ELR+ CXC chemokine signalling in cartilage homeostasis.

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ELR+ CXC CHEMOKINE SIGNALLING IN CARTILAGE HOMEOSTASIS

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Acknowledgements

Declaration

The work presented in this thesis is less than 100,000 words and was performed and analysed by the candidate except for the harvesting of mouse knee joints which was performed by Dr Laura Brandolini. Preliminary experiments leading to the study, including the discovery of upregulation CXC chemokine expression in response to mechanical cartilage injury, were performed by Dr Francesco Dell'Accio.

Dr Francesco Dell'Accio closely supervised the project providing scientific guidance and advice regarding experimental design and planning as well as interpretation of the results and critical review of the manuscript.

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Abstract

The production of ELR+ CXC chemokines is widely studied in arthritis and has been postulated to contribute to the inflammatory phenomena that eventually lead to cartilage breakdown. Healthy articular chondrocytes also express CXC chemokines and chemokine receptors, however their purpose within cartilage is unclear because chondrocytes are encased within a dense avascular extracellular matrix and are not known to migrate *in vivo*. This study reveals a novel homeostatic function of signalling via CXCR1 and CXCR2 in articular cartilage.

Confocal microscopy confirmed the localisation of CXCR1/2 in both *in vitro* cultured chondrocytes and in human articular cartilage explants at the cell membrane as well as within the cytoplasm, as expected considering the internalisation and recycling of these receptors. Calcium mobilisation assays proved that chondrocyte CXCR1/2 are functional and show a higher redundancy than that found in human neutrophils. Disruption of CXCR1/2 signalling at receptor level or by downstream G-protein inhibition resulted in a reduced extracellular matrix sulphated glycosaminoglycan content, and reduced expression of the cartilage differentiation markers COL2A1, Aggrecan, and SOX9, showing that CXCR1/2 signalling is required for the phenotypic stability of adult articular chondrocytes. In normal cartilage, CXCL6 and CXCL8 are present within the cartilage matrix. CXCL8 is bound to heparan sulphate proteoglycans, whilst CXCL6 is sequestered by an as of yet unidentified alternative matrix interaction, contributing to the determination of the chemokine signalling domain. *In vivo* analysis of CXCR2 knockout mouse knee joints revealed that mice lacking CXCR2 have significantly thinner epiphyseal growth plates and medial tibial plateaus, suggesting that CXCR signalling may be required in cartilage during periods of high chondrocyte turnover. Pharmacological modulation of the CXCR1/2 signalling pathway may allow for the selective inhibition of catabolic inflammatory responses whilst preserving CXCR1/2 maintained chondrocyte phenotypic homeostasis in articular cartilage.

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Abbreviations

ACI	Autologous chondrocyte implantation
ADAMTSs	A disintegrin and metalloproteinase with thrombospondin motif
Bcl-2	B-cell lymphoma 2
BMP	Bone morphogenetic protein
COMP	Cartilage oligomeric matrix protein
CSPG	Chondroitin sulphate proteoglycan
DMM	Destabilisation of the medial meniscus
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EP-AHAC	Early passage adult human articular chondrocytes
ERK	Extracellular signal regulated kinase
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GDF-5/CDMP1	Growth and differentiation factor 5 / cartilage-derived morphogenetic protein 1
GPCR	G-protein coupled receptor
HA	Hyaluronic acid
HIF	Hypoxia inducible factor
HSPG	Heparan sulphate proteoglycan
IGD	Interglobular domain
IGF-1	Insulin-like growth factor 1
Ihh	Indian Hedgehog
IL-1Ra	Interleukin-1 receptor anatagonist
iNOS	Inducible nitric oxide synthase
JSD	Joint surface defect

kDa	Kilodaltons
KO	Knockout
LP-AHAC	Late passage adult human articular cartilage
MAPK	Mitogen activated protein kinase
MSCs	Mesenchymal stem cells
MMP	Matrix metalloproteinase
NO	Nitric oxide
OA	Osteoarthritis
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PTHrP	Parathyroid hormone related protein
PTX	Pertussis toxin
RA	Rheumatoid arthritis
RT-PCR	Reverse transcription polymerase chain reaction
Runx2	Runt related transcription factor 2
SOX9	Sry related HMG-box gene 9
SZP	Superficial zone protein
TGFβ	Transforming growth factor β
TIMPs	Tissue inhibitors of metalloproteinases
TNFα	Tumour necrosis factor α
VEGF	Vascular endothelial growth factor
WNT	Wingless type MMTV integration site family

Chapter 1 – Introduction

Background

ELR+ CXC chemokine signalling has been widely studied for its role in neutrophil migration during inflammation (Bacon and Camp, 1990;Hauser et al., 1999). The ELR+ CXC chemokine receptors CXCR1 and CXCR2 and their ligands are shown within published literature to be expressed in both normal and inflamed articular cartilage and synovial membrane; however a migratory role for this signalling pathway in adult chondrocytes is doubtful since adult chondrocytes have never been shown to migrate *in vivo*.

In addition to the intriguing expression of these chemotactic molecules and their receptors in non mobile cells, a number of additional preliminary data prompted me to explore an alternative role for these molecules in the articular cartilage. In particular: i) studies have revealed alternative roles for ELR+ CXC chemokine signalling within angiogenesis and cancer biology (Singh et al., 2009;Strieter et al., 2005;Vandercappellen et al., 2008); ii) preliminary data produced by Dr. F. Dell'Accio suggested that CXCL8 mRNA may be upregulated as part of a molecular response to mechanical cartilage injury; ii) CXCL1 and CXCL8 induced phenotypic modulation of chondrocytes (Merz et al., 2003); iii) CXCR1 and CXCR2 are upregulated during osteoarthritis (Borzi et al., 2000); iv) a microarray screening identification of ELR+ CXC chemokine ligands as positive predictors of the capacity of *in vitro* expanded chondrocytes to form stable cartilage *in vivo* (PCT WO2005014026). This last result in particular suggested a potential role for ELR+ CXC chemokine signalling within cartilage homeostasis, which is the main subject of my thesis.

Before presenting my results investigating this hypothesis, this introduction will provide a background of cartilage biology, osteoarthritis (OA) and chemokine biology, upon which my thesis is founded.

Embryonic Development of the Skeleton and Synovial Joints

Endochondral bone formation and patterning

Endochondral bone formation begins with mesenchymal cells originating from the lateral plate mesoderm migrating and colonising developing embryonic limb buds, giving rise to the skeletal and muscle progenitors of the developing limb. Skeletal progenitor cells undergo condensation into uninterrupted rods within the centre of the developing limb, controlled firstly via the coordination of FGF, Wnt, BMP and hedgehog signalling activity (Goldring et al., 2006), and mediated by factors including cell adhesion molecules and extracellular matrix (ECM) interactions (DeLise et al., 2000). Cells within the centre of mesenchymal condensations differentiate under the control of the SOX-9 transcription factor into proliferating chondrocytes (Bi et al., 1999;Lefebvre et al., 1998), characterised by their expression of type II collagen and proteoglycans (Archer et al., 2003;Eyre, 2004). These cells then cease proliferation and further differentiate into Indian hedgehog (Ihh) expressing prehypertrophic chondrocytes, and subsequently hypertrophic chondrocytes, expressing type X collagen, alkaline phosphatase and matrix metalloproteinases (MMPs). Hypertrophic chondrocytes, characterised by Runx2 expression (Inada et al., 1999;Kim et al., 1999), undergo apoptosis whilst their surrounding ECM becomes vascularised, with osteoclasts and osteoblasts controlling the matrix degradation and calcification of the tissue to form bone (Karsenty 2002, Hayes 2001). Parathyroid hormone-related protein (PTHrP) expressing chondrocytes which form the articular cartilage continue to produce an ECM which is resistant to vascular invasion and mineralisation, whereas chondrocytes within the epiphyseal growth plates undergo hypertrophic differentiation to express type X collagen, alkaline phosphatase and matrix metalloproteinases (MMPs).

The proliferation of chondrocytes and onset of hypertrophic differentiation during endochondral bone formation is regulated via a negative feedback loop of Ihh and PTHrP

(Vortkamp et al., 1996). Ihh induces the expression of PTHrP in periarticular chondrocytes, which acts to retain the cells within their proliferative state. In addition, Ihh regulates BMP gene expression within the perichondrium, which causes an increase in proliferation, leading to some chondrocytes being pushed into areas outside of the PTHrP signalling range where they become prehypertrophic, thus restricting the area of proliferating chondrocytes and consequently endochondral bone length (Minina et al., 2001). BMP induced Ihh upregulation outside of the PTHrP signalling range promotes subsequent hypertrophic differentiation (Mak et al., 2008).
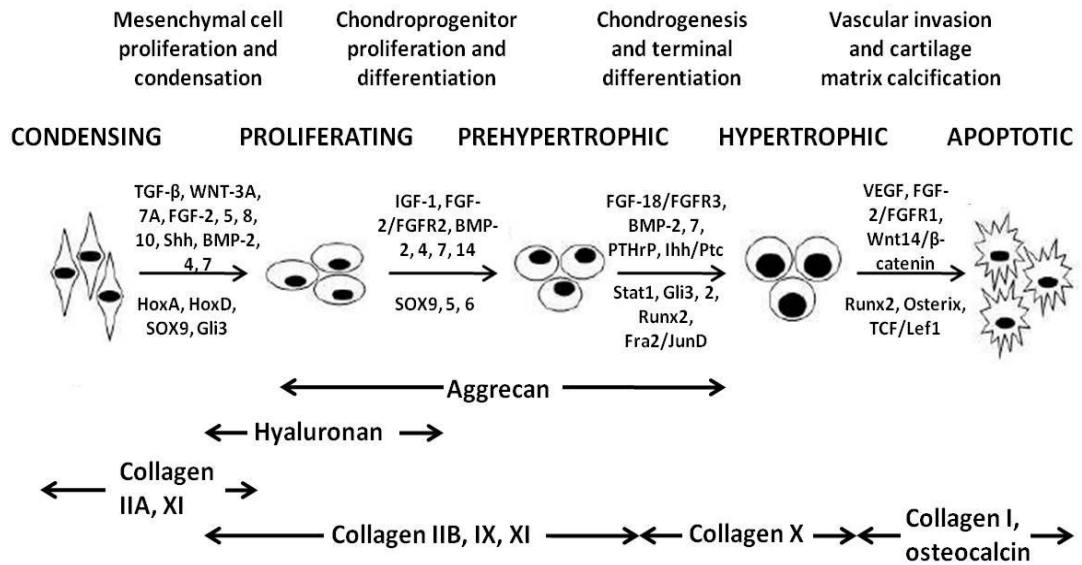
Figure 1. **Cellular events during endochondral bone development.** Mesenchymal condensation within the developing limb bud gives rise to type II collagen expressing proliferating round chondrocytes. Within the centre of the developing cartilage anlagen, cells undergo hypertrophic differentiation and express type X collagen, whilst the BMP expressing perichondrium surrounds the developing cartilage. Development of the primary ossification centre is marked by vascular invasion, osteoblast activity and matrix calcification. Each stage of chondrogenic differentiation and endochondral bone formation is characterised by the action of different signalling pathways, changes in cell phenotypes, and expression of varying ECM components. Figure edited from (Goldring et al., 2006).

Joint formation

Synovial joint formation occurs through the segmentation of the continuous mesenchymal anlagen which results in the separation and morphogenesis of the future articular surfaces and synovial tissues. Segmentation begins with the appearance of a high cell density area formed of flattened cells connected by gap junctions between the two developing articular surfaces which is known as the interzone (Archer et al., 2003). Cells within the interzone express specific markers including Growth differentiation factor-5 (GDF-5)/Cartilage-derived morphogenetic protein-1 (CDMP-1) (Francis-West et al., 1999), Wingless type MMTV integration site family (Wnt)9A, Wnt16, Wnt4 (Guo et al., 2004;Hartmann and Tabin, 2001), Noggin (Brunet et al., 1998), CD44 (Edwards et al., 1994), lubricin (Rhee et al., 2005), autotaxin (Hartmann and Tabin, 2001) the COL2A1-A splicing isoform (Koyama et al., 2008;Nalin et al., 1995) and matrilin-1 (Hyde et al., 2007) are known to differentiate into chondrocytes when cultured *in vitro* (Pacifici et al., 2006). The interzone further separates into three layers, with the centre layer expressing increased levels of the glycosaminoglycan hyaluronan (HA) following mechanical stimulation to the developing limb (Dowthwaite et al., 1998;Dowthwaite et al., 2003;Pitsillides et al., 1995). The high concentration of HA leads to the saturation of the cell surface receptor CD44, leading to cell separation and later apoptosis (Toole, 1991). The cells remaining either on either side of the cavitated area further differentiate, becoming articular chondrocytes, synovial membrane and ligaments (Koyama et al., 2008).
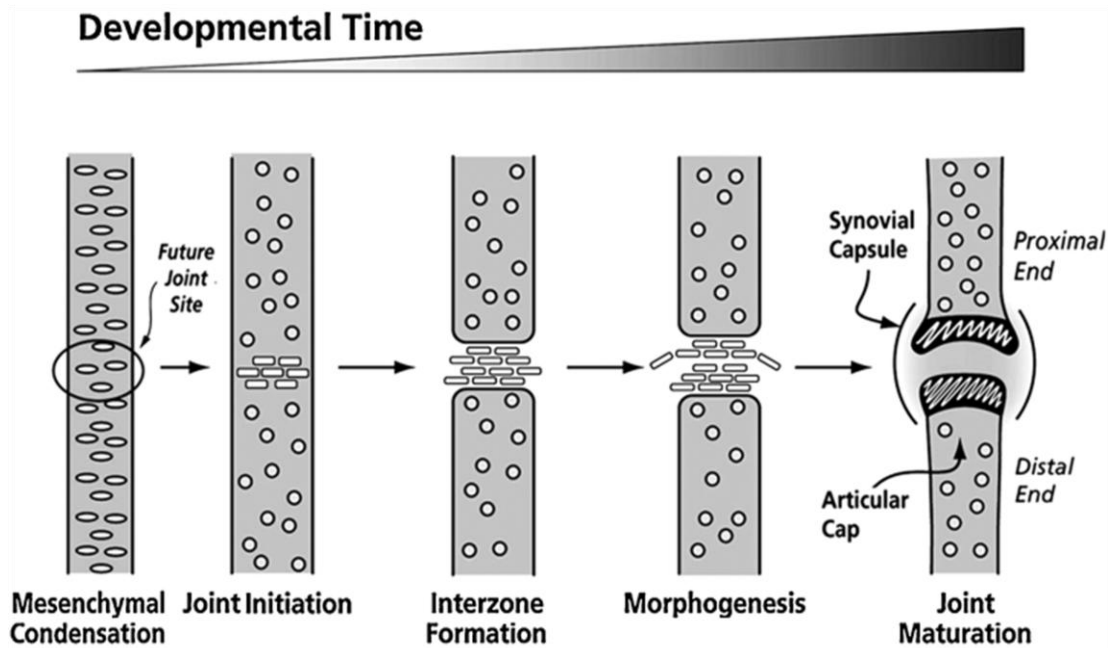
Figure 2. Schematic stages of synovial joint development. Joint initiation begins within the condensation of mesenchymal cells at the site of future joint formation, perpendicular to the direction of anlagen growth. Cells become flattened and give rise to the interzone. Joint cavitation results in the separation of adjacent skeletal anlagen and formation of the synovial cavity. Further morphogenesis results in the formation of opposing articular cartilage surfaces, joint capsule, menisci and ligaments, leading to the development of a mature joint. Figure from (Pacifici et al., 2006).

Structure and Function of Articular Cartilage

Articular cartilage is an avascular and aneural tissue which covers the surfaces of synovial joints providing a smooth surface to facilitate frictionless motion within the musculoskeletal system. It allows for the distribution of mechanical loads within weight bearing joints, minimising focal pressure upon the subchondral bone, whilst resisting compression, thereby acting as a shock absorber. Articular cartilage consists predominantly of a heavily hydrated ECM produced by chondrocytes which are the only cell type found within cartilage, occupying between 1% and 10% of total articular cartilage tissue volume depending upon species and joint location (Maroudas and Schneiderman, 1987). The dense ECM consists of a complex framework of structural macromolecules which vary in proportion and organisation between the superficial and deeper cartilage layers (Poole et al., 2001).

Both morphological and biochemical differences characterise the ECM content and chondrocyte distribution within the different layers of articular cartilage (Figure 3). The uppermost superficial layer contains chondrocytes which have become flattened amongst a tightly packed network of collagen filaments parallel to the articular surface. This structure provides resistance to shear forces occurring during normal joint movement whilst also maintaining the vital tensile properties of cartilage. Chondrocytes within the superficial layer express superficial-zone protein (SZP), an O-linked glycosylated protein which acts to lubricate the surface of the articular cartilage whilst inhibiting the overgrowth of synovial cells (Rhee et al., 2005;Schumacher et al., 1994). The transitional zone contains a lower density of predominantly spherical chondrocytes amongst a dense ECM consisting of arched large diameter collagen fibres and a high concentration of proteoglycans. Chondrocytes are arranged into columns perpendicular to the cartilage surface within the deep zone, which contains the highest concentration of highly

sulphated negatively charged proteoglycans, required to maintain the high water content providing cartilage with its resistance to compression (Bhosale and Richardson, 2008). Underlying the deep zone is the calcified layer, where chondrocytes undergo hypertophic differentiation resulting in an increased expression of type X collagen and alkaline phosphatase (Reichenberger et al., 1991;Schmid and Linsenmayer, 1985) . Collagen fibres arranged perpendicular to the cartilage surface, extend from the deep zone into the calcified layers in order to provide anchorage for the articular cartilage onto the subchondral bone below (Poole et al., 2001).
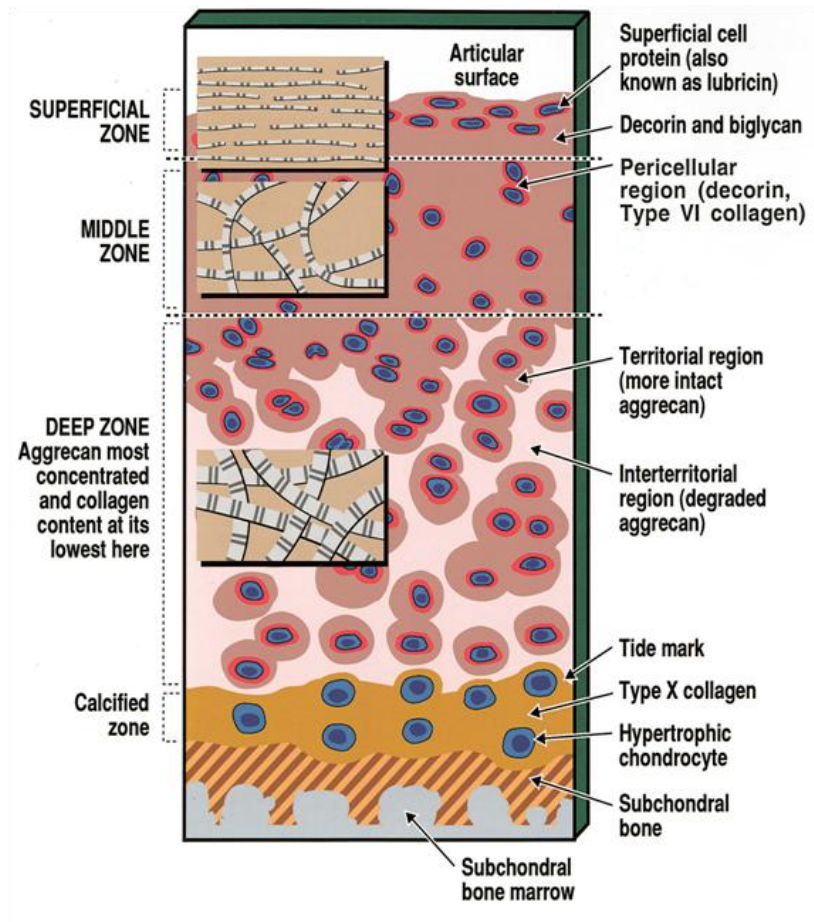
Figure 3. Cross-sectional schematic view of human articular cartilage. Figure from (Poole et al., 2001).

The Chondrocyte

Articular chondrocytes are the sole cell type found within articular cartilage and are responsible for the synthesis and maintenance of the cartilage ECM. Because cartilage is not vascularised, and chondrocytes are almost exclusively cytoplasmically isolated, chondrocytes must rely on diffusion from the articular surface or underlying subchondral bone for nutrient and metabolite exchange. As a result, chondrocytes obtain the majority of their energy requirement through glycolysis, allowing the cells to operate at low oxygen tensions of between 6% in the superficial layer, to less than 1% in the deeper zones. In fact, *in vitro* studies have shown a number of chondrogenic genes to be upregulated at lower oxygen tensions (Henderson et al., 2010;Rajpurohit et al., 1996).

Chondrocyte mophology and gene expression is known to change with increasing age. Type II collagen synthesis begins to decrease upon adult maturity (Hollander et al., 1995), whilst smaller and less glycosylated proteoglycans are produced in older individuals which are likely to alter the compressive strength of articular cartilage (Buckwalter et al., 1994).

As opposed to the epiphyseal chondrocytes that form the cartilage skeletal anlagen and are replaced by bone throughout endochondral bone formation, articular chondrocytes are phenotypically stable throughout life and are resistant to vascular invasion and endochondral bone formation. This apparent similarity of the adult articular cartilage with the zone of resting chondrocytes of the growth plate led to speculation that the articular cartilage may represent the residue of growth plate "spared" after the arrest of endochondral bone formation. In reality, articular chondrocytes display distinct molecular characteristics including lubricin, the ERG transcription factor (Iwamoto et al., 2001;Pacifici et al., 2006), and PTHrP. Importantly, recent lineage tracking studies using cre-LoxP technology have indeed confirmed that the articular cartilage has a disinct embryological

origin from the epiphyseal chondrocytes, which is defined very early on in skeletal development (Koyama et al., 2008;Rountree et al., 2004).

This phenotypic stability of the articular chondrocytes is also maintained during *in vitro* culture, at least during early passage cell expansion. Benya et al. thoroughly studied this phenomenen and showed that such dedifferentiated chondrocytes may reestablish their chondrocyte phenotype, including expression of type II collagen, in anchorage independant conditions (Benya and Shaffer, 1982). Dell'Accio et al. subsequently extended these findings *in vivo*, showing that whereas early passage adult human articular chondrocytes (EP-AHAC) can form stable cartilage implants when ectopically implanted into the muscle of immuno deficient mice, expanded late passate chondrocytes are unable to form stable ectopic cartilage *in vivo*, but rather tend to respond to molecular environmental stimuli and differentiate towards the muscle lineage (Dell'accio et al., 2001;Dell'accio et al., 2003). Although the chondrogenic phenotype of late passage cells cultured within agarose gels is partially rescued *in vitro* (Benya and Shaffer, 1982), their *in vivo* capacity to form cartilage remains compromised (Dell'accio et al., 2001).

The Extracellular Matrix

Macromolecules contributing to the ECM produced and maintained by chondrocytes are generally described as one of two major components: collagens which provide structural support to the cartilage via their arrangement into a fibrous network, and proteoglycans which attract water via their highly sulphated glycosaminoglycan chains, enabling a high swelling pressure within the tissue to counteract external compressive force.

The composition of cartilage ECM varies according to the distance from chondrocytes with three distinct regions observed within healthy articular cartilage. The pericellular matrix

forms the sphere of ECM immediately surrounding each chondrocyte. It is formed mainly from type VI collagen (Alexopoulos et al., 2009;Poole et al., 1992) and the heparan sulphate proteoglycan (HSPG) perlecan (Costell et al., 1999) along with cell membrane associated proteins such as fibronectin which serves to anchor the ECM to the cell membrane (Chang et al., 1997). Clusters of chondrocytes and areas of high cell density are enclosed by the terratorial matrix. This consists primarily of type II collagen and chondroitin sulphate proteoglycans (CSPG) which form a complex mesh in order to protect chondrocytes. The inter-territorial matrix accounts for the largest area within the articular cartilage, particularly contributing to areas of lower cell density. Type II collagen fibres and proteoglycans are arranged as previously described for each macroscopic layer within the tissue.

Collagens

Collagen macromolecules account for two thirds of articular cartilage dry weight with type II collagen the most abundant form (Eyre, 1991;Eyre et al., 2002). Polymerised type II collagen provides the fibrillar framework required for the tensile strength of cartilage and is cross-linked and stabilised by fibril associated collagens, type IX and type XI. Type VI collagen is found within the pericellular matrix of cartilage where it contributes to chondrocyte attachment and ECM interactions (Poole et al., 1992). Type X collagen is expressed by hypertrophic chondrocytes and is hence found within the calcified layer where it may be used as a marker of endochondral bone formation (Shen, 2005).

Collagen fibres are formed from 3 α-polypeptide subunits arranged into a triple helix known as tropocollagen, measuring 280nm in length and 1.5nm in diameter. α-Polypeptide subunits are synthesised with additional non-helical peptides attached to both the carboxy and amino termini forming procollagen. Until the telopeptides can be

removed from the α-polypeptides by the enzyme procollagen peptidase upon transportation out of the chondrocyte, tropocollagens are unable to precipitate within the cell or form premature collagen fibres. The type of collagen produced is determined by the combination of α-polypeptide subunits arranged within the helix. Type II collagen consists of 3 α1 chains, whereas type VI is formed from 3 different chains: α1, α2 and α3. Collagen fibrils aggregate into large bundles which form the structural fibres providing articular cartilage with tensile strength and mechanical resistance (Eyre et al., 2002;Mendler et al., 1989).
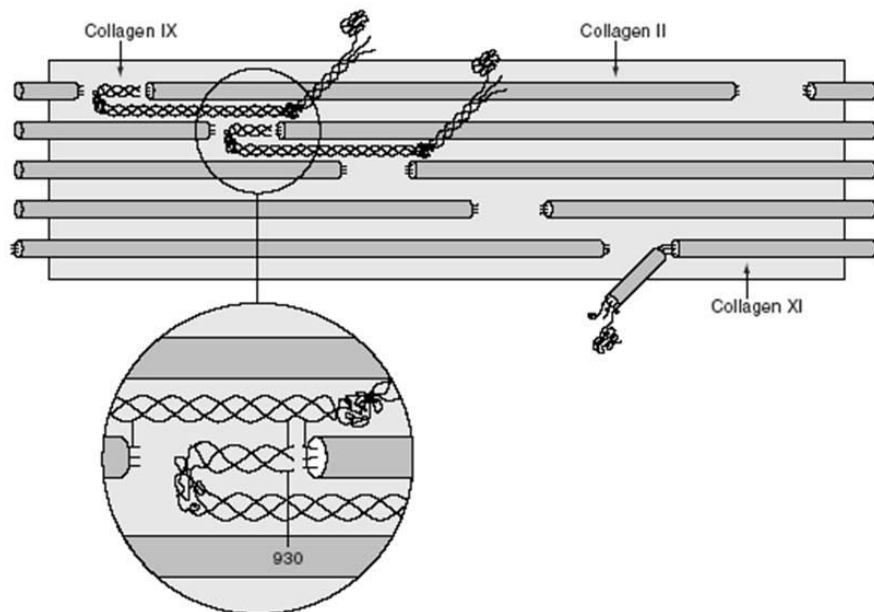
Figure 4. The collagen II, IX, XI heterofibril. Molecular diagram illustrating the interactions of type II and IX collagens within articular cartilage collagen fibrils. Type IX collagen is cross-linked to the surface of type II collagen fibrils. Type XI collagen fibrils cross-link to one another via N-telopeptide-to-helix sites, contributing to heterofibril structure and limiting lateral expansion (Blaschke et al., 2000). Diagram from (Eyre, 2004).

Proteoglycans

Proteoglycans are complex glycoproteins formed from a protein core with one or more attached glycosaminoglycan (GAG) chains. They form up to one third of articular cartilage dry weight. GAGs are long unbranched polysaccharide chains formed of repeating dissacharides each carrying an amino sugar. The high negative charge of each GAG molecule is provided by either a carboxylate or sulphate group attached to each dissacharide within the GAG chain. This negative charge attracts cations including Na^{2+}, which increase the osmolarity of the cartilage. Water molecules attracted into the tissue are retained within the macromolecular network of GAG chains amongst the collagen network, providing articular cartilage with its high compressive strength.

The most abundant proteoglycan found in articular cartilage is aggrecan. The protein core of aggrecan is constructed from three globular (G1, G2 and G3) and two extended interglobular domains (E1 and E2). The E2 interglobular domain is attached to many GAGs (chondroitin sulphate and keratin sulphate) (Doege et al., 1991;Roughley, 2006). The C-terminal G1 domain is linked to hyaluronic acid (Roughley and Lee, 1994) which may attach to as many as 100 individual GAG chains, resulting in the formation of large aggregates of aggrecan. The attachment to HA allows aggrecan to bind to the chondrocyte cell surface via the CD44 receptor (Knudson et al., 1996). In fact, this interaction may allow chondrocytes a method of regulation of ECM properties through the altered CD44 phosphorylation activity upon HA binding, which in turn is thought to lead to HA internalisation (Knudson and Loeser, 2002;Uff et al., 1995).
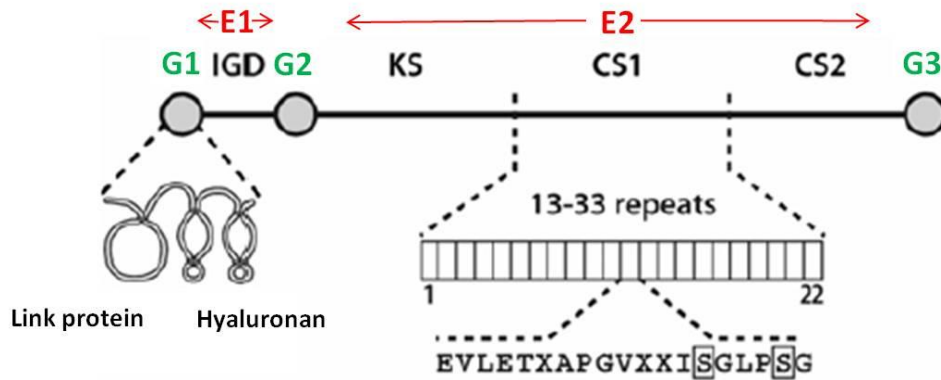
Figure 5. Structure of aggrecan. The core aggrecan protein consists of three cysteine rich globular domains (G1-3), two interglobular domains (E1, E2).   E2 contains multiple attachment regions for keratan sulphate and chondroitin sulphate chains, including the CS1 domainwhich exhibits length polymorphism leading to variation in the number of available chondroitin sulphate attachment sites.   The G1 domain is responsible for interaction with hyaluronic acid and stabilising link proteins.   Diagram adapted from (Roughley, 2006).

Less abundant proteoglycans found within articular cartilage include the dermatan sulphate proteogylcans, biglycan and decorin, the keratan sulphate proteoglycan, fibromodulin, and the heparin sulphate proteoglycan, perlecan, which all together contribute around 3% of the cartilage proteoglycans by mass. Due to the smaller size of these proteogylcans, they do not contribute directly to the cartilage mechanical properties provided by larger macromolecules. Instead, they bind to collagens in order to stabilise the matrix (Knudson and Knudson, 2001a). Decorin and fibromodulin bind to type II collagen, whereas biglycan is found primarily within the pericellular matrix and is thought to interact with type VI collagen (Roughley and Lee, 1994).

In addition to their structural function, an increasing body of evidence has revealed that cartilage proteoglycans have important functions in chondrocytes signalling, either directly (Echtermeyer et al., 2009) or indirectly through interactions with growth factors and chemokines, allowing for the formation of morphogenetic gradients. FGFs have been shown to bind to perlecan, which may contribute to its release following cartilage damage or loading, thus suggesting a mechanism of proteoglycan regulated mechanotransduction (Vincent et al., 2002;Vincent et al., 2007). Biglycan, decorin and fibromodulin interact with the chondrogenic growth factor, transforming growth factor beta (TGFβ), allowing for its sequestration within the ECM (Hildebrand et al., 1994), with slight variations in the binding properties of different proteogylcans to growth factors allowing for the regulation of growth factor activity (Kolb et al., 2001). HSPGs including syndecans are known to modulate BMP2 activity during cartilage differentiation (Fisher et al., 2006) and ELR+ CXC chemokines have been shown to interact with syndecans on endothelial cells and synovium (Halden et al., 2004;Patterson et al., 2005). The expression of extracellular heparan sulphatase enzymes within cartilage may contribute to the regulation of growth factor signalling via the release of sequestered ligands and inhibitors upon homeostatic proteoglycan turnover, or during OA (Otsuki et al., 2008;Otsuki et al., 2010).

Non-Collagenous Structural Proteins

Smaller subgroups of non-collagenous proteins and glycoproteins are found within cartilage, occupying roles within ECM-chondrocyte interactions and within the ECM framework. Cartilage oligomeric protein (COMP) is a glycoprotein found mainly within the territorial matrix of chondrocytes. COMP has been shown to bind to type I and type II collagens via zinc-dependant interactions, as part of the collagen cross-linking mechanism (Rosenberg et al., 1998). Chondronectin is a high molecular weight glycoprotein involved in the adherance of chondrocytes to the ECM via the interactions of chondronectin with chondroitin sulphate, hyaluronic acid and type II collagen (Chevalier, 1993). Fibronectin is

a large polypeptide dimer linked together by disulphide bonds. It is localised to the pericellular matrix, has multiple ECM binding sites and is linked to chondrocytes via integrins. Although fibronectin synthesis is known to increase during OA, following cyclical loading and following exposure to growth factors such as TGFβ (Homandberg, 1999), increased proteinase activity during OA results in the generation of fibronectin fragments that have been shown to activate catabolic pathways, including the upregulation of MMPs and aggrecanases (Xie and Homandberg, 1993).

Cartilage Remodelling and Homeostasis

In healthy cartilage, chondrocytes synthesise both ECM proteins, such as collagens and proteoglycans, and the enzymes responsible for their degredation (Cawston et al., 1999). The control of a slow and balanced turnover of these molecules is vital in maintaining the structure and function of the cartilage ECM. In normal conditions an equilibrium is achieved between the anabolic and catabolic activities, which is ideally controlled by a balance between the synthesis of new ECM macromolecules and the activity of ECM proteinases and their inhibitors. The remodelling of cartilage which is normally tightly controlled, is accelerated during conditions such as OA (Bay-Jensen et al., 2011). The major classes of enzymes controlling the turnover and remodelling of cartilage are the matrix metalloproteinases (MMPs) and the A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs). Both of these may be inhibited by tissue inhibitors of metalloproteinases (TIMPs).

MMPs

MMPs are a family of 23 proteases that together, are capable of degrading all ECM proteins and play a key role in cartilage turnover. Each molecule carries a central zinc atom required for catalytic activity. MMPs are initially sythesised as inactive pro-MMPs

(zymogens) with a pro-peptide domain that must be cleaved for the enzyme to become functionally active. A C-terminal haemopexin-like domain is reponsible for protein-protein interaction and consequently, substrate specificity. The catalytic and C-terminal domains are connected via a hinge region (Nagase et al., 2006).

Figure 6. Structure of matrix metalloproteinases (MMPs). Figure from (Yong et al., 2001).

MMPs can be divided into 4 main groups: collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs). Collagenases (MMP-1, MMP-8, MMP-13 and MMP-18) are the only MMP subgroup capable of degrading triple-helical collagens at a neutral pH. Once cleaved, gelatinases (MMP-2 and MMP-9) are able to further degrade the generated collagen fragments. Stromelysins (MMP-3, MMP-10 and MMP-11) have a broader substrate base and are able to cleave proteoglycans and fibronectins. They have also been implicated in the activation of other MMPs (Murphy et al., 2002;Visse and Nagase, 2003), suggesting that they may play an important role within cartilage homeostasis.

In addition to matrix remodelling, the degradation of the ECM by MMPs is necessary to allow for cell migration and tissue architecture during development and tissue repair, and

for the release and activation of ECM-bound morphogens and growth factors (Page-McCaw et al., 2007;Werb, 1997;Whitelock et al., 1996). Both the elevation and inhibition of MMP levels within cartilage have been implicated as factors in the arthritis associated destruction of cartilage. Increased levels of MMPs have been observed within cartilage from OA patients (Tetlow et al., 2001). In mice, the constitutive expression of MMP-13 under the cartilage-specific type II collagen promotor showed increased cartilage degradation in comparison to wild type litter mates when subjected to the destabilisation of the medial meniscus (DMM) model of OA (Glasson et al., 2007), whilst the selective inhibition of collagenases allowed for the significant protection from cartilage damage in STR/Ort mice (Brewster et al., 1998). However, MMP-9 null mutant mice challenged in the same DMM model also showed increased OA-like cartilage degradation in comparison to the wild type (Glasson et al., 2007). This suggests that a regulated level of MMP activity is in fact required for the remodelling and homeostasis of cartilage, and might explain the failure of MMP inhibitors as a target for clinical intervention in arthritis (Clark and Parker, 2003;Drummond et al., 1999).

ADAMTSs

ADAMTSs are a family of 19 proteases that are closely related to MMPs, containing a catalytic zinc atom within their active site (for review see (Jones and Riley, 2005)). Unlike MMPs, ADAMTSs exhibit a narrow substrate specificity due to the presence of various exosites close to the C-terminus which allow for specific protein recognition and matrix localisation. The identification of ADAMTS cleaved TEGE[373] aggrecan fragments within the synovial fluid of both OA and inflammatory arthritis patients, confirmed the activity of ADAMTSs during cartilage destruction (Lohmander et al., 1993). Although aggrecan may be cleaved by both ADAMTSs and MMPs (Lark et al., 1997), mice resistant to MMP cleavage of the IGD remained susceptible to cartilage degradation whereas mice resistant to ADAMTS cleavage of the IGD were protected from cartilage erosion in experimentally

induced arthritis, suggesting that ADAMTS activity is required for aggrecan breakdown (Little et al., 2007).

Two subgroups of ADAMTSs have been shown to have specific roles in cartilage. Procollagen N-propeptidases (ADAMTS-2, ADAMTS-3 and ADAMTS-14), specifically ADAMTS-3 in cartilage, is involved in the processing of zymogen during collagen biosynthesis (Fernandes et al., 2001). Hyalectanases (ADAMTS-1, -4, -5, -8, -9, -15 and -20), otherwise described as 'aggrecanases', cleave aggrecan between the G1 and G2 domains at the Glu-373-- Ala-374 site (IGD) close to the N-terminus, and at 4 specific sites within the CS-2 chondroitin sulphate domain at the C-terminus (Nagase and Kashiwagi, 2003;Tortorella et al., 2000). ADAMTS-4 has also been shown to cleave other cartilage macromolecules including COMP and fibromodulin, a small proteoglycan which interacts with type II collagen (Kashiwagi et al., 2004).

ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) were the first ADAMTSs to be identified (Abbaszade et al., 1999;Tortorella et al., 1999). Subsequently ADAMTS-5 was implicated as the main constitutively expressed ADAMTS within human cartilage (Tortorella et al., 2002;Bau et al., 2002), whereas ADAMTS-4 expression was observed to increase following exposure to proinflammatory cytokines, TNFα and IL-1β (Pratta et al., 2003). ADAMTS-4 was shown *in vitro* to cleave aggrecan at a higher rate than that seen using ADAMTS-5 (Bondeson et al., 2006), suggesting that ADAMTS-4 may provide the greater contribution to OA pathogeneisis in humans. Meanwhile in mice, ADAMTS-5 rather than ADAMTS-4 is seen to be stimulated by Interleukin-1 (IL-1) (Tortorella et al., 2002). ADAMTS-5 knockout mice subjected to the surgically induced DMM OA model showed a significant reduction in disease severity compared to controls, whereas no improvement was observed in ADAMTS-4 knockouts (Glasson et al., 2004;Glasson et al., 2005). Furthermore, within a model of inflammatory arthritis, ADAMTS-5 but not

ADAMTS-4 knockout mice were protected from proteoglycan loss (Stanton et al., 2005), suggesting ADAMTS-5 to be more involved in OA pathogenesis in mice. However, mice with a knockout of the catalyic domain of either ADAMTS-4 or ADAMTS-5 were found to be indistinguished from wild type littermates, suggesting that the enzymes may be redundant during development at least.

TIMPs

Four TIMPs have been characterised in humans (TIMP-1, -2, -3 and -4) that inhbit the activity of MMPs, and to a lesser extent ADAMTSs (Baker et al., 2002). In healthy cartilage, TIMPs act to regulate the homeostatic balance of MMP and ADAMTS activity required to maintain cartilage turnover, which is disrupted during OA where MMPs are shown to be upregulated relative to TIMPs (Dean et al., 1989). TIMP-3 is regarded as a protective factor against cartilage degradation, as demonstrated by the spontaneous development of an osteoarthritic phenotype in TIMP-3 knockout mice (Sahebjam et al., 2007).

Cartilage homeostasis

The articular cartilage maintains its integrity through the maintenance of a homeostatic balance of catabolism and anabolism. In physiological conditions, the turnover of cells and extracellular matrix is extremely slow, however, following injurious events (including trauma, biomechanical challenges, or inflammation), a rapid response of catabolic events, balanced with anabolic mechanisms, simultaneously or in coordinated succession, re-establish homeostasis (Dell'accio and Vincent, 2010).

Although such mechanisms were previously thought to be active during disease, to be ultimately futile, and to lead to osteoarthritis, it is now well recognised, not only through animal studies, but also in human observational studies, that such homeostatic re-adjustments, and even regeneration of full thickness defects often take place, frequently unnoticed and asymptomatically, and are a regular phenomenon throughout the adult life (Dell'accio and Vincent, 2010;Messner and Maletius, 1996;Nakamura et al., 2008).

The molecular pathways and signalling systems contribute to these homeostatic mechanisms are currently being unveiled thanks to the availability of disease models of osteoarthritis and cartilage injury in mice, and the use of mouse genetics with such models (Dell'accio et al., 2006;Dell'accio et al., 2008;Lories and Luyten, 2005;Vincent et al., 2002). The most important findings of this body of research are summarized hereafter.

Catabolic factors and the role of inflammation

The balance between matrix proteinase activity and their inhibitors is essential for the control of ECM turnover. However, the mechanisms by which cells achieve homeostasis in normal conditions, or activate excessive ECM proteolysis during arthritis are not yet well understood. A number of pro-inflammatory molecules which are present during arthritis

can induce the breakdown of cartilage matrix. IL-1 and TNFα have been shown to increase the expression and activation of MMPs and ADAMTSs (Rowan et al., 2001;Saklatvala, 1986). The presence of antagonists of these cytokines in OA cartilage explants resulted in a reduction of MMP gene expression and in a decrease in type II collagen cleavage and in GAG release (Kobayashi et al., 2005). More recently, ADAMTS-4 and ADAMTS-5 have been shown to be induced following IL-1 stimulation (Bondeson et al., 2007). MMP and ADAMTS-4 gene expression was induced in chondrocytes by the hypoxia-inducible factor 2α (HIF2α), suggesting a clear relevance of oxygen availability within the transcriptional control of ECM catabolism (Yang et al., 2010;Saito et al., 2010).

Interestingly, contrasting reports have emerged detailing the susceptibility of IL-1 knockout mice to OA. Clements et al. demonstrated that IL-1 knockout mice developed OA lesions within unoperated knees, however Glasson et al. showed that IL-1 knockout mice within the DMM surgically induced model developed less severe OA (Clements et al., 2003;Glasson et al., 2007). Notably, a similar varying outcome of OA cartilage destruction is seen in Interleukin-6 (IL-6) knockout mice, where an inhibition of DMM induced OA severity is observed in IL-6 knockout mice, but the absense of IL-6 is shown to lead to advanced OA damage upon ageing (Glasson et al., 2007;Ryu et al., 2011;de Hooge et al., 2005). These data suggest firstly that different mechanisms of disease induction active within different experimental models may require contributions from different catabolic or homeostatic signalling mechanisms to either accelerate or prevent disease progression. Furthermore, IL-1 and IL-6 demonstrate that such catabolic stimuli should not be regarded as only pathogenic mechanisms. Their activity may be required in order to activate homseostatic mechanisms in healthy cartilage, and may therefore become pathogenic only when their regulation is lost, when they are no longer balanced alongside anabolic mechanisms, or when continuous injury (e.g. during joint instability) results in their continuous activation and ultimately futile activity.

Anabolic Factors

Anabolic signalling molecules act to counteract the effects of cartilage breakdown through the promotion of ECM synthesis and the inhibition of matrix degradation. Growth factors such as TGFβ and insulin growth factor 1 (IGF-1) are able to block the cytokine induced ECM breakdown by a potential suppression of MMPs. TGFβ was also shown to induce TIMP-1 and TIMP-3 expression which in turn, inhibit MMP activity (Glasson et al., 2007;Hui et al., 2001). FGF and BMP signalling molecules stimulate an upregulation of ECM synthesis and may be upregulated in response to cartilage damage in an attempt to counteract catabolic matrix activity (Dell'accio et al., 2006;Glasson et al., 2007;Vincent et al., 2002).

Mechanical loading is widely regarded as an important anabolic stimulus within healthy cartilage matrix turnover. Cyclical loading has been shown to induce both protein and proteoglycan synthesis in cartilage explants (Larsson et al., 1991;Valhmu et al., 1998), whereas the immobilisation of weight bearing joints resulted in the rapid loss of articular cartilage proteoglycan content (Setton et al., 1995). COMP and fibronectin expression have also been seen to increase during periods of cyclical loading (Wong et al., 1999).

Osteoarthritis

Osteoarthritis is the most common form of arthritis and is a highly prevelant disease associated with chronic disability for which there is currently no cure. More than 6 million people in the UK suffer from OA in one or both knee joints. The high impact on working capacity of the population and economic costs upwards of £5.7 billion annually to the UK make OA a priority in medicine (Arthritis Research UK, 2010). Although the disease may affect younger people, OA is predominantly associated with the older population. Whereas inflammatory arthritis, including rheumatoid arthritis (RA), is an autoimmune condition, OA is a degenerative disease that has been associated with mechanical cartilage damage, obesity and with a genetic predisposition.

The major hallmarks of the clinical disease include the progressive loss of articular cartilage and alterations to the underlying subchondral bone. Cartilage damage begins with the formation of fibrillations within the superficial layer which progressively extend down through the middle zone and into the deep zone. The proliferation of chondrocytes close to fibrillations has been observed to increase, causing the OA characteristic of chondrocyte clustering (Mankin and Lippiello, 1971). This activity may be due to the increased accessibility to proliferative factors available within the synovial fluid following the degradation of the collagen network (Lee et al., 1993). Progression of the disease leads to an apparent overall decrease in extracellular matrix content and cell number (Hulth et al., 1972). Full thickness loss of articular cartilage in focal weightbearing areas is a typical feature of the end-stage disease. Ongoing modifications within the subchondral bone during OA include osteosclerosis, edema formation, and the growth of boney and cartilagenous outgrowths at the joint margin known as osteophytes (Aigner et al., 1995;Day et al., 2004).

Original theories regarding the causes of OA involved the reduced lubrication of the articular surface and thickening of the subchondral bone which were thought to lead to increased wear and tear of the articular surface through higher mechanical strain and friction (Walker et al., 1969;Simon et al., 1972). Later studies correlated the progression of OA pathology with the reduced, or lack of expression of lubricin (Rhee et al., 2005;Teeple et al., 2008;Young et al., 2006), demonstrating the importance of the protection of the articular surface against biomechanical strain and friction. Changes within the articular cartilage itself were linked to the OA pathogenesis following studies which exposed the weakening and breakdown of the collagen network (Maroudas, 1976;Pelletier et al., 1983).

More recent studies have begun to unravel the molecular events thought to contribute to the biochemical and structural changes associated with OA. The contribution of inflammatory cytokines and the activity of matrix degrading proteases are now known to disrupt the balance of cartilage ECM turnover, resulting in the progressive breakdown of ECM macromolecules which leads to the loss of structural resistance during normal weight bearing activity. The exact events driving the early progression of OA are not yet fully understood, however their identification is likely to lead to novel diagnostic tools, such as biomarkers, and the subsequent development of therapies designed to intervene prior to the disruption of the cartilage homeostatic balance.

Chondrocyte phenotypic modulation in OA

In normal articular cartilage, chondrocytes maintain a low rate of turnover of cartilage matrix macromolecules. During early OA, an increased synthetic activity is evident, suggesting an attempt from the chondrocytes to regenerate the ECM through the production of type II, VI, IX and XI collagens and aggrecan (Lorenz et al., 2005). However, this unregulated behaviour of OA chondrocytes results in the disorganisation of the ECM

and appearance of fibrillations (Pritzker et al., 2006). In some cases this is followed by cellular changes similar to those observed within the hypertrophic zone of the growth plate, including type X collagen expression, alkaline phosphatase and collagenase activity (von der et al., 1992). Type X collagen, VEGF and MMP-13 are direct transcriptional targets of HIF2α, which is upregulated within OA cartilage and may be activated following either an increase of oxygen availability during vascularisation, or by proinflammatory stimuli (Saito et al., 2010;Yang et al., 2010). However, the expression of hypertrophic markers is shown not to be a reliable marker for cartilage degeneration (Brew et al., 2010). Isolated chondrocytes cultured in monolayer quickly lose the expression of key transcription factors including SOX 9, which drive the expression of type II collagen and aggrecan. This may not in fact be a step of differentiation reversal, but a modulation of the chondrocyte phenotype in response to a change in extracellular environment and growth factor availability. It is possible for OA chondrocytes to reinitiate matrix production following transduction with SOX 9 (Tew et al., 2005), however even cells with a re-established expression of chondrocyte phenotypic markers may not fully support the maintenance of cartilage growth *in vivo* (De Bari et al., 2004;Dell'accio et al., 2001), possibly due to the permenant disruption to autocrine/paracine chondrocyte homeostatic signalling mechanisms.

The modulation of chondrocyte phenotype during OA follows the influence and feedback of mechanical, inflammatory and proteolytic factors and contributes significantly to the disruption of the homeostatic balance of ECM turnover.

Matrix turnover in OA

The balance between the synthesis of new ECM macromolecules and the breakdown of the ECM network by MMPs and ADAMTSs is vital to the homeostasis of healthy cartilage. Although both type II collagen and aggrecan have been shown to be upregulated during OA (Hermansson et al., 2004;Lorenzo et al., 2004), evidence suggests that the upregulation of protease activity results in an overall catabolism of matrix macromolecules. Furthermore, the increased expression of COL2A1 in late OA cartilage may ultimately be futile, due to the inability of severely damaged cartilage to replicate the collagen arrangement laid down during development.

A number of studies have reported the presence of increased levels of MMPs in OA joints. Elevated levels of the stromelysin MMP-3 were found within the synovial fluid obtained from OA joints (Lohmander et al., 1993). MMP-3 mRNA levels were found to be increased in the middle and deep zones of human OA articular cartilage, whilst collagenases MMP-1, -8 and -13 mRNAs and the gelatinases MMP-2 and -9 were found to be upregulated within the superficial zone (Freemont et al., 1997;Tetlow et al., 2001). Both MMP generated VDIPEN[341] and ADAMTS generated NITIGE[373] aggrecan fragments are found within OA cartilage (Lark et al., 1997), leading to debate as to which group of proteases plays the major role in cartilage degradation. Cartilage explant studies have suggested that aggrecanases play an early role in aggrecan cleavage, with MMP activity increasing at a later stage (Little et al., 2002).

Aggrecan fragments found within synovial fluid from OA joints first indicated the activity of aggrecanases during the early stages of OA development (Sandy et al., 1991). ADAMTS-5 was found using immunohistochemistry within canine joint cartilage following anterior cruciate ligament transection, whilst in human femoral head cartilage, ADAMTS-5 was found in OA but not in healthy samples (Boileau et al., 2007;Roach et al., 2005). Although

ADAMTS-5 was identified as the primary aggrecanase in both inflammatory and OA model (Glasson et al., 2005;Stanton et al., 2005), the use of RNA interferance technology to knock down either or both aggrecanases in human cartilage explants has suggested that both ADAMTS-4 and ADAMTS-5 may contribute to the structural damage associated with OA in humans (Song et al., 2007).

Although we do not fully understand the initial signals triggering the increased catabolic activity within OA cartilage, recent studies have revealed a novel mechanism by which ECM breakdown may be promoted. Transgenic mice lacking syndecan-4 were shown to be protected from proteoglycan loss via a marked decrease in ADAMTS-5 activity. Since syndecan-4 is associated with the hypertrophic chondrocyte phenotype, it is suggested that the alteration in the chondrocyte phenotype may trigger early aggrecan degradation through the increased retention of aggrecanases (Bertrand et al., 2010;Echtermeyer et al., 2009).

Inflammatory mediators of OA

The increased activity of inflammatory molecules during OA is postulated to be an important factor leading to the degradation of the ECM and phenotypic modulation of chondrocytes (Loeser, 2006). Although joint inflammation is generally considered to be secondary to cartilage degradation, evidence from a number of *in vivo* and *in vitro* studies indicate that chondrocytes may both produce and respond to cytokine and chemokines found within OA joints.

In addition to the production and secretion into the synovial fluid of IL-1, TNFα and IL-6 by OA synoviocytes (Sakkas et al., 1998), OA chondrocytes, especially those found in clonal clusters, were shown to express IL-1, IL-1β converting enzyme, IL-1 receptor type I, TNFα

and IL-6 (Guerne et al., 1990;Moos et al., 1999). IL-1 is found at suitable concentrations to induce MMP and ADAMTS expression (Tetlow et al., 2001). Cartilage explant studies have demonstrated that exposure to IL-1 causes the rapid depletion of ECM proteoglycan content (Saklatvala, 1987). IL-1β has been shown to suppress a number of genes associated with the differentiated chondrocyte phenotype including COL2A1, whilst also upregulating the expression of other proinflammatory cytokines and chemokines, including IL-6 and IL-8 (Goldring and Goldring, 2004). OA cartilage may be particularly susceptible to the influence of TNFα signalling since both p55 TNFα receptors and the TNFα convertase enzyme (TACE) are seen to be upregulated at mRNA level, in correlation with the susceptibility of cartilage explants to TNFα induced proteoglycan loss (Webb et al., 1997).

IL-1 and TNFα are potent stimulators of nitric oxide (NO) and prostaglandin production in cartilage (Palmer et al., 1993). Nitric oxide synthase (iNOS) is upregulated in OA cartilage compared to both normal and RA cartilage (Amin et al., 1995;Melchiorri et al., 1998) and results in persistent biological effects including post-translational modifications of the collagen type II network (Hughes et al., 2010). IL-1 induced NO activity inhibits aggrecan synthesis, however the blockade of NO production also resulted in an increase in proteolysis suggesting that IL-1 and NO activity may have a homeostatic role (Abramson, 2008;Taskiran et al., 1994). The recombinant human IL-1 receptor anatagonist (IL-1Ra), anakinra, had been demonstrated to reduce joint inflammation and cartilage erosion in RA patients (Jiang et al., 2000), whilst several *in vitro* and animal models of OA were subjected to anakinra treatment which suggested a beneficial effect of IL-1 blockade on cartilage structural integrity. Intra-articular injection of a human recombinant IL-1Ra into a canine OA model resulted in the decrease in osteophyte and cartilage lesion formation together with a reduction in collagenase activity in comparison with placebo treated controls (Caron et al., 1996). However, in a multi-centre trial for the use of anakinra in the treatment of

OA, no significant improvements were observed in knee pain or cartilage matrix turnover (Chevalier et al., 2009). In fact, further evidence from mice show that gene deletion of IL-1, IL-1 converting enzyme, iNOS or stromelysin-1 accelerates the development of cartilage lesions in surgical models of OA (Clements et al., 2003). This evidence again suggests that low levels of IL-1 may be required for the maintenance of homeostatic ECM turnover in normal cartilage.

Conflicting evidence from IL-6 studies have revealed that the IL-1 induced cytokine may also have a homeostatic role in cartilage. IL-6 is found to be upregulated in OA cartilage and was shown to reduce proteoglycan synthesis in normal cartilage *in vitro* (Guerne et al., 1990;Nietfeld et al., 1990). Furthermore, IL-6 is regarded as a key mediator of inflammatory arthritis (de Hooge et al., 2000). However, *in vitro* studies using human OA chondrocytes suggested that IL-6 may in fact reduce IL-1 induced proteoglycan breakdown, and induce expression of TIMP-1 (Silacci et al., 1998). Most importantly, IL-6 knockout mouse studies by Van de Loo et al., and de Hooge et al. have shown that mice lacking IL-6 undergo higher proteoglycan losses during the onset of zymogen-induced arthritis (ZIA), and during age-related OA (de Hooge et al., 2005;van de Loo et al., 1997). This evidence may be indicative of an impaired repair response in IL-6 -/- mice, thus suggesting a protective homeostatic role for IL-6 in cartilage prior to the upregulation of catabolism during OA.

Introduction

Mechanical Load and Joint Surface Trauma

The articular cartilage surface plays an essential role in the distribution of load transfer through the joint. Evidence suggests that conditions resulting in the increased and/or altered load patterns applied to the cartilage surface may accelerate the initiation and development of OA (Kerin et al., 2002). Injuries to the knee menisci or ligaments are known to predispose patients to OA through the destabilisation of joint alignment (Englund et al., 2009;Neuman et al., 2009) . Chondrocytes respond to direct biomechanical stimuli by upregulating their synthetic activity and their production of inflammatory cytokines. It is widely regarded that impact loading stimulates the depletion of cartilage proteoglycan content, whereas cyclic compressive loading may upregulate matrix synthesis (Guilak et al., 2004). Chondrocytes may respond to mechanical stimuli through a number of receptors including integrins, which are also receptors for ECM components such as type II collagen fragments and fibronectin, known to trigger an increased expression of ECM proteases (Pulai et al., 2005).

Post-traumatic secondary OA is known to develop in some individuals following damage to the articular cartilage surface (Dell'accio and Vincent, 2010;Ding et al., 2006;Ding et al., 2008). It had long been believed that joint surface defects (JSD) were unable to heal and would lead to inevitable cartilage degeneration and OA (Dell'accio and Vincent, 2010), however more recent studies have revealed that spontaneous healing does occur in certain individuals (Messner and Maletius, 1996;Shelbourne et al., 2003). The varying outcomes following JSDs in different patients can be partly explained through the consideration of underlying risk factors such as age, obesity, defect size and depth and joint malalignment. However, *in vitro* and *in vivo* models of joint surface injury suggest the involvement of underlying morphogenetic and inflammatory mechanisms which may influence healing outcome. The response of articular cartilage to sharp injury was first studied by Meachin et al. and Mankin et al., who showed that cartilage injury resulted in

interterritorial proteoglycan loss, and necrosis followed by increased proliferation of chondrocytes close to the site of cartilage damage respectively (Mankin, 1982;Meachim, 1963). This evidence suggested that chondrocytes were responsive to injury.

Recent studies have revealed the activation of morphogenetic signalling mechanisms following cartilage injury. Vincent et al. have described the release of perlecan-bound FGF-2 from the pericellular matrix following mechanical injury, which subsequently upregulated MAPK activity (Vincent et al., 2002;Vincent et al., 2007). Dell'Accio et al. used an adult human ex vivo cartilage injury model to demonstrate an increase in both BMP and Wnt signalling activity (Dell'accio et al., 2006). BMPs are well studied as anabolic mediators within cartilage and have been shown to be required for stable cartilage homeostasis (Erlacher et al., 1998;Lories and Luyten, 2005;Rountree et al., 2004). In addition, BMP signalling is an important modulator of chondroprogenitor recruitment during embryonic skeletogenesis (Tsumaki et al., 1999), and is associated with *in vivo* chondrocyte phenotypic stability (Dell'accio et al., 2001). These findings indicate that ECM production, progenitor cell recruitment and chondrocyte phenotypic preservation are all potential roles for BMP signalling during JSD repair. However, the upregulation of BMP-2 following cartilage explant exposure to IL-1 and TNFα, which are also produced following cartilage injury, suggests that any anabolic response of BMP-2 may be disrupted by injury induced inflammatory factors (Fukui et al., 2003).

Ex vivo cartilage injury resulted in the consistent downregulation of the Wnt inhibitor FRZB and the increase in Wnt transcriptional targets Axin2 and c-Jun, consitent with studies revealing an association of a FRZB loss of function polymorphism with hip OA in humans (Loughlin et al., 2004). Genetic studies targeting FRZB have since been supported with *in vivo* evidence from mice in three models of OA, which show an increase in Wnt signalling, proteoglycan loss, MMP-3 activity and cortical bone thickness following the targetted

deletion of FRZB (Lories et al., 2007). Further work revealed the upregulation of WNT-16 in ex vivo injured cartilage explants, and in human OA cartilage confirming that molecular responses to cartilage injury may be replicated during OA (Dell'accio et al., 2008). Wnt activity involved in limb regeneration and wound healing is subject to tight regulation, so it is reasonable to expect the involvement of canonical Wnts in cartilage repair would also need to be highly controlled. It is possible that unregulated Wnt signalling may increase the risk of OA development through the supression of chondrogenesis and cartilage destruction (Enomoto-Iwamoto et al., 2002;Zhu et al., 2008), or via increased but unregulated chondrogenesis (Yano et al., 2005;Zhu et al., 2009).

ELR+ CXC Chemokine Signalling

Chemokines are a family of structurally related chemotactic cytokines ranging from 8 to 10kDa in size that are involved in chemotaxis, proliferation, maturation, differentiation, apoptosis and malignant transformation and angiogenesis. Chemokines are classified into 4 subgroups, according to the arrangement of cysteines found at the N-terminus (CC, CXC, CX3X and XC). Chemokine receptors are 7-transmembrane G-protein coupled receptors (GPCR) and are classified accordingly into CCR, CXCR, CX3CR and CR families (Murphy et al., 2000;Murphy, 2002). CXC chemokines are futher divided into ELR+ (angiogenic) and ELR- (angiostatic) subgroups, based on the presence or absence of glutamic acid-leucine-arginine (ELR) sequence motif following the first N-terminus cysteine residue (Balkwill, 2004;Strieter et al., 1995;Thelen, 2001). At present, more than 30 chemokines have been identified which have been shown to interact with at least 18 different seven-transmembrane domain G-protein coupled receptors in humans (Figure 7).

Figure 7. Chemokine receptors and ligands. The chemokine wheel depicts the ligand-binding patterns of G-protein coupled seven-transmembrane domain chemokine receptors. CXCR1 may be activated by CXCL6 and CXCL8 only, whereas CXCR2 interacts with CXCL1, 2, 3, 5, 6, 7 and 8. Figure from (Balkwill, 2004).

Seven human ELR+ CXC chemokines bind to the chemokine receptor CXCR2 and activate neutrophil granulocytes. Two of these ligands, CXCL6 and CXCL8 also bind to CXCR1. Competition binding assays and calcium flux measurements have shown that CXCR2 is relatively non selective for CXCL8 versus other ELR+ CXC chemokines however CXCR1 is shown to be highly selective for CXCL8 against CXCL1 (Ahuja and Murphy, 1996;Lee et al., 1992), whilst CXCL6 is an equipotent agonist to both (Wuyts et al., 1997). Genes encoding all ELR+ CXC chemokine ligands are found clustered within the human chromosome 4 (O'Donovan et al., 1999). Until recently, it was believed that neutrophil migration in the

mouse was controlled through only one receptor, the murine homolog of CXCR2, which had been shown to bind with high affinity to the mouse ELR+ CXC chemokines which are homologs of human CXCL1. Since homologs of human CXCL8 are thought to be missing in the mouse, it was assumed that only one ELR+ CXC chemokine receptor was required. However, two different groups reported the cloning and characterisation of a mouse CXCR1 homolog and it has since been shown to be functional and may be activated using human CXCL6 and CXCL8 (Bozic et al., 1994;Fan et al., 2007;Fu et al., 2005). Since the CXCR2-/- mutant mouse does have detectable phenotypes in wound healing and neutrophil chemotaxis (Cacalano et al., 1994;Devalaraja et al., 2000), it is unclear whether these phenotypes are due to only a partial redundancy of function or to a sort of "haploinsufficiency", whereby the CXCR1 ortholog cannot compensate fully for the absence of CXCR2, particularly in conditions of challenge.

Upon secretion, the potency of chemotactic activity of CXC chemokines is increased by the proteolytic modification at the N-terminus. More than 10 different modified forms of CXCL8 have been found to occur naturally, including truncated, elongated and dieminated forms (Proost et al., 2008;Van et al., 1989). Neutrophil accumulation upon intra-air pouch injection of CXCL8 is less prominent in MMP-8 deficient mice in comparison with wild type. Neutrophil responsiveness remained unaffected in the absence of MMP-8 upon the injection of a truncated form of CXCL8 suggesting that MMP-8 activity has a role in chemokine processing and activity regulation (Mortier et al., 2011;Tester et al., 2007).

Chemokines can form robust interactions with GAG chains within the ECM and on endothelial cells (Kuschert et al., 1999). Heparan sulphate GAG chains (HSPGs) have a high sequence diversity that allows for interactions with many proteins including FGFs, Wnts and BMPs (Kronenberg, 2003). Chemokines are able to interact with HSPGs on the cell surface and within the ECM (Handel et al., 2005). There are several ways by which HSPGs

may modulate chemokine activity, primarily studied in inflammation. Firstly, it is suggested that chemokines may be unable to function efficiently during inflammation in the absence of HSPGs. Interactions may protect chemokines from proteolysis, and induce their oligomerisation that has been shown to maximise their signalling activity (Hoogewerf et al., 1997;Proudfoot et al., 2003;Webb et al., 1993). Secondly, HSPGs play a vital role in immobilising chemokines upon the cell surface and may act to establish chemokine gradients on the vascular endothelium. This HSPG mediated chemokine display is required for the directional migration of leukocytes through the blood vessel walls (Middleton et al., 1997;Wang et al., 2005).

CXCR1/2 Signalling Pathway

The biological effects of ELR+ CXC chemokines are mediated through the GPCRs CXCR1 and CXCR2. These receptors share a considerable similarity in their structure, suggesting that gene duplication may be responsible for the presence of two CXCL8 receptors in humans. Ligand binding induces conformational changes in CXCR1/2 which exposes epitopes on the intracellular loops and C-terminal of the receptor that interact with G-proteins. This change causes the release of the α and the βγ subunits from the G-protein complex which in turn activate additional downstream intracellular signalling pathways (Ribeiro-Neto and Rodbell, 1989). The classical chemotactic response of cells to CXC chemokine signalling is reduced in the presence of the Gαi inhibitor, pertussis toxin (PTX) suggesting that the Gαi subunit is largely responsible for chemokine induced cell migration. Other responses to CXCL8 signalling are unaffected by PTX, however other Gα subunit forms, and the release of the βγ G-protein subunits are now regarded as important activators of numerous intracellular pathways (Neptune et al., 1999;Schraufstatter et al., 2001).
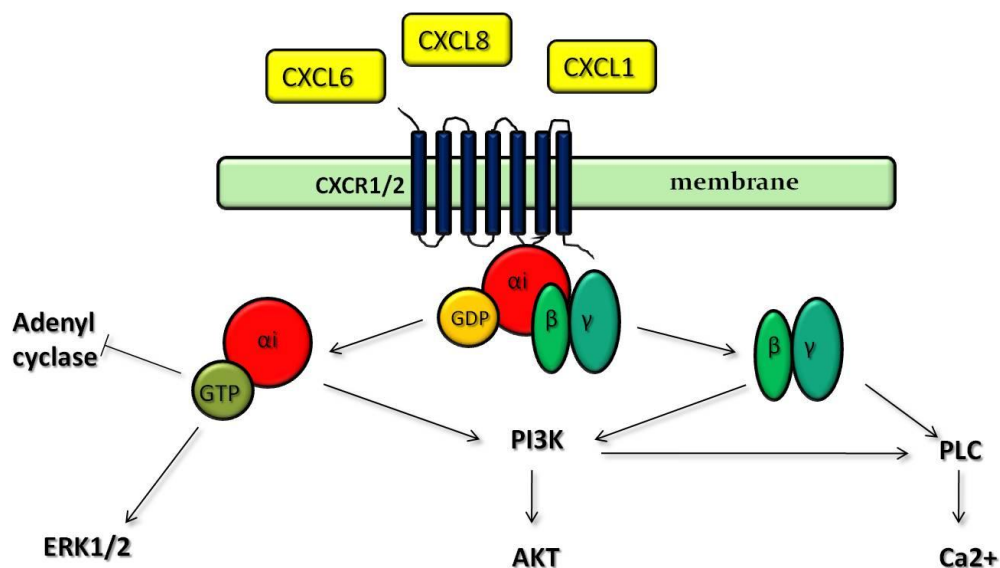
Figure 8. CXCR1/2 downstream signalling pathways. ELR+ CXC chemokines including CXCL1, 6 and 8 bind to CXCR1 and/pr CXCR2 receptors. G-protein α and βγ subunit dissociation activate PI3K, MAPK and PLC mediated signalling pathways, driving downstream signalling cascade including ERK and AKT phosphorylation, and intracellular Ca^{2+} release.

ELR+ CXC chemokines have a wide range of intracellular signalling targets (reviewed in (Waugh and Wilson, 2008)). Phosphatidylinositol-3 kinase (PI3K) is a principal target and is a principal effector of CXCL8 induced neutrophil chemotaxis, resulting in the increased phosphorylation of PKB/Akt (Knall et al., 1997). The mitogen-activated protein kinase (MAPK) serine/threonine kinase signalling cascade is activated in response to CXCL8 ligand binding. Downstream phosphorylation of ERK1/2 has been detected in both neutrophils and in cancer cells, linking CXCR1/2 signalling to E2F and activator protein transcription factors, whose function is to upregulate genes implicated in cell proliferation (Luppi et al., 2007). Gα proteins also link CXC chemokine signalling to phospholipase C, which promotes the conversion of the membrane associated lipids to diacylglycerol and inositol

trisphosphate. This in turn results in calcium mobilisation and the phospholylation of protein kinase C (PKC) (Richardson et al., 1998).

The novel concept of 'functional selectivity' is currently under investigation within the pharmacological study of GPCR signalling (for review see (Allegretti et al., 2008)). It is predicted that, for example, different CXC chemokine ligands may induce different receptor conformations which may consequently activate varying downstream signalling pathways (Baker and Hill, 2007;Mailman, 2007). The functional effects of ligand-receptor binding may be determined by the type of Gα subunit associated with the receptor and by the three-dimensional changes in the receptor C-terminus following binding. Lane et al. demonstrated that the inhibition of PI3K significantly inhibits CXCL8-induced chemotaxis in neutrophils, whereas the blockade of chemoattractant-induced Ca^{2+} release in fact increased leukocyte chemotaxis, suggesting that chemotaxis is dependant upon the downstream PI3K/AKT pathway and not on calcium signalling (Jiang et al., 1997;Lane et al., 2006), providing an opportunity for additional selectivity of phentypic outcome. This type of pathway specific behaviour is leading to the increased study of allosteric inhibition of GPCRs, whereby some but not all downstream signalling activities may be modulated (Kenakin, 2007).

Following activation of CXCR1 and CXCR2, receptors are internalised and are either recycled back to the cell surface or are targeted for degradation, providing an additional level of regulation for CXCR1/2 signalling. Exposure of cells to below saturation ligand concentrations results in the movement of the receptor into clathrin-coated pits which are directed through the early endosome and onto the recycling endosome where they may be trafficked back to and re-expressed on the cell surface. Prolonged saturation of the receptors causes a significant portion of CXCR1/2 to be directed to the late endosome, followed by movement into the lysosome where they are degraded (Richmond et al.,

2004). The chemotactic response following CXCR2 activation may be inhibited following an internalisation-imparing mutation to CXCR2, indicating that receptor recycling is necessary for chemotaxis. A study by Feniger-Barish et al., revealed an interesting mechansim by which CXCR1 and CXCR2 are differentially regulated following the binding of CXCL6, a ligand for both receptors. CXCL8 is regarded as a potent activator of both CXCR1 and CXCR2 and is seen to induce rapid internalisation of both receptors. On the other hand, CXCL6 had a low ability to induce internalisation of CXCR1 in comparison with CXCR2, correlating with previous data showing that CXCL6 is a less potent activator of CXCR1 in comparion with CXCR2 (Feniger-Barish et al., 2000;Wuyts et al., 1998). The divergent abilities of CXCL6 and CXCL8 to induce chemotaxis and receptor internalisation may represent a fine-control mechanism by which different CXC chemokine ligand/receptor interations may mediate both homeostatic and inflammatory responses.

ELR+ CXC Chemokines in Inflammatory and Non-Inflammatory Roles

CXCL8 was first identified as a neutrophil activating factor, triggering chemotaxis, degranulation and superoxide formation during the inflammatory response (Thelen, 2001). It is produced in large amounts in response to inflammatory stimuli such as IL-1 and TNFα, and is present in high quantities in synovial tissue and fluid from RA patients (Endo et al., 1991;Koch et al., 1991). A single intra-articular injection of CXCL8 can initiate an inflammatory arthritis similar to RA in rabbits in a dose dependant manner, mediated by an increase in neutrophils and mononuclear cells within joints (Kraan et al., 2001). The GAG binding properties of CXC chemokines allows for the creation of a chemotactic gradient upon endothelial cells which increases the rate at which neutrophil infiltration may occur.

Introduction

CXCL1 and CXCL8 have been shown to be strong angiogenic factors (for review see (Strieter et al., 2005)). VEGF activation of endothelial cells promotes the anti-apoptotic molecule Bcl-2, which in turn promotes endothelial expression of CXCL8. CXCL8 functions in an autocrine/paracrine manner to promote further CXCL8 expression and to enhance endothelial cell survival and proliferation (Nor et al., 2001;Heidemann et al., 2003). Since all ELR+ CXC chemokines are pro-angiogenic, it is accepted that CXCR2 is the primary receptor involved in endothelial cell migration and survival (Addison et al., 2000).

As potent mediators of chemotaxis and angiogenesis leading to the recruitment and activation of leukocytes and pro-inflammatory mediators, CXC chemokines are prime targets for study within cancer biology (for review see (Balkwill, 2004)). CXC chemokines have been linked to a wide range of roles within cancer biology which are fundamental to the survival and metastasis of tumour cells, from which many were originally purified (Proost et al., 1993;Richmond and Thomas, 1986). Cell survival in hypoxic conditions was shown to be promoted by HIF-1 induced CXCR1 and CXCR2 expression in prostate cancer (Maxwell et al., 2007). The upregulation of MMP expression has been strongly linked to CXCR1/2 activity, whereby protease activity is required for ECM breakdown, subsequently required for angiogenesis and tumour cell invasion (Galvez et al., 2005;Li et al., 2003). CXCL8 has been implicated as an autocrine growth factor for human colon carcinoma cells *in vitro* (Brew et al., 2000). This wide range of CXC chemokine mediated cellular responses suggests that any pharmaceutical targeting of chemokine signalling towards the aim of reducing their pro-inflammatory activities, must carefully examine other potential outcomes of CXC chemokine signalling inhibition, particularly in cellular phenotypic control and survival patterns.

ELR+ CXC chemokines are active in embryonic development prior to the maturation of the adult immune system. CXC chemokines have been implicated in gamete migration, whilst

CXCL1 was shown to participate in the control of spatial and temporal organisation of oligodendrocyte precursor proliferation (Kunwar et al., 2006;Robinson and Franic, 2001). Although these functions are largely based upon chemotaxis, it is evident that CXC chemokine signalling must not be solely regarded as a mediator of inflammation.

CXC Chemokines in Cartilage Biology

Human chondrocytes have been shown to express functional CXC chemokine receptors (Borzi et al., 2000) and ligands which are believed to be upregulated in both OA and RA chondrocytes (Borzi et al., 1999), most likely due to the influence of pro-inflammatory stimuli including IL-1 and TNFα (De Ceuninck et al., 2004). The possible increase in CXCR1 in OA cartilage, leading to increased MMP activity, suggested that chemokines within cartilage may contribute to a potential catabolic mechanism during arthritis. A phenotypic response to CXCL1 and CXCL8 was revealed by Merz et al., whereby markers of chondrocyte hypertrophy including type X collagen, alkaline phosphatase and MMP-13 expression were upregulated *in vitro* in CXCL1 and CXCL8 treated normal chondrocytes (Merz et al., 2003). Silvestri et al. reported that a relatively high percentage of human normal and OA chondrocytes express CXCR1 and CXCR2, suggesting that they may be implicated in normal physiological processes such as matrix remodelling (Silvestri et al., 2003). The reduced receptor levels found in OA samples however, suggests that this signalling pathway may be involved in a phenotypic maintenance that is lost during pathology. The study suggests that CXC chemokine signalling, in combination with other CC chemokine signalling pathways, may respond via either an anabolic or catabolic pathway, depending upon ligand type, dose and exposure duration (Silvestri et al., 2003). Within this study, a large variation was observed between different OA samples. Since OA may be regarded as a syndrome, where cartilage destruction results from the cumulation

of a number of genetic, mechanical and biochemical factors, it should be expected that variation in the inflammatory contribution to each case of disease may account for such differences in chemokine signalling activity, and its contribution to pathogenic mechanisms.

Preliminary data leading towards this study

Cell based cartilage repair and the role of chondrocyte phenotypic stability in outcome determination

Autologous chondrocyte implantation (ACI) is a cell based technique used to achieve biological repair of articular cartilage defects. The technology requires the harvesting of articular chondrocytes from a healthy non-load bearing area, which are enzymatically digested from the cartilage ECM and expanded *in vitro* to provide a ten-fold increase in cell number. Up to five million chondrocytes are then injected into the cartilage defect and covered with a periosteal flap, harvested from the medial tibia. Dell'Accio et al. linked the expression of a number of molecular markers to the capacity of *in vitro* expanded chondrocytes to form ectopic cartilage explants when implanted into the quadriceps of nude mice (Dell'accio et al., 2001). These findings were subsequently linked to improved clinical outcome in ACI (Saris et al., 2008), thereby establishing the importance of the capacity of articular chondrocytes to retain their phenotypic stability in culture in determining the potency of expanded chondrocyte preparations used in ACI.

Interestingly, the molecular marker profile characterising the stable chondrocyte phenotype included the CXC chemokine ligands CXCL8, CXCL6 and CXCL1. Their presence, together with the expression of the corresponding chemokine receptors CXCR1 and CXCR2 on chondrocytes raised the question and promted us to explore whether CXC chemokine signalling has a functional role in the maintenance of the phenotypic stability through culture expansion.

Chapter 2 – Hypothesis and Aims

Hypothesis

This project tests the hypothesis that ELR+ CXC chemokine signalling via CXCR1 and CXCR2 receptors is required for the phenotypic stability of articular chondrocytes and articular cartilage homeostasis

Aims of the study

The overall aim of this study was to investigate the function of ELR+ CXC chemokine signalling in articular cartilage. ELR+ CXC chemokine signalling is primarily regarded as pro-inflammatory and pro-angiogenic, however the loss of expression of CXCL1, CXCL6 and CXCL8 during *in vitro* chondrocyte expansion correlate with the dedifferentiation of chondrocytes, measured by the loss of phenotypic molecular marker expression and the ability of articular chondrocytes to form stable ectopic cartilage *in vivo*, suggesting that it may be required for the maintenance of the chondrocyte phenotype.

The specific aims for this project are as follows:

1. To confirm the expression of functional ELR+ CXC chemokine receptors in human articular chondrocytes.

2. To investigate whether CXCR1/2 mediated signalling is required for the maintenance of the stable chondrocyte phenotype.

3. To investigate whether CXC chemokine ligands may be produced and retained within healthy articular cartilage, thereby preventing the activation of pro-inflammatory responses within the synovial joint.

4. To characterise the articular cartilage phenotype of CXCR2 null mutant mice.

Experimental scheme

CXCR1/2 expression

Human primary chondrocytes   - *PCR*

- *Western blot*

- *Immunofluorescence microscopy*

Human cartilage explants   - *Immunofluorescence microscopy*

C28/I2   - *PCR*

JJ012   - *PCR*

CXCR1/2 activity

Human primary chondrocytes   - *Intracellular calcium mobilisation*

- CXCR1/2 Blocking antibodies

- CXCR1/2 siRNA

- Pertussis toxin

CXCR1/2 loss of function

Human primary chondrocytes   - *Alcian blue staining*

- CXCR1/2 blocking antibodies

- *RT-qPCR*

- CXCR1/2 blocking antibodies

- Pertussis toxin

JJ012   - *Alcian blue staining*

- CXCR1/2 blocking antibodies

- CXCR1/2 siRNA

Porcine primary chondrocytes   - *Alcian blue staining*

- Pertussis toxin

CXCR2-/- mutant mouse   - *Safranin-O staining and histomorphometry*

CXCL6/8 matrix localisation

Human cartilage explants   - *Immunofluorescence microscopy*

- Proteoglycan digestion

Chapter 3 - Materials and Methods

Cartilage samples

Human cartilage was obtained from the femoral condyles of patients undergoing knee joint replacement surgery (ethics approval from the East London & The City Ethics Committee 3). Condyles were rinsed in 2X antibiotic/antimycotic complete DMEM (10% FBS, 2% antibiotic/antimycotic, 1% sodium pyruvate, see Appendix) and full thickness cartilage was dissected from relatively preserved areas using a scalpel. Full thickness slices measuring approximately 10mm in length and 2mm in width were immediately placed into a 4% paraformaldehyde fixation solution in preparation for histology. Remaining cartilage was then finely minced and rinsed again in 2X antibiotic/antimycotic complete DMEM. Porcine cartilage was collected from the metacarpophalangeal joints of pigs from a local abattoir and was washed and minced as descibed above.

Cartilage digestion

Chondrocytes were released from cartilage tissue following a 2-stage digestion protocol. Tissue was first incubated for 30 minutes at 37°C in a complete DMEM solution supplemented with 1mg/ml pronase (Roche), followed by an overnight incubation at 37°C in 0.2% collagenase IV (Gibco, Invitrogen) in complete DMEM. The chondrocyte containing solutions were filtered through 40μm cell strainers (Falcon), separated from the medium by centrifugation and resuspended in 1ml of complete DMEM for cell counting. Cells to be stored in liquid nitrogen were suspended at a density of 250,000 cells/ml of complete DMEM medium which was then mixed with an equal volume of freezing medium (complete DMEM, 20% DMSO, 30% FBS) and stored as 1ml aliquots in cryovials.

Monolayer cell culture

For monolayer culture experiments, human primary chondrocytes and the JJ012 human chondrosarcoma cell line (kindly provided by Professor Joel Block, Chicago) were plated in complete DMEM (5×10^4 cells/cm^2) and cultured at 37°C in a humidified atmosphere containing 5% CO2 until confluent. Once confluent, cells were either used for blockade experiments, or released and replated as follows: medium was removed from the culture vessel and cells were washed twice in sterile PBS (BioWhittaker, Lonza) at room temperature (RT). Cells were covered in 0.25% trypsin-EDTA (Invitrogen) for 4 minutes at 37°C before cells were resuspended and replated in complete DMEM.

Micromass cell culture

For micromass culture, JJ012 or primary human or porcine chondrocytes were plated in 20μl drops of complete DMEM at a cell density of 20million cells/ml into a 24 well plate. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO$_2$ for 3 hours, before 1ml of complete DMEM was slowly added to each well.

CXCR1 and CXCR2 blocking antibody treatment

Chondrocyte confluent monolayer or micromass culture medium was replaced with DMEM supplemented with DMEM supplemented with 1% heat-inactivated FBS, 1% pyruvate and 1% antibiotic-antimycotic solution (see Appendix). After 24 hours incubation, blocking antibodies for the chemokine receptors CXCR1 and/or CXCR2 (R&D systems) were added at a total concentration of 10 μg/ml unless otherwise noted. Monolayer and micromass cells were then cultured for 4 days before phenotypic analysis. Isotype matched mouse IgG (Dako) at an equal concentration was used as a negative control.

CXCR1 and CXCR2 siRNA

Knockdown of CXCR1/2 was achieved using RNA interference. siRNA sequences obtained

from Invitrogen Stealth™ were:

CXCR1 sense 5'-GGAGUUCUUGGCACGUCAUCGUGUU-3'

antisense 5'-AACACGAUGACGUGCCAAGAACUCC-3',

CXCR2 sense 5'-ACCGAGAUUCUGGGCAUCCUUCACA-3'

antisense 5'-UGUGAAGGAUGCCCAGAAUCUCGGU-3'.

A Stealth™ RNAi negative control duplex of medium GC content was used as a negative

control (Invitrogen).

Lipofectamine™ transfection

24 hours prior to transfection, culture medium was removed and replaced with an

antibiotic free medium (Appendix 1). For each transfection well of a 24-well plate, siRNA

oligomer-Lipofectamine™ complexes were prepared separately as follows. 50pmol of

siRNA oligomer was diluted in 50µl of Opti-MEM® I reduced serum medium (Gibco®,

Invitrogen) and mixed gently. The required volume of Lipofectamine™ reagent

(Invitrogen) was diluted in 50µl of Opti-MEM® medium (Gibco, Invitrogen), mixed gently

and incubated at RT for 5 minutes. The diluted oligonucleotide was then combined with

the diluted Lipofectamine™ reagent and incubated at RT for 20 minutes to allow for

transfection complex formation. The oligomer-Lipofectamine™ complexes were then

added into each well containing chondrocytes and mixed by gently rocking the plate back

and forth. A Stealth™ RNAi negative control duplex of medium GC content was used as a

negative control. Cells were incubated at 37°C for 24 hours before kncokdown efficiency

was assessed by RT-PCR and Western blot. Transficiency was optimised using BLOCK-iT™

fluorescent oligo (Invitrogen) as a positive control for siRNA transfection shown to correlate with Stealth™ siRNA uptake.

jetPRIME™ transfection

siRNA was used at a total concentration of 50nM in complete DMEM using jetPRIME™ transfection reagent (Polyplus). Briefly, siRNA was added to the jetPRIME™ buffer at a concentration of 50nM, before jetPRIME™ reagent was added and vortexed to mix. Typically for each well of a 24-well plate, 2μl of reagent was combined with 50μl of buffer containing siRNA. After a 15 minute incubation at RT, 50μl of the transfection mix was added to each well containing 500μl of complete DMEM. A Stealth™ RNAi negative control duplex of medium GC content was used as a negative control. Gene and protein knockdown were assessed by RT-PCR and Western blot after 24 hours. Transfection optimisation was achieved using BLOCK-iT™ fluorescent oligo as a positive control for siRNA transfection.

G-protein blockade

Intracellular CXCR signaling was inhibited at the G-protein level using pertussis toxin from *Bordetella pertussis* (Sigma). Chondrocyte culture medium was replaced with DMEM supplemented with 1% heat-inactivated FBS, 1% pyruvate and 1% antibiotic-antimycotic solution (see Appendix) 24 hours before the cells were treated with pertussis toxin. Although all reagents used were purchased as endotoxin-free, before addition of the pertussis toxin, polymyxin B sulphate (Sigma) was added at a concentration of 50mg/l in order to neutralize any endotoxins. Pertussis toxin was added to the culture medium at a concentration of 1μg/ml and the cells were cultured for a further 4 days before phenotypic analysis. Cells treated with an equal concentration of PBS vehicle and polymyxin B sulphate were used as a negative control.

Calcium mobilisation assay

CXCR1/2 G-protein dependant calcium mobilisation was measured using the Fluo-4 Direct™ calcium assay (Molecular Probes, Invitrogen). Half of the 100µl of culture medium from each well of cells plated in a 96-well plate was removed and replaced with 50µl of reagent, made by adding 10ml of Fluo-4 Direct™ assay buffer and 200µl of 250mM probenecid stock solution to one bottle of Fluo-4 Direct™ reagent and vortexing to mix. Plates were incubated for 30 minutes at 37°C followed by a further 30 minutes at RT. CXCL8 (10ng/ml) was added as a stimulus 5 minutes before the fluorescence was measured with an excitation wavelength of 495nm and emission wavelength of 530nm using XFluor™ (Tecan Group Ltd.). The difference in fluorescence between CXCL8 stimulated and unstimulated cells was calculated and used to assess CXCL8 induced calcium mobilisation.

Total RNA extraction

Culture medium was removed from 24 well plates in which cells were growing and 1ml of TRIzol® reagent (Invitrogen) was immediately added into each well. Cells were homogenised into the reagent using a syringe and 26G needle and 1ml was transferred into a sterile RNAse free Eppendorf tube. The suspension was kept on ice for 15 minutes and was then certrifuged at 10000g for 10 minutes to remove debris. 200µl chloroform was added to each 1ml of TRIzol®, the sample was shaken vigorously for 20 seconds and left on ice for 2 minutes. Samples were then centrifuged at 10000g for 15 minutes at 4°C in order for the separation of the sample into distinct phenol and aqueous layers. The top red phenolic layer containing RNA was removed and placed into a new Eppendorf tube. The lower clear aqueous layer containing protein and cloudy interphase containing DNA was discarded. 500µl of ice cold isopropan-2-ol was added into each sample. Tubes were vigorously shaken and put on ice for 20 minutes prior to centrifugation at 10000g for 30

minutes at 4°C to precipitate RNA. The supernatant was removed leaving a small white pellet which was washed in 1ml of 70% ethanol in RNAse-free water and spun for 5 minutes at 10000g. The ethanol supernatant was removed and the pellets were left to air dry for 20 minutes before being resuspended in 12µl of RNAse-free water and stored at -80°C. 1µl was used to measure RNA concentration using a NanoDrop spectrophotometer (Thermo Scientific). The purity of the RNA was estimated using the ratio between the absorbance at 260/280 nm, where a ratio of 2 indicates pure RNA, and ratios of less than 1.6 indicates poor RNA quality.

Reverse transcription cDNA synthesis

cDNA was produced by reverse transcription using the Thermoscript™ RT-PCR System for first strand cDNA synthesis (Invitrogen, UK). For each sample, 1µg of total RNA, diluted into 1µl of RNAse-free water, was mixed with 1µl of Oligo(DT)20 primers (50mM) and 2µl of dNTPs (10mM) and made up to a total volume of 12µl with RNAse-free water in a PCR tube. Sample mixes were incubated at 65°C for 5 minutes before immediately being placed on ice. 8µl of a reaction master mix containing (per sample) 4µl of 5X cDNA synthesis buffer, 1µl ThermoScript™ reverse transcriptase (15U/µl), 1µl of 0.1M DTT, 1µl of RNAse OUT™ ribonuclease inhibitor (40U/µl) and 1µl of DEPC-treated water. Samples were mixed and spun down in a centrifuge and incubated for 1 hour at 50°C in a PCR machine (Applied Biosystems 9700), followed by 5 minutes at 85°C in order to inactivate the reverse transcriptase. To clear the resulting cDNA of any remaining RNA, 1µl of *E.coli* RNAse H (2U/µl) was added to each sample before a 20 minute incubation at 37°C. Samples were made up to a total volume of 60µl in RNAse-free water and stored at -20°C.

Quantitative real time PCR

For quantitative real time analysis of target gene expression, primers were designed using

VectorNTI™ (Invitrogen, UK) (

Table **1**) for the following properties:

- Resulting amplicon size of 100-300 base pairs

- GC base pair content of 45-65%

- Proximity to the poly-A sequence

- A target annealing temperature of 60°C.

Table 1. Sequences of the primers used for PCR reactions and their product sizes.

Genes	Sense Primer	Antisense primer	Product Size (bp)
Col2A1	5'-CTGCTCGTCGCCGCTGTCCTT-3'	5'-AAGGGTCCCAGGTTCTCCATC-3'	511
Aggrecan	5'-GTTGTCATCAGCACCAGCATC-3'	5'-ACCACACAGTCCTCTCCAGC-3'	509
SOX 9	5'-GAACGCACATCAAGACGGAG-3'	5'-TCTCGTTGATTTCGCTGCTC-3'	631
β-actin	5'-CACGGCTGCTTCCAGCTC-3'	5'-CACAGGACTCCATGCCCAG-3'	134
CXCL6	5'-AAAATTGCCCAGTCTTCAGC-3'	5'-CCGACACCTAAAGCATACCT-3'	360
CXCL8	5'-CATTGCCAGCTGTGTTGGTA-3'	5'-AGTTTCAACCAGCAAGA-3'	112
CXCR1	5'-GGAAAGAATAACCAACACCC-3'	5'-ATCAGAGCACACAGAGCCAC-3'	410
CXCR2	5'-GCAGAAGACAGTATGGCAGC-3'	5'-CCATTAAACCGTCACTTCCC-3'	459

To prepare the reaction mix, 1μl of sample cDNA was added per well using a 384-well PCR plate (Applied Biosystems) on ice before 0.5μl of forward and 0.5μl of reverse primers and 8μl of a reaction master mix consisting of 6.61μl of RNAse-free water, 0.04μl of dNTPs (10mM), 1μl of 10X PCR buffer, 0.05μl of Hot Start Polymerase, 0.1μl of 10X SYBR Green (Sigma) and 0.2μl of ROX reference dye (Invitrogen, UK) were added. For each sample, PCRs for each target gene were plated in triplicate alongside a standard curve serial dilution of a positive control sample (1:1, 1:32, 1:1024) in triplicate and a template free water negative control completing each row of wells. The plate was covered with adhesive film (Applied Biosystems), was spun down and kept at 4°C.

The PCR was run using the 7900HT Taqman real time PCR machine (Applied Biosystems) with thermal cycling conditions summarised in Table 2. Results were then analysed using 7900HT Sequence Detection System 2.3 (SDS2.3) software. Within the software, the standard curve of positive control serial dilutions allowed for the conversion of target gene Ct values into absolute gene expression. An average value of gene quantity was taken from each triplicate which was then normalised for β-actin to give a quantitative measurement of target gene expression in relation to the β-actin housekeeping gene expression. Analysis of the melting curve provided within the SDS2.3 readout provided confirmation of specific gene target amplification.

Table 2. Optimal PCR cycling conditions

	Equilibration	Enzyme Activation	40 CYCLES			Primers
			Denaturation	Annealing	Extension	
3 Step 60			30 seconds 96°C	30 seconds 60°C	1 minute 30 seconds 72°C	COL2A1, SOX9, CXCL6, CXCL8, β-actin
2 Step 68	2 minutes 50°C	15 minutes 96°C	30 seconds 96°C	1 minute 30 seconds 68°C		Aggrecan
2 Step 60			30 seconds 96°C	1 minute 30 seconds 60°C		CXCR1, CXCR2

Semi-quantitative PCR

For the testing of primer specificity and for semi-quantitative analysis of target gene expression, the same reaction volumes were prepared as for the real time RT-PCR and were amplified in separate PCR tubes using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems) using the conditions described in Table 2. To analyse, PCR products were run on a 1% agarose gel prepared in 1X Tris acetic acid EDTA (TAE) buffer (Invitrogen) supplemented with Gelred nucleic acid stain (Biotium) diluted 1:10000 into the agarose gel. DNA bands were visualised using a Gel doc 1000 imager (Biorad). Amplicon band sizes in positive control samples were measured by comparison to a 1kb DNA ladder (New England Biolabs).

Alcian Blue staining and quantification

For quantitation of cartilage-specific, highly sulphated glycosaminoglycans I used the alcian blue method as previously described (De Bari et al., 2001). Briefly, micromasses were rinsed twice with PBS, fixed in methanol for 30 minutes at -20°C, washed gently in distilled water and immersed in Alcian blue at pH 0.2 (0.5% Alcian blue 8 GS [Carl Roth, Karlsruhe, Germany] in 1N HCl) overnight at RT. After 24 hours, micromasses were washed three times for 10 minutes each in water. Alcian blue was extracted with 200µl of 6M guanidine HCl in Baxter water for 6 hours at RT. The absorbance of the extracted dye was measured at 630nm using XFluor™.

The quanitification of highly sulphated proteoglycans was then normalised for total micromass protein content using the bicinchoninic (BCA) assay (Pierce, ThermoScientific) using XFluor™. Briefly, 25µl of each sample of guanidine HCl solution retrieved following the extraction of Alcian blue, was added to wells of a 96-well plate. 250µl of BCA solution, prepared by combining the two supplied solutions in a ratio of 50 parts of Reagent A to 1

part of Reagent B, was added onto each sample and mixed thoroughly using a pipette. The

reaction plate was then incubated at 37° for 30 minutes. Absorbance was read at 570nm

using XFluor™ and protein concentration was quantified by comparison to a standard

curve of bovine serum albumin (BSA) diluted into 6M guanidine HCl on the same plate.

Western Blotting

Cells were enzymatically removed from culture and lysed in RIPA buffer (10nM NaCl, 1%

Triton X-100, 0.5% sodium deonycholate, 0.1% SDS, 50nM Tris pH8) containing PhosSTOP

protease inhibitor cocktail tablets (Roche) and Complete Mini EDTA–free phosphatase

inhibitor cocktail tablets (Roche). Total protein concentration of cell lysates was

determined using the BCA assay, requiring a standard curve of BSA diluted into RIPA

buffer. Lysates were prepared for blotting in sample buffer (see Appendix) containing 5%

2-β mercaptoethanol, and run at 150V on a 10% Tris-glycine gel (Invitrogen) contained

within a running buffer (see Appendix) and transferred onto nitrocellulose membrane (GE

Healthcare) for 1 hour at 100V within transfer buffer (see Appendix). Membranes were

blocked for 3 hours in 5% non-fat milk, 0.1% Tween20 PBS solution, then treated with

mouse anti-human CXCR1 or CXCR2 primary antibodies (R&D) at 1:200 dilution in blocking

solution at 4°C overnight, washed in 0.1% Tween20 PBS and incubated with goat anti-

mouse IgG-HRP (Santa Cruz Biotechnology), 1:2000 dilution in blocking solution at RT for

30 minutes. After further washing, HRP bound protein bands were detected using the

chemiluminescent SuperSignal® West Dura Extended Duration Substrate system (Thermo

Scientific). Following instructions from the supplier, 2.5ml of the stable peroxide buffer

was combined with 2.5ml of luminol enhancer. Membranes were submerged in the

solution and incubated at RT for 3 minutes. Membranes were then washed three times for

10 minutes in PBS, blocked for 3 hours in 5% non-fat milk, 0.1% Tween20 PBS and stained

for β-actin for normalisation using rabbit anti-human β-actin primary antibody (Cell Signaling) 1:1000 dilution in blocking solution overnight at 4°C, followed by goat anti-rabbit IgG-HRP (Santa Cruz) 1:2000 dilution in blocking solution for 30 minutes at RT before the membrane was developed as previously described.

Processing of histological samples

CXCR2-/- and wild type BALB/C mouse knee joints (provided by Dr L. Brandolini, Dompé S. P. A.) were dissected immediately after the animals were killed and fixed in formalin. The joints were then washed and decalcified in a formic acid decalcifying buffer (33% formic acid, 13.5% tri sodium citrate) for 24 hours at RT. They were then washed under running water for 24 hours before being placed in formalin prior to paraffin embedding.

Human cartilage explants were fixed in 4% paraformaldehyde in PBS and then processed for paraffin embedding.

Paraffin Embedding

Following fixation and, when appropriate decalcification, the tissues were washed in PBS, placed into embedding cassettes and processed in a Leica TP 1050 tissue processor through the following steps:

1) 70% ethanol, 1 hour x2

2) 80% ethanol, 1 hour x2

3) 95% ethanol, 1 hour x2

4) 100% ethanol, 1 hour x3

5) Xylene, 1 hour x3

6) Paraffin wax (56-58°C), 1 hour 30 mins x2

Following processing, the tissue samples were embedded into liquid paraffin in the desired orientation and placed onto a cold plate for 1 hour to set. Cartilage explants were embedded with their lateral side facing down to ensure sections include the full thickness of the articular cartilage. Mouse knees were orientated into the 'kneeling position' with the anterior aspect of the tibia facing downwards, and the femur angled at 90° and directed upwards. This orientation allowed for the presentation of the patello-femoral and both lateral and medial compartment of the tibio-femoral joints in each section.

Sample sectioning

Paraffin embedded mouse knee joints and human articular cartilage explants were placed onto ice to cool for 30 minutes before they were cut into 5µm serial sections using a Leica RM 2135 microtome (Knowlhill, UK). Each section was floated on a warm water bath (40°C), attached to a Superfrost slide and placed onto a hot plate at 50°C to dry for a minimum of 1 hour. Slides were then stored at RT before histology.

Immunofluorescence staining and imaging

Monolayer chondrocytes

Chondrocytes were plated onto 13mm glass coverslips, sterilised in 70% ethanol and left to dry within the cell culture hood before being placed into 24-well plates. Upon reaching 80% confluency, cells were fixed in 4% paraformaldehyde for 30 minutes at RT, washed in PBS, quenched in PBS supplemented with 50nM ammonium chloride and blocked in protein block (Dako) for 30 minutes. Chondrocyte coverslips were incubated with mouse anti-human CXCR1 or CXCR2 antibodies (R&D) 1:200 diluted in blocking solution at RT for 1 hour in a humid atmosphere, washed extensively in PBS containing 0.2% Triton X-100 and incubated with Cy2 conjugated goat anti-mouse IgG (Santa Cruz) 1:100 dilution for 1 hour at RT in the dark, washed in PBS 0.2% Triton X-100 three times for 10 minutes each in the

dark, counterstained with 10μg/ml propidium iodide for 5 minutes at RT in the dark, washed in PBS three times for 10 minutes in the dark, and mounted in Mowiol.

Cartilage explant sections

Cartilage explant paraffin sections were deparaffinized as follows:

1) Slides with sections were heated on a hot plate at 50°C for 30 minutes.

2) Slides were placed into xylene for 5 minutes x2

3) Slides were placed into 100% ethanol for 5 minutes x2

4) Slides were placed into distilled water for 2 minutes and left to air dry for 40 minutes at RT.

5) Sections were fixed using 4% paraformaldehyde for 10 minutes at RT, followed by two 10 minute washes in PBS.

Sections were placed for 7 min in prewarmed 0.2N HCl in slide mailers in a waterbath at 37°C. The HCl was then replaced with a pre-warmed solution of 100mg/ml pepsin in 0.2N HCl for 45 minutes at 37°C. Each slide was carefully removed, dipped individually into PBS for 2 seconds, followed by distilled water for 2 seconds and left to dry at RT. Sections were post-fixed in buffered 4% paraformadehyde for 10 minutes at RT, washed in PBS, quenched in two 5 minute washes of NH_4Cl and blocked for 1 hour in a blocking solution consisting of PBS with 20% FBS and 0.2% Triton-X100 at RT.

Fixed and digested paraffin sections were incubated overnight at 4°C with mouse anti-human CXCR1 or CXCR2 antibodies (R&D, 1:20 dilution into blocking solution), washed extensively in PBS 0.2% Triton-X and treated with Cy2 conjugated goat anti-mouse IgG secondary antibody (1:100 dilution in blocking solution) for 1 hour at RT, washed three times for 10 minutes each in PBS 0.2% Triton-X, counterstained with 10μg/ml propidium

iodide for 5 minutes at RT, washed three times for 10 minutes in PBS. Coverslips were then mounted using mowiol. Images were acquired at 22°C by either Leica DM5500 Q Confocal microscope using 40X magnification/0.75 numerical aperture, or Olympus BX61 microscope with a fixed exposure using either 10X/0.4 or 20X/0.7 objective lenses, using Cell-P software. Aquisition parameters were set using positive and negative control sections in order to achieve maximum sensitivity and specificity, which was then kept constant during the aquisition of all images in the set. Specifically, the autogain facility was switched off, exposure time was selected as to allow for the negative control to show a barely visible fluorescence, whilst the gain setting was selected using the section exhibiting the strongest fluorescent activity, using the upper limit to prevent saturation and consequent loss of sensitivity. Images were enhanced using Adobe Photoshop for better rendering without altering the relationship of target to control images.

Table 3. Summary of antibodies used for immunofluorescence staining

Target Antigen	Primary Antibody			Secondary Antibody		
	Antibody	Supplier	Dilution	Antibody	Supplier	Dilution
CXCR1	Mouse monoclonal anti-human CXCR1	R&D Systems (Cat No. MAB330)	1:200	Cy2 conjugated goat anti-mouse IgG	Santa Cruz Biotechnology	1:100
CXCR2	Mouse monoclonal anti-human CXCR2	R&D Systems (Cat No. MAB331)	1:200			
CXCL8	Mouse monoclonal anti-human CXCL8	R&D Systems Cat No. MAB208)	1:20			
CXCL6	Mouse monoclonal anti-human CXCL6	R&D Systems Cat No. MAB333)	1:20			

Explant digestion and CXC chemokine immunohistochemistry

Cartilage explant paraffin sections were deparaffinised and digested for 1 hour at 37°C in 10mg/ml pepsin in 0.2N HCl as described previously. For removal of carbohydrate chains, sections were treated with 5mU/ml heparitinase (Seikagaku) in Tris-HCl pH8.0 to remove heparan sulphate, or with 5mU/ml chondroitinase ABC (Sigma) to remove chondroitin sulphate. Sections were post-fixed in buffered 4% paraformadehyde for 10 minutes at RT, washed three times in PBS for 5 min each, quenched in NH$_4$Cl and blocked as previously described. Paraffin sections were incubated overnight at 4°C with either mouse anti-human CXCL8 primary antibody (R&D), or mouse anti-human CXCL6 primary antibody (R&D), 1:20 dilution in blocking buffer, washed extensively in PBS 0.2% Triton-X and incubated for 1 hour at RT with goat anti-mouse Cy2 secondary antibodies (Santa Cruz, 1:100 dilution), counterstained using propidium iodide and mounted as described for CXCR1/2 receptor staining.

Histological staining

Toluidine Blue

In order to identify when the correct level within the sample had been reached during cutting, a fast Toluidine blue stain was applied to the slides. Slides were individually dried above a flame, deparaffinased in xylene for 1 minute and dehydrated in 100% ethanol for 1 minute. Slides were then washed in running water and covered with a drop of Toluidine blue (see Appendix) for 10 seconds. Slides were rinsed in running water again and viewed under the microscope. Slides were left to air dry, cleared in xylene and mounted in DPX.

Safranin O

To stain the highly sulfated, negatively charged glycosaminoglycans within the cartilage, Safranin O staining was used on mouse knee sections. Slides were deparaffinised and dehydrated as described for immunohistochemistry and covered in 0.2% Safranin O solution (see Appendix) for 13 minutes. Slides were washed in distilled water and differentiated in 100% ethanol twice for 5 minutes, cleared in xylene twice for 5 minutes, then air dried and mounted in DPX.

Histomorphometry

Histomorphometric analysis was performed on sections obtained from wild type and CXCR2-/- null mutant mouse knees. Non-consecutive sections from each sample were used to evaluate variability across sections and between animals. In this analysis, articular cartilage thickness of femoral condyles and tibial plateaux and the density of Safranin O staining and hence sulphated proteoglycan content were compared using Cell-P (Olympus, UK) and ImageJ software. To this end, ImageJ was used to firstly measure the thickness of articular cartilage from each compartment and the growth plate (Figure 32). To measure Safranin-O staining intensity, a cross section of each image was selected within Image J, positive colour intensity was plotted across the section as demonstrated in Figure 33, and the area above a standard threshold measured for each individual image was recorded for each cartilage compartment. Safranin-O staining density was calculated by dividing the area of positive staining plotted above the threshold, by the thickness recorded previously. The staining density of each articular cartilage compartment was then normalised for staining density of the growth plate in that section, thus accounting for staining intensity variation between sections from the same knee.

Histological scoring

The structural integrity of the joint surface in mice was assessed using a well validated Chambers histological scoring system which is summarised in Table 4. Scoring was performed independantly by two observers (Dr Dell'Accio and myself).

Table 4. Chambers Score

Description	Points
No damage	0
Loss of metachromatic staining without structural changes	0.5
Roughened articular surface and small fibrillations	1
Fibrillations down to the layer immediately below superficial layer	2
Loss of surface lamina and fibrillations extending down to the calcified cartilage	3
Major fibrillations and cartilage erosion down to the subchondral bone	4
Major fibrillations and erosion of up to 80% of the cartilage	5
More than 80% loss of cartilage	6

Chambers et al., 2001

Statistical analysis

Parametric data were subjected to the student T-test. For dose response analysis, ANOVA analysis was used with the Dunnet post test applied. P values of less than 0.05 were considered significant, * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

Chapter 4 – Results

Expression of functional ELR+ CXC chemokine receptors and ligands in human articular chondrocytes

Experimental layout.

Previous studies have revealed the expression of the CXC chemokine receptors, CXCR1 and CXCR2, in articular chondrocytes (Borzi et al., 2000;Merz et al., 2003). In preparation for my investigation into the function of these receptors within cartilage, it was important to establish whether both ELR+ CXC chemokine receptors are expressed at mRNA and protein level by chondrocytes cultured within our lab, and to examine the distribution of these receptors both in subcellular compartments and within cartilage tissue. To this end, we began by using reverse transcription PCR and Western blotting to confirm CXCR1 and CXCR2 expression in monolayer cultured human chondrocytes. Immunohistochemistry was used to detect CXCR1 and CXCR2 in human cartilage explants in order to verify that receptor expression occurs within cartilage tissue.

As an indication of the importance of any CXCR1/2 signalling activity in relation to chondrocyte stability, real time RT-PCR was used to compare the expression of CXCL6 and CXCL8 mRNA in early and late passage chondrocytes. Late passage chondrocytes are known to lose the expression of key molecular markers of chondrocyte stability, notably type II collagen, aggrecan, BMP2, FGFR3 and SOX9, in correlation with the failure of passaged cells to form stable cartilage explants when implanted *in vivo* (Dell'accio et al., 2001).

With the aim of revealing an indication into the possible functions of CXCR1/2 signalling in articular chondrocytes, immunofluoresence microscopy and confocal microscopy was used to examine the cellular distribution of CXCR1 and CXCR2 in human early passage monolayer chondrocytes.

Following this, it was necessary to establish whether any CXCR1 and CXCR2 found within the *in vitro* cultured chondrocytes were functional. A calcium mobilisation assay was used as a downstream readout of CXCR1/2 activation following exposure to CXCL8. This approach was also used to assess the efficiency of CXCR1/2 blocking methods in preparation for later experiments, and to determine the redundancy of each receptor within chondrocytes.

ELR+ CXC chemokine receptor and ligand expression in human articular chondrocyte monolayers

The ELR+ CXC chemokine receptors, CXCR1 and CXCR2, are primarily studied during neutrophil recruitment during inflammation (Baggiolini et al., 1995;Taub et al., 1996), and in the regulation of angiogenesis and metastasis in various cancers (Balkwill, 2004;Singh et al., 2009;Xie, 2001). Chondrocytes are not known to migrate *in vivo*, therefore it was important to confirm the presence and functional activity of CXCR1 and CXCR2 in articular chondrocytes in culture in our laboratory before investigating any possible functions of the CXCR1/2 signalling pathway using receptor blocking methods.

RNA extracted from human primary chondrocytes cultured in monolayer to full confluence was subject to reverse transcription and semi-quantitative PCR (Figure 9), which confirmed the expression at mRNA level of both CXCR1 and CXCR2 at levels comparable to those found in human neutrophils. Western blotting on cell lysates extracted from confluent human primary chondrocyte monolayers confirmed the expression of CXCR1 and CXCR2 at protein level (Figure 9B).
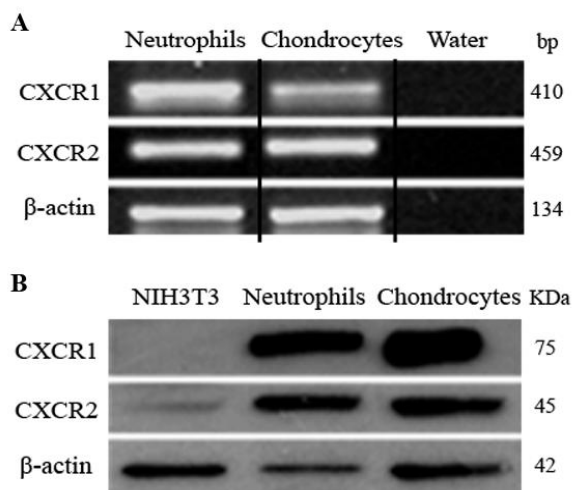
Figure 9. CXC chemokine receptor expression in human chondrocytes. (A) Semi quantitative RT-PCR shows the expression at mRNA level of CXCR1 and CXCR2 in early passage AHAC in comparison to human neutrophils and a water negative control. Human β-actin is included as a loading control. (B) Western blot shows the expression of CXCR1 and CXCR2 at protein level in freshly isolated AHAC in comparison to human neutrophil positive control and NIH3T3 negative control. Data presented are representative of 3 individual donors.

Quantitative real time RT-PCR was used to compare CXC chemokine ligand mRNA levels in early and late passage human articular chondrocyte monolayers (Figure 10). CXCL6 and CXCL8 were found to be expressed in early passage chondrocytes thereby suggesting the presence of a possible autocrine or paracrine signalling mechanism. Interestingly, this expression was found to be lost in late passage chondrocytes in correlation with the loss of phenotypic molecular markers and their capacity to form cartilage *in vivo* (Dell'accio et al., 2001).
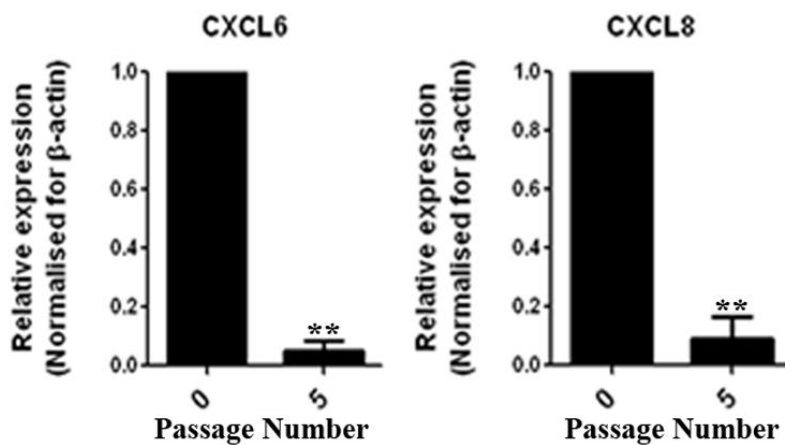
Figure 10. CXC chemokine ligand expression is lost during *in vitro* cell expansion. Real time RT-PCR comparison of CXCL6 and CXCL8 expression in EP and LP-AHAC demonstrates the loss of CXC chemokine ligands during *in vitro* chondrocyte culture. n = 3 **P < 0.01.

CXCR1 and CXCR2 subcellular expression

Confocal microscopy was used to examine the subcellular localisation of CXCR1 and CXCR2 in monolayer cultured human articular chondrocytes. Immunocytochemistry performed on permeabilised cells allowed for the visualisation of receptors both upon the surface of chondrocytes and within the cytoplasm (Figure 11B). Activated receptors are known to be recycled within the cytoplasm before being returned to the cell membrane (Feniger-Barish et al., 2000;Matityahu et al., 2002). In addition to demonstrating receptor recycling, the relative abundance of receptors present within the cytoplasm further supports the hypothesis of a possible autocrine CXCR signalling mechanism within chondrocytes.
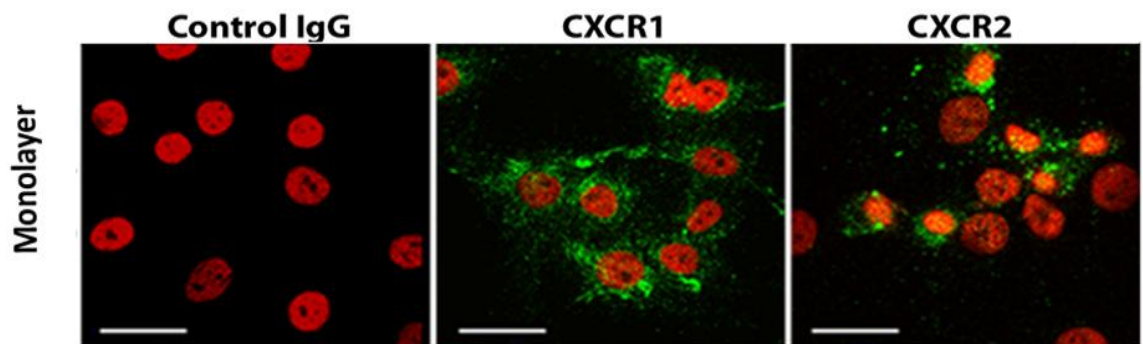
Figure 11. CXC chemokine receptor expression in monolayer chondrocytes. Confocal microscopy of immunofluorescent staining of CXCR1 and CXCR2 in freshly isolated human articular chondrocytes plated in monolayer. Receptors are found to be present within the cytoplasm as well as localised to the cell membrane. Nuclei are counterstained with propidium iodide (red). n = 3. Bar, 20µm.

CXCR1/2 expression in human articular cartilage explants

It is possible that CXCR1 and CXCR2 expression is induced in chondrocytes due to the monolayer culture conditions. Immunohistochemistry was used to identify receptors in full thickness human articular cartilage explants obtained from healthy control donors (Figure 12). CXCR1 was found to be more abundant than CXCR2 within these explants, in keeping with studies demonstrating a higher rate of degradation of CXCR2 following exposure to CXC chemokine ligands in comparison to that of CXCR1 (Chuntharapai and Kim, 1995;Haringman et al., 2004). Similar patterns of expression were found in cartilage explants taken from relatively preserved areas of cartilage obtained from OA donors, suggesting that CXCR1 and CXCR2 expression is not lost during OA pathology, at least at an early stage of disease.
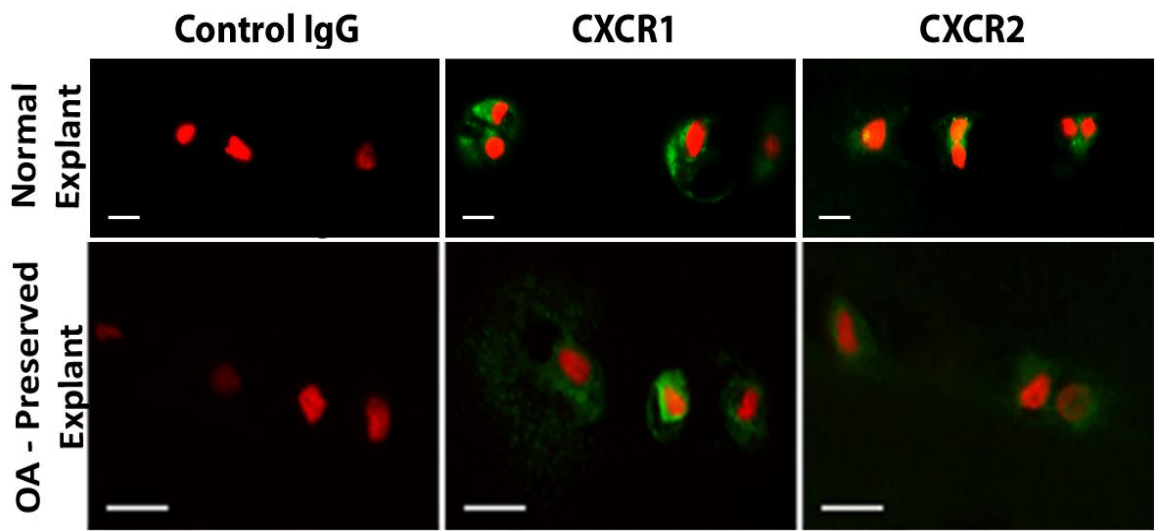
Figure 12. CXC chemokine receptor expression in human articular cartilage explants.

Immunofluorescent staining of CXCR1 and CXCR2 in human articular cartilage explants 5µm paraffin sections from femoral condyles of both healthy and osteoarthritis donors. Nuclei are counterstained with propidium iodide (red). Data presented are represenative of 3 donors. Bar, 20µm.

Interestingly, a clear patterning of CXCR2 was obvserved within the preserved cartilage explant. The receptor was found to be relatively absent within the superficial layer of the articular cartilage, whereas it was present in chondrocytes throughout the middle and deep zones of the cartilage (Figure 13).
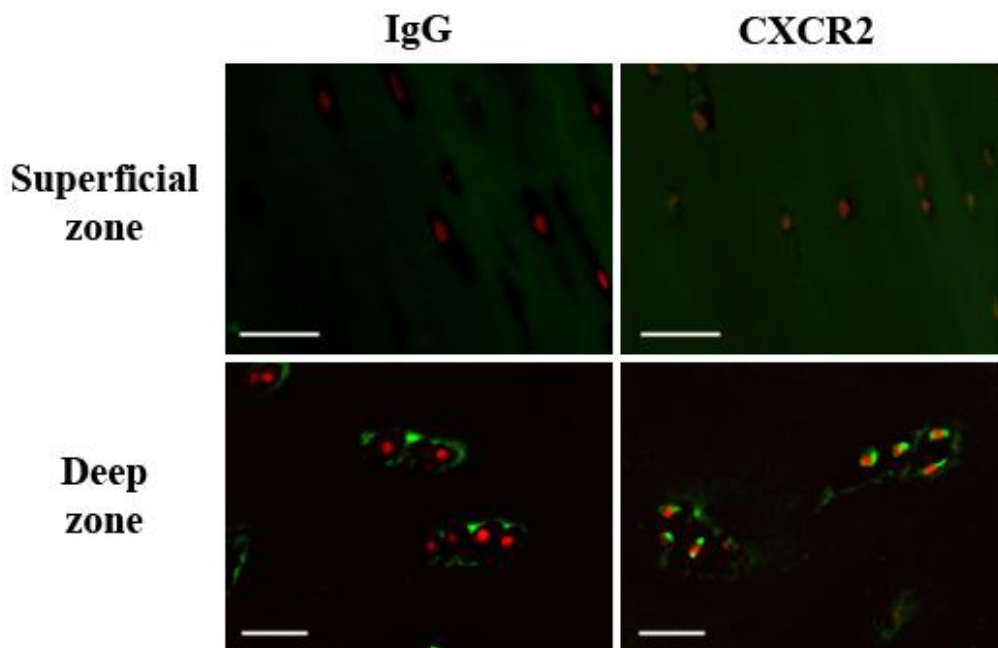
Figure 13.   CXC chemokine receptor distribution within articular cartilage. Immunofluorescent staining comparing CXCR1 and CXCR2 expression between the superficial and deep zones of preserved articular cartilage from an osteoarthritis donor. Nuclei are counterstained with propidium iodide (red). n = 3 Bar, 20μm.

CXCL8 induces calcium mobilisation in articular chondrocytes which is CXCR1/2 and G-protein dependant

Having established that articular chondrocytes express CXCR1/2 in monolayer and cartilage explants, we set out to ascertain whether the CXC chemokine receptors found to be expressed in human articular chondrocytes were functionally active. G-protein coupled ELR+ CXC chemokine receptor activation is known to stimulate calcium mobilisation (Lee et al., 1992;Wu et al., 1993). A calcium mobilisation assay was used as a method of measurement for CXCR1/2 activation (Bacon and Camp, 1990;Wuyts et al., 1997).

Initial time course experiments showed a significant increase in calcium mobilisation within human articular chondrocytes following CXCL8 stimulation, confirming that CXCR1/2 present in monolayer cultured chondrocytes are functionally active. This increase was observed to be greatest at 5 minutes following ligand addition (Figure 14), providing a standard time point for all following calcium mobilisation readings.
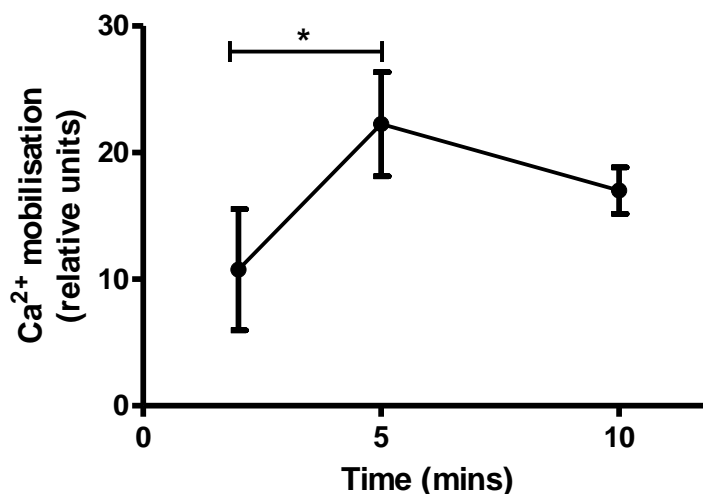
Figure 14. Time course of calcium mobilisation in chondrocytes. Fluo-4 calcium mobilisation in EP-AHAC following the CXCL8 induced release of intracellular Ca^{2+}. Time course analysis from 2 minutes post ligand addition. n = 3 * P < 0.05.

In order to confirm whether calcium mobilisation in human articular chondrocytes is CXCR1/2 dependant, we first used CXCR1 and CXCR2 blocking antibodies to inhibit CXCR1/2 signalling at ligand-receptor level, whilst measuring CXCL8 induced calcium mobilisation. To confirm this data we then used CXCR1 and CXCR2 siRNA to inhibit at specific receptor level. To investigate if CXCL8-induced calcium mobilisation conducted via a G-protein dependant signalling pathway, Pertussis toxin was used to inhibit signalling at intracellular level.

To select concentrations of CXCR1 and CXCR2 blocking antibodies and Pertussis toxin for use in calcium mobilisation analysis, and in later chondrocyte phenotypic analysis, dose response experiments were conducted around previously published concentrations for human neutrophil studies (Becker et al., 1985;Ginestier et al., 2010;Sturm et al., 2005;Wuyts et al., 1997) (Figure 15). Doses of CXCR1/2 lower than the normal published

concentration of 10µl/ml showed the same inhibition of calcium mobilisation of around 50%, whereas a dose dependant response was observed following G-protein inhibition upto the published concentration of 1µl/ml.
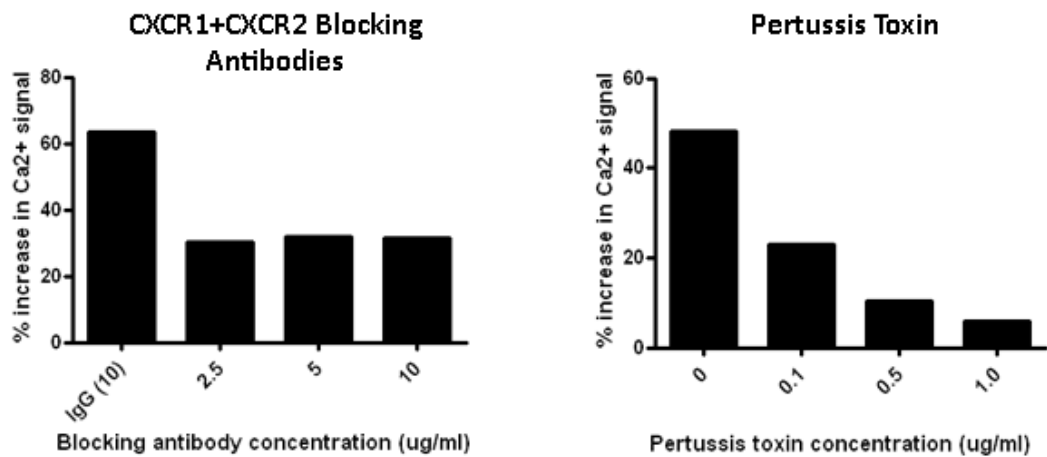
Figure 15. Dose response of CXCL8-induced calcium mobilisation following CXCR1/2 or G-protein blockade. Fluo-4 calcium mobilisation in EP-AHAC following the CXCL8 induced release of intracellular Ca^{2+}, (A) following specific inhibition of CXCR1 and CXCR2 using blocking antibodies and (B) following G-protein inhibition using Pertussis toxin. n = 1.

Calcium mobilisation was inhibited following the exposure of primary chondrocytes to CXCR1 and CXCR2 blocking antibodies at a total concentration of 10µg/ml. Analysis revealed a blocking efficiency of approximately 40% of CXCL8-induced calcium mobilisation activity for blocking antibody treatment (Figure 16A).
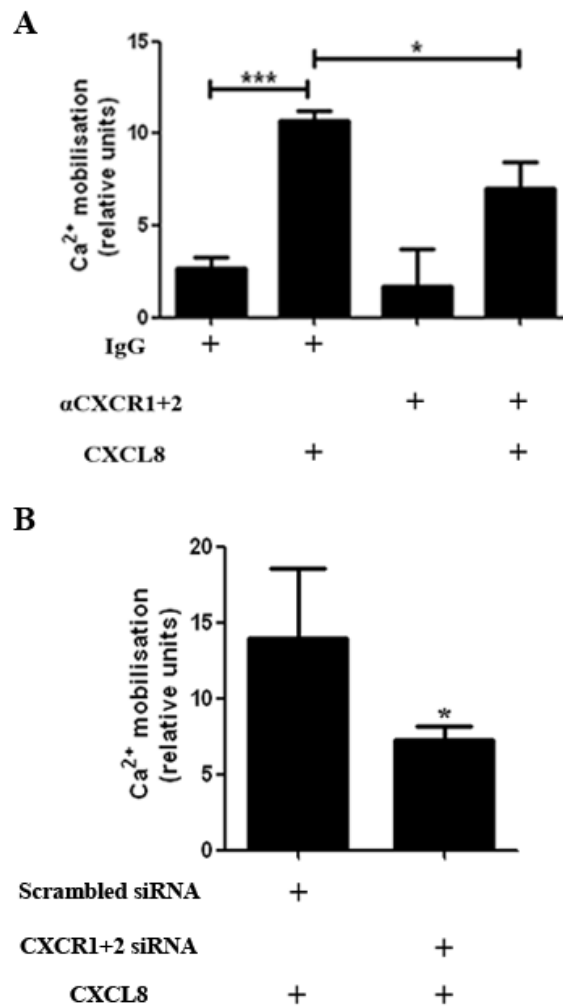
Figure 16. CXCR1/2 activity in EP-AHAC. Fluo-4 intracellular calcium mobilisation in EP-AHAC following incubation with (A) αCXCR1 and αCXCR2 blocking antibodies, with and without CXCL8 stimulus. Mouse non-specific IgG used as negative control. (B) CXCR1 and CXCR2 siRNA, following CXCL8 stimulus. Scrambled siRNA used as negative control. *P < 0.05, ***P < 0.001. Data show the activation of downstream calcium mobilisation following CXCL8 stimulation, which is significantly reduced during CXCR1+2 inhibition.

To confirm the inhibition of CXCL8-induced calcium mobilisation following CXCR1 and CXCR2 blockade using a second method, CXCR1 and CXCR2 were knocked down in human chondrocytes using specific siRNA (see Page 102 for knockdown optimisation). Calcium mobilisation was induced at 48 hours following siRNA transfection. Calcium mobilisation in CXCR1 and CXCR2 siRNA treated chondrocytes was shown to be 50% lower than in cells transfected with the scrambled control siRNA (Figure 16B), thereby confirming the decrease in calcium mobilisation found in cells treated with CXCR1 and CXCR2 antibodies.

CXCR1 and CXCR2 are G-protein coupled receptors known to regulate calcium mobilisation via the pertussis toxin sensitive release of βγ subunits from G_i class of G proteins (Wu et al., 1993;Wu et al., 1996). This allowed for pertussis toxin to be used to inhibit CXCR1/2 signalling at G-protein level, giving a blocking efficiency of more than 50% in CXCL8 stimulated cells. An inhibition of calcium influx was also seen in non-stimulated cells following pertussis toxin treatment, indicating the presence of underlying autocrine/paracrine activity either via CXCR1/2 which are inaccessible to blocking antibodies, possibly through internalisation, or via the activity of other G protein coupled receptors.
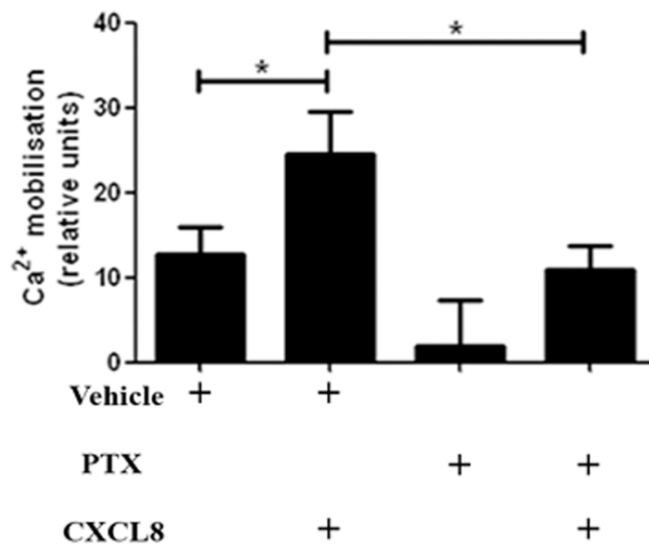
Figure 17. **G-protein dependant CXCR1/2 activity in EP-AHAC.** Fluo-4 intracellular calcium mobilisation in EP-AHAC following incubation with Pertussis toxin, with and without CXCL8 stimulus, vehicle negative control. $*P < 0.05$. Data show the activation of downstream calcium mobilisation following CXCL8 stimulation, which is significantly reduced during G-protein inhibition.

CXCR1 and CXCR2 are functionally redundant in human articular chondrocytes

Chemokine induced neutrophil activation and migration requires signalling via both CXCR1 and CXCR2 (Jones et al., 1997). The blockade of each receptor individually using blocking antibodies specific to either CXCR1 or CXCR2 was used to investigate whether the simultaneous activation of CXCR1 and CXCR2 is required for CXCR signalling measured via calcium influx in human articular chondrocytes.

CXCR1 and CXCR2 activation by CXCL8 was found to be sufficient to induce calcium mobilisation in monolayer cultured early passage chondrocytes (Figure 16). The inhibition of only one receptor did not result in a calcium influx response of significant difference to cells treated with a negative control mouse IgG antibody, whereas the blockade of both receptors simultaneously resulted in a significant decrease (Figure 18). These data suggest that CXCR1/2 signalling in chondrocytes may only be pharmacologically modulated through the blockade of both ELR+ CXC chemokine receptors, whilst the inhibition of only one receptor would be required to affect neutrophil migration during inflammation.
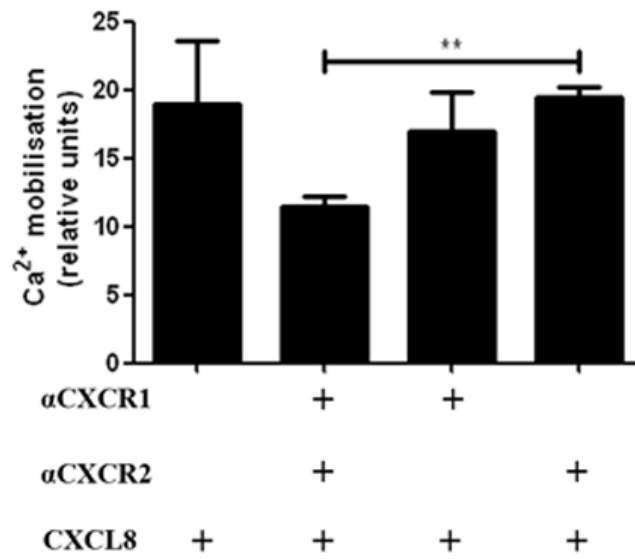
Figure 18. Redundant functional activity of CXCR1 and CXCR2. Fluo-4 intracellular calcium mobilisation in EP-AHAC following incubation with either CXCR1 or CXCR2 specific blocking antibodies. **$P < 0.01$. Data show the activation of downstream calcium mobilisation following CXCL8 stimulation, which is not significantly reduced during individual blockade of either CXCR1 or CXCR2.

CXCR1 and CXCR2 expression in human chondrocyte cell lines

Due to the periodic difficulty in obtaining well preserved human articular cartilage samples, it was necessary to obtain a suitable cell line for use in CXCR1/2 inhibition experiments on a larger scale than possible using the available primary cells. Two immortalised human chondrocytic cell lines, C28/I2 and JJ012 were analysed for their expressions of CXCR1 and CXCR2 at mRNA level in order to test for their suitability for CXCR1/2 blockade experiments.

C28/I2 mRNA was taken from monolayer cells cultured both in the presence of 10% FBS and in serum-free medium supplemented with ITS+ (Insulin-Transferrin-Selenium, 1%) for 4 days and analysed using semi-quantitative RT-PCR. CXCR2 only was found to be expressed in cells cultured in ITS+ supplemented medium, whilst neither receptor was found in standard culture conditions, deeming C28/I2 largely unsuitable for blockade experiments (**Figure 19**A). The JJ012 human chondrosarcoma cell line was then compared using RT-PCR, with both CXCR1 and CXCR2 found to be expressed at mRNA level in standard culture conditions (Figure 19B).
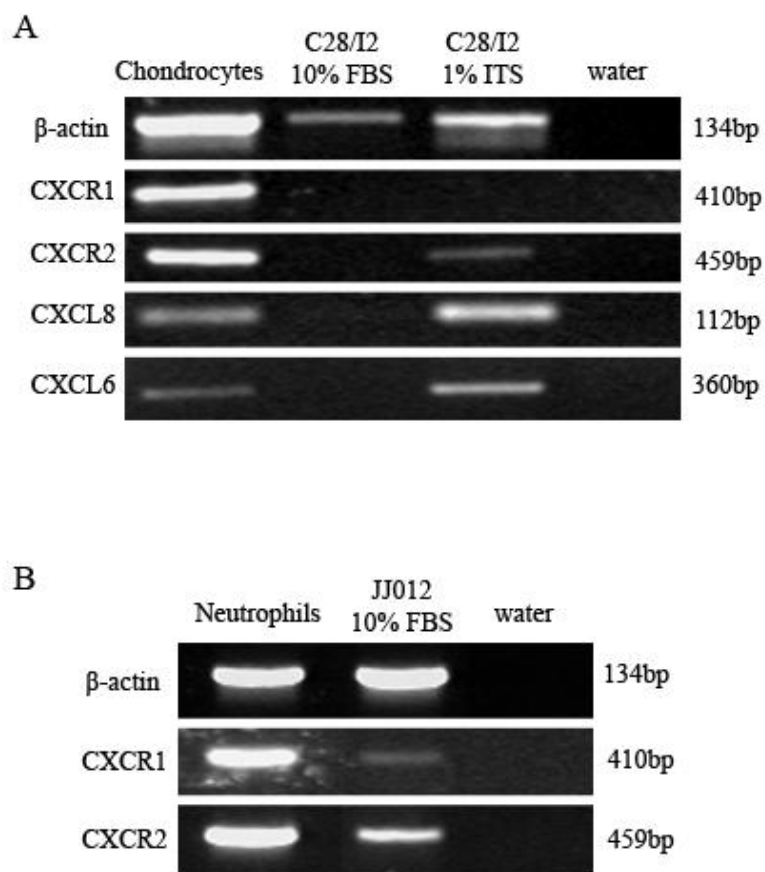
Figure 19. CXCR1 and CXCR2 expression in human chondrocyte cell lines. Semi-quantitative PCR for CXCR1 and CXCR2 in (A) C28/I2 human immortalised chondrocytes cultured in either 10% FBS supplemented culture medium or in 1% ITS+ supplemented culture medium, or (B) JJ012 human chondrosarcoma cells cultured in 10% FBS supplemented culture medium.

Discussion

ELR+ CXC chemokine signalling is primarily regarded as a mediator of neutrophil recruitment during inflammation and has been shown to be upregulated during arthritis. It is also known to regulate angiogenesis and tumour growth and survival in cancer (Singh et al., 2009;Strieter et al., 2005), suggesting that non-conventional roles aside from chemotaxis should be considered when targetting CXCR signalling during therapy. ELR+ CXC chemokines have been implicated in joint inflammation during arthritis; however, recent data suggest that ELR+ CXC chemokines may play a role in joint surface homeostasis. ELR+ CXC chemokines are expressed in normal and inflamed articular cartilage and synovial membrane (Borzi et al., 1999;Patterson et al., 2002), exogenous CXCL1 and CXCL8 have been shown to promote hypertrophic differentiation of articular chondrocytes (Merz et al., 2003), and CXCL8 has been shown in our laboratory to be upregulated in human articular cartilage explants following injury.

Before investigating a possible homeostatic role of CXC chemokine signalling using a loss of function approach, it was vital to validate the expression of ELR+ CXC chemokine receptors in human primary articular chondrocytes and potential cell lines used in our laboratory. CXCR1 and CXCR2 were first shown to be expressed at mRNA level and at protein level in monolayer cultured early passage articular chondrocytes at levels similar to those found in the human neutrophil positive control. The CXCR1/2 ligands, CXCL6 and CXCL8, were expressed in early passage, but not late passage monolayer cells, following patterns observed for chondrocyte phenotypic marker gene expression (Dell'accio et al., 2001). During *in vitro* chondrocyte expansion, the expression of type II collagen, aggrecan and SOX9 is reduced, in correlation with the loss of the ability of chondroyctes to form stable ectopic cartilage explants *in vivo*. We have demonstrated a significant reduction in CXCL6 and CXCL8 expression in late passage chondrocytes, suggesting that CXCR1/2 signalling may be linked to this maintenance of phenotypic stability in chondrocytes.

 The possibility that CXC chemokine receptor expression was induced during the monolayer seeding of chondroyctes was addressed using immunohistochemistry, which confirmed the presence of CXCR1 and CXCR2 in both normal and preserved osteoarthritic human articular cartilage explants. We cannot exclude however, that in challenge conditions, such as following tissue digestion, components of this signalling pathway including receptors and ligands may be upregulated, perhaps in an attempt to re-establish homeostasis. In fact, upregulation of CXCL8 in OA is well established (Borzi et al., 1999). It is tempting to speculate that this attempt, however, is ultimately futile due to the ECM breakdown typical of OA.

Interestingly, CXC chemokine receptors are noted within previous literature as being found only in osteoarthritic cartilage (Borzi et al., 2000). This discrepancy may be explained by the use in my study of more efficient antigen retrieval methods that have allowed for the digestion of the denser extracellular matrix of the healthy cartilage, whereas, in OA cartilage, matrix breakdown related to the disease may have facilitated detection with milder retrieval methods. Indeed, without the aggressive pepsin retrieval method used here, CXCR1, CXCR2 and CXCL8 were undetectable in the healthy or preserved cartilage explants (Figure 34). In addition, the strong detection of CXCR1 and CXCR2 at mRNA level and also at protein level by western blotting confirms our data that normal or relatively healthy chondrocytes express CXCR1 and CXCR2.

The measurement of calcium mobilisation following CXCL8 stimulation, and its reduction following various methods of CXCR1/2 signalling has demonstrated the functional activity of CXCR1 and CXCR2 in human articular chondrocytes following exposure to the ligand CXCL8. These data have also provided an insight into the efficacy of each CXCR1/2 signalling blockade approach used during my analysis of chondrocyte phenotypic stability.

Chemokines can activate several downstream signalling components including PI3K, intracellular Ca^{2+} accumulation and MAPK. One unresolved problem is the determination of which signalling components activated by chemokines may mediate the chondrocyte phenotype. PLCβ2 mediates chemokine induced Ca^{2+} release. Mice deficient in for the gene encoding PLCβ2 have impaired chemoattractant-induced intracellular Ca^{2+} release however their leukocyte migration was not affected (Jiang et al., 1997). Therefore, Ca^{2+} release does not appear to be required for chemotactic activity. However, Ca^{2+} mobilisation in chondrocytes was shown previously to modulate the chondrocyte phenotype. Intracellular calcium increases, driven by IGF1 and PI3K signalling, have a strong anabolic effect on chondrocytes (Poiraudeau et al., 1997), however, other studies have suggested that calcium mobilisation may result in the loss of chondrocyte differentiation markers including SOX9, COL2A1 and aggrecan (Alford et al., 2003;Kulyk et al., 2000;Lee et al., 2007).

The discrepancies found between the conclusions of these studies may be explained by examining the downstream molecular cascades mediating the Ca^{2+} release. The specific activation of PI3K modulated Ca^{2+} release, known to be a downstream signalling target of CXCR1/2, was shown to increase the anabolic activity of chondrocytes.

This evidence suggests that the inhibitory effects of CXCR1/2 or G-protein blockade on calcium mobilisation are likely to replicate any patterns of modulation on chondrocyte phenotypic stability seen in later experiments. The quantification of Ca^{2+} mobilisation therefore allows for the evaluation of the likely efficacy of each method of CXCR1/2 inhibition used for phenotypic analysis later in this study.

Whereas G protein blockade or receptor knockdown by siRNA resulted in a very efficient blockade of signalling, blockade using anti CXCR1 and CXCR2 blocking antibodies achieved a relatively modest inhibition in Ca^{2+} mobilisation by comparison. This may be explained

by unavailability of the internalised pool of CXCR1/2 for antibody binding. Indeed, the confocal microscopy data indicate that a large proportion of the receptor was present within intracellular vesicles. The rate of recycling and degradation of CXCR1 and CXCR2 varies as a function of ligand concentrations, however the presence of this threshold of inhibition, the amount of internalised receptors and the fact that the same cells express both ligands and receptors raises the question as to whether secretion of the ligands is required for signalling or whether autocrine production of CXC chemokines is sufficient to activate the receptors within intracellular vesicles without secretion.

CXC chemokine mediated neutrophil migration requires the activation of both CXCR1 and CXCR2 (Jones et al., 1997). My data indicate that CXCL8-induced calcium mobilisation in chondrocytes only requires the activation of one of the two receptors, suggesting at least a partial redundancy between CXCR1 and CXCR2 not seen in neutrophil activation. This suggests that alternative downstream signalling pathways, and hence functional outcomes, may depend upon the activation of either one or both receptors in chondrocytes. This provides a potential method of pharmalogical modulation of CXCR1/2 signalling in inflammatory neutrophils, via the inhibition of only one ELR+ CXC chemokine receptor, whereby chondrocyte function should remain unaffected.

In the next chapter, I aim to determine the functional role of CXCR1/2 signalling in articular chondrocytes using a series of loss of function experiments, using my findings that CXCR1/2 and G-protein inhibition disrupts calcium mobilisation, which has previously been shown to be involved in the maintenance of the chondrocyte phenotype.

CXCR1/2 signalling is required for articular chondrocyte phenotypic stability.

Experimental Layout

Having demonstrated that articular chondrocytes express functional CXCR1/2 receptors and their respective ligands, the next stage of the project was to identify the function of such molecules in chondrocytes, which are not known to migrate *in vivo*. Cartilage is an avascular tissue, therefore possible explanations involving chemotaxis or angiogenesis are unlikely to be relevant within this context.

The primary function of chondrocytes is to produce and maintain a specialised extracellular matrix, providing articular cartilage with its resistance and compressive biomechanical properties required for weight bearing and joint motion. I have shown that CXC chemokine ligand expression decreases significantly during *in vitro* cell expansion, in correlation with the loss of markers of the stable chondrocyte phenotype, including type II collagen and aggrecan (Dell'accio et al., 2001). To this end, I have hypothesised that CXC chemokines may be required for the maintenance of chondrocyte phenotypic stability and ECM production.

I have investigated this hypothesis using a series of loss of function experiments whereby CXCR1/2 signalling was inhibited at both receptor level and downstream G-protein level in human and porcine primary chondrocytes and in the JJ012 chondrosarcoma cell line. The ability of chondrocytes to produce large amounts of ECM when cultured in 3 dimensional micromasses was the first readout used to measure the influence of CXCR1/2 signalling on the maintenance of ECM production. These results were then supported using real-time RT-qPCR to measure the expression of key marker genes specific to the stable chondrocyte phenotype.

The present data in CXCR2-/- mice are in agreement with a previous study which did not report differences in cartilage phenotype (Bischoff et al., 2011).

Optimisation of CXCR1 and CXCR2 knockdown using siRNA

One of the problems found during our previous experiments using blocking antibodies to inhibit CXCR1/2 signalling was that internalised receptors were not accessible to the antibodies, resulting in only a partial inhibition of signalling using this technique. To circumvent this problem, siRNA oligonucleotide sequences were used to specifically knockdown the specific activity of the receptors, thus restricting any effect of internalised receptors which may be protected from exposure to blocking antibodies. The following series of experiments detail how the optimisation of CXCR1/2 knockdown in primary chondrocytes and JJ012 cells was achieved.

As a first approach, a recommended dose of positive control fluorescent oligonucleotides was transfected into human primary chondrocytes using lipofectamine 2000 reagent at increasing concentrations, with the aim of finding a concentration at which efficient transfection may be achieved without the toxic effects of lipofectamine compromising chondrocyte viability (Figure 20). A peak of transfection efficiency was observed using 1µg/ml of lipofectamine reagent, which was used for subsequent lipofectamine transfections. Concentrations above this level resulted in altered cell morphology.
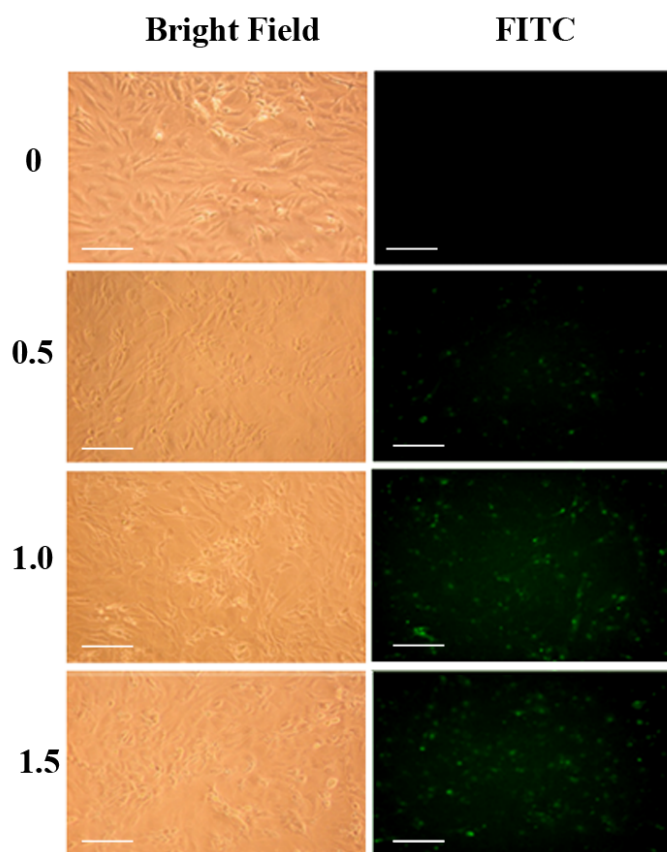
Figure 20. Lipofectamine™ dose response of fluorescent oligonucleotide transfection.

Bright field and FITC fluorescent imaging of EP-AHAC transfected with 50nM green

fluorescent oligonucleotide using an increasing concentration of Lipofectamine™. Bar,

200µm

Three individual CXCR1 and two individual CXCR2 specific siRNA oligonucleotide sequences were obtained and transfected into human primary chondrocytes in order to select the sequence providing the most effective gene knockdown. Semi-quantitative PCR normalised for β-actin expression was used as a readout to assess the knockdown at gene expression level. Figure 21 demonstrates the reduction in CXCR1 and CXCR2 mRNA obtained using each siRNA sequence relative to a scrambled control. CXCR1-oligo I was selected for use in later siRNA experiments.

To confirm the selection of CXCR2-oligo I as the most efficient sequence and to assess the translation of mRNA knockdown into protein expression in human primary chondrocytes, a Western blot was performed on cell lysates obtained from CXCR2-oligonucleotide I and CXCR2-oligonucleotide II treated cells in comparison with untreated and scrambled oligonucleotide transfected chondrocytes. After 48 hours, protein expression levels confirmed CXCR2-oligonucleotide I as the preferred oligo for CXCR2 knockdown (Figure 22), however the overall reduction in receptor expression was not satisfactorily comparable to that observed at mRNA level. This pattern was repeated in CXCR1 knockdown when comparing siRNA oligo concentration in order to select an optimal dose for protein knockdown (Figure 23). We argued that this discrepancy was due to the well known recycling of the CXCR1/2 receptors and the consequent long half-life of each individual molecule. Long-term knockdown experiments are not feasible with primary chondrocytes, which lose their phenotype with prolonged passaging. In spite of these limitations, Western blotting confirmed that an siRNA dose of 50nM was preferred, as recommended in previous literature (Klatt et al., 2007), however a maximum reduction in protein levels of 50% was achieved. As described in the previous chapter, this reduction in receptor expression was sufficient to reduce CXCL8-induced calcium mobilisation by approximately 50%.
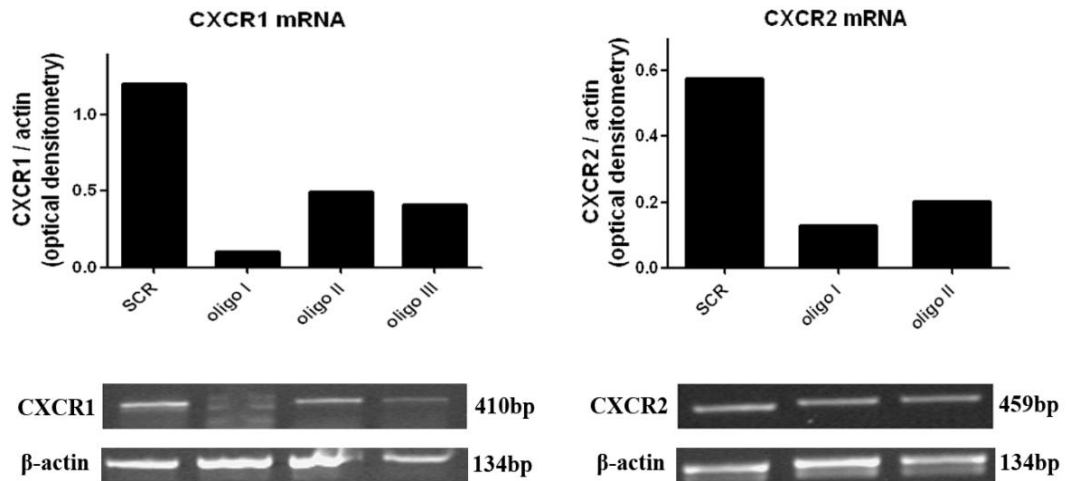
Figure 21. Selection of CXCR1 and CXCR2 siRNA oligonucleotides. Semi quantitative RT-PCR comparison of EP-AHAC transfected with either a CXCR1 (left) or CXCR2 (right) specific siRNA oligonucleotides or scrambled negative control siRNA oligonucleotide. Knockdown efficiency 48 hours post transfection was quantitatively measured by optical densitometry, normalising each CXCR1 or CXCR2 PCR band intensity for β-actin band intensity.
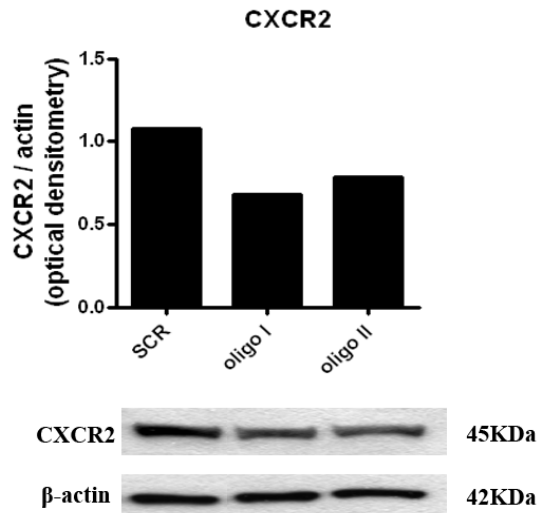
Figure 22. Confirmation of CXCR2 siRNA oligonucleotide selection. Western blot comparison of EP-AHAC transfected with CXCR2 specific siRNA oligonucleotides or a scrambled negative control siRNA oligonucleotide. Knockdown efficiency 48 hours post transfection was quantitatively measured by optical densitometry, normalising each CXCR2 band intensity for β-actin band intensity.
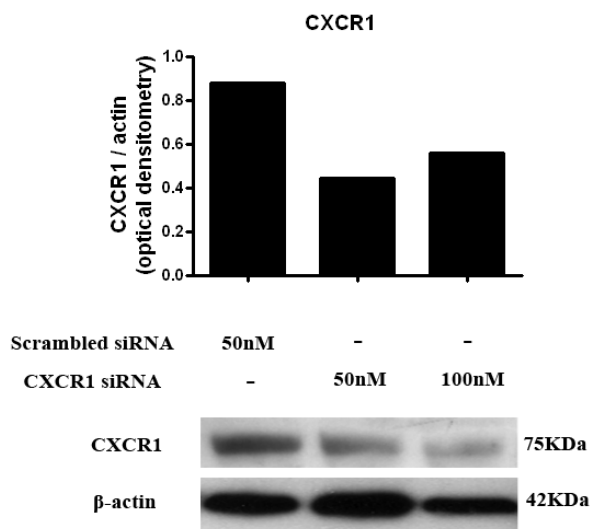
Figure 23. CXCR1 siRNA dose response. Western blot analysis of CXCR1 knockdown efficiency by CXCR1 specific siRNA in comparison to a scrambled negative control siRNA. Knockdown efficiency 48 hours post transfection was quantitatively measured by optical denstiometry, normalising each CXCR1 band intensity for β-actin band intensity.

In order to achieve a more significant functional readout at phenotype level, I decided to

assess whether a higher receptor knockdown may be achieved in the JJ012 human

chondrocyte cell line, which, since they proliferate faster than primary chondrocytes, will

be more dependent on transcription to maintain their "physiological" amount of CXCR1

and CXCR2. The choice of JJ012 was driven by preliminary experiments examining the

CXCR1 and CXCR2 expression profile of human chondrocyte cell lines, described previously

(Figure 19). In a direct comparison to fluorescent oligonucleotide transfection using

lipofectamine, jetPRIME™ (Polyplus, UK) was found to give an increased transfection

efficiency in viable cells (Figure 24).
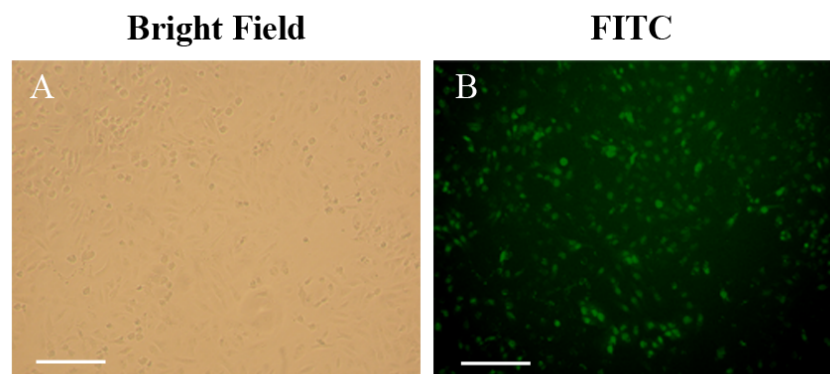
Figure 24. JetPRIME™ fluorescent oligonucleotide transfection. (A) Bright field and (B)

FITC fluorescent imaging of JJ012 cells transfected with 50nM green fluorescent

oligonucleotide using jetPRIME™ reagent. Bar, 200µm.

Using the same siRNA oligos for CXCR1 and CXCR2 shown to be most efficient in primary chondrocytes (Figure 21), JJ012 were transfected with siRNA for both receptors simultaneously using jetPRIME™ reagent and mRNA and cell lysate was collected after 24 hours. A knockdown of CXCR1 and CXCR2 at mRNA level of around 75% was observed (Figure 25A). At protein level, Western blotting indicated a clear knockdown of both receptors, particularly in CXCR2 (Figure 25B). The high availability of the JJ012 cell line, and the increased viability of cells transfected using jetPRIME™ led me to continue with this experimental approach in order to support the following loss of function data obtained using blocking antibodies and Pertussis toxin upon human and porcine primary chondrocytes.
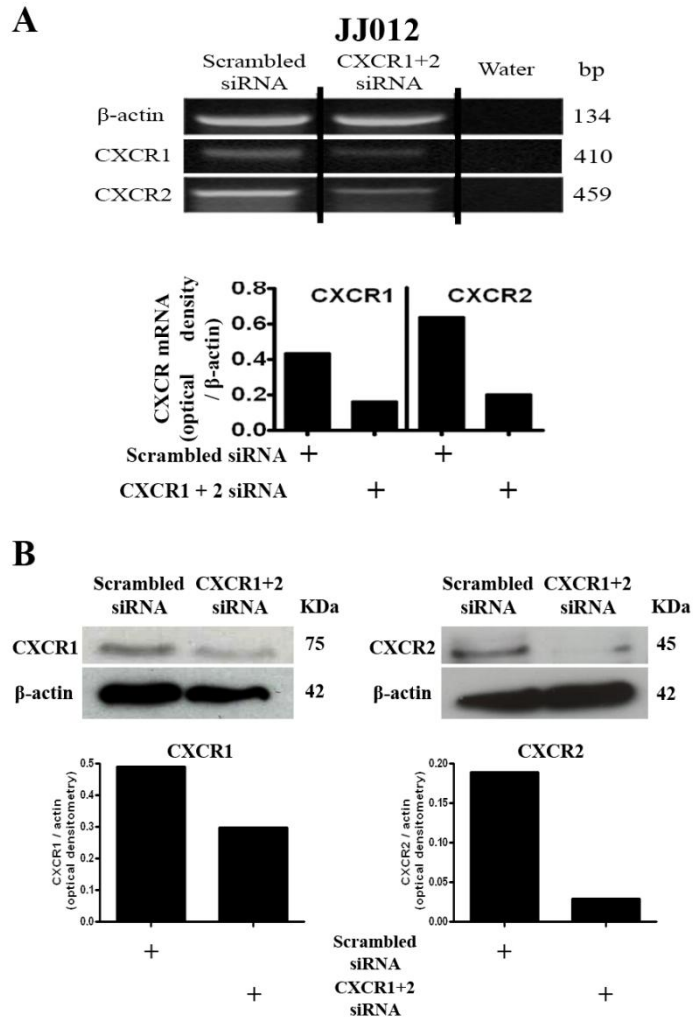
Figure 25. CXCR1 and CXCR2 knockdown efficiency in JJ012 human chondrosarcoma cell line. (A) Semi quantitative RT-PCR for CXCR1 and CXCR2 in JJ012 monolayer cells 24 hours after transfection with either CXCR1 and CXCR2 specific siRNA or scrambled negative control siRNA.   β-actin was used as a loading control.   Knockdown efficiency was quantitatively analysed by optical densitometry, normalising each CXCR1 or CXCR2 band intensity for β-actin band intensity. (B) Western blot for CXCR1 (left) and CXCR2 (right) in JJ012 monolayer cells 24 hours after transfection with either CXCR1 and CXCR2 specific siRNA or scrambled negative control siRNA.   β-actin was used as a loading control. Knockdown efficiency at protein level was quantitatively analysed using optical densitometry, normalising each CXCR1 or CXCR2 band for β-actin band intensity.

G-protein coupled receptor signalling is required for the maintenance of chondrocyte extracellular matrix

Chondrocytes are not known to migrate *in vivo* and their main function is to maintain the homeostatic equilibrium of the extracellular matrix (ECM). Therefore, I set out to test whether CXC chemokine signalling plays a role in the maintenance of the phenotype of articular chondrocyte ECM using a loss of function approach. As an initial readout, I stained chondrocyte micromass cultures with Alcian blue at pH 0.2. At this pH, Alcian blue very specifically stains highly sulphated GAGs, which are unique to the cartilage matrix. The staining can be quantified by spectrophotometry following guanidine extraction (De Bari et al., 2001).

The specific inhibition of CXCR1 and CXCR2 using blocking antibodies resulted in a statistically significant decrease in the accumulation of highly sulphated GAGs in early passage adult human articular chondrocytes (Figure 26A) and in the JJ012 human chondrocytic cell line in micromass cultures (Figure 26B). These data were supported by the similar depletion in highly sulphated GAGs obtained when CXCR1 and CXCR2 were knocked down by siRNA in JJ012 chondrocytic cells (Figure 26C), thereby confirming the requirement of the CXCR1/2 signalling pathway for the accumulation of the cartilage-specific extracellular matrix. Interestingly, the siRNA-mediated receptor knockdown resulted in a greater decrease in GAG content than treatment with anti CXCR1/2 blocking antibodies. One possible explanation is that receptors that are internalised as shown in Figure 11 may not be accessible to blocking antibodies and may support baseline signalling through autocrine availability of CXCL8.
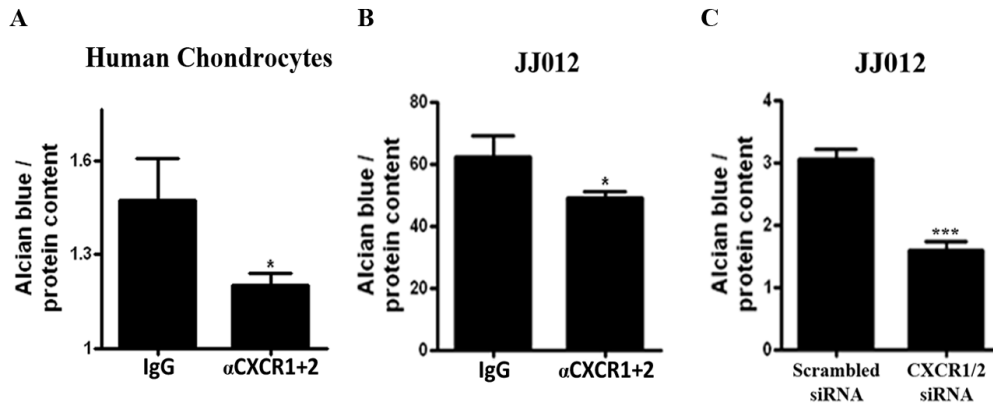
Figure 26. CXCR1/2 signalling is required for maintenance of ECM. Guanidine extraction and quantification of Alcian blue sulphated proteoglycan staining normalised for total protein content of (A) human EP-AHAC and (B) JJ012 human chondrosarcoma cell line micromass cultures incubated with αCXCR1 and αCXCR2 antibodies. Mouse non-specific IgG negative control. (C) Alcian blue staining quantification of CXCR1 and CXCR2 siRNA treated JJ012 micromass cultures normalised for total protein content. Scrambled siRNA used as negative control. *P < 0.05, ***P < 0.001. Data show CXCR1/2 signalling is required for the maintenance of chondrocyte ECM sulphated proteoglycan content in micromass cultures of human primary chondrocytes and a human chondrocyte cell line.

G protein blockade by PTX resulted in a statistically significant, dose-dependent reduction in the accumulation of highly sulphated GAGs in adult porcine chondrocytes in micromass culture (Figure 27), demonstrating that the CXCR1/2-dependent accumulation of sulphated GAGs is mediated by G proteins (most likely Gαi) and that the G-protein dependant modulation of chondrocyte phenotype may be conserved across species. A decrease was also observed in the absence of exogenous CXCL8 (Figure 27), thereby suggesting that the endogenous production of CXCR1/2 ligands is sufficient for autocrine/paracrine signalling.

Porcine Chondrocytes
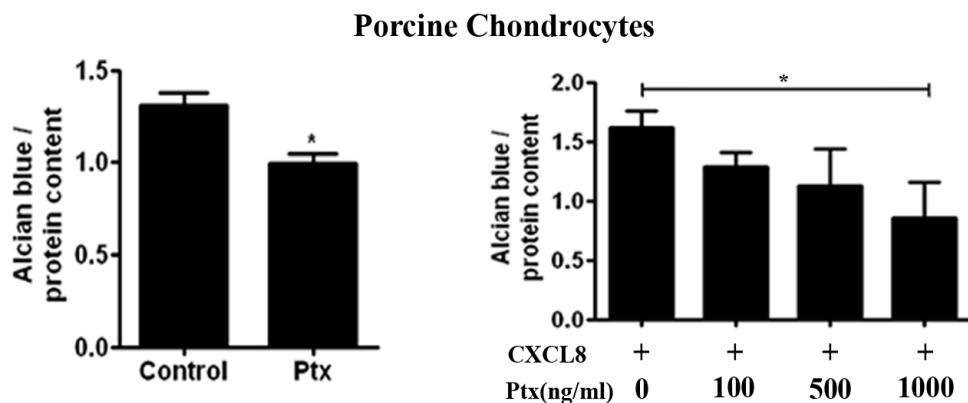
Figure 27. G-protein signalling is required for maintenance of ECM. (A) Alcian blue staining quantification of EP porcine chondrocyte micromasses normalised for total protein content treated with 1µg/ml Pertussis toxin or vehicle control, and (B) treated with an increasing dose of Pertussis toxin before 4 day incubation with CXCL8. *P < 0.05. Data show the requirement of G-protein signalling for maintenance of ECM sulphated proteoglycan content of porcine chondrocyte micromass cultures.

CXCR1/2 signalling is required for the expression of chondrocyte differentiation markers.

Next, I asked whether the decrease in cartilage specific extracellular matrix was associated with specific changes in the chondrocyte phenotype. To this end, I compared the expression of genes associated with the articular cartilage phenotype in human articular chondrocytes treated with either CXCR1 and CXCR2 specific blocking antibodies or a mouse IgG control. CXCR1/2 blockade resulted in a statistically significant decrease in the expression of COL2A1 and aggrecan mRNA (Figure 28).
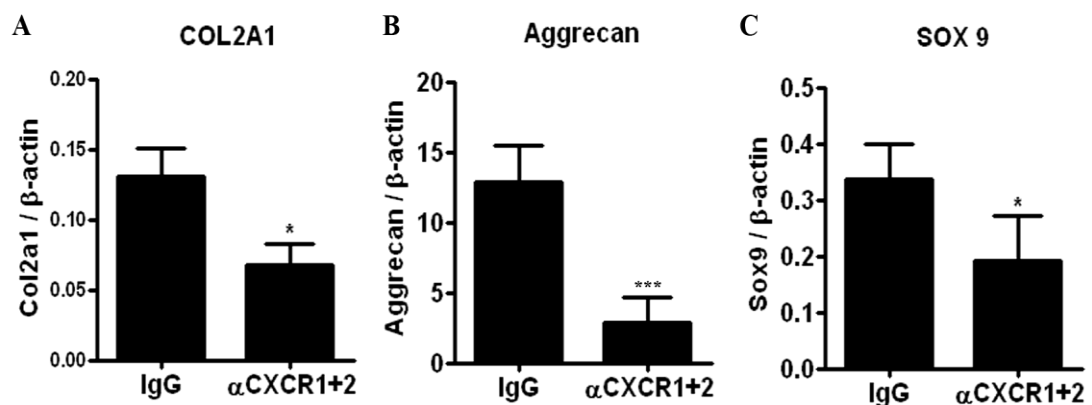
Figure 28. CXCR1/2 signalling is required for chondrocyte phenotypic marker expression. Real time RT-PCR for (A) α1(II)collagen (COL2A1), (B) Aggrecan and (C) SOX9 in EP-AHAC incubated with αCXCR1 and αCXCR2 blocking antibodies for 4 days. Mouse non-specific IgG used as negative control. . *P < 0.05, ***P < 0.001.

G protein blockade using PTX fully confirmed downregulation of COL2A1 and aggrecan mRNA (Figure 29). Taken together, these data show that CXCR1/2, G protein-dependent chemokine signalling supports the synthesis of cartilage-specific extracellular matrix at least in part by increasing aggrecan and COL2A1 mRNA accumulation. In addition, both CXCR1/2 and G protein blockade resulted in a statistically significant reduction of the expression of the transcription factor SOX9 (Figure 28 and Figure 29), which is known to support chondrocyte differentiation, including COL2A1 expression (Bi et al., 1999;Lefebvre et al., 1998), demonstrating that CXCR1/2 signalling is required for the phenotypic stability of articular chondrocytes.
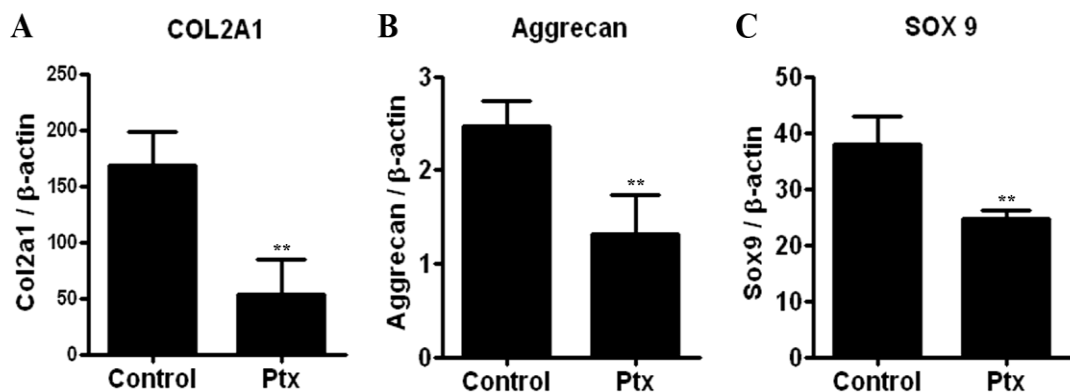
Figure 29. G-protein signalling is required for chondrocyte phenotypic marker expression. Real time RT-PCR for (A) α1(II)collagen (COL2A1), (B) Aggrecan and (C) SOX9 in EP-AHAC incubated with Pertussis toxin or vehicle control for 4 days. **$P < 0.01$. CXCR1/2 via G-protein signalling is required for the maintenance of key chondrocyte phenotypic stability marker genes in monolayer culture.

Exogenous CXCR1/2 ligands are not sufficient to rescue the expression of stable chondrocyte molecular markers in late-passage AHAC.

The blockade of CXCR1/2 signalling revealed that ELR+ CXC chemokine signalling is required for the phenotypic stability of early passage AHAC. During the *in vitro* expansion of chondrocytes, the expression of both CXC chemokine ligands CXCL8 and CXCL6, and the expression of key phenotypic marker genes including SOX9 are lost. In order to test whether the re-activation of CXCR1/2 signalling may be sufficient to rescue the expression of SOX9, type II collagen and aggrecan, late passage (second passage) were treated with either 10ng/ml CXCL8, 10ng/ml CXCL6, or both in combination, for 4 days before mRNA was retrieved for real time RT-PCR analysis for phenotypic marker genes. No significant rescue of type II collagen, aggrecan or SOX9 mRNA expression was found after 4 days of treatment (Figure 30).
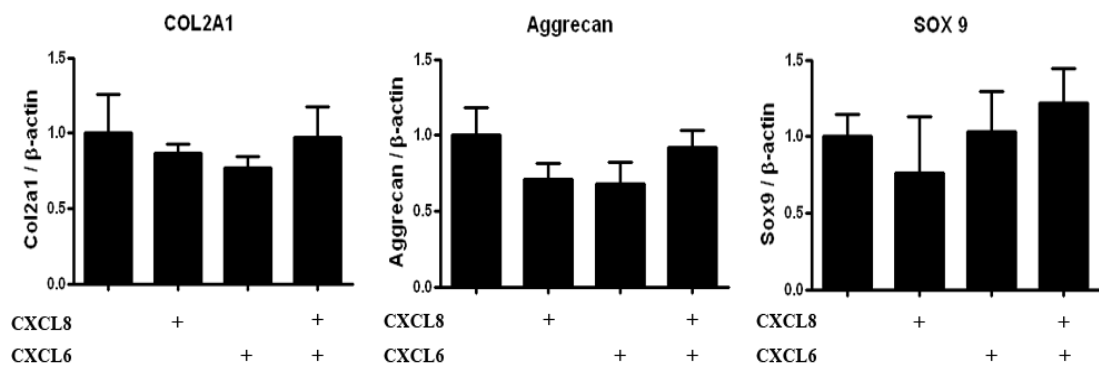
Figure 30. Addition of CXC chemokine ligands to LP-AHAC. CXCL6 and/or CXCL8 are not sufficient to rescue the expression of COL2A1, Aggrecan and SOX 9 in late passage monolayer de-differentiated AHAC. Q-PCR for COL2A1, Aggrecan and SOX 9 in late passage AHAC following 4 day culture in complete DMEM containing 10ng/ml CXCL6 and/or CXCL8.

Phenotypic analysis of CXCR2 -/- mutant mice knee joints

In order to investigate whether CXCR1/2 signalling is required for cartilage homeostasis *in vivo*, in unchallenged conditions, 8 week old CXCR2 knockout mutant mice knee joints were characterised and compared to wild-type BALB/C mice, firstly by using the Chambers osteoarthritis score to measure articular cartilage integrity, and secondly by measuring the thickness of articular cartilage on both femoral condyles and the tibial plateau, normalised by epiphysial growth plate thickness. This was followed by a quantification of Safranin-O cartilage staining intensity in order to measure the proteoglycan content of the cartilage.

In the mouse, it has long been regarded that ELR+ CXC chemokine mediated neutrophil chemotaxis is activated through one receptor, mCXCR2, following the binding of mouse CXCL1 and CXCR2 homologues, MIP2α and KC (Bozic et al., 1994;Luan et al., 2001). Recently, however, a mouse homologue of human CXCR1, capable of being activated by the human CXCL6 ligand has been discovered (Fan et al., 2007). The availability of the CXCR2 -/- knockout mouse therefore poses more detailed questions then first expected as to whether, in the mouse, the lack of CXCR2 may compromise the phenotype of articular cartilage. My data presented previously has revealed that in human chondrocytes, CXCR1 may compensate for the inhibition of CXCR2 at calcium mobilisation level. It may therefore be hypothesised that a similar redundancy of chemokine receptors in mouse articular cartilage may allow for sufficient cartilage homeostasis in normal conditions. However, this prediction is uncertain, firstly because of the large variation between human and mouse chemokine profiles, and secondly because the absence of CXCR2 in mice has been shown to be sufficient to impair neutrophil chemotaxis (Lee et al., 1995).

Comparison of the Chambers score of both medial and lateral femoral condyle and tibial plateau surfaces (Figure 31A) indicate that no spontaneous cartilage destruction is acquired consistently in CXCR2 -/- mice in comparison with wild type controls. However in

4 out of the 15 CXCR2 -/- joints examined, evidence of spontaneous damage to the articular surface was found (Figure 31B).
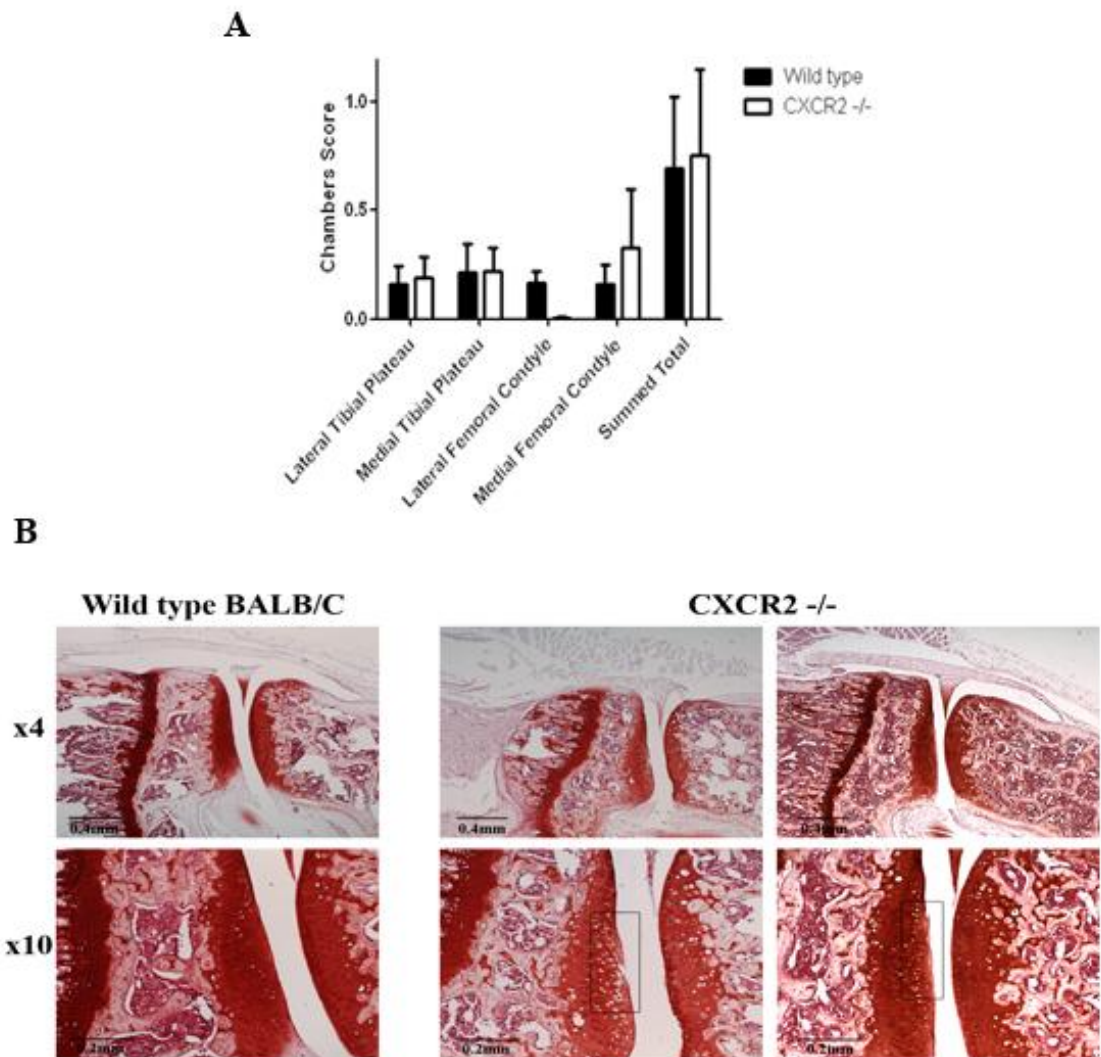
Figure 31. **Histological analysis of CXCR2-/- articular cartilage.** (A) Chambers osteoarthritis score comparing the structural integrity of cartilage from each articular surface in 8 week old CXCR2-/- null mutant mice and BALB/C wild type. (B) Safranin-O staining of tibiofemoral joint surfaces in CXCR2-/- and wild type mice. Rectangular selections in x10 images highlight superficial articular surface irregularities in CXCR2-/- sections.

CXCR2 knockout mice have been noted as being smaller than their wild type littermates in early studies on CXCR2 function (Luan et al., 2001;Padovani-Claudio et al., 2006), however a recent paper published by Bischoff et al. (Bischoff et al., 2011) has revealed that CXCR2 -/- mice exhibit an altered skeletal phenotype consisting of a decreased bone density, mineral content and repair capacity, hypothesised to be a result of a lack of CXCR regulated angiogenic activity rather than a disruption of immune cell migration or of an osteocyte phenotype. No analysis of articular cartilage or endochondral bone formation was included in the study.

Measurement of the thickness of articular cartilage in each compartment, as demonstrated in Figure 32, revealed that despite the smaller overall size of CXCR2 -/- mice reported by Bischoff et al., only medial tibial plateau cartilage thickness was significantly smaller than that in the BALB/C wild type controls. Epiphyseal growth plate cartilage was significantly thinner in CXCR2-/- mice than in wild types, in correlation with the differences observed in femur bone length, total size and weight of 6 and 12 week old mice reported previously. These data suggest that the lack of CXCR2 may result in the modulation of cartilage development, particularly affecting the epiphyseal growth plate and consequently longitudinal bone growth.
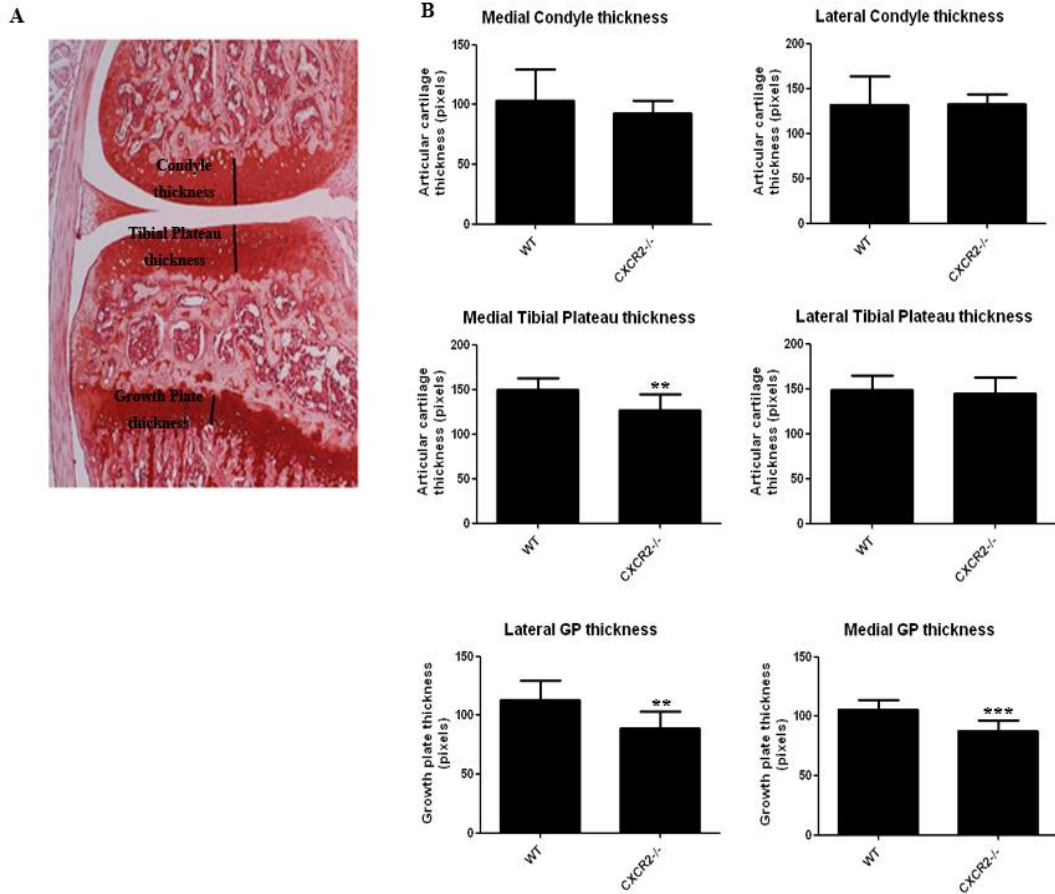
Figure 32. ImageJ analysis of articular cartilage and epiphyseal growth plate thickness.

(A) Sulphated proteoglycan rich cartilage thickness, identified by Safranin-O staining, was measured using ImageJ. (B) Comparison of cartilage thickness of 15 CXCR2-/- and 15 wild type mouse knee joints. **$P < 0.01$, ***$P < 0.001$.

I then investigated whether CXCR2 function is required to maintain the high proteoglycan content of articular cartilage by comparing the intensity of Safranin-O proteoglycan staining measured by densitometry (Figure 33). The intensity of staining of a cross section of each articular surface was plotted as demonstrated in Figure 33B. The areas under the curve, above a threshold created using the growth plate staining density were calculated, normalised for cartilage thickness, with Safranin-O staining densities for each surface of articular cartilage then normalised for Safranin-O staining density of their respective growth plate. A statistically significant decrease in proteoglycan content in the medial femoral condyle articular cartilage of CXCR2 -/- mice was observed, however no significant changes in other surfaces were found (Figure 33C).
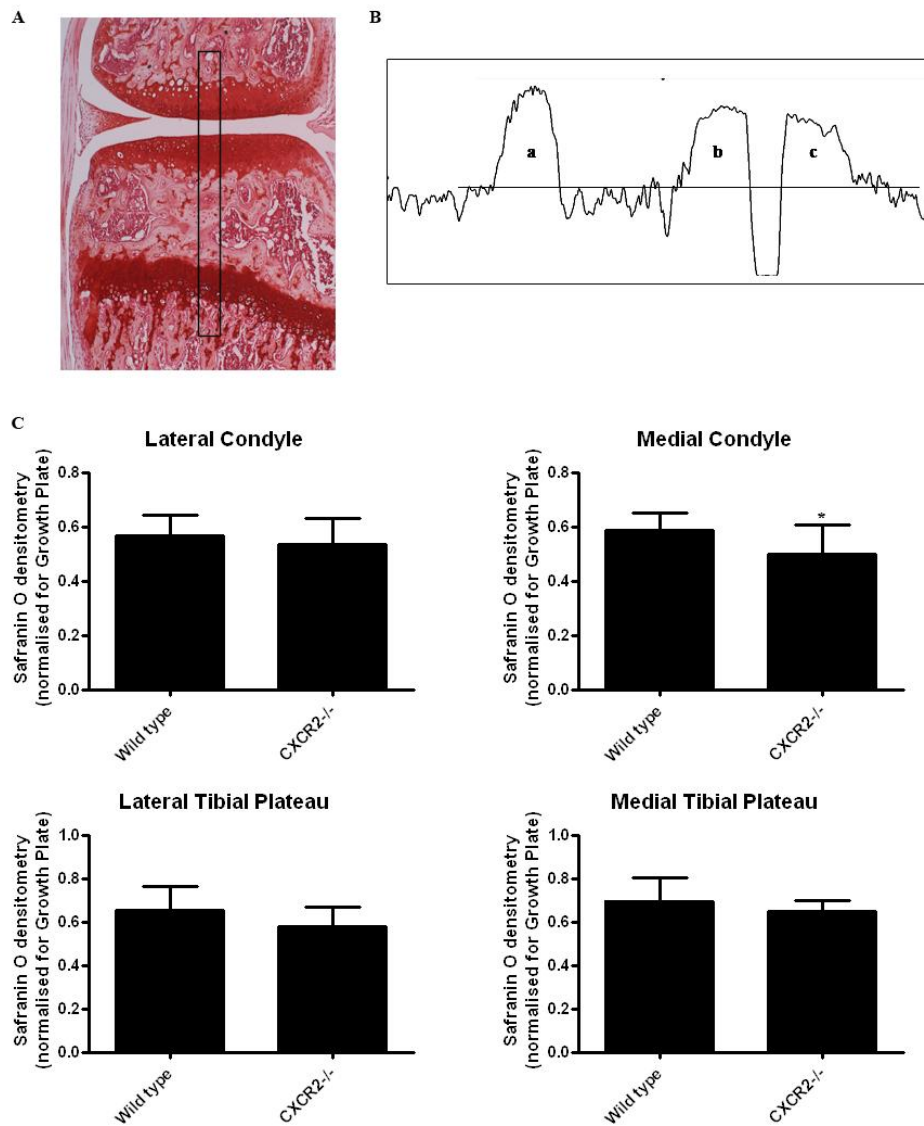
Figure 33. ImageJ analysis of articular cartilage sulphated proteoglycan content. (A) A cross section of each tibiofemoral joint was analysed for Safranin-O staining intensity as demonstrated in (B). Articular cartilage staining intensity in the tibial plateau (a) and femoral condyle (b) were each normalised firstly for cartilage thickness, and secondly for staining intensity of the corresponding growth plate (c). (C) Comparison of Safranin-O staining intensity of 15 CXCR2-/- and 15 wild type mouse knee joints. *P < 0.05.

Discussion

The data presented in this chapter reveal a new role for ELR+ CXC chemokines and their downstream CXCR1/2 modulated signalling pathway. Although CXCR1/2 signalling is primarily regarded as inflammatory and catabolic, or required for cell migration, my results suggest that a basal level of autocrine/paracrine CXCR1/2 signalling is required for the phenotypic stability of human articular chondrocytes.

The blockade of CXCR1/2 signalling at both receptor level and at G-protein level results in a significant decrease in the ability of primary chondrocytes to maintain their highly sulphated proteoglycan rich extracellular matrix. This observation has been replicated in the human JJ012 chondrosarcoma cell line following CXCR1/2 blockade, and in porcine primary chondrocytes following G-protein inhibition suggesting that this functional activity may be conserved across species.

In correlation with my previous calcium mobilisation data, the decrease in Alcian blue staining of sulphated proteoglycan ECM content was more pronounced following siRNA receptor knockdown or G-protein inhibition than in micromass cultures treated with CXCR1 and CXCR2 blocking antibodies. Although some variation in matrix composition and rates of ECM synthesis and breakdown may be expected between chondrocytes from different species, it must be considered that the blocking activity of CXCR1 and CXCR2 antibodies may be limited by the reduced accessibility of internalised receptors.

CXCR1/2 signalling inhibition at both extracellular (blocking antibodies) and intracellular (Pertussis toxin) level resulted in the significant decrease in the expression of well validated molecular markers of chondrocyte phenotypic stability. The expression of the key ECM components, type II collagen and aggrecan, and of the key cartilage-specific transcription factor, SOX9, appears to be compromised when CXCR1/2 signalling is inhibited, despite the cells not having been cultured in the presence of additional ligands,

suggesting that an autocrine or paracrine CXC chemokine signalling mechanism may be active, taking advantage of the autologous expression of chemokine ligands previously discussed.

By comparing the relatively modest levels of phenotypic modulation observed by measuring ECM proteoglycan content in micromass cultures with molecular marker expression measured using real time RT-PCR, a number of possible limitations with the micromass culture assay have been identified. Firstly, the 3-dimensional ECM structure of the chondrocyte micromass cultures may prevent the blocking antibodies from accessing receptors upon the surface of cells enclosed within the micromass, resulting in a number of cells not being reached by blocking antibody treatment, a hypothesis supported by the more significant loss of proteoglycan content in JJ012 micromasses treated with CXCR1/2 siRNA in comparison to blocking antibodies. Additionally, the measurement of micromass sulphated proteoglycan content at the same 4 day timepoint used for gene expression readouts results only in the measurement of previously established ECM GAG content which was not able to be homeostatically maintained during the assay time period.

It was hypothesised that since CXCR1/2 signalling activity was required for the maintenance of articular chondrocytes phenotypic stability, the addition of CXC chemokines to late passage dedifferentiated chondrocytes *in vitro* may re-establish the expression of molecular markers including type II collagen and aggrecan. No statistically significant rescue was observed following addition of CXCL8 or CXCL6, however it may not be ruled out that other chemokines signalling via CXCR1 and CXCR2, including CXCL1 may be required either alone, or in combinantion with other factors missing from the culture conditions. Furthermore, the unavailability of CXC chemokine ligands throughout the duration of *in vitro* expansion may result in the irreversible loss of additional signalling mechanisms required in combination with CXCR1/2 signalling for articular chondrocyte

phenotypic maintenance (Luyten 1992 JBC, Harrison 1992 *in vitro* cell dev biol, Benya 1982 cell).

Mice deficient in CXCR2 are not reported to have spontaneous features of OA (Bischoff et al., 2011), at least in physiological conditions. This is not surprising since, as demonstrated in Chapter 3, CXCR1 can functionally compensate for the absence of CXCR2 at calcium mobilisation level in the context of cartilage biology (Figure 18). Functional CXCR1 has been recently identified in the mouse, binding prevalently to mCXCL6. A double knockout will address whether the absence of the two genes *in vivo* results in an articular cartilage phenotype.

Interestingly, a recent study has reported that mice lacking CXCR2 display growth retardation (Bischoff et al., 2011). Whilst the Bischoff et al. paper suggests that the disruption of bone mineral density and repair capacity is most likely linked to CXCR2 mediated angiogenesis, my observations of significantly reduced tibial growth plate thickness in CXCR2 knockout mice suggests that an epiphyseal cartilage specific phenotype may be involved. Whereas in the resting articular cartilage, which has a very slow turnover, CXCR2 function may be sufficiently compensated, in the growth plate it is not, possibly due to the much higher biological activity of this cartilage tissue. Whether in conditions of challenge requiring an increased anabolic activity from chondrocytes the absence of CXCR2 is sufficient to accelerate articular cartilage breakdown is currently under investigation in our laboratory using, as a model of OA, the destabilisation of the medial meniscus (DMM).

CXCL8 is linked to heparan sulphate within healthy cartilage matrix

Experimental layout.

The CXC chemokines CXCL8 and CXCL1 have been shown to be upregulated in articular cartilage during osteoarthritis and have been implicated as inducers of the hypertrophic differentiation of chondrocytes via the increase in type X collagen and MMP-13 expression, alkaline phospahatase activity and matrix calcification (Merz et al., 2003). Although CXC chemokine ligands are widely accepted as being expressed by healthy articular chondrocytes, I have shown that CXCL8 and CXCL6 expression in particular is lost during chondrocyte cell expansion in correlation with dedifferentiation (Figure 10), and that CXCR1/2 signalling is in fact required for chondrocyte phenotypic stability.

ELR+ CXC chemokines are inflammatory cytokines well known for their ability to attract inflammatory cells including neutrophils. Therefore, if ELR+ CXC chemokines are produced in cartilage and are required for the maintenance of cartilage homeostasis, what prevents these molecules from activating local inflammation in physiological conditions? The opposing anabolic and catabolic outcomes of homeostatic and inflammatory CXCR1/2 signalling suggest that an additional level of modulation of chemokine activity may occur specifically within cartilage in order to prevent ligands from acting as inflammatory stimuli within the joint. Studies within the field of inflammation have shown that heparan sulphate proteoglycans (HSPGs) may act to both immobilise chemokines upon the cell surface and ECM, and to facilitate oligomerisation and interaction with other signalling molecules thus optimising chemokine signalling efficiency (Halden et al., 2004;Hoogewerf et al., 1997;Proudfoot et al., 2003;Webb et al., 1993). The high concentration of proteoglycans within articular cartilage indicates that chemokine-GAG chain interactions

may be involved in the regulation of CXCR1/2 signalling in cartilage (Knudson and Knudson, 2001b).

In this chapter I present my investigation into the patterning of ELR+ CXC chemokine distribution within cartilage explants. Immunofluorescence staining was used to examine the relative expression of CXCL8 and CXCL6 within normal articular cartilage explants. Cartilage explants were then pre-incubated with either chondroitinase ABC or heparitinase enzymes, in order to identify the type of proteoglycan found to actively bind chemokine ligands in cartilage. Finally, samples of cartilage from relatively preserved and damaged areas of OA cartilage were compared for ligand presence, leading to the formulation of a novel model of OA disease progression.

CXCL6 and CXCL8 are expressed in normal human articular cartilage

Cartilage explant immunofluorescence was optimised using sections of relatively preserved human articular cartilage explant. By comparing CXCL8 immunostaining in pepsin digested to nondigested sections, it is observed that CXCL8 expression is more apparent following cartilage pre-digestion antigen retrieval (Figure 34), suggesting that aggressive and optimized antigen retrieval is necessary to unmask CXCL8 within the cartilage ECM.
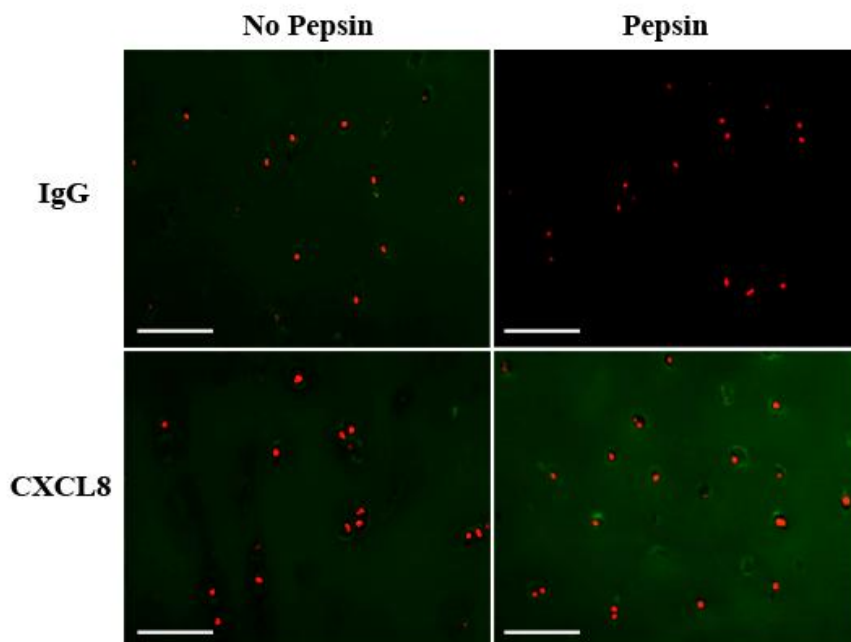
Figure 34. Pepsin retrieval is required for the demonstration of CXCL8 in human articular cartilage. CXCL8 (green) immunofluoresence staining of preserved articular cartilage explants following pepsin digestion, or following incubation in 0.02M HCl. Sections stained with IgG represent negative control. Nuclei are counterstained with propidium iodide (red). Bar, 100µm.

Before examining any chemokine-ECM interaction, I examined the expression of CXCL8 and CXCL6 in articular cartilage. Immunofluorescence in pepsin-digested cartilage explant sections from healthy donors revealed distinct distribution patterns for the two ligands. CXCL8 was found to be consistently located throughout the inter-territorial ECM with a low level found in close proximity to each chondrocyte lacunae. On the other hand, CXCL6 was found in abundance in the territorial and pericellular matrices surrounding each chondrocyte, and was largely absent within the inter-territorial space (Figure 35).
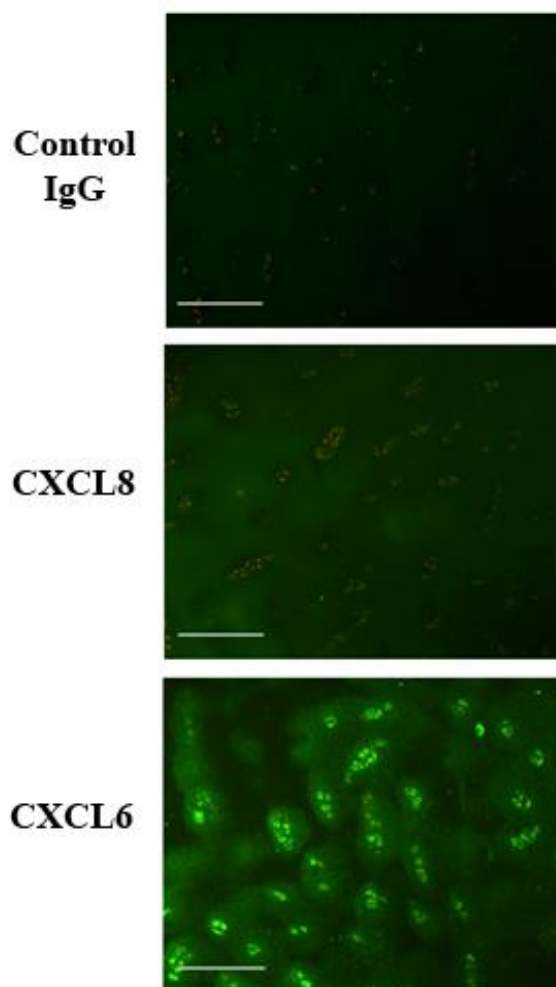
Figure 35. CXCL6 is found in high abundance in healthy human articular cartilage. CXCL6 immunofluorescence staining of healthy articular cartilage explants following pepsin digestion, in comparison with CXCL8 staining in the same explant. IgG represents negative isotype matched control. Nuclei are counterstained with propidium iodide (red). Data presented are representative of 3 donors. Bar 100μm.

CXCL8 is linked to heparan sulphate within healthy cartilage matrix

ELR+ CXC chemokines are known to avidly bind to heparan sulphate proteoglycans (HSPG) in blood vessels (Halden et al., 2004;Hoogewerf et al., 1997). Since the articular cartilage ECM is very rich in HSPGs (Knudson and Knudson, 2001b), I hypothesised that HSPGs may be responsible for retaining ELR+ CXC chemokines within the cartilage matrix, making them available for autocrine/paracrine signalling in chondrocytes. Consistent with this hypothesis, CXCL8 was detected by immunofluorescence amongst the ECM of relatively preserved (Mankin score <4) human articular cartilage from OA patients. To test whether CXCL8 was bound to either chondroitin or heparan sulphate proteoglycans, I performed the same staining following digestion of the slides with chondroitinase ABC or heparitinase. Chondroitinase digestion did not abolish the staining, and in fact further improved it by acting as a further method of antigen retrieval. In contrast, heparitinase digestion nearly completely abolished the matrix staining thereby demonstrating that binding to heparan sulphate chains is required for the localisation of CXCL8 to the cartilage ECM (Figure 36).   In keeping with my data, CXCL8 was shown to bind to HSPGs but not to chondroitin sulphate in blood vessels, and *in vitro* (Kaneider et al., 2002).
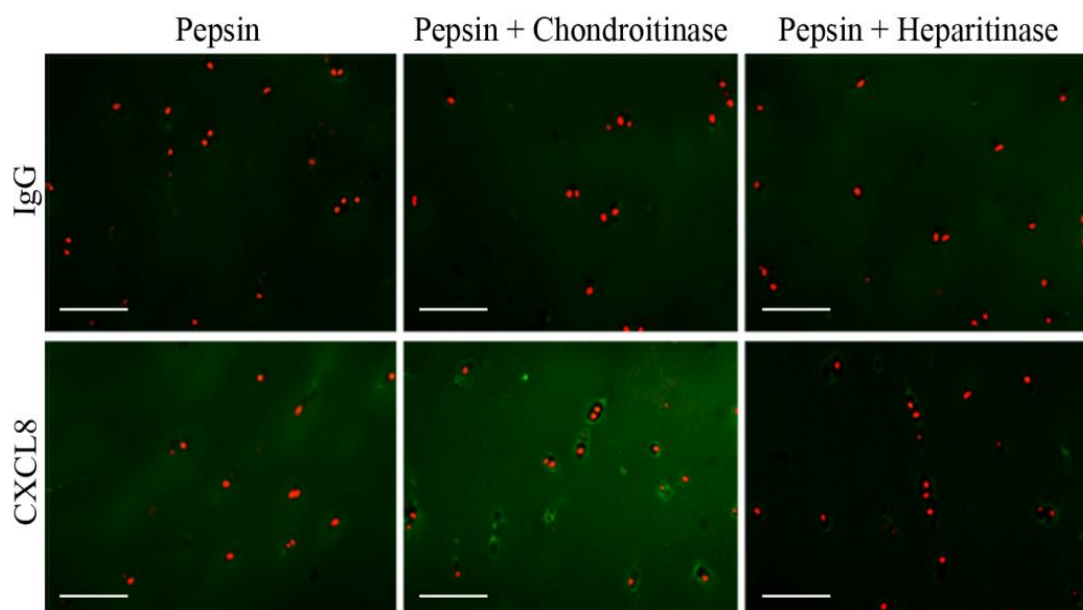
Figure 36. CXCL8 is bound to HSPGs within articular cartilage extracellular matrix. CXCL8 (green) immunofluorescence staining of preserved articular cartilage explants digested with pepsin, pepsin followed by chondroitinase ABC, or pepsin followed by heparitinase. Sections stained with IgG represent the negative control. Nuclei are counterstained with propidium iodide (red). Data presented are representative of 3 donors. Bar, 100µm.

In order to establish whether the altered distribution pattern of CXCL6 in comparison with CXCL8 is caused by CXCL6 interacting with either a different HSPG or different proteoglycan class entirely, immunofluorescence was used to detect CXCL6 in heparitinase pre-treated cartilage, which was then compared with CXCL8. CXCL8 was once again observed to be released following heparitinase treatment, however CXCL6 distribution remained unchanged (Figure 37), suggesting that CXCL6 within articular cartilage is not HSPG bound.

CXCL6 and CXCL8 distribution is altered in areas of OA damaged cartilage

Finally, cartilage samples from areas with severe OA damage (Mankin score > 6) were compared to those from relatively preserved areas (Mankin score < 4) from the same joint. In preserved samples, CXCL8 staining was more intense in proximity to the cells, whereas an opposite pattern with depletion of CXCL8 from the matrix immediately adjacent to the chondrocytes was observed in highly damaged samples (Figure 37 and Figure 38), suggesting that ECM depletion by OA chondrocytes may lead to failure of the ECM to retain CXCL8 *in situ*.

I therefore propose a model (Figure 39) in which, under physiological conditions, CXCL8 is retained in the cartilage ECM bound to HSPGs, which determine its signalling domain, and activate a homeostatic autocrine/paracrine mechanism. In OA, ECM breakdown results in failure to retain CXCL8 locally, in loss of the anabolic local autocrine/paracrine CXCR-dependent signaling. It is reasonable to speculate that the loss of anchoring HSPG may also result in the release of inflammatory chemokines into the joint space where they contribute to local inflammation which perpetuates and amplifies the damage.
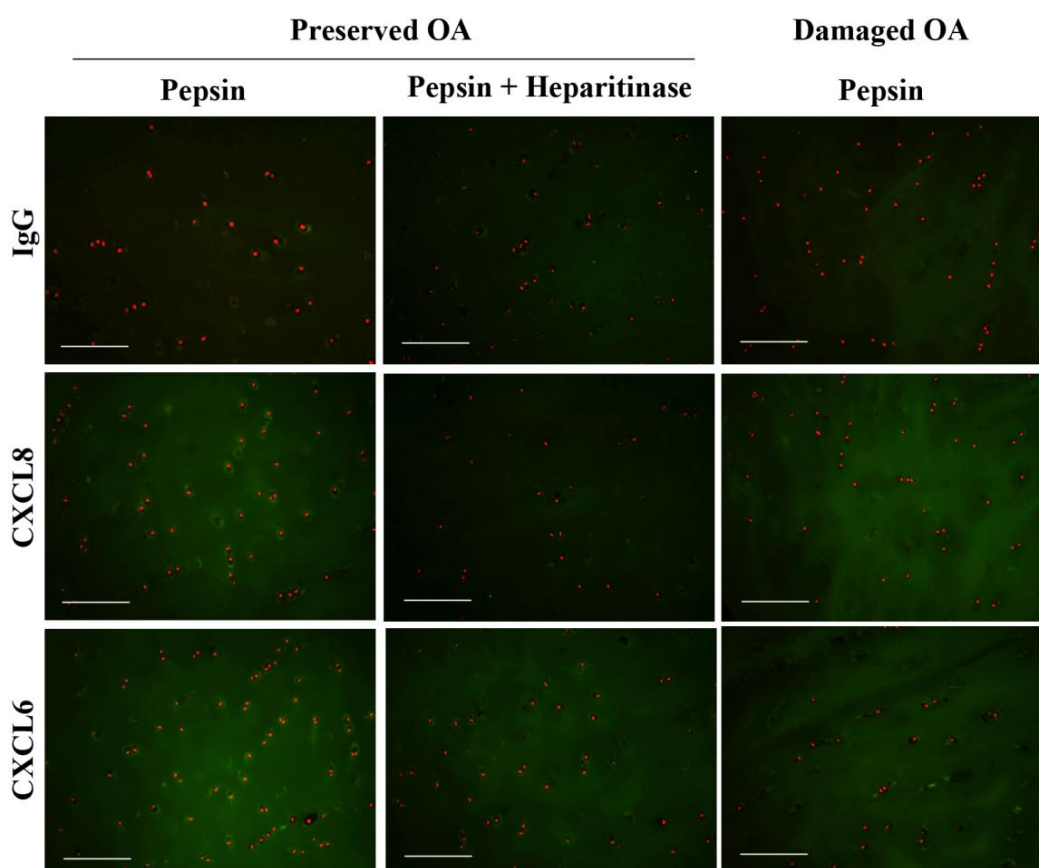
Figure 37. CXCL6 and CXCL8 show different matrix binding patterns. CXCL6 or CXCL8 (green) immunofluorescence staining of preserved and damaged articular cartilage explants from an OA donor digested with pepsin alone, or pepsin followed by heparitinase. Sections stained with IgG represent the negative control. Nuclei are counterstained with propidium iodide (red). Data presented are representative of 3 donors. Bar, 100μm.
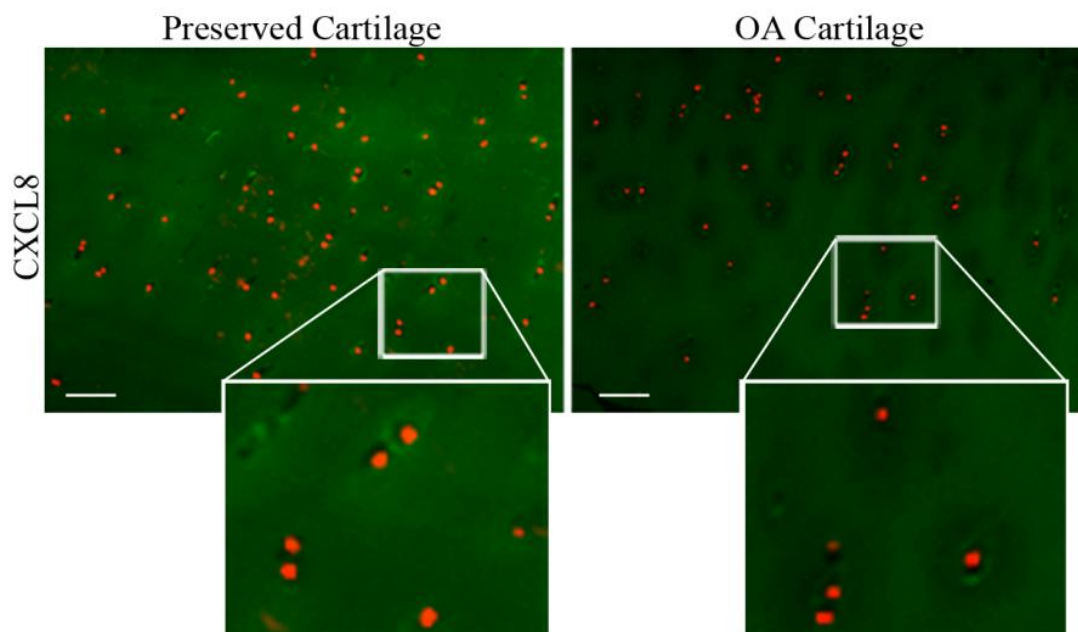
Figure 38. CXCL8 is lost from articular cartilage extracellular matrix during osteoarthritis pathology. CXCL8 immunofluorescence of pepsin-digested cartilage samples obtained either from preserved (Mankin score < 4) or from severely damaged (Mankin score > 6) areas of femoral condyles affected by OA. Nuclei are counterstained with propidium iodide (red). Data presented are representative of 3 donors. Bar, 100µm.
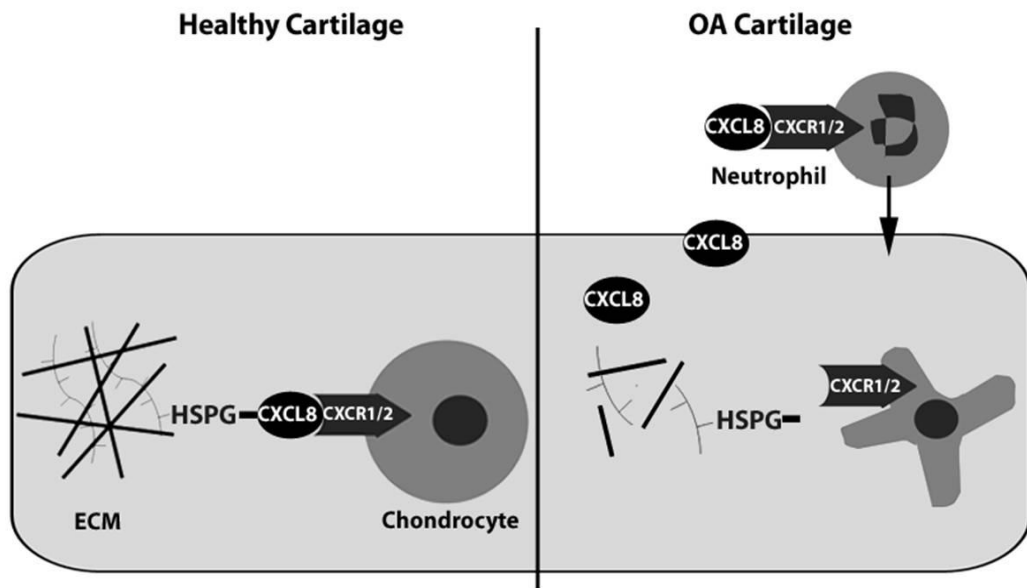
Figure 39. Autocrine/Paracrine homeostatic CXCR1/2 signalling in articular cartilage. In healthy articular cartilage, CXCL8 is expressed by chondrocytes, is retained within the ECM by HSPGs and is available and required for signaling via CXCR1 and CXCR2 supporting chondrocyte phenotypic stability and cartilage homeostasis. In osteoarthritis, breakdown of HSPGs within the ECM results in the release of CXCL8, the disruption of the homeostatic ELR+ CXC chemokine signaling mechanism required for phenotypic stability, and in the diffusion of CXCL8 in the synovial space, which leads to recruitment of inflammatory cells.

Discussion

Previous studies have, on the whole, regarded ELR+ CXC chemokine ligand and receptor expression to be upregulated during OA, resulting in the upregulation of inflammatory pathways, hypertrophic differentiation and cartilage destruction. The data presented in this chapter supports my findings from Chapter 5, that CXCR1/2 signalling is required for chondrocyte phenotypic stability, by developing a model in which chemokines may be produced by normal articular chondrocytes and retained within the cartilage ECM, where they contribute to chondrocyte homeostasis.

Firstly, I have optimised an antigen-retrieval method which enables the detection of chemokines in healthy articular cartilage explants. Previous reports of increased detection of CXCL8 in OA cartilage fail to account for the increased accessibility of chemokines in OA cartilage to antibody detection due to the breakdown of the ECM. Following pepsin digestion pre-treatement upon all cartilage explant sections, we see that chemokines are not only present in healthy tissue, and in the case of CXCL6, found in abundance.

Although previous studies have noted the upregulation of CXCL8 and CXCL1 in OA (Borzi et al., 1999;Merz et al., 2003), no data has been previously presented regarding the presence of CXCL6 in either normal or OA cartilage. My data suggests that CXCL6 may be present in high levels within healthy articular cartilage, making it a likely contributor to chondrocyte homeostasis. In OA cartilage explants, including those that are relatively preserved, CXCL6 levels were found to be much lower, indicating that the ECM associations retaining this chemokine within the tissue are disrupted at an early stage of OA pathology. Additionally, CXCL8 is known to be more potent as a mediator of human neutrophil chemotaxis than CXCL6 (Fox et al., 2005). Producing CXCL6 as a homeostatic mediator within cartilage is therefore less likely to result in an inflammatory response as would be expected from the release of a similar concentration of CXCL8, since both ligands signal via both CXCR1 and

CXCR2, although the relative potency of each ligand in chondrocyte phenotypic modulation is not yet known.

The binding of endogenous ELR+ CXC chemokines to HSPGs, or other cartilage matrix components, may play an important part in the modulation of their functional activity. Specifically, oligomerisation of chemokines when bound to GAG chains within the ECM, may allow for the presentation of subunit receptor binding sites thus actively increasing signaling activity (Proudfoot et al., 2003). In this way, ligands shown to be relatively redundant *in vitro*, including CXCL6, may in fact aid in the functional activity of other more potent chemokine ligands. Alternatively, CXCL6, which is 100 times less potent as an inflammatory chemokine as CXCL8 (Fox et al., 2005), may have a dominant negative effect over CXCL8 in cartilage. This type of agonist/antagonist system amongst chemokine ligands in cartilage would ensure sufficient signalling activity to maintain cartilage homeostasis without resulting in a pro-inflammatory or pro-angiogenic response.

Chapter 5 - General discussion and Future Directions

Discussion

We have discovered that, in physiological conditions, ELR+ CXC chemokines and their downstream CXCR1/2 signalling pathway constitute an autocrine/paracrine mechanism supporting chondrocyte phenotype and cartilage ECM homeostasis. We have shown that chondrocytes express both CXCR1 and CXCR2 and their ligands CXCL8 and CXCL6 in monolayer and cartilage explants, that endogenous ligands activate the receptors locally, and that signalling through both CXCR1/2 and G proteins is required for the maintenance of the stability of the mature articular cartilage phenotype, of the maintenance of a highly sulfated GAG-rich ECM, and for the expression of the transcription factor SOX9.

ELR+ CXC chemokines are best known as inflammatory molecules mediating leukocyte migration and angiogenesis. Although it is known that some CXC chemokines have a role in embryonic morphogenesis including gamete and oligodendrocyte migration (Kunwar et al., 2006;Robinson and Franic, 2001), these functions take place before the immune system is mature and are still mediated by the known function of these molecules to guide cell migration. Their role as mediators of homeostasis in adulthood is completely novel and unexpected, firstly, because this role is not related to cell migration, but rather to maintain cell differentiation, and secondly because it occurs outside of the context of inflammation, in an immunologically mature organism.

In nature there are a number of examples of molecules that have completely different functions within different biological contexts, particularly highly evolutionarily conserved morphogens including Wnts and BMPs which are the building blocks of embryonic morphogenesis, but is less common in more specialised and more evolutionarily recent molecules including inflammatory cytokines and chemokines. The novel homeostatic function of ELR+ CXC chemokines is made possible in this case by the peculiarity of the articular cartilage being an avascular tissue, which is immunologically "privileged", and by a sequestration of ligands including CXCL8 onto matrix molecules. Interestingly, this

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property is shared by other morphogens which also play a role in cartilage homeostasis such as Wnts (Bishop et al., 2007;Shortkroff and Yates, 2007) and BMPs (Ohkawara et al., 2002;Otsuki et al., 2010;Ruppert et al., 1996). In this regard, a crosstalk between these molecular pathways is an interesting possibility and is being investigated in our laboratory.

We cannot exclude the possibility that CXC chemokines may play additional roles in cartilage, such as attract mesenchymal stem cells (Ringe et al., 2007) or even regulate a postulated migration of chondrocytes across the different cartilage layers (Hayes et al., 2001). Of course these functions are not mutually exclusive.

Mice deficient in CXCR2 do not have spontaneous features of OA (Jacobs et al., 2010), however this is not surprising since in our experiments, CXCR1 can functionally compensate for the absence of CXCR2 in the context of cartilage biology (Bischoff et al., 2011). Interestingly, however, Bischoff et al have recently reported that CXCR2 deficient mice have a skeletal growth defect and low bone mineral density. Since the bone phenotype is prevalently related to the cortical bone, which does not develop through endochondral bone formation, it has been argued that this phenotype is due to defects of angiogenesis rather than cartilage development (Bischoff et al., 2011). Nevertheless, since bone collar formation is directly dependent on Ihh signaling from the prehypertrophic chondrocytes of the growth plate (Chung et al., 2001;Vortkamp et al., 1996), it therefore cannot be excluded that a defect in the rate of maturation of epiphyseal chondrocytes may be responsible for the bone defect in these mice. In this context, it is interesting that we have discovered that even in adulthood, CXCR2-/- mice display a significant decrease in epiphyseal growth plate thickness. Therefore we are currently exploring the hypothesis that, at least in part, the thinner cortical bones observed in these mice results from deficient Ihh signalling within the growth plate. I am currently testing this hypothesis in our laboratory using *in vitro* and *in vivo* systems. This will include addressing whether

hedgehog signalling is dependent on CXCR signaling, whether CXCR2-/- chondrocytes have deficient Ihh signalling, and if exogenous Ihh can rescue the bone phenotype.

The majority of the results included in this thesis has been obtained in *in vitro* settings and partly contrasts with previous literature showing a pathogenic role of CXCR signaling in various forms of arthritis, although most functional studies were in inflammatory arthritis, where the role played by inflammatory chemokines is clearly different. The effects of CXCR1/2 signalling intervention in attenuation of inflammatory arthritis did not include the analysis of cartilage integrity (Cunha et al., 2008;Jacobs et al., 2010). The formal testing of the role of CXCR signalling in models of OA is still missing, and is a priority for the future development of this study.

The rationale for such experiment is that although the analysis of articular cartilage of CXCR2-/- mice, which in resting conditions has a relatively slow cell turnover, revealed no significant pathology, CXCR2 specific activity may be required in order to maintain the anabolic activity of chondrocytes in conditions of challenge. A functional ortholog of CXCR1 has been recently identified in mice (Fan et al., 2007). CXCR1 activity may compensate for the loss of CXCR2 signalling to a certain extent, shown firstly at downstream calcium mobilisation level, and secondly by the apparent maintenance of articular cartilage in CXCR2-/- mice, but subtle differences between the behaviours of the two receptors found in human studies suggest that distinct functional roles may exist for each receptor. These include differences in receptor recycling, and in the selection of chemokine ligands able to act via each receptor in both humans and mice. We aim to investigate the extent to which predicted CXCR1 activity may sufficiently compensate for CXCR2 by challenging the CXCR2-/- mice in the DMM (destabilisation of the medial meniscus) OA model. The surgically induced instability, shown previously to induce articular cartilage destruction (Glasson et al., 2007), is likely to lead to accelerated cartilage

breakdown should the increased anabolic activity required to counteract the activated destructive pathways within the cartilage become too great to be supported through CXCR1 mediated homeostasis alone.

An interesting aspect of our study is that the same molecule can have a homeostatic function in physiological conditions and a pathogenic one in osteoarthritis. In fact, CXCR2 blockade has been shown to attenuate the outcome of inflammatory arthritis *in vivo* in mice. Other examples of such double function of inflammatory chemokines include IL-1 and IL-6. These two inflammatory cytokines are currently established therapeutic targets in arthritis (Cohen et al., 2002;Yokota et al., 2005;Smolen and Maini, 2006), but mice deficient in IL-1 or IL-6 are more susceptible to OA, either surgically induced (Clements et al., 2003) or spontaneous (de Hooge et al., 2005).

The dual function of some inflammatory cytokines represents an important pharmacological challenge for the use of chemokine inhibitors as a therapeutic strategy for treatment of arthritis. The homeostatic importance of ELR+ CXC chemokine signalling in chondrocytes, whereby CXCR1/2 signalling is required in order to maintain phenotypic stability and ECM production, suggests that the inhibition of this pathway aimed at reducing neutrophil infiltration during inflammatory arthritis (Barsante et al., 2008;Coelho et al., 2008) may in fact result in chondrocyte toxicity accentuating cartilage damage. At the same time, this project has presented an opportunity for the development of targeted strategies for cytokine blockade that preserve homeostatic mechanisms within the cartilage compartment.

Immunohistochemical analysis of healthy human articular cartilage within this study has revealed that CXCL8 in particular is specifically bound to HSPGs within the cartilage ECM. It is therefore reasonable to expect that homeostatic maintenance of articular chondrocytes by CXC chemokines is driven by ligands bound and localised within

the ECM. This will need experimental testing, however, if this is true, therapeutic intervention aimed at inhibiting the effects of chemokine driven joint inflammation, particularly during the early stages of disease when the cartilage remains relatively intact, should therefore be developed with the aim of being unable to infiltrate into the cartilage tissue itself. The dense, highly sulphated ECM and avascular nature of articular cartilage should present ideal opportunities for the design of suitable compounds.

The concept of "functional selectivity", whereby the activation of common receptors by different ligands, or in different cell types, results in the activation of specific downstream signalling pathways, suggests that inflammatory CXCR1/2 signalling may be specifically blocked within the joint following the elucidation and inhibition specific pathways. A number of CXCR1/2 allosteric inhibitors, shown to inhibit AKT phosphorylation and directional migration in neutrophils represent an important opportunity to develop therapeutic compounds (Allegretti et al., 2008;Leach et al., 2007). Allosteric inhibition is able to alter the three-dimensional receptor conformation, thus modulating which G-proteins are able to associate with the receptor. By evaluating the relative contributions of downstream signalling targets, including calcium mobilisation and PI3K/AKT signalling to chondrocyte phenotypic maintenance, and selecting only compounds which avoid modulation of these pathways, specific outcomes of CXCR1/2 activation may be pharmacologically targetted in order to avoid potential cartilage toxicity.

With the same aim, we have the opportunity to further investigate whether the different ligands found within healthy articular cartilage modulate specific functional outcomes. CXCL6 is a significantly less potent mediator of neutrophil migration than CXCL8, however it is found to be extremely abundant in healthy articular cartilage, suggesting that it is of greater importance to cartilage homeostasis. In this regard, we do not currently know if CXC chemokines are required in combination for cartilage

homeostasis, or whether a level of functional redundancy exists that would allow for elimination of ligands found to more actively promote inflammation, without compromising the chondrocyte phenotype.

Appendix: Reagents

Complete culture medium

500ml DMEM/F-12 + GlutaMAX™-1 (Gibco, Invitrogen, UK)

10% FBS (Gibco, UK)

1% Sodium pyruvate 100nM (Sigma, UK)

1% Antibiotic Antimycotic 100X (Gibco,UK)

HS-FBS medium

500ml DMEM/F-12 + GlutaMAX™-1

1% FBS pre-heated to 57°C for 30 minutes

1% Sodium pyruvate 100nM

1% Antibiotic Antimycotic 100X

Antibiotic free medium

500ml DMEM/F12 + GlutaMAX™-1

10% FBS

1% sodium pyruvate

Freezing medium

7.5ml FBS

5ml DMSO

Make up to 25ml with complete DMEM

Sterilise by filtration

To freeze, cells resuspended in complete DMEM are diluted 1:1 into DMSO freezing medium and placed immediately into -80°C freezer for 48 hours, before transfer into liquid nitrogen.

<u>10X PBS</u>

80g Sodium chloride (NaCl)

2g Potassium chloride (KCl)

14.4g Sodium phosphate diphasic (Na$_2$HPO$_4$)

2.4g Potassium phosphate monobasic (KH$_2$PO$_4$)

Make up to 1 litre with distilled water. Adjust pH to 7.4.

<u>Western blot sample buffer</u>

63mM Tris HCl

10% glycerol

2% SDS

0.0025% bromophenol blue

pH6.8

1. To prepare 10ml of 2X solution mix the following:
    a. 0.5M tris HCl, pH6.8   2.5ml
    b. Glycerol   2ml
    c. 10% (w/v) SDS   4ml
    d. 0.1% bromophenol blue   0.5ml
2. Adjust the volume to 10ml with ultrapure water
3. Store at 4°C. buffer is stable for 6 months

Western blot running buffer

25mM Tris base

192mM glycine

0.1% SDS

pH8.3

1. To prepare 1litre of 10X solution, dissolve the following into 900ml of ultrapure water

   Tris base   29g

   Glycine 144g

   SDS   10g

2. Mix well and adjust volume to 1000ml with ultrapure water
3. Store at RT. The buffer is stable for 6 months.
4. For electrophoresis, dilute the buffer to 1X with water, pH will still be pH8.3, do not adjust with acid/alkali.

Western blot transfer buffer

12mM Tris base

96mM glycine

1. To prepare 500ml of 25X solution dissolve the following in 400ml of ultrapure water
   a. Tris base   18.2g
   b. Glycine   90g
2. Mix well and adjust the volume to 500ml with ultrapure water
3. Store at RT, buffer is stable for 6 months
4. For blotting dilute this buffer to 1X (pH8.3):
   a. Tris-glycine transfer buffer (25X)   40ml
   b. Methanol   200ml
   c. Deionized water   760ml

Toluidine blue

0.1% Toluidine blue (Sigma) in 0.1M acetate buffer pH5.

Safranin-O

0.2% Safranin-O in 0.2M acetic acid pH3.8.

Alcian blue 8GS

0.5% Alcian blue 8GS (Carl Roth, Karlsruhe, Germany) in 1N HCl pH0.2.

Publications

1. Nalesso, G., **J.Sherwood**, J.Bertrand, T.Pap, M.Ramachandran, C.De Bari, C.Pitzalis, and F.Dell'accio. 2011. WNT-3A modulates articular chondrocyte phenotype by activating both canonical and noncanonical pathways. *J. Cell Biol.* 193:551-564.

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