucleophilic addition, forming a Schiff base. Through addition of a reducing agent (NaCNBH₃), the final stable reaction product is formed by reductive amination. The samples are stable for several months at -20°C. Reproduced with modifications from Plaas et al (2001).
Figure 2.3 Analysis of 2-AB labeled disaccharides using YMC polyamine II column. Chromatogram of the analysis of 2-AB labeled CS/DS disaccharide standards (~ 1 ng each) using an YMC polyamine II column (A). Fluorescence emission was detected at 420 nm. Labeled disaccharides eluted according to their apparent charge with increasing NaH₂PO₄ concentration (dotted line). The analysis was performed at a constant temperature of 30°C. To remove excess 2-AB dye from the sample containing disaccharide units, two methods are described: chloroform precipitation (B) and paper chromatography (C). A mix of differentially sulphated ΔHexA-GalNAc disaccharides were labeled with 2-AB and excess dye removed using the two methods. Paper chromatography proved to be more effective for 2-AB removal (red box) compared to chloroform extraction. Free 2-AB eluted around 5 min, whereas all labeled disaccharides eluted after 10 min.
2.3.3 HPLC disaccharide analysis using 2-aminoacridone labeling and reversed phase chromatography

An additional method was established where a different fluorophore, 2-aminoacridone (AMAC), was used to label disaccharides using the same principal mechanism of reductive amination (Fig. 2.2). 2-AB and AMAC differ strongly in their properties, with AMAC being more hydrophobic due to its cyclical ring structure. As a result, AMAC-labeled disaccharides can be separated using reversed phase HPLC (Deakin and Lyon 2008). Here, a linear gradient of increasing methanol concentration was applied to separate AMAC-labeled disaccharides on a C-18 column. An advantage of using AMAC to label disaccharides compared to 2-AB is that there is no post-labeling cleanup step necessary, because free AMAC is eluting later than all disaccharides at approximately 20 min. However, crystalline AMAC powder supplied by Sigma Aldrich needed to be repurified by hydrophobic interaction chromatography as described in section 2.2.6 before use, because it contained impurities that eluted in the same region as the labeled disaccharides (Fig. 2.4 B). A repurification method described by Deakin et al. (2008) proved to be very effective in removing a compound eluting around 12 min from the AMAC preparation. The purified AMAC preparation was then used for all further labeling reactions.

The retention time of each labeled disaccharide standard was individually determined using 1 ng disaccharide and eluted in the following order (Fig. 2.4 A): Δ2,4,6triS (12.8 min), Δ2,4diS (14.6 min), Δ4,6diS (16.6 min), Δ2,6diS (17.0 min), Δ2S (18.7 min), Δ4S (19.2 min), Δ6S (20.7 min), Δ0S (23.6 min). The detection limit of AMAC-labeled disaccharides was determined to be around 10 pg. Because of the superior sensitivity and the excellent separation of the free dye from the labeled disaccharides using the Kinetex C-18 column, AMAC-labeling of disaccharides was used for all further GAG chain disaccharide analyses in this study.
Figure 2.4 Analysis of AMAC-labeled disaccharides using a Kinetex C-18 column

A: Chromatogram of the analysis of AMAC labeled CS/DS disaccharide standards (~1 ng each) using a Kinetex C-18 column. Fluorescence emission was detected at 520 nm. Labeled disaccharides eluted according to their hydrophobicity with increasing methanol concentration (dotted line). Highly charged disaccharides eluted first and weakly charged last. The analysis was performed at a constant temperature of 40°C.

B: Repurification of AMAC. To remove a contaminant within the AMAC reagent supplied by Sigma Aldrich, a solution of AMAC was purified using a MacroPrep tert-butyl HIC matrix. Subsequent analysis on Kinetex C-18 column showed that the contaminant eluting at around 12.5 min (black line) was effectively removed from the reagent solution (red line). Commercially supplied AMAC and purified AMAC are shown as a black and red line, respectively.
2.3.4 Decorin purification from human skin fibroblast conditioned medium

After establishing a highly sensitive HPLC method for CS/DS disaccharide analysis, decorin was purified from human skin fibroblast and human chondrocyte progenitor cell conditioned media. The aim was to use the purified proteins for the analysis of their respective CS/DS chain composition.

Firstly, decorin was purified from conditioned medium derived from human skin fibroblasts essentially as previously described (Fiedler 2007). The purification process involved a two-stage ion exchange chromatography (IEC) procedure. One liter of conditioned medium was initially applied to a DEAE-Cellulose matrix to remove the majority of serum proteins. After washing the column with a buffer containing 400 mM NaCl, an ionic strength sufficient to elute most glycoproteins, proteins with a strong negative charge, including proteoglycans, were eluted with 1 M NaCl (Fig. 2.5 A).

The pooled and dialyzed (20 mM Tris-HCl pH 7.4, 400 mM NaCl) fractions (X7, X8) from the first IEC step were applied to a column containing Source-15Q matrix. This is a strong anion exchanger and allows for more high resolution separation of proteins eluting at different NaCl concentrations. The column was washed with up to 600 mM NaCl and decorin was eluted with 1 M NaCl (Fig. 2.5B). Fractions containing the respective protein peak (fraction 26 and 27, total volume after pooling was 2 ml) were pooled for further analysis.
Figure 2.5 Purification of decorin from human skin fibroblast conditioned media
A: Conditioned media (1 liter) was applied on a column containing DEAE 52 cellulose (15 ml column volume) equilibrated in buffer containing 150 mM NaCl. Weakly bound proteins were removed by washing the column with 400 mM NaCl. Strongly negatively charged proteins, e.g. proteoglycans like decorin, were eluted from the column using 1 M NaCl. The fractions (X7, X8) were pooled and extensively dialyzed against 20 mM Tris-HCl, pH 7.4, 400 mM NaCl.
B: The dialyzed sample was applied to a column containing Source 15Q matrix (6 ml column volume), which was equilibrated in Tris buffer containing 400 mM NaCl (not shown). The salt concentration was gradually increased to 600 mM NaCl over 20 min to remove contaminating proteins. Decorin was eluted by increasing the concentration to 1 M NaCl, represented by a sharp peak with absorbance at 280 nm. Fractions collected during the peak elution were pooled.
2.3.5 **Analysis of purified decorin**

The protein concentration in the pooled fractions was ~43 µg/ml, as determined by BCA assay. Therefore, ~86 µg total protein was isolated from one liter of conditioned medium. This is in line with data from others who have purified decorin from fibroblast-conditioned medium (Schönherr et al. 1995; Fiedler 2007). For analysis of the purified decorin by SDS-PAGE and Western blotting, a sample was digested with chABC to analyze the size of the proteoglycan core protein. For SDS-PAGE and subsequent silver staining, 4 µg decorin was digested with 10 mU chABC. For Western blotting, 2 µg decorin was digested with 5 mU chABC. An equal amount of undigested decorin and also chABC were separated alongside chABC-treated samples.

In order to collect decorin core protein from the dilute samples after chABC digest, it was ethanol precipitated. All samples were reduced for analysis. In the lane of undigested decorin in the silver stained SDS-PAGE gel (Fig. 2.6 A), a smear of 70-100 kDa is visible. This smear could be resolved into distinct bands through digestion with chABC and therefore belonged to a proteoglycan. Two major bands of approximately 43 and 45 kDa became visible after enzymatic processing and also a band of 97 kDa, which belonged to chABC. Two faint bands of about 90 kDa (marked with arrowhead) and 38 kDa, respectively, were also visible. The latter one corresponds to one of the units of chABC.

The presence of decorin was further confirmed by Western blotting with decorin-specific antibodies (Fig. 2.6 B). For detection of decorin, a polyclonal antibody against human decorin was used which was raised against chABC digested human decorin (Glössl et al. 1984). This antibody also recognizes dermatan stubs which still reside on the core protein (Voss et al. 1986). In the lane containing the undigested decorin, a smear with a size of 70-100 kDa was detected. The chABC digested decorin showed two distinct bands with sizes of about 43 and 45 kDa. These bands correspond to core proteins with either 2 or 3 N-linked glycans (Glössl et al. 1984). Again, a band of about 90 kDa was visible. It corresponds to a dimer of covalently cross-linked decorin core protein as shown previously using mass spectrometry (Fiedler, 2007). The membrane was subsequently reprobed with an antibody raised against biglycan. Biglycan
has been reported to be synthesized by fibroblasts and has similar physicochemical properties (Schönherr et al. 1995). No band could be detected, indicating that there was no contamination with biglycan (Fig. 2.6 C). Therefore, decorin was purified from conditioned media from human skin fibroblasts to a high degree of purity.

To ensure that the purified decorin was exclusively derived from the cells and not from serum used in culture media, 2 μg decorin were analyzed by mass spectrometry (MS). For this purpose, decorin was digested with chABC as described above, separated on a SDS-PAGE gel, and stained with Coomassie Brilliant Blue R-250. The bands of 43 and 45 kDa were cut out, reduced and alkylated, and digested with trypsin. Tryptic digests were subjected to MALDI-TOF (matrix-assisted laser desorption ionization - time of flight) and MS/MS analysis (Cardiff Biotechnology Service). The data gave a Mascot score of 93 for human decorin, with three peptides being identified (Fig. 2.7). Alignment of the amino acid sequence of human and bovine decorin using the ClustalW software suite (Larkin et al. 2007) showed that the identified peptides are only present in human decorin. Therefore, contamination of the decorin preparation with bovine decorin or biglycan could be excluded.
Figure 2.6 Analysis of purified decorin from human skin fibroblast conditioned media

A: SDS-PAGE analysis followed by silver staining of decorin purified from conditioned medium of human skin fibroblasts. 4 μg decorin before or after digestion with chABC was separated in 4-20% SDS-PAGE gels under reducing conditions. Left-hand lane shows native decorin with intact GAG-chain as a smear of about 70-100 kDa in size. Enzymatic cleavage of the GAG-chain by chABC resolved the smear into two bands of about 43 and 45 kDa (middle lane). These constitute decorin core protein with either 2 or 3 attached N-glycans (Glössl 1984). The arrowhead points to an additional faint band of ~90 kDa. chABC alone shows a band of 97 kDa in size (middle and right-hand lane). Migration of molecular weight marker is indicated on the right.

B: Western blotting using a polyclonal antibody raised against decorin confirmed the successful purification of decorin. A smear of ~70-100 kDa with the undigested decorin and two bands of 43 and 45 kDa, respectively, with chABC-digested sample confirm that the silver-stained proteins correspond to decorin. The band at around 90 kDa is recognized by the anti-decorin antibody. Previous work revealed that this band constitutes a stable decorin-dimer (Fiedler, 2007).

C: Western blotting using a polyclonal antibody raised against biglycan. No signal could be detected even after prolonged exposure to the photographic film.
Figure 2.7 Analysis of decorin peptide sequence using MALDI-TOF
A: To verify the purification of human and to rule out any cross-contamination with bovine decorin from FCS, decorin core protein was subjected to trypsin cleavage followed by tandem mass spectrometry analysis. Identified peptides are shaded in yellow. The identified peptides are consistent with the human but not bovine decorin sequence.
B: Summary of the ion scores and expect values for the identified decorin peptides. The ion score is a measure for the matching of the stated peptide with the observed peak in the MS/MS spectrum. A value of >46 is considered to be significant. The expect value is a measure of the probability of the peak observed matching the peptide sequence by chance. A value <0.1 is considered to be confident (Takegawa et al. 2006).
2.3.6 Decorin purification from human chondrocyte progenitor conditioned media

Similar to the purification of decorin from human skin fibroblasts, decorin was purified from one liter of conditioned media collected from human chondrocyte progenitor cells during culturing and culture expansion. Briefly, these cells originate from non-osteoarthritic knee articular cartilage and were collected post-mortem from a 56 year old male. The cells were isolated from the superficial zone of cartilage using differential fibronectin adhesion (Jones and Watt 1993). They are characterized by a high expression of SOX9 and Notch1 even after extensive expansion in monolayer culture and can be differentiated into cartilage when cultured in a 3D environment using appropriate culture medium (Williams et al. 2010; Dowthwaite et al. 2004) (see chapter 3, section 3.2.2 and 3.2.5).

The medium (containing protease inhibitor cocktail, 5 mM Tris-HCl, 0.1% Triton X-100) was applied to a column containing DEAE cellulose to remove the majority of serum proteins. Highly charged proteins were eluted using 1 M NaCl and collected fractions were pooled for further purification. After extensive dialysis against 20 mM Tris-HCl, pH 7.4, 400 mM NaCl, the dialyzed sample was applied to a Source 15Q column. Again, the column was washed using moderate salt concentrations, before applying a buffer containing 1 M NaCl, upon which a sharp peak absorbing at 280 nm was eluting (Fig. 2.8 A). Three fractions (13, 14, and 15) of 1 ml were collected and used for further analysis. Fraction 13 contained 112 µg, fraction 14 contained 208 µg and fraction 15 contained 135 µg protein, as determined by BCA assay. 4 µg protein from each faction were used for SDS-PAGE analysis followed by silver staining and 2 µg for Western blot analysis. To detect decorin core protein, an aliquot of each fraction was digested using chABC.

In the silver-stained SDS-PAGE gel, several bands were visible in the lanes of the undigested samples (Fig. 2.8B). There was no predominant characteristic smear as seen before for the intact skin fibroblast decorin (Fig. 2.6 A). However, after digestion with chABC, two major bands with a size of approximately 45 kDa and 66 kDa appeared. The upper band corresponds to BSA which was a component of the chABC preparation used. The lower band
appears only after digestion with chABC and therefore corresponds to one or more proteoglycan core proteins. This data confirms that the predominant component of the sample was a CS/DS-bearing proteoglycan of ~45 kDa. The presence of decorin was further confirmed by Western blotting (Fig. 2.8 C). Each fraction contained decorin. Undigested samples showed a small smear at around 100 kDa, indicative of highly glycanated decorin. The core protein of decorin with a size of ~45 kDa was only observed in the digested samples as expected. In chABC digested samples of fraction 14 and 15, an additional band at around 97 kDa is visible which corresponds to dimeric decorin as previously observed in Fig. 2.6 B. The membrane was then reprobed for biglycan. Weak bands with a size of ~45 kDa were observed only in the chABC digested samples, indicating a contamination of the sample with biglycan (Fig. 2.8D). However, this is below the limit of detection for the intact proteoglycan and hence present in substantially lower amounts than decorin. It is also possible that other SLRPs like fibromodulin or lumican were present. These proteoglycans contain a KS chain, and therefore the core proteins would not be visible after digestion with chABC. For all further analysis, fraction 13 was used because it showed the lowest amount of biglycan contamination from all three fractions.
Figure 2.8 Purification of decorin from human chondrocyte progenitor cell conditioned media.

Chondrocyte progenitor cells (PRO3 cell line) were grown in the presence of 10% FCS in monolayer culture and media was collected when the cells reached about 70% confluency. Most serum proteins were removed using DEAE-cellulose ion exchange chromatography. The proteoglycan fraction eluted with 1 M NaCl was pooled, dialyzed (400 mM NaCl containing buffer) and subjected to Source 15Q ion exchange chromatography. Small proteoglycans eluted at 1 M NaCl (A). The fractions collected during elution of the peak were analyzed using SDS-PAGE followed by silver staining (B). Western blot analysis of the elution fractions showed the presence of decorin (C) and biglycan (D). Samples were digested with chondroitinase as indicated (+/- chABC). The lane marked chABC represents the lyase preparation itself as a control.
2.3.7 Comparison of decorin purified from different cell sources

In addition to the purified decorin from skin fibroblasts and chondrocyte progenitor cells, recombinantly expressed human decorin purified from HEK293 cells (EMP Genetech, Ingolstadt, Germany) was used for analysis. To compare the different decorin preparations with each other, 2 µg of each sample was analyzed using SDS-PAGE, with or without chABC digestion.

In the silver-stained gel, intact decorin derived from chondrocyte progenitor cell medium showed a higher average molecular mass than skin fibroblast or HEK293 intact decorin (Fig. 2.9 A). Decorin purified from HEK293 cells showed the broadest smear with a size of approximately 80-120 kDa indicating a large degree of variation in GAG chain length. After digestion with chABC, bands of approximately 43 and 45 kDa corresponding to decorin core protein were the predominant components. Interestingly, compared to decorin purified from skin fibroblasts, an additional band of about 40 kDa was observed for decorin from chondrocyte progenitor cells and HEK293 cells, indicating that decorin with reduced N-glycosylation may be present. A band with a size of approximately 90 kDa belonged to chABC.

Western blot analysis (Fig. 2.9 B) showed that the bands for skin fibroblast and HEK293 decorin visible after probing the membrane with an anti-decorin antibody are matching up with the bands visible in the silver-stained gel as expected. In addition, dimeric decorin is visible for the skin fibroblast decorin after digestion with chABC. For chondrocyte progenitor cell decorin, the bands for decorin are weaker than expected from the silver stained gel and only partially overlap. Interestingly, only a single band was detected for the decorin core protein, likely corresponding to decorin carrying 3 N-glycans, which was not expected given the broad band observed in the silver-stained gel. The faster migrating band may be biglycan as shown in Fig. 2.8. In contrast, chABC-digested HEK293 decorin showed a broad band with a size of approximately 40-45 kDa, indicating that decorin without N-glycosylation may be present. Collectively, these data show that both N and O-linked glycosylation are cell type specific.
Figure 2.9 Comparison of decorin purified from different cell types.
2 µg of decorin from human skin fibroblasts, human chondrocyte progenitor cells (fraction 13) and recombinantly expressed in HEK293 cells were separated in 4-20% SDS SDS-PAGE under reducing conditions followed by silver staining (A) and Western blotting using an antibody raised against human decorin (B). Where indicated, samples were digested with chondroitinase (+/- chABC). Lane marked as ‘chAC’ shows the chondroitinase preparation on its own as a control.
2.3.8 Disaccharide analysis of decorin from different cell types
To analyze the disaccharide composition of the CS/DS chain attached to decorin purified from skin fibroblast and chondrocyte progenitor cell conditioned media as well as from recombinant decorin purified from HEK293 cells, 4 µg decorin from each sample was digested using 5 mU of chABC, chAC-I and chB. The disaccharides were labeled using AMAC and analyzed using reversed phase HPLC. Fig. 2.10 shows representative HPLC chromatograms of AMAC-labeled disaccharides from skin fibroblast decorin after digestion with the respective chondroitinases. Chromatograms for the other decorin preparations from chondrocyte progenitor cells and HEK293 cells after digestion with chondroitinases were of similar quality (not shown).
After complete depolymerization of the GAG chains with chABC, which cleaves both CS and DS, the total disaccharide composition was obtained (Fig. 2.11 A). The comparison of the disaccharide composition of the respective CS/DS chains from the different decorin preparations showed that there are substantial differences in the relative amounts of monosulphated disaccharides. The most abundant disaccharides were \( \Delta 4S \) and \( \Delta 6S \), and the relative levels of these differed significantly between the different decorin GAG chains (Fig. 2.11 A). Skin fibroblast decorin contained about 74.8% \( \Delta 4S \) and 12.8% \( \Delta 6S \) whereas chondrocyte progenitor cell decorin contained about 30.5% \( \Delta 4S \) and 57.3% \( \Delta 6S \) and HEK293 decorin contained about 22.9% \( \Delta 4S \) and 50.3% \( \Delta 6S \). In addition, \( \Delta 0S \) was also highly abundant in HEK293 decorin (17.0%), whereas it contributed only a small fraction to the composition of the GAG chains of the other decorins. After enzymatic digestion using chAC-I, the disaccharide composition of the CS type disaccharide within the GAG chains could be determined (Fig. 2.11 B). The ratios of the relative amount of \( \Delta 4S \) to \( \Delta 6S \) for chondrocyte progenitor cell and HEK293 decorin were not substantially changed (0.53 to 0.63 and 0.46 to 0.5, respectively), whereas it changed from 5.8 to 9.4 for skin fibroblast decorin. A much lower relative amount of tri- and disulphated disaccharides (0.7 to 1.29% and 1.3 to 1.75%, respectively) was observed compared to the chABC digest (0.8 to 1.5% and 3.3 to 6.0%, respectively), suggesting that these are largely not made of CS but DS.
Figure 2.10 Reversed phase HPLC analysis of the CS/DS chain disaccharide composition of decorin purified from human skin fibroblasts.

4 µg decorin were digested with chondroitinase ABC (A), AC-1 (B) or B (C). Disaccharides were labeled with AMAC and analyzed using reversed phase HPLC as described in Fig. 2.2. The elution positions of the disaccharide standards are indicated with arrows. Identified disaccharides within the samples are indicated in red. Note, the relative abundance of disaccharides in the different digestions requires different levels of sensitivity (scale on y-axis).
Digestion of the GAG chains using chB (specifically cleaving only DS disaccharides) showed that the major DS disaccharide was Δ4S (90.1 – 98.1%), followed by Δ2,4diS (1.9 – 7.8%) and Δ2,4,6triS (0-1.5%) (Fig. 2.11 C). In contrast to digestion with chABC and chAC-I, no Δ6S and Δ0S was observed. The reason for this is that chB requires a 4-O-sulphated GalNAc residue to perform the cleavage of the galactosaminidic bond (Yoshida et al. 1993). By comparing the relative amounts of each disaccharide species, a substantial increase in the relative amount of Δ2,4diS between the chB and chABC digest can be observed for the skin fibroblast decorin. This shows that the majority of Δ2,4diS is made from DS, which is supported by the absence of this disaccharide after chAC-I digest. The same is true for Δ2,4,6triS. Conversely, Δ4,6diS can only be observed after digestion with chABC and chAC-I, which suggests that this is mostly made from CS.

By comparing the relative disaccharide amounts for decorin from chondrocyte progenitor cells after digestion with different enzymes, a marked increase of Δ2,4,6triS and Δ2,4diS was observed after chB digest, suggesting that here as well the majority of these disaccharides was constituted by DS. Decorin produced by HEK293 cells did not have any Δ2,4,6triS, but Δ2,4diS appeared to be of DS type as well.
Figure 2.11 Quantitative analysis of the GAG disaccharide composition from decorin isolated of different cell types.

For each sample, 4 µg protein was digested using chondroitinases ABC (A), AC-I (B) or B (C), followed by labeling with AMAC and reversed phase HPLC analysis. Peaks for individual disaccharides were integrated and the result expressed at the percentage of the total disaccharide content. The data shown in the graphs is representative for one batch of decorin of each source, which was analyzed in triplicate. Data is shown as mean ± SEM.
2.4 Discussion

2.4.1 Establishing a method for disaccharide analysis by HPLC

Differentially sulphated disaccharides derived from GAG chains can be successfully analyzed using strong anion exchange HPLC (Midura et al. 1994). In order to set up the method on the Dionex ICS-3000 HPLC system (Dionex, Idstein, Germany), several parameters needed to be optimized before analyzing GAG samples of unknown disaccharide composition. These parameters are the precise retention times and detection limit of individual disaccharides.

For determining the retention times, each disaccharide standard was run separately on the system. Comparison between the order in which the different non-sulphated, mono- and disulphated CS/DS disaccharides eluted on the Dionex HPLC system and CarboPac PA1 column to the data in the literature showed almost complete agreement. Only the elution order of Δ4,6diS and Δ2,4diS was reversed. Generally, these two disaccharides elute very close to each other and therefore minor changes in the column matrix could easily account for such a change in affinity.

In order to assess the detection limit for CS/DS disaccharides, several different concentrations were analyzed. It was found that an amount of 110 ng of each standard was very close to the detection limit (Fig. 2.1 B), but still allowed the resolution into single peaks. This is in good agreement with the proposed detection limit of about 50 ng using UV detection (absorbance at 232 nm) as described by Midura and colleagues.

Even though the detection limit of this method was in the 100 nanogram range, it was unlikely to be sensitive enough for the analysis of small amounts of biological samples. Therefore, the fluorescent labeling of disaccharides using 2-AB and AMAC was evaluated. This allowed to significantly increase sensitivity by a factor of $10^3$ (2-AB) and $10^4$ (AMAC), respectively. Firstly, the fluorescent labeling of disaccharides with 2-AB was established (Kinoshita and Sugahara 1999). 2-AB is a relatively hydrophilic compound and therefore allows for separation of unlabeled disaccharides using an ion exchange HPLC. Despite the high sensitivity (picogram range), the method had a substantial
disadvantage which was the post-labeling cleanup that was required. Two different methods for the cleanup were evaluated, showing that paper chromatography was most effective. Nevertheless, an incomplete recovery of labeled disaccharides from the filter paper is a concern, especially of low-abundant disaccharide species. In addition, the Δ4S and Δ6S disaccharides eluted too close to each other for confident discrimination, even after optimization of the buffer gradient. Kinoshita and coworkers did not observe this problem. They could fully resolve every disaccharide, even though Δ4S and Δ6S were also quite close to each other. One reason for this could be the column used for separation. In their study, an amine-bound silica PA-03 column (250 x 4.6 mm, YMC Co., Kyoto, Japan) was used (which is not commercially available anymore), that may have slightly different properties compared to the column YMC-Pack Polyamine II (250 x 4.6 mm, YMC Europe GmbH, Dinslaken, Germany) employed here.

Another commonly used fluorophore, AMAC, was then used for disaccharide labeling (Deakin and Lyon 2008; Volpi 2010; Kitagawa et al. 1995; Jackson 1994). The separation of AMAC-labeled sugars follows a completely different principle. Here, the hydrophobicity of AMAC is exploited and separation of disaccharides achieved using reversed phase HPLC with a C-18 column (Fig. 2.4 A). Post-labeling cleanup was not necessary because free AMAC is more hydrophobic than labeled disaccharides and therefore elutes last. After establishing the AMAC-labeling and HPLC analysis method, a good separation of each disaccharide was achieved.

In conclusion, sensitive HPLC-based methods for GAG disaccharide analysis were compared. The HPLC methods and determined sensitivity are summarized in Table 2.4. AMAC-labeling of disaccharides was the most sensitive method and showed the best separation of individual disaccharide species.
Table 2.4 Comparison of HPLC disaccharide analysis methods

<table>
<thead>
<tr>
<th></th>
<th>unlabeled</th>
<th>2-AB labeled</th>
<th>AMAC labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection mode</td>
<td>Absorbance (232 nm)</td>
<td>Fluorescence</td>
<td>Fluorescence</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\lambda_{EX}$: 330 nm</td>
<td>$\lambda_{EX}$: 427 nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\lambda_{EM}$: 420 nm</td>
<td>$\lambda_{EM}$: 520 nm</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>~100 ng</td>
<td>~100 pg</td>
<td>~10 pg</td>
</tr>
<tr>
<td>Column</td>
<td>CarboPAC PA1 (250 x 4 mm, Dionex)</td>
<td>Polyamine II column (250 x 4.6 mm, YMC)</td>
<td>C-18 column (100 x 4.6 mm, Kinetex)</td>
</tr>
<tr>
<td>Separation method</td>
<td>Normal phase: ion exchange</td>
<td>Normal phase: ion exchange</td>
<td>Reverse phase: hydrophobicity</td>
</tr>
<tr>
<td>Gradient</td>
<td>Increasing NaCl</td>
<td>Increasing NaH$_2$PO$_4$</td>
<td>Increasing methanol</td>
</tr>
<tr>
<td>Labeling conditions</td>
<td>None</td>
<td>Reductive amination, 2h, 65$^\circ$C</td>
<td>Reductive amination, 16h, 37$^\circ$C</td>
</tr>
<tr>
<td>Post-labeling cleanup</td>
<td>None</td>
<td>Paper chromatography, chloroform extraction</td>
<td>None</td>
</tr>
</tbody>
</table>

2.4.2 Characterization of decorin preparations from different cell types

Decorin from human skin fibroblast media was purified using a two-step anion exchange chromatography method without adding denaturing agents and without precipitation of proteins from the media. Purity was assessed through SDS-PAGE analysis followed by silver staining and Western blotting and was high as described in previous studies (Glössl et al. 1984; Schönherr et al. 2005; Fiedler et al. 2008). After chABC digestion, in addition to the core protein at 43 and 45 kDa, a band at around 90 kDa was also recognized by a decorin-specific antibody. A previous study in our lab analyzed the band using mass spectrometry and provided evidence for it to be a cross-linked decorin-dimer (Fiedler 2007).

Purification of decorin using anion exchange chromatography reported the contamination of decorin with biglycan (Pearson and Gibson 1982) and $\alpha_2$HS glycoprotein (Sugars et al. 2002). However, the method employed here differs and no biglycan contamination was detected using Western blotting with a biglycan specific antibody or mass spectrometry of the bands constituting the core protein. Furthermore, the skin fibroblast cell line used as a source for
decorin was reported to lack an efficient biglycan synthesis (Schönherr et al. 1995). Also, no contamination with $\alpha_2$HS glycoprotein (59 kDa) was noticed in the silver-stained SDS-PAGE gel. It was reported earlier that $\alpha_2$HS glycoprotein co-elutes with decorin using anion exchange chromatography. However, $\alpha_2$HS glycoprotein was shown to elute at salt concentrations below 0.35 M NaCl on a strong anion exchange matrix (Sugars et al. 2002), and therefore could be separated from decorin which eluted at higher NaCl concentrations (from Source 15Q matrix at > 0.7 M NaCl).

Decorin was also purified from the conditioned media of human chondrocyte progenitor cells. For this, the same purification protocol as for the skin fibroblast medium was used. The purity of the isolated proteoglycan(s) was assessed using SDS-PAGE followed by silver staining and Western blotting. After treatment with chABC, a predominant broad band with a molecular mass of ~45 kDa appeared, which is the expected size of the decorin core protein, and confirmed that the preparation was composed of > 95% CS/DS bearing proteoglycan. The presence of decorin was confirmed by probing with an anti-decorin antibody. However, a weak band of approximately 45 kDa appeared as well after reprobing the membrane with an antibody raised against human biglycan. Contamination with biglycan was not surprising given that the chondrocyte progenitor cells are derived from cartilage and as biglycan like decorin is highly abundant in cartilage (Heinegård and Saxne 2011). Biglycan and decorin share a high degree of homology and have similar properties. Their core proteins have a similar size and since biglycan can have one or two GAG chains attached, they elute at similar NaCl concentrations. However, unlike decorin, the amount of biglycan seemed to be only a small fraction of the total protein content. In order to minimize the effect of biglycan content on HPLC disaccharide analysis, the first fraction eluting from the Source Q15 matrix was used for downstream analysis as the Western blot showed that it had the lowest biglycan content.

The comparison of the different decorin preparations in combination with recombinantly expressed decorin isolated from HEK293 cells revealed differences in GAG chain length and also in the N-linked glycosylation. This might be a result of different tissue specific expression levels of
glycosyltransferases (Little et al. 2008). The data showed that the glycosylation of decorin depends on the cell type and possibly adapts its biological function depending on the tissue. Similar observations were made by Viola and coworkers, who found that CS/DS chains of decorin of various different bovine tissues have a unique disaccharide composition (Viola et al. 2006).

2.4.3 GAG analysis of decorin from different cell types
Decorin has long been subject to detailed disaccharide analysis because of its relative simple glycosylation (1 site for O-linked and 3 sites for N-linked oligosaccharides) and biological availability, be it purified from animal tissues or through recombinant expression. The major feature being that decorin comprises only one GAG chain which makes it well suited for developing novel GAG analysis methods because the analytical results can be directly related to this simple GAG chain structure (Laremore et al. 2010; Zamfir et al. 2011; Zhao et al. 2013; Viola et al. 2006). Proteoglycans with multiple GAG chains (e.g. aggrecan) could potentially be differentially sulphated on different domains of the core protein depending on the biological context. Several studies have analyzed the disaccharide composition of decorin, either recombinantly expressed in Chinese hamster ovary cells (Kitagawa et al. 1997), HEK293 cells (Laremore et al. 2010) or purified from skin tissue (Zhao et al. 2013). Even though the human skin fibroblast cell line used in this study was used in many previous studies on decorin GAG chain structure (Seidler et al. 2007; Zamfir et al. 2009; Zamfir et al. 2003; Glössl et al. 1984; Greve et al. 1990; Hausser et al. 1992; Schönherr et al. 1995; Hausser et al. 1989), none of the publications described the total disaccharide composition. In these studies, the skin fibroblast decorin GAG chain was mainly used for developing mass spectrometry techniques for GAG chain analysis. Interestingly, in one of these studies, the composition of a pentasulphated hexasaccharide that interacts with FGF2 was identified (Zamfir et al. 2003). More recent work gave insights into the architecture of the GAG chain (Zhao et al. 2013).

The disaccharide composition of the decorin GAG chains analyzed here differed greatly between the different cell types in line with observations in
previous studies (Jianyun Yan et al. 2011; Koźma et al. 2011; Li et al. 2013). Skin fibroblast decorin had $\Delta 4S$ as its main component (75%), whereas for the other ones, $\Delta 6S$ was the main disaccharide followed by $\Delta 4S$. Although $\Delta 0S$ contributed only a small part to the disaccharide composition of skin fibroblast and chondrocyte progenitor cell decorin, it was highly abundant within the HEK293 decorin (17%). The high amount of non-sulphated disaccharides was not unexpected as decorin expression in these cells is engineered. The amount of sulphotransferases located in the Golgi system may not have been sufficient to perform complete sulphation of the disaccharides because of the artificially high expression level of decorin. Incomplete posttranslational processing of decorin when expressed recombinantly has previously been reported (Laremore et al. 2010). Laremore and coworkers investigated the disaccharide composition of recombinant decorin purified from HEK293 cells using native PAGE and LC-MS. Using these techniques combined with digestion of the GAG chain with different chondroitinases, they proposed a rough map of decorin’s GAG chain. They suggested that it is comprised of CS and DS blocks of various lengths and disaccharide compositions. In contrast to the results described here, the major disaccharide was $\Delta 4S$ (63%), followed by $\Delta 6S$ (23%) and $\Delta 0S$ (12%) after digestion with chABC (Laremore et al. 2010). Nevertheless, they also made the observation that the relative amount of $\Delta 2,4diS$ increased when comparing chABC to chB digested GAG, indicating that this disaccharide is mostly contributed to by DS, which is in agreement with the results obtained here. Given that only one type of decorin from HEK293 cells grown in a single condition was used for their study, differences in the disaccharide composition compared to HEK293 decorin in the experiments described here could be the result of many factors, including culture media, expression system, and expression levels. An overview of the differences in disaccharide composition of decorin isolated from different cell types from this study and from others is shown in Table 2.5.

To conclude, these results show that decorin is a highly versatile proteoglycan, which can have substantially different modifications in its glycosylation, be it the amount or structure of N-linked glycans or the length and the disaccharide
composition of its CS/DS chain, depending on the cell type or biological context in which it is expressed. Different disaccharide compositions could have an influence on the biological function of decorin within a specific tissue. Especially highly sulphated disaccharides, e.g. Δ2,4,6triS and Δ2,4diS, can have an effect on binding of growth factors or to cell surface receptors (Sugahara and Kitagawa 2000).
Table 2.5 Disaccharide composition of CS/DS chains of decorin isolated from different cell types. Disaccharides are shown as relative percentage of the total amount.

<table>
<thead>
<tr>
<th>Disaccharide subunit</th>
<th>Human skin fibroblasts</th>
<th>Human chondrocyte progenitor</th>
<th>HEK293</th>
<th>HEK293 (Laremore et al 2010)</th>
<th>CHO (Nomura et al 2006)</th>
<th>Porcine skin (Zhao et al 2013)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ2,4,6triS</td>
<td>0.7</td>
<td>0.8</td>
<td>1.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Δ2,4diS</td>
<td>1.0</td>
<td>3.9</td>
<td>2.2</td>
<td>1.0</td>
<td>4.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Δ2,6diS</td>
<td>0.1</td>
<td>1.8</td>
<td>1.6</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Δ4,6diS</td>
<td>2.2</td>
<td>0.4</td>
<td>0</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Δ2S</td>
<td>2.8</td>
<td>2.0</td>
<td>4.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Δ4S</td>
<td>74.7</td>
<td>30.5</td>
<td>22.9</td>
<td>63.0</td>
<td>88.0</td>
<td>88.0</td>
</tr>
<tr>
<td>Δ6S</td>
<td>12.8</td>
<td>57.3</td>
<td>50.3</td>
<td>23.0</td>
<td>-</td>
<td>5.5</td>
</tr>
<tr>
<td>Δ0S</td>
<td>5.7</td>
<td>3.2</td>
<td>17.0</td>
<td>12.0</td>
<td>8.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Chapter 3:

Development and optimization of a novel human 3D cartilage model
3 Development and optimization of a novel human 3D cartilage model

3.1 Introduction

To investigate the molecular changes taking place in cartilage in developing OA, a model system of normal human cartilage would be of great advantage for the following reasons:

- investigating human cartilage and therefore detecting relevant events as there may be species specific differences in pathogenesis
- minimizing variability as a consequence of tissue origin
- studies conducted in tightly controlled environments

Human cartilage from a non-arthritic joint can only rarely be obtained, as cases (accidental death) where such tissue can be harvested are relatively rare and therefore are not a reliable source for a steady supply of cartilage for meaningful experimental studies. Data obtained from studies on tissue harvested from unaffected areas of osteoarthritic joints, e.g. collected from total knee arthroplasties, must to be viewed with caution. Even though the cartilage may look histologically normal, the chondrocytes nevertheless were exposed to the inflammatory conditions within the joint and therefore osteoarthritic changes could be imprinted. Hence, tissue engineered human cartilage is the obvious choice to analyse chondrocyte metabolism in a controlled and defined environment.

To generate cartilage-like tissue in vitro, various techniques using different approaches have been developed (Chung and Burdick 2008; Chen et al. 2014). Tissue scaffolds based on proteins (collagen, fibrin), polysaccharides (alginate, chitosan, HA) and synthetic polymers (polylactic acid, poly(lactic-coglycolic acid)) have been used for cartilage tissue engineering (Chung and Burdick 2008; Vinatier et al. 2009). They provide a support to which
chondrocytes/chondrogenic cells can adhere and produce extracellular matrix. In addition, scaffold-free approaches have also been developed where the resulting cartilage construct is the product solely of the extracellular matrix secreted by the cells. Because of the absence of artificial compounds, scaffold-free constructs are probably as close to native cartilage as possible. However, it takes considerable time before such constructs develop mechanical integrity and resilience to loading.

A typical scaffold-free model are micromass cultures, where cells are collected by centrifugation in a conical tube, leading to formation of a small ball-shaped cartilaginous tissue (Johnstone et al. 1998). In spite of the formation of cartilaginous tissue, a disadvantage of micromass cultures is the inhomogeneous differentiation of cells into chondrocytes. Incomplete chondrogenic differentiation can occur depending on the location of the cells within the pellet and therefore, be influenced by access to medium components and growth factors. Such micromass pellet cultures are also size limited and cell death may occur in the centre of the construct due to lack of sufficient nutrient diffusion. Moreover, mechanical loading experiments are difficult to perform since the cells are experiencing different amounts of compression depending on their location in the pellet. The use of transwell inserts with a semipermeable membrane to generate disc-shaped cartilage constructs provides a different approach for micromass cultures (Murdoch et al. 2007; Tew et al. 2008). This method has the advantage that the cells have access to nutrients from two sides independently, resulting in more homogenous chondrogenic differentiation and an overall architecture more akin of joint cartilage.

Independent of the culture system employed, the success of cartilage tissue engineering ultimately depends to a large extent on the cell type used. Chondrocytes harvested from articular cartilage can be expanded in monolayer culture but quickly change their phenotype and loose the ability to form a cartilaginous matrix (Schnabel et al. 2002; Lin et al. 2008). However, it was reported that dedifferentiated primary human chondrocytes are able to re-
differentiate in a 3D environment, e.g. alginate beads or micromass culture, after losing their chondrogenic potential during monolayer expansion (Schrobbback et al. 2011; Domm et al. 2002). The main problem is the limited number of chondrocytes that can be harvested from a single cartilage sample of each patient. This considerably limits the number of cartilage constructs that can be generated. Therefore, these cells are not suitable for large scale cartilage tissue engineering. Consequently, people have looked for alternative cell sources.

Bone marrow derived mesenchymal stem cells (BMSC) are commonly used for cartilage tissue engineering (Johnstone et al. 1998; Yoo et al. 1998; Solchaga et al. 2011; Murdoch et al. 2007). The cells form a cartilage-like matrix in micromass cultures, but show expression of terminal differentiation markers after a few weeks in culture (Johnstone et al. 1998). Adipose-tissue derived stem cells show mesenchymal cell characteristics and can be differentiated into chondrocytes. However, in comparison to BMSCs, these cells show a reduced chondrogenic potential, resulting in cartilage constructs with a lower collagen type II and GAG content (Huang et al. 2005; Im et al. 2005). It was also demonstrated that stem cells isolated from synovium (Kurth et al. 2007), periosteum (Fukumoto et al. 2003), muscle (Adachi et al. 2002) and umbilical cord tissue (Harris 2013) can be differentiated into cartilaginous tissue. A cell type that has thus far only rarely been investigated for cartilage tissue engineering are chondrocyte progenitor cells. They are a cell population showing mesenchymal stem cell properties (Archer et al. 2012), residing in the surface zone of articular cartilage (Dowthwaite et al. 2004). They are capable of differentiating into chondrocytes, osteoblasts or adipocytes, respectively in vitro and in vivo. Chondrocyte progenitor cells were recently isolated from human articular cartilage, using selection based on differential fibronectin adhesion (Williams et al. 2010). In contrast to normal full-depth chondrocytes which loose their chondrogenic potential after 8 population doublings (PD) (Dell’Accio et al. 2001; Schnabel et al. 2002; Cournil-Henrionnet et al. 2008), these chondrocyte progenitor cells keep their chondrogenicity and maintain the expression of the chondrocyte specific transcription factor Sox9 even after
extensive expansion in monolayer culture and after high numbers of PD (Williams et al. 2010).
Based on this background it was considered that the chondrocyte progenitor cells were the ideal cell-type for establishing an in vitro cartilage model.

3.1.1 Aims for this chapter:
1. To establish a methodology that supports chondrogenic differentiation of human articular cartilage progenitor cells and subsequent cartilage-like ECM deposition in in vitro culture.

2. To optimize the medium formulation to yield reliable homogenous and maximal cartilage matrix formation.
3.2 Materials and methods

3.2.1 Materials
Cell culture media and other cell culture reagents were purchased from Life Technologies (Paisley, UK). All other chemical reagents and proteins were purchased from Sigma Aldrich (Gillingham, UK) unless otherwise stated. Recombinant human transforming growth factor (TGF)-β2 and recombinant human basic fibroblast growth factor (FGF)-2 were purchased from Peprotech (London, UK). Recombinant human BMP-2 was a kind gift from Genetics Institute (Cambridge, MA, USA).

3.2.2 Cell culture
Human chondrocyte progenitor cells were isolated as described (Williams et al. 2010). For monolayer expansion, cells were cultured in a 1:1 mixture of Dulbecco’s minimal essential medium and Ham’s F12 (DMEM/F12, without L-glutamine, Gibco, Cat #21331-046), containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 50 μg/ml L-ascorbic acid-2-phosphate, 1 mg/ml D-glucose, 1 ng/ml TGF-β2 and 5 ng/ml FGF-2. Medium was changed every third day. Cells were maintained in a 1:1 mixture of fresh medium and conditioned medium. The growth factors were added fresh to the medium on the day when medium was changed. Passaging of cells was performed as described in chapter 2, section 2.2.8.

3.2.3 Coating of transwell membranes with collagen type II or fibronectin
Collagen type II was isolated from bovine nasal cartilage by acid extraction and pepsin digestion, and human serum fibronectin was obtained from Sigma (Cat #F1056). Collagen type II was solubilized in 0.5 M acetic acid at a concentration of 5 mg/ml. For coating, a solution with a concentration of 0.5 mg/ml was prepared by diluting the stock solution with sterile filtered 0.5 M acetic acid. 70 μl was added directly onto the membrane for each transwell
insert, followed by addition of 10µl 0.2 M Tris-HCl, pH 7.4, to neutralize the solution. After incubation overnight at 4°C, the excess solution was carefully removed and the transwell membrane dried in a cell culture hood. Human fibronectin stock solution was diluted in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.3) to a concentration of 40 µg/ml. To each transwell insert, 70µl of the solution were added and incubated overnight at 4°C. The solution was then carefully removed and the transwell membrane dried.

3.2.4 Chondrogenic differentiation of human chondrocyte progenitor cells

Cells were cultured as a monolayer in 75 cm$^2$ tissue culture flasks until about 80% confluency was reached. The cells were passaged using trypsin/EDTA solution (0.05% v/v, Gibco) and seeded into Millicell hanging transwell culture inserts (PET membrane, 0.4 µm pore size, 6.5 mm diameter, Millipore, Watford, UK) pre-coated with human fibronectin. A total cell number of 0.5 to 14 x 10$^6$ was used per construct depending on the experiment. Prior to seeding into the transwell inserts, the cells were washed in the respective medium formulations to be evaluated (Table 3.2). Cells were finally resuspended in 500 µl medium containing the respective growth factors (TGF-β2, BMP-2, bovine insulin) as indicated and seeded into the inserts which were placed into 24-well cell culture plates (Sarstedt, Nümbrecht, Germany). The plates containing the inserts were either placed directly in the incubator or centrifuged for 5 min at 250 x g prior to that. 1 ml of the respective medium containing growth factors was applied into the wells to immerse the bottom of the transwell with medium. The constructs were cultured for 14 to 28 days (depending on the experiment) and the medium from inside the transwell insert and bottom well was changed every 2 days with fresh medium.
Table 3.2 Media formulations described in the literature for the differentiation of chondrocytes, chondrocyte progenitor cells, and mesenchymal stem cells in 3D culture systems.

<table>
<thead>
<tr>
<th>Literature reference</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Williams et al. (2010)</td>
<td>DMEM/F12 10% heat-inactivated FCS 50 µg/ml ascorbate-2-phosphate 5.5 mM D-glucose 2 mM L-glutamine 1 ng/ml TGF-β2</td>
</tr>
<tr>
<td>monolayer expansion medium (without FGF-2)</td>
<td>DMEM/F12 10% heat-inactivated FCS 50 µg/ml ascorbate-2-phosphate 5.5 mM D-glucose 2 mM L-glutamine 1 ng/ml TGF-β2</td>
</tr>
<tr>
<td>Hayes et al. (2004)</td>
<td>DMEM/F12 20% heat-inactivated FCS 100 µg/ml L-ascorbic acid-2-phosphate 5 ng/ml TGF-β2</td>
</tr>
<tr>
<td>Williams et al. (2010)</td>
<td>DMEM/F12 2% heat-inactivated FCS 50 µg/ml L-ascorbic acid-2-phosphate 5.5 mM D-glucose ITS (10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium) 100 nM dexamethasone 10 ng/ml TGF-β2</td>
</tr>
<tr>
<td>Murdoch et al. (2007)</td>
<td>DMEM 25 mM D-glucose 4 mM L-glutamine 1 mM sodium pyruvate 100 U/ml penicillin and 100 µg/ml streptomycin non-essential amino acid mixture (1x) ITS supplement 100 nM dexamethasone 50 µg/mL L-ascorbic acid-2-phosphate 10 ng/ml TGF-β2</td>
</tr>
</tbody>
</table>

3.2.5 Immunocytochemistry
Chondrocyte progenitor cells (1 x 10^5 cells/well) were seeded into wells of a 24 well-plate containing circular glass coverslips coated with 40 µg/ml human fibronectin. Cells were cultured for 48 h, washed in PBS and either fixed with 4 % paraformaldehyde (PFA, Sigma Aldrich) in PBS for 30 min for staining with anti-Sox9 antibody (Abcam, Ab3697, 4 µg/ml) or ice cold methanol/acetone (1:1) for 20 min for staining with anti-Notch-1 antibody (bTAN-20, cell culture supernatant, Developmental Studies Hybridoma Bank, Iowa, USA). Permeabilization of the cells using 0.1 % Triton X-100 / 0.5 % PFA in PBS for 5 min was performed in addition for slides stained for Sox9. The coverslips
were washed twice in PBS and nonspecific binding sites were blocked with 1% BSA in PBS for 30 min at room temperature. Afterwards, the primary antibody was diluted in 1% BSA in PBS to the appropriate concentration and added, and coverslips incubated overnight at 4°C. Coverslips were washed 3x with TBS (50 mM Tris HCl, pH 7.4, 150 mM NaCl) and incubated with a compatible AlexaFlour488-conjugated secondary antibody (1 µg/ml in 1%BSA in TBS, Life Technologies) for 1 h at room temperature in the dark. Diluted antibodies were centrifuged for 5 min at 10,000 x g to remove any aggregated antibodies before application to the coverslips. Afterwards, the coverslips were washed 3x with TBS and then mounted with Vectashield containing DAPI (Vectorlabs, UK). Antibody controls were performed by replacing primary antibodies with non-specific mouse IgG or rabbit IgG (4 µg/ml, Jackson ImmunoResearch Laboratories Inc., West Grove, USA).

3.2.6 Histological methods
Cartilage constructs were fixed for 24 h using 4% (w/v) PFA in PBS. After extensive washing in PBS, the fixed constructs were paraffin-embedded and cut into sections with a thickness of 5 µm. Sections were deparaffinised in xylene and rehydrated with a series of decreasing ethanol-concentrations. Staining with haematoxylin and eosin was performed using a Leica autostaining machine (Oral Pathology Lab, UHW). Haematoxylin and eosin are commonly used to assess the tissue and cell morphology. Haematoxylin stains nuclei blue to purple and eosin stains cytoplasmic structures pink to red. Cartilage ECM structures are stained bluish. Safranin-O and toluidine blue (both from Sigma Aldrich) staining were performed as described (Schmitz et al. 2010). Briefly, slides were deparaffinised and rehydrated as described above. For toluidine blue staining, slides were immersed in staining solution (0.04% (w/v) in 200 mM sodium acetate, pH 4) for exactly 5 min and immediately washed using tap water afterwards. Afterwards, sections were dried, washed once in xylene and subsequently mounted using DPX mounting solution (Sigma Aldrich). Nuclei stain dark blue and structures rich in negatively charged GAG chains stain violet. Other structures stained in
different shades of light blue. Safranin-O staining involved staining of slides using Fast green solution (0.001% (w/v) in H₂O) for 5 min, followed by a washing step in 1% (v/v) acetic acid solution. In the next step, slides were stained for 5 min in Safranin-O staining solution (0.1% (w/v) in H₂O). After several washing steps in pure ethanol, the slide was washed once in xylene followed by mounting with DPX. With this staining protocol, cartilage matrix stained red to orange and underlying bone stained green. Other cellular components stain grey to green (Schmitz et al. 2010).

3.2.7 Biochemical analysis of 3D constructs
Constructs were washed in PBS and frozen on dry ice after all residual liquid was removed. Until further analysis, the constructs were stored at -80°C. For determination of GAG content, the constructs were transferred into a 1.5 ml tube. To release the GAG chains, constructs were digested in 200 µl of 500 µg/ml papain (specific activity > 10 units/mg protein, Sigma Aldrich) for 16 h at 65°C in 2 mM cysteine, 50 mM sodium phosphate, pH 6.5, 10 mM EDTA. Afterwards, the sample was mixed with 400 µl 0.1 M NaOH and incubated for 20 min at room temperature, followed by addition of 400 µl neutralizing buffer (4 M NaCl, 100 mM Na₂HPO₄, 0.1 M HCl, pH 7.2) as described (Solchaga et al. 2011). The mixture was centrifuged for 10 min at 14,000 x g and the supernatant transferred to a fresh 1.5 ml tube for further analysis.

3.2.8 DMMB assay
DMMB assay was essentially performed as described (Farndale et al. 1986). Briefly, 250 µl dimethyl methylene blue (Sigma Aldrich) reagent was mixed with 10 µl sample in a 96 well plate using a multi-pipette. The absorbance was measured instantly (within ~2 min) at 525 nm using a Spectrostar Omega (BMG Labtech, Ortenberg, Germany). A standard curve from 10 to 50 µg/ml was prepared in parallel using CS from bovine trachea (Sigma Aldrich). The curve was fitted by linear regression to derive GAG concentrations in samples.
3.2.9 Mechanical loading of cartilage constructs

2 x 10⁶ cells/insert were seeded on fibronectin-coated transwell membranes and cultured for 28 days to form cartilage constructs in serum-free chondrogenic medium containing growth factors. Immediately before mechanical loading, the constructs were removed from the transwell insert and were kept in a 24 well-plate well in differentiation medium.

Unconfined mechanical loading was performed using a Bose Electroforce® 3200 loading rig with a Bose low force axial transducer loading cell (220 N force capacity; Bose GmbH, Friedrichsdorf, Germany) attached to it (Fig. 3.1). To immobilize the cartilage construct for loading, 200 µl of 1% low melting point agarose was added into the well of a 24 well plate and after setting, a hole using an 8 mm biopsy punch was made. The agarose plug was then carefully removed and the well immersed in 1 ml chondrogenic differentiation medium (without growth factors). The cartilage construct was placed in the central excised area and was subjected to compression as detailed in the results section. Data was analysed using WinTest® software suite (Bose). This experiment was done in collaboration with Dr Emma Blain, School of Biosciences, Cardiff University.

Determination of Young’s modulus (a measure for the stiffness of an elastic material during unconfined compression) was determined by placing 28 day old constructs (removed from the transwell inserts and without being immersed in medium) on a metal base and compressing them for several minutes using an SMT1-5N load cell (Interface Inc., Scottsdale, AZ, USA) mounted on a loading rig (Servocon Systems, Daventry, UK). Constructs were compressed with 100 mN and the resistive force generated by the constructs was monitored. This experiment was done in collaboration with Professor Sam Evans, School of Engineering, Cardiff University.
Figure 3.1 Setup for mechanical loading of cartilage constructs. Mechanical properties of 28 day old cartilage constructs were examined using a Bose Electroforce® 3200 loading rig (A) together with a Bose low force axial transducer loading cell (A, inside red box; magnified in B). The cartilage construct was loaded inside a well of a 24 well plate which contained medium (C).
3.2.10 RT-PCR gene expression analysis
Isolation of total RNA, generation of cDNA and PCR analysis was performed as described in chapter 4, sections 4.2.6 and 4.2.7. The primers used for experiments in this chapter are described in Table 3.1.

Table 3.1 Primer sequences used for PCR analysis

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence of forward (top) and reverse primers (bottom) (5´ - 3´)</th>
<th>Reference sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-fos</td>
<td>GGCAAGGTGGAACAGTTATCTC CTTCTCCTTCAGCAGGTTGG</td>
<td>NM_005252</td>
</tr>
<tr>
<td>h36B4</td>
<td>AGATGCAGCAGATCCGCAT ATATGAGGCAGCAGTTTCTCCAG</td>
<td>(Wagener et al. 2001)</td>
</tr>
</tbody>
</table>

3.2.11 Statistical analysis
Statistical analysis was performed using the Prism software suite (Graphpad Software Inc., La Jolla, CA, USA). Data was analyzed by one-way analysis of variance (ANOVA) with post hoc analysis using Tukey's multiple comparison test. P values < 0.05 (95% confidence) were considered as significant.
3.3 Results

3.3.1 Development of cartilage-like tissue in an in vitro 3D culture system

Human knee articular cartilage chondrocyte progenitor cells (PRO2N, post-mortem derived from a 40 year old male with no evidence of OA pathogenesis) were the cell type of choice for developing the tissue model, in order to have a well-defined and theoretically at least, unlimited supply of cells. Using chondrocytes isolated from full depth articular cartilage would have required a regular supply of “normal” human tissue and resulted in having a broad mixture of cells in different differentiation stages in the model. Another reason for not using chondrocytes was that expected variations between cell isolates would likely be a major issue for the planned downstream studies. While having its own limitations, using a defined chondrocyte progenitor population and strictly controlled conditions for the studies will help in regards to reproducibility of any findings. The progenitor cell isolates used in this study were a kind gift by Professor C. Archer and Dr R. Williams (School of Biosciences, Cardiff University, UK). For expansion of the cells in monolayers, supplementing the culture medium with fibroblast growth factor-2 (FGF 2) (5 ng/ml) and transforming growth factor-β2 (TGF-β2) (1 ng/ml) was essential to keep the cells in their progenitor state (Williams et al., 2010). In monolayer, the cells showed a characteristic spindle shaped morphology (Fig. 3.2 A). Cells cultured without these growth factors dedifferentiated, adopted a different morphology and could not be used for tissue engineering (not shown).

To analyse whether the chondrocyte progenitor cells expanded in monolayer culture still possessed their chondrogenic potential as reported in the literature (Williams et al. 2010), cells were fixed, permeabilized and incubated with anti-Sox9 antibody and anti-Notch1 antibody, respectively (Fig. 3.2 B). The cells showed clear staining for the transcription factor Sox9 in the nucleus. Strong staining for Notch1 was observed on the cell membrane and also in cytoplasm, as previously described and taken as evidence of progenitor status (Hiraoka et al. 2006; Dowthwaite et al. 2004; Grogan et al. 2009). The cytoplasmic staining of Notch1 is most likely a result from staining of Notch1 being
synthesized in the ER/Golgi system at the time of permeabilization/fixation. No such labeling could be detected in the negative control, where primary antibodies were replaced with rabbit IgG or mouse IgG as appropriate. Maintenance of Sox9 expression in monolayer culture is related to a persistent chondrogenic potential in monolayer culture (Williams et al., 2010). Consequently, the cells displayed the criteria for chondrocyte progenitor and could be used for cartilage tissue engineering experiments.

Figure 3.2 Characterization of human articular cartilage progenitor cells
Phase contrast image of human chondrocyte progenitor cells in monolayer culture (A, bar = 100 µm). Immunocytochemical staining of fixed and permeabilized progenitor cells showed expression of Notch1 and Sox9 after expansion in monolayer (B, bar = 50 µm). Control antibody showed no staining. Nuclei are stained with DAPI (blue) in control to highlight that cells are present.
3.3.2 Comparison of different media formulations for chondrogenic differentiation

A high density insert cell culture system (Fig. 3.3) was chosen to generate 3D constructs from the chondrocyte progenitor cells, essentially according to Hayes and co-workers who successfully seeded full depth bovine chondrocytes into transwell inserts and thereby generated cartilage-like tissue (Hayes et al. 2007). Because of the comparatively low proliferation rate of PRO2N cells, the cell number was a limiting factor for the experiments which was also the reason why some conditions were only tested once in the early phase of method development. Further difficulties encountered were the long time frame before the outcome of an experiment could be evaluated (3-5 weeks) and that in the absence of cartilage formation, the constructs were difficult to recover from the transwell insert for analysis.

Initially, PRO2N cells were seeded at a density of $4 \times 10^6$ per insert into a collagen type II coated transwell insert in the same medium as for the expansion of the cells in monolayers except lacking FGF-2 (Williams et al. 2010). Medium was changed every second day for 28 days in total. Afterwards, the construct was fixed, excised from the transwell insert (but still attached to the membrane) and analysed using histological staining. Fig. 3.4 shows a section of the construct stained with haematoxylin & eosin (H&E) (Fig. 3.4 A) and Safranin-O/Fast Green (Fig. 3.4 B). The analysis revealed that the cells had formed fibrous tissue, and no evidence of chondrogenesis was apparent. Additionally, contraction of the cell construct was observed after 72 h in culture (not shown) which is indicative of the presence of myofibroblast-like cells.

Given that chondrocytes had been differentiated under similar conditions by others, the complete lack of differentiation came as a surprise. Therefore, chondrogenic media used by Hayes et al. (2007) and Williams et al. (2010) (Table 3.2) were evaluated because bovine chondrocytes and human chondrocyte progenitor cells, respectively, had been differentiated to chondrocytes successfully in 3D culture systems using these conditions.
Figure 3.3 Schematic depicting culture system.

Human articular cartilage chondrocyte progenitor cells were seeded at high density into a transwell insert with a coated membrane. The cells were cultured for up to 35 days in chondrogenic medium containing various growth and differentiation factors. Medium was changed every second day. Differentiation of constructs was analyzed histologically and biochemically. Variables that were tested over the course of this work included cell seeding density, nature of membrane coating, and medium composition.
For inserts cultured with chondrogenic media by Hayes et al. (2007), a transwell insert culture was set up with $1.4 \times 10^7$ cells and a collagen type II-coated membrane (Fig. 3.4 C, D). Another insert culture with collagen type II-coated membrane containing $1.0 \times 10^7$ cells was cultured as described by Williams et al. (2010) (Fig. 3.4 E, F). Media were changed every second day and the cells fixed after 21 days. Sections of the paraffin-embedded construct were stained with H&E and Safranin-O and revealed that neither culture conditions showed clear evidence of cartilage matrix formation, and therefore making it necessary to evaluate individual parameters one by one to find conditions that supported chondrogenesis. An important finding was the absence of cell contraction in insert cultures with higher cell seeding densities.
Figure 3.4 Evaluation of different chondrogenic differentiation media. H&E staining (A, C, E) and Safranin-O staining (B, D, F) of 5 µm paraffin sections of 3D constructs cultured in different chondrogenic differentiation media formulations. Initial work evaluated conditions described in the literature: 1., cells were seeded at a density of $4 \times 10^6$ cells/insert and cultured for 28 days in monolayer culture medium supplemented with 1 ng/ml TGF-β2 (A, B); 2., cells were seeded at a density of $1.4 \times 10^7$ cells/insert and cultured in chondrogenic medium according to Hayes et al. (2007) for 21 days (C, D); 3., cells were seeded at a density of $1.0 \times 10^7$ cells/insert and cultured in chondrogenic media formulation described by Williams et al. (2010) for 21 days. Bar = 100 µm.
3.3.3 **Effect of serum concentration on in vitro cartilage-formation**

As some factors present in serum may influence chondrogenesis, the effect of different serum concentrations on cartilage formation was explored. Insert cultures were seeded with a cell density of $1.0 \times 10^7$ cells per construct using the chondrogenic media formulation by Hayes et al. (2007), but containing 5%, 10% or 20% FCS. The constructs were cultured for 21 days and subjected to histological analysis (Fig. 3.5). By looking at the H&E stained sections, it is apparent that the constructs cultured with 5% and 10% serum were thicker than the construct cultured with 20% serum. Staining of extracellular matrix using Safranin-O showed that the construct with 5% serum showed the strongest staining when compared to the others. 20% serum did not lead to any meaningful proteoglycan-rich matrix deposition and hence resulted in the absence of Safranin-O staining. Taken together, these data suggested that high concentrations of serum were detrimental to chondrogenesis, at least when differentiating human articular cartilage progenitor cells.

However, even though the chondrogenic media formulation from Hayes and co-workers showed some promising results, the constructs were very fragile and did not produce a proper extracellular matrix as is found in cartilage which was very unsatisfactory. An additional chondrocyte progenitor cell isolate (PRO3N) was provided by Dr R. Williams to facilitate the supply of sufficient cells for subsequent experiments and to ensure that lack of differentiation was not cell strain specific. Like the previously used cell isolate PRO2N, PRO3N was isolated post-mortem from normal knee cartilage of a 56 year old male using selection by differential fibronectin-adhesion (Williams et al. 2010).
Figure 3.5 Effect of serum concentration on chondrogenic differentiation and matrix formation. H&E staining (left side) and Safranin-O staining (right side) of 5 µm paraffin sections of 3D constructs (1.0 x 10^7 cells/insert) cultured for 21 days in chondrogenic medium according to Hayes et al. (2007), but containing 5% serum (A), 10% serum (B) and 20% serum (C). Bar = 100 µm.
3.3.4 Chondrogenic differentiation of progenitor cells using serum free media

Several protocols for the chondrogenic differentiation of mesenchymal stem cells in which a serum-free media formulation is used have been described (Murdoch et al. 2007; Tew et al. 2008; Solchaga et al. 2011; Johnstone et al. 1998). Especially BMSCs are a well-established cell model for creating cartilage-like tissue \textit{in vitro}. Serum-free culture media have the advantage of not being affected by lot-specific differences in serum and this reduces variability. Furthermore, it was reported by Murdoch and co-workers (2007) that the creation of a dense cell layer within the transwell culture insert was essential for chondrogenic differentiation of BMSCs. This is similar in concept to pellet micromass cultures where cells are collected by centrifugation within a conical tube, forming a small pellet of cartilaginous tissue when cultured in chondrogenic media (Solchaga et al. 2011; Johnstone et al. 1998). It is suspected that the formation of a high density cell mass mimics the process of mesenchymal condensation in embryonic limb development (Johnstone et al. 1998). To create a dense cell layer inside the transwell insert, it was centrifuged at 250 $x$ g for 5 min immediately after applying a cell suspension into it as previously described (Murdoch et al. 2007).

To investigate the effect of using serum-free culture medium (Table 3.2) for chondrogenic differentiation of chondrocyte progenitor cells, it was critical to remove any residual serum from the cells before seeding which was achieved by washing the cells in serum free medium. Different seeding densities, from $1 \times 10^6$ to $4 \times 10^6$ cells per insert, were used to identify a suitable cell density for further experiments. In the initial experiment, cells were seeded in a transwell insert with an uncoated membrane. Approximately 24 h after seeding, the cell layer contracted and peeled off from the membrane. This resulted in a construct with a spherical shape. After 14 days in culture, the constructs were harvested for histological analysis (Fig. 3.6 A). In the following experiments, different membrane coatings were used to see whether this could prevent the contraction of the cell layer. For this, the membranes were either coated with collagen type II, the major fibrillar collagen found in cartilage ECM, or fibronectin. As the chondrocyte progenitor cells were selected based on
fibronectin adhesion during the initial isolation (Williams et al. 2010), it was a logical choice to use this protein to enhance cell adhesion. The high affinity for fibronectin is most likely due to abundant expression of \( \alpha 5\beta 1 \) integrin on the cell surface (Williams et al. 2010). The constructs were again harvested after 14 days of culture for histological analysis.

When using collagen type II, the constructs again contracted about 24 h after seeding (Fig. 3.6 B) similar to the constructs seeded on uncoated membranes. In contrast, fibronectin-coating of the transwell insert membrane led to the formation of a flat disc of tissue (Fig. 3.6 C). H&E staining of sections showed that the contracted constructs (Fig. 3.6 A, B) had differentiated into two layers similar to what has previously been reported in micromass cultures. This is likely reflecting limited diffusion of nutrients and growth factors into the center of the construct, particularly in larger constructs. Using fibronectin-coated membranes (Fig. 3.6 C), there was no contraction at any of the seeding densities, with an increase in thickness when increasing cell numbers from \( 1 \times 10^6 \) to \( 2 \times 10^6 \) but not from \( 2 \times 10^6 \) to \( 4 \times 10^6 \). Therefore, coating with fibronectin at a concentration of 40 µg/ml was used with a seeding density of \( 2 \times 10^6 \) cells/insert for all further construct experiments.
Figure 3.6 Coating of transwell membrane with fibronectin prevented contraction of constructs. H&E staining of 5 µm paraffin sections of 3D constructs cultured in serum-free chondrogenic medium supplemented with 10 ng/ml TGF-β2 for 14 days. Different cell numbers were seeded on uncoated (A) and collagen type II coated (B) membranes, resulting in contraction. Coating with fibronectin prevented contraction, resulting in a disc-shaped 3D construct (C). Bar = 100 µm.
3.3.5 The combination of TGF-β2 and BMP-2 for progenitor cell stimulation leads to increased formation of cartilaginous matrix

To achieve better chondrogenesis, several different growth factor combinations were then tested. Constructs were seeded at a density of 2 x 10^6 cells per insert and cultured in serum-free chondrogenic medium for 21 days with different growth factor combinations as follows: with 10 ng/ml TGF-β2 alone (Fig. 3.7 A, B), 10 ng/ml TGF-β2 and 50 µg/ml insulin (in addition to ITS supplement) (Fig. 3.7 C, D), 10 ng/ml TGF-β2 and 100 ng/ml BMP-2 (Fig. 3.7 E, F), 10 ng/ml TGF-β2, 100 ng/ml BMP-2 and 50 µg/ml insulin (Fig. 3.7 G, H), or 10 ng/ml TGF-β2, 100 ng/ml BMP-2, 50 µg/ml insulin and 5% serum. At the end of the culture period, the constructs were harvested and processed for histological analysis. Toluidine blue staining of the sections from each condition showed that there was an increase in proteoglycan content as soon as TGF-β2 and BMP-2 were used in combination (Fig. 3.7 F, H, J), whereas only weak toluidine blue staining was observed for TGF-β2 alone or in combination with additional insulin (Fig. 3.7 B, D). Interestingly, the increased toluidine blue staining was only observed in the top and bottom regions of the constructs suggesting that growth factor concentrations may be limiting. Moreover, the addition of insulin resulted in a marked increase in construct size, but only when TGF-β2 and BMP-2 were added at the same time suggesting that these growth factors had a synergistic effect on either cell proliferation or matrix synthesis. The addition of serum gave no overt improvement and resulted in a more variable differentiation. As a result, it was decided to stay with a chemically defined chondrogenic medium without serum. Therefore, the culture medium for chondrogenic differentiation was supplemented with TGF-β2, BMP-2 and a high concentration of insulin for all further experiments.
Figure 3.7 TGF-β2 in combination with BMP-2 and insulin resulted in cartilage-like matrix formation. H&E and toluidine blue staining of 5 µm paraffin sections of 3D constructs cultured for 21 days in serum-free chondrogenic medium supplemented with different growth factor combinations. Arrows are pointing to regions with increased toluidine blue staining, indicating increased GAG deposition into the matrix. (A, B) 10 ng/ml TGF-β2; (C, D) 10 ng/ml TGF-β2 + 50 µg/ml insulin; (E, F) 10 ng/ml TGF-β2 + 100 ng/ml BMP-2; (G, H) 10 ng/ml TGF-β2 + 50 µg/ml insulin + 100 ng/ml BMP-2; (I, J) 10 ng/ml TGF-β2 + 50 µg/ml insulin + 100 ng/ml BMP-2 + 5% FCS; Note, synergistic action of TGF-β2 and BMP-2 promotes chondrogenic differentiation, whereas insulin supports growth. Bar = 100 µm.
3.3.6 Optimization of growth factor concentrations for cartilage matrix formation

A serum-free differentiation medium supplemented with TGF-β2, BMP-2 and a high concentration of insulin was successful in driving the chondrogenic differentiation of human chondrocyte progenitor cells, leading to the formation of cartilage-like tissue. However, the constructs showed a toluidine blue staining intensity characteristic for cartilage only in the top and bottom regions which were in direct contact with the culture medium. It was speculated that the weakly stained region in the middle of the construct was probably a result of a diffusion gradient of the growth factors across the tissue, leading to insufficient availability of growth factors in the middle part. To test this hypothesis, a series of increasing TGF-β2 concentrations (10 – 160 ng/ml) was applied to a set of constructs while keeping the BMP-2 concentration constant at 100 ng/ml (Fig. 3.8 A). Additionally, another set of constructs was cultured in medium containing increasing concentrations of BMP-2 (100 – 1600 ng/ml) while keeping the concentration of TGF-β2 constant at 10 ng/ml (Fig. 3.8 B). The constructs were cultured for 28 days which was considered sufficient for cartilage matrix formation based on the previous experiments and as reported in the literature (Murdoch et al. 2007; Tew et al. 2008). Staining of sections with toluidine blue showed that the weakly stained region in the middle of the construct was decreasing with increasing TGF-β2 concentrations (Fig. 3.8 A). Furthermore, toluidine blue staining intensity substantially increased with increasing TGF-β2 concentration. The strongest toluidine blue staining and most homogenous tissue morphology was achieved with a concentration of 80 ng/ml TGF-β2. Increasing the concentration of BMP-2 had only little effect on the construct architecture or toluidine blue staining intensity. It was clear from these results, that TGF-β2 had the strongest effect on the chondrogenic differentiation. Based on these results, the chondrogenic differentiation medium was modified to an increased TGF-β2 concentration of 80 ng/ml which was used for all further experiments with cartilage constructs.
Figure 3.8 Titration of effective concentrations of TGF-β2 and BMP-2 for cartilage-like matrix formation. Toluidine blue staining of 5 µm paraffin sections of cartilage constructs grown in serum-free chondrogenic medium containing different concentrations of TGF-β2 and BMP-2 for 28 days. 2 x 10^6 cells/insert were seeded into fibronectin coated inserts. One set of constructs was cultured in medium containing 100 ng/ml BMP-2 and increasing concentrations of TGF-β2 (10 – 160 ng/ml) (A), whereas another set of constructs was cultured in 10 ng/ml TGF-β2 and increasing concentrations of BMP-2 (100 – 1600 ng/ml) (B). A representative set of pictures from two independent experiments is shown in this figure. Bar = 100 µm.
3.3.7 Biochemical and histological characterization of the effect of TGF-β2 and BMP-2 on cartilaginous matrix formation

For a better understanding of the role of the different growth factors in matrix formation, constructs were cultured with different growth factor combinations and subjected to histological and quantitative biochemical analysis. The culture medium contained either no growth factors (control), 80 ng/ml TGF-β2 or 100 ng/ml BMP-2 alone, or both in combination. The wet weights of the constructs cultured in different media showed significant differences (Fig. 3.9 A). While control constructs had an average wet weight of 4.7 mg, the presence of 80 ng/ml TGF-β2 alone resulted in an increase to 7.4 mg. BMP-2 alone at a concentration of 100 ng/ml resulted in a doubling in average wet weight to 14 mg. The highest wet weight of 23.4 mg was achieved by combining TGF-β2 and BMP-2, which was nearly twice the weight compared to BMP-2 alone. Analysis of the total GAG content of the constructs using the DMMB assay showed that the control constructs had very little GAG (~11 µg) (Fig. 3.9 B). Addition of TGF-β2 resulted in an 8-fold increase to 84 µg, whereas BMP-2 addition led to an average of 66 µg GAG per construct (Fig. 3.9 B). In line with wet weight analysis, the combination of BMP-2 and TGF-β2 resulted in a further substantial increase (~3 fold), with an average GAG content of 253 µg. By looking at sections of the different constructs stained with toluidine blue, very weak staining was observed in the control construct (Fig. 3.9 C). Moreover, it had a morphology akin of fibrous tissue unlike the constructs cultured with growth factors which resembled cartilage, particularly when TGF-β2 and BMP-2 were combined. The strongest staining was observed in constructs cultured with TGF-β2, either alone or in combination with BMP-2 in line with the results of the DMMB assay. Taken together, the data show that after optimizing various parameters like transwell insert membrane coating, cell seeding density, chondrogenic medium formulation and growth factor supplementation, a protocol for successful generation of cartilaginous tissue in vitro based on differentiation of human chondrocyte progenitor cells was established.
Figure 3.9 Combination of TGF-β2 and BMP-2 led to increased wet weight, GAG content and cartilaginous tissue morphology. Cells were seeded at a density of 2 x 10^6 cells/insert and cartilage constructs grown for 28 days in serum-free chondrogenic differentiation medium (including 50 µg/ml additional insulin) containing 80 ng/ml TGF-β2 only, 100 ng/ml BMP-2 only, both in combination, or neither growth factor. Wet weight was determined immediately after harvesting of constructs (A). Total GAG content was determined using DMMB assay (B). Toluidine blue staining of 5 µm paraffin sections of cartilage constructs grown under the described conditions showed abundant GAG deposition with medium supplemented with TGF-β2 alone or TGF-β2 + BMP-2, and more modest staining for GAG with medium containing BMP-2 alone. (C) Little if any staining was observed in constructs grown in control medium without any growth factors. Data is shown as mean ± SEM derived from three independent experiments with one sample each. Statistical significance of changes was evaluated with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. *P< 0.05, **P<0.01. Bar = 100 µm.
3.3.8 Characterization of mechanical properties of 3D cartilage constructs

Cartilage constructs grown for 28 days in the optimized chondrogenic medium had a disc shape with an opaque white to beige colour similar to joint cartilage. The discs had a diameter of 6 mm and a height of around 1 mm (Fig. 3.10 A). The construct comprised of a strong extracellular matrix which allowed for elastic deformation without permanently changing the construct shape. To demonstrate this, the construct was bent using a forceps and after releasing the pressure, regained its flat disc shape immediately (Fig. 3.10 B). This showed that the construct had properties of resilience similar to articular cartilage. Preliminary experiments were performed to evaluate mechanical properties using a Bose Electroforce® 3200 loading rig (Fig. 3.1). A construct (height: 0.93 mm) was compressed stepwise by 10, 20, 30 and 40% of its height with a frequency of 1 Hz for 1 min each, resulting in pressures from 11 – 261 kPa (Fig. 3.10 C). A higher relative compression of 50% resulted in construct failure. This is similar to pressures observed for newborn bovine cartilage, which were from 22 to 84 kPa after compression by 10 to 30%, respectively (Klein et al. 2007). To analyze whether the cartilage construct responded to loading as expected in cartilage, a construct was loaded with a physiologically relevant load of 10% relative compression for 1 min at 1 Hz. The construct was immersed in TRIzol 15 min after loading and subsequently stored at -20°C until RNA isolation was performed. RT-PCR analysis showed that there was an increase in the expression of the mechanosensitive gene c-fos (Bougault et al. 2012) in the loaded sample compared to the unloaded control construct (Fig. 3.10 D). For further characterization of the mechanical properties of the cartilage constructs, the Young’s modulus was determined. This is a measure of the elasticity of an object. It is often used for the characterization of the biomechanical properties of tissues (Abrahamsson et al. 2010; Martin et al. 2000; Mauck et al. 2006; Little et al. 2011). Using indentation testing, a Young’s modulus of 39.05 ± 2.09 kPa was determined for 28 day old cartilage constructs (n=5; mean ± SEM). Based on these results,
the cartilage constructs created from human chondrocyte progenitor cells could potentially be used for further experiments to analyze the effect of mechanical loading on changes in human chondrocyte gene expression.

Figure 3.10 Physical properties of 3D cartilage constructs established from adult human articular cartilage progenitor cells. Human chondrocyte progenitor cells were seeded into transwell culture inserts (2 x 10^6 cells/insert) coated with fibronectin and differentiated for 28 days in chondrogenic differentiation medium containing 80 ng/ml TGF-β2, 100 ng/ml BMP-2, and 50 µg/ml additional insulin. A: Top view (left) and lateral view (right) of the construct after removal from the membrane; B: Bending of the construct held within a forceps to demonstrate resilience of the tissue; C: Cartilage constructs were mechanically loaded to 10 - 40% relative compression for 60 sec at 1 Hz at each step. D: c-fos expression was analyzed by RT-PCR in unloaded constructs as well as constructs subjected to loading (10% compression, 1Hz, 60 sec) 45 min after stimulation. Expression of the housekeeping gene h36B4 was analyzed in parallel as a control. Data confirmed mechanoresponse of cells. Data is shown from one single experiment using one construct.
3.4 Discussion

A major aim of the project was to develop an *in vitro* articular cartilage-like model system which can be used for analysing the carbohydrate synthesis machinery in chondrocytes in the context of normal and disease conditions such as inflammatory conditions or aberrant mechanical loading, as it occurs in osteoarthritis.

In this chapter, the development of a novel human 3D cartilage model system is described. This model is based on human chondrocyte progenitor cells, which were isolated post-mortem from normal adult articular knee cartilage. These cells have the advantage of maintaining a progenitor differentiation status after extensive expansion in monolayer (Williams et al. 2010). The cells used for experiments in this study had a high expression of SOX9, a transcription factor that is a master regulator for cartilage formation and maintaining a chondrogenic phenotype (Bi et al. 1999). In addition, the cells also showed a high expression of Notch1, a putative stem cell marker found in progenitor cells (Hiraoka et al. 2006; Dowthwaite et al. 2004; Grogan et al. 2009). These features allow for the use of a cell population isolated from a single individual to be used for generating a large number of cartilage constructs for experimental studies, without having to consider variation due to genetic background. Instead of generating pellet micromass cultures, a culture model based on transwell culture inserts was chosen as described by Hayes and coworkers (Hayes et al. 2007). Generating comparably thin disc-shaped cartilage constructs resulted in a more homogenous cell differentiation compared to pellet cultures and better reflects the physiological situation where articular cartilage sits on a solid support (bone). Furthermore, the use of a porous membrane as a support allows for asymmetric stimulation of constructs from top and bottom with different supplements, and therefore, for stratification of the tissue. It has recently been suggested that epigenetic information should be considered in cartilage tissue engineering (Hong and Reddi 2012; Polo et al. 2010). Unlike cartilage derived from BMSC’s, target tissue derived progenitor cells as used here therefore constitute a suitable cell source for tissue engineering–based approaches.
In initial experiments, different media formulations for chondrogenic differentiation of cells described in the literature were evaluated. A TGF-$\beta$ concentration of 5 or 10 ng/ml appears to be sufficient for the formation of cartilaginous tissue when using primary chondrocytes (Hayes et al. 2007) or BMSCs (Johnstone et al. 1998; Murdoch et al. 2007), respectively. However, the chondrocyte progenitor cells did not form cartilaginous tissue when cultured in serum-free chondrogenic medium supplemented with 10 ng/ml TGF-$\beta$2. An explanation for this could be a lower responsiveness of the cells to TGF-$\beta$2. In addition, the thickness of the construct resulted in the generation of a diffusion gradient across the construct, whereby the cells in the inside of the construct were exposed to little or no TGF-$\beta$2. In order to enhance the chondrogenic differentiation of the constructs, several different growth factor combinations were tried based on literature evidence for factors regulating chondrogenesis. Previous studies have shown that addition of insulin or IGF-1 to the culture medium can enhance the chondrogenic differentiation and cartilage formation of primary bovine chondrocytes (Kellner et al. 2001) and human BMSCs (Mueller et al. 2013). IGF-1 is about 100 fold more potent in the activation of the IGF-1 receptor than insulin. Both human recombinant IGF-1 and bovine insulin were evaluated with similar results (not shown). As IGF-1 is also a much more expensive reagent and when used for the generation of a large amount of cartilage constructs associated with a very substantial cost, insulin was included in the final formulation of the differentiation medium over IGF-1. To compensate for the lower potency, a high insulin concentration was used. The induction of chondrogenesis using a combination of IGF-1 and BMP-2 in vivo in an ectopic differentiation model was previously reported by our laboratory (Bulpitt and Aeschlimann 1999). Chondrogenesis of chondroprogenitor cells was indeed achieved when TGF-$\beta$2 and BMP-2 were present at the same time and supplementation with insulin had a pronounced synergistic effect. To determine the effective concentration of TGF-$\beta$2 and BMP-2 respectively, two concentration series with either increasing concentrations of TGF-$\beta$2 or BMP-2 were performed. Increasing TGF-$\beta$2 led to an increase in toluidine blue staining and reduction of un-stained middle zone in the constructs, whereas increasing BMP-2 had no significant effect,
indicating that TGF-β2 availability was limiting. Based on these results, the concentrations for TGF-β2 and BMP-2 were adjusted to 80 ng/ml and 100 ng/ml, respectively, to achieve homogenous differentiation and maximal matrix formation. A major finding in the development of a suitable chondrogenic differentiation medium for the chondrocyte progenitor cells was that the addition of BMP-2 together with TGF-β2 dramatically increased the chondrogenic differentiation of the cells (Fig. 3.9).

Shintani and coworkers described a similar synergistic effect on chondrogenesis by culturing fetal bovine synovium explant cultures in a medium containing 10 ng/ml TGF-β1 and 200 ng/ml BMP-2 (Shintani et al. 2013). Furthermore, this treatment arrested hypertrophic differentiation of the cells at an early stage. This is an important finding, which is also of importance for this study where hypertrophic differentiation is undesirable. In contrast to this study, Shintani and coworkers reported that BMP-2 alone was sufficient to form cartilaginous tissue which however, was not very homogenous and terminally differentiated. While BMP-2 alone increased both wet weight and GAG content in this study, tissue morphology was not articular cartilage-like, indicating incomplete differentiation.

Since overlapping expression patterns of the TGF-β and BMP receptors and respective ligands are found in the developing growth plate during skeletal development, an interaction between the different signaling pathways could take place (Keller et al. 2011). TGF-β signaling depends on the receptor Smads (R-Smad) 2 and 3, whereas BMP signaling involves R-Smad 1, 5, and 8. Both have a common downstream target, Smad4 (Miyazono et al. 2001). Using the chondrogenic ATCD5 cell line, Keller and coworkers found that TGF-β1 strongly enhanced BMP signaling, while BMP-2 signaling reduced TGF-β signaling (Keller et al. 2011). Several explanations for this were proposed. For example, competition over Smad4 between the pathways could take place. Furthermore, BMP signaling could induce inhibitory Smad6 and 7, which negatively regulates TGF-β signaling. Yet another possibility is an interplay between canonical TGF-β/BMP signaling depending on Smads and non-canonical signaling depending on p38, JNK and Erk1/2 signaling pathways.
Considering these results, BMP-2 is expected to be the driving force in chondrogenic differentiation. However, the results identify a critical role for TGF-β signaling as no consistent cartilage formation occurred in the presence of BMP-2 alone. It is possible that the respective growth factors are required at successive stages in the differentiation program of the cells rather than their synergistic simultaneous action. Testing this was unfortunately beyond the scope of this project. Therefore, the exact mechanism of the synergistic effect of TGF-β2 and BMP-2 in chondrogenic differentiation remains elusive. After all, the optimal composition of chondrogenic differentiation medium strongly depends on the cell type used for tissue engineering.

To further characterize the engineered cartilage-like tissue, the mechanical properties of 28 day constructs were determined by measuring the Young’s modulus. This is a measure for the stiffness of an elastic object and helps to compare cartilage matrices from different species, at different stages of development, and different tissue engineering approaches (Little et al. 2011). Human adult articular cartilage has a Young’s modulus of 0.45 to 0.8 MPa (Mansour 2009; Jurvelin et al. 2003). In contrast, tissue engineered constructs mostly have a much lower Young’s modulus and are strongly influenced by the scaffold material used (Yamaoka et al. 2006; Hoenig et al. 2013). Few literature data are available on the Young’s modulus for scaffold-free cartilage constructs. Using porcine chondrocytes, Hoenig and colleagues created scaffold-free cartilage constructs over 21 days in culture for mechanical stimulation (Hoenig et al. 2011). They determined a modulus of about 35 kPa, which is comparable to the value of ~39 kPa determined for the constructs used in this study. The extracellular matrix of a 28 day old construct is more likely to resemble immature cartilage matrix, which could greatly affect the mechanical properties (Klein et al. 2007). Despite of the differences in the mechanical properties between native adult cartilage and tissue engineered cartilage constructs, mechano-responses seen in cartilage can be replicated in this model albeit by applying reduced forces (Fig. 3.9), suggesting that it constitutes a good model for the planned downstream studies.
In conclusion, the results presented in this chapter show that human cartilage progenitor cells are a good cell type to be used in cartilage tissue engineering. The generated cartilage constructs are an ideal model system for furthering the overarching aim of this project to investigate the effects of external stimuli on changes in GAG chain synthesis and modification related to osteoarthritis.
Chapter 4:

Characterization of human 3D cartilage model system
4 Characterization of human 3D cartilage model system

4.1 Introduction

Chondrogenesis describes the differentiation of mesenchymal stem cells into chondrocytes. During skeletal development, this leads to the formation of cartilage anlagen, which are the precursors of long bones. Initially, mesenchymal condensation occurs, where mesenchymal chondroprogenitor cells aggregate into precartilage condensations (Fell 1925). The mesenchymal cells produce an extracellular matrix rich in HA, fibronectin, collagen type I, and collagen type II A variant (Sandell et al. 1994). In case of limb development, those cells originate from the lateral plate mesoderm (Olsen et al. 2000; Tickle and Münsterberg 2001). Signals for cell-cell and cell-matrix interactions are crucial to initiate condensation. Neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM) play important roles in maintaining these interactions and are therefore highly expressed in precartilage condensations. Other matrix proteins, such as tenascin C and cartilage oligomeric matrix protein (COMP) are also expressed and interact with cell adhesion molecules. This leads to the transition of the chondroprogenitor cells into fully differentiated chondrocytes through activation of intracellular signaling pathways (DeLise et al. 2000).

Sox9, the master chondrogenic transcription factor, is controlling the development of embryonic cartilage and is absolutely essential for cell survival and chondrocyte differentiation (Akiyama et al. 2002; Bi et al. 1999; Moskalewski et al. 2013). It is also required for the expression of collagen type II. Two other members of the Sox family, L-Sox5 and Sox6, are not expressed during mesenchymal condensation but during chondrogenic differentiation (Lefebvre et al. 1998). They are responsible for the expression of other matrix molecules, e.g. collagen type IX, aggrecan, and cartilage link protein, as well as collagen type II (Smits et al. 2001).
During differentiation, chondrocytes switch from collagen type II A to II B expression. Type II B, which lacks exon 2, is solely expressed in mature chondrocytes and is therefore a reliable marker for chondrocyte differentiation (Zhu et al. 1999). In addition, collagen type I as well as N-CAM and N-cadherin expression is lost during chondrogenic differentiation (Oberlender and Tuan 1994). The chondrocytes are starting to produce other important cartilage matrix proteins like aggrecan, link protein, collagen type IX and XI (DeLise et al. 2000; Mendler et al. 1989). Eventually the cartilage undergoes endochondral ossification to form the long bones. In this process, chondrocytes undergo hypertrophy, and this precedes the mineralization of the tissue. Collagen type X expression is increasing, whereas collagen type II expression decreases. Sox9 expression decreases as well and expression of Runx2, a major osteogenic transcription factor, increases. Moreover, hypertrophic chondrocytes start to express osteonectin, osteocalcin and osteopontin, which are core constituents of bone (van der Kraan and van den Berg 2012). Chondrocyte hypertrophy is mechanistically linked to vascular invasion which ultimately leads to bone deposition. Hypertrophic differentiation and cartilage mineralization moves outward to the distal ends of the developing bone, leaving a cartilage growth plate on each end, which is required for postnatal long bone growth. This process is regulated by indian hedgehog (IHH) and parathyroid hormone-related peptide (PTHrP) (DeLise et al. 2000). Finally, expression of VEGF leads to vascularization of the calcified cartilage matrix and bone formation.

In the previous chapter, the development and optimization of a novel human 3D cartilage model system based on chondrocyte progenitor cells was described. To use the cartilage construct for further experiments investigating changes in cartilage metabolism in response to external stimuli, the cartilage constructs required characterizing in detail to establish a body of information at baseline conditions.
4.1.1 **Aims for this chapter:**

1. Investigate the chondrogenic differentiation process
2. Analyse the biochemical composition of the cartilage constructs
3. Analyse the organization of cells with regards to differentiation stage within the constructs
4. Investigate the capacity for chondrogenic differentiation in relation to cell source

4.2 **Materials and methods**

4.2.1 **Chondrogenic differentiation of human chondrocyte progenitor cells**

Cells were cultured as monolayer in 75 cm$^2$ tissue culture flasks until about 80% confluency was reached (described in chapter 3, section 3.2.2). The cells were passaged using trypsin/EDTA solution (0.05% v/v, Gibco) and seeded into Millicell hanging transwell culture inserts (PET membrane, 0.4 µm pore size, 6.5 mm diameter, Millipore, Watford, UK) pre-coated with human fibronectin (see section 3.2.3). A total cell number of 2 x 10$^6$ was used per construct. Prior to seeding into the transwell inserts, the cells were washed in serum-free chondrogenic differentiation media (DMEM, 25 mM D-glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin, non-essential amino acid mixture, ITS supplement (all from Gibco), 100 nM dexamethasone, 50 µg/mL ascorbic acid-2-phosphate (both from Sigma Aldrich)) to remove any residual serum. Cells were finally resuspended in 400 µl serum-free chondrogenic media containing growth factors (80 ng/ml TGF-β2 (Peprotech), 100 ng/ml BMP-2 (Genetics Institute, Boston, USA.), 50 µg/ml bovine insulin (Sigma Aldrich)) and seeded into the inserts, which were placed into 24-well cell culture plates (Sarstedt, Nümbrecht, Germany). The plates containing the inserts were centrifuged for 5 min at 250 x g. After that, 1 ml of the above medium containing growth factors was applied into the respective wells to immerse the bottom of the transwell with medium. 500 µl medium were applied inside the transwell. The constructs
were cultured for at least 28 days by changing the top and bottom medium every 2 days.

4.2.2 Immunohistochemical staining

After tissue fixation, paraffin embedding, deparaffinization and rehydration (described in chapter 3, section 3.2.6), sections used for immunohistochemical staining were incubated in 1% (v/v) H₂O₂ in methanol for 40 min to block endogenous peroxidase activity. Treatment with 40 mU/ml chondroitinase ABC (chABC) for 1 h at 37°C (Seikagaku, Associates of Cape Cod, East Falmouth, MA, USA) in 50 mM Tris-HCl, pH 8.0, 60 mM sodium acetate, was performed to unmask epitopes for antibodies when appropriate. For antibodies binding to collagen type I, II and X, the sections were incubated with 0.1 % (w/v) pepsin (Sigma Aldrich) in 0.1 M acetic acid at 37°C for 1 h, followed by 1 mg/mL bovine testicular hyaluronidase (Sigma Aldrich) in TBS at 37°C for 1 h. To prevent unspecific antibody binding, sections were blocked with 1% BSA (w/v) in TBS for 30 min. Slides were washed three times for 5 min in TBS after primary antibody and secondary antibody incubations. Primary antibodies were incubated overnight at 4°C, and secondary antibodies for 1 h at room temperature. Antibody controls were performed by replacing primary antibodies with mouse IgG or rabbit IgG (whole molecule, Jackson ImmunoResearch Laboratories Inc., West Grove, USA), respectively. Antibodies used for immunohistochemical staining in this chapter are described in Table 4.1.

Immunocytochemical staining of JF012OA cells using an anti-SOX9 antibody was performed as described in chapter 3, section 3.2.4.
Table 4.1 Antibodies used for immunohistochemical staining of cartilage constructs

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution Factor</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-1 (collagen type I), mouse</td>
<td>1:2000</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>monoclonal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIIC1 (collagen type II), mouse</td>
<td>1:5</td>
<td>Developmental studies hybridoma bank, University</td>
</tr>
<tr>
<td>monoclonal</td>
<td></td>
<td>of Indiana, USA</td>
</tr>
<tr>
<td>R220-X (collagen type X), rabbit</td>
<td>1:300</td>
<td>Dr Thomas Schmid, Rush University, Chicago, USA</td>
</tr>
<tr>
<td>polyclonal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMP, rabbit polyclonal</td>
<td>1:100</td>
<td>Dr Frank Zaucke, University of Cologne, Germany</td>
</tr>
<tr>
<td>Aggrecan, rabbit polyclonal</td>
<td>1:300</td>
<td>Prof Daniel Aeschlimann, Cardiff University, UK</td>
</tr>
<tr>
<td>Decorin, rabbit polyclonal</td>
<td>1:100</td>
<td>Dr Elke Schönherr, Cardiff University</td>
</tr>
<tr>
<td>Bs-11175R (lubricin), rabbit</td>
<td>1:300</td>
<td>Bioss Inc., Woburn, MA, USA</td>
</tr>
<tr>
<td>polyclonal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1B5 (chondroitin-0-sulphate), mouse</td>
<td>1:20</td>
<td>Prof Bruce Caterson, Cardiff University</td>
</tr>
<tr>
<td>monoclonal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2B6 (chondroitin-4-sulphate), mouse</td>
<td>1:100</td>
<td>Prof Bruce Caterson, Cardiff University</td>
</tr>
<tr>
<td>monoclonal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3B3(+) (chondroitin-6-sulphate),</td>
<td>1:100</td>
<td>Prof Bruce Caterson, Cardiff University</td>
</tr>
<tr>
<td>mouse monoclonal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>1 µg/ml</td>
<td>Jackson ImmunoResearch Laboratories Inc., West</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grove, PA, USA</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>1 µg/ml</td>
<td>Jackson ImmunoResearch Laboratories Inc., West</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grove, PA, USA</td>
</tr>
<tr>
<td>Rabbit anti-mouse IgG/HRP conjugate</td>
<td>1:100</td>
<td>Dako, Cambridgeshire, UK</td>
</tr>
<tr>
<td>Porcine anti-rabbit IgG/HRP conjugate</td>
<td>1:100</td>
<td>Dako, Cambridgeshire, UK</td>
</tr>
</tbody>
</table>

4.2.3 DNA measurement using Hoechst 33258 dye
To determine the DNA content of a cartilage sample digested with papain (chapter 3, section 3.2.7), 100 µl sample were mixed with 100 µl Hoechst 33258 (1 µg/ml, Sigma Aldrich) and emission monitored at 460 nm using BMG Fluostar Omega with excitation at 320 nm. A standard curve from 0.25 to 2 µg/ml was prepared using Herring sperm DNA (Sigma Aldrich).

4.2.4 Hydroxyproline assay
Hydroxyproline is an amino acid typically found in collagen molecules. From the amount of hydroxyproline, the amount of fibrillar collagen can be
extrapolated. From papain digested samples (chapter 3, section 3.2.7), 100 µl were mixed with 100 µl concentrated HCl in a 2 ml Apex® microcentrifuge tube with screw cap (Alpha Laboratories, Eastleigh, UK) and incubated for 16 h at 110°C. This step was necessary to chemically depolymerize the collagen into its single amino acids. The samples were dried in a vacuum concentrator. In the next step, the samples were reconstituted in 100 µl H₂O. 30 µl sample were added into a well of a transparent 96 well plate (Greiner, Bio-One, Stonehouse, UK), followed by the addition of 70 µl diluent (2 volumes of propan-2-ol and 1 volume H₂O) to it. Then, 50 µl oxidant solution (0.7 g chloramine T, 10 ml H₂O, 50 ml stock buffer; stock buffer: 28.5 g sodium acetate, 18.75 g sodium citrate dihydrate, 2.75 g citric acid, 200 ml propan-2-ol) were added and the plate placed on an orbital shaker at room temperature for 5 min. In the last step, 125 µl color reagent (7.5 g dimethylaminobenzaldehyde, 9.64 ml perchloric acid (70%), 62.5 ml propan-2-ol, and 1.61 ml H₂O) were added and the plate incubated at 70°C for 10 min. Absorbance at 550 nm was measured immediately. A standard curve from 10 to 50 µg/ml was prepared using L-hydroxyproline (Sigma Aldrich). A conversion factor of 10 was applied to calculate the amount of collagen, assuming an hydroxyproline content of 10% (w/w) per collagen molecule (Murdoch et al. 2007).

4.2.5 Stimulation of cartilage constructs to induce hypertrophic differentiation

To induce hypertrophic differentiation of chondrocytes, cartilage constructs differentiated for 35 days were cultured in a modified chondrogenic differentiation medium for a further 14 days. The medium consisted of DMEM, 25 mM D-glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin, non-essential amino acid mixture, 50 µg/mL ascorbic acid-2-phosphate, ITS supplement, 1 nM dexamethasone, and 1 nM T3 (3,3',5-Triiodo-L-thyronine, Sigma Aldrich). No additional growth factors were added to the medium. Hypertrophic differentiation medium was added inside the transwell and the bottom well. Medium was changed every second day.
4.2.6 RNA isolation and generation of cDNA
Cartilage constructs were washed in PBS and subsequently dissociated with 500 µl TRIzol® reagent (Life Technologies) per construct. They were snap frozen in TRIzol using liquid nitrogen and stored at -80°C. At the time of analysis, samples were thawed and homogenized in an RNase free 1.5 ml tube (Eppendorf, Hamburg, Germany) using a sterile RNase free pestle. Subsequently, 120 µl chloroform was added and the sample mixed to extract nucleic acids. The organic and RNA-containing aqueous phase were separated using Heavy Phase Lock Gel tubes (5 Prime, Hamburg, Germany). The aqueous phase was transferred to a fresh RNase free 1.5 ml tube and RNA was precipitated by adding an equal volume of 2-propanol and incubation at -20°C for 20 min. The precipitated RNA was collected by centrifugation at 12,000 x g for 10 min at 4°C. The pellet was washed in 750 µl of 75% ethanol. After an additional centrifugation step at 7,500 x g for 5 min at 4°C, the supernatant was removed and the pellet briefly air-dried. Finally, the RNA pellet was resuspended in 40 µl RNase free H₂O (Promega, Southampton, UK). cDNA was generated from 1 µg total RNA using SuperScript® II Reverse Transcriptase (Life Technologies) following the manufacturer's protocol using oligo(dT) 15 oligonucleotides (Promega) for priming. The PCR reactions were carried out on a GeneAmp PCR System 9700 (Applied Biosciences, Paisley, UK). cDNA was stored at -20°C until further use. Remaining total RNA was precipitated by adding 1 µl glycogen (5 mg/ml, Roche Diagnostics GmbH, Mannheim, Germany), followed by one tenth of the sample volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of nuclease-free ethanol. The sample was stored at -80°C. To recover the precipitated RNA, it was collected by centrifugation at 12,000 x g at 4°C for 20 min, washed in 500 µl 80% ethanol and collected again by centrifugation at 4°C for 10 min. The pellet was briefly air-dried and subsequently dissolved in nuclease-free water to the desired concentration.

4.2.7 PCR analysis and Q-PCR analysis
PCR analysis of collagen type II A/B gene expression was carried out using GoTaq® Hot Start Polymerase (Promega), 250 nM forward and reverse primer,
200 µM dNTPs, 2 mM MgCl\(_2\) and nuclease-free water in a final volume of 50 µl. In the first step, the sample was incubated for 2 min at 94°C. The second step was comprised of sample denaturation at 94°C for 30 sec, primer annealing at 60°C for 60 sec and DNA elongation at 72°C for 72 sec. This was repeated for 40 cycles. In the last step, the sample was incubated at 72°C for 7 min. PCR products were analyzed on 1% agarose gels, following ethidium bromide staining.

For quantitative gene expression analysis, 100 ng cDNA were mixed with Power SYBR® Green Master Mix (Life Technologies) and 300 nM of forward and reverse primer. The primers used for experiments in this chapter are described in Table 4.2. Nuclease-free H\(_2\)O was added to obtain a final volume of 25 µl. Analysis was performed using an ABI PRISM® 7000 Sequence Detection System and data was analyzed with the provided software suite (Applied Biosystems). In the first step of the reaction, the samples were heated to 95°C for 10 min to activate the AmpliTaq Gold® DNA polymerase. Following this, 40 cycles were performed comprised of 95°C for 10 sec for sample denaturation and 60°C for 60 sec for primer annealing and elongation. At the end of each analysis run, a dissociation run was carried out to determine the melting temperature and homogeneity of the resulting PCR products. Since the SYBR Green dye intercalates with any double stranded DNA, also unwanted PCR products, e.g. primer dimers, can give a fluorescent signal and therefore contribute to the overall result. This could lead to an overestimation of the respective gene expression level. The \(\Delta\Delta\text{Ct}\) method was used to estimate relative transcript levels, while employing H36B4 as housekeeping gene for normalization of gene expression between different samples. This gene is encoding for the acidic ribosomal phosphoprotein P0 (Laborda 1991) and was found to have a more consistent expression level in various tissues compared to other commonly used housekeeping genes, e.g. β-actin, and glyceraldehyde 3-phosphate dehydrogenase (Akamine et al. 2007). However, using only one housekeeping gene as reference can implicate the problem.

For calculation of p-values, the respective \(\Delta\Delta\text{Ct}\) values were used. Fold change in gene expression was determined from the respective \(2^{-\Delta\Delta\text{Ct}}\) values.
Table 4.2 Primer sequences used for PCR and Q-PCR analysis of cartilage constructs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence of forward (top) and reverse (bottom) primers (5’ - 3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type I (COL1A1)</td>
<td>GTGCTAAAGGTGCCAATGGT CTCCTCGTTCCTCCTCTCT</td>
<td>228</td>
<td>NM_000088</td>
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<tr>
<td>Collagen type II (COL2A1)</td>
<td>TGTGAAGACGTGAAGACTGCC TCACCTTTGTCAACCAGGATCC</td>
<td>221</td>
<td>NM_001844</td>
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<tr>
<td>Collagen type II A/B</td>
<td>CTCGCTGTCGCCCGGCTGTCYT AAGGGTCCCAGGTTCTCCATCTC</td>
<td>432/225</td>
<td>(Johnstone et al. 1998)</td>
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<tr>
<td>Collagen type X (COL10A1)</td>
<td>CACTACCAACCAAGACACAG CACACGTCGAGGTGTATCTCAACAGCG</td>
<td>230</td>
<td>NM_000493</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>CTCCTGGGAAACCAGGTGTT AAACAGGTGTCAGGACTCTCT</td>
<td>174</td>
<td>NM_001135</td>
</tr>
<tr>
<td>Versican</td>
<td>GAGACCCAACCAAGCAGCAAGTC TCTTCATTCCCAAAGCAGG</td>
<td>283</td>
<td>NM_004385</td>
</tr>
<tr>
<td>Matrilin 3</td>
<td>GTTGCGGCACTGAGGACACAG GCTGTCACATTTTCAGGTGAG</td>
<td>208</td>
<td>NM_002381</td>
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<tr>
<td>Chondromodulin-1</td>
<td>AACTCTGGTGATGACCTCC CTGCCCTCTGCTGCTGATGT</td>
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<tr>
<td>Osteonectin</td>
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<td>HAS 2</td>
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<td>GDF 5</td>
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<td>HIF2α</td>
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<td>Tenascin C</td>
<td>GGTGAAGTCAGGAGCAGCATGTCAGGTGTCATCTTCAGG</td>
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<td>NM_002160</td>
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<td>N-cadherin</td>
<td>ATCATTGCATCCTGTCCTGC TCAGGCTCAGTGCTCAGG</td>
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<td>MMP13</td>
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<tr>
<td>H36B4</td>
<td>AGATGCAGCAGATCCGC ATATGAGGCACGAGGTTCTCCAG</td>
<td>130</td>
<td>(Wagener et al. 2001)</td>
</tr>
</tbody>
</table>
4.3 Results

In the previous chapter, the development of the cartilage model system was described. An optimized chondrogenic medium formulation was developed, yielding a small disc of cartilaginous tissue. In this chapter, the thorough characterization of the human 3D cartilage model system using histological, biochemical and molecular biology methods is described.

4.3.1 Cartilage construct: time course of differentiation

To investigate the chondrogenic differentiation of human chondrocyte progenitor cells, constructs were seeded and cultured for a maximum of 35 days. This was important to better understand the dynamics of the differentiation from progenitor cells to chondrocytes and the production of a cartilaginous extracellular matrix. Cultures were analysed at day 1, 7, 14, 21, 28 and 35 post seeding. From each set of constructs, the wet weight was determined immediately after removing the constructs from the transwell inserts (Fig 4.1 A). Tissue architecture was analysed after fixing the construct and embedding it in paraffin wax. Sections of 5 µm were stained using toluidine blue to show the relative GAG content of extracellular matrix, which stained violet (Fig. 4.1 B). Toluidine blue is a cationic dye, similar to Safranin-O, which binds to the negatively charged sulphate groups within GAG chains and is therefore a simple method to identify cartilaginous ECM (Schmitz et al. 2010). During culture in chondrogenic media, the wet weight of the constructs steadily increased (Fig. 4.1 A). Starting from approx. 6 mg, the weight of the constructs slowly doubled to about 12 mg at day 21. From then on, the construct mass increased in much bigger steps than before. The increase in wet weight was reflected by the staining of the cartilage construct with toluidine blue (Fig. 4.1 B). This suggests that as a result of increased GAG synthesis by the cells, water was attracted due to the resulting negative charge, which increased the tissue weight. This is consistent with a substantial volume increase from 6 mg to 28 mg in the absence of extensive cell proliferation (see Fig. 4.2 B). To determine the dry weight, constructs were frozen on dry ice and subsequently placed into a freeze-dryer for 16 h. By comparing the wet and dry weight after
35 days in culture, the water content of the constructs was determined to be approx. 90%. Toluidine blue staining was weak up to day 14, but increased rapidly from day 21 until the whole construct stained strongly at day 35. Interestingly, the top half of the construct showed stronger toluidine blue staining than the bottom half. Also, cartilage lacunae typical for cartilage tissue proper were more apparent in the upper part. However, this was somewhat variable between experiments (compare with Fig. 4.3 and Fig. 4.10) and likely reflects a gradient of diffusion of growth factors across the constructs. Collectively, these data suggest that while chondrogenesis occurs rapidly in these cultures, a further 3-4 weeks are required to establish a cartilage-like ECM.
Figure 4.1 Analysis of human chondrocyte progenitor cell differentiation into cartilaginous tissue over 35 days. A: Tissue wet weight of constructs differentiated for 1 to 35 days. Results are displayed as mean ± SEM of two independent experiments with one sample per experiment. B: Paraffin sections of constructs stained with toluidine blue after the indicated time in differentiation culture medium. Bar = 100 µm. Statistically significant changes to sample from day 1 were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. *P< 0.05.
4.3.2 Biochemical analysis of cartilage constructs

To extend these findings and estimate to what degree this reflects a cartilage ECM, biochemical analysis of the constructs was performed by measuring the total GAG content, DNA content and collagen content. For this, the constructs were completely digested with papain. The GAG content was determined using the DMMB assay. DMMB is a cationic dye which binds quantitatively to the negatively charged GAG chains. The assay showed that there was a steady increase in the GAG content every 7 days (Fig. 4.2 A). Similar to the wet weight, a slow increase was observed until day 21. From then on, the GAG content nearly doubled each week, showing a final average GAG amount of 433 µg.

The DNA content of the constructs was measured using the intercalating fluorescent dye Hoechst 33258. No significant difference in the DNA content during the time course was observed, showing that the cell proliferation after seeding the constructs was very low (Fig. 4.2 B). The average DNA content slightly decreased within the first 2 weeks from an average 19 µg to 14 µg and then increased until day 35 to 19.5 µg again. By normalizing the GAG amount to the DNA content, a substantial increase was observed from day 7 to day 28, which then further increased at a lower rate until day 35 (Fig. 4.2 C).

During the whole culture period, the conditioned medium from the cartilage constructs was collected from inside the transwell and bottom well and pooled each time. This medium was used to determine the increase of released GAG chains during the chondrogenic differentiation (Fig. 4.2 D). A steady increase in the GAG content of the medium was observed throughout the whole culture period, with the highest average GAG release of 28.5 µg per day at day 35.

The total collagen content of the constructs was measured using the hydroxyproline assay. Because hydroxyproline is mostly present in fibrillar collagens, this assay gives a good estimate for amounts of collagens of type I, II, IX and XI present. Similar to the increase of GAG chains within the construct, the amount of collagen fibrils deposited in the ECM of the constructs only slowly increased within the first 21 days (Fig. 4.2 E). After that, the collagen amount nearly doubled every 7 days, reaching a maximum of 2 mg on day 35. By normalizing the collagen amount to the DNA content, a similar trend was
observed with the highest value of 101.8 µg collagen per 1 µg DNA on day 35 (Fig. 4.2 F). The fact that the rate of increase in both GAG and collagen deposition was reducing from 28 to 35 days suggests that the matrix is starting to reach a saturation point. However, it is likely that at least 7 to 8 weeks would be required to reach this point or possibly substantial longer time periods which was not feasible in this project.
Figure 4.2 Biochemical composition of cartilage constructs at different stages of differentiation. After the indicated time of differentiation, constructs were digested with papain to break down the tissue for further analysis. A: Total GAG amount per construct as measured by DMMB assay; B: Total DNA per constructs as measured by Hoechst assay; C: Total GAG amount normalized to DNA content; D: GAG content of conditioned media collected during culturing period (from transwell and bottom well); E: collagen content measured by hydroxyproline assay (conversion factor 1:10). Results are displayed as mean ± SEM of three independent experiments with one sample each. Statistically significant changes to samples from day 1 were determined with one-way ANOVA with post hoc analysis using Tukey's multiple comparison test. *P< 0.05, **P<0.01, ***P<0.001.
4.3.3 **Immunohistochemical analysis**

To investigate the presence of matrix proteins that are characteristic for cartilage ECM, paraffin section of 35 day old cartilage constructs were stained with antibodies raised against various matrix proteins, i.e. collagen type I, II, X, cartilage oligomeric matrix protein (COMP), aggrecan, decorin, lubricin, and CS epitopes $\Delta 0S$ (C-0-S), $\Delta 4S$ (C-4-S), $\Delta 6S$ (C-6-S) (Fig. 4.3). Toluidine blue staining showed a homogenous violet staining of the whole construct, indicating the abundant presence of GAG chains. Staining for collagen type I and X showed the absence of collagen type X and that little collagen type I was present. The strongest staining was found in the middle part of the construct, where tissue transformation may be less complete due to limited growth factor diffusion. In contrast, collagen type II, the major collagen found in cartilage, was highly abundant throughout the construct, with the highest expression in the top region. The absence of collagen type X expression confirms the absence of hypertrophic differentiation in line with the morphological appearance of the tissue (see H&E section). The cartilage matrix proteins COMP, aggrecan and decorin were also highly expressed in the constructs. COMP and aggrecan were evenly distributed in the construct as expected, whereas decorin showed the strongest staining in the top region. Interestingly, lubricin staining was restricted to the surface in the top part of the construct. This is in good agreement with the expression pattern of lubricin in articular cartilage, where it is expressed in the superficial zone (Schumacher et al. 1994). The CS epitopes C-0-S, C-4-S and C-6-S were all evenly distributed throughout the cartilage matrix. Staining was strongest for C-4-S, followed by C-6-S and weakest for C-0-S. However, the staining intensity is not a quantitative measurement for the relative amount of each disaccharide, but is indicative of the last disaccharide present just before the GAG chain linker region, that cannot be cleaved by chABC. A digest of the section with chB followed by probing with an antibody detecting C-4-S did not result in any staining, showing that there were no DS stubs present on cartilage proteoglycans as expected. Taken together, these data not only confirm the presence of typical cartilage matrix constituents but a degree of stratification
of the constructs that mimics that of articular cartilage and also the absence of hypertrophic differentiation.

Figure 4.3 Immunohistochemical staining of a 35 day old cartilage construct. 5 µm paraffin sections of a 35 day old cartilage construct were stained with H&E and toluidine blue to visualize the tissue morphology and ECM deposition. Constructs were stained with antibodies raised against cartilage matrix proteins and disaccharide epitopes (Table 4.1). Sections stained for collagen type I, II and X were digested with 0.1% pepsin (w/v) for 1 h at 37°C, followed by 1 mg/ml hyaluronidase for 1 h at 37°C prior to primary antibody incubation. Sections stained for COMP, aggrecan, decorin, lubricin, C-0-S, C-4-S, C-6-S, and DS were digested with 40 mU chondroitinase ABC for 1 h at 37°C. Primary antibodies were incubated for 16 h at 4°C, whereas secondary antibodies were incubated at room temperature for 1 h. Rabbit and mouse IgG controls were treated with both pepsin/hyaluronidase and chondroitinase pre-treatments and did not show any staining. Bar = 100 µm.
4.3.4 HPLC analysis of total CS chain disaccharide composition of cartilage constructs

The composition of CS chains produced during chondrogenic differentiation of cartilage constructs was determined using reversed phase HPLC analysis of AMAC-labeled disaccharides. From each time point, 1 µg GAG chains from papain-digested samples was digested using chABC in combination with chAC-1. No tri-sulphated disaccharides were detected for any sample (Fig. 4.4 A). Δ2,6diS and Δ2,4diS disaccharides were only present on day 1 prior to chondrogenic differentiation. The major disaccharide subunits were Δ4S and Δ6S and Δ0S. The relative amounts of these disaccharides within the CS chains from different time points during cartilage differentiation were compared (Fig. 4.4 B). On day 1, the proportions of Δ4S and Δ6S were nearly equal. Δ0S was only marginally present. However, this ratio changed after 7 days in culture and thereafter, the relative amount of Δ6S increased steadily to 68.8%, whereas for Δ4S it fluctuated between 20.0 – 33.3% and for Δ0S between 7.9 – 13.4%. An increase in Δ6S is consistent with a mature cartilage matrix where Δ6S reaches 80% in adult articular cartilage (Bayliss et al. 1999).
Figure 4.4 Analysis of GAG chain disaccharide composition of cartilage constructs during differentiation. A: 1 µg total GAG (derived from papain digested cartilage constructs) was digested with chABC + chAC-I and labeled with AMAC. Analysis was performed using reversed phase HPLC with a C-18 column. Chromatograms of day 1 to 35 are overlaid. Arrows indicate the elution position of labeled CS disaccharides. B: Comparison of the relative amounts of the most abundant disaccharides $\Delta4S$, $\Delta6S$, $\Delta0S$, on each time point during the chondrogenic differentiation time course. Data is shown as mean ± SEM from three independent experiments with one sample each. Statistical analysis using one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test showed no significance for the changes of each respective disaccharide amount from day 7 – 35 compared to day 1. * = sample unrelated components derived from AMAC labeling.
4.3.5 Gene expression analysis during cartilage construct differentiation

While the biochemical, immunohistochemical and HPLC analysis confirmed that a cartilage-like ECM was formed, this provided little information on the state or path of cell differentiation. Therefore, gene expression analysis of markers of cell differentiation was carried out at the indicated times during the differentiation process. The analysis was performed using SYBRGreen and relative quantification derived from the \( \Delta \Delta \text{Ct} \) method. The advantage of this is that it is relatively easy to perform and economical. However, also undesired PCR products, e.g. primer dimer, cause a positive signal. Therefore, besides confirming the presence of a single product through melting analysis, samples were further analyzed with agarose gel electrophoresis after the Q-PCR run was complete to confirm that the products generated were of expected size. Firstly, the expression of the major cartilage matrix proteins collagen type II and aggrecan during differentiation was analyzed as well as collagen type I (Fig. 4.5). Matrilin 3 was also investigated as an example of a non-collagenous cartilage matrix protein that is present in permanent cartilage remaining throughout life, as opposed to temporary cartilage anlagen formed in development and subsequently undergoing joint cavitation (Pullig et al. 2002). Collagen type II showed no expression in the monolayer cells before seeding into the transwell culture inserts. In contrast, collagen type I was expressed, as seen in the agarose gel (Fig. 4.5 A). One day post seeding, collagen type I expression was substantially increased (40-fold) which was likely due to the culture conditions (3D environment, ascorbic acid-2-phosphate supplementation) rather than cell differentiation. No substantial change in collagen type I expression was seen thereafter. After 7 days, collagen type II expression was increased approx. 1000-fold compared to day 1 and by day 14 reached a plateau at > 50,000-fold increase (Fig. 4.6 B). The expression of the major cartilage proteoglycan aggrecan was increased 6-fold on day 1 and increased further after 7 days to approx. 800-fold (Fig. 4.6 C). The expression level slowly reached a plateau with the highest value of 2,720-fold change on day 35 compared to monolayer. Matrilin 3, a protein involved in formation of filamentous networks in cartilage extracellular matrix (Klatt et al. 2011; Budde...
et al. 2005), showed increased expression levels during the whole time course. Expression was very low in monolayer and on day 1, but then increased about 10-fold on day 7 (Fig. 4.6 D). This was followed by a gradual increase to about 200-fold by day 28 where it reached a plateau. Taken together, these data indicate that the time period taken to reach a steady state level of expression differs between genes, with matrilin 3 requiring 28 days, suggesting that an extended time period is required for cell differentiation and not just for matrix synthesis.
Figure 4.5 Constructs expressed cartilage matrix proteins during differentiation. Cartilage constructs (2 x 10⁶ cells/construct) were differentiated in chondrogenic medium to the indicated time points. Total RNA was isolated and converted into cDNA. 100 ng cDNA per sample were used for Q-PCR gene expression analysis using SYBRGreen. Above each graph, a representative set of samples was analysed after Q-PCR using agarose gel electrophoresis to illustrate the resulting PCR products used for expression analysis. A logarithmic scale of the y-axis was applied when the fold changes in expression exceeded a value of 1000. Data is shown as mean ± SEM derived from three independent experiments (independently grown cartilage constructs). Each sample was measured in duplicate. Statistically significant changes compared to monolayer (M) samples were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. *P< 0.05, **P<0.01, ***P<0.001.
During the chondrogenic differentiation of human progenitor cells, several genes developmentally associated with the formation of mesenchymal condensation, which had a high expression in the monolayer cells were down regulated over 35 days. The expression level of N-cadherin for example significantly decreased (about 3.4-fold) within 14 days of culture in chondrogenic differentiation medium (Fig. 4.6 A). It is known to be involved in cell-adhesion and is highly expressed in mesenchymal condensation during skeletal development and disappears in differentiated chondrocytes (Oberlender and Tuan 1994; DeLise et al. 2000; Goldring et al. 2006). In the agarose gel electrophoresis performed after Q-PCR analysis, a band of approx. 50 bp appeared in every sample (arrow) in addition to the expected product for N-cadherin. These bands were most likely the result of primer dimers that formed because of a low amount of N-cadherin cDNA. While the C\textsubscript{T} values are likely to be minimally affected as template amplification precedes primer dimer formation, these results should be considered with caution and ideally would require repeating with inclusion of a TaqMan probe for confirmation.

Tenascin C expression also decreased significantly over the course of construct differentiation (Fig. 4.6 B). On day 35, it was down regulated 10-fold compared to monolayer cells. Tenascin C is also highly expressed during the stage of mesenchymal condensation and in cartilage anlagen, but absent in mature cartilage (Mackie and Murphy 1998).

HAS-2, one of the major HA synthases in cartilage (Hiscock et al. 2000), showed significant down regulation as well (Fig. 4.6 C). The expression level decreased gradually every week and was the lowest at day 35 with an approx. 30-fold change compared to monolayer. This was somewhat unexpected but likely indicates a switch in use of HAS enzymes from HAS-2 to HAS-3.
Figure 4.6 Expression of transient cartilage markers decreased during differentiation. Cartilage constructs (2 x 10^6 cells/construct) were differentiated in chondrogenic medium to the indicated time points. Total RNA was isolated and converted into cDNA. 100 ng cDNA per sample were used for Q-PCR gene expression analysis using SYBRGreen. Above each graph, a representative set of samples was analysed after Q-PCR using agarose gel electrophoresis to illustrate the resulting PCR products used for expression analysis. Data is shown as mean ± SEM derived from three independent experiments. Each sample was measured in duplicate. Statistically significant changes compared to monolayer (M) samples were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. *P< 0.05, **P<0.01, ***P<0.001.
As mentioned above, collagen type II is the predominant fibrillar collagen present in cartilaginous ECM. However, two different splice variants exist, the expression of which is differentially regulated during chondrogenic differentiation (Zhu et al. 1999). In precartilage condensations, collagen type II A variant is the most abundant form and in mature cartilage, the B variant is solely expressed. The difference between the two splice variants is the presence of exon 2 which can only be found in the A variant. The amplified product covered the end of exon 1 to the beginning of exon 3. Thus, the PCR product containing exon 2 is longer (432 bp) than the one without (225 bp). Through analysis of the cDNA derived from cartilage constructs at different stages of differentiation, the relative expression levels for each splice variant could be determined (Fig. 4.7 A). The intensity of the bands was estimated by densitometry using the ImageJ software package (Abramoff et al. 2004) to derive the relative percentages of variant A and B of the total collagen type II expression level. Directly after seeding the chondrocyte progenitor cells at day 1, neither variant A nor B variant were detectable as expected (see Fig. 4.5 B). From day 7 onwards, both variants were detected throughout the last time point at day 35. On day 7, variant B was the predominant splice variant with approx. 70% compared to variant B with approx. 30%. On day 14, both variants were equally expressed. From day 21 to 35, a steady decrease of expression of variant A accompanied with the increase of variant B was observed. At day 35, more than 90% of the expressed collagen type II was variant B.

GDF-5, a critical signaling molecule for cartilage development, both during mesenchymal condensation as well as chondrocyte proliferation in later stages of skeletal development (Francis-West et al. 1999), was already expressed in monolayer cells. Its expression level stayed constant during the whole culturing period (Fig. 4.7 B). Versican expression levels decreased about 2-fold after seeding cells into the transwells. Again, the expression level stayed relatively constant over the culture period (Fig. 4.7 C).

Chondromodulin-1 was absent in monolayer, but increased rapidly in transwell environment, reaching a 100-fold increase by day 7 compared to day 1 and approaching a plateau of 5,000 to 10,000-fold from day 14 onwards (Fig. 4.7D). Chondromodulin-1 is a cartilage matrix protein with anti-angiogenic properties.
It has been suggested that chondromodulin-1 expression is an important factor of maintaining the avascularity of cartilage (Hiraki et al. 1997; Hayami et al. 2003). Moreover, it stimulates chondrocyte proliferation and synthesis of cartilage proteoglycans (Hiraki et al. 1991) and is therefore a key component of persistent cartilage.

In addition to markers indicating the process of cartilage matrix formation, markers indicating chondrocyte hypertrophy were evaluated as well. Collagen type X, deposition of which is a hallmark for chondrocyte hypertrophy, could not be detected in the ECM (Fig. 4.3). However, it was expressed already in monolayer cells (Fig. 4.8 A). Its expression level gradually increased from day 1 to day 35, a feature which has previously been seen in association with chondrogenic differentiation. However, as no protein was detectable, the significance of this remains unclear. Hypoxia-inducible factor 2α (HIF2α), a very important transcription factor involved in terminal differentiation of chondrocytes and transactivation of collagen type X and MMP13 (Saito et al. 2010), was transiently increased to about 10-fold on day 1 and decreased thereafter. It is likely that this change was a result of the change in culture conditions as this gene is known to respond rapidly to environmental changes (Fig. 4.8 B). Differences in the expression level of MMP13, a member of the matrix metalloproteinase family and highly expressed in hypertrophic cartilage, were also observed during the time course. It was expressed in monolayer cells and up regulated on day 1 (Fig. 4.8 C). Expression decreased on day 7 and was very low on both day 14 and 21, but increased moderately thereafter. Although the changes did not reach significance, it is possible that MMP13 contributed to matrix remodeling at these later stages of tissue formation.

Osteonectin, a protein present in bone as well as in terminally differentiated cartilage (Goyal et al. 2010; van der Kraan and van den Berg 2012), showed a gradual increase in expression. Notably, the expression level was relatively high already in monolayer cells, as seen in the agarose gel analysis of the Q-PCR products (Fig. 4.8 D). Neither the immunohistochemical analysis nor the gene expression analysis provided evidence for hypertrophic differentiation of the cartilage constructs.
Figure 4.7 Constructs expressed maturation related variant of collagen type II and markers associated with permanent joint cartilage. Cartilage constructs (2 x 10^6 cells/construct) were differentiated in chondrogenic medium to the indicated time points. Total RNA was isolated and converted into cDNA. 100 ng cDNA per sample were used for PCR analysis of collagen type II A/B gene expression (A). Densitometrical analysis of PCR bands was performed using the ImageJ software package. 100 ng cDNA per sample were used for Q-PCR gene expression analysis of GDF 5, versican, and chondromodulin 1 using SYBRGreen (B, C, D). Above each graph, a representative set of samples was analysed after Q-PCR using agarose gel electrophoresis to illustrate the resulting PCR products used for expression analysis. A logarithmic scale of the y-axis was applied when the fold changes in expression exceeded a value of 1000. Data is shown as mean ± SEM derived from three independent experiments. Each sample was measured in duplicate. Statistically significant changes compared to day 7 for collagen type II A/B analysis and monolayer (M) samples for GDF 5, versican and chondromodulin 1 were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. *P<0.05, ***P<0.001. n.d. = not detectable.
Figure 4.8 Analysis of expression of cartilage hypertrophy markers. Cartilage constructs (2 x 10^6 cells/construct) were differentiated in chondrogenic medium to the indicated time points. Total RNA was isolated and converted into cDNA. 100 ng cDNA per sample were used for Q-PCR gene expression analysis using SYBRGreen. Above each graph, a representative set of samples was analysed after Q-PCR using agarose gel electrophoresis to illustrate the resulting PCR products used for expression analysis. A logarithmic scale of the y-axis was applied when the fold changes in expression exceeded a value of 1000. Data is shown as mean ± SEM derived from three independent experiments. Each sample was measured in duplicate. Statistically significant changes to monolayer (M) samples were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. *P< 0.05, **P<0.01; ***P<0.001.
In summary, while many cartilage matrix proteins are already expressed in monolayer cells including aggrecan, these are highly up regulated over the first 14 days of culture. Significantly, neither collagen type II nor chondromodulin were present in monolayer cells and the up regulation of their expression correlates with signs of tissue transformation as evidenced by morphological changes and toluidine blue staining of ECM visible by day 14 (Fig. 4.1). This suggests that collagen type II and chondromodulin play a critical role in matrix assembly. However, a mature chondrocyte phenotype was not reached until at least day 28 as evidenced by the switch in collagen type II isoform expression and altered expression of matrilin 3.

4.3.6 Induction of hypertrophy in cartilage constructs
Hypertrophic differentiation is a well-known issue in cartilage tissue engineering (Johnstone et al. 1998; Murdoch et al. 2007). It is commonly observed that cartilaginous tissue made from mesenchymal stem cells derived from bone marrow aspirate, for example, undergoes terminal differentiation spontaneously (Johnstone et al. 1998; Abrahamsson et al. 2010). Staining for collagen type X, a hallmark for chondrocyte hypertrophy, did not show any collagen type X deposition in the matrix of the cartilage constructs produced here after 35 days in culture (Fig. 4.3), even though there was a substantial increase of collagen type X mRNA expression observed over the 35 day culture period (Fig. 4.8A). To answer the question of whether the cells and culture conditions used in this study prevented hypertrophic differentiation or the absence of collagen type X deposition was for unknown reasons not reflecting cell differentiation, fully differentiated 35 day old cartilage constructs were cultured further in medium which is expected to stimulate hypertrophic differentiation of cartilage (Mueller and Tuan 2008; Böhme et al. 1995). This medium was based on the standard serum-free chondrogenic differentiation medium but did not contain any of the growth factors (TGF-$\beta$2, BMP-2, additional insulin), had a reduced amount of dexamethasone and contained thyroid hormone T3. T3 hormone regulates chondrocyte terminal differentiation through the Wnt/\(\beta\)-catenin signaling pathway by activation of
Wnt-4 expression (Wang et al. 2010). Control constructs were cultured in normal chondrogenic medium containing growth factors. The constructs were cultured for an additional 14 days, with medium change every 2 days. Staining of sections with an antibody against collagen type X showed a marked difference between the control construct (Fig. 4.9 A, C) and the construct cultured in hypertrophic medium (Fig. 4.9 B, D). In the latter one, strong staining for collagen type X was evident in the area directly adjacent to the transwell membrane (arrow). Magnification of this part showed an increased size of the cartilage lacunae (Fig. 4.9 D) indicative of hypertrophic differentiation of cells.

Analysis of gene expression (Fig. 4.9 E) showed that Runx2, an osteogenic differentiation marker, was surprisingly down regulated. Collagen type X expression levels were unchanged. The biggest difference in gene expression was observed for MMP13, with an average fold change of 7.4. Other hypertrophy markers, HIF2α and IHH, were slightly up regulated compared to control. Osteonectin expression was down regulated, which was again not expected, as it is typically up regulated in hypertrophic and OA-cartilage (Goyal et al. 2010). However, localized changes in expression in the cell layer near the filter membrane may be underestimated or masked by the fact that gene expression of the entire construct rather than the hypertrophic layer was examined.
Figure 4.9 Stimulation of hypertrophic differentiation with T3 induced increased matrix deposition of collagen type X and significantly increased MMP13 gene expression. Cartilage constructs (2 x 10^6 cells/construct) were differentiated for 35 days using chondrogenic differentiation medium. Hypertrophy was induced by culturing constructs in medium without growth factors, but containing thyroid hormone T3 for a further 14 days. Control constructs were cultured in chondrogenic medium containing growth factors. Afterwards, constructs were fixed and embedded in paraffin. 5 µm sections of control constructs (A) and hypertrophic culture constructs (B) were stained with an antibody against collagen type X. The arrow points to the region with enhanced collagen type X staining. Magnified parts of the sections (red box) are shown in C & D, respectively. 100 ng cDNA were used for Q-PCR analysis of marker genes for hypertrophic differentiation (E). Data is shown as mean ± SEM from two independent experiments with one sample each. Each sample was measured in duplicate. Statistical significance of changes to control samples (dotted line) were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. *P< 0.05, **P<0.01, ***P<0.001. Bar = 100 µm.
4.3.7 Chondrogenic differentiation of chondrocyte progenitor cell strains established from different individuals

To demonstrate that the protocol used for the chondrogenic differentiation of the PRO3 human chondrocyte progenitor cell strain also worked for progenitor cells generated from different donors under similar conditions, the PRO2 and PRO5 cell line were used for chondrogenic differentiation. These were a kind gift from Dr Rebecca Williams (School of Biosciences, Cardiff University). PRO2 cells were post mortem derived from the normal knee articular cartilage of a 56 year old male and PRO5 from a 75 year old male. The provided cell aliquots were at a similar population doubling (around 25) and cultured using the same monolayer expansion medium as the PRO3 cells. The cells were seeded at a density of $2 \times 10^6$ cells per insert and cultured for 28 days in serum-free chondrogenic medium containing 50 µg/ml bovine insulin, 80 ng/ml TGF-β2 and 100 ng/ml BMP2 as before. At the end of the culture period, the constructs were embedded in paraffin and 5 µm sections were prepared. The sections were stained with H&E and toluidine blue to assess the chondrogenic tissue morphology and deposition of ECM. This confirmed the capacity for chondrogenic differentiation of both the PRO2 and PRO5 cell strains under these conditions (Fig. 4.10, left pictures). This showed that the differentiation protocol and medium developed and used in this study can be used to differentiate human chondrocyte progenitor cells into cartilage-like tissue independent of the age and genetic background of the donor. This could be potentially useful for future applications of cartilage tissue engineering using this particular cell type.
Figure 4.10 Chondrogenic differentiation of other human chondrocyte progenitor cell clones. Cartilage constructs (2 x 10^6 cells/construct) from each progenitor cell line were differentiated for 28 days using an identical chondrogenic differentiation medium formulation. 5 µm sections from each construct were stained with H&E (left) and toluidine blue (right). Bar = 100 µm.
4.3.8 Chondrogenic differentiation of OA-cartilage derived progenitor cells

In addition to the chondrogenic differentiation of human chondrocyte progenitor cells isolated from normal knee cartilage, the chondrogenic differentiation of a progenitor cell line isolated from OA-cartilage was investigated. This cell line, JF012 OA, was derived from knee cartilage of a 65 year old male undergoing total knee arthroplasty. Again, the cells were a kind gift from Dr Rebecca Williams. The provided cell aliquot was expanded in monolayer culture the same way as the “normal” human progenitor cell strains PRO2, PRO3, and PRO5.

The expression of Sox9 was investigated by immunocytochemistry. Cells were seeded onto a fibronectin-coated glass cover slip and stained with a rabbit polyclonal antibody against Sox9 as described (chapter 3, section 3.2.4). JF012OA cells showed strong staining for Sox9 in the nucleus (Fig. 4.11 A). Based on this result, it was expected that the cells were capable of differentiating into cartilage similar to the other cell lines as shown in the previous paragraph.

Constructs were seeded with a seeding density of $2 \times 10^6$ cells per construct and cultured for 28 days in serum-free chondrogenic medium containing the same growth factor cocktail and concentrations as used for the other cell lines. Constructs were fixed in 4% PFA/PBS for histological analysis and immunohistochemical staining of matrix components. At the time of harvest, it was obvious that the resulting construct was much smaller and less rigid than constructs made from non-OA cartilage derived progenitor cells. By comparing the JF012OA-construct with a 28 day old PRO3-construct, it was clearly visible that the PRO3-construct (left) was more than twice the size of the JF012OA construct (right) (Fig. 4.11 C). H&E and toluidine blue staining (Fig. 4.11 D, E) showed that the tissue had a more fibrous morphology for the OA cell construct but some toluidine blue staining of the ECM was evident. Immunohistochemical staining using antibodies against collagen type I, II and X (Fig. 4.11 F, G, H, respectively) showed that collagen type I was more abundant and present throughout compared to PRO3 derived constructs (Fig. 4.3). Interestingly, staining for collagen type II was much reduced and staining
for collagen type X was evident. The significance of collagen type X deposition is unclear as tissue morphology showed neither typical features of cartilage nor hypertrophy. However, as induction of hypertrophy in articular chondrocytes is a feature of OA it is possible that the progenitor cells have acquired properties of terminally differentiated chondrocytes and are therefore unable to synthesize a cartilage matrix proper.

Analysis of the wet weight at the time of tissue collection showed that the JF012OA constructs were 2.3 times lighter than the PRO3 constructs (Fig. 4.11 K). Interestingly, the average wet weight of 9 mg was almost as low as for the PRO3 constructs at day 1, which was around 6 mg. Given that the same cell seeding density was used, this indicates that the weight did not increase much during the course of differentiation. The average total GAG content of the JF012OA constructs (83.2 µg) was significantly lower than that of the corresponding PRO3 constructs (250.3 µg) (Fig. 4.11 L) but at the same time higher than PRO3 constructs after 7 days in culture, suggesting that some matrix deposition did occur.

The analysis of the gene expression levels showed that collagen type II expression was about 10-fold lower than control (PRO3 constructs at day 28) (Fig. 4.11 M). In contrast, an almost identical expression level of aggrecan was observed. Furthermore, collagen type X expression was much lower, as was MMP13. This could indicate that there is a feedback loop that connects matrix deposition of collagen type X to gene regulation. Interestingly, the expression of the pro-inflammatory cytokine IL-8 was significantly up regulated with an average fold change of > 1,000 fold (Fig. 4.11 M). This suggests that an inflammatory state had been imprinted into OA-derived cells prior to their isolation.
Figure 4.11 Human chondrocyte progenitor cells isolated from OA cartilage (JF012OA cell strain) showed a decreased ability of chondrogenic differentiation and changes in behavior akin of inflammation associated cell differentiation. Sox9 expression in JF012OA cells was analyzed using immunocytochemistry of monolayer cells seeded on fibronectin-coated glass cover slides (A, bar = 40 µm). Control with non-specific IgG is also shown (B). H&E staining of 5 µm paraffin sections of PRO3 (left) and JF012OA constructs (2 x 10⁶ cells/construct) cultured for 28 days with the same chondrogenic medium formulation revealed substantial difference in tissue height (C). Staining of sections with H&E (D) and toluidine blue (E) showed fibrocartilaginous nature of tissue. Immunohistochemical staining
showed the presence of collagen type I (F), type II (G) and type X (H). Controls using rabbit IgG (I) or mouse IgG (J) showed no staining. Bar = 100 µm. Analysis of wet weight (K) and total GAG content with DMMB assay (L) of the constructs substantiated differences seen in histology. Gene expression analysis using SYBRGreen Q-PCR of selected cartilage matrix proteins and OA markers (M) was carried out with 100 ng cDNA. Data is expressed relative to expression levels determined for PRO3 constructs. Data is shown as mean ± SEM from two independent experiments with one sample each. Each sample was measured in duplicate. Statistical significance of changes to PRO3 day 28 control were determined using two-tailed Student’s t-test (K, L) and one-way ANOVA with post hoc analysis using Tukey's multiple comparison test (M). *P< 0.05, **P<0.01, ***P<0.001. Bar = 100 µm.
4.4 Discussion

Following the development and optimization of the human 3D model of articular cartilage in the previous chapter, the resulting cartilage construct needed to be thoroughly characterized with regards to cell differentiation and biochemical composition. The best way to characterize the cartilage constructs was to carefully investigate the changes associated with tissue formation. An extracellular matrix which showed cartilaginous tissue morphology and characteristic staining with toluidine blue was established over a 35 day period. In parallel to the increase in tissue height, the wet weight was increasing which was presumably a result of the increased water content of the constructs.

Examination of the total GAG and collagen content using DMMB assay and hydroxyproline assay revealed that those two major cartilage components gradually increased over 35 days. Over the first 2 weeks, the increases were only slow. But then after day 21, the GAG and collagen amounts deposited in the ECM doubled each week. In contrast, the DNA content did not significantly change during the differentiation process. This indicated that the cell number stayed relatively constant. Murdoch and coworkers (2007) used a similar approach for the generation of cartilage constructs. In their model, human bone marrow mesenchymal stem cells (MSCs) were seeded into transwell culture inserts and cultured for 28 days. They observed an eight-fold increase of DNA within the first 7 days, corresponding to three cycles of cell division. This could be explained by a much more proliferative activity of MSCs compared to chondrocyte progenitor cells. Analysis of the released GAG chains into the conditioned medium showed that there was a gradual increase in rate of release over time in our system. In contrast, cartilage constructs based on MSCs showed an increase in rate of release of GAG chains up to day 7 in culture, after which release was constant. This could indicate that at day 7, the collagen and aggrecan network was fully established and therefore a state of homeostasis reached with matching rates of synthesis and catabolism (Professor Tim Hardingham, Manchester University, personal communication). Relating this to the results in this study, chondrogenesis of the articular progenitor cells appears to proceed at a slower rate and therefore,
building the ECM meshwork is progressing slower as well. Articular cartilage was reported to have a water content of about 70-80% of its total weight. The dry weight is comprised of about 60% collagen and 10% GAG chains (McDevitt 1973). In comparison, the water content of the cartilage constructs was slightly higher with 90%, as well as collagen (~70%) and GAG (~15%) content of the dry weight. This indicates that the overall biochemical composition of the cartilage constructs was similar compared to articular joint cartilage.

Immunohistochemical staining of fully differentiated cartilage constructs showed the presence of the major cartilage components collagen type II and aggrecan, as well as non-collagenous glycoproteins including COMP, decorin, fibronectin and lubricin. Lubricin, also known as superficial zone protein and proteoglycan 4, is expressed in the superficial layer of cartilage and has been proposed to be part of the lubricant of the joint surface (Schumacher et al. 1994). It was very encouraging that lubricin was predominantly expressed in the “surface” layer of the cartilage constructs. Since the cells originate from the superficial zone of the donor cartilage (Dowthwaite et al. 2004; Williams et al. 2010), one would have not been surprised if lubricin would be homogenously expressed in the constructs. This shows that the chondrocytes within the construct are influenced by other factors independent from the growth factors supplied in the medium, and are attempting to re-establish the tissue architecture seen in joint cartilage. One of these factors could be hydrostatic pressure, which is induced by culturing the cells in the transwell and adding excess medium on top of them. Moreover, given the size of the constructs, concentration gradients of supplements will be established across the tissue. Since lubricin is only produced by superficial zone chondrocytes, it could indicate that a stratification within the cartilage construct took place with chondrocyte progenitor cells at the surface and normal chondrocytes in the remaining parts of the constructs. Specific sulphation patterns have been reported for the cartilage stem cell niche (Hayes et al. 2008). The monoclonal antibodies 3B3 (-), 7D4, and 4C3 were used because they were reported to bind CS motifs present in the niche, but no specific staining was observed
(data not shown). Therefore, it remains to be seen whether the chondrocyte progenitor cells are restricted to the superficial layer of the constructs. Staining of the constructs with antibodies against disaccharide epitopes generated after chABC digest showed strong staining for $\Delta 4S$ and $\Delta 6S$ and weak staining for $\Delta 0S$. Digestion with chB followed by staining for $\Delta 4S$ resulted in no staining, indicating the absence of DS in the construct as expected. However, the staining was no indication for the relative content of these disaccharides in the GAG chains, since the antibodies only recognize the “stubs” of the GAG chains on the core proteins.

HPLC analysis of AMAC labeled disaccharides generated after exhaustive cleavage of the cartilage construct GAG chains revealed that the disaccharide composition changed during differentiation. Initially at day 1, the GAG chains comprised an approx. equal amount of $\Delta 4S$ (51.4%) and $\Delta 6S$ (46.3%) and a small amount of $\Delta 0S$ (2.4%). Within the first week of differentiation, the disaccharide composition of the constructs changed substantially and then gradually thereafter. The GAG composition was characterized by $\Delta 6S$ being the most abundant disaccharide, followed by $\Delta 4S$ and $\Delta 0S$, reaching 69%, 20% and 11% by day 35.

Bayliss and coworkers (1999) investigated the disaccharide composition of total GAG chains from human cartilage explants with different donor age. They observed that the amounts of $\Delta 4S$ and $\Delta 6S$ within the GAG chains were very similar at young age, but then changed in adulthood to $\Delta 6S$ being the main disaccharide (~80%), followed by $\Delta 4S$ (~15%). Only about 5% of the disaccharides were non-sulphated (Bayliss et al. 1999). They also observed di- and trisulphated disaccharides, which contributed less than 1% to the total amount. This change in the ratio of $\Delta 6S:\Delta 4S$ is similar to the changes observed in the differentiation of the cartilage construct in this study. However, the time scale is of course not comparable. The cartilage constructs cultured for up to 5 weeks could not possibly undergo the spectrum of matrix maturation observed during postnatal development and growth in humans over decades.
Using aggrecan purified from the cartilage explants for Western blotting analysis, Bayliss and coworkers (1999) showed increased staining of the membrane after sample digest with chABC using the antibody 3B3 (recognizing Δ6S stubs) with increasing patient age. This indicates that aggrecan was probably the main source for the increased Δ6S content.

To gain further insight on cell differentiation, the expression of marker proteins during chondrogenic tissue formation was analyzed using Q-PCR. As expected, the expression of collagen type II and aggrecan increased over the time course very substantially. Matrilin-3 expression also increased over 35 days, but much later than the other matrix proteins with maximal expression not seen until day 28. Matrilins play a role as adapters between collagen fibrils and aggrecan. They help to form the ECM meshwork predominantly made from these matrix proteins (Klatt et al. 2011). One reason why it took about 28 days to form the proper cartilaginous ECM, even though collagen type II and aggrecan were already highly expressed from day 7, could have been the relatively late increase in matrilin-3 expression.

In contrast to collagen type II, collagen type I was expressed in monolayer culture. Interestingly, collagen type I expression was seen throughout the time course, although it may not have been expressed by all cells as indicated by regional restriction of collagen type I staining to the centre of the constructs by immunohistochemistry. Expression of collagen type I was also observed during chondrogenic differentiation of MSCs by others (Murdoch et al. 2007).

During the time course of differentiation of the cartilage constructs, a switch from collagen type II A to type II B was observed. Type II A is the isoform expressed by chondrocyte progenitor cells during mesenchymal condensation (Sandell et al. 1994; Goldring et al. 2006). The type II B splice variant lacks exon 2, which encodes a von Willebrand factor type C domain in the N-terminal propeptide that was shown to interact with TGF-β1 and BMP-2 in vitro (Zhu et al. 1999). This growth factor binding property of type II A collagen might be particularly important for chondrogenesis. After 35 days in culture, PCR analysis showed that > 90% of the total collagen type II was the B isoform,
indicating that the vast majority of progenitor cells had differentiated into mature chondrocytes. Overall, the analysis showed that the switch from II A to II B progressed gradually over 4 weeks, which was relatively slow compared to the chondrogenesis of MSCs. Murdoch and coworkers showed that in their cartilage model, the switch from collagen type II A to B occurred within 4 to 5 days after seeding (Murdoch et al. 2007). As discussed above, this showed again that MSCs much more readily differentiated into mature chondrocytes than human knee cartilage derived progenitor cells.

By evaluating the gene expression levels of hypertrophic markers, an increase in the expression of collagen type X was observed. However, no collagen type X deposition in the cartilage construct was detected after 35 days in culture. In normal healthy articular cartilage, no collagen type X expression is detected (van der Kraan and van den Berg 2012), but it is commonly observed in tissue engineered cartilage constructs and linked to spontaneous hypertrophic differentiation of cells in such engineered tissue constructs (Johnstone et al. 1998; Murdoch et al. 2007; Yoo et al. 1998; Barry et al. 2001).

Mueller and Tuan (2008) investigated the effects of known hypertrophy inducing factors, e.g. decreasing dexamethasone concentration, adding thyroid hormone and removing TGF-β from the culture medium, on chondrogenic differentiation of MSCs. They observed that while in control constructs the expression levels of collagen type X mRNA and protein were much higher than in constructs stimulated to undergo hypertrophy, there was no collagen type X deposition in the matrix of control constructs. Collagen type X deposition was strictly associated with chondrocyte hypertrophy (Mueller and Tuan 2008). This very interesting observation made it clear that a high level of collagen type X expression by itself does not inevitably lead to chondrocyte hypertrophy. In a similar experiment in this study, fully differentiated constructs were stimulated with a medium promoting hypertrophy for 14 days. T3 hormone was added into the medium to stimulate hypertrophic differentiation (Wang et al. 2010). This treatment led to collagen type X deposition in the bottom most part of the construct next to the supporting
filter, a tissue zone which also showed morphological changes akin of hypertrophy. Gene expression analysis showed that the mRNA levels of collagen type X were unchanged, but the expression of MMP13 was about 7 fold increased. In addition, HIF2α and indian hedgehog (IHH) were also slightly up regulated. However, the expression of Runx2 and osteonectin was higher in the control constructs. In a recent study, Yang and colleagues found that matrilin 3 is a novel binding partner for BMP-2 and may prevent premature chondrocyte hypertrophy through suppression of BMP-2/Smad1 activity (Yang et al. 2014). In the cartilage constructs, matrilin 3 expression was strongly increased on day 28 and 35 (Fig. 4.5 D). The resulting increased presence of matrilin 3 could have therefore resulted in the sequestration of BMP-2 in the ECM and inhibited the hypertrophic differentiation of cartilage constructs. Taken together, these results indicate that the cartilage constructs derived from chondrocyte progenitor cells formed stable cartilage without terminal differentiation under normal culture conditions and when induced to undergo terminal differentiation, display an architecture mimicking that of joint cartilage with a superficial zone at the top and hypertrophic zone at the bottom.

Using progenitor cells established from different individuals (PRO3, PRO2 and PRO5), the efficacy of the developed chondrogenic differentiation protocol was demonstrated. The successful differentiation of two additional cell strains showed that the protocol can be applied to progenitor cells in general, independent of patient age. In contrast, differentiation of a progenitor cell strain isolated from OA-knee cartilage resulted in a fibrocartilaginous tissue with a significantly reduced size, wet weight and GAG content compared to our standard PRO3 cell strain. Furthermore, extensive deposition collagen type X in the matrix was revealed by immunolocalization. This indicates a hypertrophic-like phenotype of the chondrocytes. Up regulation of MMP13 or collagen type X was not observed, which would be expected for terminally differentiated chondrocytes. In fact, expression levels were lower than control. Interestingly, IL-8 was highly up regulated. IL-8 is a pro-inflammatory cytokine and produced by chondrocytes upon stimulation with IL-1β and TNF-α (Lotz, Terkeltaub, et al. 1992). It is also present in the synovial fluid of patients with
knee osteoarthritis (Pierzchala et al. 2011). Given that culture conditions for PRO3 control cells and JF012OA cells were identical, it is suspected that the OA cells had an intrinsically high expression level of proinflammatory cytokines. Since the cells were derived from OA knee cartilage, they must have been exposed to an inflammatory environment present during OA development. Epigenetic modifications of a number of genes in chondrocytes from OA cartilage have been described (Barter and Young 2013; Moazedifuerst et al. 2014). This environment could have caused epigenetic changes in these cells, affecting gene expression long after isolation from the tissue and extensive expansion in monolayer culture. This further highlights epigenetic information as an important factor in cartilage tissue engineering.

In conclusion, the results presented in this chapter show that cartilaginous tissue generated with progenitor cells had similar biochemical and histological features as articular cartilage and could be used for further investigation of cartilage metabolism. Although chondrogenesis of these cells was slower compared to MSCs widely used for cartilage tissue engineering, the advantages were that the cells did not undergo terminal differentiation and formed stable cartilage. In addition, a single cell strain was used to generate an enormous amount of cartilage constructs, whereas MSCs can only be expanded in monolayer to a limited extend.
Chapter 5:

Changes in carbohydrate biosynthesis associated with exposure of cartilage to an OA-like inflammatory stimulus
5 Changes in carbohydrate biosynthesis associated with exposure of cartilage to an OA-like inflammatory stimulus

5.1 Introduction
The perception of OA being solely caused by wear induced degeneration of joint cartilage has changed in recent years. Increasingly, evidence indicates that inflammation of the synovium (synovitis) plays an important role in the development of OA (Berenbaum 2012; Goldring and Otero 2011). Upon induction of inflammation, the synovial membrane gets infiltrated by mononuclear cells and inflammatory factors are secreted into the synovial fluid. These factors include interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α) and various chemokines (Sellam and Berenbaum 2010). Although the origin of synovitis is not entirely clear, several theories have been put forward. One reason for the development of synovitis could be the release of cartilage fragments into the synovium which leads to activation of synovial cells and production of inflammatory factors. These factors can induce the expression of catabolic enzymes, e.g. matrix metalloproteases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), in the superficial layer of joint cartilage leading to matrix break-down. Moreover, these factors can promote synovial angiogenesis and induce an increased expression of inflammatory factors by the synovial fibroblasts themselves and thereby drive a catabolic cycle (Sellam and Berenbaum 2010). Another theory describes synovial tissue as the initiator of OA development. In a mouse model of OA, depletion of synovial macrophages before inducing collagenase-mediated joint instability prevented the generation of MMP-generated neo-epitopes (Blom et al. 2007; Blom et al. 2004). The important role of these cells was further underlined by a decreased incident of osteophyte formation (Blom et al. 2004). Furthermore, an increased interleukin 17 (IL-17) level in human OA synovial tissue could be related to increased IL-17 expression in synovial mast cells (Suurmond et al. 2011).
IL-1β, a member of the interleukin cytokine superfamily, is one of the predominant inflammatory cytokines involved in inflammatory joint disease (Goldring and Otero 2011). It is a key pro-inflammatory cytokine secreted by innate immune cells but can also be produced by many other cells including synovial fibroblasts and chondrocytes. Upon binding to its receptor (IL-1R1), it can initiate several signaling pathways, leading to activation NF-κB, Erk1/2, p38, and JNK (Fig. 5.1) (Weber et al. 2010; Daheshia and Yao 2008). This results in increased expression of several catabolic enzymes, including MMP1, MMP3, and MMP13 (Fan et al. 2005; Inoue et al. 2005; Kobayashi et al. 2005; Mix et al. 2001; Tetlow et al. 2001), as well as ADAMTS-4 and ADAMTS-5 (Fan et al. 2005; Cortial et al. 2006). Furthermore, chondrocytes also increase expression and secretion of IL-1β and another inflammatory cytokine, IL-6, resulting in an autocrine-positive feedback loop (Fan et al. 2007) driving catabolic activity. IL-1β (and TNF-α) signaling leads to the suppression of expression of proteins responsible for maintaining the chondrocyte phenotype, e.g. aggrecan and collagen type II (Goldring and Otero 2011).

Oncostatin M (OSM), a member of the interleukin-6 protein family, was reported to be involved in inflammatory joint disease (Cawston et al. 1995; Langdon et al. 2000). It is secreted by monocytes, activated T cells and neutrophils (Tanaka and Miyajima 2003). OSM alone causes mild catabolic responses in chondrocytes (Goldring and Otero 2011). However, it signals synergistically together with IL-1β, leading to enhanced collagen (Cawston et al. 1995) and aggrecan (Cawston et al. 1998; Durigova et al. 2011) degradation through the up-regulation of MMPs (Blain et al. 2010; Hui et al. 2003) and ADAMTSs (Hui et al. 2003; M Durigova et al. 2008; Durigova et al. 2011). In particular, OSM is a potent inducer of MMP13 in articular cartilage (Blain et al. 2010; Fearon et al. 2006; El Mabrouk et al. 2007), and therefore plays an important part in the pathological process of cartilage degeneration (Goldring and Goldring 2007). OSM binds to its plasma membrane receptor which is composed of a heterodimer of the signal transducer receptor chain gp130 (glycoprotein 130) and OSMRβ (OSM-receptor β) (Heinrich et al. 2003). OSM can also bind to a receptor heterodimer composed of gp130 and LIFRβ
(leukemia inhibitory factor receptor $\beta$). Signal transduction is primarily mediated through the JAK/STAT signaling pathway (Fig. 5.1) (Tanaka and Miyajima 2003; Heinrich et al. 2003). Alternatively, in some contexts signaling can also be transduced through the MAPK (mitogen activated protein kinase) pathway, leading to phosphorylation of Erk1/2 (extracellular regulated kinase 1/2), p38, and JNK (c-Jun N-terminal kinase) (Heinrich et al. 2003). Recently, Gilbert and coworkers found that in primary bovine articular chondrocytes, co-stimulation of IL-1$\beta$ and OSM leads to increased expression of MMP9, ADAMTS-4, and ADAMTS-5 through activation of protein kinase R (Gilbert et al. 2012).

Proposed biochemical biomarkers for pre-radiographic OA are mostly cartilage matrix turnover and degradation products as already detailed in the introduction to this thesis. However, it is not yet established whether any of these markers for disease currently available are reliable for clinical diagnostic (Mobasher and Henrotin 2011; Kraus et al. 2011). Moreover, the baseline level for some of the markers has a large variability between patients which makes it difficult to establish diagnostic thresholds. Identification of one or more qualitative markers for early OA, for example an epitope specific for OA, should prove to be a more reliable indicator of disease than simply assessing the quantity of a certain metabolic product.

Using the novel human cartilage model based on chondrocyte progenitor cells (described in chapter 3 and 4), global changes in gene expression of enzymes involved in glycan synthesis and modification associated with inflammatory stimulation can be analyzed using microarray technology. Such an approach might provide clues from which disease-associated biosynthetic changes in glycosylation patterns can be predicted, and subsequently tested in follow-up biochemical studies. In addition to inflammatory stimulation of cartilage constructs, dermal equivalents were generated and stimulated with the same inflammatory regimen. The purpose of this was to use a reference model, helping to identify changes in gene expression which are chondrocyte specific and those that are a general tissue response to the cytokine treatment.
5.1.1 **Aims for this chapter:**

1. Identify relevant concentrations for inflammatory insult in cartilage model system and verify that expected catabolic changes take place, i.e. establish an *in vitro* model of osteoarthritis.

2. Identify changes in gene expression using microarray technology in combination with a robust experimental design likely to provide answers to central questions concerning cartilage-specific changes in protein glycosylation mediated by an inflammatory environment.

3. Select genes of interest from microarray data and verify changes in gene expression using Q-PCR to validate data set and identify candidate genes of interest for further studies.
Figure 5.1 Overview of signalling pathways of IL-1β and OSM that play a major role in OA. IL-1β binds to the IL1R1 receptor, leading to activation of NFκB, Erk1/2, p38 MAPK, and JNK signalling. OSM can bind to two different receptor subtypes which are either composed of a dimer of gp130 / OSMRβ or gp130 / LIFRβ. Signalling mainly occurs through the JAK/STAT pathway, but may also involve other pathways including Erk1/2, p38 MAPK, and JNK signalling. Both, IL-1β and OSM signalling lead to an increase in the expression of catabolic enzymes and inflammatory mediators which are found in OA cartilage. The information presented in this schematic diagram was obtained from Weber et al. (2010), Heinrich et al. (2003), and Dey et al. (2013).
5.2 Materials and methods

5.2.1 Inflammatory stimulation of cartilage constructs
Cartilage constructs were developed for 35 days in chondrogenic differentiation medium as described in chapter 4, section 4.2.1. Before inflammatory stimulation, the constructs were cultured for 72 h in chondrogenic basal medium (chondrogenic differentiation medium without dexamethasone, bovine insulin, TGF-β2, and BMP-2). The medium was changed in both the bottom (1 ml) and top (500 µl) of the transwell insert at all steps. This was done to remove any anabolic growth factors endogenously synthesized by the constructs which could potentially influence the chondrocyte response to the inflammatory cytokines. In the next step, cartilage constructs were cultured in basal medium (control), basal medium containing 1 ng/ml IL-1β or basal medium containing 1 ng/ml IL-1β and 10 ng/ml OSM. For each condition, three constructs were cultured at the same time for 48 h while replacing the medium after 24 h. Subsequently, the cartilage constructs were washed twice with PBS before being removed from the transwell insert and transferred into a 1.5 ml tube containing 500 µl TRIzol. The tubes were frozen immediately in liquid nitrogen and stored at -80°C until RNA extraction was performed. Analysis of the GAG content in the conditioned medium was determined by DMMB assay (described in chapter 3, section 3.2.8).

5.2.2 Inflammatory stimulation of dermal constructs
To determine the effective dose of IL-1β for Bonacker fibroblasts, 1 x 10⁴ cells were seeded per well of a 24 well plate and cultured in 2 ml medium per well containing 10% serum (chapter 2, section 2.2.8). After reaching approximately 70% confluency, the serum concentration in the medium was reduced to 4%. Different concentrations of IL-1β from 0.1 to 30 ng/ml were added to the medium and the cells cultured for 72 h without medium change. At the end of the incubation period, the medium was removed and the cells were washed with PBS. 35 µl cell lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2% glycerol, 1% Triton X-100, containing protease inhibitor cocktail (Sigma Aldrich, Cat. No. P8340; final concentrations: 1.04 mM AEBSF, 0.8 µM...
aprotinin, 40 mM bestatin, 14 mM E-64, 20 µM leupeptin, 15 µM pepstatin A) were added to each well and the cells lysed on ice while shaking for 30 min. Subsequently, the remaining cells were scraped from the well. The suspension was centrifuged for 10 min at 14,000 x g and 4°C and the supernatant collected and stored at -20°C. The protein concentration in the cell extracts was determined using the Pierce BCA assay kit. For Western blot analysis, 15 µg protein were loaded per lane on a 4-20% Tris-glycine PA gel. SDS-PAGE and Western blotting were performed as described (chapter 2, sections 2.2.13 and 2.2.15). The membrane was initially probed with an antibody raised against cyclooxygenase 2 (COX2), and afterwards with an antibody against β-tubulin as a loading control (Table 5.1). The ratio between the amount of COX2 and β-tubulin for every condition was calculated from densitometric analysis using the ImageJ software package (Abramoff et al. 2004).

For microarray analysis, hyperconfluent cultures of Bonacker fibroblasts were prepared and grown in the presence of ascorbate-2-phosphate. Under these conditions, fibroblasts are known to establish an endogenous extracellular matrix containing collagen and fibrillin fibrils (Kielty et al. 1994). Cells were seeded with a density of 1.5 x 10^5 cells/well into 24 well plates, and after 24 h switched to minimal essential medium (MEM) containing 10% heat-inactivated fetal bovine serum, non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, and 50 µg/ml ascorbic acid-2-phosphate. The medium (1 ml per well) was changed every 2 days. After 22 days in culture, the ECM layer was washed with PBS, and the serum concentration in the culture medium reduced to 4% and cytokines added. For each condition, three constructs were cultured in parallel for 48 h in either control medium, medium containing 1 ng/ml IL-1β or medium containing 1 ng/ml IL-1β and 10 ng/ml OSM. Medium was replaced after 24h. At the end of the inflammatory stimulation, cells were washed in PBS twice. Subsequently, 500 µl TRIzol were added per well and cells lysed at room temperature for 10 min, and the ECM eventually dislodged from the plastic surface using a cell scraper. The resulting suspension was frozen on dry ice and stored at -80°C until RNA extraction was performed.
5.2.3 Purification of total RNA for microarray analysis
Total RNA was purified using TRIzol as previously described (chapter 4, section 4.2.6). Because highly pure RNA is required for microarray analysis, the purified RNA was further processed to remove any remaining genomic DNA contamination as well as proteins. The precipitated total RNA pellet from the initial purification was reconstituted in 70 µl RNase-free H₂O and digested with 4 µl (4 units) recombinant RNase-free DNase I (Promega) in a total volume of 100 µl for 30 min at 37°C. To remove DNA fragments generated by the DNase digest as well as aggrecan which co-purifies with RNA during TRIzol extraction (McKenna et al. 2000), the samples were applied to RNEasy mini spin columns (Qiagen). The total RNA was purified using the RNEasy Mini kit according to the manufacturer’s instructions. Afterwards, the RNA was precipitated in ethanol as described (chapter 4, section 4.2.6) and stored at -80°C until further use.

5.2.4 RNA quality control
The quality of the purified total RNA from cartilage and dermal constructs was assessed using an Agilent 2100 Bioanalyzer (Agilent). This was performed by Dr Claudia Consoli at Central Biotechnology Services (CBS, School of Medicine, Cardiff University). Briefly, approx. 250 ng of total RNA were applied to Eukaryote Total RNA Nano assay chips (Agilent). The RNA quality was assessed based on the amount of 18S rRNA and 28S rRNA in the sample, as well as the overall electrophoretic sample profile expressed as the RIN score (RNA Integrity Number) which gives a verified measure of the degree of RNA degradation. The RIN score range is from 1 to 10, whereby 1 is very poor RNA quality with a high degree of degradation and 10 is very good RNA with essentially no degradation. The recommended threshold for a good quality RNA preparation is a RIN score of 6 or higher. In addition, RNA purity was also assessed spectrophotometrically as the absorbance at 260 nm to 280 nm ratio using a NanoVue™ Plus Spectrophotometer (GE Healthcare). A value higher than 1.8 was considered to be acceptable. Any samples that did not meet either of these criteria were excluded from the analysis.
5.2.5 RNA storage using RNAstable tubes
For shipping of RNA to the Scripps Research Institute (La Jolla, CA, USA), 3 µg total RNA were applied to RNAstable tubes (Biomatrica, San Diego, CA, USA) and dried using a vacuum concentrator. The RNAstable tubes allow storage of RNA at ambient temperatures without increasing the degree of degradation over extended time periods. This was considered to be more suitable for shipping of RNA samples than on dry ice, because unexpected delay in the shipping of dry ice samples may compromise RNA quality. To recover the RNA from the tubes, RNase-free H2O was added to give the desired RNA concentration, and a good yield of recovery (> 90%) experimentally verified.

5.2.6 Microarray analysis of gene expression
The gene expression of cartilage and dermal samples was analysed using GLYCO v4 gene chips which are custom-designed Affymetrix chips (Affymetrix, Santa Clara, CA, USA) made for the Consortium of Functional Glycomics (CFG, www.functionalgenomics.org). It contains probes for 1260 human transcripts related to glycosylation. For each of the three conditions, cartilage constructs or dermal equivalents were prepared in three independent experiments. Synthesis of labelled cRNA and hybridization of the microarrays was performed by Dr Steven Head and his colleagues at the DNA Array Core Facility at the Scripps Research Institute (La Jolla, CA, USA). For this, the total RNA was recovered from RNAstable tubes and transcribed into DNA in a two-step process. In the first step, cDNA was generated using Superscript II reverse transcriptase at 42°C for 1h. In the second step, the cDNA was added to a mixture of *E. coli* DNA ligase, *E. coli* DNA polymerase I, *E. coli* RNase H and incubated at 16°C for 2 h, followed by addition of T4 DNA polymerase and incubation for another 5 min at 16°C. To purify the cDNA from the cDNA/protein mixture, phenol/chloroform extraction was used. The aqueous phase containing the cDNA was separated using phase lock gels and concentration of the cDNA was performed using ethanol precipitation. The generation of biotinylated cRNA from cDNA was performed using *in vitro* transcription using the MessageAmp™ II Biotin Enhanced aRNA Amplification
Kit (Ambion/Life Technologies) according to the manufacturer’s instructions. After sample clean-up using the RNeasy mini kit (Qiagen), cRNA was fragmented using fragmentation buffer containing MgOAc and KOAc. Hybridization and analysis of the chip was performed on a GeneChip Scanner 3000 7G (Affymetrix) using standard Affymetrix protocols based on methods originally described by Lockhart et al. (Lockhart et al. 1996).

The initial microarray data analysis was performed by Suzanne Papp and Lana Schaffer at the DNA Array Core Facility (Scripps Research Institute). Quality control of microarray chips was performed using Bioconductor routines (Gentleman et al. 2004). The data obtained from the chips was normalized separately for cartilage and dermal samples, resulting in 755 annotated probe sets (Bolstad et al. 2003; Irizarry 2003; Carvalho and Irizarry 2010). Before comparison of the data sets, the 40% variable transcripts with lowest signal intensity were excluded from the data set. The fold changes and standard errors were calculated using the LIMMA (Linear Models for Microarray Data) package (Smyth 2004) by fitting a linear model for each gene. The fold changes are expressed as the average log$_2$ of the array signal raw data. This was done because the log scale is the relevant scale for microarray analysis starting with log transformed data, taking into account the distribution of noise in the intensity measurements as well as the transformation to a normal distribution. Smoothing of standard errors was performed with empirical Bayes methods. The changes in gene expression between the different experimental conditions within each tissue model are presented as fold change, p-value (not shown), and adjusted p-value. The adjusted p-value is derived from the p-value after adjustment for multiple testing with the Benjamini and Hochberg’s method of controlling the false discovery rate of 0.15 or less (Benjamini and Hochberg 2009). The threshold for a transcript to be recognized as differentially expressed was set as a fold change > 1.4 and an adjusted p-value < 0.15. Heat maps of the chips were generated using the dChip software package (Li and Wong 2001). Shades of red reflect increased and blue decreased fold change levels relative to the mean expression of the control group for each transcript, respectively.
5.2.7 Verification of microarray results using Q-PCR

Verification of changes in expression of the genes of interest identified from the microarray data was performed as described (chapter 4, sections 4.2.6 and 4.2.7). The primers used for this analysis are described in Table 5.2. Statistical analysis was performed as described (chapter 3, section 3.2.11). For calculation of p-values, the respective \( \Delta \Delta Ct \) values were used. The fold change in gene expression was determined from the respective \( 2^{-\Delta \Delta Ct} \) values.

Table 5.1 Antibodies used in this chapter for Western blotting

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<th>Source</th>
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<td>Santa Cruz Biotechnology</td>
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<tr>
<td>Gene name</td>
<td>Sequence of forward (top) and reverse (bottom) primers (5' - 3')</td>
<td>Amplicon size (bp)</td>
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</table>
5.3 Results

5.3.1 Inflammatory stimulation of cartilage and dermal constructs

In the previous chapter it was shown that the human chondrocyte progenitor cells fully differentiated into chondrocytes in the developed cartilage model system. Using this model system, the effects of inflammatory stimulation on gene expression should be investigated, using the cytokines IL-1β and OSM. These cytokines are leading to the up-regulation/activation of key catabolic enzymes, including MMP1, MMP13, ADAMTS-4, and ADAMTS-5 which are responsible for degradation of the cartilage extracellular matrix in osteoarthritis. It was shown before that combined treatment with IL-1β and OSM has an even stronger catabolic effect on cartilage than IL-1β alone (Barksby et al. 2006; M. Durigova et al. 2008; Pelletier and Martel-Pelletier 2003). For that reason, it was decided to have a group stimulated with IL-1β alone and another group stimulated with IL-1β and OSM in combination. From previous studies looking at the effects of cytokines on cartilage, it was known that treatment with OSM alone had little effect in terms of driving cartilage degradation (Barksby et al. 2006; Hui et al. 2003; Cawston et al. 1998). Therefore, the treatment with OSM alone was excluded from the experimental setup in the following experiments. In addition to the inflammatory stimulation of the cartilage model, a dermal model consisting of a 3D matrix generated by skin fibroblasts was subjected to the same conditions. A comparison of fibroblast and chondrocyte responses to cytokine stimulation will facilitate identification of changes in gene expression that may be tissue specific.

Initially, the effective dose of IL-1β for stimulation of fibroblasts was determined. As a readout, the expression of COX-2 was determined using Western blotting (Fig. 5.2 A). COX-2 expression is induced by IL-1β through NFκB signaling (Weber et al. 2010). For analysis, densitometry was used whereby the signal intensity of COX-2 was normalized to the signal intensity of the loading control β-tubulin (Fig. 5.2 B). COX-2 expression showed a classic dose-response behavior. At a concentration of 1 ng/ml IL-1β, COX-2 expression was increased about 60-fold compared to control. Higher concentrations of IL-1β up to 30 ng/ml did not further increase COX-2
expression substantially (Fig. 5.2 B). 1 ng/ml was considered a physiologically relevant concentration, and was chosen for further evaluation in the cartilage model.

Figure 5.2 Analysis of IL-1β regulated COX-2 expression in human dermal fibroblasts.

To identify the effective dose of IL-1β for regulation of expression, Bonacker cells were stimulated with different concentrations of IL-1β. Cells were incubated for 72 h and expression levels of COX-2 and β-tubulin were analyzed using Western blotting (A). Densitometrical analysis of Western blot bands was performed using the ImageJ software package (B). Data is presented as the fold increase in COX-2 signal intensity relative to the corresponding β-tubulin loading control signal intensity compared to untreated cells. Results are displayed as mean ± SEM of two independent experiments with one sample set each. Data was fitted with a dose response curve. Statistical significance of changes relative to untreated control were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. *P< 0.05.
Identification of an effective concentration of OSM was attempted using a cell proliferation assay, as OSM had been suggested to have a mitogenic effect on fibroblasts (Ihn and Tamaki 2000). However, it was not possible to stimulate proliferation with OSM in the range of 0.1 to 30 ng/ml in Bonacker cells. For inflammatory stimulation of cartilage constructs, an IL-1β concentration of 1 ng/ml and an OSM concentration of 10 ng/ml was used. The OSM concentration was derived from the literature, where 10 ng/ml had proven effective in previous studies (Barksby et al. 2006; Gilbert et al. 2012; Blain et al. 2010; M. Durigova et al. 2008). To ensure that the cytokines had the expected effect on cartilage and dermal constructs, RT-PCR was performed using primers for MMP13, ADAMTS-4, and ADAMTS-5 (cartilage constructs) and MMP1 (dermal constructs) (Fig. 5.3). The housekeeping gene h36B4 was used as a control for the quantity/quality of the cDNA. In the unstimulated control cartilage constructs, MMP13, ADAMTS-4 and ADAMTS-5 were barely detectable, whereas upon addition of IL-1β for 48 h, strong bands for each of the genes could be observed. This was also observed for the combined treatment with IL-1β and OSM. For the dermal constructs, MMP1 expression was analyzed after inflammatory stimulation. In the control constructs, weak expression was observed. After addition of IL-1β alone and in combination with OSM, a strong up-regulation of expression was observed. Analysis of the GAG released from the cartilage constructs into culture supernatant during inflammatory stimulation using the DMMB assay further substantiated these findings and showed that the amount of GAG chains released was 7-fold higher on day 1 and about 5-fold higher on day 2 compared to control when treated with IL-1β alone (Fig. 5.4). Combination of OSM and IL-1β resulted in an even stronger GAG chain release which was 19-fold higher on day 1 and 11-fold higher on day 2 compared to unstimulated control.

In conclusion, this showed that the cells were responding to the inflammatory stimulation as expected and the respective cytokine concentrations were therefore chosen for the microarray gene expression profiling.
Figure 5.3 Cytokine treatment of cartilage constructs and dermal equivalents resulted in up-regulation of matrix degrading enzymes. Cartilage constructs were grown in differentiation medium for 35 days and then maintained in medium without TGF-β2, BMP-2, additional insulin and dexamethasone for a further 3 days prior to stimulation (A). A confluent fibroblast layer was grown in ascorbate-2-phosphate for 22 days to establish a dermal tissue like 3D extracellular matrix (B). Cytokines (1ng/ml IL-1β, 10 ng/ml OSM) were added to the culture media every day for 2 days. Afterwards, total RNA was purified from cartilage constructs and dermal-like tissue. The expression of known target genes up-regulated by IL-1β and OSM was investigated using PCR. Addition of IL-1β alone and in combination with OSM to cartilage constructs resulted in an increase of MMP-13, ADAMTS-4 and ADAMTS-5 expression (A). In dermal fibroblast cultures, up-regulation of MMP-1 by cytokines was confirmed (B). Each figure shows a representative analysis from three independent experiments with 3 repeats each.

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<tr>
<td>H36B4</td>
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</table>
Figure 5.4 GAG chain release from cartilage constructs upon stimulation with inflammatory cytokines. Fully differentiated cartilage constructs were cultured in medium without cytokines, or supplemented with IL-1β (1 ng/ml) alone or in combination with OSM (10 ng/ml) for a total of 2 days. Medium was replaced every day and fresh cytokines were added. Released GAG accumulating in the conditioned medium was measured using the DMMB assay. Data is shown as the mean ± SEM of three independent measurements.
5.3.2 Preparation of RNA for microarray analysis and evaluation of RNA quality

Cartilage constructs were grown in differentiation medium for 35 days and were then cultured in medium without TGF-β2, BMP-2, additional insulin, and dexamethasone for 3 days prior to stimulation with IL-1β alone or IL-1β in combination with OSM for 48 h. Dermal equivalents were obtained by growing hyperconfluent fibroblast cultures for 22 days in the presence of ascorbate-2-phosphate and were subsequently exposed to the same cytokine regimen. Total RNA was extracted using TRIzol and purified over several stages as outlined in Materials and Methods. RNA was isolated from 3 pooled constructs, and the samples for an n = 3 were grown independently.

The quality and quantity of the isolated RNA was evaluated. The amount of RNA was determined spectrophotometrically using absorbance at 260 nm. Cartilage samples contained between 4.3 and 11.3 µg RNA, and dermal samples between 6.2 and 20.9 µg RNA. Analysis of RNA integrity using the Agilent Bioanalyzer showed that there was very little RNA degradation in the samples (Fig. 5.5). The peaks representing 18S rRNA and 28S rRNA were the biggest peaks observed in the density plots created from the micro agarose gel electrophoresis on the chips. By integration of the area under the curve from each peak, the RNA integrity number (RIN) can be derived resulting in the RIN score. This score is not simply based on 28S/18S rRNA ratio but considers a wider range of elements of the electropherogram within the calculations for assessing RNA quality (Schroeder et al. 2006). It is generally accepted that RNA samples used for microarray analysis should have a RIN score not lower than 6, because otherwise RNA fragmentation by RNases could negatively affect the results. The RIN scores determined for the cartilage and dermal construct samples were all above 6 and are shown in table 5.3.

From each sample, 3 µg total RNA were dried into RNAstably tubes (Biomatrica) using a vacuum concentrator and shipped to the Scripps Research Institute, San Diego, USA, for preparation of labeled cRNA and hybridization.
<table>
<thead>
<tr>
<th>Sample name</th>
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</tr>
<tr>
<td>cartilage 1 IL-1β only</td>
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<tr>
<td>cartilage 1 IL-1β/OSM</td>
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<tr>
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<tr>
<td>cartilage 3 IL-1β only</td>
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<td>skin 1 IL-1β/OSM</td>
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</tr>
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</tr>
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<td>skin 3 IL-1β only</td>
<td>7.8</td>
</tr>
<tr>
<td>skin 3 IL-1β/OSM</td>
<td>7.8</td>
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Figure 5.5 Analysis of RNA integrity after isolation from cartilage (A, C, E) and dermal (B, D, F) constructs. Total RNA samples isolated from cartilage and dermal control constructs (A, B), IL-1β only treated cartilage and dermal constructs (C, D), and IL-1β/OSM treated cartilage and dermal constructs (E, F) were analysed using Agilent 2100 Bioanalyzer chips. Density plots of the gel electrophoresis analysis (right hand side next to respective plot) showed sharp peaks for 18S rRNA (1874 nucleotides) and 28S rRNA (4718 nucleotides). A representative set of samples from each treatment group is shown in this figure.
5.3.3 **Microarray analysis and chip data quality control**

The total RNA was processed for microarray analysis at the DNA Array core facility at the Scripps Research Institute. Initially, the quality parameters for the chip data were determined. The chip diagnostics recommended by Affymetrix were performed using the Bioconductor software suite (Gentleman et al. 2004; Kauffmann et al. 2009).

In order to assess the efficiency of cDNA synthesis by the reverse transcriptase and the degree of RNA degradation, the ratio of 3’ and 5’ human GAPDH control probes was analyzed. A ratio of less than 1.8 between the 3’ and 5’ probe sets on the arrays was considered to be acceptable. The ratios for all cartilage and dermal samples were below this threshold. Most samples including all cartilage samples had a ratio close to 1 which is excellent (Table 5.4, 5.5). A high ratio was measured for only one sample which was the second repeat of the IL-1β/OSM-treated dermal sample with 1.75 (Table 5.5).

<table>
<thead>
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<th>Sample</th>
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Table 5.5 GAPDH 3’/5’ ratios for dermal microarray samples

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<td>skin 2 control</td>
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<tr>
<td>skin 3 IL-1β/OSM</td>
<td>1.32</td>
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Furthermore, the raw hybridisation intensity values for all genes of the chips were compared to identify chips which are substantially different from others in the same data set. Density profile plots showed that there were no outlier chips within these data set (Fig. 5.6). Additional quality checks of the chip data included analyzing the expression values and the respective residuals from the fitting procedures using the relative log expression (RLE) and the normalized unscaled standard error (NUSE). The RLE was determined by calculating the ratio between the expression of a probe set and the median expression of this probe set across all chips of the experiment for each single probe. Assuming that the majority of the genes are not differentially expressed across the arrays, the log ratios should all be centered near zero and have a similar spread. For the cartilage and dermal construct samples, only the spread of the first cartilage sample treated with IL-1β alone (Cart_Treat1_1) and the second dermal sample treated with IL-1β/OSM (Skin_Treat2_2) were marginally larger than the other samples within the respective groups (Fig. 5.7).
Figure 5.6 Density plots of raw hybridization intensity values. Superimposition of densities from cartilage arrays (A) and dermal equivalent arrays (B) show that all chips had a similar density and distribution of signals. Plots were created using the Bioconductor software package together with the R software suite by Suzanne Papp and Lana Schaffer, Scripps Research Institute, La Jolla, CA, USA. Samples are named as follows: Cart_Ctrl_1-3 = cartilage control samples 1-3, Cart_Treat1_1-3 cartilage IL-1β alone samples 1-3, Cart_Treat2_1-3 = cartilage IL-1β + OSM samples 1-3, Skin_Ctrl_1-3 = skin control samples 1-3, Skin_Treat1_1-3 = skin IL-1β alone samples 1-3, Skin_Treat2_1-3 = skin IL-1β + OSM samples 1-3
The NUSE is based on the standard error estimates obtained for each gene from the normalized fit. The standard error estimates are then normalized by dividing with the median standard error so that the median of the box is at 1.0. The medians of all samples were similar except for cartilage IL-1β sample 1 (Cart_Treat1_1) and skin IL-1β/OSM sample 2 (Skin_Treat2_2) (Fig. 5.7 C, D). Taken together, none of the samples showed consistent deviation from the expected quality thresholds and therefore all chips were considered valid data sets for further analysis.

Finally, similarity in gene expression between the individual chips within the cartilage and dermal sample sets was calculated from the centered correlation and average linkage of the expression data from each chip. The resulting cluster dendrograms (Fig. 5.8) showed that for the cartilage samples, the treated groups clustered separately from the control group, but not discretely from each other. This indicates an overlap in gene expression between the IL-1β alone and IL-1β/OSM treatment. This suggests that while having a synergistic effect on cartilage breakdown (Fig. 5.4), OSM does not substantially alter the gene expression profile from that induced by IL-1β. In contrast, the dermal samples were instead clustering discretely between each group without any overlap.
Figure 5.7 Analysis of probe-set homogeneity. RLE (relative log expression) plots of cartilage constructs (A) and dermal equivalent (B) samples show the relative gene expression values to the mean expression values as a box plot. NUSE (normalized unscaled standard error) plots of cartilage (C) and dermal (D) construct samples show the standard error estimates obtained for each gene from the normalized fit divided by the median standard error as a box plot. RLE and NUSE plots were created using the Bioconductor software package together with the R software suite by Suzanne Papp and Lana Schaffer, Scripps Research Institute, La Jolla, CA, USA. Samples are named as follows: Cart_Ctrl_1-3 = cartilage control samples 1-3, Cart_Treat1_1-3 cartilage IL-1β alone samples 1-3, Cart_Treat2_1-3 = cartilage IL-1β + OSM samples 1-3, Skin_Ctrl_1-3 = skin control samples 1-3, Skin_Treat1_1-3 = skin IL-1β alone samples 1-3, Skin_Treat2_1-3 = skin IL-1β + OSM samples 1-3
Figure 5.8 Cluster dendrograms of array data. Hierarchical clustering is calculated from the centered correlation and average linkage of the expression data between the arrays which is then transformed into a tree. It gives information on the similarity in gene expression between the different arrays. A: Arrays from cartilage construct samples; B: Arrays from dermal equivalent samples; Samples are named as follows: Cart_Ctrl_1-3 = cartilage control samples 1-3, Cart_Treat1_1-3 cartilage = IL-1β alone samples 1-3, Cart_Treat2_1-3 = cartilage IL-1β + OSM samples 1-3, Skin_Ctrl_1-3 = skin control samples 1-3, Skin_Treat1_1-3 = skin IL-1β alone samples 1-3, Skin_Treat2_1-3 = skin IL-1β + OSM samples 1-3. Plots were created using the Bioconductor software package together with the R software suite by Suzanne Papp and Lana Schaffer, Scripps Research Institute, La Jolla, CA, USA.
3.4 Analysis of microarray data for experimental groups

After the chip quality controls showed that all samples used for each set of experiments were within acceptable range, the changes in gene expression between the treatment groups and respective controls were analyzed. Bioconductor analysis transformed the raw gene expression data into fold change and associated adjusted p-values. Genes were considered differentially regulated when the magnitude of change was >1.4 and the adjusted p-value was < 0.15.

Differences and similarities in gene expression between the groups were determined for each sample set. Compared to the control group, cartilage constructs treated with IL-1β alone contained 152 differentially expressed genes and IL-1β in combination with OSM contained 187 differentially expressed genes (Fig. 5.9 A). Within the treatment groups, 37 genes were unique to the IL-1β only group and 72 genes were changed specifically when OSM was added. Both treatment groups had 115 differentially regulated genes in common. In dermal equivalents, expression of 101 genes changed when IL-1β only was added and 182 changed when the dermal constructs were treated with IL-1β and OSM together (Fig. 5.9 B). A comparison of the two treatments revealed that 30 gene changes were unique to IL-1β treatment whereas 111 were differentially expressed only in response to OSM stimulation. Both treatment groups had 71 changed genes in common. Therefore, the overall magnitude of changes induced by cytokine treatment is similar in cartilage and dermal constructs. Heat maps of the genes differentially expressed in response to treatment regimens in cartilage and dermal constructs are shown in Fig. 5.10. This illustrates the consistency of differential regulation of these genes across the repeat chip sets.

In the following paragraphs, differentially expressed genes are grouped into different categories for more detailed discussion. These include inflammatory response genes, proteoglycans, CS/DS synthesis, heparin/HS, HA, KS synthesis and modification, and N-linked glycan synthesis and modification. This should help to give a better overview of the effects of inflammatory stimulation on each type of glycosylation. A table listing the gene name
abbreviations together with the full names of the corresponding proteins can be found in Table 5.6.

**Table 5.6 Abbreviations of gene names and their corresponding translated proteins with full names.** Full names were obtained from the UniProt Knowledgebase database (www.uniprot.org).

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<td>Alpha-1,2-glucosyltransferase 10B</td>
</tr>
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<td>UDP-N-acetylglucosaminyltransferase subunit</td>
</tr>
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<td>BGN</td>
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<td>Macrophage inflammatory protein 3 alpha</td>
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<td>Glypican 3</td>
</tr>
<tr>
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<td>Glypican 4</td>
</tr>
<tr>
<td>GPFPT2</td>
<td>Glutamine--fructose-6-phosphate aminotransferase 2</td>
</tr>
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<td>Hyaluronic acid synthase 2</td>
</tr>
<tr>
<td>HAS3</td>
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<td>Interleukin 8</td>
</tr>
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<td>Transcription factor jun-B</td>
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<td>LUM</td>
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<td>OGN</td>
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<td>Serglycin</td>
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<tr>
<td>UGCGL2</td>
<td>UDP-glucose:glycoprotein glucosyltransferase 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full name of protein</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
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<td>Uronyl 2-sulfotransferase</td>
</tr>
<tr>
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<td>Versican</td>
</tr>
<tr>
<td>XYLT1</td>
<td>Xylosyltransferase 1</td>
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Figure 5.9 Venn diagrams showing differences in gene expression in response to cytokine stimulation. The diagrams show the number of transcripts found to be differentially expressed for either IL-1β alone (red) or IL-1β + OSM co-stimulation (blue) for cartilage constructs (A) or dermal equivalents (B). The number of genes which were responding to both treatments are shown as a overlapping area (purple). Diagrams were created using the Bioconductor software package together with the R software suite by Suzanne Papp and Lana Schaffer, Scripps Research Institute, La Jolla, CA, USA.
Figure 5.10 Heat maps of cartilage and dermal construct microarrays. The heat maps show the mean-scaled expression of individual transcripts which were identified to be differentially expressed in both treatments compared to control for cartilage samples (A) and dermal samples (B). Heat maps were generated using the dChip software package by Suzanne Papp and Lana Schaffer, Scripps Research Institute, La Jolla, CA, USA. Red shows increased and blue shows decreased expression relative to control. Colour shades reflect relative fold change over control based on log₂ signal intensity values (see scale at bottom of panel). Samples are named as follows: Cart_Ctrl_1-3 = cartilage control samples 1-3, Cart_Treat1_1-3 cartilage = IL-1β alone samples 1-3, Cart_Treat2_1-3 = cartilage IL-1β + OSM samples 1-3, Skin_Ctrl_1-3 = skin control samples 1-3, Skin_Treat1_1-3 = skin IL-1β alone samples 1-3, Skin_Treat2_1-3 = skin IL-1β + OSM samples 1-3.
5.3.4.1 Genes associated with inflammatory response

Inflammatory stimulation resulted in the up-regulation of cytokines and chemokines in cartilage (Table 5.7) and dermal constructs (Table 5.8) as expected. The biggest changes in expression were observed for genes encoding the small chemokines CXCL1, 3 and 5. They are members of the CXC chemokine family and induce attraction of neutrophils and monocytes (Persson et al. 2003; Smith et al. 2005; Moser et al. 1990) by signaling through CXCR2. Furthermore, the chemokines CCL2 and CCL20 were highly up-regulated. Likewise, a substantial increase in IL-8 and LIF gene expression was observed. These proteins are known to be up-regulated upon IL-1β stimulation (Carlson et al. 1996; Campbell et al. 1993; Lotz, Moats, et al. 1992) and IL-8 and CCL20 expression was further investigated as a positive control of genes known to be highly up-regulated by IL-1β. Q-PCR analysis of IL-8 and CCL20 gene expression confirmed the up-regulation of both genes, although the actual magnitude of change in expression determined in this more sensitive method was substantially higher compared to the data from microarrays (Table 5.6, 5.7).
Table 5.7 Inflammatory stimulation of cartilage constructs led to increased expression of chemokines and cytokines. Microarray results are shown as the magnitude (fold change) and adjusted p-value for each gene for treatment with IL-1β alone or in combination with OSM. Validation of changes in expression of selected genes was performed using SYBRgreen Q-PCR. Here, data is shown as mean ΔΔCt ± SEM and corresponding fold change \(2^{\Delta\Delta C_t}\) ± SEM derived from three independent experiments with duplicates. P-values are based on ΔΔCt values. Statistically significant changes to untreated control samples were determined with one-way ANOVA with post hoc analysis using Tukey's multiple comparison test. n.s. = not significant.

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</thead>
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<td>CXCL5</td>
<td>NM_002994</td>
<td>103.2</td>
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<tr>
<td>CCL20</td>
<td>NM_004591</td>
<td>81.5</td>
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<tr>
<td>CCL2</td>
<td>NM_002982</td>
<td>54.2</td>
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<tr>
<td>IL8</td>
<td>NM_000584</td>
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<tr>
<td>LIF</td>
<td>NM_002309</td>
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<table>
<thead>
<tr>
<th>Q-PCR</th>
<th>IL-1β alone</th>
<th>IL-1β + OSM</th>
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</thead>
<tbody>
<tr>
<td>Gene</td>
<td>ΔΔCt</td>
<td>p-value</td>
</tr>
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<tr>
<td>CCL20</td>
<td>-11.1 ± 1.4</td>
<td>&lt;0.01</td>
</tr>
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</table>
Table 5.8 Inflammatory stimulation of dermal constructs led to increased expression of chemokines and cytokines. Microarray results are shown as the magnitude (fold change) and adjusted p-value for each gene for treatment with IL-1β alone or in combination with OSM. Validation of changes in expression of selected genes was performed using SYBRgreen Q-PCR. Here, data is shown as mean ΔΔCt ± SEM and corresponding fold change \(2^{ΔΔCt}\) ± SEM derived from three independent experiments with duplicates. P-values are based on ΔΔCt values. Statistically significant changes to untreated control samples were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. n.s. = not significant.

### Microarray

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<tr>
<th>Gene</th>
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</thead>
<tbody>
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<td></td>
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### Q-PCR

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<th>Fold change</th>
<th>ΔΔCt</th>
<th>p-value</th>
<th>Fold change</th>
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</thead>
<tbody>
<tr>
<td>IL8</td>
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<td>2192.2 ± 723.8</td>
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<td>&lt;0.01</td>
<td>196.6 ± 74.0</td>
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<tr>
<td>CCL20</td>
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<td>11.2 ± 6.6</td>
<td>-0.9 ± 0.6</td>
<td>n.s.</td>
<td>2.2 ± 0.9</td>
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</table>
5.3.4.2 Proteoglycans

Stimulation of cartilage constructs with IL-1β in the presence and absence of OSM affected the expression of various proteoglycans (Table 5.9). The biggest change in gene expression was observed for serglycin, being up-regulated 17-fold (IL-1β alone) and 14-fold (IL-1β/OSM). Genes encoding endocan, agrin, perlecan and lumican were moderately up-regulated (up to about 4-fold). Down-regulation was observed for genes involved in maintaining the chondrocyte phenotype (aggrecan, lubricin, collagen type IX α2 chain) as well as versican which belongs like aggrecan to the large aggregating proteoglycans present in cartilage. Moreover, proteoglycans not known to be associated with articular cartilage, osteoglycin and phosphacan, were identified and found to be down-regulated. Several proteoglycans were only identified as changed in one of the two treatments. Endocan (ESM1) and perlecan (HSPG2) were only up-regulated when IL-1β alone was added, whereas lumican and collagen type IX α2-chain were only changed in the presence of both IL-1β and OSM.

Validation of the microarray results was performed for serglycin and lumican. The results showed that serglycin was even more substantially up-regulated than expected from the microarray data. The expression was 130-fold increased for IL-1β alone and 51-fold increased for IL-1β in combination with OSM (Table 5.9). The differences between the two methods were not as big for lumican. It was 6-fold increased for IL-1β alone and 4-fold increased for IL-1β together with OSM (Table 5.9).

In the dermal constructs, serglycin was only marginally up-regulated during IL-1β treatment (Table 5.10). Moreover, in contrast to cartilage, lumican was down-regulated by treatment of dermal equivalents with IL-1β. Mostly syndecans and glypicans were differentially expressed in dermal samples (Table 5.10). In addition, fibromodulin expression increased with both treatments and biglycan expression increased 2-fold when IL-1β and OSM were added to the medium.
Table 5.9 Effect of inflammatory stimulation on cartilage proteoglycan gene expression.

Microarray results are shown as the magnitude (fold change) and adjusted p-value for each gene for treatment with IL-1β alone or in combination with OSM. Validation of changes in expression of selected genes was performed using SYBRgreen Q-PCR. Here, data is shown as mean $\Delta\Delta$Ct ± SEM and corresponding fold change ($2^{-\Delta\Delta\text{Ct}}$) ± SEM derived from three independent experiments with duplicates. P-values are based on $\Delta\Delta$Ct values. Statistically significant changes to untreated control samples were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. n.s. = not significant.

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<tr>
<th>Gene</th>
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<td>Magnitude (FC)</td>
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<td>-</td>
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<td>-</td>
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<td>LUM</td>
<td>-1.7 ± 1.1</td>
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Table 5.10 Effect of inflammatory stimulation on dermal proteoglycan gene expression.
Microarray results are shown as the magnitude (fold change) and adjusted p-value for each gene for treatment with IL-1β alone or in combination with OSM.

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5.3.4.3 Genes associated with CS/DS biosynthesis

The expression of enzymes involved in the synthesis and modification of CS/DS chains was influenced by inflammatory stimulation of cartilage constructs. XYLT1 (xylosyltransferase 1) is responsible for the initiation of the chain synthesis by adding a xylose to serine/threonine. It was down-regulated 2 and 3-fold for IL-1β alone and in combination with OSM, respectively (Table 5.11). CHPF (chondroitin polymerizing factor) and CHSY3 (chondroitin sulphate synthase 3), both glycosyltransferases involved in the synthesis of the repeating GlcA/GalNAc disaccharide subunits, were down-regulated by both treatments. Besides GAG chain synthesis, also enzymes involved in the sulphation of CS chains were affected (Table 5.11). PAPSS1 was down-regulated about two fold by each treatment. This enzyme is responsible for the synthesis of PAPS which serves as the donor molecule for sulphate groups used by sulphotransferases. Expression of CHST3 and CHST15, both sulphotransferases with different specificity, was changed by inflammatory treatment. CHST3 is adding a sulphate group to the C6-atom of GalNAc and CHST15 is adding a sulphate group on the C6-atom of GalNAc-4-sulphate. In contrast to all other genes being down-regulated during inflammation, CHST15 was up-regulated when IL-1β and OSM were added to the medium at the same time (Table 5.11). Q-PCR analysis subsequently revealed that an increase in expression also occurred for treatment with IL-1β alone. CHST11 expression was also further investigated as it was reported to be up-regulated in OA cartilage compared to normal cartilage (Karlsson et al. 2010). However, data obtained from Q-PCR analysis showed that its expression was unchanged in both treatment groups. Taken together, these data indicate reduced CS synthesis combined with increased GalNAc4,6diS content.

Interestingly, the CS/DS synthesis machinery in dermal constructs was not as negatively affected by cytokine stimulation as in cartilage constructs (Table 5.12). While XYLT1 expression levels were essentially unchanged, CHGN and CHPF2 expression levels were increased for each treatment. Similar to CHPF, both proteins are glycosyltransferases which are involved in the synthesis and elongation of CS/DS chains (Mikami and Kitagawa 2013). CHPF2 up-regulation was confirmed by Q-PCR, showing that its expression was about 3-
fold (IL-1β alone) and 5-fold (IL-1β/OSM) increased (Table 5.12). In contrast to cartilage, microarray and follow up Q-PCR data also showed that CHST11, encoding a chondroitin 4-O sulphotransferase, was highly up-regulated. Its expression was more than 7-fold (IL-1β alone) and 35-fold (IL-1β/OSM) increased compared to control (Table 5.12).
Table 5.11 Effect of inflammatory stimulation on CS/DS synthesis and modification in cartilage constructs. Microarray results are shown as the magnitude (fold change) and adjusted p-value for each gene for treatment with IL-1β alone or in combination with OSM. Validation of changes in expression of selected genes was performed using SYBRgreen Q-PCR. Here, data is shown as mean ΔΔCt ± SEM and corresponding fold change \((2^{-ΔΔCt}) ± SEM\) derived from three independent experiments with duplicates. P-values are based on ΔΔCt values. Statistically significant changes to untreated control samples were determined with one-way ANOVA with post hoc analysis using Tukey's multiple comparison test. n.s. = not significant.

<table>
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<td>-3.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PAPSS1</td>
<td>NM_005443</td>
<td>-2.4</td>
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<td>-2.0</td>
<td>&lt;0.15</td>
</tr>
<tr>
<td>CHSY3</td>
<td>NM_175856</td>
<td>-3.6</td>
<td>&lt;0.01</td>
<td>-4.1</td>
<td>&lt;0.001</td>
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Q-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>ΔΔCt</th>
<th>p-value</th>
<th>Fold change</th>
<th>ΔΔCt</th>
<th>p-value</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHST15</td>
<td>-1.5 ± 0.6</td>
<td>n.s.</td>
<td>4.2 ± 0.6</td>
<td>-2.2 ± 0.5</td>
<td>&lt;0.5</td>
<td>6.6 ± 1.0</td>
</tr>
<tr>
<td>XYLTI1</td>
<td>-0.2 ± 0.6</td>
<td>n.s.</td>
<td>1.3 ± 0.4</td>
<td>-1.2 ± 0.8</td>
<td>n.s.</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>CHST11</td>
<td>-0.2 ± 0.3</td>
<td>n.s.</td>
<td>1.2 ± 0.2</td>
<td>0.2 ± 0.6</td>
<td>n.s.</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>CHSY3</td>
<td>1.6 ± 0.4</td>
<td>n.s.</td>
<td>0.4 ± 0.1</td>
<td>2.5 ± 0.7</td>
<td>&lt;0.5</td>
<td>0.2 ± 0.1</td>
</tr>
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</table>
Table 5.12 Effect of inflammatory stimulation on CS/DS synthesis and modification in dermal constructs. Microarray results are shown as the magnitude (fold change) and adjusted p-value for each gene for treatment with IL-1β alone or in combination with OSM. Validation of changes in expression of selected genes was performed using SYBRgreen Q-PCR. Here, data is shown as mean ΔΔCt ± SEM and corresponding fold change ($2^{\Delta\Delta C_t}$) ± SEM derived from three independent experiments with duplicates. P-values are based on ΔΔCt values. Statistically significant changes to untreated control samples were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. n.s. = not significant.

<table>
<thead>
<tr>
<th>Microarray</th>
<th>IL-1β alone</th>
<th>IL-1β + OSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Accession no.</td>
<td>Magnitude (FC)</td>
</tr>
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<td>CHGN</td>
<td>NM_018371</td>
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</tr>
<tr>
<td>CHST11</td>
<td>NM_018413</td>
<td>2.1</td>
</tr>
<tr>
<td>XLYT1</td>
<td>NM_022166</td>
<td>-</td>
</tr>
<tr>
<td>CHPF2</td>
<td>NM_019015</td>
<td>-</td>
</tr>
<tr>
<td>PAPSS2</td>
<td>NM_001015880</td>
<td>-</td>
</tr>
<tr>
<td>UST</td>
<td>NM_005715</td>
<td>-</td>
</tr>
<tr>
<td>PAPSS1</td>
<td>NM_005443</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Q-PCR</th>
<th>IL-1β alone</th>
<th>IL-1β + OSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>ΔΔCt</td>
<td>p-value</td>
</tr>
<tr>
<td>CHST11</td>
<td>-1.8 ± 1.3</td>
<td>n.s.</td>
</tr>
<tr>
<td>CHPF2</td>
<td>-0.1 ± 2.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>XLYT1</td>
<td>-0.1 ± 1.8</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
5.3.4.4 Genes associated with heparin/heparan sulphate and keratan sulphate biosynthesis

In addition to changes observed in the expression levels of CS/DS chains, genes encoding for proteins involved in synthesis and modification of heparin/HS, HA and KS were affected by the inflammatory stimulations. However, the number of genes was more limited compared to CS/DS biosynthesis. For reasons of relevance to the main topic of the PhD, only changes in cartilage constructs are presented and discussed.

Microarray results showed that except for HS2ST1, a sulphotransferase adding a sulphate group to the C-2 atom of iduronic acid in HS, all genes found to be differentially expressed were down-regulated (Table 5.13). This included EXTL2 which is a key enzyme that adds the first GlcNAc residue to the GAG linkage region and thereby initiates heparin/HS synthesis (Sugahara and Kitagawa 2000). EXTL1 is another glycosyltransferase involved in HS chain synthesis (Kim et al. 2001) and this was down-regulated as well.

Two genes coding for enzymes known to modify KS were found to be affected by the inflammatory stimulation (Table 5.13). One was ST3GAL1, the expression of which was increased more than 4-fold by IL-1β alone and 3-fold by IL-1β together with OSM. Q-PCR analysis verified the microarray data, showing that the increase was about 9-fold for IL-1β and 2-fold for IL-1β/OSM treatment (Table 5.13). This enzyme is a sialic acid transferase that transfers sialic acid to the non-reducing terminal end of KS. The other enzyme, CHST6, is a KS specific GlcNAc 6-O sulphotransferase which selectively acts on terminal GlcNAc residues in KS (Stanley and Cummings 2009). It was down-regulated almost 5-fold by IL-1β and 3-fold by IL-1β/OSM (Table 5.13). In addition to CHST6, sulphation in KS can also occur through CHST3 which sulphates chain internal galactose residues. CHST3 was down-regulated by IL-1β/OSM but not by IL-1β alone as already discussed (Table 5.11). Taken together, this points towards an altered pattern of sulphation in KS upon inflammatory stimulation.
5.3.4.5 Genes involved in hyaluronic acid synthesis
HA synthases (HAS) 2 and 3 were both differentially expressed upon inflammatory stimulation. Both enzymes are located in the plasma membrane and directly secrete HA chains into the extracellular space (Weigel and DeAngelis 2007). HAS2 is the major HA synthase in cartilage (Hiscock et al. 2000). Microarray data showed that it was 2-fold down-regulated by IL-1β stimulation (Table 5.13). HAS3 which has a low expression level in cartilage under normal conditions, was increased by IL-1β stimulation. In contrast to the other HAS enzymes, the chain synthesized by HAS3 has a lower molecular weight (Tammi et al. 2011; Oguchi and Ishiguro 2004). Such a change may affect average chain length of HA molecules synthesized.
Table 5.13 Effect of inflammatory stimulation on heparin/HS, HA, and KS synthesis and modification in cartilage constructs. Microarray results are shown as the magnitude (fold change) and adjusted p-value for each gene for treatment with IL-1β alone or in combination with OSM. Validation of changes in expression of selected genes was performed using SYBRgreen Q-PCR. Here, data is shown as mean ΔΔCt ± SEM and corresponding fold change ($2^{-\Delta\Delta C_t}$) ± SEM derived from three independent experiments with duplicates. P-values are based on ΔΔCt values. Statistically significant changes to untreated control samples were determined with one-way ANOVA with post hoc analysis using Tukey's multiple comparison test. n.s. = not significant.

<table>
<thead>
<tr>
<th>Microarray</th>
<th>IL-1β alone</th>
<th>IL-1β + OSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin/HS</td>
<td>Accession no.</td>
<td>Magnitude (FC)</td>
</tr>
<tr>
<td>HS2ST1</td>
<td>NM_012262</td>
<td>1.5</td>
</tr>
<tr>
<td>HS3ST3A1</td>
<td>NM_006042</td>
<td>-1.6</td>
</tr>
<tr>
<td>EXT1</td>
<td>NM_001033025</td>
<td>-1.8</td>
</tr>
<tr>
<td>EXT1</td>
<td>NM_004455</td>
<td>-3.2</td>
</tr>
<tr>
<td>HS3ST1</td>
<td>NM_005114</td>
<td>-</td>
</tr>
</tbody>
</table>

| KS | | |
| ST3GAL1 | NM_003033 | 4.7 | <0.01 | 3.0 | <0.05 |
| CHST6 | NM_021615 | -4.2 | <0.05 | -4.4 | <0.05 |

| HA | | |
| HAS3 | NM_005329 | 1.5 | <0.05 | - | - |
| HAS2 | NM_005328 | -2.0 | <0.15 | - | - |

<table>
<thead>
<tr>
<th>Q-PCR</th>
<th>IL-1β alone</th>
<th>IL-1β + OSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>ΔΔCt</td>
<td>p-value</td>
</tr>
<tr>
<td>ST3GAL1</td>
<td>-4.5 ± 1.3</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>
5.3.4.6 Genes involved in N- and O-linked glycosylation of proteins

Not only GAG synthesis was affected by inflammatory stimulation, but also the synthesis and modification of N-linked glycans. N-linked glycans are attached to the core protein through asparagine glycans. Several enzymes involved in the formation of N-linked oligosaccharide precursors were changed in their expression (Table 5.14). ALG genes are coding for glycosyltransferases involved in the build-up of the branched oligosaccharides consisting of GlcNAc, mannose and glucose. ALG14 and ALG10B which are involved in the initial and late stages of glycan precursor formation, respectively, were both down-regulated by IL-1β/OSM co-stimulation. In contrast, the mannosyltransferase ALG9 was up-regulated by both treatments. Using Q-PCR analysis, the down-regulation of ALG14 by IL-1β/OSM treatment could be confirmed (Table 5.14). For IL-1β alone, the expression was unchanged. Furthermore, DDOST is unchanged by treatment with IL-1β alone and down-regulated by IL-1β/OSM. This is a key enzyme in the N-linked glycosylation pathway as it is responsible for the ‘en-bloc’ transfer of the oligosaccharide precursor to the asparagine residue of the protein. Taken together, these changes may lead to reduced N-glycosylation.

O-linked glycans are attached to the core proteins through serine/threonine residues. Their synthesis occurs in the Golgi system and is initiated by the transfer of a GalNAc catalyzed by N-acetylgalactosaminyltransferases (GALNTs). Several of these enzymes were either down-regulated (GALNT1, 3, 4, 5, 6, 10) or up-regulated (GALNT2, 4) upon inflammatory stimulation (Table 5.15). The increase in GALNT4 expression was confirmed by Q-PCR (Table 5.15). Moreover, the fucosyltransferase FUT4 was found to be increasingly expressed with both treatments (Table 5.15). O-linked glycans can be modified by sulphation, sialysation, fucosylation and acetylation (Van den Steen et al. 1998).
Table 5.14 Effect of inflammatory stimulation of cartilage constructs on expression of enzymes involved in N-linked glycosylation. Microarray results are shown as the magnitude (fold change) and adjusted p-value for each gene for treatment with IL-1β alone or in combination with OSM. Validation of changes in expression of selected genes was performed using SYBRgreen Q-PCR. Here, data is shown as mean ΔΔCt ± SEM and corresponding fold change \((2^{\text{ΔΔCt}})\) ± SEM derived from three independent experiments with duplicates. P-values are based on ΔΔCt values. Statistically significant changes to untreated control samples were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. n.s. = not significant.

<table>
<thead>
<tr>
<th>Microarray</th>
<th>IL-1β alone</th>
<th>IL-1β + OSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Accession no.</td>
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</tr>
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<td>GFPT2</td>
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</tr>
<tr>
<td>ALG9</td>
<td>NM_024740</td>
<td>2.1</td>
</tr>
<tr>
<td>ALG10B</td>
<td>NM_032834</td>
<td>-</td>
</tr>
<tr>
<td>ALG14</td>
<td>NM_144988</td>
<td>-</td>
</tr>
<tr>
<td>DDOST</td>
<td>NM_005216</td>
<td>-</td>
</tr>
<tr>
<td>UGCGL2</td>
<td>NM_020121</td>
<td>-1.9</td>
</tr>
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<table>
<thead>
<tr>
<th>Q-PCR</th>
<th>IL-1β alone</th>
<th>IL-1β + OSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>ΔΔCt</td>
<td>p-value</td>
</tr>
<tr>
<td>ALG14</td>
<td>-0.1 ± 0.7</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
Table 5.15 Effect of inflammatory stimulation of cartilage constructs on expression of enzymes involved in O-linked glycosylation. Microarray results are shown as the magnitude (fold change) and adjusted p-value for each gene for treatment with IL-1β alone or in combination with OSM. Validation of changes in expression of selected genes was performed using SYBRgreen Q-PCR. Here, data is shown as mean ΔΔCt ± SEM and corresponding fold change ($2^{\Delta\Delta Ct}$) ± SEM derived from three independent experiments with duplicates. P-values are based on ΔΔCt values. Statistically significant changes to untreated control samples were determined with one-way ANOVA with post hoc analysis using Tukey's multiple comparison test. n.s. = not significant.

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Microarray IL-1β alone</th>
<th>Microarray IL-1β + OSM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Magnitude (FC)</td>
<td>Adjusted p-value</td>
<td>Magnitude (FC)</td>
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<td>NM_013382</td>
<td>2.0</td>
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<td>GALNT2</td>
<td>NM_004481</td>
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<td>&lt;0.15</td>
</tr>
<tr>
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<td>1.4</td>
<td>&lt;0.15</td>
</tr>
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<td>GALNT6</td>
<td>NM_007210</td>
<td>-1.5</td>
<td>&lt;0.15</td>
</tr>
<tr>
<td>GALNT10</td>
<td>NM_017540</td>
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<td>&lt;0.15</td>
</tr>
<tr>
<td>GALNT3</td>
<td>NM_004482</td>
<td>-1.8</td>
<td>&lt;0.15</td>
</tr>
<tr>
<td>GALNT1</td>
<td>NM_020474</td>
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<td>&lt;0.15</td>
</tr>
<tr>
<td>GCNT1</td>
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<td>&lt;0.15</td>
</tr>
<tr>
<td>GALNT5</td>
<td>NM_014568</td>
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<td>&lt;0.01</td>
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</table>

Q-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Q-PCR IL-1β alone</th>
<th>Q-PCR IL-1β + OSM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔΔCt</td>
<td>p-value</td>
</tr>
<tr>
<td>GALNT4</td>
<td>-1.5 ± 0.7</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

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5.4 Discussion

In this chapter, cartilage and dermal constructs were stimulated with IL-1β alone and in combination with OSM. The effects on gene expression were analyzed using GlycoChips developed by the International Consortium for Functional Glycomics. These are Affymetrix microarrays containing a probe set designed for investigating genes involved in glycosylation and related proteins. The aim of these experiments was to investigate changes in the expression of glycosyltransferases and sugar modifying enzymes established by an inflammatory environment in cartilage and dermal constructs to identify characteristic patterns that may aid development of biomarkers for OA.

The importance of inflammation in early OA is now generally accepted (Goldring and Otero 2011). Previous studies used cartilage model systems to investigate the effects of catabolic factors, e.g. IL-1β, TNF-α, on cartilage metabolism (Barksby et al. 2006; Aigner et al. 2005; Sun et al. 2011; Vincenti and Brinckerhoff 2001). The microarray chips used in these studies contained probe sets for the whole human genome and essentially revealed increased expression of genes associated with the major catabolic pathways. In addition, the up and down-regulation of specific cartilage matrix proteins was observed. However, no fundamental new discoveries were made and no novel biomarker identified. The advantage of the chips used in this study compared to previous studies was the use of a restricted probe specifically designed to look only at a selected type of metabolic pathway, namely glycosylation. This was considered to be a promising approach to gain new insights into posttranslational modifications of GAG chains, and glycosylated proteins more generally in OA. Furthermore, the cell model employed here allowed for strict control of experimental conditions which is paramount in any microarray-based screening approach. As previously described (Barksby et al. 2006; Aigner et al. 2005), IL-1β alone and in combination with OSM resulted in the up-regulation of IL-8, LIF, CCL2, and CXCL2, as well as other proteins involved in chemoattraction. No significant difference between cartilage and dermal constructs was observed in our study for most inflammatory mediators, indicating that the response to IL-1β through up-regulation of cytokines and
chemokines is not tissue specific. The combination of IL-1β and OSM had an even more severe effect on cartilage degradation compared to IL-1β alone (Fig. 5.4) in line with previous studies (Barksby et al. 2006; Gilbert et al. 2012). However, little mechanistic information on the reason for this synergy is published. By comparing the signaling pathways triggered by either IL-1β or OSM (Fig. 5.1) it becomes clear that an overlap exists. Both cytokines are known to also activate Erk1/2, p38 MAPK and Jnk1/2/3 signaling. Therefore, one explanation for the synergistic effect on cartilage degradation could be enhanced signaling through these alternative pathways. A different explanation could be the effect on IL-6 production. Using mouse synovial fibroblasts, Le Goff and coworkers could show that OSM induced IL-6 expression even at low concentrations, and further enhanced IL-6 expression when combined with IL-1β or TNFα (Le Goff et al. 2014). Furthermore, they showed that OSM increased IL-1R1 expression, and could therefore lead to increased or sustained sensitivity of synovial fibroblasts to IL-1β. However, IL-6 was not detectable in our microarray experiment even after IL-1β or continued IL-1β/OSM stimulation.

Proteoglycan expression was changed by the inflammatory stimulation. Down-regulation of aggrecan and collagen type IX α2 chain after treatment with IL-1β alone or in combination with OSM was recently described (Gilbert et al. 2012), and therefore an additional control for the validity of our model system. Interestingly, serglycin showed the highest up-regulation in cartilage constructs and was several fold higher expressed in cartilage than in dermal constructs at baseline. Serglycin is a proteoglycan traditionally thought to be expressed by cells of the hematopoietic lineage. The GAG chains attached to serglycin can be of heparin, HS, or CS type. Only little information about serglycin in chondrocytes is available. Recently, Zhang and colleagues identified serglycin as a binding partner for MMP-13 C-terminal domain (Zhang et al. 2010). Using conditioned medium from human articular chondrocytes (isolated after joint arthroplasty) embedded in alginate beads, serglycin was isolated using an affinity column containing the MMP-13 C-terminal domain.
Immunocytochemical staining of serglycin in chondrocytes showed a granular distribution in the cytoplasm, similar to MMP-13 in the same cells. However, no co-localization was reported. In addition, the same observation was made when full-depth cartilage was stained for serglycin and MMP-13, but this time the two stainings did co-localize. Since serglycin is known to be involved in intracellular packaging and inactivation of proteases in secretory granules in hematopoietic cells (Stevens and Adachi 2007; Niemann et al. 2004), the authors suggested that serglycin may be involved in the regulation of MMP-13 secretion in chondrocytes. Our observation of a >50-fold up-regulation of serglycin upon inflammatory stimulation of chondrocytes is novel and identifies this proteoglycan as a potentially important player in the context of OA.

In addition to serglycin, lumican expression was increased in cartilage constructs upon inflammatory stimulation, although more modestly, whereas it was down-regulated in dermal equivalents. Lumican belongs to the group of small leucine-rich proteoglycans (SLRP) and carries a KS chain (Nikitovic et al. 2008). SLRPs in cartilage are known to be involved in growth factor binding, cell adhesion and proliferation, and organization of fibrillar collagens (Iozzo 1999; Svensson et al. 2000). Using a lateral meniscectomy sheep model of OA, Young and coworkers found a significant increase in lumican expression in induced OA compared to non-operated control. The magnitude of change in expression was the biggest increase of all investigated SLRPs (Young et al. 2005). This result is in line with the increase in lumican expression found in this study. Lumican was recently also proposed as a potential novel biomarker for OA (Fernández-Puente et al. 2011). This was based on a study where the authors were analyzing proteins in synovial fluid of OA patients using mass spectrometry (Melrose et al. 2008).

Analysis of gene expression changes in enzymes responsible for CS/DS synthesis and sulphation showed that in cartilage constructs, most of the genes were down-regulated. This involved chain initiation (XYLT1), elongation (CHPF, CHSY3) and sulphation (CHST3). In early OA, the cartilage tries to repair itself through increased anabolic activity of chondrocytes (Goldring and Goldring 2007). This is accompanied by an increased GAG chain synthesis, in
parts mediated by up-regulation of XYLT1. Venkatesan and coworkers (2012) found that XYLT1 expression in cartilage was increased by TGF-β1. In addition, regulation of other CS/DS modifying enzymes by TGF-β1, EGF, and PDGF has been described in human lung fibroblasts (Tiedemann et al. 2005). Furthermore, a recent study using a model of vascular calcification identified XYLT1-mediated GAG synthesis controls TGF-β1 activity and thereby establishes a positive feedback loop controlling XYLT1 expression and TGF-β1 activity (J Yan et al. 2011). In contrast, IL-1β down-regulates XYLT1 expression, leading to a decreased amount of GAG chains attached to cartilage proteoglycans (Venkatesan et al. 2012; Venkatesan et al. 2009), consistent with the findings here.

Despite the likely reduction in overall GAG chain synthesis, changes in expression of individual sulphotransferases could lead to the generation of GAG chain modifications that could potentially serve as a biomarker. The increase of CHST15 (GalNAc4S-6ST) expression upon inflammatory stimulation in cartilage constructs was very interesting. GalNAc4S-6ST is a sulphotransferase that catalyzes the transfer of a sulphate group to the C-6 atom of GalNAc-4S (Ohtake et al. 2003; Ohtake et al. 2001; Ito and Habuchi 2000). This type of sulphation was first identified in squid cartilage CS and named CS-E (Suzuki et al. 1968). This CS species comprises significant biological activity. For example, it was shown that it enabled Lewis lung carcinoma cell metastasis (Mizumoto et al. 2013; Mizumoto et al. 2012). These cells express CS chains containing the CS-E motif on their surface which leads to binding of the receptor of advanced glycation end product (RAGE) expressed in pulmonary tissue. Administration of an anti-RAGE antibody into mice as well as gene knockdown of GalNAc4S-6ST resulted in a markedly lower colonization of the lungs by these carcinoma cells. An increase in GalNAc4S-6ST expression could potentially be a lead for a novel OA biomarker. The fact that GalNAc4S-6ST was the only sulphotransferase gene being up-regulated during inflammatory stimulation of cartilage constructs further highlights the potential of this. In addition, this was not found in dermal constructs, showing that the differential regulation could be tissue specific. Therefore, OA-specific CS epitopes may be generated by this enzyme.
However, more experimental evidence would be necessary to support such a notion. It was shown that GalNAc4S-6ST sulphates internal as well as terminal disaccharides on the non-reducing end (Mikami and Kitagawa 2013; Midura et al. 1995). Plaas and coworkers identified changes in the GAG chain fine structure in OA cartilage (Anna H Plaas et al. 2001). They observed a reduction in the amount of CS-4,6diS as the terminal disaccharide on the non-reducing end of CS chains from 60% in normal cartilage to 30% in OA cartilage. This is not consistent with the observed up-regulation of GalNAc4S-6ST in our model system. A possible explanation for this observation could be that because the cartilage used by Plaas et al was obtained from knee arthroplasties, gene expression could have changed during the course of disease, with joint replacement reflecting end-stage disease. In this study, the early changes in gene expression directly after inflammatory stimulation was analyzed. Furthermore, it is possible that GalNAc4S-6ST showed a preference for internal rather than terminal chain disaccharides. Interestingly, previous studies also found that during mast cell maturation, GalNAc4S-6ST, C4ST-1 (CHST11) and serglycin expression was increased at the same time (Duelli et al. 2009; Ohtake et al. 2008). A high degree of sulphation of GAG chains attached to serglycin, i.e. its strong negative charge, is necessary for maintaining the storage capacity for basic granule compounds (Pejler et al. 2009). Furthermore, mast cells from GalNAc4S-6ST knockout mice showed reduced granule-associated protease activity (Ohtake-Niimi et al. 2010), possibly because protease storage in the granules was impaired. Similarly, mast cells from serglycin knockout mice also displayed defects in storage of various mast cell proteases (Abrink et al. 2004). Based on these observations, we hypothesize that serglycin CS/DS chains are the main target of GalNAc4S-6ST in chondrocytes. Therefore, further studies are aimed at investigating changes in serglycin GAG chain structure associated with inflammation and OA.

In addition to the possible connection between serglycin and GalNAc4S-6ST expression, a different plausible scenario for GalNAc4S-6ST up-regulation could be a role related to the ability of CS-4,6diS to bind growth factors. In a recent study by Kawamura and colleagues (2014), the effect of different CS
types (CS-4S, CS-6S, CS-4,6diS) on the chondrogenic differentiation of ATDC5 cells was investigated. They found that the addition of CS-4,6diS to the culture media increased the expression of aggrecan, collagen type II, Sox9 during chondrogenic differentiation of the cells induced by insulin (Kawamura et al. 2014). For the other CS types, the expression levels remained unchanged compared to control. The authors suggested that the effect could be a result of enhanced retention of endogenously expressed BMP-4 which is an important growth factor involved in chondrogenic differentiation. The increased expression of GalNAc4S-6ST during cartilage inflammatory stimulation discovered here could possibly be involved in a similar mechanism. An increased secretion of CS-4,6diS attached to proteins could also lead to the retention of growth factors which have anabolic activity and this could therefore be an attempt of cartilage at repairing itself.

The inflammatory stimulation of dermal constructs had the opposite effect on the expression of genes involved in CS/DS synthesis and sulphation. Several glycosyltransferases responsible for GAG chain elongation (CHPF2, CHGN) as well as XYLT1 which initiates GAG chain synthesis were up-regulated. Moreover, CHST11 expression was substantially increased which indicates that CS/DS chains are likely to contain a higher amount of GalNAc-4S. The altered CS/DS chain synthesis during inflammation could be associated with the wound healing response of the fibroblasts. Kuwaba and colleagues found that the CS/DS chain of decorin in fibroblasts was elongated in healing skin, allowing enlarged interfibrillar gaps between collagen fibrils to form (Kuwaba et al. 2001).

Besides CS/DS, also the expression of enzymes involved in heparin/HS synthesis or modification was changed by inflammatory stimulation of cartilage constructs. Two glycosyltransferases involved in heparin/HS synthesis were down-regulated. This may not imply necessarily that heparin/HS synthesis was down-regulated as the expression of major glycosyltransferases involved in chain elongation, EXT1 and EXT2 (Esko et al. 2009), was apparently unchanged (not shown). HS2ST-1, a sulphotransferase responsible for the
sulphation on the C-2 atom of iduronic/glucuronic acid, was modestly up-regulated during inflammatory stimulation. This could potentially result in an increased 2-O sulphation in heparin/HS.

KS synthesis was not apparently changed by IL-1β or IL-1β/OSM, but expression of enzymes known to modify KS were affected. CHST6 expression was more than 4-fold decreased which could have an effect on the degree of KS sulphation and therefore change the properties of proteoglycans containing KS, e.g. lumican. The increase in ST3GAL1 expression could affect the degree of sialic acid content of KS. However, KS is not the only substrate for this enzyme (Rao et al. 2009).

In terms of N-glycosylation, mostly enzymes responsible for the build-up of the glycan precursor were affected, namely ALG9, 10B, 14 and DDOST by inflammatory stimulation of the cartilage model. However, there was no uniform up- or down-regulation of gene expression that would clearly indicate a generalizable change in glycan structure. In addition, the changes were only modest, leading to the assumption that N-linked glycosylation might not be overtly altered by inflammatory stimulation. The only gene which was increased by both treatments more than 2-fold was GFPT2. This protein is known to protect cells against reactive oxygen species (ROS) induced cytotoxicity (Zitzler et al. 2004) which could occur as a consequence of inflammatory stimulation.

Otherwise, no general tendency for a reduction or increase in the amount of O-linked glycans attached to proteins in response to inflammatory stimulation of cartilage was observed. Interestingly, an increase in the expression of the fucosyltransferase FUT4 was observed for both treatments which could indicate that O-linked glycans contain more fucose residues than in normal cartilage. Increased fucosylation was found in synovial proteins from patients with arthritis (Ferens-Sieczkowska et al. 2007) which supports this finding.
In summary, analysis of gene expression of cartilage and dermal constructs stimulated with IL-1β alone or IL-1β together with OSM showed that genes involved in the glycosylation of proteins are effected in different ways. Importantly for our overall aims, some of these alterations appear to be tissue specific. Our data show that many different types and aspects of glycosylation are influenced, from synthesis of basic sugar units, synthesis of precursors, build-up of glycan structures to post-synthesis modifications of glycans by various enzymes. Such changes can dramatically affect the physiological properties of glycosylated proteins. Most importantly, several interesting candidate genes were found, including serglycin, lumican, and GalNAc4S-6ST. Further analysis will need to confirm that the observed gene expression changes translate to the protein level. This could then lead the way for potential experiments using the identified proteins or specific epitopes thereof, or products of respective enzyme activity as markers in patient samples.
Chapter 6:

Characterization of changes induced in tissue models by inflammatory mediators
6 Characterization of changes induced in tissue models by inflammatory mediators

6.1 Introduction

In the previous chapter, cartilage constructs generated from human chondrocyte progenitor cells were stimulated with pro-inflammatory cytokines and expression of genes involved in glycan synthesis and modification were analyzed using microarray and Q-PCR. In addition, dermal constructs based on human skin fibroblasts were stimulated with the same treatment. This identified changes in the cytokine response that are solely found in chondrocytes. Many different genes were differentially regulated during inflammatory stimulation. Although not all of them were highly up- or down-regulated, one has to keep in mind that also small changes in expression can make a huge difference.

In this chapter, it should be analyzed whether selected changes in gene expression observed in the microarray experiments translated to changes in the ECM of stimulated cartilage constructs on a protein level. This was very important with respect to a potential use of these markers for the diagnosis of early OA in the future. It would strengthen the scientific case to justify further development, and ultimately it would enable analysis of changes in cartilage homeostasis resulting from inflammation in chondrocytes using common clinical tools like ELISAs.

Two proteoglycans, serglycin and lumican, were identified to have an increased expression as a result of inflammatory stimulation. These two proteins were chosen for further analysis in the following experiments. While lumican expression and function in cartilage is well characterized (Grover et al. 1995; Roughley 2006), little is known about the role of serglycin in cartilage. It is predominantly found in cells derived from the hematopoietic lineage, e.g. monocytes, macrophages, megakaryocytes, platelets, lymphocytes, mast cells, and neutrophils (Scully et al. 2012). It plays a major role in the genesis
and homeostasis of intracellular granules and secretory vesicles in these cells. More recently, serglycin was also found in chondrocytes (Zhang et al. 2010), endothelial cells (Meen et al. 2011), and smooth muscle cells (Lemire et al. 2007).

The core protein of serglycin contains a characteristic region rich in Ser-Gly repeats, which gave rise to the name “serglycin” (Bourdon et al. 1985). The GAG chains attached to the serine residues can be either of heparin, HS or CS type. These are so densely packed, that the whole molecule is resistant to proteolytic degradation (Kolset and Pejler 2011). Interestingly, inflammatory mediators have been shown to substantially alter serglycin modification with glycans (Chang et al. 2012).

6.1.1 **Aims for this chapter:**

1. Investigate changes in expression and composition of serglycin and lumican in cartilage constructs stimulated with inflammatory factors at the protein level to substantiate results from gene expression analysis.

2. Investigate whether inflammatory stimulation impacts on posttranslational modification of decorin in line with expected changes in carbohydrate biosynthesis from gene expression screening in the absence of changes in expression of decorin itself.
6.2 Materials and methods

6.2.1 Inflammatory stimulation of cartilage constructs
Cartilage constructs were treated with 1 ng/ml IL-1β alone or in combination with 10 ng/ml OSM as described in chapter 5 (section 5.2.1). Constructs were cultured for a total of 6 days in medium containing cytokines, with replacing the medium every day. The conditioned medium was collected from the inside of the transwell and from the bottom well during media change, pooled for each construct and stored at -20°C. GAG content of the media was determined using the DMMB assay described in chapter 3 (section 3.2.8). For histological analysis, constructs were collected at day 2, 4 and 6. At the time of harvest, the constructs were washed in PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 24 h at 4°C. Prior to that, the wet weight was determined. Processing of the sample for histology was performed as described in chapter 3 (section 3.2.5). For protein extraction, constructs were harvested at day 2, 4 and 6. At the end of the experiment, the cartilage constructs were removed from the transwell culture inserts, washed with PBS and subsequently snap frozen in liquid N₂. The constructs were stored at -80°C until further processing. Each condition was analyzed in triplicate with one construct used per experiment.

6.2.2 OA cartilage tissue samples
Osteochondral knee joint specimens were obtained from patients undergoing total knee arthroplasty. Specimens were dissected from macroscopically undamaged areas of cartilage using an 8 mm disposable biopsy punch and subsequently fixed in 4% PFA in PBS for 24 h, followed by embedding in paraffin. 5 µm sections were cut from the specimens and used for histological staining and immunohistochemistry as described in chapter 3 (section 3.2.5) and chapter 4 (section 4.3.3).
6.2.3 Extraction of ECM proteins from cartilage constructs
For the extraction of ECM proteins, the constructs were placed into a 1.5 ml tube and immediately mixed with 200 µl extraction buffer (6 M guanidine HCl, 50 mM sodium acetate, 100 mM EDTA, pH 5.8) containing protease inhibitors (100 mM aminohexanoic acid, 10 mM benzamidine, 1 mM PMSF, 1 mM NEM). The constructs were crushed with a disposable plastic pestle on ice into a homogenous solution. The tubes were sealed and incubated at 4°C for 16 h on a rotary shaker to maximize efficiency of extraction. Afterwards, the samples were centrifuged at 12,000 x g for 5 min to remove residual insoluble material. The supernatant was collected. The protein concentration was determined using the Pierce™ BCA assay kit (Thermo Scientific) as described (chapter 2, section 2.2.11).

6.2.4 Analysis of cartilage extracts and conditioned media using Western blotting
Proteins extracted from the cartilage constructs were precipitated by mixing one volume of sample with 9 volumes of ice-cold ethanol, followed by incubation for 16 h at 4°C. Afterwards, the precipitated protein was recovered by centrifugation at 14,000 x g for 30 min at 4°C. The resulting pellet was dissolved in 7 µl 8 M Urea and 7 µl SDS sample buffer (2x stock, containing β-mercaptoethanol) and boiled for 5 min. 20 µg protein from each sample were separated on a 4-20% Tris-glycine gel (Life Technologies). For the analysis of proteins released into the conditioned medium during inflammatory stimulation, 100 µl from each time point were used. The samples were dried using a vacuum concentrator (RVC 2-25 CDplus), and the resulting pellet was resuspended in urea and sample buffer as above, and boiled for 5 min. After electrophoresis and transfer to nitrocellulose/PVDF membranes, the membrane was blocked with 5% skimmed milk in PBS and probed with antibodies (Table 6.1) as previously described (chapter 2, section 2.2.15). For detection of lumican, a nitrocellulose membrane was used for Western blotting as normal, whereas for detection of serglycin, a PVDF membrane was used as this protein was reported to bind poorly to nitrocellulose. For digestion of
the samples with chABC or heparinase 2 prior to electrophoresis, 20 µg protein from each sample were dried using a vacuum concentrator. The resulting pellet was either resuspended in 100 µl of 50 mM Tris-HCl, pH 8.0, 60 mM sodium acetate containing 10 mU chABC (Seikagaku, Associates of Cape Cod, East Falmouth, MA, USA) or resuspended in 100 µl of 20 mM sodium acetate, pH 7.0, 2 mM calcium acetate containing 10 mU heparinase 2 (Sigma Aldrich) and incubated for 1 h at 37°C. Note, heparinase 2 can cleave both heparin and HS. Afterwards, the reaction mixture was heated to 100°C for 5 min to inactivate the enzyme. The samples were dried using a vacuum concentrator and mixed with Urea and SDS sample buffer for SDS-PAGE and Western blotting as before.

6.2.5 Immunohistochemical analysis of cartilage constructs after inflammatory stimulation

The tissue was embedded in paraffin and 5 µm sections were prepared. Deposition of lumican and serglycin within the extracellular matrix was visualized as described (chapter 4, section 4.3.3). The antibodies used are described in Table 6.1.

Table 6.1 Antibodies used for Western blotting (WB) and immunohistochemical staining of cartilage sections (IHC)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody name, type and dilution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumican</td>
<td>LUM-1, mouse monoclonal, 1:500 in 5% skimmed milk/TBS (WB), 1:20 in 1% BSA/TBS (IHC)</td>
<td>Bruce Caterson, Cardiff University</td>
</tr>
<tr>
<td>Serglycin</td>
<td>Ab76512, mouse monoclonal, 1:500 in 5% skimmed milk/TBS (WB), 1:100 in 1% BSA/TBS (IHC)</td>
<td>Abcam</td>
</tr>
</tbody>
</table>
6.2.6 Inflammatory stimulation of human skin fibroblasts and analysis of changes in decorin GAG chain disaccharide composition

Bonacker cells were cultured in monolayer culture as described (chapter 2, section 2.2.8). When approx. 70% confluency was reached, the serum concentration was reduced to 4% and cytokine treatment was started. Cells were cultured with medium containing either 1 ng/ml IL-1β alone or in combination with 10 ng/ml OSM. The cells were cultured for 72 h without medium change. Afterwards, the medium was collected and centrifuged at 500 x g for 5 min to remove any cells or cell debris. The medium was supplemented with Tris-HCl pH 7.5 to a final concentration of 5 mM and stored at -20°C until further use. Decorin was purified from the conditioned medium as described (chapter 2, section 2.2.9). Afterwards, the disaccharide composition of CS/DS chains of decorin was analyzed using AMAC-labelling and reversed phase HPLC as described (chapter 2, section 2.2.5, 2.2.7).

6.2.7 Deglycosylation of decorin samples using PNGase F

Peptide-N-glycosidase F (PNGase F) is a protein isolated from Flavobacterium meningosepticum which allows for specific removal of N-linked glycans from proteins. 2 µl PNGase F (corresponding to 1000 U, New England Biolabs, Ipswich, MA, USA), 2 µl NP40 (10% v/v) and 2 µl G7 buffer (10X stock) were added to 14 µl sample and incubated for 2 h at 37°C. For Western blotting, the reaction mixture was afterwards dried using a vacuum concentrator and reconstituted in 7 µl 8 M urea and 7 µl SDS sample buffer (from 2X stock, containing β-mercaptoethanol).
6.3 Results

6.3.1 Changes associated with inflammatory stimulation of cartilage constructs: Wet weight and GAG release

In the previous chapter, gene expression changes of cartilage constructs upon stimulation with IL-1β alone or in combination with OSM was analyzed. To follow up on these experiments, similar fully differentiated constructs were here stimulated with both treatments, the difference to the previous experiments being that the inflammatory stimulation was carried out for 6 days. The reason for this was to get a better picture of the more long-term effects of the inflammatory stimulation on cartilage constructs and to allow for the changes in gene expression to have enough time to become detectable on a protein level. A set of constructs was collected every 2 days to monitor the progression of the catabolic events and conditioned medium collected every day for 6 days (Fig. 6.1 A). Analysis of the wet weight showed that no obvious changes were present after 2 or 4 days of stimulation. After 6 days, tissue loss was evident for constructs treated with IL-1β/OSM in that they were lighter compared to untreated and IL-1β only treated constructs (Fig. 6.1 B). Analysis of the amount of GAG chains released into the conditioned media showed that for both treatment groups, release was increased already within the first day of stimulation (Fig. 6.1 C). In line with previous observations (Fig. 5.4), constructs treated with IL-1β/OSM substantially showed enhanced GAG chain release compared to IL-1β alone. GAG chain release peaked during the first 2 days. Thereafter, the GAG release by the constructs treated with IL-1β/OSM or IL-1β gradually returned to a similar level as observed for control. As GAG release differed substantially between the IL-1β/OSM and IL-1β only groups, GAG depletion from the tissue was not the main reason for the reduced GAG release seen in the later phases of the experiment, at least for IL-1β only treated constructs.
Figure 6.1 Inflammatory stimulation of cartilage constructs

Fully differentiated cartilage constructs (35 days) were cultured for 3 days in medium without differentiation factors prior to stimulation with IL-1β (1 ng/ml) alone or in combination with OSM (10 ng/ml) for a total of 6 days. Medium was replaced every day and fresh cytokines were added. Cartilage constructs were collected for analysis after 2, 4 and 6 days in culture (A). The wet weight was determined immediately after removing the constructs from the transwell inserts (B). Released total GAG from the conditioned media was measured using the DMMB assay (C). Data is presented as mean ± SEM of two independently grown constructs. Statistically significant changes compared to control samples were determined with one-way ANOVA with post hoc analysis using Tukey's multiple comparison test. **P<0.01, ***P<0.001.
6.3.2 **Histological analysis of cartilage constructs stimulated with inflammatory cytokines**

To analyze the effects of inflammatory stimulation of cartilage constructs on a histological level, constructs collected after 2, 4 and 6 days of treatment were stained with H&E and toluidine blue and also used for immunohistochemical staining for serglycin and lumican. Histological analysis of untreated control constructs showed a cartilaginous tissue morphology and high amount of GAG chains as indicated by toluidine blue staining (Fig. 6.2 B). Using a monoclonal antibody raised against serglycin and lumican showed that both proteins were present. Serglycin staining was strong directly around or associated with chondrocytes throughout all layers of the construct but not in the ECM. Staining for lumican was faint and was found pericellularly around chondrocytes as well as in the matrix proper in the upper half of the construct.

![Histological analysis of an untreated control cartilage construct at day 6](image)

Figure 6.2 Histological analysis of an untreated control cartilage construct at day 6
5 µm paraffin sections of a cartilage construct cultured without inflammatory cytokines were stained with H&E (A) and toluidine blue (B) to visualize tissue morphology and matrix content. Sections were also stained with antibodies raised against serglycin (C) or lumican (D). Non-specific mouse IgG was used as a control (E). Bar = 100 µm.
The detrimental effect of adding IL-1β to the culture medium of cartilage constructs was visible after staining of the constructs with H&E and toluidine blue (Fig. 6.3). Toluidine blue staining of cartilage constructs stimulated with IL-1β alone showed that the GAG chains within the ECM were gradually lost over time. At day 2, GAG depletion was restricted to the superficial layer (Fig. 6.3). After 4 days, bands of weak (top) and strong (bottom) toluidine blue staining ECM were observed, which showed that the release of GAG chains proceeded slowly from the surface layer to the inside of the construct. After 6 days, the ECM was substantially depleted of GAG chains throughout all layers of the construct. Also, the resilience to mechanical challenge of the construct was much reduced at this stage (not shown). Immunostaining for serglycin showed that there was an increase in staining over the treatment period. While at day 2, the staining was relatively evenly distributed across the construct, staining in the GAG depleted top area was increased at day 4 and further enhanced after 6 days. At day 6 and to a lesser extent at day 4, the very superficial layer of the construct was staining particularly strongly for serglycin, which indicated that serglycin may have a unique role in superficial zone cells. Immunostaining for lumican did not show similar changes. On day 2, staining was comparable to control. Staining of the superficial layer ECM was increased marginally on day 4, but did not further increase by day 6.

It would have been expected from the measurement of the released GAG chains into the conditioned medium that the ECM lost the bulk of its GAG chains on day 1 and 2. Strangely, the cartilage ECM after 2 days was still rich in GAG chains. This may indicate that intact GAG chains are released initially but at later stages broken down into smaller components that are not detected in the DMMB assay.
5 µm paraffin sections of a cartilage construct cultured with 1 ng/ml IL-1β alone for 2, 4 and 6 days were stained with H&E, toluidine blue, and antibodies raised against serglycin and lumican. Non-specific mouse IgG was used as a control. Bar = 100 µm.
Stimulation of cartilage constructs with IL-1β in combination with OSM showed an even more pronounced catabolic effect on tissue architecture (Fig. 6.4). Very weak toluidine blue staining was observed already after 2 days of treatment indicating almost complete GAG depletion. Similarly, constructs cultured for 4 days in IL-1β/OSM had the same weak staining. Interestingly, there was a slight increase in toluidine blue staining around cells in the upper part of the construct after 6 days. A reason for this could potentially be an increased GAG chain synthesis of the chondrocytes as an attempt to repair the ECM. Similar to the construct stimulated with IL-1β alone, serglycin staining was substantially increasing during the inflammatory treatment. However, by day 6 a general increase throughout the construct was observed in addition to very strong staining in the superficial zone (Fig. 6.4). Lumican staining was observed mainly in the superficial layer on day 2 and 4 (Fig. 6.4). On day 6, this staining was marginally enhanced. A notable observation was that the constructs collected after 6 days of IL-1β/OSM treatment were very fragile. This showed that the ECM integrity was completely lost as a result of the catabolic events triggered by the cytokine stimulation.
Figure 6.4 Histological analysis of cartilage constructs stimulated with IL-1β and OSM
5 µm paraffin sections of a cartilage construct cultured with 1 ng/ml IL-1β and 10 ng/ml OSM
for 2, 4 and 6 days were stained with H&E, toluidine blue, and antibodies raised against
serglycin and lumican. Non-specific mouse IgG was used as a control. Bar = 100 µm.
Human cartilage isolated from waste tissue of total knee arthroplasty was used to compare the expression of serglycin and lumican in patient OA cartilage with that observed in the \textit{in vitro} model. Samples were collected from macroscopically intact areas of joint cartilage plate and were left attached to the subchondral bone plate for processing.

Patient #1 was an 88 year old male, and Patient #2 was a 76 year old female. Both samples showed some degree of tissue degradation in the superficial layers (Fig. 6.5). These regions showed reduced toluidine blue staining, indicating GAG chain loss likely as a result of progression of OA. Immunostaining for serglycin showed a similar pattern as observed in the \textit{in vitro} model with increased staining of the superficial zone as well as staining of chondrocytes in deeper layers (Fig. 6.5). Lumican staining was faint but nevertheless present in the same region, although only few chondrocytes were positive (Fig. 6.5). These distributions are remarkably similar to the ones observed for cartilage constructs stimulated with IL-1β alone (Fig. 6.3).

Unfortunately, no healthy control cartilage was available which would have been useful for comparison with the OA cartilage samples. Therefore, it remains unclear whether these proteins are increased in OA knee cartilage or not.
Figure 6.5 Analysis of human OA-cartilage specimens
5 µm paraffin sections from knee cartilage of two patients with OA were stained with H&E, toluidine blue and antibodies raised against serglycin and lumican. Mouse IgG was used as a control. Bar = 100 µm.
6.3.3 Analysis of changes in serglycin and lumican caused by inflammatory stimulation of cartilage constructs

In addition to immunostaining of cartilage sections, matrix proteins were extracted from cartilage constructs treated with pro-inflammatory cytokines for 2, 4, and 6 days. The samples were used for Western blotting followed by staining with an antibody raised against serglycin (Fig. 6.6) and lumican (Fig. 6.7). For the analysis of serglycin, samples were digested with chABC (Fig. 6.6 A), heparinase 2 (Fig. 6.6 B) or with both enzymes at the same time (Fig. 6.6 C, D, E) prior to SDS-PAGE. This was done because serglycin can carry CS and HS chains. Probing of the Western blots with an anti-serglycin monoclonal antibody resulted in multiple bands for all conditions (Fig. 6.6 A, B, C). After chABC digestion, a high molecular weight band larger than 100 kDa showed the strongest signal (Fig. 6.6 A [1]). After digestion of the samples with heparinase 2, a predominant band with a molecular weight of around 80 kDa appeared (Fig. 6.6 B [2]). Again, multiple smaller bands were observed, whereby the lowest band had a molecular weight of ~30 kDa. However, this was not consistent with the calculated molecular mass of the serglycin core protein which is approx. 17 kDa. Interestingly, after digesting the samples with both chABC and heparinase 2, both bands seen before appeared at the same time (Fig. 6 C [1] and [2]). Assuming that the monoclonal antibody was specific for serglycin as stated by the manufacturer, two different forms of serglycin with either CS or HS were present in cartilage. On the other hand, given that the molecular mass is not consistent with serglycin, it is also possible that the antibody cross-reacts with another CS/HS proteoglycan and this requires further investigation.

Western blot analysis of the conditioned media collected during inflammatory stimulation of the constructs over 6 days showed that a ~25 kDa protein detected by the anti-serglycin antibody was highly up-regulated after stimulation with IL-1β alone from day 1 to 4 (Fig. 6.6 D). The migration position of this band is consistent with that reported for de-glycanated serglycin in the literature (Chang et al. 2012). This band was not observed in the control samples. Surprisingly, Western blot analysis of the conditioned media from constructs stimulated with IL-1β/OSM did not reveal a similar up-regulation of
the respective protein. Only weak bands that corresponded in size to the intense bands from Fig. 6.6 D were observed after 1 and 2 days of treatment. This could indicate differential regulation of serglycin by the different inflammatory regimens, or more likely, indicate that secreted serglycin is rapidly undergoing proteolytic degradation (personal communication, Prof. Niels Borregaard, University of Copenhagen, Denmark) given that it is up-regulated by both treatments as determined by Q-PCR (Table 5.9) and by immunohistochemistry (Fig. 6.3, 6.4). Enhanced proteolysis is also consistent with the more rapid breakdown of cartilage after IL-1β/OSM treatment compared to IL-1β alone (Fig. 6.3, 6.4).

In the control samples, a band >100 kDa was reproducibly observed at day 0 (before starting the treatment). The nature of this band is unclear but it may be related to band 1 previously discussed in the cartilage extracts.
Figure 6.6 Western blot analysis of cartilage matrix and conditioned media for serglycin after inflammatory stimulation

Guanidine HCl-extracts of cartilage constructs (A – C) cultured for up to 6 days under inflammatory stimulation (20 µg per lane) or respective conditioned media (D – E) were probed with a monoclonal antibody raised against serglycin. Proteins were digested with chABC (A), heparinase 2 (B) or chABC and heparinase 2 (C, D, E) prior to SDS-PAGE separation and Western blotting. Migration of molecular weight markers is indicated on the left.
Western blot analysis of lumican extracted from cartilage constructs showed that there was a small increase in band intensity (around 66 kDa) observed between control samples and samples from cartilage stimulated with cytokines. Lumican was detected as a broad band of ~50 - 75 kDa as it is substituted with KS. By day 4, the bands of samples from both inflammatory treatments had a similar intensity but were more intense than the one from control (Fig. 6.7 A). By day 6, no signal was present in the IL-1β/OSM sample, whereas the band detected in control and IL-1β samples had a similar intensity. As samples prepared from the cartilage construct ECM contained also newly synthesized matrix proteins, this could suggest that lumican expression was increased as a result of inflammatory stimulation in line with gene expression data (Table 5.9).

Analysis of the conditioned media showed that lumican release was increased in cartilage constructs stimulated with IL-1β only on day 1, 2 and 3 (Fig. 6.7 B). The release decreased on day 4 and no lumican could be detected on day 5 and 6. Similarly, conditioned media from constructs stimulated with both IL-1β/OSM also showed increased release on day 2 and 3, which was reduced on day 4 and 5 and was absent on day 6 (Fig. 6.7 C). Unfortunately, the intensity of the signal was suboptimal in this experiment and therefore requires repetition.

HPLC-based disaccharide analysis of cartilage extracts prepared from papain-digested samples was unfortunately not successful. The reason for this was that the background of the samples was unexpectedly high and no conclusive data could be obtained. This was unfortunate as it would have been interesting to see whether there would be a difference in the disaccharide compositions of GAG chains after inflammatory stimulation, especially as it was known from the microarray and Q-PCR analysis that CHST15 (GALNAC4S-6ST) was up-regulated by cytokine treatment. It would have been important to confirm differences in the relative amounts of its reaction product (GlcA-GalNAc4,6diS). However, it is also possible that such changes would have been masked due to the massive release of GAG chains immediately after addition of cytokines.
Figure 6.7 Western blot analysis of cartilage matrix and conditioned media for lumican after inflammatory stimulation

Guanidine HCl-extracts of cartilage constructs (A) cultured for up to 6 days under inflammatory stimulation (20 µg per lane) or respective conditioned media (B, C) were probed with a monoclonal antibody raised against lumican. Proteins were digested with chABC prior to SDS-PAGE separation and Western blotting. Migration of molecular weight markers is indicated on the left.
6.3.4 Effects of inflammatory stimulation on decorin secreted by skin fibroblasts

Decorin purified from the conditioned media of human skin fibroblasts stimulated with IL-1β alone or in combination with OSM was analyzed to investigate changes that may have occurred in its CS/DS chain. This should be considered separately from the cartilage inflammatory stimulation and the search for biomarkers and be seen more as a general investigation of the hypothesis that GAG chain synthesis and modification can be affected by inflammatory factors. After SDS-PAGE of the samples followed by silver staining, a difference in the molecular weight between decorin purified from untreated and treated cells was observed (Fig. 6.8 A). Decorin purified from untreated cells showed a smear with a molecular weight around 97 kDa (see also Fig. 2.6 A). Addition of IL-1β resulted in a higher molecular weight of decorin compared to untreated control and addition of IL-1β/OSM resulted in an even larger decorin molecule than when IL-1β was added alone (Fig. 6.8 A). Furthermore, each sample contained a high molecular weight band, the size of which was increasing correspondingly. Western blotting using a polyclonal antibody raised against decorin (described in Table 2.3) showed that both bands were decorin. The higher molecular weight band are likely to be a decorin dimer as discussed previously (Fig. 2.6 B).

To investigate whether the increase in molecular weight was resulting from alterations in the GAG chain attached to the decorin core protein, the N-linked glycans attached to decorin or the core protein itself, each sample was digested with chABC alone (as described in chapter 2, section 2.2.10) or in combination with PNGase F. Depolymerization of the GAG chain by chABC showed that all decorin core proteins had a similar molecular weight of ~44 kDa independent of the treatment (Fig. 6.8 B). Further digestion with PNGase F showed that the size of decorin core protein was unchanged across the conditions (Fig. 6.8 C). Taken together, this indicates that there is no difference in the amount of N-linked glycans. From this experiment it was concluded that cytokine stimulation only affected the length of the CS/DS chain of decorin.
Figure 6.8 Inflammatory stimulation of skin fibroblasts leads to increased GAG chain size on secreted decorin

Human skin fibroblasts (Bonacker cells) were cultured in monolayer with medium supplemented with IL-1β alone or in combination with OSM for 72h. Decorin was purified from the conditioned medium and analyzed using SDS-PAGE and silver staining (A, left) or Western blotting (A, right) using the polyclonal antibody raised against decorin. The asterisk marks an immunoreactive band that likely corresponds to a decorin dimer. Digestion of the samples with chABC alone and in combination with PNGase F (B) showed that there was no difference in the amount of N-linked glycans attached to the core protein between the different culture conditions.
The previous experiment showed that the GAG chain length was increased after inflammatory stimulation of human skin fibroblasts. To further investigate changes in the GAG chain composition, CS/DS chains derived from decorin were analyzed using AMAC-labelling followed by reversed phase HPLC (Fig. 6.9). This showed that there was a difference in the amount of $\Delta 4S$, $\Delta 6S$ and $\Delta 0S$ disaccharides between the different samples after complete GAG chain depolymerization with chABC (Fig. 6.9 A). While the relative amount of $\Delta 4S$ increased from 74.6% in control samples to 81.8% in IL-1$\beta$ only and 85.7% in IL-1$\beta$/OSM stimulated samples, the amount of $\Delta 2S$, $\Delta 6S$ and $\Delta 0S$ decreased from 2.8%/12.8%/5.7% in control samples to 1.3%/9.2%/4.1% in IL-1$\beta$ only and 0.7%/7.6%/3.6% in IL-1$\beta$/OSM stimulated samples, respectively. Differences in the relative amount of di- and tri-sulphated disaccharides between the different treatments were much smaller. Digestion of the CS/DS chains using chAC-I showed only little difference in the relative amounts of $\Delta 4S$, $\Delta 6S$ and $\Delta 0S$ (Fig. 6.9 B). In contrast, chB-digestion showed that decorin from cells stimulated with IL-1$\beta$/OSM had a 4% higher content of $\Delta 4S$, which was made up of DS (Fig. 6.9 C). Differences were also observed for the disulphated $\Delta 2,4\text{diS}$, which was increased for IL-1$\beta$ only and decreased in IL-1$\beta$/OSM co-stimulation compared to control.

Taken together, these results suggest that stimulation of skin fibroblasts with IL-1$\beta$ alone or in combination with OSM affected the length of GAG chains and also influenced the sulphation of disaccharide subunits. In both, chABC or chB digestion of GAG chains, an increase in $\Delta 4S$ disaccharides was detected. Furthermore, these disaccharides appear to be largely made of DS.
Figure 6.9 Quantitative analysis of the disaccharide composition of decorin isolated from human skin fibroblasts stimulated with 1 ng/ml IL1-β alone or in combination with 10 ng/ml OSM.

From each sample, 4 µg protein was digested using chondroitinases ABC (A), AC-I (B) or B (C), followed by labelling with AMAC and reversed phase HPLC analysis. Data is shown as mean ± SEM from three independent experiments. Statistical significance of changes compared to control samples were determined with one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test. None of the changes was large enough to reach significance with the confidence interval set to 95%.
6.4 Discussion

In this chapter, the effect of inflammatory stimulation of cartilage constructs using 1 ng/ml IL-1\(\beta\) alone or in combination with 10 ng/ml OSM was analyzed using histological methods and Western blotting as well as HPLC-based disaccharide analysis. The results from the experiments showed that serglycin was up-regulated in cartilage constructs upon stimulation with IL-1\(\beta\) alone or together with OSM. Additionally, lumican protein expression was also increased by both treatments, although to a lesser extent. Analysis of decorin CS/DS chains purified from human skin fibroblasts cultured in media containing inflammatory factors showed that GAG chain length was increased as well as disaccharide sulphation changed as a result of the cytokine addition. Most of the changes observed in this chapter can be directly linked to the changes in gene expression identified using the microarray and Q-PCR analysis in the previous chapter 5.

This chapter shows in some instances preliminary data, which may not fully support a final conclusion and some aspects clearly need to be further investigated. This work was still in progress at the end of this PhD project, but should not be omitted from the thesis in order to provide a comprehensive overview on the work that has been carried out and the direction of future work that was enabled by it.

It was shown previously (chapter 5, Fig. 5.4) that the combination of 1 ng/ml IL-1\(\beta\) and 10 ng/ml OSM had an even more dramatic effect on cartilage ECM integrity than addition of IL-1\(\beta\) alone as determined from GAG release. This was confirmed here by toluidine blue staining of cartilage constructs (Fig. 6.4), showing that the ECM was almost completely depleted of GAG chains already after 2 days. This was not surprising as it is known that the combination of IL-1\(\beta\) and OSM results in a much higher expression and activity of matrix degrading enzymes, for example MMP13, ADAMTS-4, ADAMTS-5 (Barksby et al. 2006; Gilbert et al. 2012), than IL-1\(\beta\) alone. Nevertheless, it confirmed that our model faithfully reproduced the expected sequence of events associated with OA.
The staining intensity of serglycin in the immunohistochemical analysis of cartilage constructs seemed to be time dependent rather than influenced by the addition of OSM, because the staining increased in both treatment groups at a similar rate over time. The strongest staining was observed in the superficial layer of the constructs, which could be explained by the fact that this layer was directly exposed to the cytokines. The discreteness of the labeling, i.e. what appears to be a single cell layer, could also indicate a specific role of serglycin in these cells that are clearly distinct and have a unique role in cell communication between cartilage proper and the fluid phase (culture medium/synovium). Lumican staining was weak in the cartilage constructs and did not give a conclusive impression whether it was increased or not. Given that lumican was abundantly expressed as determined from cartilage extracts, it is possible that a keratanase digestion would have improved immunolocalization.

Immunostaining of patient OA cartilage showed that there was an increased amount of serglycin and lumican present in the top region. This was similar to the observations made with cartilage constructs after 6 days of inflammatory stimulation. However, a proper control was not available to compare staining in OA cartilage with normal cartilage. Little is known about serglycin expression in cartilage or OA. The only publication we are aware of (Zhang et al. 2010) provides very limited information on how serglycin is involved in inflammatory processes in cartilage. From the information obtained by the staining of the patient cartilage, it can be speculated that lumican and serglycin is more abundant in areas which show reduced toluidine blue staining. It is likely that a high expression of matrix degrading enzymes can be found in these regions. If this was caused by the presence of inflammatory cytokines in the synovial fluid during development of OA, which cannot be definitely confirmed, it would be in line with the results derived from the experiments with cartilage constructs and could relate to IL-1β signaling. However, it is also possible that antigenic epitopes are more available in these regions and actual protein levels not altered.
Western blot analysis of serglycin from cartilage construct extracts was not conclusive. Firstly, there were many bands present which indicated that either the antibody showed some cross-reactivity with other proteins or that serglycin was present with various different and extensive posttranslational modifications, potentially with different amounts of GAG chains attached to it. Only after chABC or heparinase 2 digest, predominant discrete bands were observed. However, these did not apparently relate to the same protein species as determined from soluble digests. Serglycin expressed by activated macrophages and mucosal mast cells contains CS but not heparin/HS (Kolset and Tveit 2008). However, heparinase 2 and chABC digestion here indicated that HS (and possibly heparin) and CS are present in cartilage derived serglycin. Without digestion prior to Western blotting, no band was observed at all (not shown). This indicated that the molecular mass of fully intact serglycin extracted here from cartilage was either too high to enter the separation gel in the SDS-PAGE or the epitopes were masked by the GAG chains. The two different band sizes observed after chABC and heparinase 2 digest on their own could be explained when serglycin contains both CS and HS type chains. But then, a combined digest with both enzymes should have resulted in a complete removal of GAG chains from the serglycin core protein. Neither of the bands was consistent with the molecular mass of the core protein. Further experiments should repeat the Western blot analysis using a different antibody against serglycin to substantiate the specificity of these findings in a first instance. It is possible that besides carbohydrate addition, serglycin also undergoes some form of covalent cross-linking, either through the core protein such as transglutaminase cross-linking, or involving GAGs as observed in inter-α-inhibitor (Zhuo et al. 2004; Chang et al. 2012), and this may explain the observed high molecular mass components apparently containing serglycin.

Although lumican immunostaining of cartilage sections was relatively weak, Western blotting of the cartilage extracts showed that there was an increase in extractable lumican as a result of inflammatory stimulation and incubation time. Digestion of the samples with keratanase would clarify whether lumican
protein expression is increased, as it removes the KS chains attached to the lumican core protein and therefore would separate core protein changes from differential glycosylation. However, keratanase was unavailable at the time when the experiments were performed and could not be repeated due to time restraints. The strongest band was observed at around 66 kDa, but further bands running below and above were also observed. This could be explained by lumican containing KS with different chain length, or different modifications of the KS chain, for example incorporation of sialic acid (Esko et al. 2009). Western blot analysis of lumican present in the conditioned media showed firstly, that there was increased release following inflammatory stimulation and secondly, that intact protein appeared to be released. This was most likely the result of matrix degradation triggered by the cytokine treatment.

In chapter 5, an increase in expression of the GALNAC4S-6ST after inflammatory stimulation of cartilage constructs was identified. This was interesting as it indicated that specific changes in GAG chain sulphation in an inflammatory environment were likely to occur. To confirm the increase of GALNAC4S-6ST expression on a protein level, Western blot analysis using a monoclonal antibody raised against the sulphotransferase was performed using the cartilage extract samples. However, there was no protein detectable, probably because the expression levels were too low or the antibody not suitable (not shown). Attempts to purify proteoglycans from the cartilage constructs for further analysis was not successful. One of the reasons was that the cartilage material was not available in a sufficient amount. A more sensitive method which would have been useful to analyze the composition of newly synthesized GAG chains employs $^{35}$S labeled sulphate. This was previously used by researchers to quantify newly synthesized GAG chains (Carney et al. 1985). Unfortunately, this was not possible to be performed in our laboratory because of safety concerns and the lack of an extraction hood in the radiation laboratory.

The observation that the CS/DS chain attached to decorin from skin fibroblasts was larger after addition of IL-1β and also IL-1β/OSM (Fig. 6.8 A) can be
directly related to changes in gene expression observed after microarray and Q-PCR analysis of dermal constructs stimulated with the same cytokines. The increase in GAG chain length likely be the result of the increased expression of the CS-glycosyltransferases CHGN and potentially CHPF2. HPLC analysis of the GAG chain composition showed that there was an increase in the relative proportion of Δ4S disaccharides in decorin isolated from skin fibroblasts stimulated with cytokines (Fig. 6.9). This was probably caused by the increased expression of CHST11, which was about 8-fold up regulated in cells treated with IL-1β alone and about 36-fold with IL-1β/OSM. In addition, a reduction in the amount of Δ2S, Δ6S and Δ0S was observed, which was possibly because of a reduced amount of free disaccharide subunits available for other sulphotransferases.

This result showed that the GAG chain synthesis and modification machinery is sensitive to inflammatory conditions. Furthermore, it confirmed that changes in expression affecting oligosaccharide biosynthesis as determined by gene expression analysis indeed translate into altered posttranslational modification of proteins, albeit demonstrated here for a fibroblast culture rather than cartilage constructs. The resulting changes could influence the biological activity of GAG chains, for example to bind and retain growth factors or influence cell signaling.
Chapter 7:

General discussion
7 General discussion

7.1 Summary of the results

The hypothesis to be investigated in this thesis was whether the glycosylation of secreted proteoglycans changes as a result of inflammatory processes in cartilage. The resulting alterations in glycosylation may lead to specific carbohydrate epitopes that could be utilized as novel biomarkers for degenerative joint diseases like osteoarthritis (OA).

The results presented in this thesis show that substantial changes in the expression of genes involved in glycosylation and glycan modification are observed after applying inflammatory conditions. This was demonstrated using a novel 3D human cartilage model system, which served as a model for human articular cartilage and was stimulated with inflammatory factors present in osteoarthritis. In addition, dermal constructs based on human skin fibroblasts were treated with the same inflammatory cytokines. This served as a reference model to identify tissue specific changes in cartilage. The data showed that the expression of serglycin and GalNAc4S-6ST were strongly increased in cartilage constructs and much lower or absent in dermal constructs. Immunohistochemical analysis of stimulated cartilage confirmed an increase in expression of serglycin in the tissue upon inflammatory stimulation.

As a follow-on to experiments using tissue models, GAG chain analysis was performed of decorin purified from conditioned medium of human skin fibroblast. Culturing the cells in medium containing inflammatory cytokines resulted in an increased GalNAc4S content within the GAG chain. Moreover, the chain length was increased after inflammatory stimulation.

In summary, the combined results show that glycosylation in general and especially GAG chain synthesis and modification is highly sensitive for changes in cell metabolism and that identification of novel epitopes generated during pathological conditions could be used as markers for diagnosis as well as monitoring disease progression and treatment efficiency.
7.2 Conclusions

7.2.1 Formation of cartilage constructs using human chondrocyte progenitor cells: a valuable new model for study of OA pathogenesis and pharmacological intervention

For the investigation of changes in cartilage GAG chain synthesis under OA-like conditions, a supply of normal human articular cartilage was an essential requirement to perform all necessary experiments, e.g. microarray and Q-PCR analysis, protein extraction, or GAG analysis. However, completely normal human cartilage is rarely available and the relatively limited amount of normal cartilage which would be obtainable from patients undergoing joint replacement without having degraded cartilage (and therefore OA) would make experiments difficult. Furthermore, every normal or OA cartilage sample has different properties depending on the patient’s genomic background, age, gender or secondary diseases, and tissue derived from an individual with OA is likely affected by the inflammatory environment even if macroscopically normal.

To solve the issue regarding the cartilage supply for the experiments, a model system was established which could deliver the necessary amount of normal cartilage. Human chondrocyte progenitor cells (Dowthwaite et al. 2004; Williams et al. 2010) were used to generate cartilage constructs in vitro. The results showed that it was important to grow the cells in serum-free medium with a combination of TGF-β2, BMP-2, and insulin to achieve chondrogenic differentiation. Both TGF-β2 and BMP-2 are described to induce chondrogenic differentiation on their own (Johnstone et al. 1998; Murdoch et al. 2007; Shintani et al. 2013), but cartilage formation with only one of the two growth factors was not successful. The chondrogenic effect of growth factors seems to be strongly dependent on the cell type and needs to be determined each time when a so far untested cell type is used for generating cartilaginous tissue. Culturing human chondrocyte progenitor cell strains isolated from knee articular cartilage of different patients using the same chondrogenic medium formulation resulted in the formation of cartilaginous tissue thereby confirming that the optimized medium formulation could work for any other chondrocyte
progenitor cell strain derived in the future. It is important to note, however, that no similar path of differentiation was observed when progenitor cells were derived from an OA affected joint.

The advantage of this model over other established model systems, e.g. bovine cartilage explants (Hui et al. 2003; Gilbert et al. 2012; Sauerland and Steinmeyer 2007; Sauerland et al. 2003; M Durigova et al. 2008) or cartilage constructs based on stem cells derived from other tissues (bone marrow, fat tissue) (Mendelson et al. 2011; Im et al. 2005; Johnstone et al. 1998; Murdoch et al. 2007), is first of all that the model is based on cells of human origin. This is an important prerequisite to study the potentially species specific biochemical changes that take place in human osteoarthritis or other degenerative joint diseases. Secondly, the cells are derived from the correct tissue site and therefore carry appropriate epigenetic information. The chondrocyte progenitor cells are derived from articular cartilage and therefore seem to be predetermined for the formation of cartilaginous extracellular matrix. For example, the cells have a constitutively high expression of the important chondrogenic transcription factor Sox9 (Williams et al. 2010), which regulates the expression of collagen type II, IX, XI, and aggrecan (de Crombrugghe et al. 2000).

Following development and optimization of the cartilage model system, the biochemical and histological properties of the constructs were thoroughly analyzed. This was important to determine the baseline properties of the cartilaginous tissue. Biochemical, histological and gene expression analysis revealed that after 35 days in culture, the constructs showed properties that were similar to fully differentiated articular cartilage. Q-PCR analysis showed that during chondrogenesis, expression of cartilage-specific genes (collagen type II, aggrecan, matrilin-3, GDF-5, chondromodulin-1) increased. Moreover, markers of mesenchymal condensation (N-cadherin, tenascin-C) that were increased initially, but then decreased during progression of differentiation. This suggests that the progenitor cells were undergoing an accelerated embryonic development. Furthermore, a switch to expression of collagen type II B indicated the presence of progressively increasing numbers of mature chondrocytes in the cartilage constructs (Zhu et al. 1999; Sandell et al. 1994;
Johnstone et al. 1998). Likewise, GAG amount increased to a level consistent with cartilage and after 35 days, approached a level of C-6-S typically seen in articular cartilage. Hypertrophic differentiation of tissue engineered cartilage constructs is a big issue, especially when bone marrow-derived MSCs are used (Johnstone et al. 1998). No overt sign of hypertrophic differentiation was found in the cartilage constructs after 35 days. Although gene expression of collagen type X increased during differentiation, no collagen X deposition in the ECM was observed. Conversely, terminal differentiation was observed after inducing chondrocyte hypertrophy on purpose using triiodothyronine (Mueller and Tuan 2008). Based on these results, one might speculate that cartilage constructs generated from chondrocyte progenitor cells do not undergo terminal differentiation spontaneously, although this would need to be confirmed in more long-term experiments. This is a very important feature, because it indicates that constructs generated from autologous progenitor cells from patients could be used to generate functional cartilage constructs for re-implantation using the protocol described in this thesis.

The cartilage model system developed here has a lot of potential for being used to further study the effects of different conditions on OA development and progression. For example, it could be used to analyze the effect of anti-inflammatory drugs to prevent cartilage degradation. Another aspect would be to use the model for investigating the effect of aberrant mechanical load on cartilage metabolism. Although bovine cartilage is heavily used for this purpose already, cartilage constructs based on human cells might provide additional data that could be more useful for human OA research. In addition, the influence of specific components on formation of ECM or chondrogenic differentiation could be studied using the model, for example through a knockdown approach using shRNA. As a specific example, it would be interesting to investigate the effect of various sulphotransferase knockdowns in chondrocytes. Sulphation of CS plays an important role in cartilage formation, and studies by Klüppel and colleagues found that knockdown of chondroitin-4-O-sulphotransferase 1 leads to severe chondrodysplasia (Klüppel et al. 2005).
7.2.2 Stimulation of cartilage constructs with inflammatory factors alters the expression of genes involved in GAG chain synthesis

Based on the work described above, sufficient amounts of cartilage constructs could be generated for the analysis of genes involved in protein glycosylation, with and without inflammatory stimulation. Microarray analysis was performed using a custom-designed chip (GlycoChipv4), detecting only genes relevant to glycosylation. Among the many interesting observations, the data showed that genes responsible for GAG chain synthesis were generally down-regulated in cartilage. However, it also showed that the proteoglycan serglycin was highly up-regulated after addition of inflammatory cytokines and that GalNAc4S-6ST expression was substantially increased, which is a sulphotransferase specific for CS chains. Both these findings were novel. This was a promising result because the GalNAc4,6diS modification was reported to have potent biological activity (Mizumoto et al. 2013; Ohtake-Niimi et al. 2010). Subsequent Q-PCR analysis confirmed the up-regulation of both proteins, and some data on protein expression, although preliminary, revealed interesting new observations.

Further investigation should look at the purification of serglycin from conditioned medium of cartilage constructs. This would reveal changes associated with inflammatory stimulation, and the proteins could then be used to analyze the serglycin-specific GAG chain disaccharide composition. Serglycin contains a large amount of GAG chains and is therefore an interesting target to identify changes in GAG chain modification in response to inflammation. It would be interesting to see whether there is an increase in GalNAc4,6diS in one of its CS chains as a result of the up-regulation of GalNAc4S-6ST, or whether the modification is made on a CS chain of a different proteoglycan (e.g. aggrecan, decorin, or biglycan). Clearly, serglycin itself or an associated GAG epitope could be a new lead in the search for novel biomarkers for OA. However, further analysis on the potential of serglycin as a marker for OA needs to be carried out. For this, a sample bank containing synovial fluid, urine, and plasma from patients with joint disease is currently being assembled in our laboratory and could be utilized to identify serglycin or its GAG component in biological fluids. Furthermore, the link to and biological
role of GalNAc4,6diS in OA needs to be investigated. This might serve together with serglycin as a potential biomarker. However, an antibody which is specific for this particular sulphation motif would need to be developed.

In conclusion, the results from the gene expression screening experiments provide novel insight into changes in glycosylation during inflammatory stimulation of cartilage, an area which is thus far under-investigated.

7.2.3 **Stimulation of human skin fibroblasts with inflammatory cytokines leads to up-regulation of genes involved in CS chain synthesis**

Dermal constructs generated by human skin fibroblasts were stimulated with inflammatory cytokines similar to cartilage constructs. The reason why dermal constructs were chosen as a reference model to compare changes in gene expression with human cartilage was because these cells are also producing abundant extracellular matrix, but of a different type (mainly collagen type I). Similar to chondrocytes, they are also secreting proteoglycans and have therefore a similar repertoire of glycosyl- and sulphotransferases. In addition, the fibroblast model and protocols to isolate decorin, a proteoglycan of interest here, from culture media were already established in our laboratory.

Microarray analysis showed that stimulation of dermal constructs with IL-1β alone or in combination with OSM resulted in an increase in the expression of several glycosyltransferases involved in the elongation of CS/DS chains. Moreover, chondroitin-4-O-sulphotransferase 1 was up-regulated after addition of the cytokines. Both observations translated into corresponding changes in the decorin GAG chain as discussed below, thereby validating the gene expression screening approach for identification of changes in carbohydrate biosynthesis in response to cell stimulation. This was in contrast to the data obtained after cartilage construct inflammatory stimulation, where a general down-regulation of GAG chain synthesis was observed. The up-regulation of GAG chain synthesis or increased length of GAG chains could play a role in wound healing as proposed by others (Kuwaba et al. 2001). The observation that the expression of glycosyl- and sulphotransferases in dermal
constructs was so different from the gene expression in chondrocytes points to an important and tissue-specific role of glycosylation during inflammation.

7.2.4 **Decorin GAG chain composition varies between different cell types**

For the analysis of GAG chain composition, several HPLC analysis methods were evaluated for their sensitivity. Fluorescent labeling of disaccharides with 2-aminoacridone (AMAC) (Deakin and Lyon 2008; A H Plaas et al. 2001; Jackson 1994; Kitagawa et al. 1995) followed by separation using reversed phase HPLC provided the highest sensitivity of about 10 pg per disaccharide. Decorin isolated from human skin fibroblast conditioned media was used as a model protein because it has a relatively simple structure containing a single CS/DS chain. In addition, the decorin CS/DS chain composition is sensitive to changes in cell metabolism (J Yan et al. 2011) and could therefore be a sensitive indicator for changes in GAG biosynthesis. Disaccharide analysis of decorin from different sources (skin fibroblasts, human chondrocyte progenitor cells, and recombinantly expressed by HEK293 cells) showed that the CS/DS chain composition varied between different cell types in line with previous reports (Laremore et al. 2010; Nomura 2006; Zhao et al. 2013). This highlights that the GAG chain is “tailored” within each tissue to suit its biological function, e.g. binding of growth factors (Seidler et al. 2005), cytokines (Tufvesson and Westergren-Thorsson 2002), or cell signaling (Schönherr et al. 2005; Santra et al. 2002). These results show that the composition of decorin’s CS/DS chain is highly versatile and is therefore a good model to investigate changes resulting from different stimuli, e.g. inflammatory conditions.

7.2.5 **Decorin GAG chain length and disaccharide composition are changed by inflammatory cytokines**

By comparing the different decorin preparations, an increase in GAG chain length could be observed that was occurring in response to inflammatory stimulation of fibroblasts. This was in line with an increased expression of the CS chain polymerases ChGn and ChPF2.
Disaccharide analysis showed that also the composition of decorin’s CS/DS chain was altered after culturing cells in the presence of cytokines. Compared to untreated control, cytokine addition resulted in an increased amount of GalNAc4S and reduced amount of GalNAc6S and non-sulphated GalNAc. This could be a result of the observed increased expression of chondroitin-4-O-sulphotransferase 1.

These results demonstrate a direct link between changes in gene expression in a biosynthesis pathway and the respective end product at the protein level. In addition, they show that medium supplementation with IL-1β alone or together with OSM has a direct influence on the length and sulphation pattern of GAG chains. This opens up the possibility that specific sulphation motifs in GAG chains are generated as a consequence of inflammatory processes. These novel structures could be used for the generation of specific antibodies which would improve diagnosis of pathological conditions. Furthermore, additional research is required to investigate the role of altered GAG chain composition and its influence on the biochemical properties of the proteoglycan, for example facilitating growth factor binding or regulation of cell signaling pathways. An emerging body of evidence indicates that SLRPs like decorin or biglycan play an important role as signaling molecules in innate immunity (Frey et al. 2013). This would provide a better understanding on how cells are responding to an inflammatory environment.

In conclusion, the work presented in this thesis has advanced knowledge first of all in the field of cartilage tissue engineering, demonstrating that a so far unexplored cell type known as chondrocyte progenitor cells is well suited for the formation of cartilaginous tissue when cultured in an optimized chondrogenic medium. In addition, the gene expression analysis of cartilage constructs stimulated with inflammatory cytokines showed that disease-specific alterations in protein glycosylation are likely to exist.
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Appendix I: Curriculum vitae

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Former address: Matrix Biology & Tissue Repair Research Unit, School of Dentistry, Cardiff University, Heath Park, Cardiff, CF14 4XY, United Kingdom

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Education
01/2011 – present Cardiff University, Cardiff, UK
PhD student

10/2006 – 12/2010 Technical University Darmstadt (TUD), Darmstadt, Germany
Awarded degree “Diplom-Biologe” (with honours)

Research experience
07/2014 – present Zedira GmbH, Darmstadt, Germany
• Research Scientist; Transglutaminase research

01/2011 – 03/2014 Cardiff University, Cardiff, UK
• PhD student at the Matrix Biology & Tissue Repair Unit
• Work involved research in cartilage tissue engineering and glycobiology in relation to osteoarthritis

04/2010 – 12/2010 Zedira GmbH, Darmstadt, Germany
• Diploma student in collaboration with TUD;
• Work involved purification and biochemical characterization of recombinant blood coagulation factor XIII from different species

Presentations
• “A novel 3D in vitro model of human articular cartilage” at the British Society for Matrix Biology (BSMB) Autumn Meeting, Cardiff, UK, September 2013
“Potency of different inhibitors for recombinant blood coagulation factor XIII from different species” at the International FXIII Workshop, Debrecen, Hungary, September 2012

Publications

Articles:


Abstracts:

  
  o Awarded Unilever poster prize for best poster presentation (£3000 for lab funding)

- “Novel assay to show activation of GPR56 by tissue transglutaminase” Bauer L, Heil A, Aeschlimann D, Knauper V. BSODR Annual Meeting, Bath, UK, September 2013

Method description prepared in collaboration with BMG Labtech:

Appendix II: Application to the Consortium of Functional Glycomics (microarray studies)

Understanding inflammation-mediated changes in protein glycosylation in cartilage

Context
The Arthritis Research UK Centre of Excellence in Cardiff has been established to address the ‘big’ questions in joint disease. Osteoarthritis (OA) is the most common form of joint disease. However, diagnosis of OA is still confirmed by x-ray (or MRI), yet this detects gross structural changes, which occur late in the disease process, and there are no reliable diagnostic or prognostic biochemical markers that would enable early OA diagnosis or patient stratification. An OARSI/FDA consensus document [1] laid out the need for progress in this area and pointed out that developing new prognostic indicators will not only have an impact on patient diagnosis but is crucial for development of future disease modifying therapeutic agents.

Background
OA is no longer considered as mainly a degenerative disorder as it is associated with both local (synovial) and also low-level systemic inflammation [2]. For example, elevated levels of markers for inflammation such as AGE and HMGB1 were found in the synovium of the STR/ort mouse model of spontaneous OA and positively correlated with increased serum levels of IL-1β [3]. We believe that disease-specific protein modifications are likely to provide a sensitive qualitative indicator of early OA as has been seen in various immune-mediated conditions including rheumatoid arthritis [4].

Objective
This project will analyse changes in the pattern of glycosylation of specific proteins with the aim of identifying signature patterns in the carbohydrate chains that characterize disease states and therefore can be used to develop biomarker assays.

Outline
A major problem in OA biomarker studies is patient-to-patient variability. To avoid this, we have established a human cartilage in vitro model. Chondroprogenitor cells were isolated from normal superficial zone knee cartilage [5]. These cells keep their chondrogenicity even after extensive expansion in monolayer [6]. Cells are seeded into transwells at high density in serum-free medium containing a cocktail of growth factors to induce differentiation, forming cartilage disks of 6 mm diameter and 1 mm height within 28 days. These constructs have biomechanical properties similar to articular cartilage explants. This approach allows us to produce a large number of samples in a reproducible way for experimental manipulation.
The glyco-chip provided by the CFG is optimally suited to identify changes in gene expression of enzymes that play a role in posttranslational modification during protein biosynthesis. Constructs have been treated with inflammatory cytokines to mimic OA conditions or were left untreated. Total RNA from multiple samples was pooled and the experiment repeated 3 times. Additionally, we have selected an unrelated tissue model in which proteoglycans we are particularly interested in are expressed. This model was treated with the same cytokine regimen as the cartilage model. The purpose is to differentiate the changes that are a generic response to the cytokine challenge from those which are a cartilage-specific response. In total, our experimental setup therefore requires 18 microarrays (2 tissues, 3 conditions, 3 replicates). Differential expression of selected proteins as identified in microarray analysis will subsequently be confirmed in tissue from OA patients. Based on the identified altered expression of enzymes we can subsequently analyze carbohydrates for specific structural changes in a targeted fashion using a mass-spectrometry-based micro sequencing approach [7].

References