Development of model *in vitro* culture systems for studying cartilage metabolism in health and disease

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Abbreviations

ATP	Adenosine triphosphate
ADAMs	A Disintegrin and Metalloproteinase
ADAMTS	A Disintegrin and Metalloproteinase (reprolysin type) with thrombospondin motifs
Å	Angstrom unit equal to 10^{-10} metre or 0.1nm
RGD	Arginine / Glycine / Aspartic Acid sequence
ACI	Autologous Chondrocyte Implantation
AP-1	Activator Protein-1
bFGF	Basic Fibroblast Growth Factor
β-GP	*Beta-* glycerophosphate
BMP	Bone Morphogenetic Protein
BrDU	Bromodeoxyuridine
CPP	Calcium Polyphosphate
C-terminal	Carboxy terminal
CILP	Cartilage Intermediate Layer Protein
COMP	Cartilage Oligomeric Matrix Protein
CS	Chondroitin Sulphate
C-0-S	Chondroitin-0-sulphate
C-4-S	Chondroitin-4-sulphate
C-6-S	Chondroitin-6-sulphate
Col	Collagenous domain
Clq	Complement component
COX-2	Cyclo-oxygenase-2
Da	Dalton
DS	Dermatan Sulphate
DMMB	Dimethylmethylene Blue
DMEM	Dubecco's Modified Eagle Medium
ESC	Embryonic Stem Cells
ER	Endoplasmic Reticulum
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ECM	Extracellular Matrix

ERK	Extracellular signal-regulated protein kinase C
FACIT	Fibril associated collagens with an interrupted triple helix
FGF	Fibroblast Growth Factor
FLS	Fibroblast-like Synoviocytes
Fn	Fibronectin
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FT	Full thickness cartilage
GI tract	Gastro Intestinal Tract
GlcUA	Glucuronic Acid
Gln	Glutamine
GAG	Glycosaminoglycan
GPI	Glycosylphosphatidylinositol
HARE	Hyaluronan receptor for endocytosis
HABR	Hyaluronan binding region
HSP	Heat Shock Protein
HS	Heparan Sulphate
Hep	Heparin
HA	Hyaluronan
HAS	Hyaluronan Synthases
Hyal	Hyaluronidases
IdoUA	Iduronic acid
Ig	Immunoglobin
ITS	Insulin – Transferrin – Selenium
IGD	Interglobular domain
IL-1β	Interleukin-1 β
ITM	Interterritorial Matrix
iNOS	Inducible nitric oxide synthase
IGF-1	Insulin-like growth factor-1
KS	Keratan Sulphate
kDa	Kilo Dalton
LYVE-1	Lymphatic vessel endothelial HA receptor
MMPs	Matrix Metalloproteinases
mRNA	messenger Ribonucleic Acid

MSC	Mesynchymal Stem Cells
MD	Mid to deep region Cartilage
MED	Multiple epiphyseal dysplasia
GalNAc	N-acetylgalactosamine
NC	Non-collagenous domain
NFκβ	Nuclear factor-kappa-beta
OA	Osteoarthritis
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related protein
P (0-5)	Passaged cells (number of times a chondrogenic source had been culture expanded in monolayer culture)
PCM	Pericellular Matrix
PBS	Phosphate Buffered Saline
PEA	Phosphoethanolamine
PARP	Proline / arginine-rich protein
PRELP	Proline Arginine-rich and leucine rich repeat
PG	Proteoglycan
PTR	Proteoglycan tandem repeat
PSACH	Pseudoachondroplasia
PEMFs	Pulsed Electromagnetic Fields
RA	Rheumatoid Arthritis
RHAMM	Receptor for hyaluronan-mediated motility
RHT	Ruthenium hexamine trichloride
SPARC	Secreted Protein Rich in Cysteine
SLRPs	Small leucine rich proteoglycans
SEM	Standard error mean
s-GAG	Sulphated GAG
SZP	Superficial Zone Protein/ Proteoglycan
SOX 9	Sry-like (sex determining region of Y-chromosomes) HMG (High mobility group) box
TM	Territorial Matrix
T	Tesla
TSP	Thrombospondin
TIMPs	Tissue Inhibitors of Metalloproteinases

TGFβ	Transforming Growth Factor β
TNFα	Tumour Necrosis Factor α
TACE	Tumour Necrosis Factor α converting enzyme
TSG-6	Tumour necrosis factor stimulated gene – 6
UTP	Uridine Triphosphate
vWFA	von Willebrand factor A

Summary

The generation of neocartilage grafts *ex vivo* shows promise for the repair of articular cartilage defects. This thesis describes (1) the metabolism and macromolecular organisation of grafts produced utilising a Transwell culture system at biochemical and microscopic levels, (2) the effect of chondrocyte de-differentiation, induced by monolayer expansion, on resultant graft tissue architecture, and (3) how addition of exogenous hyaluronan of varying molecular weights, during and post graft generation effect chondrocyte metabolism, with a view to explaining effects of hyaluronan administration in viscosupplementation procedures. Grafts generated from primary chondrogenic sources were of hyaline nature with distinct zonal stratification as found in native articular cartilage evidenced by cell morphology, matrix organisation and immunohistochemical composition. Graft tissue produced from immature sources was more synthetically active than those produced from mature sources. Passage expansion of chondrocytes in monolayer culture caused progressive reduction in graft thickness, loss of zonal architecture, and a more fibrocartilaginous tissue histology, consistent with a de-differentiating chondrocyte phenotype. Grafts subjected to exogenous hyaluronan of smaller molecular weight than that typically found in native aggrecan aggregates (*500kDa–1000kDa*) had an increased release of proteoglycan from graft tissue. Addition of hyaluronan with significantly higher molecular weight than endogenous hyaluronan resulted in an increased retention of proteoglycan within graft tissue, suggesting that high molecular weight hyaluronan in viscosupplementation procedures may be facilitating the retention of newly synthesised 'repair aggrecan' in pathological cartilage, slowing down the rate of cartilage destruction through loss of proteoglycan from the tissue. Futhermore, the addition of 490kDa (*similar molecular weight to endogenous hyaluronan)* showed a marked increase in the amount of aggrecan synthesised. Collectively, this data suggests that viscosupplementation procedures should incorporate a mixture of hyaluronan ranging between 490kDa-3000^{+}kDa in order to provide optimal benefits of aggrecan retention and biosynthesis to osteoarthritic patients receiving this treatment modality.

1 INTRODUCTION

1.1 Articular cartilage morphology

Ideally, tissue engineered cartilage grafts should show similarities to native cartilage in cellular distribution, collagen organisation and at a macromolecular level. It is important to take all these aspects into consideration when generating cartilage grafts. Cartilage is extensively distributed within the foetus, and often considered to be an 'embryonic tissue'. Through a process termed endochondral ossification, cartilage provides a template in which skeletal elements can develop, leading to skeletal maturity. In adults, there is a restriction in cartilage distribution but it is still located in a variety of sites including trachea, nasal septum, and the focal point for this project 'the articulating joint'(Archer & Francis-West., 2003).

Articular cartilage is an avasular, aneural, alymphatic connective tissue that lines the ends of bones in diarthroidal joints, providing a frictionless surface that has impact absorbing properties (Buckwalter and Hunziker., 1999). Cartilage contains one type of cell, the 'chondrocyte'. The chondrocyte is responsible for synthesising the extracellular matrix of articular cartilage. A large percentage of the extracellular matrix is composed of collagens (***see section 1.2***), proteoglycans (***see section 1.4***) and water. Collagen constitutes up to 60% dry weight of articular cartilage and proteoglycans, immobilised within the extracellular matrix via a collagen framework, constitute 10-15% wet weight of articular cartilage. Depending on the load status of the cartilaginous tissue water can contribute 65-80% of the total cartilage weight (Jackson *et al.*, 2001).

1.1.1 Chondrocytes

There is only one cell type found within articular cartilage and it comprises only 2-5% of the tissue volume, this being the 'chondrocyte'. The chondrocytes are cytoplasmically isolated, and consequently cell-cell contact is improbable. These cells are responsible for the synthesis of extracellular matrix molecules including collagens, proteoglycans and non-collagnous proteins forming a unique, ordered

structure (Buckwalter and Hunziker., 1999). Chondrocytes are largely nourished by diffusion from adjacent synovial fluid, which fills the joint cavity. Additionally, small amounts of nutrients are derived from blood vessels that course through the calcified cartilage close to the bone (Schumacher *et al.*, 1994). As a result of chondrocytes being locked within an extracellular matrix they are unable to move. They do not actively change their shape except during hypertrophy, but they may be passively deformed during compressive loading (Skaggs *et al.*, 1993).

Typically chondrocytes can vary in shape from a spheroidal to a flattened form. Factors that influence the shape, size and characteristics of a chondrocyte are the type of cartilage in which the chondrocyte is present, the actual position of the chondrocyte within the tissue, the age of the organism in which it is present and the cellular density of the tissue (Buckwalter & Hunziker., 1999).

Chondrocytes contain cellular organelles similar to those found in other cells, e.g. a nucleus containing the DNA content of the cell, endoplasmic reticulum, golgi apparatus, lysosomes and mitochondria. There are low levels of mitochondrion, as chondrocytes are unique in that they exist in a low oxygen tension environment and most energy requirements are obtained through glycolysis (Bulkwalter & Hunziker., 1999).

The chondrocyte contains a cytoskeleton consisting of three types of protein elements; microfilaments, microtubules and intermediate filaments. The cytoskeleton of chondrocytes is important in maintaining the chondrogenic phenotype, cell division, inside-out and outside-in signalling mechanisms. The action of a chondrocyte on the extracellular matrix depends on the stimuli received and vice versa, therefore a feedback loop of signals from the extracellular matrix to the chondrocyte and the chondrocyte to the extracellular matrix is maintained (Benjamin *et al.*, 1994). Some chondrocytes also have short processes or microvilli cilium extending into the cell matrix which sense mechanical change within the extracellular matrix conveying the information back to the cell (Buckwalter and Hunziker., 1999).

1.1.2 Zonal Organisation in Articular Cartilage

Four zones of articular cartilage can be identified (**Figure 1.1**), due to morphological changes in both the chondrocytes and matrix from the articular surface to the subchondral bone. There is variance between species and among joints in the relative size and appearance of each of the zones. Chondrocytes from different zones vary in size, shape and metabolic activity (Schumacher *et al.*, 1994). The arrangement of the cells within these zones is maintained by the collagen fibre organisation known as 'Benninghoff Arcades' (Benninghoff., 1925).

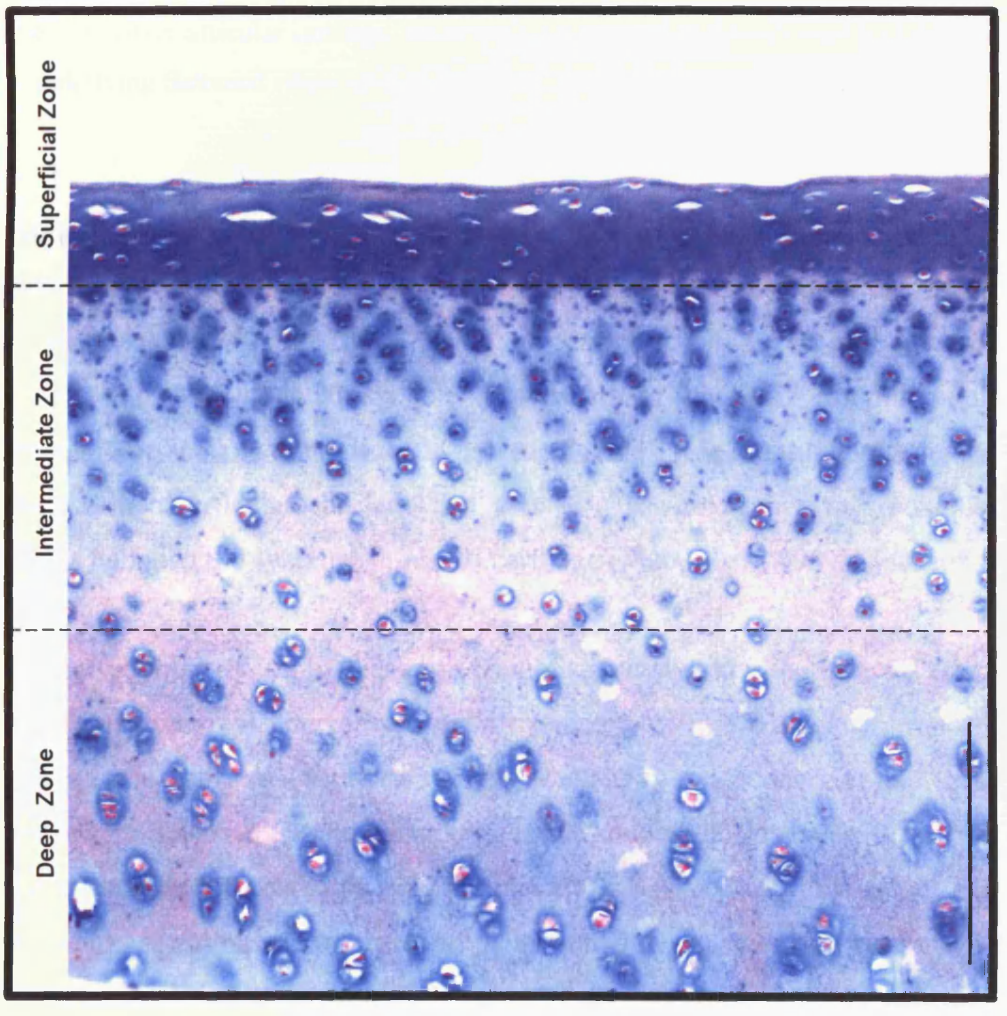

Figure 1.1 Histological section through mature (18 month) bovine native articular cartilage depicting the zonal organisation of chondrocytes. Alcian blue and haematoxylin/eosin. Scalebar=200μm

(A) Tangential layer / Superficial Zone

Figure 1.2 Histological section showing the superficial zone of mature (18 month) bovine native articular cartilage, an amorphous material at the top most surface and underlying flattened ellipsoid chondrocytes. Alcian blue and haematoxylin/eosin. Scalebar=50μm

The superficial zone typically consists of an acellular layer and a cellular layer (**Figure 1.2**). The acellular, most superficial region is referred to as the lamina splendens (Jackson *et al.*, 2001) which is composed of an amorphous material (Jurvelin *et al.*, 1996). The lamina splendens consist of fine fibrils running parallel to the articular surface and contains little polysaccharide. It is the thinnest zone and the so-called skin of articular cartilage that acts as a barrier against the movement of molecules between the synovial fluid and cartilage (MacConaill 1951) (Jackson *et al.*, 2001).

The deeper cellular region of this zone contains disc shaped chondrocytes (flattened ellipsoid chondrocytes) interspersed within a matrix of fine collagen fibrils aligned parallel to the surface (Schumacher *et al.*, 1994). Fibronectin and collagen concentrations (Buckwalter and Hunziker., 1999) are high in this layer in contrast to a low concentration of proteoglycans found within this region. However, a novel proteoglycan is synthesised by the superficial zone chondrocytes of articular cartilage called Superficial Zone Protein (SZP) and was first identified by Schumacher *et al* in 1994.

This zone has great tensile strength in comparison with deeper zones found in cartilaginous tissue (Jackson *et al.*, 2001). The superficial layers have been described as a 'tension resisting diaphragm' (Meachim & Stockwell., 1979) manifested by the

tendency of articular cartilage to curl when released from the subchondral bone (Broom & Poole., 1982).

(B) Transitional zone / Intermediate zone

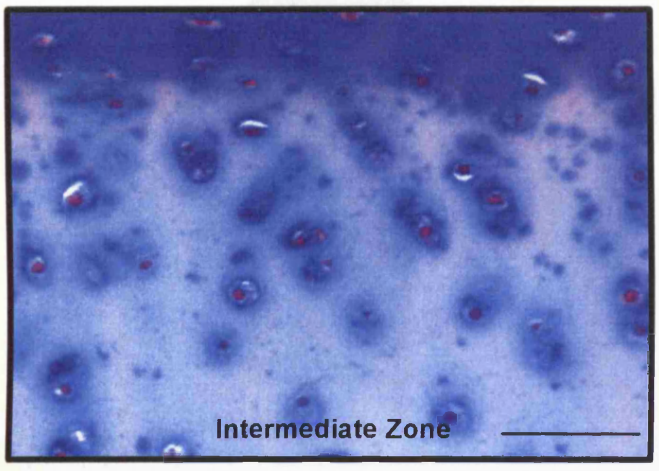

Figure 1.3 Histological section showing the intermediate region of mature (18 month) bovine native articular cartilage depicting randomly dispersed chondrocytes. Alcian blue and haematoxylin/eosin. Scalebar=50μm

The transitional zone as the name implies is an intermediate between the superficial zone and the radial zone (**Figure 1.3**). The chondrocytes in this region are slightly larger, spheroidal in appearance, prehypertrophic and widely spaced. The chondrocytes have a higher concentration of synthetic organelles than those found in the superficial layer, synthesising a matrix with a higher proteoglycan concentration. Both the water and collagen concentrations decrease within this region in comparison to the superficial zone, but the fibrils that are present have a larger diameter and they are not arranged in any clear orientation (Buckwalter and Hunziker., 1999). Unique to this area of cartilage is a molecule known as Cartilage Intermediate Layer Protein (***CILP see section 1.5.3***) (Lorenzo *et al.*, 1998).

(C) Radial zone / Deep zone

Figure 1.4 A histological section of the deep region of 18 month bovine articular cartilage clearly showing the columnar organisation of the chondrocytes. Alcian blue and haematoxylin/eosin. Scalebar=50μm

This zone contains fairly large, spheroidal chondrocytes that are arranged into columns orientated perpendicularly to the surface of the articular cartilage tissue. The course of large diameter collagen fibrils follows the orientation of the chondrocyte columns. The collagen fibres pass into the so-called tidemark, a smooth undulating junction between the radial layer and the calcified cartilage. This zone contains the lowest concentration of water and the highest concentration of proteoglycans (Buckwalter and Hunziker., 1999).

(D) Calcified cartilage layer

This zone separates the radial zone and the subchondral bone. The chondrocytes located within this region are rounded and contained within uncalcified lacunae. Some cells appear completely surrounded by calcified cartilage suggesting an extremely low metabolic activity (Buckwalter and Hunziker., 1999). The collagen fibrils are anchored within a calcified matrix (Poole 1997).

1.1.3 Circumferential Differentiation

The description of zones shows the horizontal matrix subdivisions that occur within articular cartilage. There is also a circumferential differentiation apparent within articular cartilage (**Figure 1.5**). 'Circumferential differentiation' refers to the distance from a chondrocyte to distinct regions within the extracellular matrix, which provide the biochemical and biomechanical microenvironment of the chondrocyte (Buckwalter *et al.*, 1990).

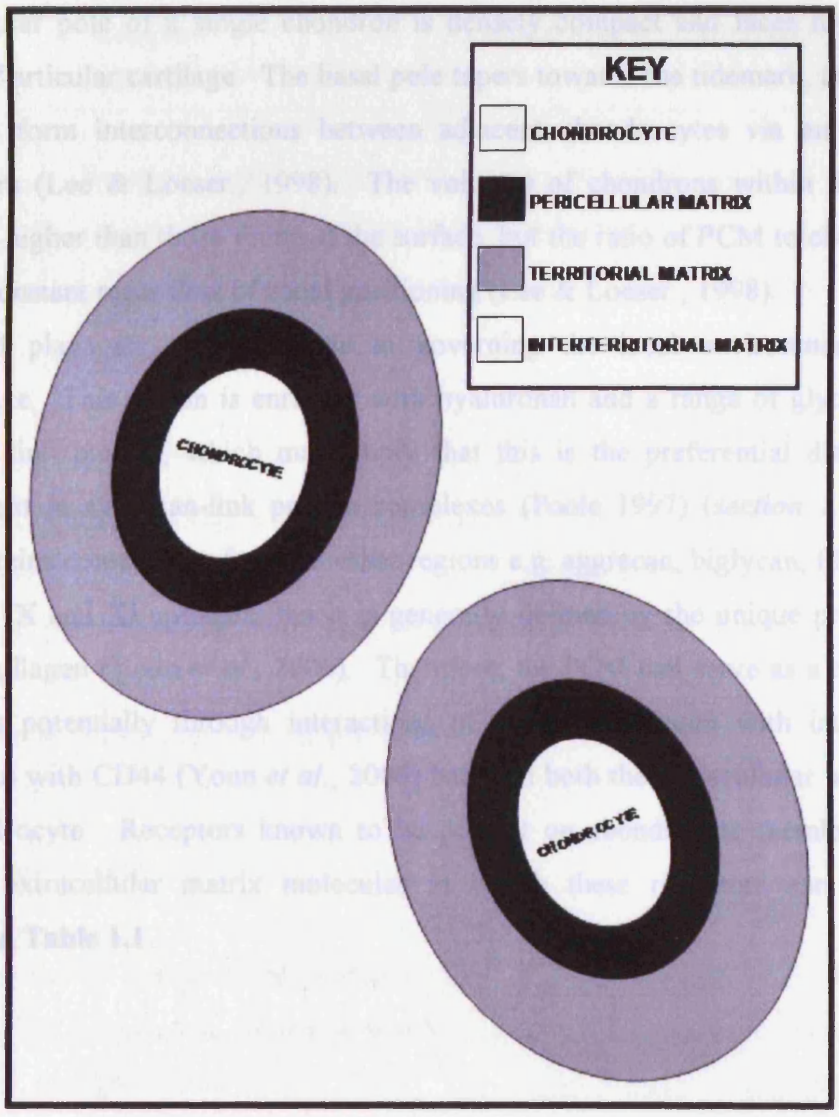

Figure 1.5 A diagram showing circumferential differentiation between chondrocytes, and certain regions within the extracellular matrix (Adapted from Buckwalter & Hunziker 1999).

(A) Pericellular Matrix

The pericellular matrix (PCM) is the immediate environment surrounding the chondrocyte. This immediate environment along with the chondrocyte has been termed the 'chondron' (Benninghoff., 1925) (Poole 1997).

The shape of a chondron reflects the collagen architecture of the interterritorial matrix, and this varies significantly with depth from surface to deep zones in articular cartilage. Chondrons may contain a single chondrocyte or several chondrocytes (Youn *et al.*, 2006). A chondron containing one chondrocyte shows matrix polarity. The articular pole of a single chondron is densely compact and faces towards the surface of articular cartilage. The basal pole tapers towards the tidemark, and has the ability to form interconnections between adjacent chondrocytes via an unknown mechanism (Lee & Loeser., 1998). The volumes of chondrons within the deeper zones are higher than those found at the surface, but the ratio of PCM to chondrocyte remains constant regardless of zonal positioning (Lee & Loeser., 1998).

The PCM plays an important role in governing the local environment of the chondrocyte. This region is enriched with hyaluronan and a range of glycoproteins including link protein, which may imply that this is the preferential distance for involvement in aggrecan-link protein complexes (Poole 1997) (***section 1.4.1***). The PCM contains constituents found in other regions e.g. aggrecan, biglycan, fibronectin, Types II, IX and XI collagen, but it is generally defined by the unique presence of type VI collagen (Youn *et al.*, 2006). Therefore, the PCM can serve as a transducer of signals potentially through interactions of type VI collagen with integrins or hyaluronan with CD44 (Youn *et al.*, 2006) between both the extracellular matrix and the chondrocyte. Receptors known to be present on chondrocyte membranes and potential extracellular matrix molecules in which these receptors can bind are depicted in **Table 1.1**.

RECEPTORS EXPRESSED ON THE SURFACE OF CHONDROCYTES	EXTRACELLULAR MATRIX MOLECULES THAT BIND TO CHONDROCYTE RECEPTORS
CD44	Hyaluronan (Knudson & Knudson 2004)
CD44 plus GAG chain	Collagens, fibronectin, laminin and Chondroitin sulphates (Goodison *et al.*, 1999, Greenfield et al., 1999)
Annexin V (*Formally anchorin cII*)	Type II & X collagen (Lucic *et al.*, 2003, Kirsch et al., 1992)
Integrins ($\alpha_1\beta_1$, $\alpha_2\beta_2$)	Types II & VI collagens (Durr *et al.*, 1993)
Integrins ($\alpha_5\beta_1$, $\alpha_3\beta_2$)	Fibronectin (Durr et al., 1993, Salter *et al.*, 1992)

Table 1.1. Receptors found on chondrocyte membranes and the molecules with which these receptors have the ability to bind.

(B) Territorial Matrix

The territorial matrix (TM) is the region surrounding the pericellular matrix. There is a more concentrated amount of chondroitin sulphate-rich proteoglycans present within this region (Meachim & Stockwell., 1979). In the middle and deep zones of articular cartilage the pericellular matrix is separated from the territorial matrix by a fibrous capsule (Lee & Loeser., 1998). Potentially the territorial matrix could have an influence on the pericellular matrix activities.

(C) Interterritorial Matrix

The interterritorial matrix (ITM) surrounds the territorial matrix. It is the largest fraction of cartilage matrix, consisting mostly of aggregating proteoglycan (aggrecan) and type II collagen (Youn *et al.*, 2006). The largest collagen fibres predominate within this region, and their compacted organisation and alignment defines the collagen arcades described previously by Benninghoff 1925. Meachim and Stockwell in 1979 reported that this region was more concentrated in keratan sulphate rich proteoglycans (Poole 1997).

1.2 Collagens

There are twenty eight members of the collagen family that have been identified to date. Collagens are a major component of articular cartilage extracellular matrices (*constituting 20% w/w*) that provide a structural role and strength to withstand tensional forces. Therefore, it is important to take into consideration the desired distribution of the collagens present in native cartilage whilst engineering this tissue.
Collagens are made up of a number of polypeptide chains that individually form left-handed helices. These chains wind around each other in a right handed super triple helix and are held together by hydrogen bonds (Stryer 1995). The triple helical structure in collagen arises from an unusual abundance of three amino acids (*glycine, proline and hydroxyproline*) forming a characteristic motif; Gly-X-Y. The amino acid proline is a cyclic structure found in the bends and folds of proteins and glycine is the only amino acid with a side chain that can fit into the crowded centre of a triple stranded helix. Hydroxyproline has been found to stabilise collagen, as the proportion of hydroxyproline containing chains increase, helix melting temperatures increase (Slatter *et al.*, 2003).
Collagens are separated into four classes; Fibril forming, Network forming, Fibril associated collagens with an interrupted triple helix (FACITs) and other groups (*e.g. transmembrane, beaded filaments and anchoring fibrils for basement membranes*). The collagens present in articular cartilage and their associated classes are discussed below.

1.2.1 Heterotypic Fibrils of Articular Cartilage

Types II, IX and XI collagens aggregate together forming organised matrix fibrils (**Figure 1.6**), where type II constitutes the bulk of the fibril, and both type IX and XI collagens are found co-distributed on the fibrils contributing to the organisation and mechanical stability of the type II fibrillar network. Other structural macromolecules such as fibromodulin and decorin participate in fibril assembly, but only the collagenous constituents will be discussed here (Oldberg *et al.*, 1989 & Pringle & Dodd., 1990).

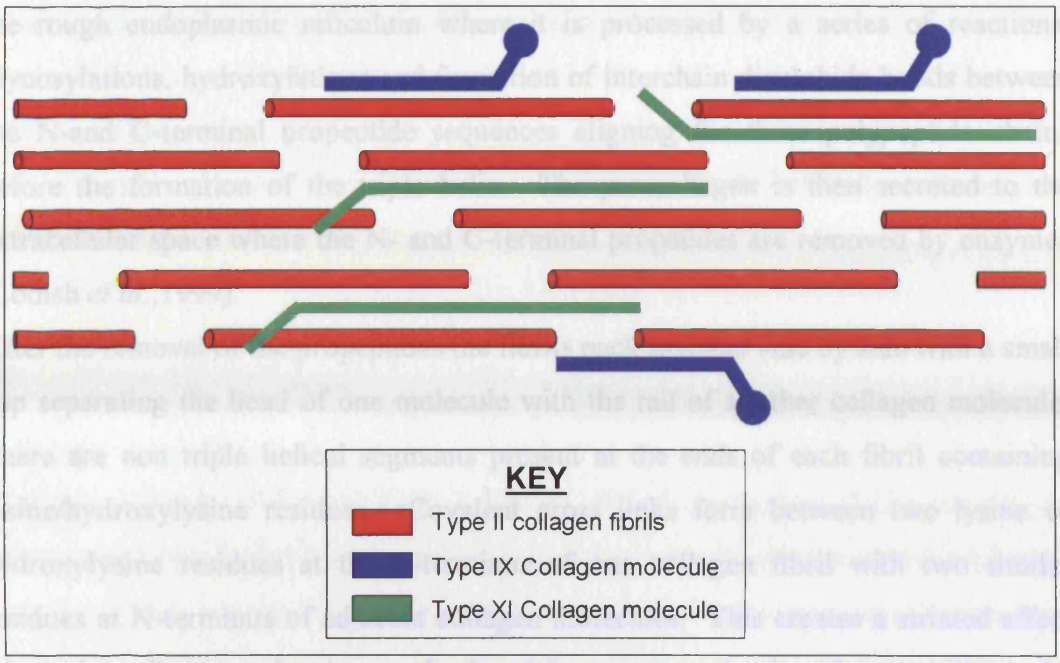

Figure 1.6. Organisation of major collagens in articular cartilage (Personal communication with Vic Duance)

(A) Type II Collagen

The major collagen predominantly, but not exclusively found in hyaline cartilage is the fibril forming type II collagen. Type II collagen is a product of a single gene, COL2A1 and two forms of COL2A1 mRNA are expressed due to variable splicing of the primary transcript. One form contains and the other lacks a cysteine rich exon 2 coding domain (Ryan *et al.*, 1990). The two splice variant pro-collagens show different tissue distributions (Sandell *et al.*, 1991); the type IIA is a transient embryonic form found in pre-chondrogenic mesenchyme and perichondrium and the type IIB dominant form is found in mature cartilage (*differentiated chondrocytes*). It has been found that IL-1β down regulates the COL2A1 gene inhibiting production of newly synthesised collagen (Chadjichristos *et al.*, 2003)

The biosynthesis of type II fibres follows the normal pathway of secreted proteins. Firstly, they are synthesised as precursors called pro-collagens by ribosomes on the surface of the rough endoplasmic reticulum. Pro-collagens contain a large central triple helical domain linked to N- and C- propeptides, by a short sequence, the telopeptides. A growing pro-collagen chain is transported into the luminal space of

the rough endoplasmic reticulum where it is processed by a series of reactions: glycosylations, hydroxylations and formation of interchain disulphide bonds between the N-and C-terminal propeptide sequences aligning the three polypeptide chains before the formation of the triple helix. The pro-collagen is then secreted to the extracellular space where the N- and C-terminal propetides are removed by enzymes (Lodish *et al.*,1999).

After the removal of the propeptides the fibrils pack together side by side with a small gap separating the head of one molecule with the tail of another collagen molecule. There are non triple helical segments present at the ends of each fibril containing lysine/hydroxylysine residues. Covalent cross links form between two lysine or hydroxylysine residues at the C-terminus of one collagen fibril with two similar residues at N-terminus of adjacent collagen molecules. This creates a striated effect where the collagen molecules are displaced from one another by 67nm stabilising the packing of collagen forming an extremely strong fibril (**Figure 1.7**) (Lodish *et al.*,1999)

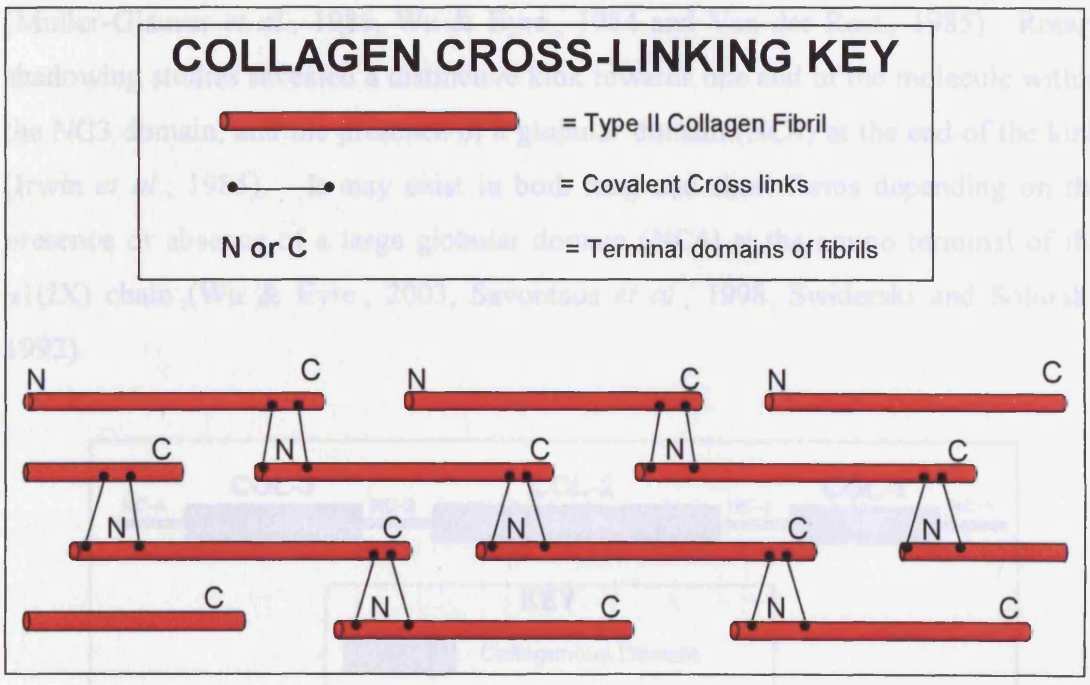

Figure 1.7. Schematic of the striated effect created by collagen cross-linking (Adapted from Lodish *et al.*,1999)

In mature cartilage, type II forms fibrillar networks with thicker fibrils in the deep layers of cartilage and finer fibrils enriched in the surface (Aydelotte & Kuettner., 1988). The collagenous fibres form a stable network that counteracts the hydrodynamic pressure generated by the highly hydrodynamic proteoglycan aggregates.

(B) Type IX Collagen

Type IX collagen (**Figure 1.8**) is a FACIT (Fibril Associated Collagen with an Interrupted Triple-helix) found covalently bound to type II collagen on the surface of heterotypic fibrils located in articular cartilage. It was found that a single amino acid substitution in the C-terminus region of type II collagen altered the affinity of type IX for type II collagen (Steplewski *et al.*, 2005).

Type IX collagen is composed of three disulphide bonded polypeptide chains [α1(**IX**)] [α2(**IX**)] [α3(**IX**)] that form together three triple helical domains (COL-1,-2 and -3) interrupted by non-helical sequences (NC-1,-2,-3 and -4) (**Figure 1.8**) (Muller-Glauser *et al.*, 1986, Wu & Eyre., 1984 and Van der Rest., 1985). Rotary shadowing studies revealed a distinctive kink towards one end of the molecule within the NC3 domain, and the presence of a globular domain (NC4) at the end of the kink (Irwin *et al.*, 1985). It may exist in both long and short forms depending on the presence or absence of a large globular domain (NC4) at the amino terminal of the α1(IX) chain (Wu & Eyre., 2003, Savontaus *et al.*, 1998, Swiderski and Solursh., 1992).

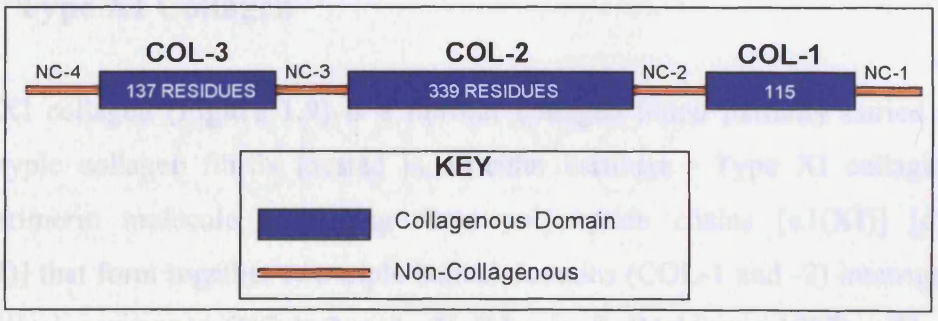

Figure 1.8. Structural organisation of a type IX collagen molecule (Adapted from Eyre *et al.*, 2006).

It is a unique member of the collagen family due to several distinct characteristics; one of the collagen helices (COL-3) has a high content of both proline and hydroxyproline resulting in an extremely stable helix with an elevated melting temperature (Irwin *et al.*, 1985). Another unique feature of type IX collagen is the apparent linkage of chondroitin sulphate and sometimes dermatan sulphate to the [α2(**IX**)] polypeptide chain in the NC-3 domain (Irwin *et al.*, 1985, Muller & Glauser., 1986, Huber *et al.*, 1986 and Irwin and Mayne 1986). The attachment of a GAG chain is both tissue and species specific, substitution has been observed the vitreous of the eye (in both chick and bovine) (Yada *et al.*, 1990, Bishop *et al.*, 1992, Ring *et al.*, 1995) and in chick cartilage (Vaughan *et al*, 1985, Bruckner *et al.*, 1985, McCormick *et al.*, 1987), but GAG substituted type IX collagen has been found to be a minor component in human and bovine articular cartilage (Diab *et al.*, 1996).
In articular cartilage, type IX collagen functions in the regulation of type II collagen fibril diameter, its presence appears to reduce the fibril diameter of heterotypic fibril aggregates (Wotton *et al.*, 1988). Type IX collagen is responsible for stabilising the fibrillar network as it is arranged along the surface of type II collagen fibrils, its N-terminal domain projects from the fibril surface encouraging interactions with other matrix components (Leppanen *et al.*, 2007). Type IX collagen is known to decrease with an increased age (Duance & Wotton., 1991), a reduction of type IX collagen affects the organisation of cartilage components at a molecular level, having a profound effect on the integrity of the cartilage matrix, leading to cartilage degradation as occurs in osteoarthritis (Hu *et al.*, 2006) and rheumatoid arthritis (Jaalinoja *et al.*, 2008).

(C) Type XI Collagen

Type XI collagen (**Figure 1.9**) is a fibrillar collagen found partially buried within heterotypic collagen fibrils located in articular cartilage. Type XI collagen is a heterotrimeric molecule containing three polypeptide chains [α1(**XI**)] [α2(**XI**)] [α3(**XI**)] that form together two triple helical domains (COL-1 and -2) interrupted by non-helical sequences (NC-1,-2 and -3) (Morris & Bächinger 1987). The NC2 domain is a hinged region within the molecule. The α1 and α2 chains resemble type

V collagen, whereas the α3 is highly homologous to an over-glycosylated [α1(**II**)] chain (Morris & Bächinger 1987 & Vaughan-Thomas *et al.*, 2001).
The cross-linking bonds formed by type XI collagen molecules are homopolymeric and molecules cross-link head to tail. There are three modes of interaction; [α1(**XI**)] N-telopeptide to [α3(**XI**)] C-terminal helix, [α2(**XI**)] N-telopeptide to [α1(**XI**)] C-terminal helix and [α3(**XI**)] N-telopeptide to [α2(**XI**)] C-terminal helix (Wu & Eyre., 1995). Also, a cross-link was identified between the amino-terminal of the α1(**XI**) triple helix and the α1(II)/α3(XI) C-telopeptide, forming a potential linkage of the type XI collagen homopolymer with the type II collagen within the heterotypic fibril (Duance *et al.*, 1999).

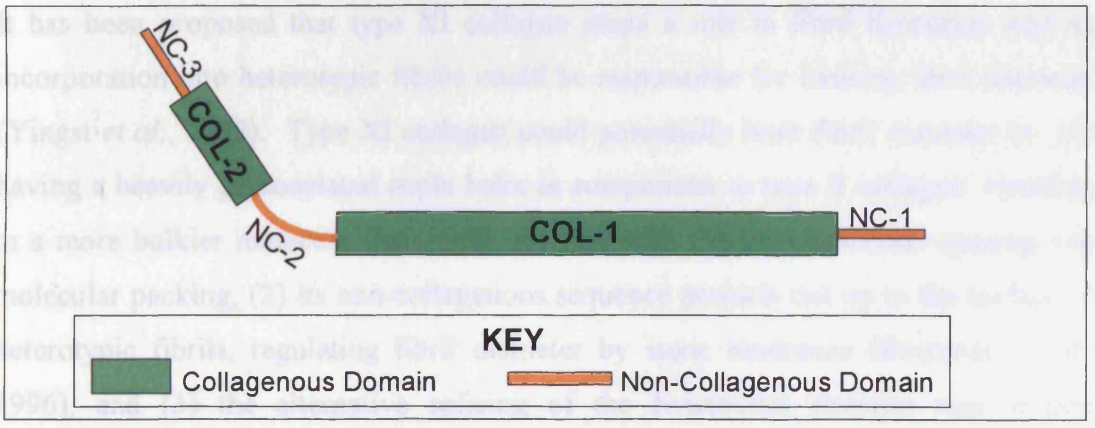

Figure 1.9. Structural organisation of a type XI collagen molecule
(Adapted from Duance *et al.*, 1999)

The most N-terminal domain consists of a module rich in basic residues, also found in FACIT collagens and is characterised by a β-stranded structure, which constitutes the proline/arginine-rich protein (PARP) (Neame et al., 1990). Nucleotide sequencing demonstrated that PARP is a fragment of the NH_2 terminal non-collagenous (NC-3) domain of collagen [α2(**XI**)] chain (Zhidkova *et al.*, 1993 & Rousseau *et al.*, 1996). Between the PARP module and the short triple helix, there is a highly acidic domain that presents a poor sequence conservation among the three chains and is variable in length (it is referred to as the variable region) (Rousseau *et al.*, 1996).
Six isoforms of type XI collagen can be generated from splicing in the [α1(**XI**)] chain; alternative splicing of this region has also been demonstrated within the [α2(**XI**)] chain (Rousseau *et al.*, 1996). BMP-1 can be held responsible for a variety of

processing of these chains (Medeck *et al.*, 2003). The functional significance of such structural diversity is yet to be elucidated, but the presence of these isoforms may indicate important roles of type XI collagen at different stages of development and growth (Duance *et al.*, 1999).

The α1(XI) and α2(XI) chains within the COL1 domain contain heparin binding sites (Smith and Brandt., 1987) (aggrecan can also bind through the same site), these binding sites can be exploited providing a method for the column purification of type XI collagen preventing contamination with type II collagen (Smith & Brandt., 1987). Type XI collagen has been reported to interact with components of cartilage extracellular matrices. Along with type IX collagen, type XI collagen potentially makes the surface of heterotypic collagen fibrils in cartilage highly interactive.

It has been proposed that type XI collagen plays a role in fibril formation and its incorporation into heterotypic fibrils could be responsible for limiting fibril diameter (Yingst *et al.*, 2008). Type XI collagen could potentially limit fibril diameter by: (1) having a heavily glycosylated triple helix in comparison to type II collagen, resulting in a more bulkier molecule that could interfere with the intermolecular spacing and molecular packing, (2) its non-collagenous sequence projects out up to the surface of heterotypic fibrils, regulating fibril diameter by steric hindrance (Rousseau *et al.*, 1996), and (3) the alternative splicing of the N-terminal domains may enable interactions to occur with other matrix molecules which stabilise the fibrils at a certain diameter (Duance *et al.*, 1999).

1.2.2 Minor collagens present in cartilage

The most predominant collagen in articular cartilage is type II collagen. All other collagens present in cartilage are found in smaller quantities in comparison to type II collagen. Therefore, the collagens discussed below (**Table 1.2**) are referred to as minor collagens.

Collagen (Type)	Information
Fibril Forming	
Type I	• Enriched in the 'lamina splendens' of the superficial region (Duance 1983), and is not present in the mid or deep regions of articular cartilage (Stanescu *et al.*, 1976 & Eyre *et al.*, 1978).
Type III	• Copolymerises and links to type II collagen (Young *et al.*, 1995 & Eyre 2002). • Found pericellularly throughout cartilage depth at a variety of ages, ranging from young to old (Wotton & Duance., 1994). • Identified in both normal (Wotton and Duance 1994) and osteoarthritic (Aigner *et al.*, 1993) human articular cartilage. • Osteoarthritic state: Found concentrated in the superficial and transitional zones (Teshima 2004).
Type V	• Present in articular cartilage, but not epiphyseal cartilage its role is unknown (Eyre *et al.*, 1978). • Located in the pericellular region of the matrix (Furuto *et al.*, 1991 & Bland & Ashhurst., 1996). • Shares sequence homology to 2 chains of type XI collagen (www.rockland-inc.com/commerce/misc/eci.jsp).
Network Forming Collagens	
These collagens self assemble into networks and have longer non-collagenous domains than fibril forming collagens (Kadler *et al.*, 1996). The monomers associate at their C-termini to form dimers and at their N-termini to form tetramers. The triple helical domains intertwine to form supercoiled structures.	
Type X	• Short chain, non-fibril-forming collagen that is a product of hypertrophic chondrocytes (Kielty *et al.*, 1985). • Possible involvement during the endochondral ossification process (Morris & Bächinger 1987). • Human osteoarthritic cartilage shows moderate mRNA expression of type X collagen in the deep zones (Aigner *et al.*, 1993).

Fibril associated collagens with interrupted triple helix (FACITs)	
The FACIT family do not form fibrils themselves, but are found attached to the surface of pre-existing fibrils of the fibril forming collagens (Shaw & Olsen 1991). The FACIT group share sequence similarities, their most conserved region being their COL1 domain. They are thought to bind collagen fibril surfaces via their COL1/NC1 domains (Eyre 2002).	
Type XII	• Located at the surface of type II fibrils associated in a non-covalent fashion, distributed in areas of cartilage with more organised fibril orientation potentially playing a role in promoting alignment or stabilising organisation (Gregory *et al.*, 2001). • Also found in: tendon, ligament, perichondrium and periosteum tissues that are rich in type I collagen; type XII collagen localises to the surface of type I fibrillar collagens (Bland & Ashhurst., 1996).
Type XIV	• Localised uniformly throughout articular cartilage, but absent in growth plate cartilage (Watt *et al.*, 1992).
Other Small groups in Cartilage (Beaded Filaments)	
Type VI	• Heterotrimer of three distinct α chains that arrange themselves to contain short triple helical domains and extended globular termini, consisting of a larger globular domain at its C-terminal and smaller one at its N-terminal (Soder *et al.*, 2002). • The microfibrils formed are of beaded appearance. The helical segment is unusual in that the Gly-X-Y sequence is interrupted approximately twenty four times with segments unable to form triple helices providing flexibility to the molecule (Knupp *et al.*, 2001). • Self-assembles into disulphide-bonded dimers and tetramers, polymerising into a filamentous network that is mostly concentrated around cells in the pericellular matrix (Eyre 2002). • Binds integrins on the chondrocyte membrane via RGD-sequences in the α1 and α2 chains, and binds other proteins of the pericellular matrix such as other collagens, decorin, fibromodulin, hyaluronan, and fibronectin providing an interface between the rigid interterritorial matrix and the chondrocyte (Marcelino *et al.*, 1995). • Involvement in cell anchoring, maintenance of chondrocyte integrity and matrix cell signalling (Poole *et al.*, 1992). • The microenvironment surrounding Type VI collagen might also be a central factor for formation of chondrocyte clusters, a characteristic feature of osteoarthritis (OA) (Soder *et al.*, 2002).

Table 1.2. The minor collagens present in articular cartilage

1.3 Glycosaminoglycan (GAGs)

There are six glycosaminoglycans (GAGs) and they are divided into two groups depending on their disaccharide unit composition. One group is the 'galactosaminoglycans' that include both chondroitin sulphate (CS) and dermatan sulphate (DS), and the other group referred to as the 'glucosaminoglycans' include heparan sulphate (HS), heparin (Hep), hyaluronan (HA) and keratan sulphate (KS). All glycosaminoglycans are un-branched linear polymers of repeated disaccharide units of varying lengths that covalently attach to a protein core forming a proteoglycan, with hyaluronan being an exception to this rule. Each disaccharide unit has at least one negatively charged carboxylate or sulphate group. Therefore, GAGs form a long string of negative charges that repel other negatively charged molecules and attract cations (Bulkwalter and Hunziker 1999). As a result of being highly charged molecules, GAGs function as a water adsorbent attracting water molecules from the surrounding environment to maintain a hydrated matrix.

Glycosaminoglycans provide essential roles in vertebrates as intracellular adhesives, signalling molecules, anti-coagulants and structural elements (Williams *et al.*, 2006). The GAGs found in cartilage include hyaluronan, chondroitin sulphate, keratan sulphate and dermatan sulphate and are discussed in more detail below. Concentrations of these molecules vary among sites within articular cartilage. Factors contributing towards this variability are age, cartilage injury and disease (Bulkwalter and Hunziker 1999).

1.3.1 Galactosaminoglycans

(A) Chondroitin Sulphate

Chondroitin sulphate (CS) is an important structural component of articular cartilage that provides much resistance to compression by attracting water molecules. It is made up of alternating sugars of N-acetylgalactosamine (galNAc) and glucuronic acid (GlcA) (**Figure 1.10**). There are eight possible isomers of CS made from repeating disaccharide units that utilise three possible combinations of estersulphation (**Figure 1.10**). The most common forms found in nature include a non-sulphated chondroitin

sulphate molecule referred to as chondroitin-0-sulphate (C-0-S), and monosulphated CS molecules (*sulphated at either carbon -4 or -6 of the N-acetylgalactosamine unit*) referred to as chondroitin-4-sulphate (C-4-S) and chondroitin-6-sulphate (C-6-S), respectively. Several other less common CS isomer repeat disaccharides also occur in nature. CS type E contains disaccharides with ester sulphate substituted in both 4- and 6-position of N-acetylgalactosamine residue. Four CS isomers have also been identified that contain ester sulphate substitution at the 2-position of the glucuronic acid residue with various sulphation patterns occurring on the N-acetylgalactosamine residue at positions 4- and 6-. The most highly sulphated isomer of CS contains sulphate in all three available positions (Caterson *et al.*, 1990).

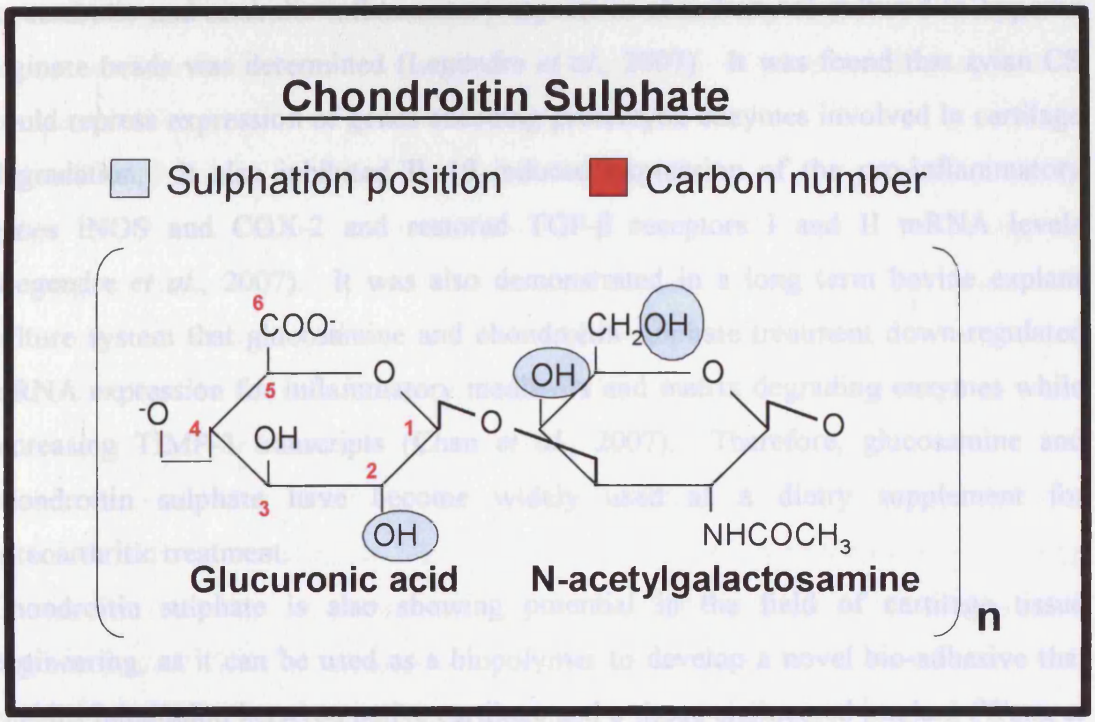

Figure 1.10. A diagrammatic representation of a Chondroitin Sulphate disaccharide unit.

The glycosaminoglycan CS can link to a hydroxyl group within a serine residue of certain proteins to form proteoglycans. The glycosylated serines of the proteins to which the CS chains can attach are often followed by a glycine residue neighboured by acidic regions, although this motif does not always predict glycosylation.

Alterations of sulphation patterns in chondroitin sulphate chains on proteoglycans have been associated with both ageing and degeneration of articular cartilage.

Sauerland & Steinmeyer (2007) reported that sulphation patterns of CS chains in bovine articular cartilage explants were also altered following mechanical loading. The internal region of the CS chain showed an increase in sulphation at both carbon 4 and 6 of the N-acetylgalactosamine unit and simultaneously less CS chains terminated with galNAc 4 and 6 sulphation following an applied mechanical load.

The loss of CS from articular cartilage is a major consequence osteoarthritis, and there is a marked increase of CS chain length present in the synovial fluid following both exercise and osteochondral injury (Brown *et al.*, 2007). Chondroitin sulphate may exert both limited chondroprotective and anti-inflammatory effects on articular cartilage that could provide long term benefits on the osteoarthritic process.

Utilising an avian model, the effect of CS on IL-1-induced expression of genes related to catabolic and anabolic inflammatory aspects in chondrocytes cultured in hypoxic alginate beads was determined (Legendre *et al.*, 2007). It was found that avian CS could repress expression of genes encoding proteolytic enzymes involved in cartilage degradation. It also inhibited IL-1β induced expression of the pro-inflammatory genes iNOS and COX-2 and restored TGF-β receptors I and II mRNA levels (Legendre *et al.*, 2007). It was also demonstrated in a long term bovine explant culture system that glucosamine and chondroitin sulphate treatment down-regulated mRNA expression for inflammatory mediators and matrix degrading enzymes while increasing TIMP-3 transcripts (Chan *et al.*, 2007). Therefore, glucosamine and chondroitin sulphate have become widely used as a dietry supplement for osteoarthritic treatment.

Chondroitin sulphate is also showing potential in the field of cartilage tissue engineering, as it can be used as a biopolymer to develop a novel bio-adhesive that enables integration between native cartilage and a tissue engineered implant (Wang *et al.*, 2007). The CS is chemically modified with methacrylate and aldehyde groups on the polysaccharide backbone to chemically bridge biomaterials and tissue proteins via two fold covalent links (Wang *et al.*, 2007).

(B) Dermatan Sulphate

Dermatan sulphate (DS) also known as Chondroitin Sulphate B, was originally called mucopolysaccharide and has been found to accumulate in many of the

mucopolysaccharidosis disorders. It is very similar in structure to CS, but some of the glucuronic acid residues are epimerized to *L*–iduronic acid (IdoA).

1.3.2 Glucosaminoglycans

(A) Keratan Sulphate

Keratan sulphate (KS) also known as keratosulphate is found in cornea, cartilage and bone and is a highly hydrated molecule in joints that can act as a cushion. It has many features in common with complex oligosaccharides found in glycoproteins and contains oligosaccharide branches, but it is still considered a GAG as it consists of repeating disaccharides of N-acetylglucosamine linked to galactose (Heinegard and Paulsson 1984).

Keratan sulphate can be found in both an N-linked form (*in the cornea*), or an O-linked form (*in cartilage*). In the N-linked form keratan sulphate attaches through an asparagine residue in the amino acid consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline. O-linked attachment occurs through the consensus sequence Glu-Glu/Lys-Pro-Phe-Pro-Ser (Barry *et al.*, 1995) where keratan sulphate attaches via the serine residue.

Keratan sulphate chains increase with ageing (Theocharis *et al.*, 1985) as do the level of KS sulphation (Greiling & Baumann 1973). The concentrations of circulating cartilage-derived KS in serum are higher in adult patients with osteoarthritis (Wakitani *et al.*, 2007). Measuring serum KS levels could be used as a diagnostic marker in joint disease (Thonar *et al.*, 1987).

(B) Hyaluronan

Hyaluronan (HA) forms strikingly viscous solutions and is unlike any other GAGs, as it does not contain sulphate groups, epimerize or occur in proteoglycan form but it is found in a free form (Kjellen and Lindahl 1991 & Stern *et al.*, 2006). Its highest concentrations are found in connective tissues and its lowest concentrations in blood and it has a relatively high turnover rate. The HA polymer is made up from repeating

disaccharide units of [D-glucuronic acid (1-β-3) N-acetyl D-glucosamine (1-β-4)]n (Meyer & Palmer, 1934) (**Figure 1.11**). The HA polymer forms stiffened helical configurations that are stabilised by hydrogen bonds parallel within the chain axis (Laurent and Fraser., 1992). Under normal physiological conditions, hyaluronan consists of 2,000-25,000 disaccharides, which corresponds to polysaccharides with relative molecular masses of 10^6-10^7 and polymer lengths of 2-25μm (Toole 2004). The large molecular mass hyaluronan is highly negatively charged and can adsorb vast amounts of water exerting a swelling pressure on surrounding tissues (Underhill *et al.*, 1998).

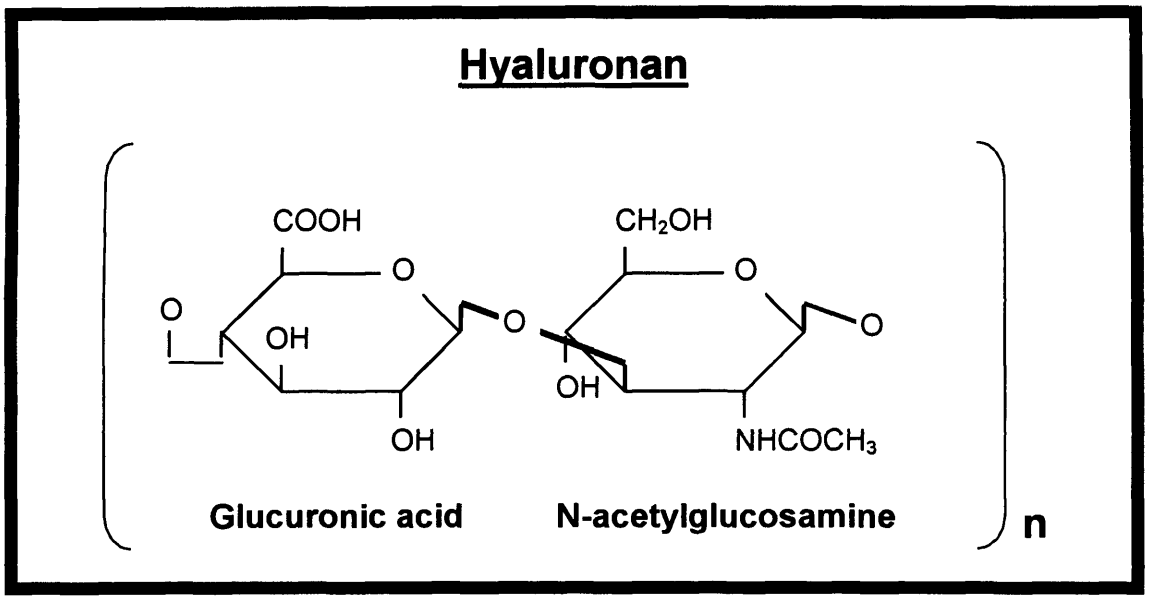

Figure 1.11. A diagrammatic representation of a hyaluronan disaccharide unit.

Hyaluronan molecules bind to HA binding proteins (*referred to as hyaladherins*), such molecules include: CD44, RHAMM, LYVE-1, TOLL4 and extracellular matrix PGs e.g. aggrecan and versican (Stern *et al.*, 2006). HA-binding proteins interact with hyaluronan via common structural domains of approximately 100 amino acids termed link modules, proposed to be related to C-type lectin modules owing to their similar topological function (Seyfried *et al.*, 2006). Hyaluronan interacts with the surface of chondrocytes in two ways: (1) through receptor (CD44, RHAMM) binding

at the surface of the chondrocyte and (2) through hyaluronan synthases (Toole., 2004).

(i) Hyaluronan Biosynthesis

Hyaluronan is synthesised in a different manner to other GAGs. It is synthesised in the plasma membrane of cells such as chondrocytes, fibroblasts and synoviocytes by enzymes referred to as hyaluronan synthases. The HA chain grows by the addition of sugars to the reducing end of the polymer, whereas the non-reducing end translocates into the pericellular matrix during synthesis (Laurent and Fraser., 1992 & Laurent and Fraser 1986). This enables an intimate relationship of HA with the cell surface and can readily participate in the creation of a pericellular hydrated zone around the cells (Toole., 2004). In some cases HA can be released from the cell surface, but the release mechanism has not been clarified (Laurent and Fraser 1992). In comparison other GAGs are synthesised by the addition of sugar units to the non-reducing end of their polymers within the endoplasmic reticulum and Golgi apparatus without the aid of hyaluronan synthases (Laurent and Fraser 1992 & Laurent and Fraser 1986).

There are at least three synthases in mammals being; HAS-1, HAS-2 and HAS-3 that produce hyaluronan products of varying lengths (Stern *et al.*, 2006). Membranes from cells expressing HAS-1 or HAS-3 synthesise hyalyronan of 0.2-20 MDa (Brinck *et al.*, 1999) and cell membranes expressing HAS-2 generate hyaluronan polymers of more than 2 MDa (Itano *et al.*, 1999). Interestingly, hyaluronan has wide biological roles some of which are size dependent; this could potentially imply that hyaluronan functions are diversely regulated through control of the activities and expression of HAS isoforms (Itano *et al.*, 2004). It has been found that alterations at two conserved amino acid residues in *streptococcus equisimilis* hyaluronan synthase (*Changes at* K^{48} *within membrane domain 2 to either Arg or Glu and* E^{327} *in membrane domain 4 to either Lys, Asp or Gln*) decreased the length of hyaluronan produced (Kumari *et al.*, 2006).

The over expression of HAS-2, leads to over expression of HA, enhancing anchorage-independent growth and tumorigenicity (Kosaki *et al.*, 1999). It has been suggested that the three HAS isoforms differ from each other in expression profiles during embryonic development (Itano *et al.*, 2004).

(ii) Hyaluronan Degradation

Hyaluronan has a high rate of turnover as the polymers are funnelled through three catabolic pathways (Stern *et al.*, 2006). The three different pathways are discussed below:

1. Cellular Turnover

Hyaluronan catabolism in cartilage explant studies revealed that unlike proteoglycan fragments, HA degradation products are found neither in culture medium nor within the tissue, suggesting an alternative mode of removal (Nicoll *et al.*, 2002). Local HA turnover is dependent on receptor (CD44, RHAMM) mediated endocytosis and delivery to low pH intracellular organelles to be degraded by a series of enzymes (Chow *et al.*, 2006). These enzymes are referred to as hyaluronidases. High molecular weight HA is cleaved progressively by a series of enzymes in which the product of one becomes the substrate for another, producing HA fragments of a decreasing size (Lepperdinger *et al.*, 1998, Csoka *et al.*, 2001). It has been shown that G1 aggrecan fragments (***section 1.4***) can also be internalised and degraded in this pathway (Embry Flory *et al.*, 2006). There are six hyaluronidase sequences in the human genome which are clustered in groups of three at two chromosomal sites (**Table 1.3**) (Csoka *et al.*, 2001).

Chromosome	Gene	Protein
3p21.3	HYAL1	Hyal-1
	HYAL2	Hyal-2
	HYAL3	Hyal-3
7q31.3	HYAL4	Hyal-4
	SPAM1	PH-20
	HYALP1	None

Table 1.3. Six hyaluronidase sequences located in the human genome, and the proteins in which these genes encode.

Three of these genes do not play a role in hyaluronan turnover in articular cartilage as HYALP1 in humans is a recently identified pseudogene that may be active in other species. PH-20 is a testicular hyaluronidase important during egg fertilisation by sperm and Hyal-4 is restricted to placenta and skeletal muscle with preliminary evidence indicating that Hyal-4 is a chondroitinase with no activity against HA (Csoka *et al.*, 2001). The hyaluronidases actively involved in the turnover of HA in cartilage are Hyal-1, Hyal-2, and Hyal-3, with Hyal-2 being the most predominantly expressed hyaluronidase in human articular cartilage (Chow & Knudson., 2005). Not much is known about Hyal-3, but strong hybridization patterns have been found in mammalian testis and bone marrow. These two tissues retain a stem cell-like state for the life of an animal, suggesting that Hyal-3 maybe important in stem cell regulation (Csoka *et al.*, 2001).

Hyal-2 is a glycosylphosphatidylinositol (GPI)-linked enzyme attached to the external surfaces of the plasma membrane. It is constitutively expressed and not regulated by catabolic agents (Chow & Knudson., 2005). This enzyme has unusual substrate specificity cleaving high molecular weight HA polymers to intermediate size fragments of approximately 20kDa (Lepperdinger *et al* 1998 & Stern., 2004). The HA fragments are then delivered to early endosomes and to lysosomes where fragmentation continues (*through the action of acid active Hyal-1*) generating predominantly tetrasaccharides (Stern *et al.*, 2004 & 2006).

Hyal-1, a 57kDa protein (*single polypeptide chain of 49kDa with approximately 8kDa post translational glycosylation*), is an acid active lysosomal enzyme that utilizes HA of any size as a substrate, generating predominantly tetrasaccharides and also cleaves chondroitin sulphate to a limited extent. There are two isoforms of Hyal-1, the first form found in plasma and the second form with a higher specific activity is found in urine. The second form is a 45kDa protein with approximately 100 amino acids deleted at the carboxyl terminal resulting in two polypeptide chains bound by disulphide linkages (Csoka *et al.*, 2001). Primary chondrocytes in monolayer culture have the ability to degrade exogenous hyaluronan (Williams *et al.*, 2005). The two isoforms of Hyal-1 have been detected in chondrocyte cultures, the first form is predominant in the culture medium (higher molecular weight form) and the smaller form is found in the cell layer (Csoka *et al.*, 2001). The modulation of native hyaluronidase activity may offer a new approach to improve the quality and quantity of hyaluronan in articular joints (Williams *et al.*, 2005).

2. Scission of Hyaluronan by Free Radicals

The scission of HA can occur via free radicals under oxidative conditions promoted by divalent cations (Stern *et al.*, 2006).

3. Tissue Turnover

Hyaluronan is released from tissue matrices and mobilised from these sites through lymphatic vessels to lymph nodes. The final degradation steps occur either in the liver, kidney or possibly the spleen. This pathway involves unique receptors such as the HA receptor for endocytosis (HARE), also stabling-2, lymphatic vessel endothelial HA receptor (LYVE)-1 and layilin (Stern *et al.*, 2006). In the liver, liver endothelial cells carry specific receptors for the endocytosis of HA. Following endocytosis, the HA is transported to lysosomes that contain enzymes for HA degradation (*e.g hyaluronidase, β-glucuronidase and β-N-acetylglucoaminidase*). Hyaluronan degradation products are the two monosaccharide units that make up the HA polymer. The pathway which the monosaccharides follow during degradation is shown in **Figure 1.12** (Laurent and Fraser 1992).

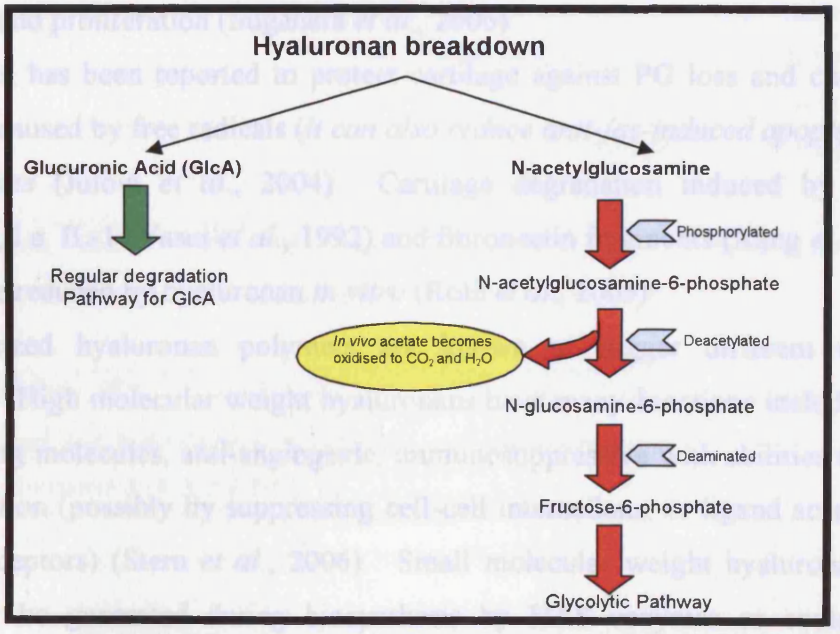

Figure 1.12. The pathway followed during hyaluronan degradation by the monosaccharides that constitute a hyaluronan polymer (Adapted from Laurent & Fraser 1992)

(iii) Functions of Hyaluronan

Hyaluronan appears to be a simple molecule, but has an extraordinarily large array of functions, some of which are contradictory (**Table 1.4**). It is hard to determine the *in vivo* functions of hyaluronan, as functions may be gained or lost upon extraction (Stern *et al.*, 2006). Hyaluronan plays an important role in development, differentiation and inflammation (Seyfried *et al.*, 2006).

Levels of hyaluronan have been found to rapidly increase as a survival mechanism in response to acute stress, shock, septicaemia, extensive burns and in sepsis, as well as increasing during tumour invasion (Csoka *et al.*, 2001). It functions as a lubricant (*providing visco-elastic properties separating most tissues that slide along each other*), a shock absorber and semi-permeable barrier regulating metabolic exchange between cartilage and the synovial fluid.

It plays an important structural role in cartilage as the proteoglycan, aggrecan, binds to hyaluronan chains stabilised by a link protein. Without this interaction proteoglycans would not be retained within cartilage. This interaction influences the hydration properties of tissues. The network of hyaluronan chains evolved within a tissue regulates the distribution and transport of plasma proteins. Hyaluronan forms a pericellular coat around cells which enables cells to participate in cell adhesion, migration and proliferation (Sugahara *et al.*, 2006).

Hyaluronan has been reported to protect cartilage against PG loss and chondrocyte cell death caused by free radicals (*it can also reduce anti-fas-induced apoptosis of OA chondrocytes* (Julovi *et al.*, 2004). Cartilage degradation induced by catabolic stimulants, i.e. IL-1 (Yasui *et al.*, 1992) and fibronectin fragments (Kang *et al.*, 1999) can also be reduced by hyaluronan *in vitro* (Roth *et al.*, 2005)

Various sized hyaluronan polymers are known to trigger different functional responses. High molecular weight hyaluronans have many functions including being space-filling molecules, anti-angiogenic, immunosuppressive with abilities to impede differentiation (possibly by suppressing cell-cell interactions, or ligand access to cell surface receptors) (Stern *et al.*, 2006). Small molecular weight hyaluronans could potentially be generated during biosynthesis by HAS enzymes or could just be degradation products of hyaluronidases. They appear to act as endogenous 'danger signals' mediating cell signalling effects (Powell & Horton., 2005). The smaller fragments are inflammatory, immuno-stimulatory and angiogenic (Stern *et al.*, 2006).

They are capable of activating macrophages (Kumari *et al.*, 2006) competing with high molecular weight hyaluronan for HA receptors and can induce proteolytic cleavage of the CD44 HA receptor from the surface of tumour cells promoting tumour migration (Sugahara *et al.*, 2006).

High Molecular Weight Hyaluronan	Low Molecular Weight Hyaluronan
500-730kDa HA: counteracts IL-1β induced effects, decreasing MMP-3 expression, decreasing nitric oxide synthase production of nitric oxide and increasing proteoglycan synthesis (Monfort *et al.*, 2005).	***Tetrasaccharides HA_4:*** Anti-apoptotic actions and inducers of heat shock proteins (Stern *et al.*, 2006). Suppress proteoglycan sulphation (Solursh *et al.*, 1980).
800kDa HA: Addition to bovine explant cultures and *in vivo* rabbit joints inhibited IL-1β production of MMP-1, -3 and 13 and the catabolic action of fibronectin fragments. Therefore having the effect of preventing breakdown of type II collagen and proteoglycan. It was found that aggrecan breakdown was delayed, but not completely blocked (Julovi *et al.*, 2004 & Kang *et al.*, 1999).	***Hexasaccharides HA_6:*** Induce HAS-2 in chondrocytes (Knudson & Knudson 2004). Induce nitric oxide synthase to produce nitric oxide (*nitric oxide is produced during inflammation, infection, disease or tissue damage*) (Iacob & Knudson., 2006) and activates a specific profile of transcription factors that are observed in cartilage e.g Retenoic acid receptor (RAR), Retenoid X receptor (RXR), SP-1, MMP-3, Type I collagen and Comp. It was demonstrated that high molecular weight HA could suppress HA_6 signalling (Ohno *et al.*, 2005).
120-1260kDa HA: Did not induce production of nitric oxide (Iacob & Knudson., 2006)	***8-50kDa HA:*** Induces angiogenesis (Sugahara *et al.*, 2006)
1000-5000kDa HA: suppresses HA synthesis (Lueke and Prehm 1999) and inhibits phagocytosis (Forrester & Balazs 1980).	***10kDa HA:*** Displaces proteoglycans from cell surfaces (Solursh *et al.*, 1980).

Table 1.4. The large array of contradictory functions identified to date by both high and low molecular weight hyaluronans.

1.4 Proteoglycans

Proteoglycans are complex macromolecules that are widely distributed in all connective tissues in varying amounts. They have a large degree of variability providing them foundation to fulfil a diversity of biological roles. They consist of a core protein of varying size to which a few to several hundred polyanionic glycosaminoglycan chains covalently attach via their reducing terminals and radiate out from the core (Heinegard & Paulsson 1984). A major source of structural variation occurs within the glycosaminoglycan chains that are attached to the core protein. The number of GAG chains, their length and sulphation patterns can change to suit different biological requirements (Hardingham & Fosang 1992). Proteoglycans are classified into a number of structurally and functionally diverse families including: Hyalectins, Small Leucine Rich Proteoglycans, cell surface proteoglycans and basement membrane proteoglycans (Vertel 1995). In this section proteoglycan families shall be discussed, with a focus on the proteoglycans relevant to this research project, paying particular attention to those present in articular cartilage.

1.4.1 Hyalectins

Members of the hyalectin family include aggrecan (present in cartilage, aorta, intervertebral disc and tendon), versican (present in fibrous and fibrocartilaginous tissues), brevican and neurocan (both predominantly found in adult brain). They are grouped into this family alternatively named for their common G1 and G3 domains. This family share the capability of interacting with hyaluronan via a proteoglycan tandem repeat (PTR) unit contained within their G1 domain at their amino termini. This region is termed the hyaluronan-binding region (HABR). Other hyaluronan binding proteins such as link protein, tumour necrosis factor stimulated gene-6 (TSG-6) and the cell surface receptor for hyaluronan (CD44) all expressed in cartilage, have been found to contain sequence homologies similar to the G1 domain or a single proteoglycan tandem repeat (PTR) loop. This family may also be referred to as the hyaluronan-binding proteins or the lecticans (Heinegard & Paulsson 1984).

(A) Aggrecan

Aggrecan is a large proteoglycan that is one of the major constituents of cartilage extra-cellular matrices and is entrapped by a 3D latticework of fibrillar collagens. The core protein of an aggrecan monomer (*Mr 210-250kDa*) is divided into multiple structural regions that serve unique functions (**Figure 1.13**). These regions consist of three globular domains (*i.e. G1, G2 and G3*) and interglobular domains (*i.e. interglobular domain (IGD), chondroitin sulphate (CS) and keratan sulphate (KS) glycosaminoglycan attachment regions*). A clear relationship has been determined between the structure of aggrecan and its function. The structure of aggrecan is not constant, but varies with species, age and site origin (Knudson and Knudson 2001).

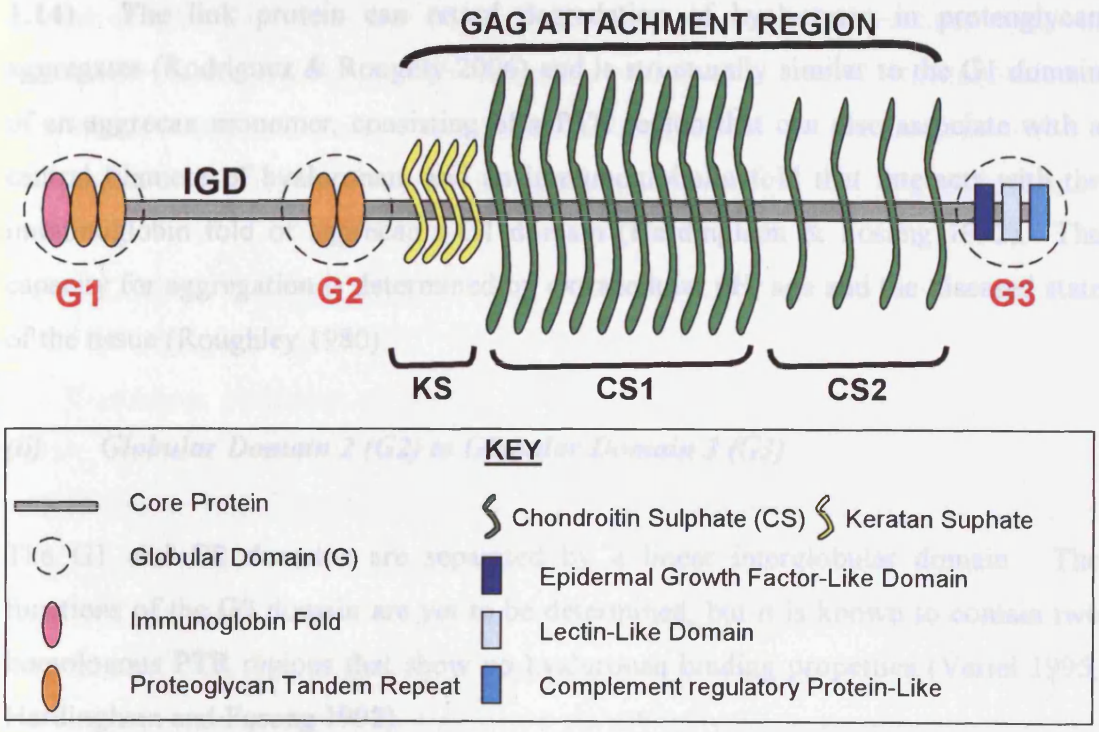

Figure 1.13. An Aggrecan Monomer (Adapted from Roughley 2006)

(i) Globular Domain 1 (G1) to Globular Domain 2 (G2)

The amino terminus of an aggrecan monomer is where the G1 domain is located. The G1 domain contains 3 sub-domains consisting of disulphide bonded loops: loop A having sequence homology with the immunoglobin superfamily, loops B and B' that share sequence homology with each other and form a double looped structure motif referred to as the proteoglycan tandem repeat (PTR) (**Figure 1.13 & 1.14**).

The PTR provides the functional site for the binding of aggrecan to hyaluronan. Due to the hyaluronan binding capacity of the PTR it is referred to as the hyaluronan-binding region (HABR). Therefore, G1 domains of up to one hundred aggrecan monomers can non-covalently associate with a central filament of hyaluronan (*typical Mr 0.5-1.0 x 10^6 Da*), self assembling into supramolecular aggregates of approximately 200 x 10^6 Da.

These aggregates are stabilised by a glycoprotein referred to as link protein (**Figure 1.14**). The link protein can retard degradation of hyaluronan in proteoglycan aggregates (Rodriguez & Roughly 2006) and is structurally similar to the G1 domain of an aggrecan monomer, consisting of a PTR region that can also associate with a central filament of hyaluronan, and an immunoglobulin fold that interacts with the immunoglobin fold of aggrecan's G1 domain (Hardingham & Fosang 1992). The capacity for aggregation is determined by extracellular pH, age and the diseased state of the tissue (Roughley 1980).

(ii) Globular Domain 2 (G2) to Globular Domain 3 (G3)

The G1 and G2 domains are separated by a linear interglobular domain. The functions of the G2 domain are yet to be determined, but it is known to contain two homologous PTR regions that show no hyaluronan binding properties (Vertel 1995, Hardingham and Fosang 1992).

Extending from the G2 to the C-terminal G3 domain is a glycosaminoglycan attachment region containing three adjacent domains; a keratan sulphate rich domain (KS) and two adjacent chondroitin sulphate rich domains (CS1 and CS2). This GAG attachment region shows a high degree of variability among species. A short proline-rich hexapeptide repeat sequence on the protein core proximal to the CS domain and located nearer to the N-terminal has been shown to contribute to keratan sulphate

substitution and has been designated the KS domain. The KS domain is responsible for the covalent attachment of up to 30-60 keratan sulphate glycosaminoglycan chains, each chain being of 20-25 repeats per aggrecan monomer. Within the CS domain there are approximately 120 serine-glycine repeat sequences on the core protein recognised for the covalent attachment of up to 100-150 chondroitin sulphate glycosaminoglycans chains of 40-50 repeats per aggrecan monomer (Vertel 1995, Knudson and Knudson 2001). The chondroitin sulphate chains in human articular cartilage are of shorter length in the CS-2 in comparison to those found in the CS-1 domain (Rodriguez *et al.*, 2006). The covalent attachment of CS, KS chains and shorter N- and O-linked oligosaccharides contribute to 90% of aggrecan's mass. (Knudson and Knudson 2001).

Glycosaminoglycans are highly sulphated and contain negatively charged carboxyl groups, as a result negative charge concentrates within the extracellular matrix. Immobilising a negative charge attracts positive counter ions, and increases the chance of attracting water molecules forming a highly hydrated resilient tissue of expanded volume. This enables the formation of a resilient layer on the epiphyses of long bones acting as a protective measure for the joint, providing cartilage with its characteristic resistance to compressive loads and mechanical stress (Murdoch 2002).

(iii) Globular Domain 3 (G3)

The C-terminus comprises of the G3 domain that consists of a number of protein motifs: epidermal growth factor (EGF) like repeats, a C-type lectin motif, a complement regulatory protein (CRP) and a short tail peptide (Knudson and Knudson 2001, Day *et al.*,1999). Alternative splicing of the mRNA encoding these motifs creates G3 domains of varied properties. The G3 domain has been reported to interact with other components within the extracellular matrix such as tenascin, fibulins and cell surface proteoglycans. The ligands for the G3 domain are not generally found in mature cartilage but in developing cartilaginous regions of the body. This suggests an important role for G3 in the assembly of the extracellular matrix and possibly an involvement in a repair process. An increase in age causes an increase of the population of aggrecan monomers that lack the G3 domain, most likely due to proteolytic cleavage in matrix turnover (Knudson and Knudson 2001).

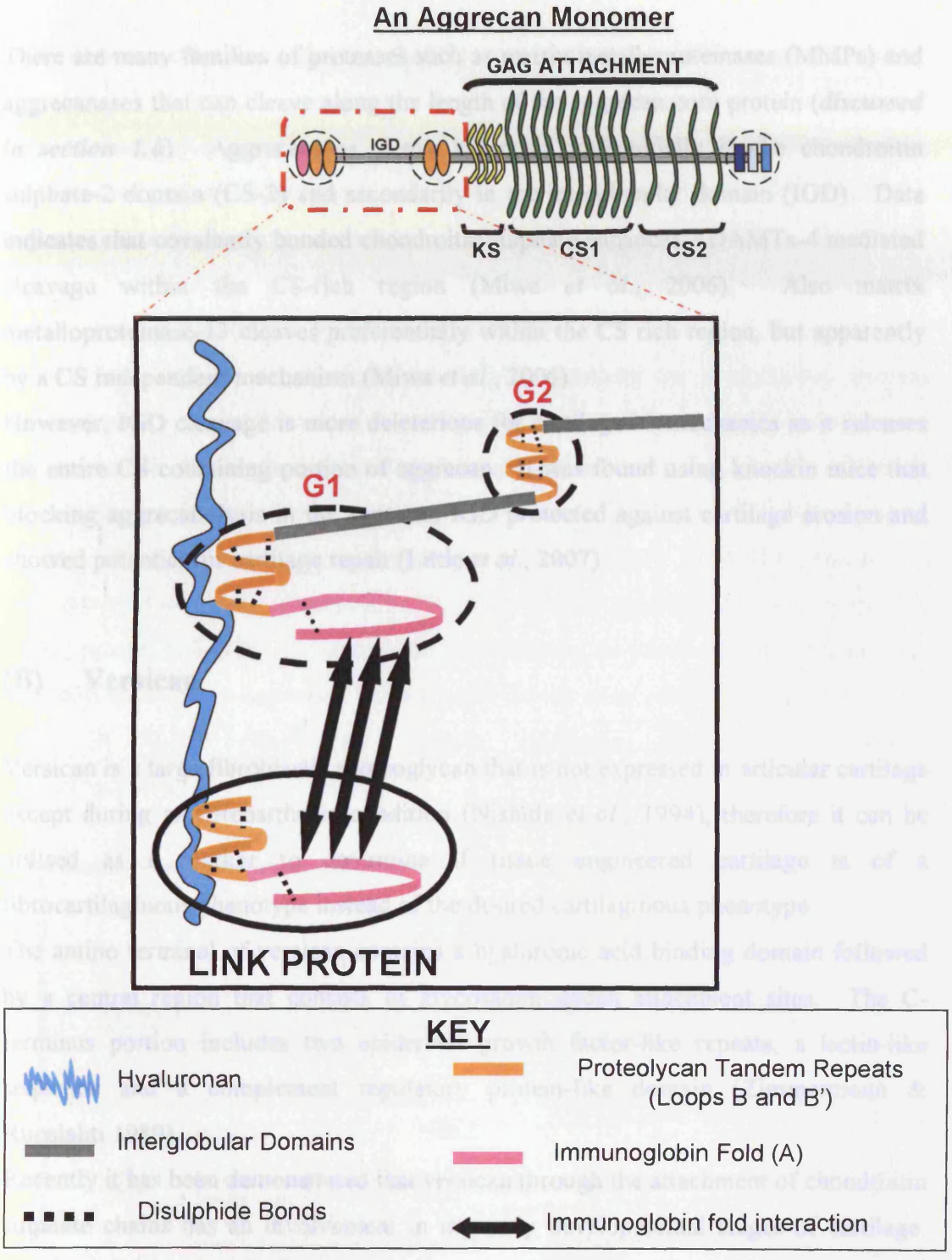

Figure 1.14. Interactions involved in the aggregation of aggrecan (*Adapted from Hardingham and Fosang 1992*).

(iv) Proteolytic Cleavage of Aggrecan

There are many families of proteases such as matrix metalloproteinases (MMPs) and aggrecanases that can cleave along the length of the aggrecan core protein (***discussed in section 1.6***). Aggrecanases cleave aggrecan preferentially in the chondroitin sulphate-2 domain (CS-2) and secondarily in the interglobular domain (IGD). Data indicates that covalently bonded chondroitin sulphate enhances ADAMTs-4 mediated cleavage within the CS-rich region (Miwa *et al.*, 2006). Also matrix metalloproteinase-13 cleaves preferentially within the CS rich region, but apparently by a CS independent mechanism (Miwa *et al.*, 2006).

However, IGD cleavage is more deleterious for cartilage biomechanics as it releases the entire CS containing portion of aggrecan. It was found using knockin mice that blocking aggrecanolysis in the aggrecan IGD protected against cartilage erosion and showed potential for cartilage repair (Little *et al.*, 2007).

(B) Versican

Versican is a large fibroblastic proteoglycan that is not expressed in articular cartilage except during an osteoarthritic condition (Nishida *et al.*, 1994), therefore it can be utilised as a marker to determine if tissue engineered cartilage is of a fibrocartilaginous phenotype instead of the desired cartilaginous phenotype.

The amino terminal of versican contains a hyaluronic acid binding domain followed by a central region that consists of glycosaminogycan attachment sites. The C-terminus portion includes two epidermal growth factor-like repeats, a lectin-like sequence and a complement regulatory protein-like domain (Zimmermann & Ruoslahti 1989).

Recently it has been demonstrated that versican through the attachment of chondroitin sulphate chains has an involvement in the early developmental stages of cartilage. Versican positively regulates mesenchymal cell condensations and the onset of chondrocyte differentiation during chondrogenesis (Kamiya *et al.*, 2006).

1.4.2 Small Leucine Rich Proteoglycan

Small leucine rich proteoglycans (SLRPs) are synthesised either as glycoproteins containing N-linked oligosaccharides or as proteoglycans containing glycosaminoglycan side chains (Iozzo 1999). The core protein of SLRPs contain a cysteine rich region followed by a main portion of leucine rich repeats that participate in protein-protein interactions flanked by disulphide loops on both sides. Apart from chondroadherin, these proteins contain divergent amino-terminal extensions with features unique for different proteins (**Figure 1.15**).

SLRPs are divided into four classes (I-IV) depending on evolutionary protein conservation, gene organisation, the number of leucine rich repeats within the core protein central domain and the presence of distinct cysteine rich clusters at the N-terminal with varying consensus sequences (Iozzo 1999). Class I SLRP members (**Table 1.5**) include decorin, biglycan and asporin. Class II SLRPs (**Table 1.6**) comprise of 5 members; fibromodulin, lumican, proline arginine-rich and leucine-rich repeat protein (PRELP), keratocan and osteoadherin, of which all except keratocan and osteoadherin are expressed in cartilage (Grover *et al.*, 1995). Members of the Class III SLRPs (**Table 1.7**) include epiphycan, mimecan and opticin. Chondroadherin is a class IV SLRP.

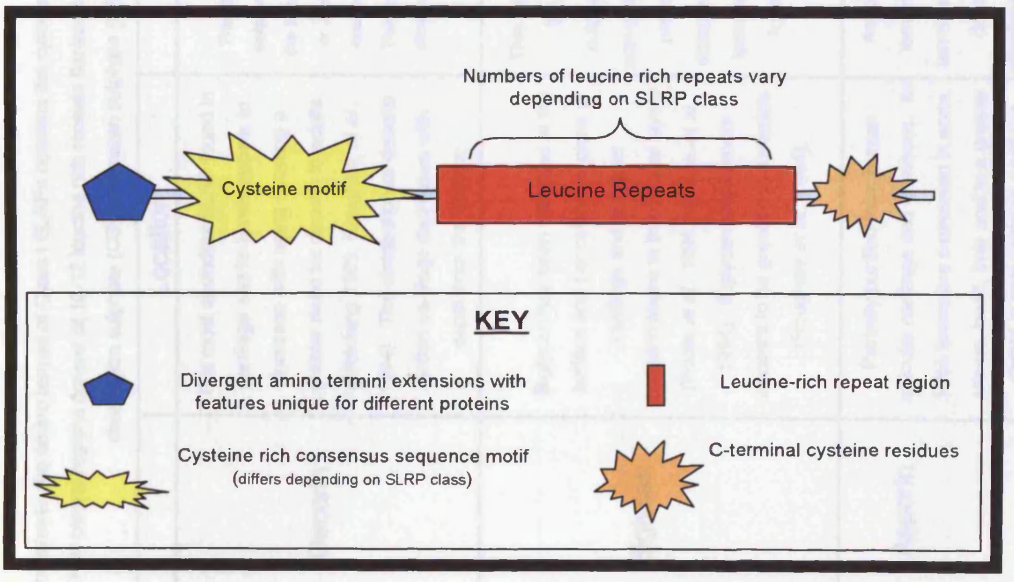

Figure 1.15. General structure of a Small Leucine Rich Proteoglycan (SLRP) (Iozzo 1999)

Class I SLRPs			
In general, the amino termini of Class I SLRPs contains the cysteine rich consensus sequence motif [CX_3CXCX_6C (C= Cysteine residue and X= intervening amino acid spacing]. Their central regions consist of 10-12 leucine rich repeats flanked by cysteine regions (Iozzo 1999). All Class I SLRPs (Asporin being an exception) are primarily substituted with chondroitin sulphate (CS) / dermatan sulphate (DS) chains that show variation with age and between connective tissues (Roughley *et al*, 1993).			
	Location	**Structure**	**Function**
Decorin	The most abundant SLRP found in cartilage and its levels appear to increase with aging showing a greater need for decorin in adults (Melching 1989, Roughley *et al.*, 1994). The distribution of decorin within cartilage decreases with depth from the surface.	The glycoprotein core of decorin harbours 12 leucine-rich sequence motifs flanked by cysteine-rich regions with 4 at the N-terminus and 2 at the C-terminus. It also has either 2 or 3 asparagine-bound oligosaccharides (*at asparagines residues 181, 232 and 273 respectively*) (Hausser 1998). The decorin proteoglycan appears to have horseshoe like dimensions and due to this unusual structure decorin can partake in a multiple of functions.	The horseshoe structure of decorin enables it to bind various ligands including; TGF β, fibronectin and various collagen molecules. It is presumed that the concave surface of decorin binds gap zones of collagen fibres maintaining fibril-fibril spacing via the GAG side chain at the N-terminus (Yamagudu 1990, Knudson & knudson 2001).
Biglycan	Biglycan has been identified at the surface and pericellular regions of cartilage and at higher concentrations in the deeper layers (Poole *et al.*, 1996, Miosge *et al.*, 1994). Biglycan requirement appears to be greatest in juveniles (Roughley *et al.*, 1994).	This SLRP is composed of a 38kDa core protein. At the amino terminus there are two chondroitin sulphate/dermatan sulphate attachment sites. The non-proteoglycan form of biglycan constitutes a minor proportion of biglycan in newborn, but is a major component in adults (Roughley *et al.*, 1993). More C-terminal of this region there is a cysteine rich region followed by a leucine rich region containing 10 repeating units (Neame *et al.*, 1989).	Biglycan has been demonstrated to interact with some of the molecules in which decorin can interact such as TGFβ. There is conflicting data as to whether biglycan can interact with fibrillar collagens or not. Biglycan doesn't interact with collagen under all conditions and primarily it interacts with type VI collagen. In Schwarm rat chondrosacoma tissue, both biglycan and decorin have been demonstrated to interact with matrilin-1 forming complexes acting as a linkage between collagen type VI microfibrils to both collagen type II and aggrecan (Wiberg *et al.*,2003). Biglycan has been indicated as a positive regulator of bone formation and bone mass (Xu *et al.*, 1998).
Asporin	Partially purified from human articular cartilage and meniscus. Its RNA levels are expressed in aorta, uterus, heart, liver and to a greater extent in osteoarthritic cartilage (Lorenzo *et al.*, 2001).	Asporin contains a putative pro-peptide, 4 amino-terminal cysteines, 10 leucine rich repeats and 2 C-terminal cysteines. Asporin is not a proteoglycan, as it doesn't contain a consensus sequence for GAG attachment and it comprises of a long stretch of aspartic acid residues in its amino terminal (Lorenzo *et al*, 2001).	The aspartic acid repeat polymorphism in asporin inhibits chondrogenesis and potentially contributes to calcium phosphate deposition in osteoarthritis (OA) (Lorenzo *et al*, 2001, Kizawa *et al.*, 2005, Ikegawa S 2006). Asporin has not been found to play a major role in the susceptibility to rheumatoid arthritis, but it may influence the disorder (Torres *et al.*, 2007).

Table 1.5. Members of Class I Small Leucine Rich Proteoglycans located in cartilage

Class II SLRPs			
In general, the amino termini of Class II SLRPs contain sulphated tyrosine residues and the cysteine rich consensus sequence motif [CX_3CXCX_9C]. Their central domains consist of 10 leucine rich repeats (Iozzo 1999). All Class II SLRPs, with the exception of PRELP (that has heparin sulphate attachment sites) are primarily substituted with polylactosamine or keratan sulphate (KS) chains.			
	Location	**Structure**	**Function**
Fibromodulin	Cartilage, tendon, skin, sclera and cornea (Oldberg *et al.*, 1989). In articular cartilage high levels of fibromodulin have been located to the interterritorial matrix region with decreasing concentrations from articular surface with depth (Hedlund *et al.*, 1994).	Fibromodulin can contain up to 4 KS chains, within its leucine rich region through N-glycosidic linkages to asparagine residues (Plaas *et al.*, 1990). Fibromodulin in juveniles and young adults exists in a proteoglycan form possessing KS chains, the size of these chains decrease with age. In mature cartilaginous tissues fibromodulin does not possess KS chains or non-sulphated polylactosamine chains, but possesses the same number of N-linked oligosaccharides (Roughley *et al.*, 1996).	Fibromodulin is localised along the surface of collagen fibrils and may possibly regulate fibril diameter (Hedbom & Heinegard 1993). It has also been shown to inhibit type I collagen fibrillogenesis through more than one binding domain within fibromodulin (Font *et al.*, 1998).
Lumican	The major location is the cornea, but it does have a wide spread distribution including muscle, kidney, lung, intestine and connective tissues including articular cartilage (Melching and Roughley 1989, Ying *et al.*, 1997).	In adult cartilage, lumican exists predominantly in a glycoprotein form lacking KS chain attachment, whereas the juvenile form of the molecule is a proteoglycan form (Grover *et al.*, 1995, Melching *et al.*, 1997).	Colocalises with fibrillar collagens in the corneal stroma, regulating assembly and diameter of collagen fibres (Blochberger *et al.*, 1992, Neame *et al.*, 2000). Modification of lumican with KS may contribute to corneal transparency (Chakravarti *et al.*, 1998 review). Lumican has been shown to interact with type II collagen (Sztrolovics *et al.*, 1999).
Proline Arginine-rich and leucine-rich repeat protein (PRELP)	Articular cartilage (predominantly in the territorial matrix) and at certain basement membranes. Also present in kidney, skin, aorta, sclera, liver, skeletal muscle, cornea and tendon.	It is the only member of the SLRPs with a basic amino-terminal region that is rich in proline and has clusters of arginine residues.	Binds the heparan sulphate proteoglycan perlecan via its positively charged amino terminal (Bengtssonn *et al.*, 2000 & 2002) and binds collagens type I and type II through its leucine-rich repeat domain suggestive that PRELP functions as a molecule for anchoring basement membranes to underlying connective tissues (Bengtssonn *et al.*, 2002).

Table 1.6. Members of Class II Small Leucine Rich Proteoglycans located in cartilage

Class III SLRPs In general Class III SLRPs contain the N-terminal cysteine rich consensus sequence [CX_2CXCX_6C] and have slightly smaller central domains consisting of only six leucine rich repeats (Iozzo 1999).			
	Location	**Structure**	**Function**
Epiphycan / PG-Lb / DSPG3	Epiphysis (Johnson *et al.*, 1997), cartilage, ligament and placental tissue (Deere *et al.*, 1996).	The core protein of bovine epiphycan is approximately 46kDa and substitution of dermatan sulphate/chondroitin sulphate of variable size ranging from 23-34 kDa at two serine residues have been found within its N-terminal acidic region. All glycosylations except one N-linked oligosaccharide occur in the N-terminal domain of the protein core.	Epiphycan functions as an immediate marker for chondrogenesis and has an involvement in the organisation of the matrix growth plate, especially the zone of flattened chondrocytes where it is abundantly located (Johnson *et al.*, 1999).
Mimecan / Osteoglycin	Found in sclera and cornea, but can also be found in non-ocular tissues as a non-sulphated glycoprotein. (Funderburgh *et al.*, 1997, Madisen *et al.*, 1990).	Exhibits only ~ 40% protein sequence identity to epiphycan (Shinomura and Kimata 1992)	Mimecan has been demonstrated to regulate collagen fibrillogenesis *in vivo* (Tasheva *et al.*, 2002). Mimecan is also a secretary product found in osteoarthritic cartilage (De Ceuninck *et al.*, 2005).
Opticin / Oculoglycan	Located in ocular tissues, cartilage, ligament, skin, muscle, and testes (Pellegrini *et al.*, 2002).	At its amino terminal region there are O-linked oligosaccharides (Reardon *et al.*, 2000) and heparin binding consensus sequences (Cardin & Weintraub 1989)	Been reported to bind to collagen fibrils (Pellegrini *et al.*, 2002).

Table 1.7. Members of Class III Small Leucine Rich Proteoglycans located in cartilage

Chondroadherin (a class IV SLRP) is a cell binding protein that is expressed at high levels in certain zones of articular cartilage within the territorial matrix, also detected in bone, tendon, bone marrow, and chondrosarcoma cells (Tasheva *et al.*, 2004). Principal features of this Class IV SLRP are that it contains a series of ten leucine rich repeats and most N terminal of these repeats is a cysteine residue (Cys63) that is not disulphide bonded, but probably involved in hydrogen bonding. Chondroadherin lacks both amino and COOH terminal extensions outside the cysteine motifs. Two major isoforms of this proteoglycan generated as a result of cleavage within the C-terminal region have been isolated from bovine articular cartilage (Neame *et al.*, 1994).

Chondroadherin has been found to bind two sites on collagen type II and has also been shown to mediate adhesion of isolated chondrocytes (Camper *et al.*, 1997). Both type II collagen and chondroadherin have been found to interact with chondrocytes via the same receptors, but the interaction of the two different molecules with the receptor give rise to different cellular responses (Mansson *et al.*, 2001).

1.4.3 Superficial Zone Protein (SZP) / Proteoglycan 4 / Lubricin

This proteoglycan was first described by Schumacher *et al* and identified in the culture medium from thin slices of superficial bovine articular cartilage (Schumacher *et al.*, 1994). The molecular weight of this proteoglycan is approximately 345kDa and it is specifically synthesised by chondrocytes located within the superficial zone of articular cartilage (Schumacher *et al.*, 1994). SZP is not synthesised by chondrocytes located in the mid or deep regions therefore functions as a specific marker for chondrocytes of the superficial zone region (Schumacher *et al.*, 1994). Accumulation of SZP in articular cartilage has been shown to increase in response to bone morphogenetic protein-7 (BMP-7) and growth factors (Khalafi *et al.*, 2007).

The molecule is substituted with keratan sulphate and chondroitin sulphate mostly at the amino or carboxy-terminal, or at both termini therefore it can be categorized as a proteoglycan (Schumacher *et al.*, 1999). Very little SZP is incorporated in the matrix surrounding the chondrocytes; most diffuses out of the tissue and can be detected in the synovial fluid (Schumacher *et al.*, 1994, Schumacher *et al.*, 1999). Not only has SZP been located at the surface of articular cartilage, it has also been found in the

synovial membrane (Schumacher *et al.*, 1999 and at the surface of fibrocartilaginous regions of tendon with an increased presence with age (Rees *et al.*, 2002).

The amino acid composition of SZP is highly conserved between human and bovine species and has been found homologous to the precursor protein megakaryocyte-stimulating factor (MSF) (Schumacher *et al.*, 2005). SZP is a multifunctional proteoglycan. Its core protein within the central region is composed of large and small mucin-like repeat domains composed of the sequences KEPAPTTT/P (76-78 repeats) and XXTTTX (6-8 repeats), respectively. The mucin-like domains are likely to be substituted with O-linked oligosaccharides, which provide lubricative and cytoprotective properties. The mid region is flanked by cysteine rich N- and C-terminal domains, which are homologous to somatomedin B and haemopexin domains of vitronectin, respectively. The potential growth promoting, cytoprotective properties have been associated with the N-terminal domain and both matrix binding and aggregating properties have been associated with the C-terminal domain (Flannery *et al.*, 1999).

1.5 Other Extracellular Matrix Macromolecules

There are a number of molecules within the extracellular matrix that are not collagens or proteoglycans that have been identified in articular cartilage. Some of these are discussed in detail below.

1.5.1 Cartilage Oligomeric Matrix Protein (COMP)

The acidic oligomeric protein COMP is a member of the thrombospondin gene family of extracellular glycoproteins, and recently has been termed thromospondin 5 (TSP-5) (Kipnes *et al.*, 2003). It is a product of a unique gene and is not formed by the alternative splicing of the thrombospondin gene (Oldberg *et al.*, 1992).

Cartilage Oligomeric matrix protein is a non-collagenous glycoprotein expressed predominantly in articular cartilage (Hedbom *et al.*, 1992), tendon (DiCesare *et al.*, 1994) and ligament. In adult cartilage, COMP has been shown to be primarily located within the interterritorial matrix (Kipnes *et al.*, 2003). Its expression has also been

found to be predominantly located in the proliferative region of the growth plate (Shen *et al.*, 1995, Ekman *et al.*, 1997) indicating a role for COMP in developing cartilage.

The structure of COMP has been identified as a pentameric bouquet like protein (~524kDa) composed of five identical subunits (**Figure 1.16**). Each subunit contains an α-helical structure located at its N-terminus which is responsible for the stabilisation of the pentameric structure due to disulphide bonds occurring between residues 20-83 (Efimov *et al.*, 1994). Each COMP subunit contains 2 N-linked oligosaccharides which differ between immature and mature cartilage. The N-terminal is followed by a flexible region of 4 type 2 epidermal growth factor (EGF) domains and 7 thrombospondin (TSP) type 3 calcium binding domains and a C-terminal globular domain (Oldberg *et al.*, 1992).

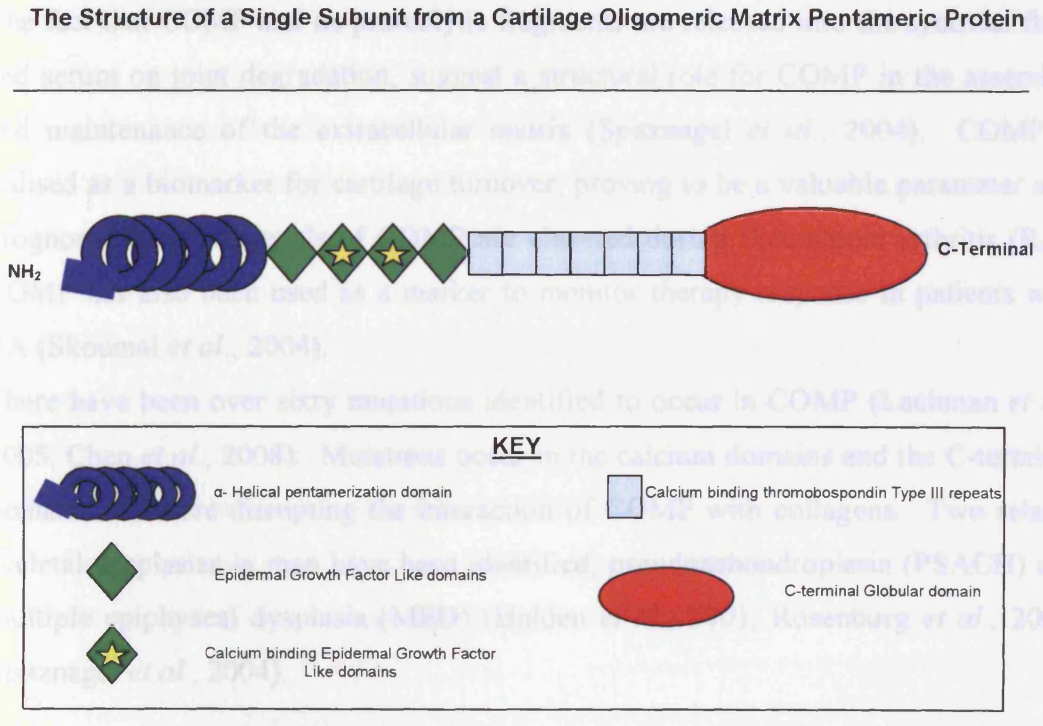

Figure 1.16. Schematic diagram representing one of the five subunits found on a COMP pentameric protein adapted from Spitznagel *et al.*, 2004.

Crystal structure analysis of the recombinant coiled coil domain of COMP revealed that within the pentameric α helical bundle there was a hydrophobic channel, 73 Å long with a diameter of 2-6 Å potentially responsible for the binding of hydrophobic molecules (Özbek *et al.*, 2002). This is suggestive that COMP plays a role in storage and delivery of regulatory molecules in metabolism (Özbek *et al.*, 2002).

COMP mediates primary chondrocyte attachment of matrix components and has a role in organising matrix assembly in cartilage (Misumi *et al.*, 2001). It had been suggested that it plays a role influencing the organisation of collagen fibrils via its C-terminal domain with collagen types I, II and IX. COMP binds collagen type I/II and pro-collagen I/II in the presence of Zn^{2+} and with the non-collagenous domains of type IX collagen (Holden *et al.*, 2001). This has a stabilising function on the collagen network in cartilaginous tissues. Potentially COMP can bind with its five C-terminals to five different collagen molecules, possibly indicating that COMP has an involvement in the organisation of collagen molecules into microfibrils (Halasz *et al.*, 2007).

The fact that COMP and its proteolytic fragments are released into the synovial fluid and serum on joint degradation, suggest a structural role for COMP in the assembly and maintenance of the extracellular matrix (Spitznagel *et al.*, 2004). COMP is utilised as a biomarker for cartilage turnover, proving to be a valuable parameter as a prognostic factor as levels of COMP are elevated during rheumatoid arthritis (RA). COMP has also been used as a marker to monitor therapy response in patients with RA (Skoumal *et al.*, 2004).

There have been over sixty mutations identified to occur in COMP (Lachman *et al.*, 2005, Chen *et al.*, 2008). Mutations occur in the calcium domains and the C-terminal domains therefore disrupting the interaction of COMP with collagens. Two related skeletal dysplasias in man have been identified; pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) (Holden *et al.*, 2001, Rosenburg *et al.*, 2001, Spitznagel *et al.*, 2004).

1.5.2 Matrilins

Four members of the glycoprotein matrilin family have been identified to date; matrilin-1, -2, -3 and -4. The first member to be discovered was matrilin-1, originally

called cartilage matrix protein (Paulsson and Heinegard 1981). Matrilins have the ability to form both homotypic and heterotypic oligomers and there are seven novel matrilin isoforms (Frank *et al.*, 2002). Comparative genomics of the syndecans has defined an ancestral genomic context associated with matrilins in vertebrates (Chakravarti & Adams 2006). Recently, mutations in the human gene encoding matrilin-3 have been associated with different forms of chondrodysplasias (Nicolae *et al.*, 2007)

All four matrilins are expressed in cartilage (Nicolae *et al.*, 2007). Matrilin-1 is expressed mainly in cartilage and matrilin-3 has a similar distribution, while both matrilins -2 and -4 occur in a wide variety of extracellular matrices (Deak *et al.*, 1999). In rabbit knee joints matrilin-1 was found within deeper epiphyseal cartilage and remained confined to the residual epiphyseal cartilages indicating that articular and epiphyseal cartilage are different from the earliest of developmental stages (Kavanagh & Ashhurst 1999).

All matrilins contain common structural motifs; they have two von Willebrand Factor A (vWFA)-like domains separated by varying numbers of epidermal growth factor (EGF)-like domain(s) (*varying from one in matrilin-1 to ten in matrilin-2*) and a coiled-coil α-helical motif (Aszodi *et al.*, 1999 & Deak *et al.*, 1999).

Matrilins are similar in their structures, but versatile in their functions. They are matrix adapter proteins that mediate interactions between collagen-containing fibrils and other matrix constituents, such as aggrecan (Wagener *et al.*, 2005 & Nicolae *et al.*, 2007). They participate in the formation of fibrillar or filamentous structures and are often associated with collagens (Wagener *et al.*, 2005 & Nicolae *et al.*, 2007). Matrilin-1 has been found to form complexes with both decorin and biglycan molecules acting as a linker with abilities to connect type VI collagen to both collagen type II and aggrecan (Wiberg *et al.*, 2003). These linkers have also been shown to connect a number of pro-collagen molecules to collagen type VI scaffolds (Wiberg *et al.*, 2003).

Both matrilins-1 and 3 have been found to be major components of the pericellular matrix and chondrocyte sensitivity to changes in the microenvironment induced by cyclic loading can be adjusted by altering the content of matrilins in the pericellular matrix, supporting a critical role of the pericellular matrix in chondrocyte mechano-transduction (Kanbe *et al.*, 2007).

All matrilins except matrilin-4 are capable of mediating cell attachment. Matrilins-1 and -3 have been found to facilitate cell attachment in a dose dependent manner, but higher doses of these matrilins are required for cell attachment to reach plateau values similar to those obtained using fibronectin (Mann *et al.*, 2007).

1.5.3 Cartilage Intermediate Layer Protein

The cartilage intermediate layer protein (CILP) gene is mapped to chromosome 15 (locus q22) (Nakamura *et al.*, 1999) and it encodes a proform of two polypeptides. The precursor protein, contains a putative signal peptide of 21 amino acids and is synthesised as a single polypeptide chain that is processed into two polypeptides upon or preceding secretion. The amino terminus corresponds to non-collagenous CILP protein. CILP has been identified as a single 92kDa polypeptide chain that contains 30 cysteine residues, 1 thrombospondin-like repeat and 6 N-glycosylation sites (*10% of its total molecular mass being attributed to N-linked oligosaccharides*) (Lorenzo *et al.*, 1998). At the C-terminal, 460 amino acids show 90% similarity to pig ectonucleotide pyrophosphohydrolase (NTPPHase); this region is preceded by a furin protease consensus cleavage site (Lorenzo *et al.*, 1998). The NTPPHase contains 10 cysteine residues and 2 putative N-glycosylation sites.

CILP is a synthetic product of chondrocytes and it is restricted to cartilaginous tissues with specific distribution in the middle layer of the tissue with deposition in the interterritorial matrix (Lorenzo *et al.*, 1998). It is not observed in the superficial or the deep regions of articular cartilage (Lorenzo *et al.*, 1998). Interactions of CILP with other extracellular matrix molecules have not been identified.

Increased synthesis of CILP has been shown in lumbar disc disease and in early OA (Mori *et al.*, 2006 & Seki *et al.*, 2005). In early OA, CILP distribution changes from a predominantly interterritorial location to a pericellular location (Lorenzo *et al.*, 1998).

CILP/NTPPHase secretion into the matrix around chondrocytes has been shown to increase with age. In this extracellular site it may generate inorganic pyrophosphate and contribute to age-related calcium pyrophosphate dihydrate crystal deposition disease (Masuda *et al.*, 2001 and Yamakawa *et al.*, 2002).

1.5.4 Tenascins

Tenascins are large multi-subunit glycoproteins that are found in numerous tissues. There are five types of tenascins that have been identified to date these being; tenascin-R (*predominantly found in the central nervous system having an impact on neurite outgrowth and synaptic function* (Chiquet-Ehrismann 2004a & b), -X (*found in connective tissue around blood vessels*), -W (*found in the extracellular matrix of bone, muscle and kidney* (Chiquet-Ehrismann 2004 a & b)), -Y and C (*produced by osteoblasts, cartilage, perichondral cells and fibroblasts* (Chiquet-Ehrismann 2004 a & b)).

There are several distinct isoforms of Tenascin-C and its expression has been found in large concentrations in articular cartilage, suggesting a tissue specific function. The smaller 220kDa isoform of tenascin-C is found in normal chondroid matrix and the expression of a larger 320kDa isoform is found in chondrosarcoma (Ghert *et al.*, 2002). Therefore, alternative isoforms of tenascin-C may lead to therapeutic approaches for chondrosarcoma (Ghert *et al.*, 2002).

The overall glycoprotein structures of the tenascin family are the same, although tenascin-C is the only one in the family known to form hexabrachions (Jones & Jones 2000). Tenascin-C contains six monomers each terminating in a globular domain that radiate from a central complex, existing as a disulphide linked multimer. Each monomer of the hexabrachion, at the N-terminal contains a group of hydrophobic residues and eight cysteine residues thought to be responsible for association of the six monomers into a homohexamer. C-terminal to this region are epidermal growth factor-like (EGF-like) repeat modules that show sequence homology with type II units found in fibronectin and several other proteins (Gulcher *et al.*, 1991).

Tenascins have been found to have adhesive and anti-adhesive properties. Evidence for adhesive domains within tenascin is that it can interact with chondroitin sulphate proteoglycans or with RGD-sensitive integrins (Gotz *et al.*, 1996, Xiao *et al*, 1997, Grumet *et al.*, 1996, Elefteriou *et al.*, 1999). Tenascin-C has been found to be modulated by mechanical stress, having an increased expression in tissue surrounding tumours, wounds and inflamed tissues (Latijnhouwers *et al.*, 1998, Chiquet-Ehrismann *et al.*, 2003 & 2004, Dang *et al.*, 2006). Here it may possibly regulate cell morphology, growth and migration by activating diverse intracellular signalling

pathways (Chiquet-Ehrismann 2004 a & b). It has been demonstrated that tenascin-C can interfere with the binding of syndecan-4 to fibronectin and block cell adhesion stimulating tumour cell proliferation (Huang *et al.*, 2001). It has been suggested that tenascins may act as 'avoidance molecules' that could act as steric blockers of cellular receptors for other extracellular matrix components (Sage and Bornstein, 1991).

1.6 Degenerative Changes in Articular Cartilage

A major degenerative pathology of cartilage is arthritis. Arthritis is a multifactorial disease with genetics, obesity, joint injury, sex and age all being possible contributors. A central event in both osteoarthritis and rheumatoid arthritis is the depletion of cartilage extracellular matrices via enzymes; this also occurs to a lesser extent during the course of normal matrix turnover.

There are several enzyme families termed matrix proteases which are responsible for matrix erosion. They include Cathepsins (Soderstrom *et al.*, 1999, Fosang *et al.*, 1992 Rantakokko *et al.*, 1996, Deiss *et al.*, 1996 and Wex *et al.*, 2001), A Disintegrin and Metalloproteinase (ADAMs) (Wolfsberg *et al.*, 1998, Black *et al.*, 1997, Primakoff & Myles., 2000 and Hartmann *et al.*, 2002), A Disintegrin and Metalloproteinase (reprolysin type) with Thrombospondin motifs (ADAMTs) (Kuno *et al.*, 1997, Flannery *et al.*, 1999, Somerville *et al.*, 2003, Tang *et al.*, 2001, Abbaszade 1999, Cal *et al.*, 2002., Bornstein 1992, Arner 2002, Tortorella *et al.*, 1999, 2000a & 2000b), and Matrix Metalloproteinases (MMPs) (Van Wart *et al.*, 1990, Gomis-Ruth *et al.*, 1996, Fosang *et al.*, 1992, Little *et al.*, 2002).

Members of the ADAMTs family (ADAMTs-1, ADAMTs-4 (Aggrecanase-1), ADAMTs-5 (Aggrecanase 2) and ADAMTs-9) have been shown to cleave at various positions along the core protein of aggrecan, the major proteoglycan found in cartilage (Tortorella *et al.*, 1999, 2000b, Abbaszade *et al.*, 1999 and Flannery *et al.*, 1999). Matrix Metalloproteinases (MMPs) that have been found to cleave aggrecan to date include MMP-1,-2, -3, -7, -9, -10 and -13 (Fosang *et al.*, 1992, Little *et al.*, 2002 and Flannery *et al.*, 1992) and the collagenases (MMP-1, -8, -13 and -14) have been found to efficiently degrade fibrillar collagens (Somerville *et al.*, 2003). Altered mechanical stresses induce chondrocytes within cartilage to make inflammatory

cytokines resulting in synthesis, secretion, and activation of these cartilage degrading matrix proteases.

Inhibitors of the MMPs have been identified, and are called Tissue Inhibitors of Metalloproteinases (TIMPs) (Henriet *et al.*, 1999). Recent studies have shown that they also have the ability to inhibit aggrecanase activity (Hashimoto *et al.*, 2001 and Gendron *et al.*, 2003). Cystatins are known inhibitors of Cathepsins (Auerswald *et al.*, 1996 and Sawicki & Warwas 1989); alterations in the Cathepsin / cystatin balance result in uncontrolled proteolysis as seen in disorders such as osteoarthritis (Soderstrom *et al.*, 1999). Consequently, these enzymes are a target for therapeutic intervention, with much research focusing on discovering various inhibitors with chondro-protective abilities.

1.6.1 Osteoarthritis

Osteoarthritis (OA), also known as osteoarthrosis, arthrosis, and degenerative joint disease is the most common age-related disorder of articular cartilage joints and bone tissue although in certain cases it has been found to affect juveniles. OA can affect different joints including hands, feet, spine, knees and hips and is characterised by progressive degeneration and loss of articular cartilage. There is no cure for this common disease and current treatments only modify the symptoms of the disease to alleviate pain and minimise joint damage (Dodge and Jimenez, 2003).

Factors that contribute to OA include obesity, joint injury and a persons' sex (O'Conner 2006, Pearson-Ceol 2007). A higher disease incidence occurs with increasing age, although OA is not an inevitable part of ageing. Occasionally OA can be found in multiple members of the same family implying a genetic role. Genetic mutations in ECM components of cartilage predispose the joints to OA either through failure of matrix integrity or of whole joint functioning. Genes associated with abnormal development of skeletal structures are found at (http://www.ncbi.nlm.nih.gov/omim). Early stage OA cannot be detected using radiographs as significant alterations must occur before its detection, therefore it is essential to identify early onset biomarkers associated with this disease (Kato *et al.*, 2007).

The early stages of osteoarthritis are non-inflammatory. Initially, articular cartilage attempts to repair and remodel itself rather than enter a degradation process, reinitiating the synthesis of some extracellular matrix proteins characteristic of early developmental stages. Increased expression levels of aggrecan, link-protein, type II collagen (some of the type II collagen is present in its embryological form, Type IIA) and type X collagen (a marker for hypertrophy) have been observed (Hardingham and Fosang 1992). The chondrocytes are stimulated to undergo cell replication which is the converse of normal non-diseased, non-proliferating chondrocytes (Sandell & Adler 1999) and they start to synthesis more ECM molecules. This remodelling of articular cartilage compromises the ability of cartilage to resist compressive loads.

Altered mechanical stresses induce chondrocytes within the cartilage to make inflammatory cytokines resulting in the activation of matrix proteases. An inflammatory reaction is also observed within the synovial membrane, and the volume of synovial fluid increases showing changes in the lubrication properties during this disease as the fluid has a reduced viscosity (Gerwin *et al.*, 2006). Matrix proteases increase both proteoglycan and collagen degradation. The initial loss of proteoglycan is from the superficial zone and its degradation compromises the ability of cartilage to imbibe water and resist compressive loads.

During the later stages of OA cellular changes in cartilage morphology occur, cells divide in their lacunae and become hypertrophic forming cells clusters (von der Mark 1995). Remodelling of chondrons within the deep zones occur, where there is a characteristic decline in pericellular fibrillar collagens (i.e type IX collagen) and an increase in type VI collagen forming a type VI rich glycocalyx to accommodate the increasing number of chondrocytes attempting to repair (Poole 1997). Eventually cartilage can be worn away exposing the underlying subchondral bone; joint space narrowing is also evident. The formation of osteophytes and thickening of the subchondral plate is observed in bone (Martel-Palletier *et al.*, 1999)

1.6.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a systemic, chronic inflammatory autoimmune disease. The immune system attacks normal tissue components as if they were invading pathogens, attacking the lining of joints causing them to be swollen, stiff and painful. The pathology of RA extends throughout the synovial joint, and in severe cases involves many other organs (e.g. skin, blood vessels, heart, lungs and muscle). There is a 3 fold predominance of RA in women that may be attributed to hormonal factors (Masi *et al.*, 2006).

The cellularity of the synovial membrane increases from 1-2 to 6-8 cells thick, comprising mostly macrophages, as it is infiltrated with cells from the blood during RA. The synovial fluid of the diseased joint also becomes more cellular in comparison to the acellular fluid that is usually present under normal conditions. It becomes predominantly enriched with neutrophils but there are also macrophages, T-lymphocytes and dendritic cells present.

The major site of irreversible tissue damage originates at the junction of the synovium lining the joint capsule with the cartilage and bone, a region termed the pannus. Pannus cells migrate over underlying cartilage and into the subchondral bone, causing erosion of these tissues (Feldman *et al.*, 1996). Fibroblast-like synoviocytes (FLS) are a cell population central to the development of the pannus. It was found that p21 was involved in regulating cell cycle, playing a role in repressing FLS migration. The loss of p21 expression that occurs during RA, may contribute to the excessive invasion and subsequent joint destruction that takes place during RA (Woods *et al.*, 2006).

Many pro-inflammatory cytokines (*including TNFα and IL-1β*), chemokines, and growth factors are expressed in diseased joints from cells within the pannus and synovial lining; inhibition of these molecules may provide a treatment in RA (Chu *et al.*, 1992). Cytokines cause synthesis, secretion and activation of matrix proteases (i.e. MMPs, ADAMTs 4 and 5) responsible for degradation of cartilage and bone within the joint. Destruction of cartilage as seen in rheumatic disease is now considered to be mostly due to the activity of matrix metalloproteinase enzymes (MMPs), produced by activated macrophages and fibroblasts in response to pro-inflammatory cytokines such as IL-1β and TNF α (Feldman *et al.*, 1996).

Recently it was established that IL-23 p19 was expressed in the synovium of RA patients, but not in OA patients. IL-1 β and to a lesser extent TNF α were found to induce and regulate IL-23 p19 expression from RA FLS via NF kappa β and AP-1 pathway. IL-23 p19 promoted an inflammatory response in RA, inducing IL-8 and IL-6 which was repressed by the addition of inhibitors for both NF kappa β and AP-1 (Liu *et al.*, 2007). Inflammatory conditions can affect the activities of glycosyltranferases, thereby modifying glycan chain synthesis. This can have detrimental effects when the function of glycoproteins present within a matrix is influenced (Yang *et al.*, 2007).

At present there are no cures for RA, just symptom modifying treatments which reduce pain and minimise joint destruction. In the past, inhibitors of TNF α have been utilised as a treatment, but frequency and degree of response was restricted. Recently it has been reported that a promising treatment of RA involves the use of three novel biological compounds; rituximab (anti-CD20), abatacept (cytotoxic T-lymphocyte antigen 4 immunoglobin) and tocilizumab (anti-interleukin 6 receptor) (Smolen *et al.*, 2007). RA FLS gene expression profiles may provide an insight into disease pathogenesis and have utility in diagnosis, prognosis and drug responsiveness (Galligan *et al.*, 2007).

1.6.3 Cartilage Defects

Cartilage defects are localised lesions of cartilage, either generated as a result of OA or joint trauma. A cartilage defect can be regarded as a bioactive chamber. Natural repair can hypothetically occur from the surrounding cartilage walls, the calcified zone, the bone marrow and migrating cells from the synovial fluid (Brittberg 1999). Cells adjacent to wound margins undergo cell death, then after 24 hours there is an increase in cell proliferation, cluster formation and increased matrix synthesis and catabolism. This response is short lived and is not sufficient to repair the defect (Redman *et al.*, 2005).

There are two types of articular cartilage defects, partial thickness defects and full thickness defects. Partial thickness defects do not penetrate the subchondral bone and are unable to heal spontaneously often undergoing progressive degeneration to osteoarthritis (OA). Full thickness defects penetrate through the zone of calcified

cartilage and into subchondral bone and elicit an intrinsic repair response generating a fibrocartilaginous repair tissue. With time there is marked degeneration of repair tissue and a continued degeneration of native articular cartilage (Redman *et al.*, 2005).

1.7 Current Cartilage Repair Strategies and Techniques

Current, non-operative treatments that are applied for pain reduction during arthritis include; anti-inflammatory medications, viscosupplementation, activity modifications, bracing and the use of orthotics (Simon & Jackson *et al.*, 2006). A number of surgical procedures such as joint realignment may provide pain relief for patients for a number of years. The only effective treatment for pain reduction and knee disability is prosthetic joint replacement, although this does not provide permanent functionality, as prostheses have a limited life span.

1.7.1 Viscosupplementation

Viscosupplementation, approved in the late 1990's involves 3-5 injections of exogenous high molecular weight hyaluronan (HA) to the intra-articular space of arthritic joints over several weeks. High molecular weight injections used to date include: (1) Hyalgan (*naturally derived sodium hyaluronate with average molecular weight (Mwt) 500-730kDa*), (2) Supartz (*naturally derived sodium hyaluronate with average Mwt 630-1120kDa, Seikagaku corpororation, Tokyo*), (3) Orthovisc (*Sodium hyaluronate with average Mwt 1900-3000kDa. Anika Therapeutics, Woburn MA*) and (4) Synvisc (*hylan G-F20, chemically modified with an average Mwt > 6000kDa, Genzyme*) (Goldberg & Buckwalter 2005).

These injections improve the visco-elastic properties of the synovial fluid encouraging lubrication, shock absorbance, the formation of a semi permeable barrier and decreased inflammatory and proliferative processes that occur within diseased synovium. Degradation by active oxygen species may reduce the protective properties of HA (Roth *et al.*, 2005).

Currently, intra-articular HA injections are indicated as symptom modifying treatments only. However, there is substantial evidence suggesting that HA can also have disease modifying activity in certain patient populations, as pain relief has been found to last considerably longer than the half life of the injected HA. In an animal trial, the half life of hyalgan injections were estimated to be as short as 18-24 hours but pain relief lasted for 26 weeks or more (Goldberg & Buckwalter 2005).
Adverse effects associated with hyaluronan injections are a mild pain and swelling at the injection area. There was a rare report of a pseudoseptic knee, where a patient developed a severe inflammatory reaction against chicken proteins present in the high molecular weight HA injection (Hamburger *et al.*, 2003).

1.7.2 Debridement and Lavage

Debridement and lavage procedures have been employed in the treatment of cartilage defects. Cartilage in and around a chondral defect is abnormal and unstable. Debridement is the arthroscopic excision of this damaged cartilage. Debridement itself has been shown to improve symptoms for up to 5 years or more (Smith *et al.*, 2005). Lavage is the irrigation of a joint during arthroscopy which removes debris and appears to alleviate pain. Both of these treatments can be successful in treating the early stages of OA, but they do not induce repair. When both debridement and lavage are used in conjunction with each other the pain relief lasts longer (Redman *et al.*, 2005).

1.7.3 Microfracture

Microfracture (Steadman *et al.*, 2001) is a procedure introduced by Steadman 20 years ago that stimulates intrinsic repair (Smith *et al.*, 2005). It is a marrow stimulating technique that is similar to abrasion arthroplasty and Pridie drilling (Redman *et al.*, 2005). This technique has acceptable clinical results of up to 5-7 years and has been found to have better results with patients less than 35 years of age (Smith *et al.*, 2005). During this technique damaged tissue is debrided down to the

subchondral bone. The subchondral bone is perforated by small awls, approximately 2-3mm apart inducing bleeding (Redman *et al.*, 2005).

Invading mesenchymal stem cells from the bone marrow space have the potential to differentiate into chondrocytes, but can produce a fibro-cartilaginous tissue containing collagen type I. Therefore, there is an unpredictable outcome of the nature and quality of the repair tissue produced from patient to patient utilising this technique (Redman *et al.*, 2005).

1.7.4 Mosaicplasty

Mosaicplasty (also known as osteochondral transfer (OATS)) is a procedure that was first described in 1993 (Hangody *et al.*, 1997 & 1998). It can be carried out arthroscopically or with an open joint to treat both chondral and osteochondral defects (Smith *et al.*, 2005). Autologous osteochondral cylindrical plugs are taken from regions considered to be relatively non-load bearing, usually the peripheries of both femoral condyles at the level of the patellofemoral joint (Smith *et al.*, 2005). However, no harvest site is truly non-load bearing; for this reason donor sites shall be referred to as low-load bearing sites.

Different sized plugs are mosaically transplanted to a debrided full depth defect in order to fill the defect. An advantage of this technique is that defects are filled immediately with mature hyaline cartilage. The disadvantages are that cell death can occur at the wound margins of the donor site, and at the margins of the ostoechondral plug leading to failure of lateral integration. Tissue from the low load bearing regions may have difficulties withstanding the stresses found in a high load bearing area (Redman *et al.*, 2005). An additional problem is the variation in thickness of the osteochondral plugs from the donor site cartilage in comparison to the cartilage found at the defect site (Smith *et al.*, 2005).

1.8 Culture Systems for Cell Based Therapies in Tissue Engineering.

There has been recent interest in the use of cell based therapies and tissue engineering long lasting hyaline-like cartilaginous replacement tissue for damaged areas of joints. However, this has proved problematic as the engineered tissue must have similar mechanical properties to native articular cartilage and also have the ability to integrate with native tissue.

Tissue engineering itself implies problems: (1) currently there are many experimental models utilised to generate articular cartilage; both structural and macromolecular properties of engineered tissue vary depending on the culture system used; and (2) the initial cell source used to engineer cartilage could be at a different stage of development, come from a different origin or even a different species giving rise to conflicting results and complicating the understanding of tissue engineering cartilage.

Culture systems enable *in vitro* cultivation of tissues and cells, allowing the analysis of chondrocytes and their extracellular matrix. Culture systems that have been utilised to date for cell based therapies in tissue engineering are discussed below.

1.8.1 Cartilage Explant Culture System

The cartilage explant culture system provides a model to analyse chondrocytes that are resident to their native matrix and their extracellular matrix *ex vivo* (Flannery *et al.*, 1999). The cartilage explants are cultured in a rich medium, often supplemented with serum, metabolites and growth factors. The cartilage explant system provides a model system for analysis of mechanical compression effects (Sah *et al.*, 1989), proteoglycan synthesis and catabolism, matrix degradation initiated by cytokines such as IL-1 and potential protective properties of varying factors (Flannery *et al.*, 1999). Chondrocytes within cartilage explant cultures maintain their differentiated state. Therefore, some of the tissue's architecture can be preserved within the explant. The chondrocytic cells of the explants remain viable for both short and long term incubation periods ranging from six hours to six weeks (Maroudas and Kuettner 1990).

1.8.2 Monolayer Culture System

A human chondrogenic cell source for use in tissue engineering is of limited supply. Therefore an unavoidable measure is to multiply the cells utilizing a process termed 'passaging' within a monolayer culture.

The initial step is the enzymatic isolation of chondrocytes harvested from a low load bearing region of a patient. This procedure proves problematic, as all cell-matrix inter-relationships and cell surface molecules are abolished, altering the metabolic activity and growth rate of the chondrocytes from what would be observed in native cartilage (Maroudas and Kuettner 1990). Hayman *et al* in 2006 analysed the effects different enzymatic procedures inflicted upon an isolated chondrocyte. They reported that a longer collagenase digestion step was preferable for obtaining a high cell yield, but a shorter collagenase digestion step was required to yield chondrocytes with gene expression closest to native chondrocytes.

The isolated chondrocytes within a media suspension are placed into flasks and allowed to multiply in an alien 2-dimensional culture system without the crucial influences of physiological cell-cell and cell-extracellular matrix interactions. As a result the chondrocytic phenotype is challenged and chondrocytes undergo a process called de-differentiation that is characterised by the transition of a chondrocytic phenotype to a more fibroblast-like phenotype.

To try minimising the occurrence of de-differentiation, one study has investigated the effects of culturing bovine articular chondrocytes on different substrates including: tissue culture plastic, fibronectin, type I collagen and type II collagen. It was reported that chondrocyte expansion on protein monolayers may not be an effective solution to prevent de-differentiation (Brodkin *et al.*, 2004).

As a result of this process there are alterations in chondrocyte morphology, gene expression, matrix formation and cytoskeletal organisation. The expression of collagen type I increases, and collagen type II decreases. The addition of factors that encourage chondrogenic behaviour during the expansion procedure may prove beneficial. Improving the basic knowledge of alterations that occur to the chondrocyte and cellular signalling during this process may provide an insight for future intervention preventing this un-wanted de-differentiated characteristic.

1.8.3 Three Dimensional Culture systems

The re-differentiation of de-differentiated chondrocytes can by encouraged by placing expanded chondrocytes from monolayer cultures into a 3-dimensional bio-mimetic culture environment that encourages the chondrocytes to lay down an extracellular matrix, generating a tissue. Culture systems that can be used for such a purpose include; (**A**) pellet, (**B**) alginate, (**C**) agarose, and (**D**) the use of scaffolds. Although a limitation of stimulating re-differentiation, is the induction of hypertrophic differentiation that leads to the undesired effect of endochondral ossification.

(A) Pellet Culture

In a pellet culture system, chondrocytes are pelleted in the base of a tube and cultured in this fashion (Boyer *et al.*, 2003). The chondrocytes are less likely to de-differentiate in comparison to monolayer culture, and cell recovery is not as problematic as it is with agarose cultures. The initial amount of extracellular matrix is essentially zero. The chondrocytes synthesise a matrix around themselves, forming a ball of cells and matrix that remain suspended in culture medium. If the culture period of pellet cultures is prolonged then the centre may differentiate further and begin to express bone markers and become mineralised (Naski & Ornitz 1998).
It has been shown in high-density bovine chondrocyte pellet cultures that the production of cartilage proteoglycan and collagen can be stimulated by extracellular ATP and UTP (Croucher *et al.*, 2000).

(B) Alginate Culture

The use of an alginate culture system to encourage re-differentiation of de-differentiated chondrocytes has been reported utilising many different species including: human (Gagne *et al.*, 2000 & Schulze-Tanzil *et al.*, 2002), rabbit (Yoon *et al.*, 2002) and bovine (Domm *et al.*, 2002). Low oxygen tensions have been reported to induce the re-differentiation of de-differentiated cells in alginate cultures (Domm *et al.*, 2002).

(C) Agarose Culture

An advantage of agarose cultures is that they can three dimensionally immobilize cells and maintain the differentiated phenotype of chondrocytes, enabling them to deposit a characteristic matrix after modulation in monolayer (Makarand & Sittinger 2002). The major advantage of these gels is a rather even distribution of cells while their mechanical behaviour is not suitable for transplantation (Sittinger *et al.*, 1999). The cultures may be exposed to catabolic stimulants, mechanical loading, enzyme inhibitors or drugs.

(D) Scaffolds

Scaffolds have been utilised as a tool to provide growth and integration for engineered articular cartilage in a 3-dimensional biomimetic environment. They function as a delivery vehicle enabling the distribution of cells to a site of injury, minimizing cell loss in transferral and enabling the treatment of relatively large defect areas. Scaffolds may also be cut into a required shape and provide guidance for desired *in vitro* and *in vivo* tissue development. Ideally scaffolds would biodegrade at the same rate that the implanted chondrocytes produce their framework (Mahmoudifar and Doran 2005). The use of scaffolds is superior to conventional 2D monolayer cultures as they help encourage and maintain the chondrogenic phenotype (Heng *et al.*, 2004). The nature of the tissue developed and molecules synthesised depends on the cell source, the type of scaffold used and the chosen factors applied to the scaffold system. A recent development in scaffold technology utilises a senate scaffold that actually monitors *in vivo* loading from within the joint over extended periods of time, having the ability to monitor healing (Geffre *et al.*, 2008).

There have been numerous matrix scaffolds that have been applied in tissue engineering cartilage. Matrices tested to date include protein based matrices (fibrin, collagen and gelatin), carbohydrate based polymers (polylactic acid, polyglycolic acid, hyaluronan, agarose, alginate and chitosan) and synthetic polymers (carbon fibres, polyethylene trephthalates and polytetrafluroethylene) (Hunziker 2001). The naturally occurring bioactive materials utilised as matrices may have problems with immunogenicity (Brittberg 1999). Therefore, smart scaffolds have been developed

incorporating inflammatory inhibitors or antibiotics. The slow controlled release of bioactive molecules encourages adaptation and maturation of new cartilage in hostile *in vivo* conditions, preventing early infections following surgery (Risbud and Sittinger 2002).

1.8.4 Autologous Chondrocyte Implantation

Autologous Chondrocyte Implantation (ACI) is a technique utilised for treating articular cartilage defects and the procedure has been employed for the last two decades. Initially, ACI was described by *Brittberg et al 1994,* using a truly committed chondrocyte cell source. This cell based therapy was approved by the USA Food and Drug Association in 1997, and is the only cell based therapy permitted to date. There are many factors that can determine the effectiveness of ACI including; defect size, previous associated surgeries (*Re-operation is frequent after ACI and is associated with a less satisfying outcome (Henderson et al., 2006))* and defect location as cartilage thickness and properties can vary with its positioning within the knee.

The ACI technique has been applied across a variety of species including rabbit (Brittberg 1996), canine (Breinan *et al.*, 1997), goat (Dell'Accio *et al.*, 2003) and human (Brittberg *et al.*, 2004). In humans, this procedure seems to be more beneficial for the treatment of femoro-tibial defects and less successful in the patello-femoral region (Brittberg *et al.*, 1994). Contradictory results have been reported using a canine model in comparison to rabbit, goat and human systems. It was perceived that in canines there were no significant differences between defects that were treated or untreated with autologous chondrocytes, indicating that the ACI procedure did not contribute to the repair response observed. These findings may be due to species differences (Breinan *et al.*, 1997).

The Steps Involved In Autologous Chondrocyte Implantation (ACI) Procedure

1. Initially, a cartilage biopsy is taken from a low load bearing region of the knee (**Figure 1.17**). In essence this can prove to be detrimental as removal of cells from a donor site would instigate cell death at the wound margins creating another cartilage defect.

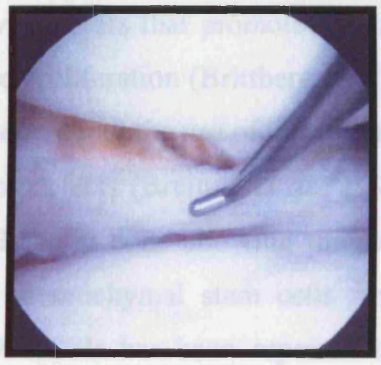

Figure 1.17. Cartilage biopsy being taken from a low load bearing region (*Kindly donated by Genzyme*).

2. Chondrocytes are enzymatically isolated from the cartilage biopsy. There is a limitation on chondrocyte numbers that can be obtained from a patient; unfortunately high cell seeding densities are required for this procedure. Therefore, the harvested chondrocyte numbers are multiplied in monolayer culture. It is a disadvantage to have to multiply the chondrocytes as they have a tendency to de-differentiate due to cytoskeletal modification in the 2D monolayer culture environment, altering their chondrogenic phenotype (Vacanti *et al.*, 2001). The passaged cells appear to be more fibrous in nature lacking the appropriate material and organisational properties of a chondrocyte. Addition of factors to stimulate re-differentiation can prove problematic as they can then induce hypertrophic differentiation of the chondrocytes.

3. At the defect site cartilage is debrided back to the healthy tissue, without puncturing the subchondral bone, as bleeding is an inevitable consequence of

surgery and it is well documented that blood induces cartilage damage (Sawatzky *et al.*, 2005).

4. A covering in the form of a periosteal patch (taken from the medial tibia) or a biodegradable membrane (e.g collagen type I/III membrane) is sutured to the wound margin. The use of a periosteal covering has been reported to provide a shield preventing wear and tear, possibly providing a source of potential stem cells and growth factors that promote the chondrogenic phenotype and enhance chondrocyte proliferation (Brittberg *et al.*, 1999). It has been shown that periosteum alone, without the use of injected autologous chondrocytes is a suitable treatment for defects (Breinan *et al.*, 1997). This may be due to an opening in the subchondral bone allowing the periosteum to exert paracrine effects on invading mesenchymal stem cells from the bone marrow space (Brittberg *et al.*, 1999). It has been reported that the use of a periosteal covering leads to graft hypertrophy post-implantation, and this is avoided by using a collagen type I/III membrane (Steinwachs & Kreuz 2007). One disadvantage is the loss of periosteal / collagen membranes during the recovery period (Driesang & Hunziker 2000).

5. The cultured chondrocytes are injected within a suspension under the sutured covering (**Figure 1.18**). The injected chondrocytes within the defect synthesise and lay down a matrix, generating a graft over time that to a certain extent covers the defect.

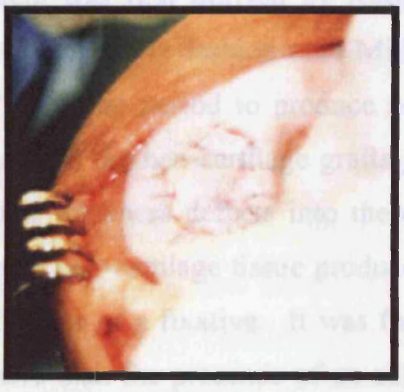

Figure 1.18. A suspension of cultured chondrocytes injected underneath a periosteal covering (*Kindly donated by Genzyme*).

The ACI procedure is capable of forming durable hyaline-like tissue *in vivo*. However, the reparative tissue consists of mixed morphologies, ranging between a hyaline-like and fibro-like cartilage. Integration of new tissue with surrounding matrix can prove unsatisfactory (Hunziker 1999, 2002). Autologous chondrocyte repairs composed of fibro-cartilage showed more morphologic abnormalties and became more symptomatic earlier on than hyaline or hyaline like cartilage repair. The hyaline articular cartilage repair had biomechanical properties comparable to surrounding cartilage and superior to those associated with fibrocartilage repairs (Henderson *et al.*, 2006).

Moriya *et al, 2007*, evaluated the reparative cartilage generated one year following ACI treatment for full thickness defects in human Osteochondritis dissecans femoral condyles. The reparative tissue was reportedly of mixed morphologies and hypertrophy of the periosteum occurred. The reparative tissue stained for type II collagen in the deep to mid regions of the grafts, but did not stain well at the surface. The glycosaminoglycan concentrations within reparative tissues were lower than those found in native, being 76.6μg/mg +/- 4.2 μg /mg and 108μg/mg +/- 11.2 μg/mg, respectively. When utilising a cell source it is important to take into consideration its cell viability. It has been reported that periosteum viability significantly decreases following implantation to a rabbit knee and chondrocyte viability did not significantly decline post-implantation (Emans *et al.*, 2006).

1.8.5 Transwell Culture Systems

The Transwell culture system was first utilized by Kandel *et al* in 1995. Rabbit chondrocytes were seeded in monolayer fashion onto Millipore filter inserts (**Figure 1.19**) and maintained over a culture period to produce neo-cartilage grafts *ex vivo*. The Kandel group investigated if the neo-cartilage grafts generated could be used to treat cartilage defects i.e. full thickness defects into the subchondral bone or intra-chondral defects. The *in vitro* neo-cartilage tissue produced was placed into a rabbit defect in the presence or absence of a fixative. It was found that the absence of an adhesive was more productive than the presence of an adhesive, enhancing grafts to remain in position within full thickness defects. However, maintaining graft

positioning within intra-chondral defects was poor and required further analysis (Kandel *et al.*, 1995).

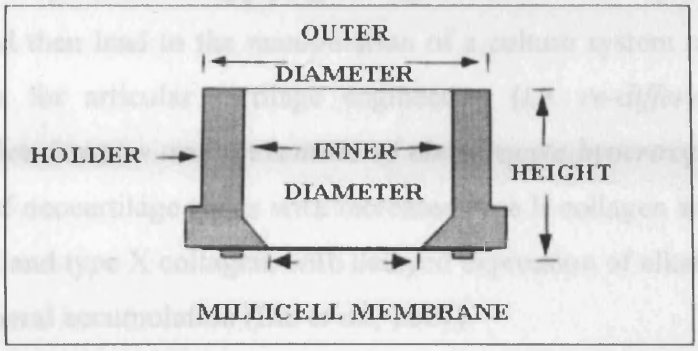

Figure 1.19. Schematic of Millicell filter insert.

Generating grafts *ex vivo* using a Transwell culture system shows potential benefits in comparison to autologous chondrocyte implantation. The surgical procedure could be simplified as it can be performed arthroscopically, reducing surgical morbidity and recovery time. There are no requirements for a periosteal flap or covering, so there is a reduction in damage to the surrounding cartilage. It can also be used to treat larger degenerative defects. The Transwell culture system is an ideal test model as all non-gaseous molecules produced during time of culture can be monitored. Therefore, the Transwell culture system is an excellent model to evaluate the efficiency of novel therapeutics for future research into cartilage metabolism in health and disease.

1.9 Signalling molecules involved in chondrogenesis and *in vivo* cartilage homeostasis and their potential in tissue engineering applications.

At a glance articular cartilage appears to be a relatively simple tissue, having no innervation, blood or lymphatic supply and containing only one cell type; 'The chondrocyte,' making this tissue a likely candidate for tissue engineering. Yet the structural simplicity of articular cartilage is superficial and there are extreme complexities that must be taken into consideration whilst engineering this tissue.

To engineer cartilage *ex vivo*, it is important to understand the initial development of cartilage *in vivo* (termed chondrogenesis) and the functions of the growth factors, signalling molecules, effector proteins and cell to cell interactions involved in this process and the importance of applying the right factor at the right moment. This knowledge could then lead to the manipulation of a culture system to encourage the ideal conditions for articular cartilage engineering (*i.e. re-differentiation of de-differentiated chondrocytes and prevention of chondrocyte hypertrophy*) resulting in the production of neocartilage tissue with increased type II collagen and proteoglycan, decreased type I and type X collagen, with delayed expression of alkaline phosphatase and calcium mineral accumulation (Liu *et al.*, 2007).

Adkisson in 2001 used an *in vitro* Transwell culture system to analyse human chondrocyte activity in a serum free environment. It was found that: (i) juvenile chondrocytes produced neocartilage discs with collagenous matrices and chondrocytes lacked cellular hypertrophy, (ii) Pre-adolescent chondrocytes recapitualated embryonic development in the absence of embryonic factors, but (iii) adult chondrocytes failed to produce grafts in serum free conditions. This emphasises the fact that an understanding of factors required to generate neocartilage grafts would be advantageous (Adkisson *et al.*, 2001).

1.9.1 Chondrogenesis

The sequential events (*labelled A-D*) involved during chondrogenesis are discussed below, and are represented by a chondrogenic timeline (**Figure 1.20**).

(A) Commitment (*Stage 1*)

Mesenchymal cells (also referred to as precursor cells) originating from the mesodermal germ layer of the blastocyst of a developing embryo are encouraged to differentiate and commit themselves to the chondrogenic lineage with the aid of growth factors such as IGF-1. Previously, these precursor mesenchymal cells have been referred to as chondroblasts. This is incorrect as the mesenchyme cells at this stage can differentiate into either chondrocytes or osteoblasts depending on the factors

to which they are subjected. It is thought that both BMP-2 and 4 support the transition of mesenchyme into the chondrogenic lineage, but they may also support proliferation and hypertrophy of chondrocytes (Sandell & Adler., 1999).

(B) Condensations (*Stage 2*)

The committed mesenchyme condense and compact into nodules differentiating to a chondrogenic phenotype. Pre-cartilaginous condensations express SOX 9, a marker for chondrogenesis in less mature chondrocytes.

(C) Proliferation (Stage 3)

The chondrocytes start proliferating rapidly, secreting cartilage specific extracellular matrix molecules. Both FGF-2 (Jingushi *et al.*,2006, Miot *et al.*, 2006) and IGF-1 (Takahashi *et al.*, 2005) have been found to stimulate proliferation. FGF-2 may also inhibit chondrocytes from progressing to the hypertrophic state (Sandell & Adler., 1999, Tchetina *et al.*, 2006). Cells proliferating and beginning to hypertrophy release a protein called Indian hedgehog (Ihh). The released Ihh protein binds to a protein within the perichondrium called patch, initiating the hedgehog signalling pathway resulting in increased Parathyroid Hormone Related Protein (PTHrp) secretion from perichondrial cells within close proximity to undifferentiated mesenchyme. Prior to the chondrocytes expressing Indian hedgehog protein they express PTHrp receptors. The released PTHrp binds to the receptors within the mesenchyme setting up a negative feedback loop (**Figure 1.20**) blocking the differentiation of remaining cells and increasing proliferation.

(D) Hypertrophy and Calcification (*Stage 4*)

Chondrocytes stop dividing and increase in volume, becoming hypertrophic. Hypertrophic chondrocytes alter the matrix in which they synthesize, secreting both type X collagen and fibronectin to surrounding extracellular matrix. They also start to produce an alkaline phosphatase enzyme essential for mineralization. BMP-6 is present in both pre-hypertrophic and hypertrophic cells enabling mineralization by calcium carbonate (Sandell & Adler., 1999). The matrix becomes calcified which is

less hydrated than hyaline cartilage creating a restriction of nutrient diffusion and waste elimination.

(E) Endochondral Ossification (*Stage 5*)

The hypertrophic chondrocytes die via apoptosis leaving cavities that are invaded by blood vessels and bone forming cells. Eventually cartilage is replaced by bone in a process termed endochondral ossification.

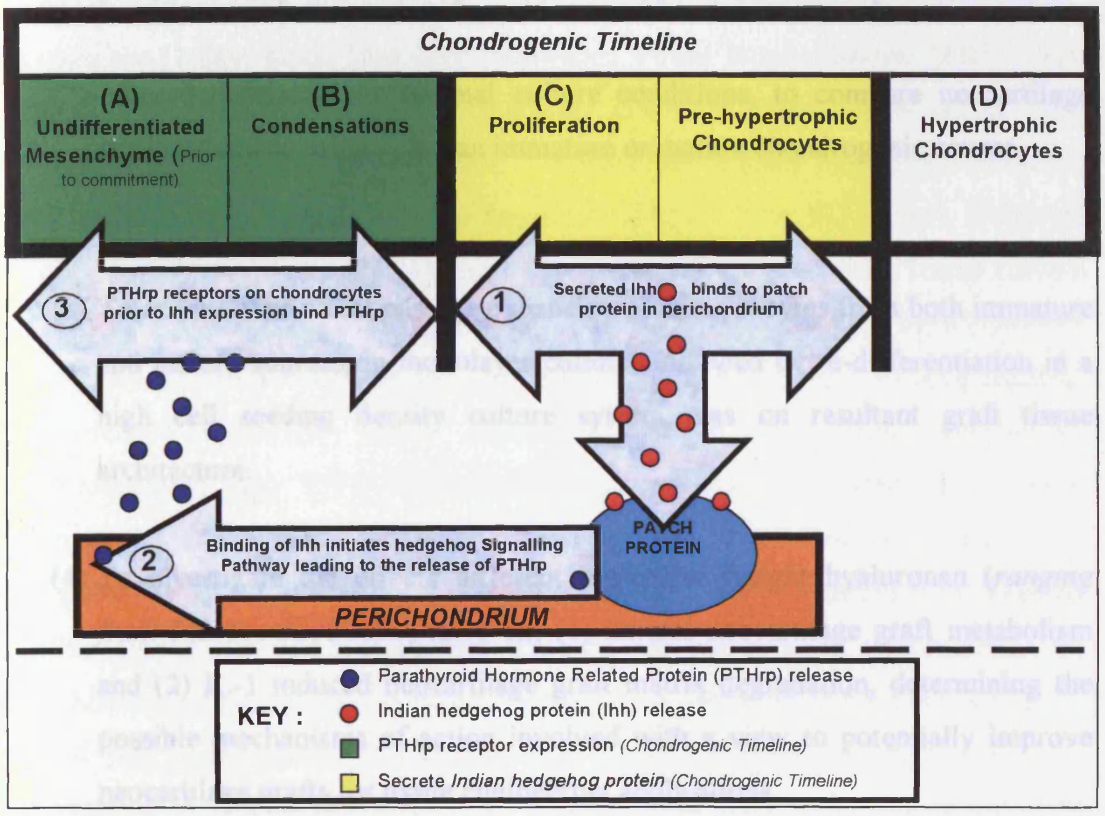

Figure 1.20. Embryological development of cartilage. The sequence of events are labelled A-D and discussed above. Arrows indicate a negative feedback control mechanism involved in the regulation of cartilage development (Adapted from Sandell & Adler, 1999).

1.10 Aims of the project

The objective was to evaluate the organisation of scaffold-free neocartilage grafts produced utilising an *in vitro* Transwell culture system that was originally described by Kandel *et al* in 1995 and use this system to investigate the effects of different Mwt HA on chondrocyte metabolism. The aims to achieve this objective were;

(1) To investigate the effects an increased cell density and culture duration have on neocartilage graft production and determine the optimal culture conditions (*i.e. cell density and culture period*) to be applied in future experimentation.

(2) After determining the optimal culture conditions, to compare neocartilage grafts produced using either an immature or mature chondrogenic source.

(3) To examine the effect passage expansion of chondrocytes from both immature and mature sources, in monolayer culture, followed by re-differentiation in a high cell seeding density culture system, has on resultant graft tissue architecture.

(4) To investigate the effects different molecular weight hyaluronan (*ranging from 5kDa – 3000^{+}kDa*) have on; (1) normal neocartilage graft metabolism and (2) IL-1 induced neocartilage graft matrix degradation, determining the possible mechanisms of action involved with a view to potentially improve neocartilage grafts for tissue engineering applications.

2.0 GENERAL METHODS AND MATERIALS

2.1 Materials

All chemicals and materials utilized in this project were obtained from Sigma-Aldrich Chemical Corporation, UK unless otherwise stated. Pronase (7u/ml) from Streptomyces griseus was obtained from Roche Diagnostics, Collagenase Type II (100u/ml) prepared from Clostridium Histolyticum was obtained from Lorne labs, Worthington Biomedical Corporation,UK. Dulbecco's Modified Eagles Medium (DMEM) without Sodium Pyruvate with 4500mg/ml Glucose with pyridoxine, Gentamycin (1000x stock) and heat inactivated Foetal Bovine Serum (FBS) were obtained from Invitrogen, UK. Biopore filter inserts (12 mm, 0.4μm pores) were obtained from Millipore, Bedford, MA. Recombinant human $TGF\beta_2$ was obtained from Peprotech, UK. 40μm Cell strainers were obtained from BD Falcon, Belgium. Phosphate buffered Saline (PBS) tablets were obtained from Oxoid. Tissue culture plates were obtained from Corning. Histobond microscope slides for both histology and immunolabelling were obtained from RA Lamb. For Immunolabelling analysis Cryo-protect/Cryo-M-Bed tissue mountant was obtained from Bright/UK. DakoCytomation pen, FITC secondary antibody, Goat and swine serum were obtained from Dakopatts Ltd, UK. Keratanase was obtained from AMS Biotechnology, UK. Propidium Iodide (0.5μg/ml) Molecular probe was obtained from Invitrogen, UK and Vectorsheild was obtained from Vector Laboratories Ltd, UK. Mayers haematoxylin and Eosin (Aqueous 1%) were obtained from RA lamb. The 96 well multiplates were obtained from Elkay Laboratory products UK, Ltd, Basingstoke,UK. Powdered DMMB was obtained from Serva. Formic acid was from BDH. Lactate assay kit was obtained from Trinity Biotech. The Combiplate 8 Eliza plates were obtained from Thermoelectron Corporation. Ethylenediaminetetraacetic Acid Disodium Salt Dehydrate, Acetic Acid, Xylene, Ethanol, sodium hydroxide, hydrochloric acid, perchloric acid, dialysis tubing and propan-2-ol were all obtained from fisher.

2.2 Preparation of scaffold free neocartilage graft production utilising an *in vitro* Transwell culture system.

2.2.1 Isolation of articular chondrocytes

Cartilage grafts were tissue engineered using a modification of the method described by Kandel *et al.*, 1995. Articular cartilage shavings were harvested from either 7 day (immature) or 18 month (mature) bovine metacarpophalangeal joints. Full depth cartilage was harvested and chondrocytes isolated using well-established asceptic techniques. Full depth cartilage was digested in 7 units/ml pronase in Dulbecco's modified Eagle medium (DMEM) containing 50μg/ml gentamycin and 5% (v/v) heat inactivated FBS for 3 hours at 37°C. The cartilage shavings were washed with an arbitrary volume of DMEM containing 50μg/ml gentamycin, then further digested in 100 units/ml collagenase type II in DMEM containing 50μg/ml gentamycin and 5% (v/v) heat inactivated FBS overnight at 37°C.

The overnight digest was filtered through a cell strainer and the cell suspension was centrifuged at 470g for 5 minutes. The supernatant was removed and the chondrocyte pellet was resuspended in DMEM containing 50μg/ml gentamycin and 10% heat inactivated FBS. The cell suspension was again centrifuged at 470g for 5 minutes. The supernatant was removed and the chondrocyte pellet was resuspended in 10ml DMEM containing 50μg/ml gentamycin and 10% heat inactivated FBS.

2.2.2 Cell Count

Isolated cells were stained with 0.4% (v/v) trypan blue to determine percentage cell viability.

- **Cell viability** (%) = Total viable cells (unstained) / Total cells (stained and unstained) **x** 100

The cells were counted using a Sigma bright-line haemocytometer and a bright field microscope. If the cell yield was high, then cell suspension was diluted with PBS prior to the cell count. This dilution factor was then taken into account when calculating the final cell numbers. The subsequent cell concentration per ml (and the total number of cells), were determined using the following calculations:

- **Cells per ml** = The **average** count of cells per square on haemocytometer (5 squares counted in total, centre square and 4 corner squares on haemocytometer grid) **x** dilution factor **x** 10^4.

- **Total cells** = Cells per ml **x** original volume of fluid from which the sample was removed.

In all the following experiments, cells were seeded at $4x10^6$cells/400µl unless otherwise stated. Therefore, the volume of media in which the cells should be resuspended to make a required concentration of cells was calculated. After this the 10ml cell suspensions were centrifuged and made to the appropriate volumes with DMEM containing 50µg/ml gentamycin and 10% heat inactivated FBS.

2.2.3 Seeding chondrocytes onto Millipore filter inserts and culture maintenance using a Transwell culture system

In the following experiments, unless otherwise stated, cells were seeded at $4x10^6$ cells/400µl onto 0.6cm^2 Millicell^{CM} filter inserts (0.4µm pores) pre-coated with 40µl of 0.5mg/ml chick type II collagen, and each filter insert positioned into a well of a 24 well tissue culture plate (**Figure 2.1**).

Figure 2.1.
Schematic depicting the positioning of a millipore filter insert into a 24 well culture plate.

A schematic of the compartmental organisation of a Transwell culture system is shown in **Figure 2.2**. Outside of the filter insert, within the well of the culture plate, 600µl DMEM containing 50µg/ml gentamycin and 20% heat inactivated FBS was pipetted. The seeded chondrocytes were left to settle for 48 hours at 37°C in a humidified atmosphere containing 5% CO_2.

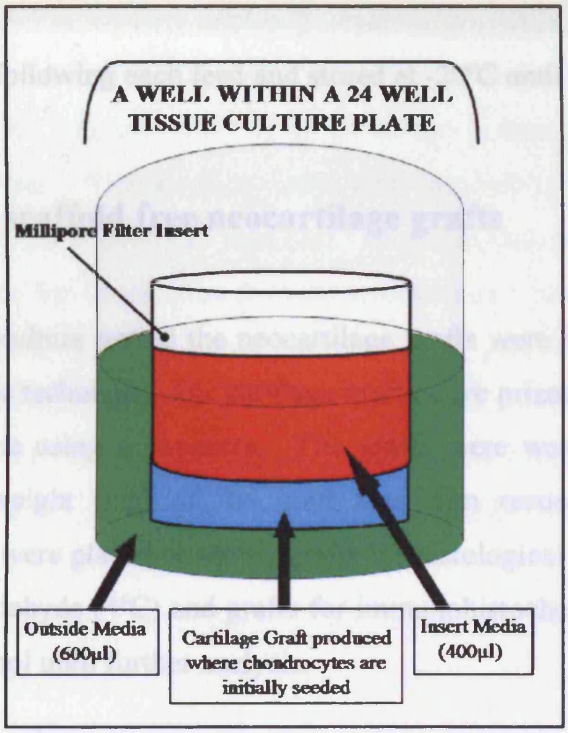

Figure 2.2. A Transwell culture system used to generate neocartilage grafts

Culture Maintenance of the Transwell culture system

- The first feed consisted of removing both the insert and outside media from the culture system following a 48 hour incubation period. DMEM containing 50µg/ml gentamycin and 20% heat inactivated FBS supplemented with 100µg/ml ascorbate and 5ng/ml TGFβ_2 was applied to both the filter insert (400µl) and outside of the well (600µl). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

- After the first feed cultures were maintained by a media change thrice weekly of DMEM containing 50μg/ml gentamycin and 20% heat inactivated FBS supplemented with 100μg/ml ascorbate and 5ng/ml $TGFB_2$. 600μl was pipetted to the outside well and 400μl into the inner insert. The cultures were maintained over a 4 weeks culture period unless otherwise stated at 37°C in a humidified atmosphere containing 5% CO_2.

Media was collected following each feed and stored at -20°C until further analysis.

2.2.4 Harvesting scaffold free neocartilage grafts

Following a 4 week culture period the neocartilage grafts were harvested. This did not require an asceptic technique. The cartilage grafts were prized from the Millipore filter insert membrane using a tweezers. The grafts were washed with PBS and blotted dry. The weight (mg) of the graft was then recorded and grafts for biochemical analysis were placed at -20°C, grafts for histological analysis were fixed using 4% paraformaldehyde (4°C) and grafts for immunohistochemical analysis were fixed using 95% ethanol until further analysis.

2.3 Immunofluorescent labelling and histological analyses of scaffold free neocartilage grafts

2.3.1 Immunofluorescent labelling

Circular 6mm tissue graft samples were harvested and stored at 4°C in a 95% ethanol solution. Prior to section cutting, individual tissue samples were put on rotation at 4°C in a PBS and 5% (w/v) sucrose solution (~5hours) to cryo-protect the grafts, then samples were snap frozen onto cryostat chucks in Cryo-M-Bed tissue mountant. Cryosections (12μm thickness) were cut using a Bright 5030 cryostat (Bright, UK). The

sections were mounted onto HistoBond microscope slides and stored at -80°C until required. An area around each individual section was circumscribed with a water repellent ring using a DakoCytomation pen. The sections were then wet in 0.05M phosphate buffered saline (PBS) (pH 7.4) containing 0.1% (v/v) Tween20 (Polyoxyethylene-Sorbitan Monolaurate) for 10 minutes. The sections were transferred to a humidified labelling box and pre-treated with appropriate enzymes to expose the neoepitope of interest or to improve antibody penetration (**Table 2.1**). This involved a pre-treatment with a cocktail of 0.4U/ml chondroitinase ABC and 0.4U/ml keratanase in a 100mM Tris Acetate solution (pH7.4) at 37°C for 1 hour to enzymatically de-glycosylate the sections. The sections were then washed in 0.05M PBS (pH7.4) containing 0.1% (v/v) Tween20 (3x5 minutes). Blocking serum at 1:20 dilution was applied to the sections for 30 minutes at room temperature. Sections that were to be stained with monoclonal antibodies were blocked using goat serum and those to be stained with polyclonal antibodies were blocked with swine serum. The blocking serum was removed and primary antibodies applied at appropriate concentrations (**Table 2.1**) overnight at 4°C. As a negative control, the primary antibody was omitted and replaced with an appropriate (Mouse/Rabbit) 'naïve' immunoglobin (10μg/ml) with or without the enzymatic digestion step. Non-specific binding of the secondary antibody was checked by omitting the primary antibody and replacing it with 0.05M PBS (pH7.4) and 0.1% (v/v) Tween20.

Following an overnight incubation, the sections were washed in 0.05M PBS (pH7.4) containing 0.1% (v/v) Tween20 (3x5 minutes). An appropriate FITC-conjugated secondary antibody (**Table 2.1**) recognising either mouse or rabbit species was applied at a 1:50 dilution for 1 hour at room temperature to individual sections. The sections were then washed in 0.05M PBS (pH7.4) containing 0.1% (v/v) Tween20 (3x5 minutes). Propidium Iodide (0.5μg/ml) was applied to sections for 5 minutes for nuclei staining, and then the sections were again washed in 0.05M PBS (pH7.4) containing 0.1% (v/v) Tween20 (3x5 minutes). Sections were mounted under a coverslip (22x50mm) using vectashield fade retarding mountant, the coverslips were held into place using nail varnish. Sections were viewed and photographed using an Olympus fluorescence microscope.

Specificity	Antibody (clone ;dilution)	Pre-treatment	Blocking serum	Secondary antibody	Source/Reference
Type I collagen	Anti-type I (poly; 1:100)	ABC/K	Swine	Swine anti-rabbit FITC	Fitzgerald Industries Int
Type II Collagen	CIICI (mono;1:5)	ABC/K	Goat	Goat Anti-mouse FITC	DSHB/ Holmdahl et al (1986)
Aggrecan IGD	6B4 (mono 1:20)	ABC/K	Goat	Goat Anti-mouse FITC	Caterson et al (2000)
Versican (HA binding region)	12C5 (mono 1:5)	ABC/K	Goat	Goat Anti-mouse FITC	DSHB/Asher et al (1991)
Unsulfated chondroitin	1B5 (mono 1:500)	ABC/K	Goat	Goat Anti-mouse FITC	Couchman et al (1984) Caterson et al (1985)
Chondroitin-4-sulfate	2B6 (mono 1:20)	ABC/K	Goat	Goat Anti-mouse FITC	Couchman et al (1994) Caterson et al (1985)
Chondroitin-6-sulfate	3B3 (mono 1:20)	ABC/K	Goat	Goat Anti-mouse FITC	Caterson et al (1985)
Decorin (core protein)	28.4 (mono 1:20)	ABC/K	Goat	Goat Anti-mouse FITC	Bidanset et al (1992)
Biglycan (core protein)	PR8A4 (mono 1:20)	ABC/K	Goat	Goat Anti-mouse FITC	Roughley et al (1993)
Keratan sulfate	5D4 (mono 1:20)	None	Goat	Goat Anti-mouse FITC	Caterson et al (1983)
Superficial zone protein (SZP)	3A4 (mono)	None	Goat	Goat Anti-mouse FITC	Schumacher *et al.*, 1999 Flannery *et al.*, 1999

Table 2.1. The required conditions for specific antibodies utilised in the immunolabeling of chief extracellular matrix molecules.

2.3.2 Histological Analysis

For histology, circular (6mm diameter) tissue samples were fixed in cold 4% paraformaldehyde, and processed for paraffin wax using standard methods. Wax sections of the cartilage grafts (8μm in thickness) were cut using a microtome (LKB rotary microtome). Sections were dewaxed and dehydrated by putting them in descending alcohol concentrations as follows: xylene (2 min), xylene (2 min), 100% alcohol (1 min), 100% alcohol (1 min), 95% alcohol (1 min), 70% alcohol (1 min). The sections were then washed in running water (1 min) followed by staining for proteoglycan in 1% (w/v) alcian blue 8GX stain pH 2.5 (20 min). The sections were washed in running water (1min) for the second time followed by a staining with Mayer's haematoxylin stain (1 min). The sections were then washed and blued by applying running tap water for the third time (3 min). A 1% (v/v) eosin stain was applied to the sections (2 min) followed by a wash in running tap water (20 sec). The sections were then re-hydrated and placed in an ascending alcohol concentration as follows: 70% alcohol (20 sec), 95% alcohol (45 sec), 100% alcohol (1 min), 100% alcohol (2 min), xylene (2 min), xylene (2 min). The sections were mounted under a coverslip using DPX mountant and allowed to dry. Tissue organisation was evaluated using a brightfield microscope. Neocartilage graft thickness was measured using a microscope equipped with digital image acquisition. Measurements of graft thickness were taken at 10 arbitrary points across the diameter of tissue for 3 sections (*i.e. 30 measurements per graft were taken*).

2.4 Biochemical analysis of scaffold free neocartilage grafts

2.4.1 Proteoglycan extraction

Matrix molecules, including proteoglycans, present in neocartilage graft tissues were extracted by the addition of 1ml guanidine extraction buffer (*4M Guanidine HCl (a chaotrophic agent), 50mM sodium acetate, 0.01M ethylenediaminetetraacetic acid disodium salt dehydrate, 0.1M amino hexanoic (caproic) acid (pH 5.8-6.8). Fresh*

inhibitors were added prior to digest: 0.005M benzamidine HCl and 0.5mM phenyl methyl sulfonyl fluoride (PMSF)) to each graft for 48hours at 4°C with constant agitation. The 4M guanidine acts to dissociate PG aggregates by breaking any non covalent bonds and removing them from their native environment into solution (Sajdera & Hascall., 1969, Heinegard & Sommarin., 1987). The mixture was centrifuged and supernatant removed and dialysed three times against MilliQ™ water. The sulphated glycosaminoglycan content that was released by guanidine extraction was analysed using Dimethylmethylene Blue Assay (DMMB) (**2.5.1**) and the collagen concentration was analysed via a hydroxyproline assay (**2.5.3**). The residual insoluble grafts were further analysed.

2.4.2 Pepsin digest

Following a guanidine extraction, the residual grafts were further digested using a pepsin digest as to release soluble type II collagen from cartilage. Pepsin is an endopeptidase that hydrolyses peptide bonds. To each sample, 0.5ml of 0.5M acetic acid containing 1mg/ml pepsin was added for 24hours at 4°C with constant agitation. The sulphated glycosaminoglycan content that was released by the pepsin digest was analysed using Dimethylmethylene Blue Assay (DMMB) (**2.5.1**) and the collagen concentration was analysed via a hydroxyproline assay (**2.5.3**). The residual grafts were again further analysed.

2.4.3 Papain digest

The residual grafts still remaining were completely digested via a papain digest. Papain is an extremely stable cysteine protease that works at elevated temperatures. It digests most protein substrates more extensively in comparison to other proteases. The addition of 1ml papain buffer (*0.05M sodium-acetate, 0.025M EDTA pH 5.6, 0.005M cysteine and 2µl/ml papain*) was added to each graft for 18hours at 65°C with occasional agitation. The sulphated glycosaminoglycan content that was released by papain extraction was analysed using Dimethylmethylene Blue Assay (DMMB)

(**2.5.1**) and the remaining collagen concentration was analysed via a hydroxyproline assay (**2.5.3**).

2.5 Assays applied for both media and graft analysis

2.5.1 Analysisng sulphated GAG concentrations using Dimethylmethylene Blue Assay (DMMB)

The proteoglycan content present in grafts and media was measured as sulphated GAG by Dimethylmethylene Blue (DMMB) assay. It works by binding to the negatively charged sulphate groups on the GAG chains forming a complex which produces a metachromatic shift in the absorbance maxima (Farndale *et al.*, 1986). Chondroitin sulphate C from shark cartilage was used as a standard ranging from 0-40μg/ml, and appropriately diluted samples were added at 40μl to a 96 well multiplate. 200μl of DMMB solution (*32mg 1,9 DMMB, 20ml ethanol, 59ml 1M sodium hydroxide, 7ml 98% formic acid and made up to 2L with water*) was added to samples and absorbance was read at 525nm on a Labsystem Multiscan MS Spectrophotometer. All standards and samples were measured in triplicate with dilution factor taken into account. When calculating percentage proteoglycan present in grafts, GAG results were: (1) converted from μg to mg, (2) multilplied by $^{10}/_{9}$ (as GAG constitutes 90% of proteoglycan present), (3) divided by the actual graft weight (w/w), and (4) multiplied by 100.

2.5.2 Media analysis for lactate concentrations

The lactate assay was carried out using the Trinity biotech assay kit (Procedure No 735). Lactate is a by product of the glycolytic pathway and is utilised as a measure to determine the anaerobic metabolic activity that occurs in a culture system. Lactic acid is converted to pyruvate and hydrogen peroxide by lactate oxidase. In the presence of the hydrogen peroxide formed, peroxidase catayses the oxidative condensation of chromogen precursors to produce a coloured dye with an absorption maximum at

540nm. The increase in absorbance at 540nm is directly proportional to lactate concentration. In a 96 multiwell plate 5µl of lactate standards of 0µg/ml, 25µg/ml, 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml and 400µg/ml were measured in duplicate. 5µl of culture medium at appropriate dilutions were measured in triplicate. To each well 250µl of lactate reagent (*lactate oxidase (microbial), peroxidase (horseradish), chromogen precursors and buffer pH 7.2*) was pipetted and then the multiwell plate was incubated at room temperature for 10 minutes. The absorbance at 540nm was read using the Labsystem Multiscan MS Spectrophotometer.

2.5.3 Hydroxyproline Assay

Hydroxyproline is unique to collagen. As a result it is used in the determination to quantify amounts (but not types) of collagenous protein present in tissue or a culture medium (Woessner.1976, Reddy & Enwemeka., 1996). The hydroxproline content generated by the Transwell culture system was analysed by amounts of collagen retained within grafts (following a Guanidine extraction, Pepsin digest and Papain digest) and within the media (both insert and outside). Firstly, samples are hydrolysed in 6N HCl for 24 hours at 110°C. The samples must be hydrolysed to generate free hydroxyproline not peptide bound for analysis. Hydroxyproline is fairly stable in 6N HCl although hydrolysis does initiate the epimerizaton of hydroxyproline, but in this colorimetric assay all epimers are determined together. After the hydrolysis process the hydrolysates are freeze dried to remove the acid. The freeze dried samples are then reconstituted with milli Q water in the same volume of media that was originally taken for hydrolysis. The reconstituted sample may contain a lot of particulate material. Therefore, samples are centrifuged at 8000rpm for 10 minutes in a microfuge to sediment insoluble material as it may interfere with the assay. In a combiplate 8 ELIZA plate, 30µl of trans–4–Hydroxy–L-proline standards; 0µg/ml, 0.5µg/ml, 1µg/ml, 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml, and 10µg/ml were measured in triplicate and 30µl of sample at appropriate dilutions were also measured in triplicate. To each well of the multiplate 70µl diluent (*100ml propan-2-ol and 50ml water)* and 50µl oxidant (*0.308M Chloramine T, 10ml water and 50ml stock buffer (Stock buffer; 0.42M sodium acetate trihydrate, 0.127M tri sodium citrate*

dehydrate, 0.025M Citric acid and 40% (v/v) Propan-2-ol)) was pipetted and the plate was mixed on a plate shaker for 5 minutes. The oxidant must be prepared fresh prior to use and will keep overnight if stored at 4°C. The addition of the oxidant oxidises the water soluble hydroxyproline to the toluene soluble pyrrole molecule, this chemical conversion is advantageous as small amounts of hydroxproline can be detected in samples containing large amounts of contaminating substances. Colour reagent that must be prepared fresh prior to use *(0.682M ρ-dimethylaminobenzaldehyde (Ehrlich's reagent) 11.25ml (60% V/V) perchloric acid and 62.5ml propan-2-ol)* was added to the multiplate at 125µl. The plate was then mixed well on a plate shaker and then incubated at 70°C for 20 minutes. The addition of ρ-dimethylaminobenzaldehyde reacts with the pyrrole molecules that were generated by oxidation and forms a complex that is detected and quantified as a chromophore (peach colour) at 550nm. A graph is plotted of absorbance versus concentration of standards to find the function for the straight line. The function is then utilised to calculate the concentration of hydroxyproline present. The content of hydroxylated proline residue was used to estimate collagen content of the cartilage using the conversion factor of 1g hydroyproline per 7g of collagen. When calculating percentage collagen present in grafts, collagen results were: (1) converted from µg to mg, (2) divided by the actual graft weight (w/w), and (3) multiplied by 100.

2.6 Statistical Analysis

Data are presented as mean ± SEM. Data was checked for normality (Shapiro-Wilk) and equal variance using SPSS 13. Where data was normal/equal variance a one-way ANOVA followed by a Tukeys post hoc test was applied. Where data was not normal a Mann-Whitney non-parametric test was applied. Significance was assumed where $P< 0.05$ (*), $P< 0.01$ (**) or $P< 0.001$ (***).

3.0 DETERMINATION OF THE OPTIMAL CULTURE CONDITIONS FOR THE GENERATION OF HIGH QUALITY ARTICULAR CARTILAGE GRAFTS USING *IN VITRO* TRANSWELL CULTURE SYSTEMS.

There is current interest in cartilage repair strategies with a particular focus on cell based, tissue engineering approaches (Kim *et al.*, 2000, Risbud and Sittinger 2002 & Kuo *et al.*, 2006). A technique initially described by Brittberg *et al* in 1994 called autologous chondrocyte implantation (ACI) is the most common cell based therapy permitted for clinical use to date. This procedure involves the injection of a truly committed autologous chondrocyte suspension into a cartilage defect under a sutured membrane covering (*i.e. periosteal patch or biodegradable membrane*).

There are several problems associated with ACI, these include: (1) loss of the sutured membrane covering during the recovery period; (2) unsatisfactory integration between native and repair tissue (Hunziker 1999 & 2002); (3) limited cartilage resurfacing; and (4) the reparative tissue generated, consists of mixed morphologies ranging between hyaline-like and fibro-like cartilage (Roberts *et al.*, 2003, Henderson *et al.*, 2007, Moriya *et al.*, 2007).

A potentially improved approach would be to engineer neo-cartilage grafts *ex vivo* using a Transwell culture system, and implant the developed graft within a defect site (Kandel *et al.*, 1995). This technique would prove advantageous over traditional cell based therapies as (1) There would be no requirement of a periosteal/collagen covering or a scaffold carrier as the graft would exist as a fully differentiated matrix; (2) The initial generation of the cartilage grafts would be highly regulated in culture, as most non-gaseous graft metabolites produced throughout the culture duration can be monitored, which is impossible to achieve in a diseased or damaged joint; and (3) potentially a larger degenerate area of the joint could be repaired and resurfaced, arthroscopically reducing a patients rehabilitation time.

In the present study, we (i) describe the effects increased cell density and culture period have on the organisation of scaffold-free neocartilage graft production utilising an *in vitro* Transwell culture system at light and electron-microscopic levels (ii) determine the optimal culture conditions (*i.e. cell seeding density and culture duration)* required for future analyses (iii) compared the quality of grafts generated

from both immature and mature bovine chondrogenic sources using optimal culture conditions at both histological and biochemical levels.

3.1 Methods

3.1.1 Effects of cell density and culture duration on neocartilage graft organisation and the determination of optimal culture conditions

Neocartilage grafts were produced, maintained and harvested following methodology described in section 2.2. A slight variation was, that chondrocytes were seeded at a range of cell densities (2, 4, 6, 8, 10 and 12 x 10^6 cells/insert) and cultured over varying periods of: 2, 4, 6 and 8 weeks.

Analyses: Graft histology was analysed as described in section 2.3.2. For ultrastructural investigation analysis was performed by Dr Anthony Hayes; grafts were processed for both scanning and transmission electron microscopy to facilitate an appraisal of both topography and internal structure, respectively. For transmission electron microscopy, small samples of graft tissue (~1mm^3) were fixed in 2% glutaraldehyde in 0.05M cacodylate buffer, pH 7.4 containing 0.7% ruthenium hexamine trichloride (RHT; Sigma-Aldrich, UK) for 2 hours at room temperature. After washing (3 x 5 mins) in buffer, samples were post-fixed in 1% OsO_4 in 0.1M cacodylate, pH 7.4 containing 0.7% RHT for 2 hours at room temperature. Samples were washed briefly in buffer, dehydrated in an ascending ethanol series and infiltrated and embedded in Spurr's resin (Agar Scientific, UK), which was allowed to polymerize overnight at 70°C. Ultra-thin sections were cut through the tissue depth on a Reichert ultra-microtome (Leica, Germany). These were stained with uranyl acetate and lead citrate before examination in a Phillips EM 208 transmission electron microscope (Phillips Electron Optics, Holland) operating at 80kV accelerating voltage. For scanning electron microscopy, RHT was omitted from the fixatives to prevent proteoglycan retention within the tissue. After the primary fixation step, samples were washed in buffer and macerated in 10% NaOH with constant agitation for 1 week at 4°C to remove the soluble proteoglycan component, and allow a better appraisal of the collagenous network. The tissue was then rinsed in buffer, passed

through an ascending ethanol series, before drying in a Tousimis Samdri 780 critical point drier (Tousmis Research Corporation, USA), and sputter coating in gold on a EMScope sputter coater (EMScope, UK). Observation of the coated specimens was in a Phillips XL-20 scanning electron microscope (Phillips Electron Optics, Holland) operating at 40kV accelerating voltage. Collagen fibre organisation was assessed by staining in 0.1% (w/v) Sirius red F3B in saturated aqueous picric acid. Samples were then visualised using bright field Normarski differential interference contrast (DIC) and polarizing optics.

3.1.2 Comparison of cartilage grafts produced using either an immature or mature chondrogenic source.

Both immature and mature chondrogenic grafts (n=3) were generated as described in section 2.2. Grafts were both histologically and biochemically analysed as described in sections 2.3 and 2.4, respectively. The assays applied for both sulphated glycosaminoglycan and collagen analysis are described in section 2.5. Statistics were applied as described in section 2.6.

3.2 Results

3.2.1 Effects of cell density and culture duration

An immature primary chondrogenic source (7 day bovine), isolated from full depth native articular cartilage produced grafts of an opaque, hyaline appearance at all cell densities and time points studied (**Figure 3.1**). The generation of neo-cartilage tissue is constrained by the walls of the culture vessel, and growth would occur predominantly in a vertical direction with a resultant increase in tissue thickness. A combination of potential factors including cell proliferation, hypertrophy and progressive accumulation of extracellular matrix material may be responsible for increased tissue thickness. Therefore, the effects of both cell density and culture duration on the production of neo-cartilage graft tissue were examined by the effect they have on tissue thickness.

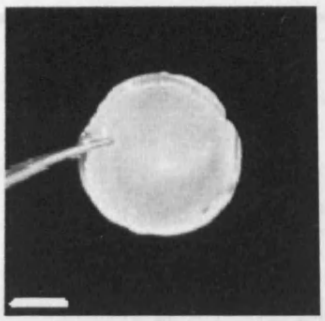

Figure 3.1. Neocartilage graft with an opaque, hyaline appearance. (Seeded at 6 x 10^6 cells and generated over a 4 weeks culture period). Bar = 2mm

Cell seeding density is known to modulate the rate chondrogenesis proceeds *in vitro* by affecting proliferation (Iwasa *et al.*, 2003), differentiation (Watt *et al.*, 1988, Seghatoleslami & Tuan., 2002), apoptosis (Kühn *et al.*,1999), matrix synthesis and accumulation (Iwasa *et al.*, 2003, Williams *et al.*, 2005). During this study it was determined that increasing cell number 2 fold from 2 x 10^6 to 4 x 10^6 cells yielded a highly significant increase ($P<0.001$) in the thickness of graft tissue produced at all time points (**Table 3.1 & Figures 3.2 & 3.3**). Increasing cellularity above 4 million cells per insert showed only subtle increases of variable significance in graft thickness (**Table 3.1 & Figures 3.2 & 3.3**). The effect of seeding 6 million cells/insert and above yielded a tissue characterised by a hypercellular appearance (**Figure 3.3**). Overall, an increased cell seeding density resulted in increased graft tissue cellularity (**Figure 3.3**).

Graft Thickness (μm)	**TIME PERIOD (Weeks)**			
CELL DENSITY	**2**	**4**	**6**	**8**
2 x10^6 cells	124.15 ± 4.08	186.37 ± 4.47	323.18 ± 5.64	353.96 ± 3.97
4 x10^6 cells	196.78 ± 7.41	308.57 ± 5.58	398.53 ± 4.66	427.56 ± 6.29
6 x10^6 cells	185.70 ± 3.54	330.51 ± 4.74	424.42 ± 5.65	472.76 ± 8.07
8 x10^6 cells	206.89 ± 3.22	339.07 ± 7.82	423.11 ± 9.54	450.07 ± 5.76
10 x10^6 cells	203.95 ± 2.84	370.36 ± 6.08	404.59 ± 4.13	434.28 ± 10.26
12 x10^6 cells	221.14 ± 3.83	364.63 ± 5.44	411.84 ± 4.81	484.33 ± 5.46

Table 3.1. Cartilage graft tissue thickness produced over time associated with different cell seeding densities. Data represented as mean ± SEM.

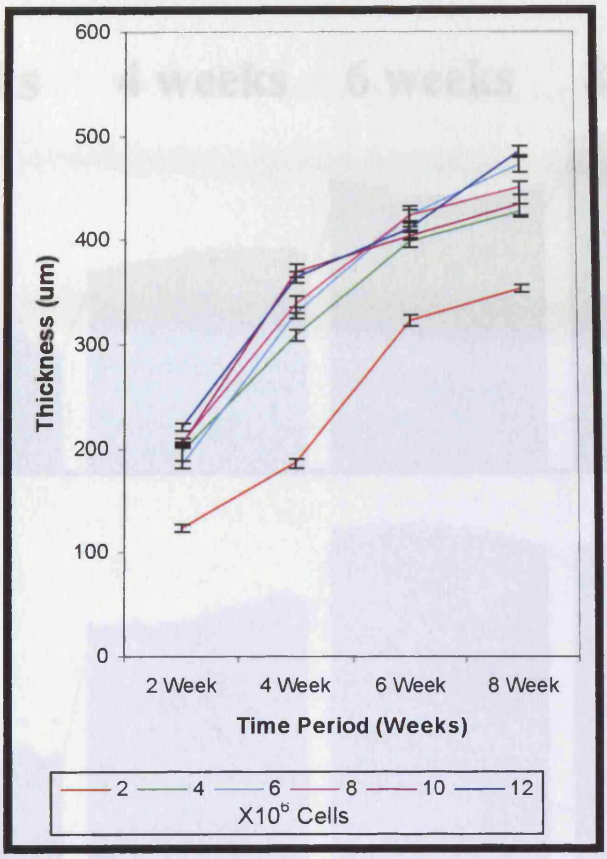

Figure 3.2. Thickness of cartilage grafts with varying cell density and culture period.

At all cell densities, increasing culture duration from 2-8 weeks showed an increase in thickness of engineered grafts (**Table 3.1 and Figures 3.2 & 3.3**). The zonal proportions of the grafts appeared constant, suggesting *in vitro* growth occurred uniformly throughout the tissue during culture (**Figure 3.3**), an increased culture period encouraged chondrocytes within the basal portion of graft tissue to acquire a more columnar organisation (**Figure 3.3**).

It was concluded from this data with respect to native articular cartilage that grafts produced from 6 x 10^6 cells and above were too hypercellular, and grafts produced from 2 x 10^6 cells were too thin. Therefore, the optimum culture conditions considered to be most promising for engineering neocartilage tissue in future experiments was a cell density of 4-6 x 10^6 cells over a 4 week culture period.

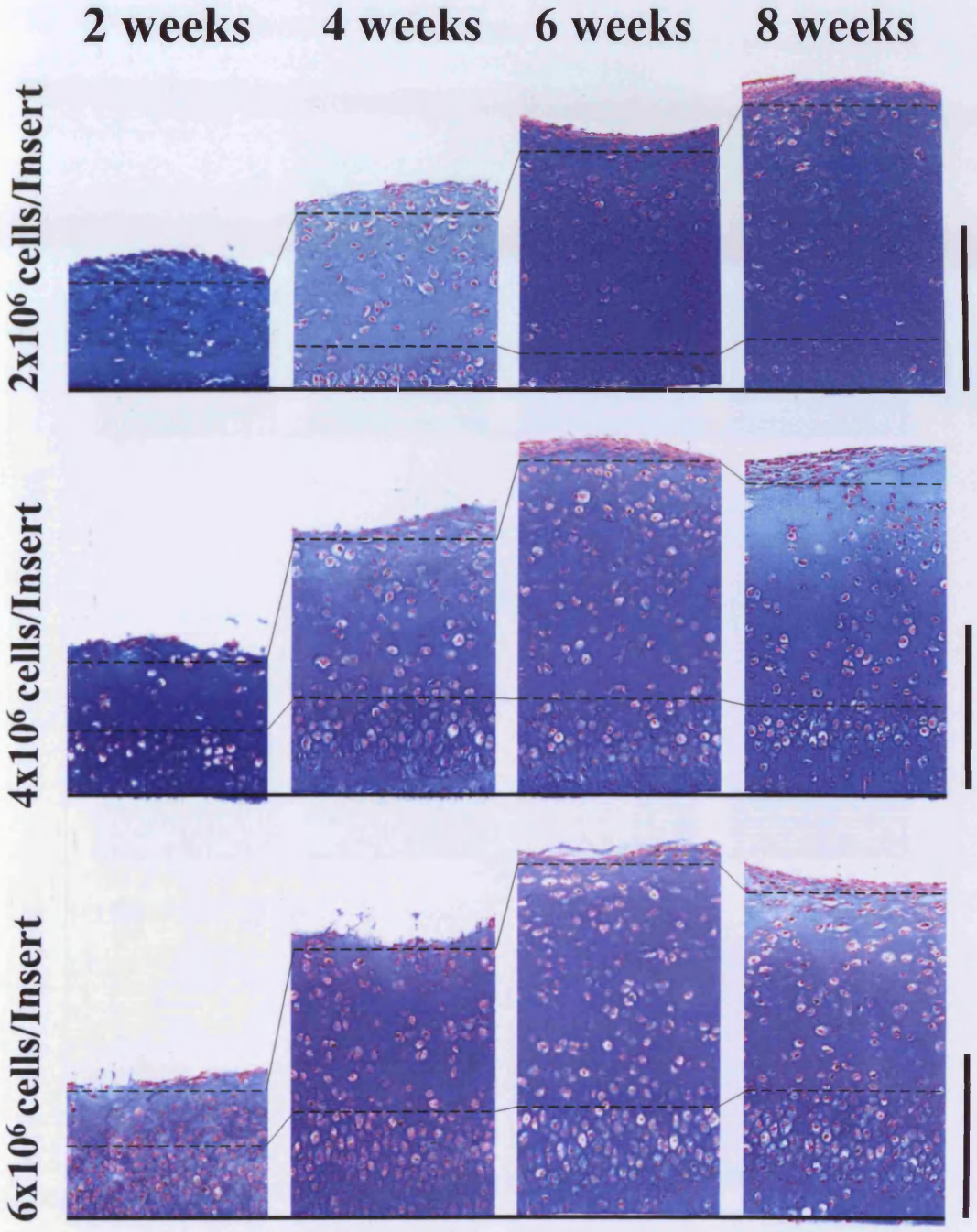

Figure 3.3. The effect of culture time and cell seeding density on neocartilage tissue histology (dashed lines demarcate boundaries between each cartilage zone). Neocartilage tissue generated from isolated immature (7 day) bovine chondrocytes. Alcian blue and haematoxylin and eosin. Scalebar = 200μm.

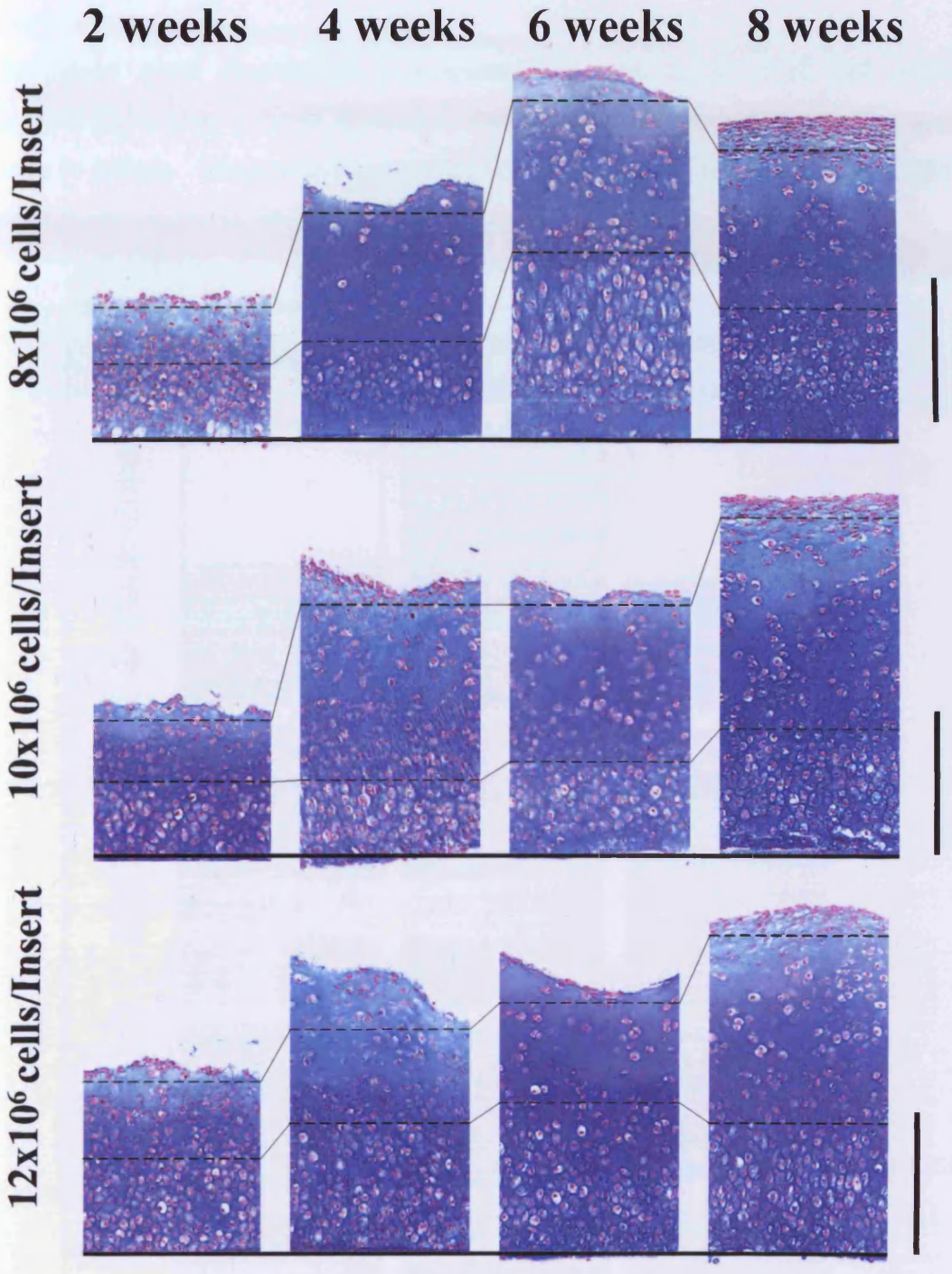

Figure 3.3. The effect of culture time and cell seeding density on neocartilage tissue histology (dashed lines demarcate boundaries between each cartilage zone). Neocartilage tissue generated from isolated immature (7 day) bovine chondrocytes. Alcian blue and haematoxylin and eosin. Scalebar = 200μm.

3.2.2 Zonal Morphology of neocartilage grafts

Histological zonal organisation was apparent in graft tissue at all cell seeding densities and culture periods showing a stratification into distinct zones after just 2 weeks in culture. These zones approximated to the surface, middle and deep regions that characterise native adult articular cartilage *in vivo* (**Figure 3.4**).

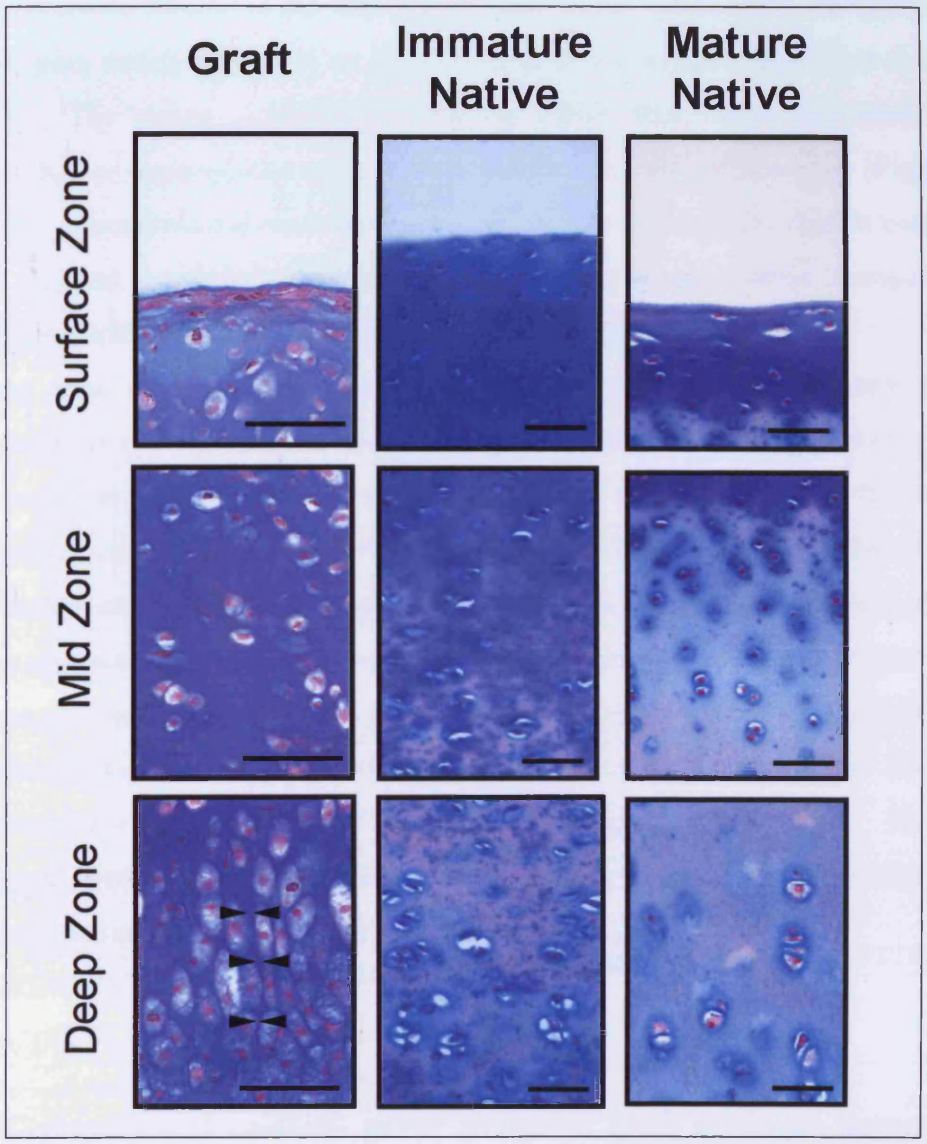

Figure 3.4. Histological sectioning through a neo-cartilaginous tissue made from chondrocytes from an immature chondrogenic source (*arrows in deep zone denote thin septa of alcianophilic matrix that separate cells, organised into a columnar array*) and an immature and mature native articular cartilage explant depicting the zonal organisation of the chondrocytes. Alcian blue and haematoxylin/eosin. Scalebar = 50μm.

The surface zone of neocartilage grafts showed weak alcian blue staining, an indication of low proteoglycan content (**Figure 3.4 & 3.5 (*A*)**). This superficial region was a few cells thick consisting of flattened/discoidal chondrocytes surrounded by a sparse, meshwork of collagen fibrils (**Figure 3.5 (*A, B & E*)**). In this zone, both cells and matrix had prominent horizontal organisation that was clearly apparent under Normarski DIC and polarising optics (*arrows in* **Figure 3.5 (*H & I*)** *denote cell and matrix orientation*).

The chondrocytes located in the mid region were small, spheroidal, pre-hypertrophic cells that were widely spaced in an alcian blue positive matrix (**Figure 3.4 & 3.5 (*A, C & F*)**). The strong alcianophilic staining within this region indicated that it contained higher proteoglycan content in comparison to the surface zone (**Figure 3.4 & 3.5 (*A*)**). Ultrastructural observation showed that its extracellular matrix comprised of well defined pericellular, territorial and interterritorial matrix compartments showing similarities to native articular cartilage (**Figure 3.5 (*C & F*)**).

The deep zone consisted of large, hypertrophic chondrocytes that were densely packed together and appeared to acquire a more columnar organisation with advance in culture period. Some cells in these columnar arrays were separated by thin septa of alcianophilic extracellular matrix (**Figure 3.4 (*denoted by arrows*) & 3.5 (*A, D & H*)**). Interterritorial matrices of these septa were evident at ultrastructural levels showing that they consisted of prominent longitudinal fibrils that were arranged vertically with respect to graft surface (**Figure 3.5 (*D & G*)**). Observation with both Normarski DIC and polarising optics indicated that tissue organisation in the deep zone was perpendicular to that occurring at the graft surface (**Figure 3.5 (*H, I, J*)**). There was no apparent calcified zone underlying the deep region in the neocartilage tissue as is observed in native articular cartilage.

3.2.3 Comparison of cartilage grafts produced using either an immature or mature chondrogenic source.

The optimal culture conditions required for future analysis to generate high quality cartilage grafts utilising an *in vitro* Transwell culture system were determined in section 3.2.1 (*i.e. 4-6 x 10^6 cells over 4 weeks culture period*).

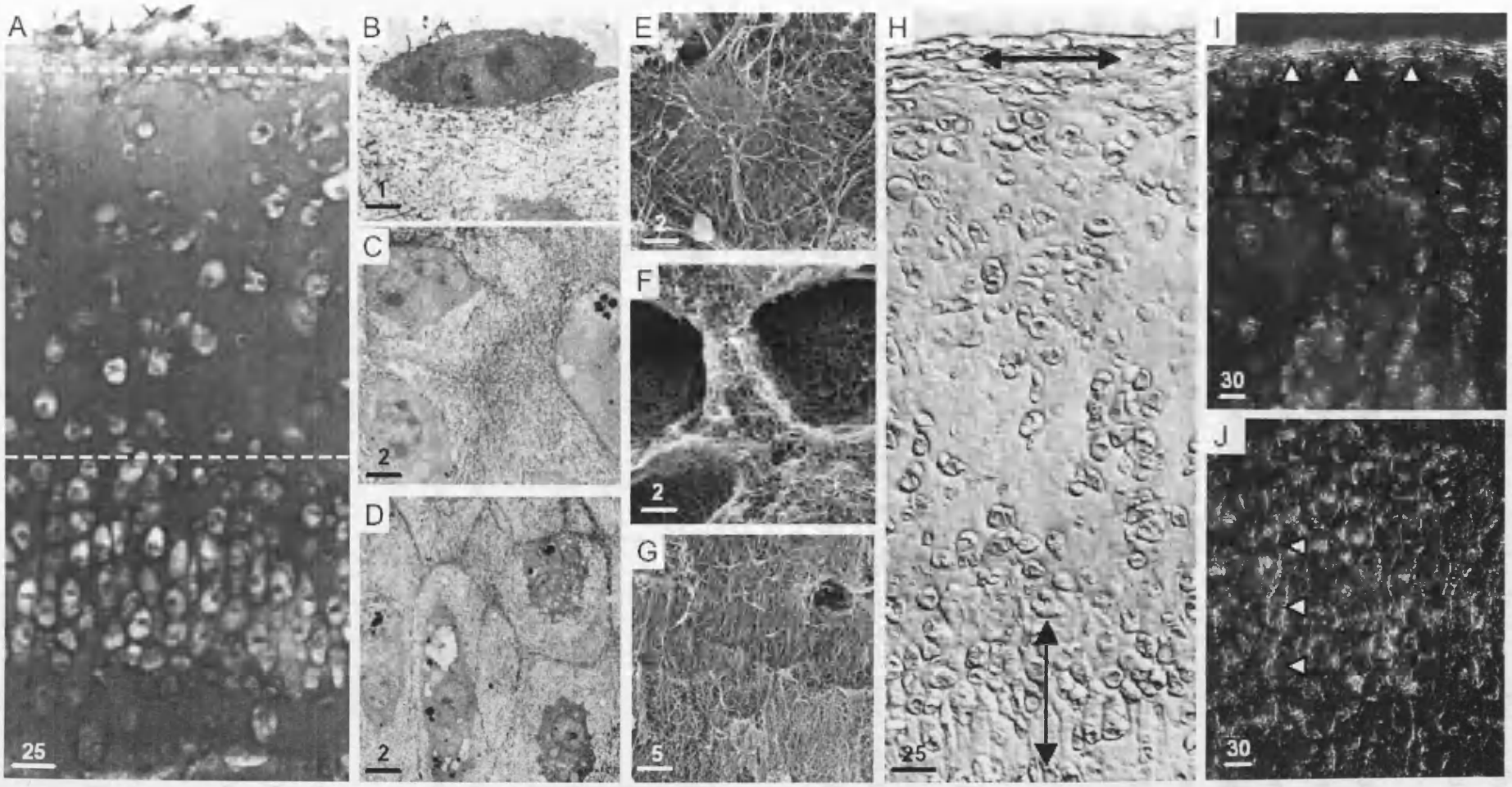

Figure 3.5. Histological and ultrastructural organisation of neocartilage tissue. *A*. Wax histology. The graft is comprised of three histologically distinct zones that approximate to the surface, middle and deep zones of native adult cartilage (dashed lines demarcate boundaries between each cartilage zone). Alcian blue and haematoxylin and eosin. *B-D*. Transmission electron micrographs demonstrating cell morphology and matrix organisation associated with each graft zone (top to bottom). *E-G*. Scanning electron micrographs showing topographic detail of collagenous organisation in graft zones (top to bottom). *H*. Nomarski differential interference contrast image showing distinct tissue organisations associated with each zonal strata (arrows denote predominant orientation of cells and matrix). Picrosirrius red. *I-J*. Polarised light microscope images showing: *I*. horizontal organisation of collagen fibre bundles at the graft surface and, *J*. vertical organisation of collagen associated with the inter-territorial matrix of deep zone chondrocytes. Scalebars represented in μm.

Neocartilaginous grafts were engineered at optimal culture conditions from both an immature and mature primary chondrogenic source, and the effects of age were compared at a histological and biochemical level.

Sulphated glycosaminoglycan (s-GAG) analysis revealed that grafts produced from the immature source generated a significantly higher ($P<0.05$) amount of total s-GAG in comparison to the mature source (**Table 3.2**). The largest proportion of s-GAG produced over the 4 weeks culture period was present in the culture media and not retained in grafts for both immature and mature sources. Interestingly, there is always more sulphated GAG in the inner insert media than outside media. Importantly, of the total s-GAG generated by each source, a significantly higher ($P<0.001$) percentage of s-GAG is released into the media of the Transwell cultures using the mature chondrocytes (92%) in comparison to the immature (63.5%) (**Table 3.2**). The literature reports that the extracellular matrix of native articular cartilage is composed of 10-15% (w/w) proteoglycan. The grafts generated were constituted with 4.33% (w/w) proteoglycan in the immature (~ 4x less than native cartilage) and 1.93% (w/w) proteoglycan in the mature (~8x less than native cartilage) (*Calculation methods described in chapter 2.5.1*).

Age	Media		Graft	Residue		Total GAG (μg)
	Insert	*Outside*	*4M GuHCl*	*Pepsin*	*Papain*	
Immature	765.04±23.01	119.83±5.43	445.50±124.24	14.60±1.71	48.13±8.89	1393.06±145.42
Mature	268.85±9.20	123.06±2.37	2.20±0.35	13.03±0.60	18.61±2.98	425.74±10.94

Table 3.2. Comparison of sulphated glycosaminoglycan (μg) generated by grafts produced from both an immature and mature bovine chondrogenic source seeded at optimal culture conditions (n=3). Data presented as mean ± SEM.

Grafts produced from an immature source versus a mature source are significantly different, and both graft weights (**Figure 3.6**) and thickness (**Figure 3.7**) support the above s-GAG conclusions. The grafts generated from the immature source were significantly heavier and thicker in comparison to those generated by the mature source. This indicates that the tissue formed by immature cells retains much more proteoglycan/aggrecan in its extracellular matrix facilitating water to be imbibed, causing the tissue to swell with a resultant increase in tissue thickness.

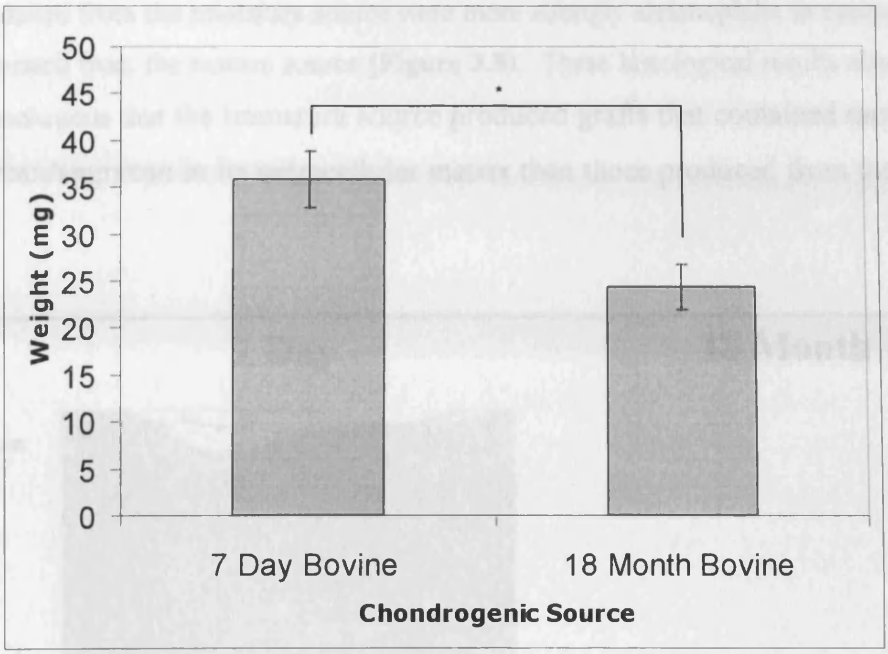

Figure 3.6. Comparison of graft weights generated from both an immature and mature bovine chondrogenic source seeded at optimal culture conditions. Data presented as mean SEM and significance was assumed where $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***) using a paired T-test.

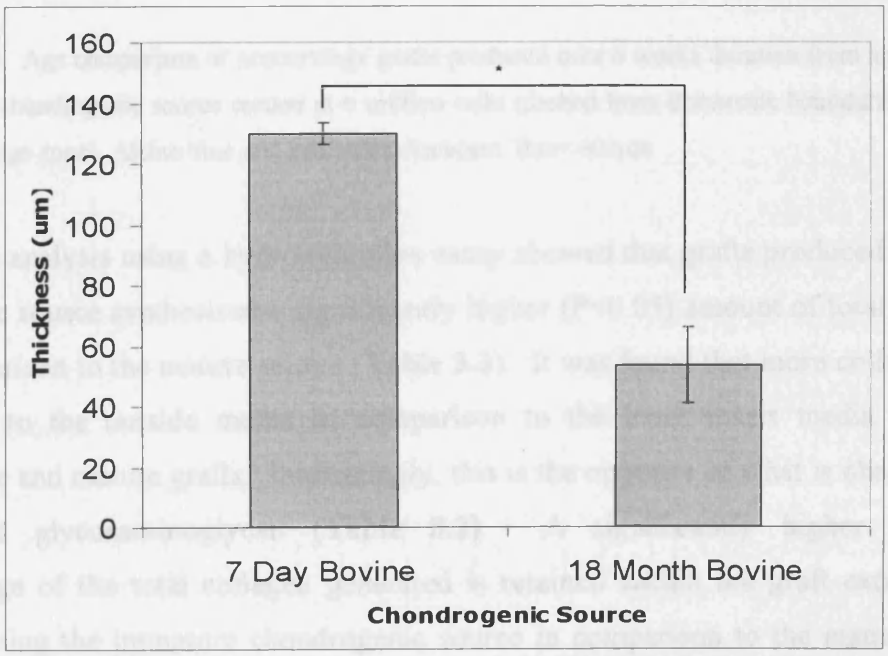

Figure 3.7. Comparison of graft thickness generated from both an immature and mature bovine chondrogenic source seeded at optimal culture conditions. Data presented as mean SEM and significance was assumed where $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***) using a paired T-test.

Grafts produced from the immature source were more strongly alcianophilic in comparison to grafts generated from the mature source (**Figure 3.8**). These histological results also support s-GAG conclusions that the immature source produced grafts that contained much more proteoglycan/aggrecan in its extracellular matrix than those produced from the mature source.

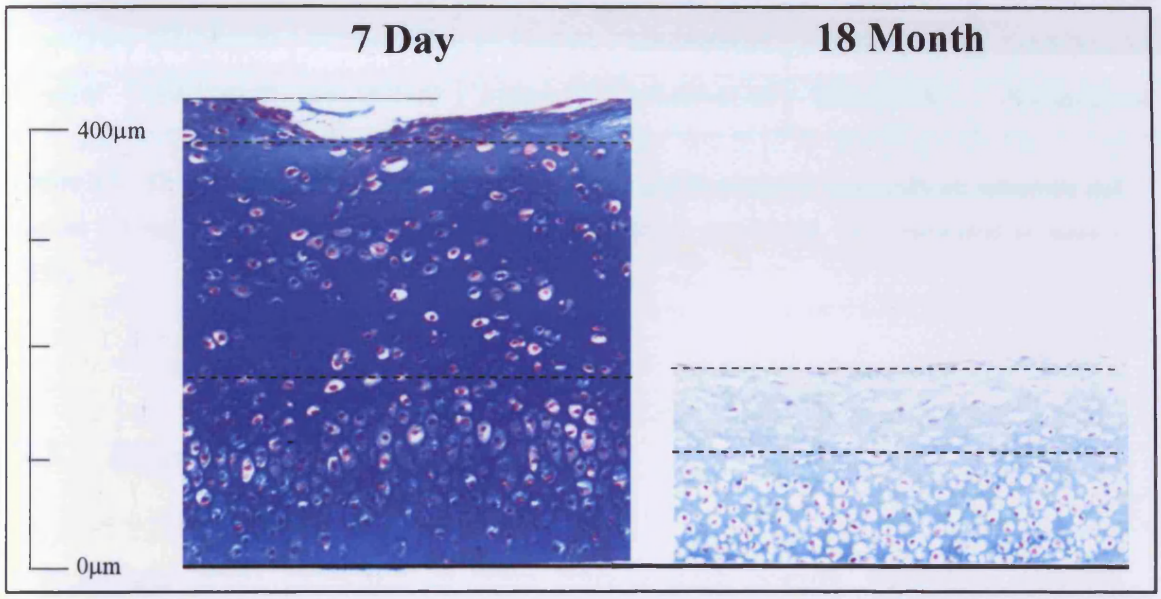

Figure 3.8. Age comparison of neocartilage grafts produced over 6 weeks duration from an immature or mature chondrogenic source seeded at 6 million cells (dashed lines demarcate boundaries between each cartilage zone). Alcian blue and haematoxylin/eosin. Bar= 400µm

Collagen analysis using a hydroxyproline assay showed that grafts produced from the immature source synthesised a significantly higher ($P<0.05$) amount of total collagen in comparison to the mature source (**Table 3.3**). It was found that more collagen was released to the outside media in comparison to the inner insert media for both immature and mature grafts. Interestingly, this is the opposite of what is observed for sulphated glycosaminoglycan (**Table 3.2**). A significantly higher ($P<0.05$) percentage of the total collagen generated is retained within the graft extracellular matrix using the immature chondrogenic source in comparison to the mature, being 68.7% and 35.4%, respectively (**Table 3.3**). The literature reports that the extracellular matrix of native articular cartilage is composed of 20% (w/w) collagen. The grafts generated were constituted with 2.63% (w/w) collagen in the immature (~

7.6x less than native cartilage) and 2.30% (w/w) collagen in the mature (~8.7x less than native cartilage) (*Calculation methods described in chapter 2.5.3*).

Age	Media		Graft	Residue		Total
	Insert	*Outside*	*4M GuHCl*	*Pepsin*	*Papain*	Collagen(μg)
Immature	124.13±2.46	168.84±2.70	14.19±2.62	324.64±56.31	303.08±118.49	934.87±63.53
Mature	122.22±0.98	242.55±3.83	1.00±0.55	169.96±12.45	28.63±14.47	564.36±25.84

Table 3.3. Comparison of collagen (μg) synthesised by grafts produced from both an immature and mature bovine chondrogenic source seeded at optimal culture conditions. Data presented as mean ± SEM.

3.3 Discussion

Neocartilage grafts produced *in vitro* from a high density, immature, primary chondrogenic source using a Transwell culture system, had the ability to reconstitute a highly organised stratified hyaline tissue showing similarities to native adult articular cartilage following just 2 weeks in culture (Kandel *et al.*, 1995) and this morphological stratification became more apparent with time and cell seeding density (Hayes *et al.*, 2007). There are numerous theories that could potentially explain the distinct strata that are observed utilising this Transwell culture system; i.e. (1) the generation of a metabolic/morphological gradient between the graft tissue surface and basal region within the culture system; (2) zone specific cell sorting, (3) effects of culture environment and (4) different levels of oxygen tension throughout the graft, all of which are discussed in detail below.

- **Metabolic/morphological gradients (*i.e. nutrient, oxygen and pH*) generated between the surface and basal regions within a developing graft tissue utilising the *in vitro* Transwell culture system.**

Oxygen tensions within grafts may be one possibility for producing graft tissue stratification. Oxygen tensions fall with distance from the articular surface to the

deeper regions. This is supported by studies in our laboratory by Dr Anthony Hayes (unpublished observations) using the hypoxia marker pimonidazole hydrochloride (Hypoxyprobe™-1; Chemicon, Millipore, UK). The oxygen gradient set up depends on the balance between the rate of oxygen transport through cartilage and the rate of oxygen consumption by the chondrocytes. This is affected by cell density and distribution, cartilage thickness and the supply of oxygen applied to the graft from both the surface and basal regions of the system.
The Transwell culture system mimics *in vivo* native articular cartilage, as nutrients and oxygen are more readily available from the top of the graft (inner insert media) representing *in vivo* the nutrient supply from the synovial fluid, and diffusion of nutrients is minimal at the base of the graft (from outside media) representing transport from the subchondral bone, where the cells within the graft are densely packed and a high degree of metabolite competition occurs.
An increased cell seeding density used in the Transwell culture system could diminish nutrient supply from culture media at a much quicker rate than a lower cell number and potentially more frequent media changes may be required when using higher cell numbers (Mauck *et al.*, 2003). However, chondrocyte metabolism involving the uptake of nutrients from culture media rapidly acidifies the media, (i.e. decreases pH) the surrounding environment of the developing grafts. Due to diffusion rates to both inner insert media and outside media of the Transwell culture well, potentially a pH gradient could be generated that is responsible for the resultant stratification within graft tissue.
Alternatively, nutrient limitation could lead to cell death. There have been studies within our laboratory by Dr Anthony Hayes into the cell viability of Transwell cartilage grafts using a fluorescent live/dead viability kit. It was shown that the majority of cells in thin (~0.25mm) living sections of graft tissue were viable after 4 weeks in culture. During the same 4 week time frame both necrotic and apoptotic cells had accumulated throughout graft matrices, but there were no distinct zones of cell death (Hayes *et al.*, 2007).

- **Zone specific cell sorting**

Cell tracker studies in our laboratory using a live/dead™ viability/cytotoxicity kit showed that following a 4 week culture period, a labelled mid zone sub-

population largely retained its intermediate position in tri-layered composites, indicating little inter-zonal movement of chondrocytes. When grafts were made by mixing a single fluorescently labelled sub population with its unlabelled counterparts from adjacent zones, in all cases the chondrocytes were randomly distributed through-out tissue depth. Therefore, there is no evidence that zone specific cell sorting is responsible in the establishment of the distinct stratification observed by neocartilage grafts produced *in vitro* (Hayes *et al.*, 2007).

- **Culture environment**

Alternatively, the establishment of stratification within the grafts could be dictated by the culture environment produced by the Transwell culture system. Studies in our laboratory using bromodeoxyuridine (BrDu) to identify proliferating cells and the role cell proliferation has on tissue organisation have shown that high levels of proliferation occur at both the graft surface and in underlying tissue. This high proliferative level reflects the known effects that transforming growth factor (a supplement in the Transwell culture system) has on chondrocyte proliferation. Therefore, the chondrocytes divide or differentiate according to their potential and position in the developing tissue. At the surface of the developing graft the conditions resemble a monolayer culture. Therefore, the chondrocytes flatten out, proliferate and differentiate expressing molecules typical to the superficial zone. The deeper regions of the developing graft contain a high density of cells, therefore close contact with neighbouring cells conduct chondrogenesis and the formation of spheriodal cells showing morphological similarities to those found within the deeper regions (Hayes *et al.*, 2007).

Although distinct cartilaginous zones were observed within the grafts, there was no identifiable calcified zone. Advances were made in 1997, chiefly by the Kandel laboratory finding that the addition of Phosphoethanolamine (PEA), β-glycerophoshate (β-GP) or adenosine triphosphate (ATP) to deep zone chondrocytes in Transwell culture systems encouraged calcification, producing a mineral identified as hydroxyapatite similar in composition and crystal size to the mineral present in native *in vivo* cartilage (Kandel *et al.*, 1997). More recent advances involve the generation of neocartilage tissue on a bone substitute calcium polyphosphate (CPP)

that has been found to improve integration with bone within cartilage defects (Waldman *et al.*, 2007, Pilliar *et al.*, 2007). The presence of the calcified zone has been found to increase both the tissue stiffness and interfacial shear properties at the cartilage-CPP interface (Allan *et al.*, 2007).

Previously, *in vitro* systems utilised to study the deep region of articular cartilage region had limitations. The Transwell culture system is ideal for studying the different properties of chondrocytes isolated from different cartilaginous zones. Waldman in 2003 isolated chondrocytes from full thickness cartilage (FT), mid to deep region cartilage (MD) and from the deep region (DEEP) and seeded each group onto the surface of a porous ceramic substrate and maintained them in culture for 8 weeks. The tissue developed from the chondrocytes taken from full thickness explants accumulated the most amount of collagen. Tissues produced from isolated chondrocytes from the deep region were thicker and accumulated a larger amount of extracellular matrix. The tissue produced by chondrocytes isolated from MD regions contained more proteoglycan and showed mechanical properties four times greater than tissue produced from deep or FT cartilage chondrocytes.

In contrast, preliminary work in our laboratory has shown a reduction in the differentiation potential of chondrocytes with increasing depth from the articular surface utilizing a Transwell culture system (Hayes *et al.*, 2007). Chondrocytes isolated from the surface zone, and to a lesser extent the mid zone have the ability to reconstitute the macromolecular organisation of native cartilage tissue throughout the depth of the resulting graft. Chondrocytes isolated from the deep zone were unable to produce a stratified graft; the grafts produced were highly disorganised and stained strongly for collagen type I and X in addition to type II collagen. This supports the finding of a progenitor cell population within the superficial zone of articular cartilage (Dowthwaite *et al.*, 2004) that allows appositional growth from the surface zone region (Hayes *et al.*, 2001). These findings suggest that chondrocytes isolated from different zones have the ability to produce engineered tissues of varying properties. Therefore, the subpopulation of chondrocytes used in tissue engineering could be dictated by the requirements at the site of injury.

In a human knee joint, articular cartilage compensates for joint incongruence and differences in mechanics throughout the joint, leading to morphological variations of the articular cartilage in a site specific manner (i.e. its thickness, cellularity and the components that contribute to its ECM). A detailed account of the structural

information of native articular cartilage throughout the joint would be beneficial to tissue engineering, as a patient with a defect could potentially be treated with engineered tissue that was manipulated to consist of the relevant properties to a site of injury or disease. Although, it must be taken into consideration that repair graft tissue must develop and function under different metabolic and biomechanical constraints in comparison to the original native cartilage predecessor. Therefore, the requirements of the repair tissue may vary in comparison to native cartilage in order to carry out its healing function (Quinn *et al.*, 2005).

This study has shown that both thickness and cellularity of engineered grafts can be regulated by increasing the culture duration and cell seeding density, respectively. In the future, multiple neocartilage grafts of varying properties could be generated for site specific treatment by: (1) applying various growth media to Transwell culture systems (e.g. neocartilage grafts subjected to different growth factors and serum concentrations)); (2) applying a mechanical load to developing neocartilage grafts as mechanical forces stimulate ECM synthesis (Waldman *et al.*, 2003, 2006 & 2007, Miyanishi *et al* 2006, Kawanishi *et al* 2007); (3) applying low energy pulsed electromagnetic fields to grafts to up-regulate glycosaminoglycan synthesis and anti-inflammatory agents (De Mattei *et al.*, 2007); (4) the use of different chondrocyte subpopulations to generate grafts (Waldman *et al.*, 2003, Kandel *et al.*,1997 and Hayes *et al.*, 2007); and (5) the use of different cell sources.

In this study it was concluded from histology data that the optimal culture conditions for neocartilage graft generation utilising the Transwell culture system for future work was a cell seeding density of 4-6 x 10^6 cells generated over a 4 week culture period. In 2007, Hayes *et al* reported that immunohistochemical labelling towards chief ECM molecules showed that Transwell neocartilage tissue generated using these optimal culture conditions had many similarities to immature native articular cartilage (Hayes *et al.*, 2007). Type I collagen labelling was predominant at the surface of the grafts and both aggrecan and type II collagen labelling were present throughout the whole depth of the graft tissue, as found in native articular cartilage (**Figure 3.9**). No labelling was observed for versican, versican being a marker for fibroblastic tissue. All the extracellular matrix components that were present in the neo-cartilage tissue produced using these optimal culture conditions are discussed to a greater length in **Table 3.4**.

Extracellular matrix molecules present in native cartilage	Composition of neo-cartilage graft tissue produced from a 7 day bovine chondrogenic source over a 4 weeks primary culture
Collagens	
Type I	Present in the surface zone, not in underlying tissue
Type II	Identifiable throughout graft tissue
Type X	*4 weeks culture*- The immaturity of the tissue is reflected in the fact that collagen type X is not detectable in grafts following 4 weeks culture (Schmid & Linsenmayer., 1985). *Extended culture period (>12 weeks)*- Weak association of type X collagen within the pericellular matrix of hypertrophic chondrocytes of the basal zone (Schmid & Linsenmayer., 1985).
Proteoglycans	
Aggrecan	One of the major constituents of cartilage extra-cellular matrices was detectable throughout graft tissue.
Small leucine rich PG (i.e. Biglycan & Decorin)	Detectable throughout graft tissue. Decorin, in particular, showed a more prominent labelling in the surface zone that diminished with depth of the tissue (Miosge *et al.*, 1994).
Surface Zone Protein (SZP)	*4 weeks culture*- Weakly associated with cells at graft surface *Extended culture period (>12 weeks)*- SZP became more organised at graft surface showing similar distribution to native articular cartilage (Schmacher *et al.*, 1994).
Glycosaminoglycans	
Chondroitin Sulphate (CS)	Unsulphated chondroitin (C-0-S) and chondroitin-4-sulphate (C-4-S) 'stubs' (i.e. following chondroitinase digestion) showed labelling throughout graft tissue suggesting an association with aggrecan. Chondroitin-6-sulphate (C-6-S) 'stubs' were not detectable in graft ECM indicative of the immaturity of the engineered graft (Cheng *et al.*, 1996).
Keratan Sulphate (KS)	Present in pericellular matrix compartments, becoming more predominant with graft tissue depth. Similar observations found in native articular cartilage where KS associates with aggrecan (Hedlund *et al.*, 1999).
Dermatan Sulphate (DS)	Located at the surface zone of graft tissue, suggesting that this molecule is associated with decorin (Choi *et al.*, 1989, Miosge *et al.*, 1994).

Table 3.4. Extracellular matrix composition of neo-cartilage grafts generated from an immature bovine chondrogenic source over a 4 weeks culture period (Hayes *et al.*, 2007).

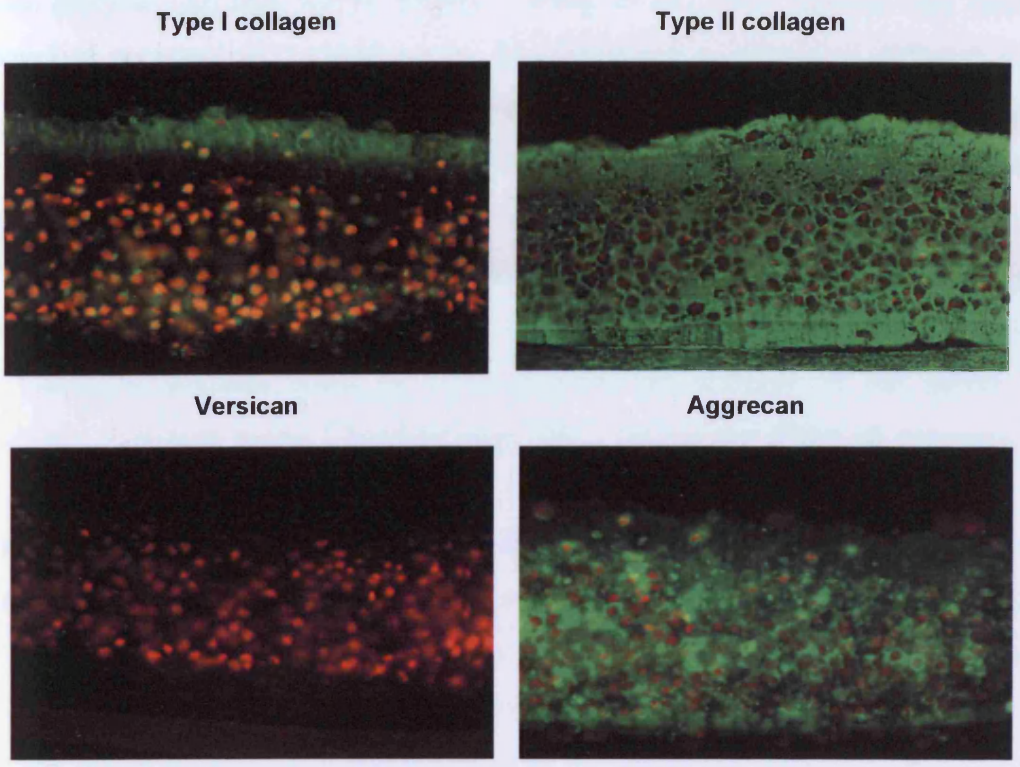

Figure 3.9. Immunohistochemical labelling of extracellular matrix components of neo-cartilage generated using an *in vitro* Transwell culture system (*Kindly donated by Dr Anthony Hayes*).

A comparable study of neocartilage grafts produced using either an immature or mature chondrogenic source at optimal culture conditions revealed that the immature source produced a more alcianophilic, highly organised, stratified, hyaline tissue of increased tissue weight and thickness than the mature source. There were marked differences in the synthetic capacity of chondrocytes obtained from both sources. Grafts produced from the immature source generated x3.3 fold more total s-GAG and x1.7 fold more total collagen than the mature source over a 4 week culture period. Interestingly, of the total s-GAG and collagen that was released to culture media from grafts generated by both an immature and mature source, a higher proportion of s-GAG was released to the inner insert media than the outside media and the opposite was observed for collagen. These results suggest that grafts produced from an immature bovine chondrogenic source, due to their highly active synthetic nature, could effectively heal partial or full thickness defects supporting the studies of Namba *et al* 1998.

However, one potential problem is the integration of neo-cartilage tissue to adjacent native articular cartilage within defects. Wang *et al.*, 2004 showed that freshly isolated or passaged (P1) chondrocytes, have potential to adhere to different joint surfaces that they could be exposed to in a cartilage defect (i.e. mid-deep zone hyaline cartilage, calcified cartilage or bone). There is potential to improve integration between repair tissue and native cartilage at a defect site, as proteoglycans carrying dermatan sulphate GAG chains are thought to inhibit cell attachment. Following the debridement of a defect site, and prior to implantation of repair tissue to the defect site, dermatan sulphate could be removed from the surfaces of the defect by enzymatic treatment (using Chondroitinase ABC) having the effect of encouraging cell attachment (Brittberg 1999).

In the literature it was reported that the production of neo-cartilage grafts in Transwell culture systems involved two phases; (1) matrix accumulation (*culture days 10-21*) and (2) maintenance of the existing matrix (*following culture day 35*). It was found that both collagen and proteoglycan content within graft tissue increased with culture time and matrix accumulation was enhanced by increasing cell density in the presence of serum and ascorbate. Newly synthesised proteoglycan retained in graft matrices increased from 20% on culture day 6 to 85% on culture day 35. Following day 35 of culture there were no further increases in proteoglycan and collagen content (Boyle *et al.*, 1995). Potentially, this study could have been improved if the effects of increased cell density and culture duration on matrix accumulation (*i.e. sulphated glycosaminoglycan and collagen content*) were taken into consideration.

Sun *et al* in 2002, utilized the Transwell culture system to generate grafts using an equine chondrogenic source and found that the tissue generated had more sulphated glycosaminoglycan and lower collagen content than native articular cartilage. An improvement upon this study would have been to analyse both the collagen and s-GAG content of the harvested native articular cartilage prior to enzymatic isolation for use of chondrocytes in neocartilage graft production, so that the biochemical content of native tissue and engineered neocartilaginous graft tissue could have been compared more accurately.

3.4 Chapter summary

- Neocartilage grafts produced from an immature bovine chondrogenic source were of an opaque, hyaline appearance at all cell densities and time points studied.

- Stratification into distinct zones approximating to the surface, middle and deep regions of native adult articular cartilage *in vivo* was evident within graft tissue following just 2 weeks of culture and this morphological stratification became more apparent with time and increased cell seeding density.

- An increased culture period and cell seeding density produced grafts of increased thickness and cellularity, respectively.

- With respect to native adult articular cartilage, grafts made from 6 x 10^6 cells or above were too hypercellular and grafts produced from 2 x 10^6 cells were too thin.

- The optimal culture conditions for future analysis within this project was found to be a cell seeding density of 4-6 x 10^6 cells over a 4 week culture duration determined by histological data alone.

- Neocartilage tissue generated from an immature chondrogenic source, utilising optimal culture conditions, showed immunofluorescent labelling of major extracellular matrix components was similar to immature native articular cartilage.

- At optimal culture conditions neocartilage grafts produced from an immature chondrogenic source were more synthetically active compared to those produced from a mature chondrogenic source, generating significantly higher amounts of both total sulphated glycosaminoglycan and collagen.

- A higher s-GAG and collagen content was retained within grafts generated from the immature chondrogenic source compared to mature; these findings were supported by graft weight, thickness and histology.

- Interestingly, more s-GAG was found to be released to inner insert media of the Transwell culture system in comparison to outside media and the opposite was observed with collagen release.

- The Transwell culture system is advantageous over many other culture models, as neocartilage graft generation can be highly regulated in culture, as most non-gaseous graft metabolites produced throughout the culture duration can be monitored.

4.0 THE EFFECT OF PASSAGE EXPANSION OF CHONDROCYTES IN MONOLAYER CULTURE, ON RESULTANT NEOCARTILAGE TISSUE ARCHITECTURE USING AN *IN VITRO* TRANSWELL CULTURE SYSTEM.

An unavoidable and necessary step for the use of human chondrocytes to assist in the healing of articular cartilage defects is cell expansion to increase the cell number because human chondrogenic sources utilized for clinical applications are of limited supply. Animal chondrogenic sources are of a more plentiful supply, but are not practical due to immuno-rejection. Therefore, human chondrocytes are isolated from harvested cartilage from a low load bearing region of a patient and multiplied in monolayer culture.

In monolayer culture the chondrocytes are placed into an alien 2-D culture environment in which modifications to the cytoskeleton occur. The chondrocytes lack the crucial influences of physiological cell-cell and cell-extracellular matrix interactions. As a result chondrocytes change in phenotype to a more fibroblastic state (**Figure 4.1**). The chondrogenic phenotype is rounded, whereas the fibroblastic phenotype is flattened and stretched out. The process in which a chondrocyte phenotype changes from chondrogenic to fibroblastic is termed de-differentiation.

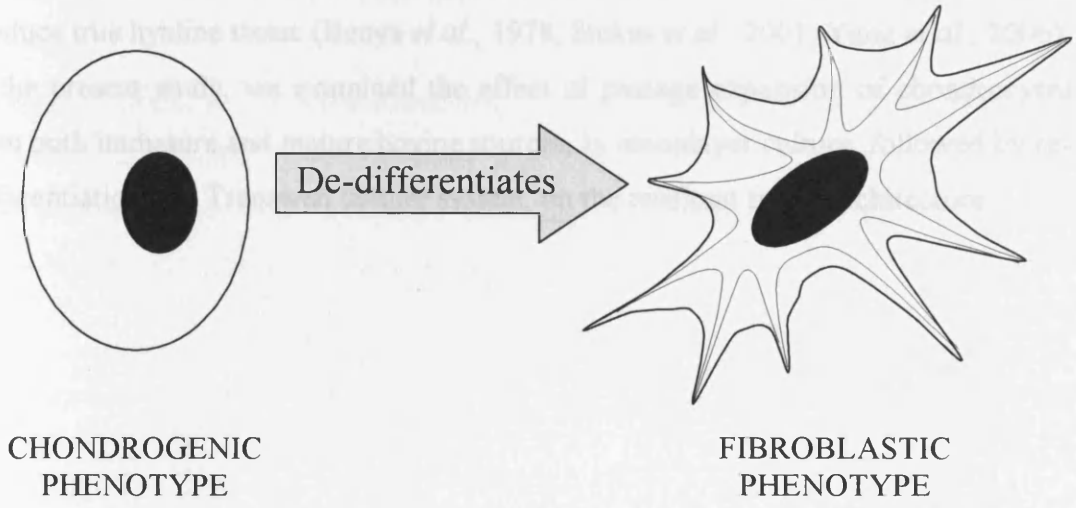

Figure 4.1. Morphological changes that occur to a chondrogenic cell source resulting from passage expansion in monolayer culture.

De-differentiation is a process in which tissues that have undergone cell differentiation (*a series of events, involving the interactions of growth factors leading to the development of specialised cells having specific structural, functional and biochemical properties*) can be made to reverse the process as to become a primordial cell again.

Like chondrocytes, fibroblastic tissue is derived from primitive mesenchyme and they express the intermediate filament vimentin, a marker illustrating mesenchymal origin. Fibroblasts are able to synthesise extracellular matrix molecules including: collagens, glycosaminoglycan, elastic fibres and glycoproteins. Fibrocartilage consists of mostly Type I collagen fibres (*unlike hyaline tissues that consist of more type II collagen in comparison*) with small amounts of type III and V collagens and less proteoglycan than hyaline cartilage (Eyre & Wu 1983).

This resultant phenotypic change of cells is a consequence of proliferation in monolayer and is accompanied by the up-regulation of type I collagen and versican (*Fibroblastic markers*) and the down regulation of aggrecan, type II collagen and specific genes SOX 5, 6 and 9 (*Cartilaginous markers*). Re-differentiation of the passaged cell source into a 3-D culture system or high cell density culture (i.e. *alginate, agarose, pellet cultures or in vitro Transwell culture systems*) may provide a solution to this problem.

However, stimulating re-differentiation of chondrocytes may encourage the undesired effect of hypertrophic differentiation and dramatically diminish chondrocyte ability to produce true hyaline tissue (Benya *et al.*, 1978, Stokes *et al.*, 2001, Yang *et al.*, 2006). In the present study, we examined the effect of passage expansion of chondrocytes from both immature and mature bovine sources, in monolayer culture, followed by re-differentiation in a Transwell culture system, on the resultant tissue architecture.

4.1 Methodology

Chondrocyte sources (*i.e. immature (7 day bovine) and mature (18 month bovine)*) designated for de-differentiation were serially passage expanded in monolayer culture using aerated 75cm^2 flasks maintained in DMEM plus 50μg/ml gentamycin, containing 10% FBS. The cells were grown to confluence, then cultures were washed to remove surplus protein using serum free DMEM plus 50μg/ml gentamycin. The media was removed and Trypsin.EDTA (5ml) was added to the monolayer flasks for 5 minutes at 37°C to allow cells to lose contact with the surface of the flasks. With a higher passage number (P4 and P5), cell removal from the flasks became more challenging, so addition of collagenase (100units/ml) was also required for 30 minutes at 37°C following a trypsin treatment.

Harvested chondrocytes were washed, counted, and seeded onto filter inserts (*immature: 6x10^6 cells/insert and mature: 4x10^6 cells/insert*) as described in section 2.2. Neocartilage grafts were produced over a 6 week (*immature*) and 4 week (*mature*) culture period and were maintained and harvested following methodology also described in section 2.2.

The remaining cells harvested from the monolayer culture, were returned to monolayer culture at low density for further rounds of expansion. This procedure was repeated until passage number 5, by which time the growth rate in monolayer was insufficient to sustain sufficient cell numbers for further graft production.

Analyses: Grafts (*i.e. immature and mature*) were histologically and biochemically analysed using methodology previously described in chapter 2. Neocartilage grafts generated from expanded immature chondrogenic sources were immuno-labelled following the methods described in section 2.3.1.

4.2 Results

4.2.1 Effect of passage expansion of immature chondrocytes, in monolayer culture, on resultant neo-cartilage tissue architecture using an *in vitro* Transwell culture system.

Immature bovine chondrocytes showed reduced ability to produce a true hyaline tissue resembling articular cartilage, using an *in vitro* Transwell culture system, following successive monolayer expansions. An increasing passage number of the cell source resulted in the generation of neo-cartilage grafts with a decreasing thickness from approximately 500μm at P0 (*primary chondrogenic source*) to ~300μm at P5 (*grafts produced from a chondrocyte cell source that had been serially passaged 5 times*) (**Figure 4.2**).

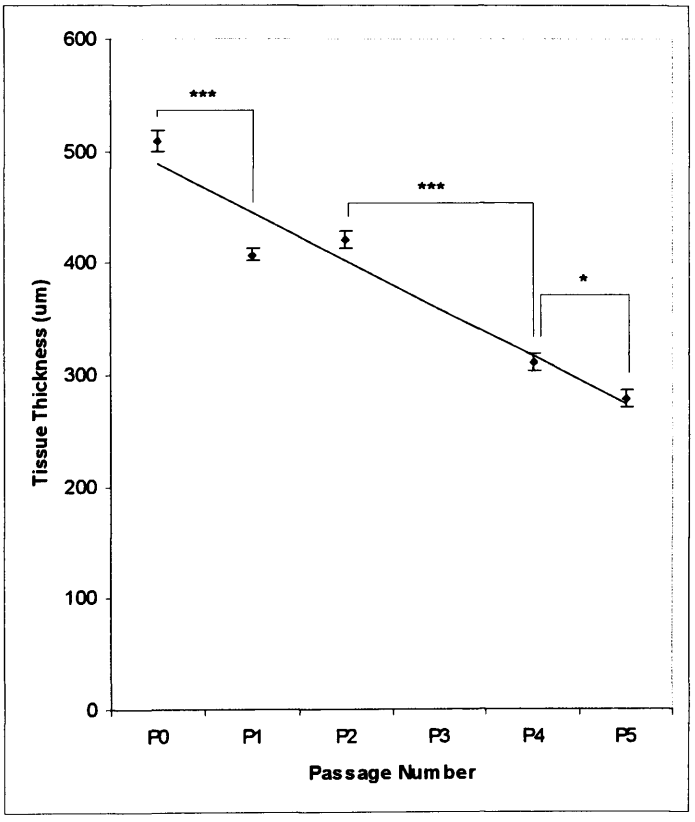

Figure 4.2. Thickness of neo-cartilage grafts produced at 6 weeks from an immature chondrogenic source with an increasing passage number. Data presented as mean SEM and significance was assumed where P<0.05 (*), P<0.01 (**) or P<0.001 (***).

Histological organisation of neo-cartilage grafts was progressively lost with an increased passage number, and alcian blue staining reduced, indicating diminished sulphated GAG content with successive passage (**Figure 4.3**). At P0, P1 and P2 grafts generated showed similarities to native adult articular cartilage; with distinct stratified regions of surface, middle and deep zones consisting of spheroidal chondrocytes in the mid to deep region and discoidal chondrocytes at the surface. Chondrocytes that had been passaged three times (P3) were unable to lay down an extracellular matrix, therefore cells were lost to culture medium at this stage resulting in a premature termination of the Transwell filter insert cultures.

However, chondrocytes that were further passaged a fourth (P4) and fifth (P5) time regained their capacity to lay down an extracellular matrix. Although the resultant neo-cartilage grafts from both P4 and P5 lacked distinct zonal organisation, and their chondrocytes appeared to be less rounded in comparison to the chondrocytes observed from grafts generated at P0 to P2. Neo-cartilage grafts generated from P4 and P5 chondrocytes contained discrete clusters of cells in their basal portions (**Figure 4.3 (*arrow heads*)**) and histologically resembled fibrocartilage. This is suggestive that a phenotypic change from chondrogenic to fibroblastic occurs at very early stages of chondrocyte cell expansion.

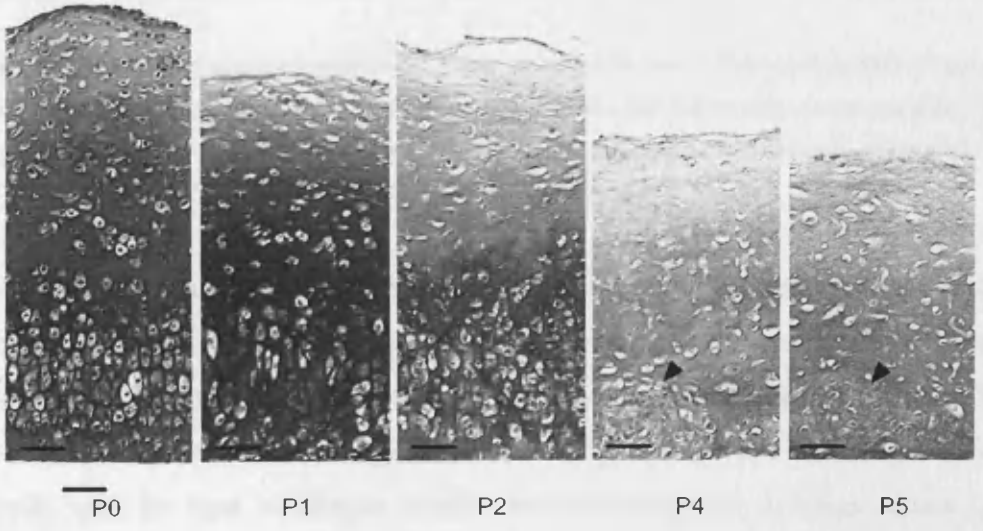

Figure 4.3. Neo-cartilaginous tissue produced using an *in vitro* Transwell culture system from immature chondrocytes that were serially passage expanded (P0-P5) in monolayer culture. Alcian blue and haematoxylin/eosin. Scalebar represents 40μm.

These histological findings were supported by s-GAG analyses in that grafts produced at all passages generated significantly less total s-GAG compared to Control (P0) treated grafts (**Table 4.1 & Figure 4.4**). Decreased glycosaminoglycan retention within neocartilage grafts was observed between P0 to P3, showing an approximate 65.6% decrease between P0 and P2 and a 100% decrease between P0 and P3 (**Table 4.1 & Figure 4.4**). However, following further passages for a 4th and 5th time, the capacity of grafts to retain s-GAG within matrices was restored to 36.5% and 41.3% of P0, respectively.

Passage Number	Media		Graft	Residue		Total GAG (μg)
	Insert	Outside	4M GuHCl	Pepsin	Papain	
P0	824.80±12.61	393.35±3.60	1191.47±126.07	278.7±179.53	132.54±100.47	2820.89±366.17
P1	731.40±12.00	341.05±10.61	824.58±105.53	70.33±9.64	26.77±3.28	1994.14±117.86 (*)
P2	272.32±14.24	364.60±8.56	482.83±115.59	42.00±21.44	26.49±3.59	1188.24±123.99 (***)
P3	30.84±4.59	25.52±2.69	-	-	-	56.36±7.23 (***)
P4	738.33±5.38	302.37±6.11	483.89±51.48	60.73±19.49	41.05±6.75	1626.37±64.58 (**)
P5	655.52±12.05	216.22±7.62	425.62±65.89	210.03±33.24	26.19±4.42	1533.58±93.26 (**)

Table 4.1. Total sulphated glycosaminoglycan (s-GAG) generated by neocartilage grafts produced using an *in vitro* Transwell culture system, from an immature chondrogenic source that was serially passage expanded (P0-P5) in monolayer culture (n=3). Data presented as mean SEM and significance was assumed where P<0.05 (*), P<0.01 (**) or P<0.001 (***). All data compared relative to the Control.

Immunohistochemical (IHC) labelling of grafts produced using successively passaged immature bovine chondrogenic sources, showed that Type II collagen was present throughout graft matrices regardless of passage number (**Figure 4.5**). Grafts generated from a primary chondrogenic source (P0) showed IHC labelling within the superficial zone for type I collagen, similar to native articular cartilage where type I collagen is a minor component enriched in the 'lamina splendins' of the superficial region (**Figure 4.5**). An increased passage number from P0 to P2 extended collagen type I labelling from the graft surfaces towards the mid region. At P3, no graft was

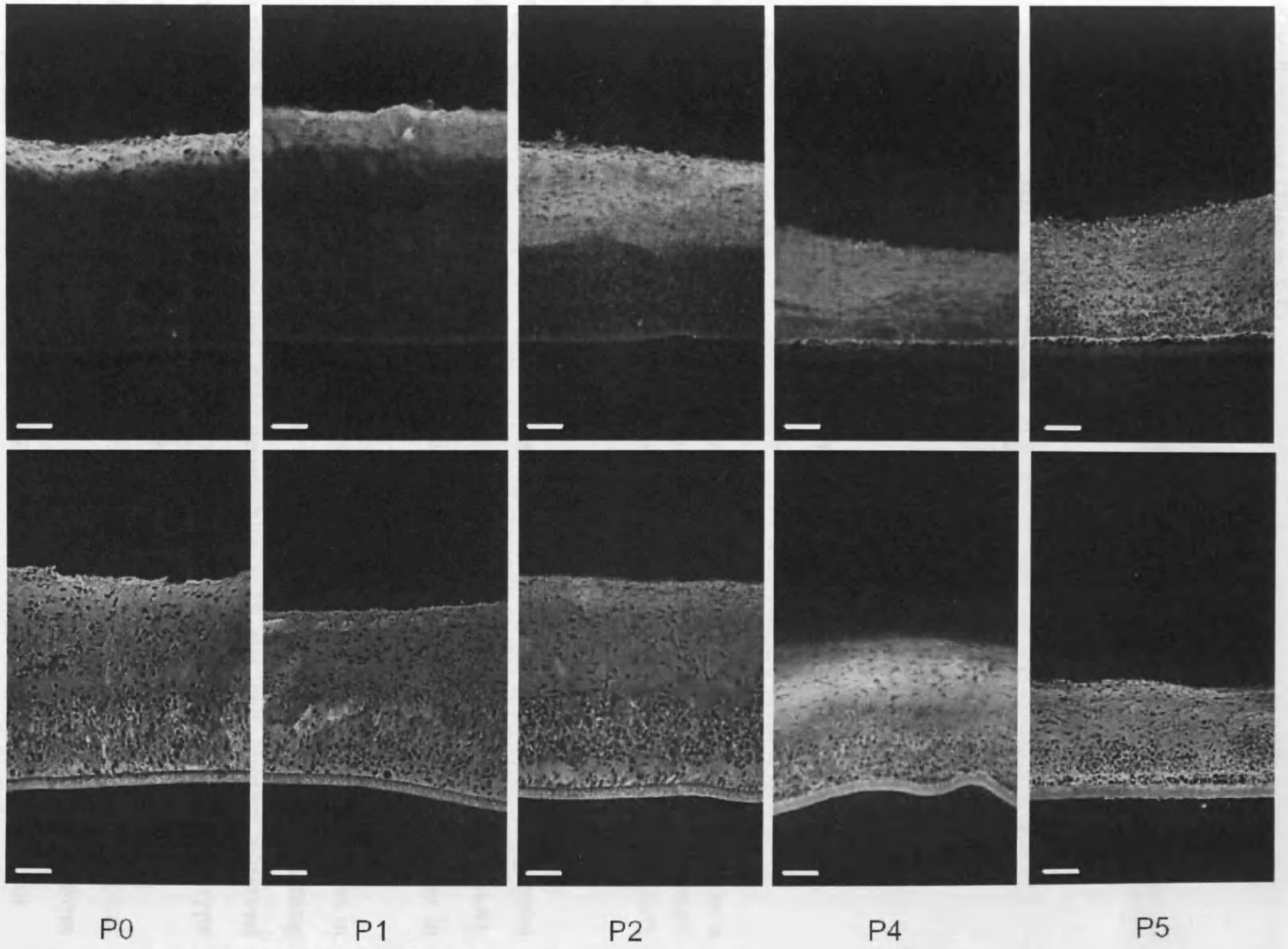

Figure 4.5. Distribution of collagen types I & II in neo-cartilaginous graft tissues produced from serially expanded (P0-P5) chondrogenic sources in monolayer culture (scalebars = 80μm)

produced as chondrocytes were unable to lay down an extracellular matrix leading to a premature termination of cultures at this stage. After chondrocytes were further passaged a 4th and 5th time, type I collagen labelling was present throughout the whole of graft matrices (**Figure 4.5**).

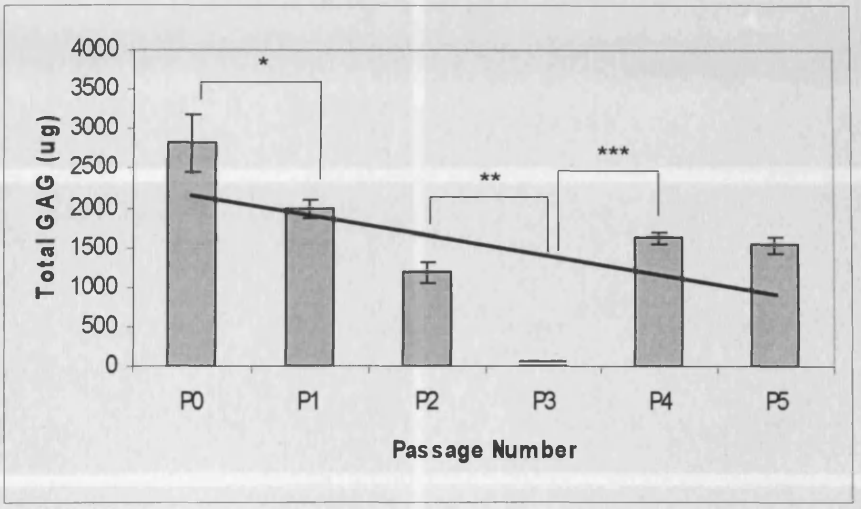

Figure 4.4. Total sulphated glycosaminoglycan (s-GAG) generated by neocartilage grafts produced using an *in vitro* Transwell culture system, from an immature chondrogenic source that was serially passage expanded (P0-P5) in monolayer culture (n=3). Data presented as mean SEM and significance was assumed where $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

Aggrecan, a major constituent of cartilage extracellular matrices was present throughout the whole of the graft matrix regardless of passage number (**Figure 4.6**). However, the staining intensity of aggrecan labelling decreased with an increase in passage number (**Figure 4.6**).

Interestingly, versican labelling (*a fibrocartilaginous marker*) became predominant in the deep zones of neo-cartilage grafts at both P4 and P5 (**Figure 4.6**). The labelling pattern for versican coincided spatiotemporally with the cell clusters observed histologically at the base of the tissue, suggesting a fibrochondrocytic cell phenotype in this tissue zone (**Figure 4.3 & Figure 4.6**).

The IHC labelling results correlate with both the histological and biochemical findings, suggestive that between P0 and P3 there is a phenotypic shift from chondrogenic to fibroblastic. This is supported by a decreased staining intensity for aggrecan labelling and an increased labelling for both collagen type I and versican in neo-cartilage grafts following successive passages.

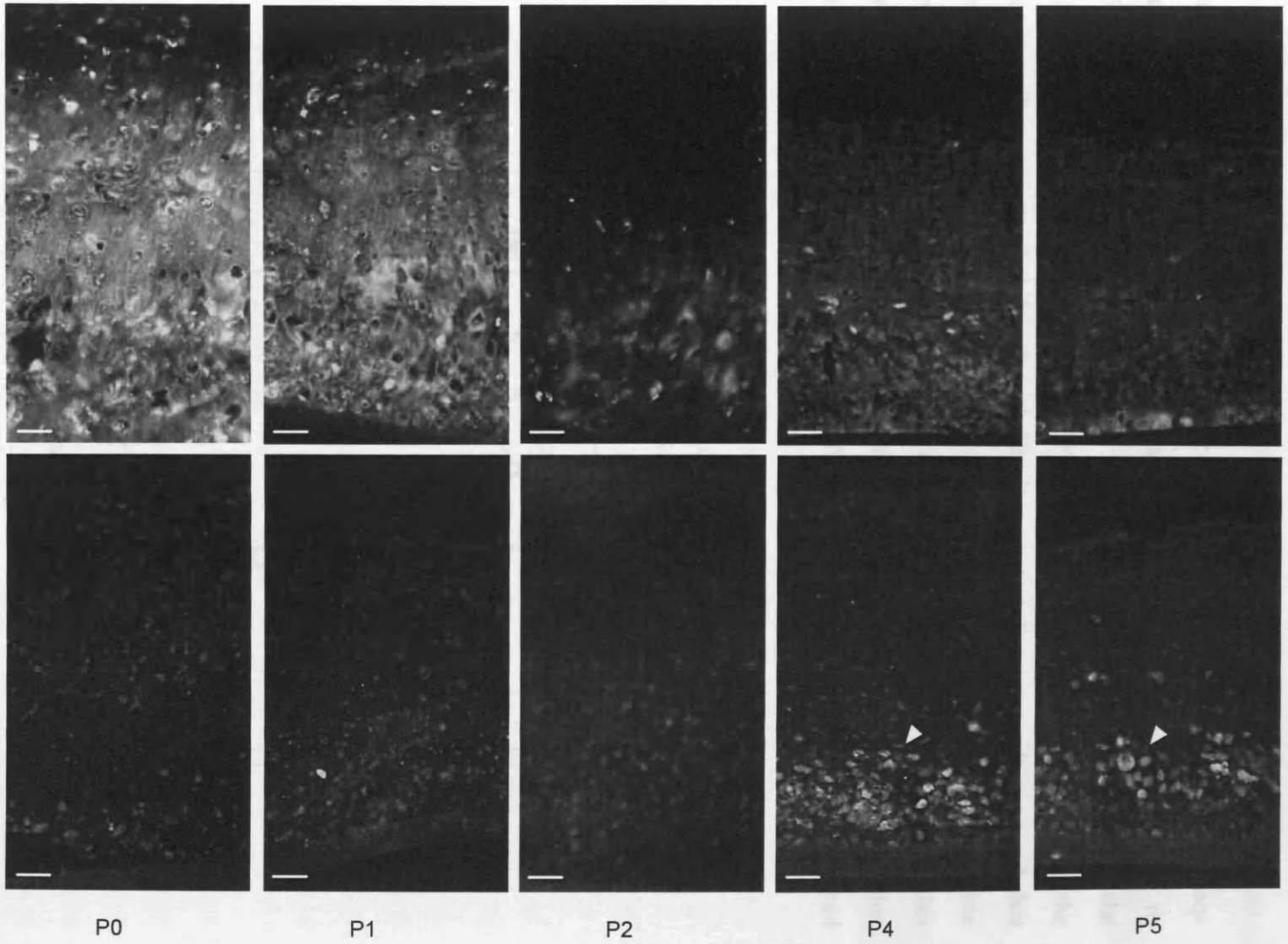

Figure 4.6. Distribution of aggrecan and versican in neo-cartilaginous graft tissue produced from serially expanded (P0-P5) chondrogenic sources in monolayer culture. Scalebars=40μm

4.2.2 Effect of passage expansion of mature chondrocytes, in monolayer culture, on resultant neo-cartilage tissue architecture using an *in vitro* Transwell culture system.

Increasing passage number of the mature cell source resulted in the generation of neo-cartilage grafts with a decreasing thickness from approximately 108μm at P0 to ~57μm at P5. Grafts at all passages were significantly thinner when compared to the Control (P0) graft, with the exception of P2. A similar trend was observed using the mature cell source, to that found with the immature cell source. It appeared that neocartilage grafts generated from a cell source that had been serially expanded three times (P3) were the thinnest, suggestive of a reduced capacity for the cells at this stage to lay down a matrix. This ability was slightly restored following further passages a 4th and 5th time, producing grafts of increased thicknesses when compared to P3.

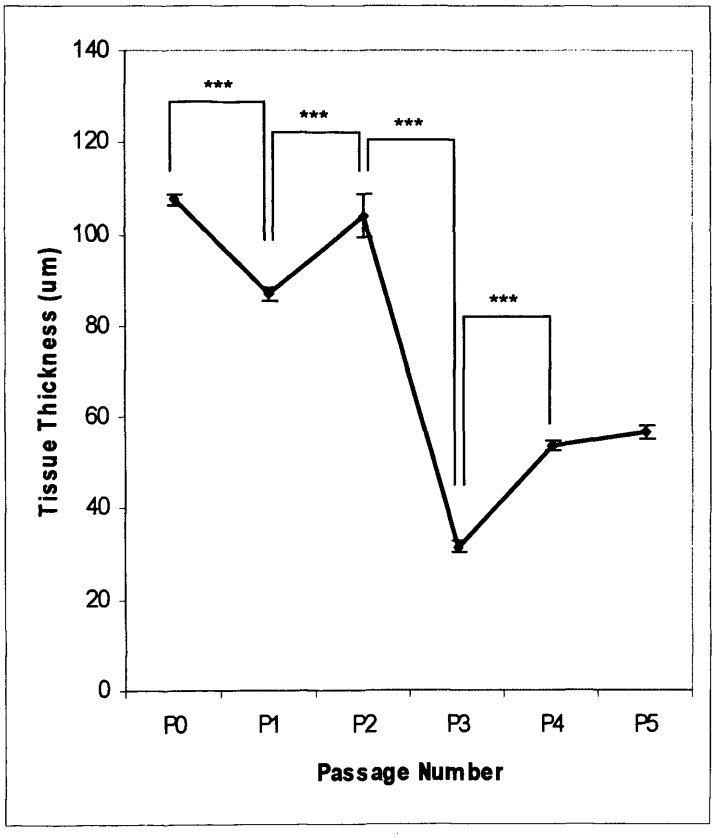

Figure 4.7. Thickness of neo-cartilage grafts generated from a mature cell source of increasing passage number. Data presented as mean SEM and significance was assumed where P<0.05 (*), P<0.01 (**) or P<0.001 (***).

The histological organisation of neocartilage grafts produced from mature cell sources, lacked the distinct zonal stratification observed in native adult articular cartilage, regardless of passage number (**Figure 4.8**). Chondrocytes present within the Control (P0) graft tissue appeared to be extremely hypercellular; all grafts appeared to be extremely cellular with very little matrix accumulation.

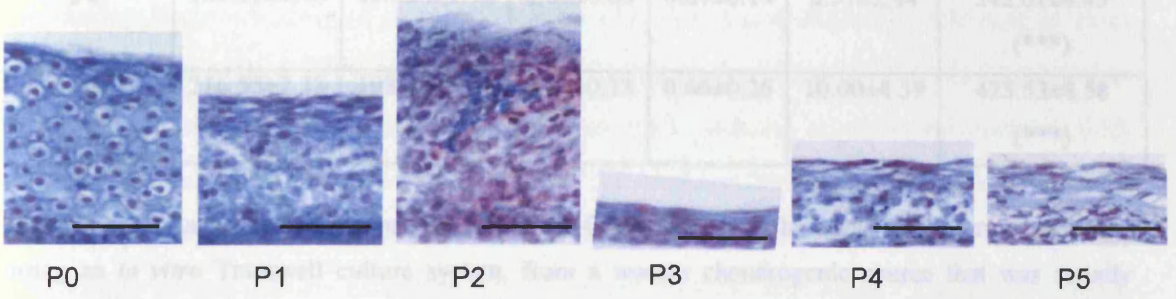

Figure 4.8. Neo-cartilaginous tissue produced using an *in vitro* Transwell culture system from mature chondrocytes that were serially passage expanded (P0-P5) in monolayer culture. Alcian blue and haematoxylin/eosin. Scale bar represents 50μm.

These histological findings were supported by s-GAG analyses in that grafts produced at all passages generated significantly less total s-GAG compared to Control (P0) treated grafts (**Table 4.2 & Figure 4.9**). A 62.7% decrease in glycosaminoglycan retention within neocartilage grafts generated from mature cells was observed between P0 to P3 (**Table 4.2 & Figure 4.9**). However, following further passages for a 4^{th} and 5^{th} time, the capacity of grafts to retain s-GAG within matrices was restored to 50.3% and 57.5% of P0, respectively.

The histological and biochemical findings for neocartilage grafts generated from serially expanded mature chondrogenic sources support a similar trend to what was observed using an immature chondrogenic source. This suggests that prior to P3 a phenotypic shift occurs within the cell source from a more chondrogenic to a more fibroblastic phenotype. After three passage expansions the chondrocytes show a reduced capacity to lay down extracellular matrices. Following a further 4^{th} and 5^{th} passage this ability is restored, however the neocartilage tissue generated appears to be more fibrocartilaginous.

Passage Number	Media		Graft	Residue		Total GAG (μg)
	Insert	Outside	4MGuHCL	Pepsin	Papain	
P0	769.15±6.10	265.68±6.83	1.26±0.47	0.90±0.08	16.61±8.19	1053.60±11.41
P1	387.28	176.85	2.31	0.07	7.17	573.68
P2	329.17±4.91	293.02±8.09	0.00±0.00	0.31±0.20	6.14±2.68	628.64±12.79 (***)
P3	229.46±2.98	204.50±1.94	0.22±0.22	0.36±0.23	6.42±4.77	440.95±1.51 (***)
P4	169.29±1.46	163.28±1.76	0.00±0.00	0.87±0.19	8.57±2.44	342.01±4.43 (***)
P5	216.55±2.13	198.19±2.51	0.13±0.13	0.66±0.26	10.00±4.39	425.53±8.58 (***)

Table 4.2. Total sulphated glycosaminoglycan (s-GAG) generated by neocartilage grafts produced using an *in vitro* Transwell culture system, from a mature chondrogenic source that was serially passage expanded (P0-P5) in monolayer culture. Data presented as mean SEM and significance was assumed where P<0.05 (*), P<0.01 (**) or P<0.001 (***). All data compared relative to the Control.

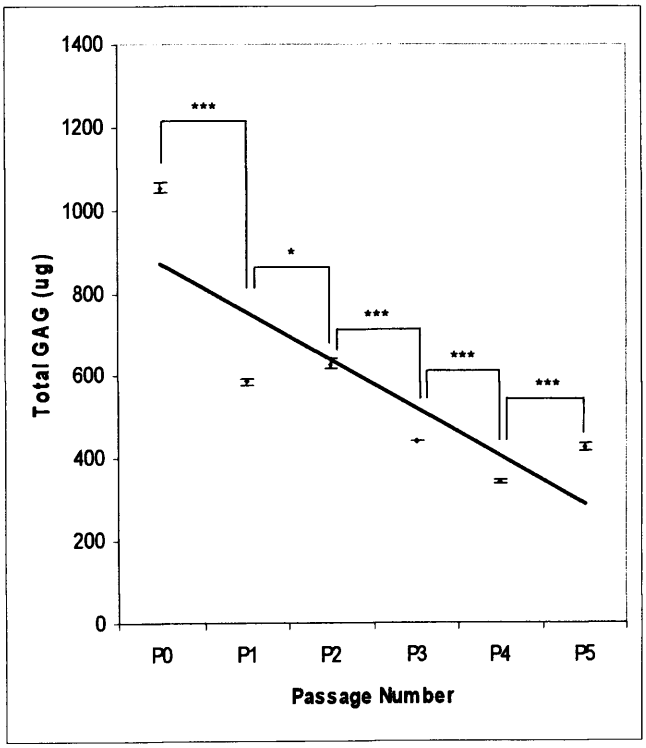

Figure 4.9. Total sulphated glycosaminoglycan (s-GAG) generated by neocartilage grafts produced using an *in vitro* Transwell culture system, from a mature chondrogenic source that was serially passage expanded (P0-P5) in monolayer culture. Data presented as mean SEM and significance was assumed where P<0.05 (*), P<0.01 (**) or P<0.001 (***).

4.3 Discussion

These experiments involved tissue engineering articular cartilage grafts using chondrocytes isolated from immature and mature bovine full depth cartilage. It was shown that with an increasing passage number of the chondrogenic source, the grafts that were generated increasingly lacked the distinct appearance and morphological stratification of native articular cartilage. The immature cell source is excellent at producing large amounts of extracellular matrix, but is not clinically relevant or even practical. The mature chondrogenic source has poor synthetic abilities in comparison to the immature cell source, and they produce grafts lacking a zonal architecture, with little matrix, appearing extremely cellular. An explanation for this possibly could be that *in vivo* the natural repair process as well as environmental factors change the phenotype of the mature chondrocyte to a more fibroblastic state. Therefore mature chondrocytes de-differentiate *in vivo* prior to the cell isolation, expansion and seeding onto filter inserts for graft production, de-differentiated cells lose their zonal differences.

After both the immature and mature chondrogenic sources had been passaged three times (P3) they lost the ability to re-differentiate. No grafts were produced from the immature cell source and grafts produced from the mature source were very thin, suggesting that at this point the chondrocytes had undergone a phenotypic change and de-differentiated. The capacity of the chondrogenic sources to lay down an extracellular matrix was restored following successive passages of the cells after P3, although the zonal organisation of hyaline cartilage was not apparent appearing more fibrocartilaginous in nature.

Parallel results in other studies show similar phenotypic changes occur in cartilage chondrocytes between P0 (*utilising a primary cell source*) and P4 (*utilising a chondrogenic source that has been passaged four times*). De Haart *et al* 1999 found that expansion of bovine articular cartilage chondrocytes showed a decreased expression of type II collagen following a third passage of the cell source. Chondrocytes isolated from human nasal septum cartilage have also been shown to exhibit a similar impairment in their ability to proliferate at P3, they begin to acquire a more fibroblastic morphology (Chua *et al.*, 2005). Honda *et al.*, 2004 reported that multiplied chondrocytes in a new scaffold: Hybrid 75:25 Poly (L-Lactide-ε-

Caprolactone) sponge, that was coated with type I collagen generated cartilage tissue up to P2 but not beyond P4. The use of subpopulations in monolayer showed that superficial zone chondrocytes had more SZP than mid/deep zones to begin with, and the mid/deep zones had more collagen type II than superficial zone to begin with. Following three passages these differences no longer existed (Darling and Athanasiou 2005). Interestingly, Fu *et al* 2005, expanded young rabbit chondrocytes to passage 3 and then seeded the source on polylactic acid scaffolds (PLA) coated with lecithin and poly lysine modulated by bFGF and they reported observing histologically cartilaginous grafts.

A study analysing the effects of monolayer expansion of human articular chondrocytes reported the down-regulation in mRNA for aggrecan and type II collagen and an up-regulation of both versican and type I collagen (Binette *et al.*, 1998). This was consistent with the immunolabelling data presented in this study.

To improve grafts generated from a passaged source it would be beneficial to optimise conditions for monolayer culture and re-differentiation encouraging chondrogenesis and inhibiting hypertrophic differentiation by adding the right factors at the appropriate moment. For example PTH/PTHrP has been found to inhibit chondrocyte hypertrophy, but insulin sends chondrocytes towards hypertrophy therefore addition of the right factors are essential (Liu *et al.*, 2007). Ideally conditions should encourage type II collagen and proteoglycan production, inhibit collagen type I, suppress type X collagen and delay the expression of alkaline phosphatase and calcium mineral accumulation (Liu *et al.*, 2007). A detailed understanding of intracellular signalling during chondrogenesis and the effects of monolayer culture on chondrocyte signalling would be advantageous, as improving basic knowledge in this area would help clinicians to intervene in attempt to prevent bio-artificial cartilage from losing its stability.

Yang *et al.*, 2006 looked at the impact expansion and re-differentiation conditions had on the chondrogenic capacity of cultured chondrocytes. During chondrocyte expansion they either supplemented media with or without bFGF, then analysed the re-differentiation abilities of the two expanded sources in either a pellet culture or filter inserts with type II collagen. An increased differentiation of bFGF supplemented chondrocytes was observed in comparison to the non supplemented chondrocytes in monolayer culture, and the re-differentiation of expanded supplemented chondrocytes was better than the non supplemented expanded

chondrocytes showing increased proteoglycan synthesis. It was found that expansion of supplemented chondrocytes and re-differentiation on collagen coated filters resulted in the most optimal chondrogenic conditions. Similar results were also observed when expanding goat chondrocytes supplemented with bFGF. There was increased chondrocyte proliferation and their post expansion chondrogenic capacity to re-differentiate onto HYAFF-M or polyactive foams was enhanced (Miot *et al.*, 2006).

Neighbouring cells can influence cell type and de-differentiation induced by passaging. A chondrogenic source that was passaged four times then mixed with a primary chondrogenic source and grown on filter inserts showed an increase in cartilage tissue formation and re-differentiation of de-differentiated chondrocytes with addition of increasing concentrations of the primary chondrogenic source. Therefore, the quality of *in vitro* cartilage tissue could be improved with the use of co-culturing both primary and passaged chondrocytes (Gan & Kandel 2007).

In vivo, articular cartilage is subjected to varying loads; mimicking *in vivo* conditions could enhance the synthesis of important matrix components *in vitro*. The influence of intermittent loading during re-differentiation of chondrocytes in alginate culture was analysed and it was observed that collagen type II and glycosaminoglycan increased encouraging re-differentiation (Heyland *et al.*, 2006). Staurosporin (*described as a protein kinase C inhibitor*) has been described as a potent re-differentiating agent of articular chondrocytes that have been sub-cultured up to passage 2 (Borge *et al.*, 1997). Chondrogenesis can also be promoted in human chondrocytes, but not fibroblasts, by responding to perlecan *in vitro* (French *et al.*, 1999) and enhancing the chondrogenic transcription factor, SOX 9 (Bell *et al.*, 1997).

The potential recovery of passaged chondrocytes to a more chondrogenic phenotype can be achieved through SOX 9 transduction to enhance the re-expression of cartilage matrix genes (Tew *et al.*, 2005). The transcription factor SOX 9 is an essential factor in chondrocyte differentiation functioning as a potent inhibitor of chondrocyte hypertrophy. It has been reported that passaged human osteoarthritic chondrocytes show an increased collagen type II expression (*in both mRNA and protein levels*) in monolayer when transduced using adenoviral or retroviral vectors of SOX 9 (Tew *et al.*, 2005).

In this study the nature of the engineered tissue generated was analysed utilizing aggrecan, versican, collagen types I and II markers. A problem is 'what can be

determined as a cartilaginous or fibroblastic specific marker?' Recently it was reported that collagen type II might be a marker for the synthetic activity of chondrocytes rather than a de-differentiation marker (Goessler *et al.*, 2006). The expression of markers that define both the chondrogenic and fibroblastic phenotype should have been looked at over the various culture stages (i.e. following chondrocyte isolation, during monolayer culture and following re-differentiation in the Transwell cultures) as should have cell viability. Ideally, terminal differentiation markers such as type X collagen, alkaline phosphatase and osteopontin should have been used so that hypertrophic differentiation of the cell source following re-differentiation in Transwell cultures could potentially be eliminated.

In 2001, it was reported that the monoclonal antibody II-fibrau was a useful marker to characterise chondrocyte differentiation, having the ability to discriminate between differentiated and de-differentiated chondrocytes by binding to de-differentiated chondrocytes only. It was found that the addition of differentiated cells to alginate culture resulted in 90-95% of the cells being II-fibrau negative, whereas the addition of de-differentiated cells to alginate in a chondrogenic favouring environment resulted in an increase in type II collagen but II-fibrau staining was positive. The placement of de-differentiated cells within a biomaterial into a nude mouse resulted in no apparent II-fibrau staining (Van Osch *et al.*, 2001).

Alternatively, maybe the use of a chondrogenic source for tissue engineering cartilage isn't the way forward. A possible avoidance measure to the obstacle that passaging creates could be the usage of an immortalised cell line. However, this could prove disadvantageous as the use of a tumour cell line would be required for this and the chondrocytes would grow not under the control of the body, but under the control of the virus. Cell sources that show potential for future use are embryonic stem cells, mesenchymal stem cells and progenitor cells. To date knowledge of these cell sources is minimal and each source provides impediments that need to be overcome, but they do provide optimism for the future.

4.4 Chapter Summary

- Neocartilaginous tissues produced from immature primary chondrogenic cell sources showed similarities to native adult articular cartilage with distinct stratified zones approximating to the surface, middle and deep regions that were progressively lost with an increased passage number.

- Neocartilaginous tissues generated using mature cell sources were extremely cellular showing little matrix accumulation and lacked the distinct zonal stratification that is observed in native adult articular cartilage.

- Increasing passage number from 0-3 times of the chondrocyte cell source (*both immature and mature*) used to generate neocartilage grafts showed a decreasing s-GAG retention within neocartilage grafts. Grafts produced from a chondrocyte source that had been serially expanded three times (P3) compared to Control (P0) treated grafts (*in both immature and mature*) showed large decreases in both graft thickness and total s-GAG generation.

- Chondrogenic cell sources (*both immature and mature*) that were serially passaged three times (P3) resulted in a reduced capacity to lay down an extracellular matrix (ECM). In the immature cultures, P3 cells were lost to culture medium resulting in premature termination of Transwell filter inserts, and in mature cultures the thinnest neocartilage grafts were produced at this stage.

- Further passage of the chondrogenic source (*both immature and mature*) a 4^{th} and 5^{th} time regained the capacity of cells to lay down an ECM. Neocartilage tissues produced from either P4 or P5 cells were thicker showing higher s-GAG retention within grafts compared to P3 generated graft tissues, but not in comparison to P0-P2 generated neocartilage tissues.

- Neocartilage grafts generated from an immature P4 or P5 chondrogenic source histologically contained discrete cell clusters within their basal portions, that

IHC labelled spatiotemporally for versican (*a fibroblast marker*) suggestive that P4 and P5 graft tissue resembled a more fibrocartilaginous tissue. This was also supported by a decreased staining intensity for aggrecan labelling and increased labelling for type I collagen.

5.0 THE EFFECTS OF DIFFERENT MOLECULAR WEIGHT HYALURONAN ON NEOCARTILAGE GRAFT METABOLISM UTILIZING AN *IN VITRO* TRANSWELL CUTURE SYSTEM.

Hyaluronan (HA) is a glycosaminoglycan made up of repeating disaccharide units of [D-glucuronic acid (β1-3) N-acetyl D-glucosamine (β1-4)]n (Meyer 1934). HA is endogenously synthesised by chondrocytes (Gillard *et al.*, 1975) and synovial membrane cells of the joint cavity (Baxter *et al.*, 1973) and has been found to have a high rate of catabolism (Laurent and Fraser., 1992, Csoka *et al.*, 2001). Hyaluronan is a relatively simple molecule, but has a wide range of biological roles within articular joints and other tissues of the body. Primarily HA provides a structural role in cartilage, as the major proteoglycan 'aggrecan' binds to HA chains stabilised by link protein, having the effect of influencing the hydration properties of cartilage (Hardingham & Muir, 1972). HA is also present within joint synovial fluid, and is involved in the regulation of metabolic exchange between cartilage and the synovial fluid functioning as a semi-permeable barrier, a lubricant and a shock absorber.

It has been reported that various sized HA polymers are known to trigger different functional responses. The low molecular weight (Mwt) hyaluronan appear to act as 'endogenous danger signals' mediating cell signalling effects (Powel & Horton, 2005). The smaller fragments are inflammatory, immuno-stimulatory and angiogenic (Sugahara *et al.*, 2006) with the capability to activate macrophages, suppress proteoglycan sulphation (Sorlursh *et al.*, 1980), displace proteoglycans from cell surfaces (Sorlursh *et al.*, 1980) and compete with high Mwt HA for receptor binding (Stern *et al.*, 2006).

High molecular weight HA is used clinically as an accepted viscosupplementation procedure in the treatment of degenerative joint diseases (e.g. Osteoarthritis–OA). Viscosupplementation has been found to improve the visco-elastic properties of the synovial fluid encouraging lubrication, shock absorbance, the formation of a semi-permeable barrier, decreasing both inflammatory and proliferative responses that occur within diseased joints. It has been found that pain reduction lasts considerably longer than the half life of injected HA, suggestive that the high molecular weight HA injections potentially may have disease modifying activity (Goldberg & Bulkwater,

2005). However, the mechanisms of action of high Mwt HA in viscosupplementation at present are unclear but potential anti-inflammatory, anti-angiogenic, immuno-suppressive effects with abilities to retard differentiation and impede cartilage matrix catabolism by certain catabolites [*e.g. IL-1 (Yasui et al., 1992, Monfort et al. 2005) and fibronectin fragments (Kang et al., 1999)*] have been implied for high Mwt HA in the literature.

The original objectives of the present study were to examine the effects different molecular weight hyaluronans (*ranging from 5kDa – 3000^{+}kDa*) had on: (1) normal neocartilage graft metabolism, and (2) IL-1 induced neocartilage graft matrix degradation utilising an *in vitro* Transwell culture system. However, under the experimental conditions used, minimal IL-1 effects were observed but interesting data was obtained from the 'Control' experimentation in this study. Thus, the focus of experimentation in this chapter will be the effects of different Mwt HA on Transwell cartilage graft metabolism.

5.1 Methods

5.1.1 Neocartilage graft Transwell culture protocol

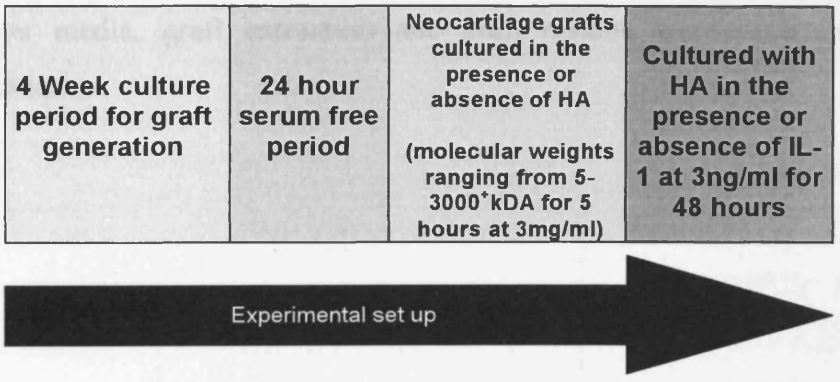

Figure 5.1. Schematic illustrating the culture protocol used to study the effect of different molecular weight hyaluronan on neocartilage grafts produced in Transwell culture.

A schematic showing the different culture times used to study the effects of different molecular weight hyaluronan (*ranging from 5kDa to 3000^{+}kDa*) on cartilage graft metabolism in the presence or absence of 3ng/ml IL-1 is shown in **Figure 5.1**. In brief, neocartilage grafts were produced using an immature bovine chondrogenic source following 4 weeks of Transwell culture as described in chapter 2.2. In most experiments these were then cultured for 24 hours in serum free media. They were then cultured for 5 hours in the absence or presence of 3mg/ml of varying molecular weight HA, followed by a 48 hour culture in the absence or presence of IL-1 (3ng/ml) and in the presence or absence of 3mg/ml HA of different molecular weights. In these experiments low Mwt HA is defined as that being lower than the size of endogenous HA (i.e. 500-1000kDa (Hardingham & Fosang, 1992) and high Mwt HA as that greater than that of endogenous HA.

5.1.2 Biochemical Analysis

Transwell culture media (*both inner and outer well*) was harvested at 48 hours and analysed for sulphated glycosaminoglycan (s-GAG) as described in section 2.5.1. Proteoglycan content was extracted from the neocartilage grafts with 4M GuHCl for 48 hours, the extract was dialysed and s-GAG determined. The remaining neocartilage residue following proteoglycan extraction was papain digested and s-GAG was determined. Percentages of s-GAG in replicate cultures (n=2 or n=3) of inner and outer media, graft extraction and graft residue were determined and subjected to statistics.

5.2 Results

5.2.1 Evaluation of how the addition of different molecular weight hyaluronan (5kDa and 3000^{+}kDa) affected normal graft metabolism and IL-1 induced neocartilage graft matrix degradation using an *in vitro* Transwell culture system.

The addition of an extremely low molecular weight (5kDa) hyaluronan (HA) to neocartilage tissue generated in Transwell cultures, resulted in a significant increase ($P<0.01$) of sulphated glycosaminoglycan (s-GAG) released into culture media (*both inner and outer wells*) in comparison to the Control (**Table 5.1**). In contrast, addition of a very high molecular weight (3000^{+}kDa) hyaluronan had an opposite effect of 5kDa HA and significantly decreased ($P<0.001$) percentage s-GAG released to culture media compared to the Control (**Table 5.1**).

TREATMENT	% Total GAG		
	INSERT MEDIA	**OUTSIDE MEDIA**	**TOTAL (Insert & Outside Media)**
Control	20.35 ± 1.28	6.30 ± 0.37	26.66 ± 1.64
Control + 5kDa HA	25.76 ± 3.44	11.02 ± 1.49 (**)	36.78 ± 2.15 (**)
Control + 3000^{+}kDa HA	3.22 ± 0.94 (***)	1.30 ± 0.34 (**)	4.52 ± 1.27 (***)
IL-1	22.58 ± 0.29	6.30 ± 0.20	28.88 ± 0.10
IL-1 + 5kDa HA	27.77 ± 1.32	9.15 ± 0.52	36.92 ± 1.80 (**)
IL-1 + 3000^{+}kDa HA	3.69 ± 0.2 (P=0.08)	2.62 ± 0.48 (*)	6.31 ± 0.25 (***)

Table 5.1. Effects of different molecular weight hyaluronan on proteoglycan release to media from neocartilage graft extracellular matrices in the presence or absence of IL-1 (n=3). Data presented as mean ± SEM. All data compared relative to Control, significance was assumed where $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

The percentage s-GAG retained within Control neocartilage grafts was 73.3% ± 1.69. The addition of an extremely low molecular weight (5kDa) hyaluronan significantly reduced sulphated glycosaminoglycan retention within grafts (**Table 5.2**). Interestingly, addition of the very high molecular weight (3000^{+}kDa) hyaluronan to grafts significantly increased sulphated glycosaminoglycan retention by ~20% compared to the Control (**Table 5.2**).

TREATMENT	% Total GAG		
	4M GUANIDINE	PAPAIN DIGEST	TOTAL RETAINED IN GRAFTS
Control	56.28 ± 2.95	17.07 ± 1.34	73.34 ± 1.69
Control + 5kDa HA	45.63 ± 3.71	17.59 ± 1.86	63.22 ± 2.15 (**)
Control + 3000^{+}kDa HA	78.87 ± 6.59 (**)	16.61 ± 5.60	95.48 ± 1.27 (***)
IL-1	57.31 ± 0.37	13.82 ± 0.28	71.12 ± 0.10
IL-1 + 5kDa HA	46.99 ± 1.60	16.09 ± 0.42	63.08 ± 1.80 (**)
IL-1 + 3000^{+}kDa HA	74.11 ± 0.64 (*)	19.58 ± 0.42	93.69 ± 0.25 (***)

Table 5.2. Effects of different molecular weight hyaluronan on proteoglycan retention in neocartilage graft extracellular matrices in the presence or absence of IL-1 (n=3). Data presented as mean ± SEM. All data compared relative to Control, significance was assumed where $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

Similar results were obtained in duplicate experiments [*Experiment 1 shown above (n=3) and Experiment 2 (n=2)*], a summation of the data from these two experiments is shown in **Figure 5.2**. It was apparent that IL-1β at 3ng/ml resulted in minimal or no effect on inducing increased graft matrix degradation following a 48hour treatment period, due to the concentration of IL-1β used being too low i.e 3ng/ml. Consequently, all IL-1 data in the following experiments of this chapter are not reported because no effects were observed due to the low concentrations.

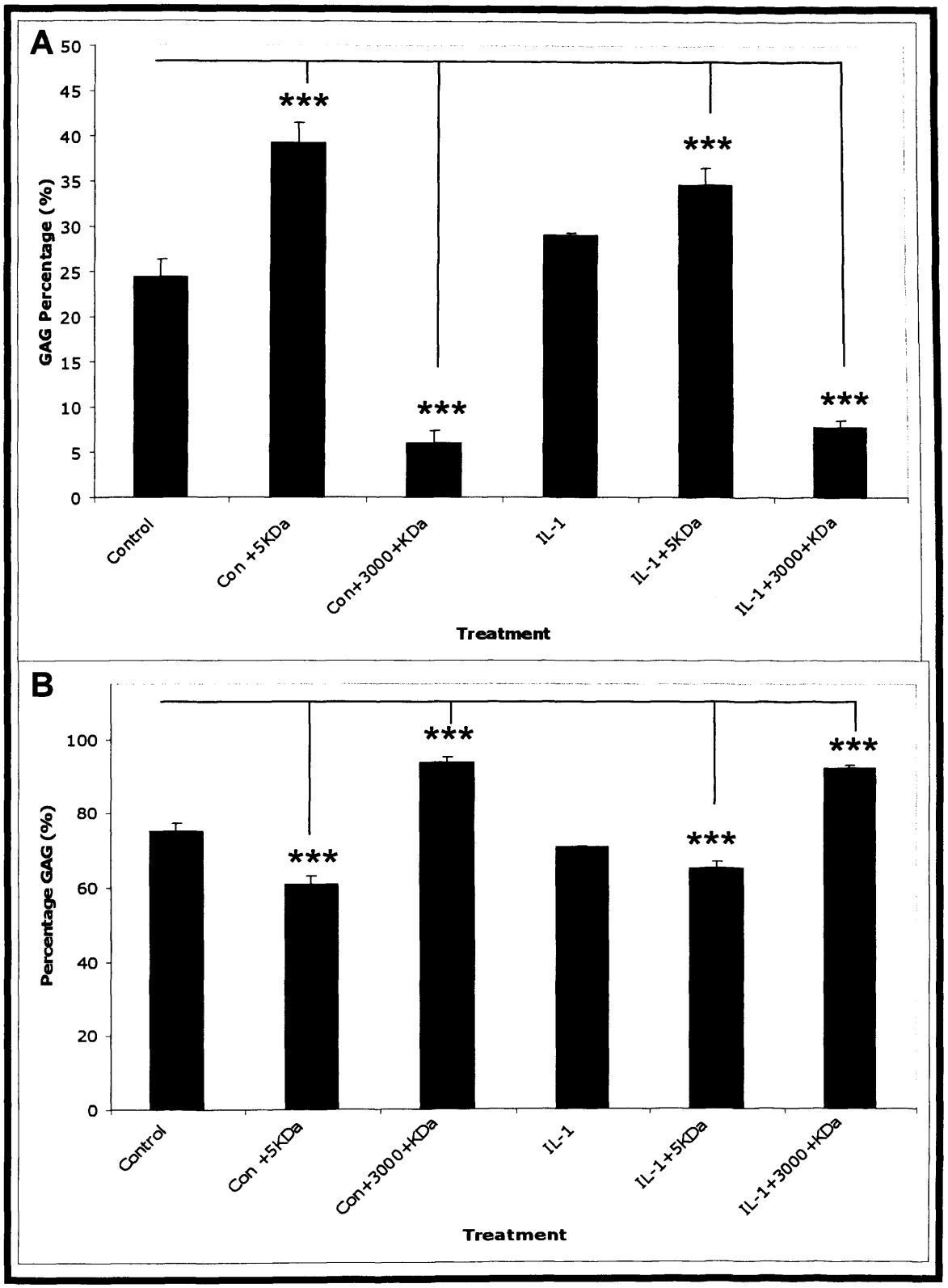

Figure 5.2. Sulphated glycosaminoglycan **(A)** released to culture media and **(B)** retained in neocartilage graft matrices, following the addition of an extremely low (5kDa) and very high (3000+kDa) molecular weight hyaluronan (n=5). Data presented as mean ± SEM. All data compared relative to Control, significance was assumed where $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

The addition of an extremely low molecular weight (5kDa) HA to neocartilaginous graft tissue significantly increased ($P<0.001$) the release of percentage s-GAG to culture media (*to both inner and outer wells*) in comparison to the Control (**Figure 5.2 (A)**). The increased amounts of s-GAG released to culture media in the presence of 5kDa HA suggest that a considerable amount of aggrecan (*a major proteoglycan of cartilage*) within the graft extracellular matrix (ECM) must be present as non-link protein stabilised aggregates; i.e. the 5kDa HA can compete for binding and retention with some of the aggrecan aggregates within the ECM thereby facilitating the 'extraction' or release of aggrecan from the graft tissue.

Addition of a very high molecular weight hyaluronan (3000^{+}kDa) significantly increased ($P<0.001$) the retention of sulphated GAG within graft extracellular matrices (**Figure 5.2 (B)**). Thus, the addition of high molecular weight HA appears to cause retention of aggrecan within the tissue. This suggests that the high molecular weight HA is able to diffuse into the graft ECM and entrap the aggrecan monomers in very large aggrecan aggregates that remain within the graft tissue. Whether or not these larger aggregates were link protein stabilised was not determined in these experiments.

5.2.2 Addition of either a very low molecular weight (100kDa) or high molecular weight (2200kDa) hyaluronan to Transwell cultured neocartilage grafts and their effects on graft metabolism.

The addition of a very low molecular weight (100kDa) hyaluronan to Transwell grafts, showed a significant increase of s-GAG release (**Table 5.3 & Figure 5.3 (A)**) to culture media (inner and outer well) and decreased s-GAG retention within neocartilage grafts compared to the Control (**Table 5.4 & Figure 5.3 (B)**). Therefore 100kDa HA appears to have similar affects on neocartilage grafts as the extremely low molecular weight (5kDa) HA described earlier. The addition of a high molecular weight HA (2200kDa) significantly decreased s-GAG release to culture media (inner and outer well) [**Table 5.3 & Figure 5.3 (A)**] and an increased s-GAG retention within grafts (**Table 5.4 & Figure 5.3 (B)**), similar to that observed with 3000^{+}kDa hyaluronan.

TREATMENT	% Total GAG		
	INSERT MEDIA	OUTSIDE MEDIA	TOTAL (Insert & Outside Media)
Control	9.13 ± 0.94	5.92 ± 0.32	15.05 ± 1.23
Control + 100kDa HA	12.79 ± 0.75	9.28 ± 0.38	22.07 ± 1.12 (**)
Control + 2200kDa HA	7.24 ± 0.55	1.50 ± 0.06	8.74 ± 0.48 (**)

Table 5.3. Effects of different molecular weight hyaluronan on proteoglycan release from neocartilage graft extracellular matrices in the absence of IL-1 (n=3). Data presented as mean ± SEM. All data compared relative to Control, significance was assumed where $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

TREATMENT	% Total GAG		
	4M GUANIDINE	PAPAIN DIGEST	TOTAL RETAINED IN GRAFTS
Control	77.15 ± 2.9	7.8 ± 1.74	84.95 ± 1.23
Control + 100kDa HA	70.61 ± 1.41	7.32 ± 1.31	77.93 ± 1.12 (**)
Control + 2200kDa HA	84.32 ± 1.26	6.94 1.10	91.26 ± 0.48 (**)

Table 5.4. Effects of different molecular weight hyaluronan on proteoglycan retention in neocartilage graft extracellular matrices in the absence of IL-1 (n=3). Data presented as mean ± SEM. All data compared relative to Control, significance was assumed where $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

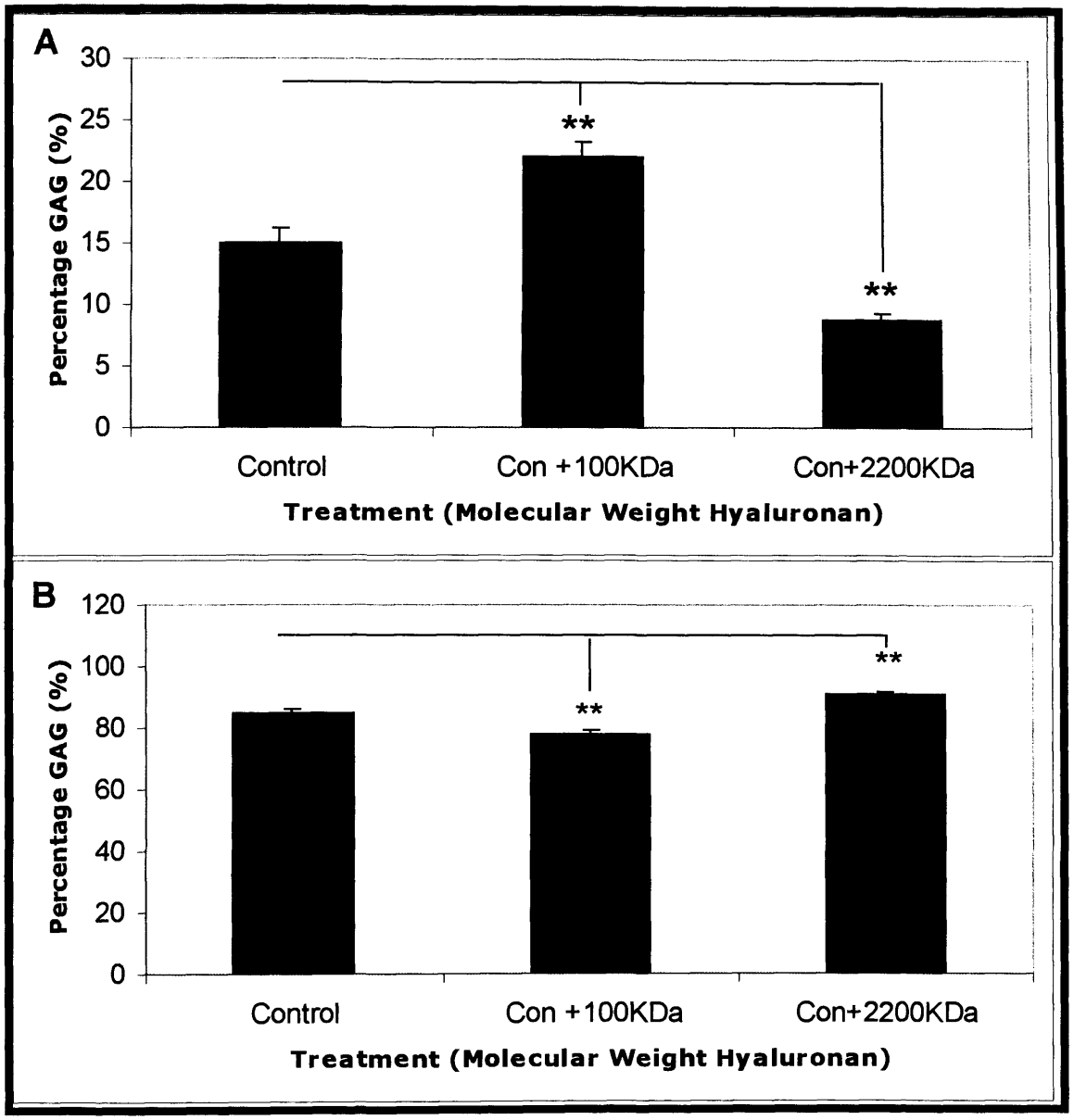

Figure 5.3. Sulphated glycosaminoglycan **(A)** released to culture media and **(B)** retained in neocartilage graft matrices, following the addition of a very low (100kDa) and high (2200kDa) molecular weight hyaluronan (n=3). Data presented as mean ± SEM. All data compared relative to Control, significance was assumed where $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

5.2.3 Addition of either a low molecular weight (250kDa) or high molecular weight (1600kDa) hyaluronan to Transwell cultured neocartilage grafts and their effects on graft metabolism.

The addition of either 250kDa or 1600kDa molecular weight hyaluronan, to neocartilage graft tissue generated in a Transwell culture system, had no significant effect on sulphated glycosaminoglycan retention within grafts (**Tables 5.5, 5.6 & Figure 5.4**).

TREATMENT	% Total GAG		
	INSERT MEDIA	OUTSIDE MEDIA	TOTAL (Insert & Outside Media)
Control	10.57 ± 0.42	10.87 ± 1.03	21.44 ± 1.12
Control + 250kDa HA	12.37 ± 1.19	13.29 ± 0.31	25.65 ± 1.33
Control + 1600kDa HA	9.98 ± 1.28	11.70 ± 0.72	21.68 ± 1.89

Table 5.5. Effects of different molecular weight hyaluronan on proteoglycan release to Transwell culture media from neocartilage graft extracellular matrices in the absence of IL-1 (n=3). Data presented as mean ± SEM. All data compared relative to Control, significance was assumed where P<0.05 (*), P<0.01 (**) or P<0.001 (***).

TREATMENT	% Total GAG		
	4M GUANIDINE	PAPAIN DIGEST	TOTAL RETAINED IN GRAFTS
Control	64.53 ± 1.94	14.03 ± 1.5	78.56 ± 1.12
Control + 250kDa HA	58.16 ± 3.78	16.19 ± 2.73	74.35 ± 1.33
Control + 1600kDa HA	62.9 ± 2.43	15.42 ± 0.56	78.32 ± 1.89

Table 5.6. Effects of different molecular weight hyaluronan on proteoglycan retention in neocartilage graft extracellular matrices in the absence of IL-1 (n=3). Data presented as mean ± SEM. All data compared relative to Control, significance was assumed where P<0.05 (*), P<0.01 (**) or P<0.001 (***).

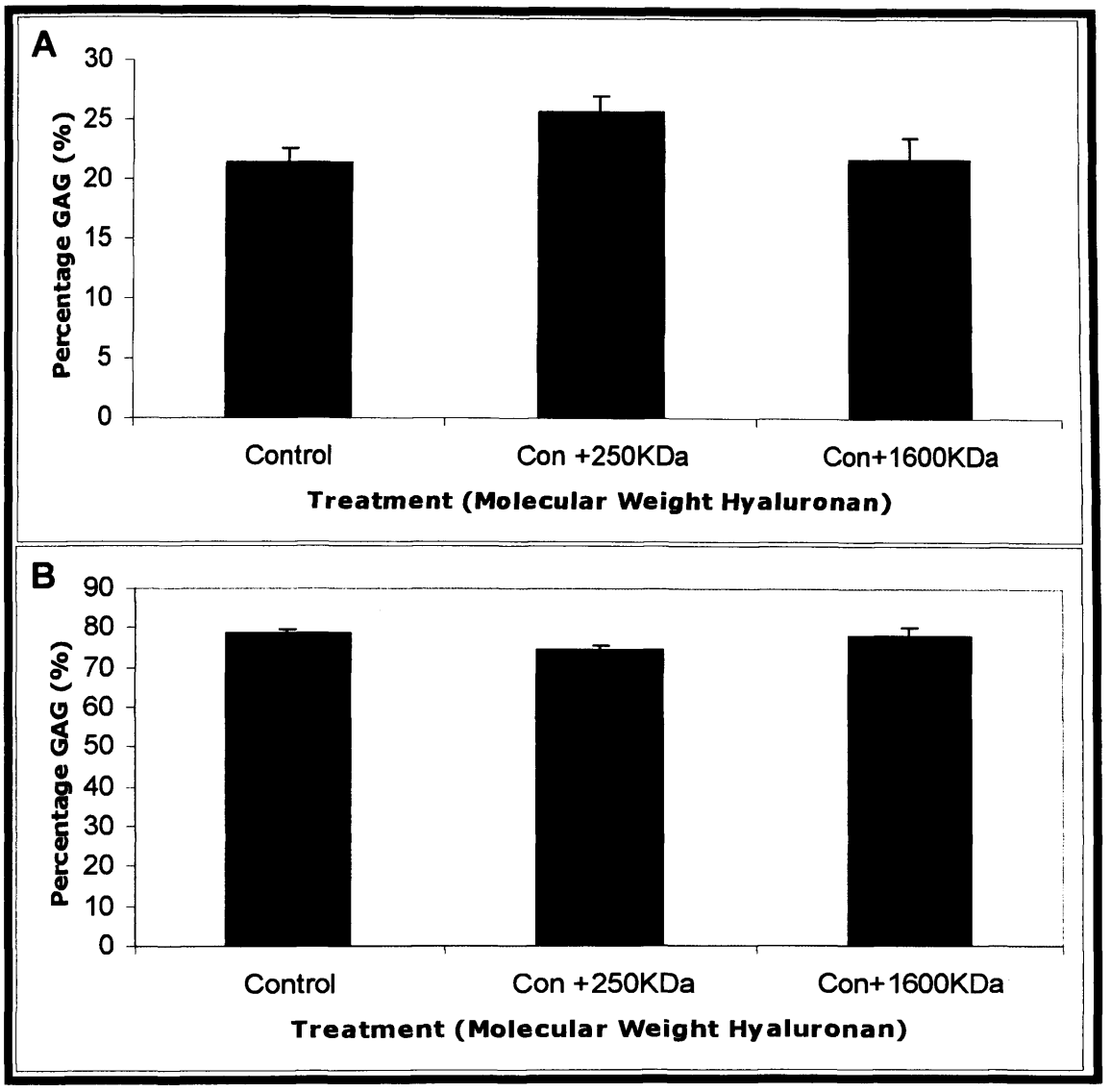

Figure 5.4. Sulphated glycosaminoglycan **(A)** released to culture media and **(B)** retained in neocartilage graft matrices, following the addition of low (250kDa) and high (1600kDa) molecular weight hyaluronan (n=3). Data presented as mean ± SEM. All data compared relative to Control, significance was assumed where $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

5.3 Discussion

During this study, interesting information as to possible mechanisms of action of exogenously added HA to Transwell neocartilage grafts, and its effects on cartilage / chondrocyte metabolism were concluded. The proteoglycan 'aggrecan' is one of the major constituents of native adult articular cartilage. Aggrecan monomers non-

covalently associate with a central filament of hyaluronan, self assembling into supramolecular aggregates that are stabilised by a glycoprotein referred to as link protein. The endogenous hyaluronan chains present within aggrecan aggregates in articular cartilage typically are of molecular weight ranging between 500 - 1000kDa (Hardingham & Fosang, 1992). The exogenous addition of different molecular weight hyaluronan to neocartilage grafts in Transwell systems that ranged above and below the typical molecular weight of endogenous hyaluronan found in aggrecan aggregates of native articular cartilage were found to have opposing effects.

The addition of hyaluronan to Transwell cultured grafts that had lower molecular weights than the typical *Mr* of endogenous HA found in native aggrecan aggregates (i.e. 5kDa, 100kDa & 250kDa HA) resulted in increased release of s-GAG to culture media (inner and outer wells) compared to experimental Controls (**Table 5.7 & Figure 5.5**). This result is consistent with the theory that 'smaller' molecular weight hyaluronan compete for newly synthesised and existing aggrecan that was either free or present in non-link protein stabilised aggregates, thereby facilitating the 'extraction' or 'release' of these aggrecan / smaller HA aggregates from graft extracellular matrices into the media of the Transwell cultures. However, the addition of HA with similar molecular weight (i.e. 1600kDa) to that found in aggrecan aggregates releases a similar amount of percentage s-GAG into culture media as the experimental Controls (**Table 5.7 & Figure 5.5**).

In contrast, addition of HA to neocartilage grafts of a significantly higher molecular weight (i.e. 2200kDa and 3000^{+}kDa HA) than the typical molecular weight of endogenous HA found in aggrecan aggregates, showed decreased s-GAG release to media, and increased s-GAG retention within graft extracellular matrices compared to experimental Controls (**Table 5.7 & Figure 5.5**). This implies that the larger molecular weight hyaluronan can diffuse into the graft matrices and more efficiently 'trap' newly synthesised and endogenous aggrecan in HA-aggrecan aggregates preventing the diffusion of this pool of aggrecan out of the cartilage graft ECM.

These results demonstrate that very high molecular weight HA can diffuse into the cartilage graft ECM and help to entrap newly synthesised and non-link protein stabilised 'free aggrecan' in its ECM. Thus the clinical use of HA in viscosupplementation procedures for OA patients may be facilitating the retention of newly synthesised 'repair aggrecan' in the pathological cartilage and thereby slowing down the rate of cartilage destruction through loss of proteoglycan from the tissue.

Molecular Weight Hyaluronan		% GAG Released to Media (*Minus IL-1β*)	Comment
5kDa	(1)	37 (27)	All hyaluronan of molecular weight <500kDa have increased % GAG released to media compared to experimental Controls
	(2)	43 (21)	
100kDa	(1)	22 (15)	
	(2)	27 (25)	
250kDa	(1)	26 (21)	
1600kDa	(1)	22 (21)	Similar to experimental Control values
2200kDa	(1)	9 (15)	All these (> 1600kDa) have decreased % GAG release to media compared to experimental Controls
	(2)	17 (25)	
3000+kDa	(1)	5 (27)	
	(2)	8 (21)	

Table 5.7. Sulphated GAG (%) released into Transwell culture media (inner and outer well) in the presence of different molecular weight HA in the absence of IL-1β. In each case the experimental number (1 or 2) is indicated and the appropriate Control for each experiment is in (brackets) next to the % GAG released to media.

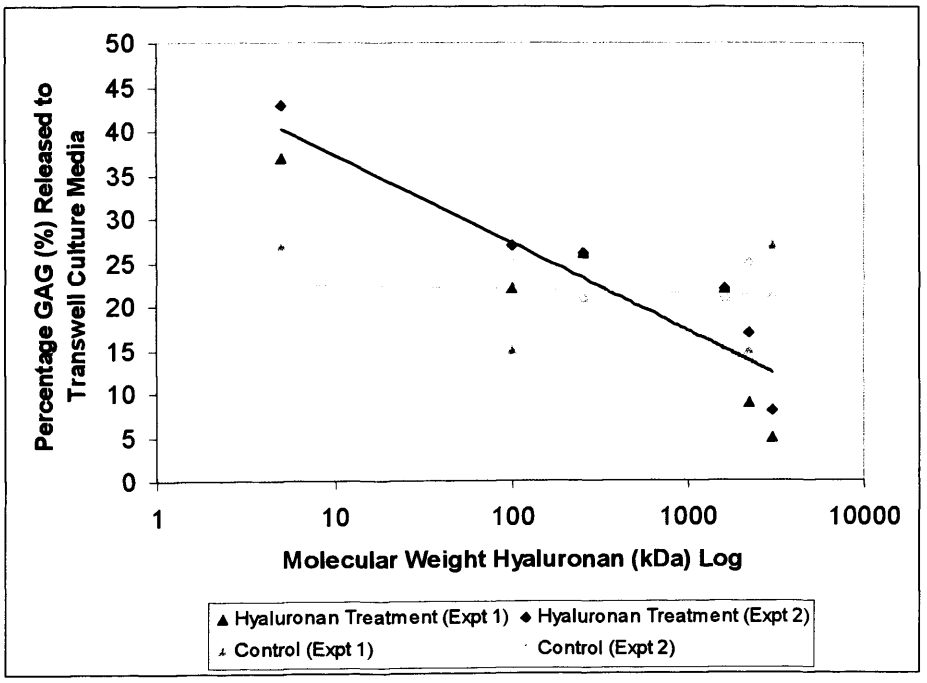

Figure 5.5. Sulphated GAG (%) released into Transwell culture media (inner and outer well) in the presence of different molecular weight HA in the absence of IL-1β. In each case the experimental number (1 or 2 as in Table 5.7) is indicated by the ▲ or ◆ symbols, respectively and the appropriate Control for each experiment is represented in grey.

All of these above conclusions were independent of the studies seeking to find out if the addition of varying molecular weight HA can abrogate IL-1β induced cartilage matrix degradation. Regarding the IL-1β component of the studies, it appears that 3ng/ml IL-1β had minimal but some effects on increasing GAG release to the media in some but not all experiments (data not shown). Where this increase was evident, there appeared to be an abrogation / reduction in the 'apparent' IL-1β induced GAG release with the addition of higher molecular weight hyaluronans (e.g. 2200 and 3000^{+}kDa). None-the-less, the protective mechanism of high molecular weight hyaluronan is unknown and will require further analysis prior to any conclusions being made.

The results of this study were obtained following a 48 hour incubation period of neocartilage grafts with the different molecular weight HA. A 24 hour incubation period appeared to be insufficient to elicit any s-GAG response, potentially a consequence of delayed aggregation. Bayliss *et al*, in 1983 reported an initial delay in the formation of link protein - stabilised aggrecan aggregates in human adult articular cartilage. At first the Bayliss results appeared to conflict with those previously found by Oegema (1980), who had suggested that synthesised proteoglycans in normal cartilage were just as capable of aggregation at 4 hours as they were at 18 hours. However, the experimental procedures in both reports determining the ability to form aggregates were different. Oegema used mixed disaggregated proteoglycans with exogenous hyaluronic acid, but Bayliss allowed re-aggregation in the presence of endogenous hyaluronic acid and link protein.

Overall, these results show similarities to recent publications, in that low molecular weight hyaluronan is more likely to have detrimental effects on extracellular matrices (Solursh *et al.*, 1980, Stern *et al.*, 2006, Iacob & Knudson, 2006, Sugahara *et al.*, 2006) and high molecular weight HA tend to have beneficial effects (Kang *et al.*, 1999, Julovi *et al.*, 2004, Monfort *et al.*, 2005).

5.4 Chapter Summary

- Interesting results were observed with 'Control (absence of IL-1)' Transwell cultures exposed to different molecular weight hyaluronan for 48 hours. The

IL-1 data has been omitted from this chapter as IL-1 at 3ng/ml resulted in minimal or no effect on inducing increased graft matrix degradation following a 48 hour treatment period, due to the concentration of IL-1β used being too low.

- When neocartilage grafts were subjected to exogenous hyaluronan of a smaller Mwt (i.e. 5kDa, 100kDa & 250kDa HA) than the typical *Mr* of endogenous HA (500kDa–1000kDa range) found in native aggrecan aggregates, an increased release of s-GAG to Transwell media and decreased s-GAG retention within neocartilage grafts was observed. This is suggestive that the 'smaller' molecular weight hyaluronan compete for newly synthesised and existing aggrecan that was either free or present in non-link protein stabilised aggregates, thereby facilitating the 'release' of these aggrecan / smaller HA aggregates from graft extracellular matrices into the media of the Transwell cultures.

- HA of Mwt 1600kDa (slightly larger than endogenously produced HA) had no effect on increasing the s-GAG content of the Transwell culture media.

- In contrast, addition of HA to neocartilage grafts of a significantly higher molecular weight (i.e. 2200kDa and 3000^{+}kDa HA) than the typical molecular weight of endogenous HA found in aggrecan aggregates, showed decreased s-GAG release to media, and increased s-GAG retention within graft extracellular matrices implying that the larger molecular weight hyaluronan can diffuse into the graft matrices and more efficiently 'trap' newly synthesised and endogenous aggrecan in HA-aggrecan aggregates preventing the diffusion of this pool of aggrecan out of the cartilage graft ECM.

- Therefore, the clinical use of HA in viscosupplementation procedures for OA patients may be facilitating the retention of newly synthesised 'repair aggrecan' in the pathological cartilage and thereby slowing down the rate of cartilage destruction through loss of proteoglycan from the tissue.

6.0 THE GENERATION OF TRANSWELL NEOCARTILAGE GRAFT TISSUE IN THE PRESENCE OF DIFFERENT MOLECULAR WEIGHT HYALURONAN.

In the previous chapter interesting changes in properties of Transwell neocartilage graft metabolism were observed from the addition of different molecular weight hyaluronan to cartilage grafts following their generation in Transwell culture systems. This chapter describes related studies where cartilage grafts were produced over a 4 week culture period whilst in the presence of different molecular weight hyaluronan with a view to investigating potential beneficial effects of hyaluronan on cartilage graft metabolism for tissue engineering applications.

6.1 Methods

Cartilage grafts were produced in Transwell culture systems using an immature chondrogenic source as described in chapter 2.2. However, when chondrocytes were added to Transwells they were incubated in Dulbecco's Modified Eagle Medium (DMEM) containing 50μg/ml gentamycin and 20% heat inactivated FBS supplemented with 100μg/ml ascorbate and 5ng/ml TGFβ_2 (Control) or in the presence of added 3mg/ml hyaluronan of different molecular weights (i.e. 5kDa, 490kDa and 3000$^+$kDa). Media (inner and outer wells) was changed and harvested thrice weekly (as described in section 2.2). The harvested media was subjected to s-GAG analyses and stored at -20°C. Following a 4 week culture period, duplicate grafts were analysed for weight (n=2). One set was subjected to histological thickness studies and immunohistochemical labelling to assess the presence of commonly found cartilage extracellular matrix molecules (described in section 2.3). The other set was subjected to 4M GuHCl extraction for 48 hours, the extract was dialysed and s-GAG determined. The remaining neo-cartilage residue following proteoglycan extraction was both pepsin and papain digested and s-GAG was determined.

6.2 Results

6.2.1 Zonal Morphology of neocartilage graft tissue produced in the presence of different molecular weight hyaluronan.

Histological zonal organisation approximating to the characteristic surface, middle and deep regions found in native adult articular cartilage were overtly evident in the Control, 490kDa HA and 3000^{+}kDa HA treated neocartilage grafts, but not in the 5kDa HA treated graft (**Figure 6.1**). Grafts produced in the presence of the low molecular weight (5kDa) HA were less alcianophilic, suggestive of lower proteoglycan content in comparison to the other treatments. Interestingly, the 'mid-zone' of the graft treated with 3000^{+}kDa HA appeared to be thicker in comparison to the other treatments (**Figure 6.1**).

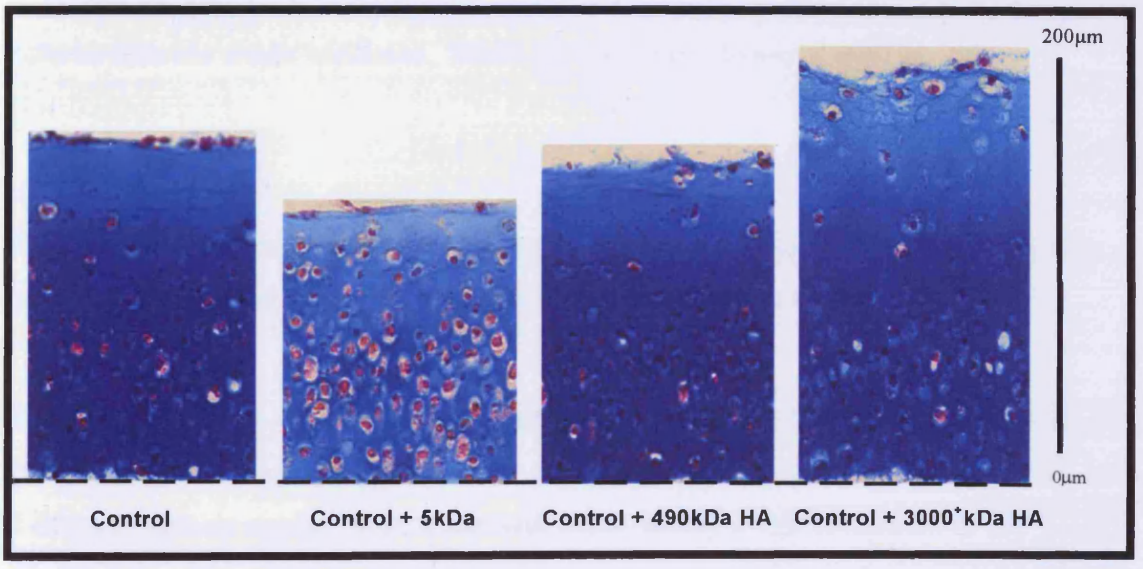

Figure 6.1. Effects of generating neocartilage grafts in the presence of different molecular weight hyaluronan upon the histological organisation of engineered constructs. Alcian blue and haematoxylin /eosin. Scale bar = 200µm.

The thinnest graft produced (~29% thinner compared to Control) was that treated with the 5kDa HA (**Table 6.1**). Neocartilage graft tissue produced in the presence of 490kDa HA was similar in weight to the Control treated graft, although it was

approximately 11% thinner than the Control (**Table 6.1**). The addition of a very high molecular weight (3000^{+}kDa) HA during graft production, resulted in an increase in graft weight (~47%) and graft thickness (10.9%) in comparison to the Control (**Table 6.1**). A potential explanation of these results could be that the extremely low molecular weight 5kDa HA is competing for and binding non-link protein stabilised aggregates present within the graft extracellular matrix, facilitating the release of very low Mwt link stabilised aggrecan HA aggregates from graft tissue into Transwell culture media.

Treatment	Average Graft Weight (*mg*) (n=2)	Graft Thickness (μM) (n=1)
Control	22.00 ± 2.30	188.71
Control + 5kDa HA	14.90 ± 4.10	133.42
Control + 490kDa HA	22.40 ± 8.40	168.00
Control + 3000^{+}kDa HA	32.35 ± 8.15	209.26

Table 6.1. Weights (n=2) and thicknesses (n=1) of neocartilage grafts produced in the presence of different molecular weight hyaluronan. Where applicable data represented as mean ± SEM.

6.2.2 Glycosaminoglycan metabolism in neocartilage graft tissue produced in the presence of different molecular weight hyaluronans.

Grafts produced in the presence of both 5kDa and 490kDa molecular weight HA resulted in an increased release of sulphated glycosaminoglycan (s-GAG) into Transwell culture media (both inner and outer wells) when compared to the Control (**Table 6.2**). The addition of the 490kDa HA had the greatest effect resulting in 5.23 fold increase in s-GAG release to media compared to the Control (**Table 6.2**). The presence of the very high molecular weight (3000^{+}kDa) HA showed a decrease in s-GAG released to media in comparison to the Control graft (**Table 6.2**). Following all treatments, more s-GAG was released to the inner insert well compared to the outer well of the Transwell culture system over the 4 weeks culture period. An exception was with neocartilage grafts generated in the presence of 490kDA HA, where slightly more s-GAG was released into the outer well (**Table 6.2**).

Transwell Culture Treatment	GAG (μg)			Total GAG release compared to Control (%)
	Inner Well	Outer Well	Total	
Control	731.68	388.76	1120.44	–
5kDa HA	1207.20	504.37	1711.57	52.8 ↑
490kDa HA	2868.94	2992.63	5861.57	423.4 ↑
3000^{+}kDa HA	520.13	438.03	958.16	14.5 ↓

Table 6.2. Sulphated glycosaminoglycan (μg) released to Transwell culture media (inner and outer well) over a 4 weeks duration by grafts produced in the presence of different molecular weight hyaluronan (n=1).

Neocartilaginous graft tissue produced in the presence of both 5kDa and 490kDa HA retained less s-GAG than Control treated grafts by approximately 70.2% and 7.4%, respectively (**Table 6.3 & Figure 6.2**). Grafts generated in the presence of a high molecular weight (3000^{+}kDa) HA showed a 122.45% increase in s-GAG retention compared to the Control (**Table 6.3**). The presence of all molecular weight hyaluronan treatments (i.e. 5kDa, 490kDa & 3000^{+}kDa) resulted in an increased synthesis of total s-GAG (**Table 6.3 & Figure 6.2**). Interestingly, grafts produced in the presence of 490kDa HA showed a massive increase (x3.7 fold) in total s-GAG synthesis compared to the Control, suggestive that 490kDa HA inclusion within Transwell culture systems enhances proteoglycan biosynthesis (**Table 6.3 & Figure 6.2**).

Transwell Culture Treatment	GAG (μg)					Total GAG compared to Control (%)
	Media Total	Total in 4M GuHCl	Residue		Total	
			Pepsin	Papain		
Control	1120.44	570.60	26.04	23.92	1741	100
5kDa HA	1711.57	144.45	15.47	24.86	1896.35	8.92 ↑
490kDa HA	5861.57	524.96	23.45	26.08	6436.06	269.68 ↑
3000^{+}kDa HA	958.16	1328.25	28.96	23.15	2338.52	34.32 ↑

Table 6.3. Total sulphated glycosaminoglycan (μg) and GAG retention (μg) within neocartilage grafts produced over a 4 weeks culture period using a Transwell system in the presence of different molecular weight hyaluronan (n=1).

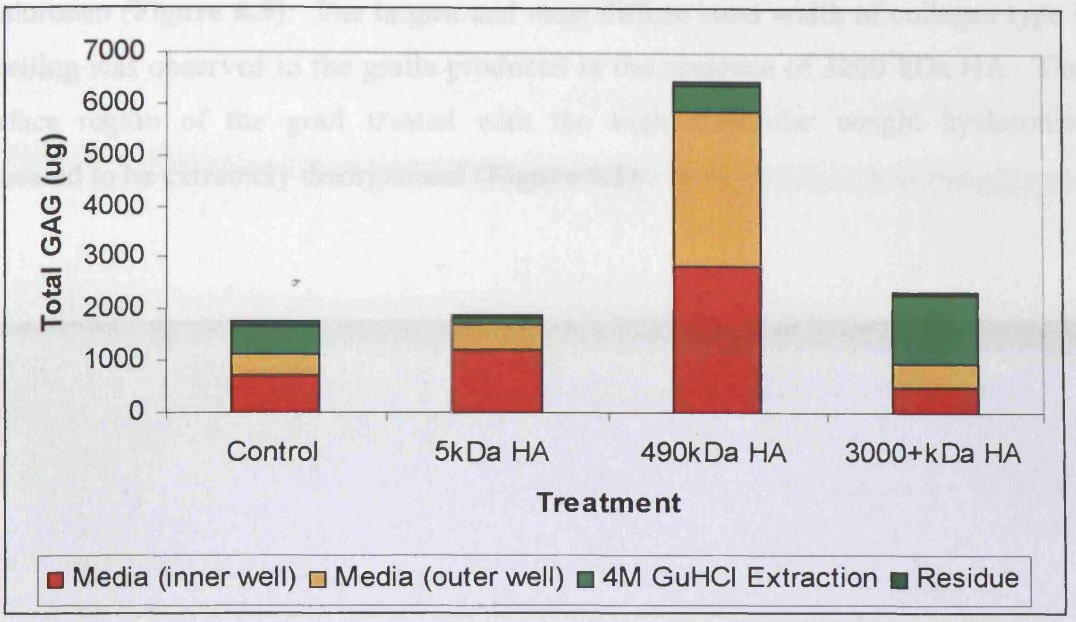

Figure 6.2. Total sulphated glycosaminoglycan (μg) generated over 4 weeks culture duration by grafts produced in the presence of different molecular weight hyaluronan utilising a Transwell system (n=1).

6.2.3 Biochemical and immunochemical composition of neocartilage graft extracellular matrices generated in the presence of different molecular weight hyaluronans.

Negative Controls showed no non-specific labelling with either primary or secondary antibodies (**Figure 6.11**). Immuno-histochemical labelling of grafts generated in the presence of varying molecular weight hyaluronan revealed that the graft matrix had many similarities to immature native articular cartilage. A summary of these findings are provided below.

A. Collagens

Collagen type I labelling was present at the surface and not in the underlying regions of all grafts regardless of treatment (**Figure 6.3**), showing similarities to native articular cartilage (**Figure 6.10A**). The band width of type I collagen labelling within the surface region of the grafts, increased with an increasing molecular weight

hyaluronan (**Figure 6.3**). The largest and most diffuse band width of collagen type I labelling was observed in the grafts produced in the presence of 3000^{+}kDa HA. The surface region of the graft treated with the high molecular weight hyaluronan appeared to be extremely disorganised (**Figure 6.3**).

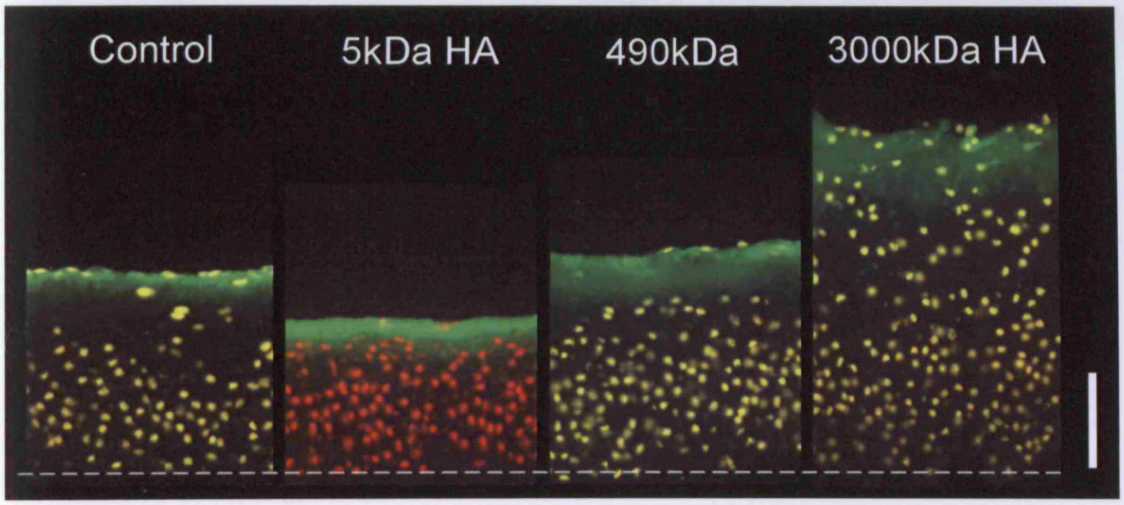

Figure 6.3. The effects of varying molecular weight hyaluronan present during graft generation on the distribution of Type I collagen labelled with polyclonal Ab #70-XR90 in neocartilage graft tissue. Scalebar represents 100μm.

Type II collagen labelling was present throughout graft matrices following all treatments (**Figure 6.4**) as is observed in native articular cartilage (**Figure 6.10B**), confirmatory of the tissues hyaline nature. However, type II collagen labelling appeared more intense in the surface and deep zones, with less labelling within the mid zone (**Figure 6.4**). This was more apparent with the addition of the higher molecular weight hyaluronan (490kDa & 3000^{+}kDa), potentially suggesting that the higher Mwt HA could be masking collagen type II labelling. However, all treatments were chondroitinase ABC and keratanase digested prior to labelling, eliminating this possibility showing that labelling is not affected by epitope masking.

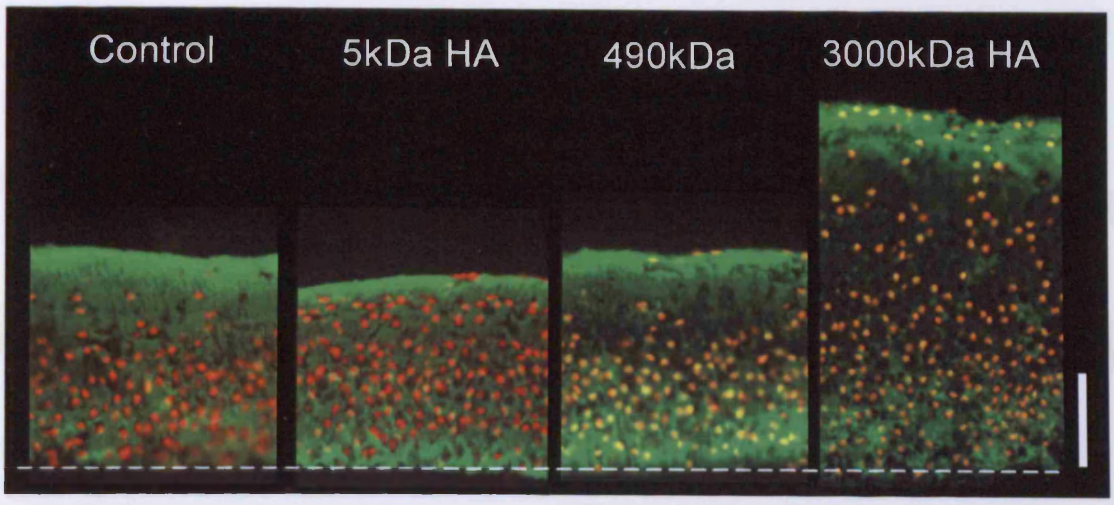

Figure 6.4. The effects of varying molecular weight hyaluronan present during graft generation on the distribution of collagen type II labelled with mAb CIICI in neocartilage tissue. Scalebar represents 100μm.

(B) Glycosaminoglycans

It would be expected that the chondroitin sulphate glycosaminoglycans would show similar distribution to aggrecan, suggestive of their association with the large proteoglycan. Chondroitin-0-sulphate (C-0-S) labelling on chondroitinase generated neoepitope 'CS-stubs' (Caterson *et al.*, 1990) were present throughout the whole of the grafts, especially in the graft produced in the presence of 5kDa HA (**Figure 6.5**). Although, labelling of all grafts show a more intense labelling within the deep zone that occurs to a lesser extent in the 5kDa HA treated graft. This shows similarities to native articular cartilage as the highest concentration of proteoglycan (i.e. aggrecan) in which CS associates is found within the deeper regions.

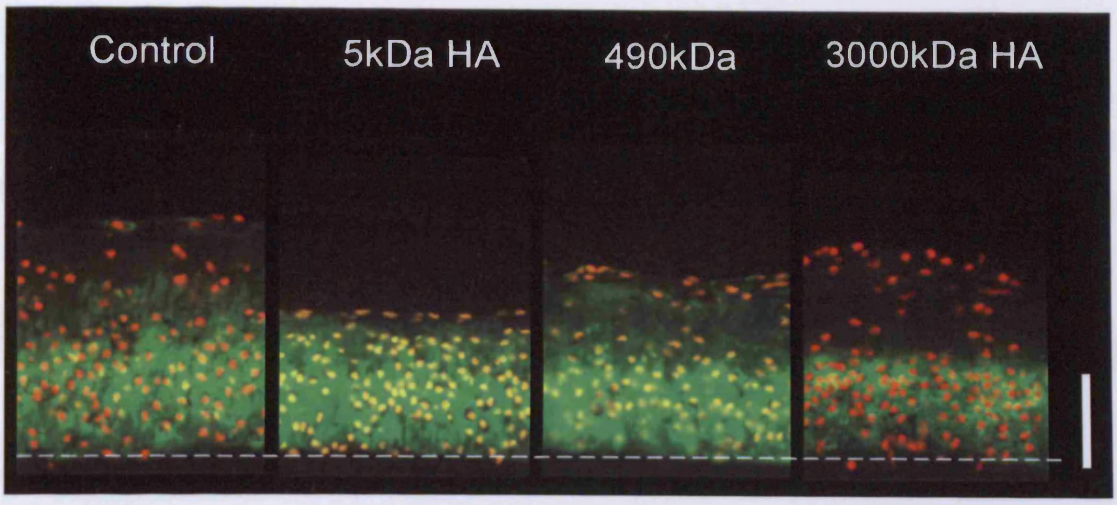

Figure 6.5. The effects of varying molecular weight hyaluronan present during graft generation on the distribution of GAG (C-0-S) labelled with mAb 1-B-5 in neocartilage tissue. Scalebar represents 100μm.

Immuno-labelling for chondroitin-4-sulphate (C-4-S) on chondroitinase generated neoepitope 'CS-stubs' was evident throughout the entire graft ECM, but appeared weaker in the presence of 5kDa HA (**Figure 6.6**). This is expected, as the GAG (CS) would show similar distribution to aggrecan suggestive of GAG association with large proteoglycans (**Figure 6.10G**).

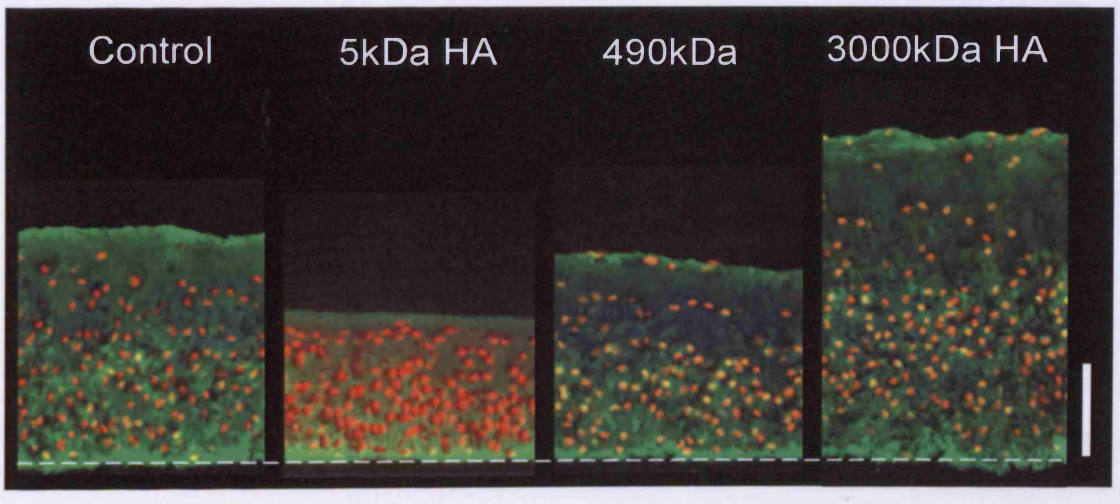

Figure 6.6. The effects of varying molecular weight hyaluronan present during graft generation on the distribution of GAG (C-4-S) labelled with mAb 2-B-6 in neocartilage graft tissue. Scalebar represents 100μm.

Chondroitin-6-sulphate (C-6-S) 'CS-stubs' showed weak labelling throughout graft tissue extracellular matrix regardless of treatment, with a more predominant staining within the deep region (**Figure 6.7**). This is an indicative of the immaturity of the neocartilage grafts and shows similar labelling as would be observed in immature native articular cartilage (**Figure 6.10H**).

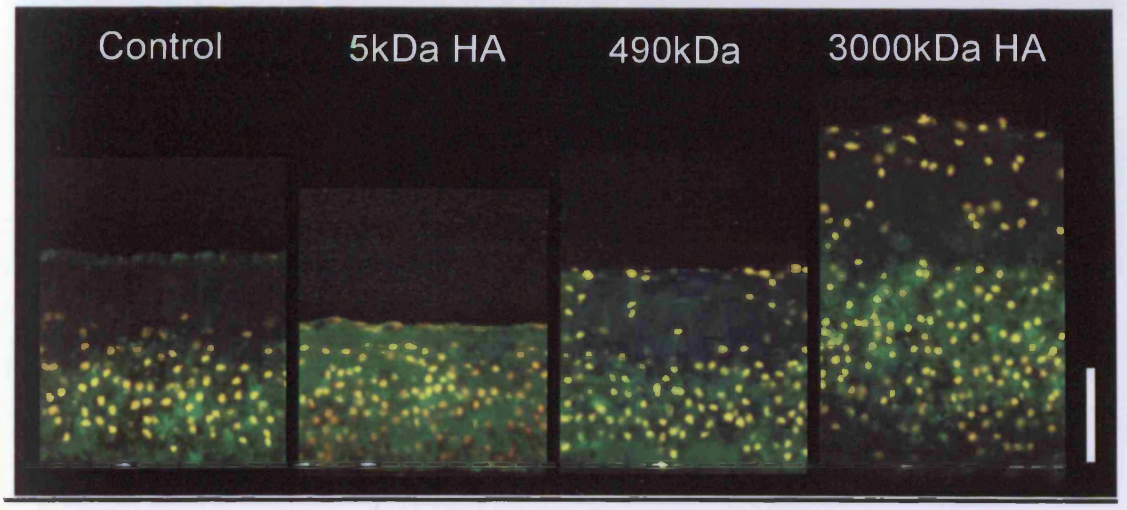

Figure 6.7. The effects of varying molecular weight hyaluronan present during graft generation on the distribution of GAG (C-6-S) labelled with mAb 3-B-3 in neocartilage tissue. Scalebar represents 100μm.

(C) Small Leucine Rich Proteoglycans (SLRPs)

The class I small leucine rich proteoglycans, decorin (**Figure 6.8**) and biglycan (**Figure 6.9**) were detectable throughout graft tissue. Both SLRPs, but decorin in particular, were more predominant in the surface zone of the tissue and diminished with depth, as occurs in native cartilage (**Figures 6.10C&D**) (Miosge *et al.*, 1994). The grafts generated in the presence of 3000^{+}kDa HA showed a more intense thicker band width labelling at the surface zone for both decorin and biglycan in comparison to the other treatments (**Figure 6.8** & **Figure 6.9**). The distribution of decorin is similar to that observed for Type I collagen (**Figure 6.3**) labelling, suggestive that decorin maybe associating with this collagen.

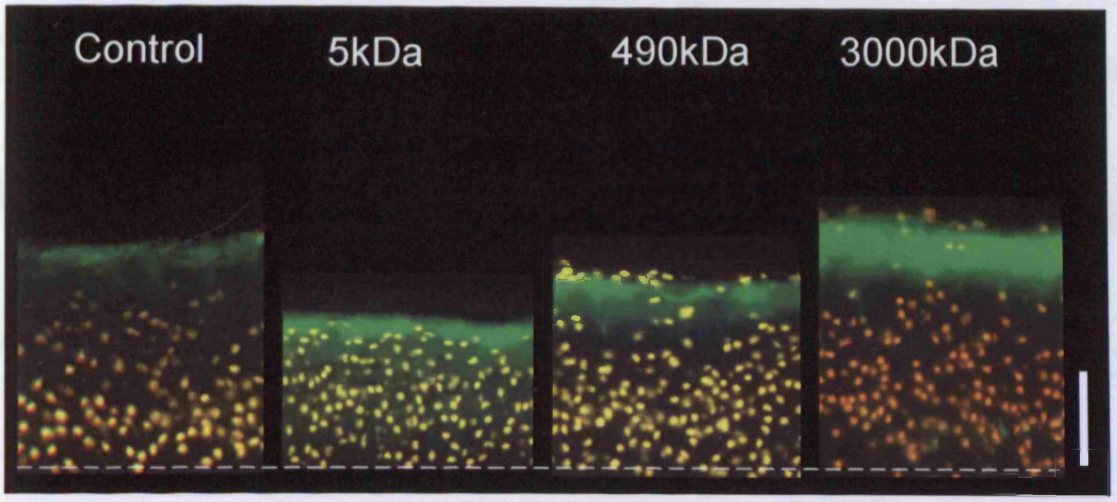

Figure 6.8. The effects of varying molecular weight hyaluronan present during graft generation on the distribution of decorin labelled with mAb 28.4 in neocartilage tissue. Scalebar represents 100μm.

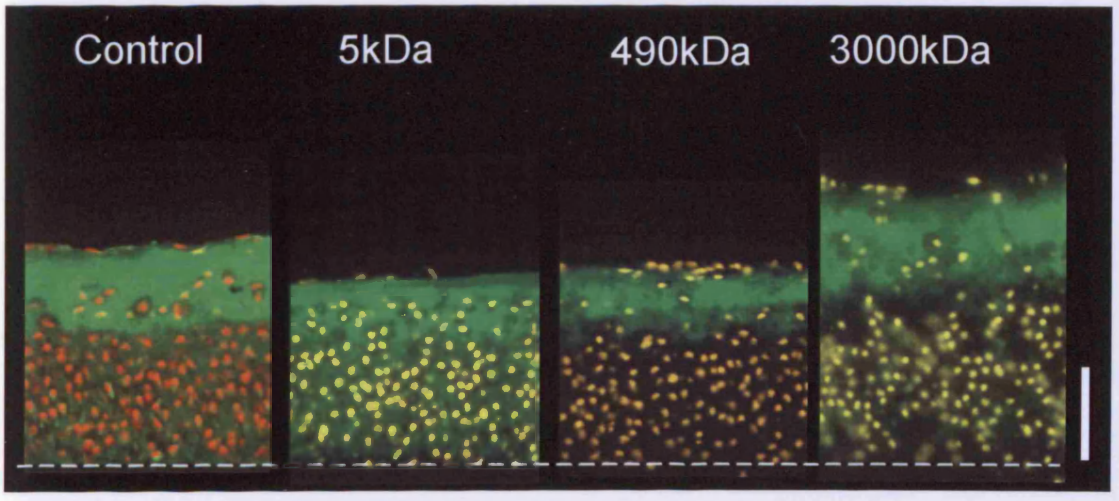

Figure 6.9. The effects of varying molecular weight hyaluronan present during graft generation on the distribution of biglycan labelled with mAb PR8A4 in neocartilage tissue. Scalebar represents 100μm.

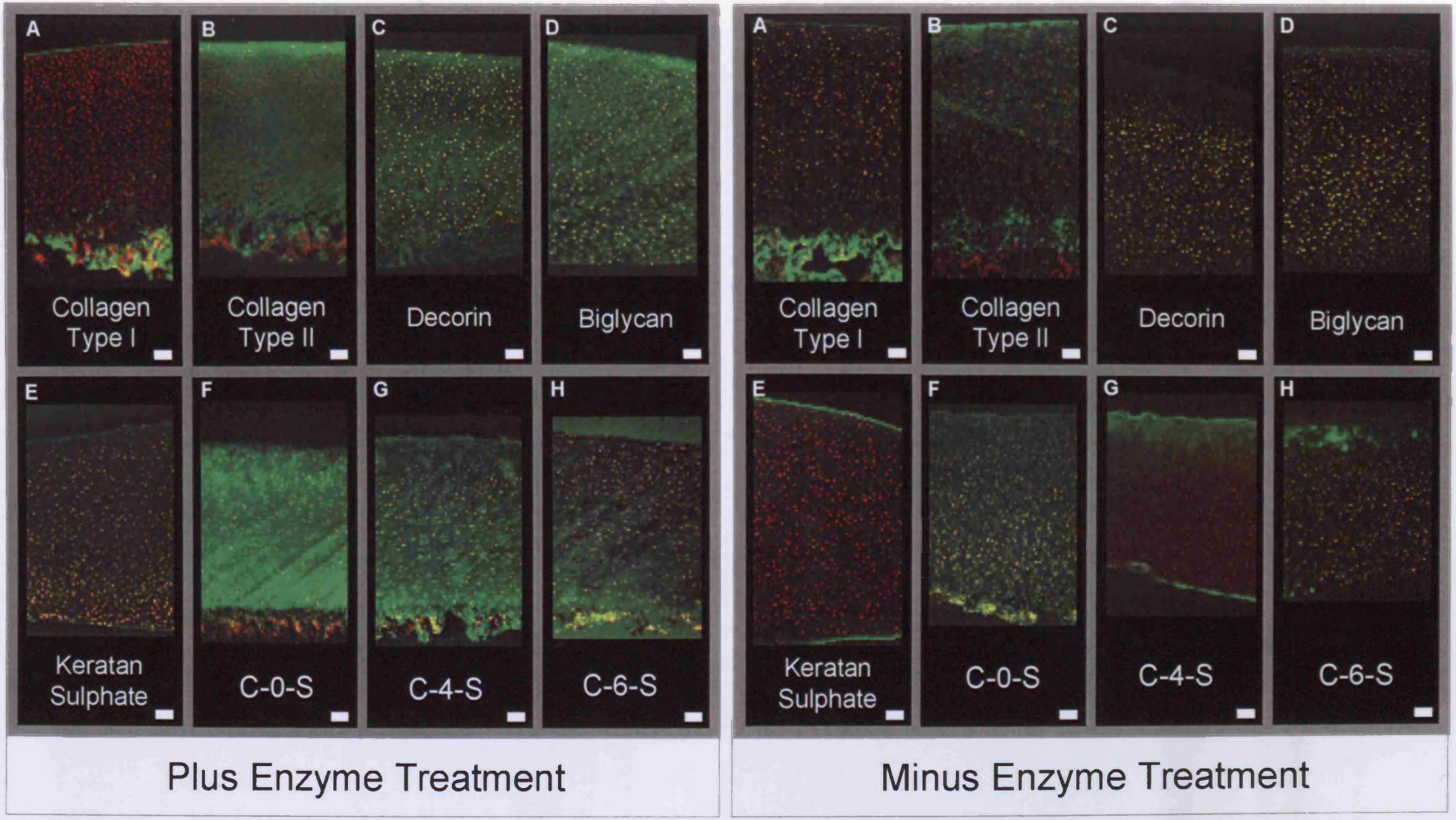

Figure 6.10. Positive Controls. Immunohistochemical composition of immature Native Bovine Articular Cartilage ECM. Sections have been labelled (green) with a panel of antibodies towards chief structural components of the extracellular matrix, i.e. Collagens (A&B), Proteoglycans (C&D) and glycosamnoglycans (E-H) in the presence or absence of chondroitinase enzyme. Cell nuclei have been counterstained red with propidium Iodide. C-0-S, Chondroitin-0-sulphate. C-4-S, Chondroitin-0-sulphate . C-6-S, Chondroitin-6-sulphate. Scalebars = 100μm.

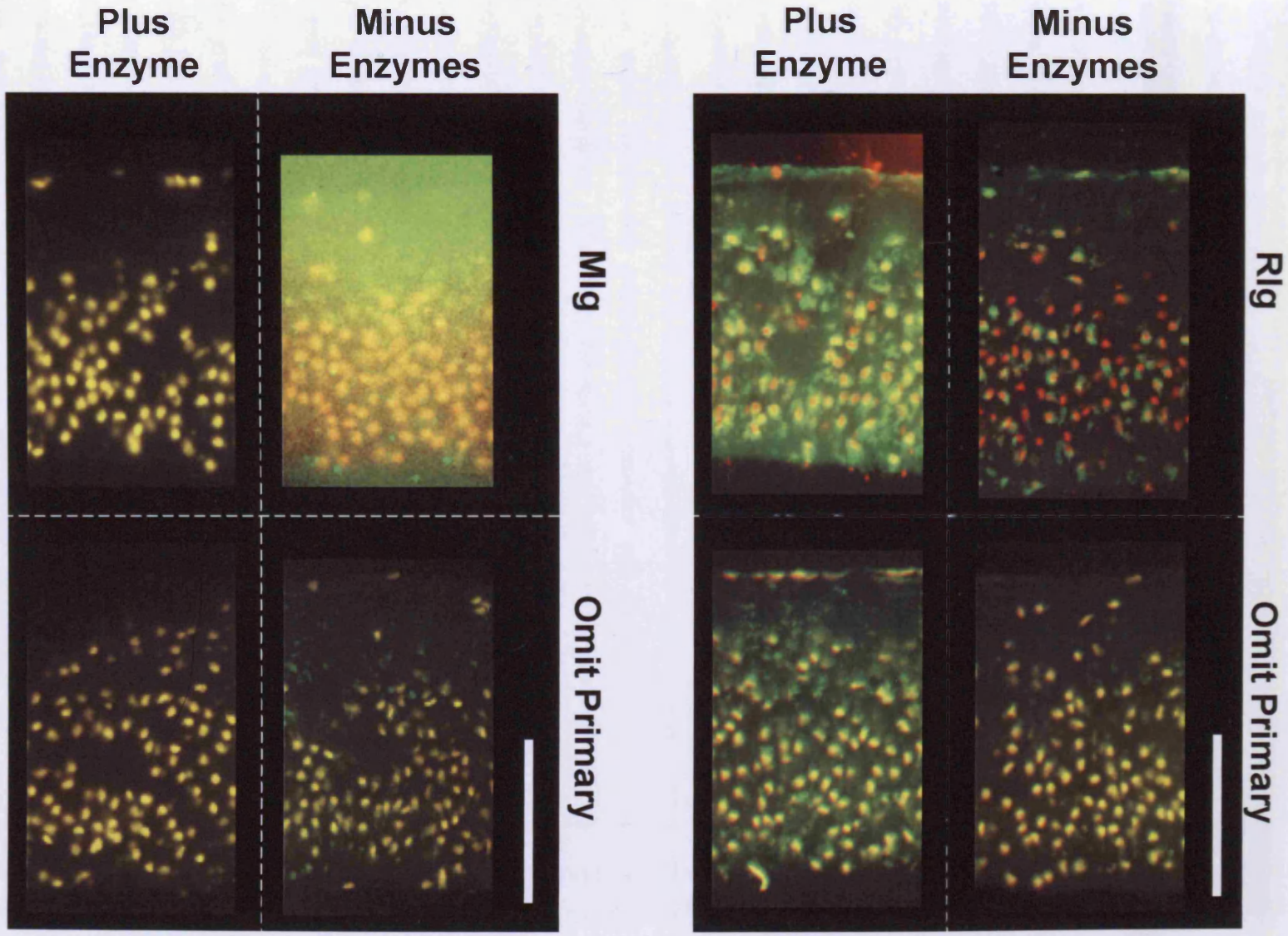

Figure 6.11. Negative Control neocartilage sections were incubated with PBS (Omit Primary), 10µg/ml mouse immunoglobulins (Mlg), or Rabbit immunoglobulins (Rig) instead of Primary antibody, with or without the enzymatic digestion step. Scalebar represents 100µm

6.3 Discussion

During this study, due to limited n number of replicates it was difficult to make definitive conclusions of any significance from the data obtained. However, grafts produced in the presence of different molecular weight hyaluronan utilising a Transwell culture system were found to have profound differences in graft extracellular matrix composition and biosynthetic capacity.

All treatments (i.e. Control, 490kDa HA and 3000^{+}kDa HA) with the exception of 5kDa HA produced grafts that were stratified into well-defined cartilaginous zones that approximated with the superficial, middle and deep layers of adult articular cartilage. However, neocartilage tissue generated in the presence of the 3000^{+} kDa HA appeared to have a thicker 'mid zone' in comparison to other treatments, with a more disorganised surface zone.

Immunofluorescent labelling patterns obtained with a panel of antibodies towards commonly found cartilage extracellular matrix molecules suggested that regardless of treatment, all grafts showed a similar immunohistochemical distribution of; (1) collagens (Types I and II); (2) Glycosaminoglycans (C-0-S, C-4-S and C-5-S); and (3) The small leucine-rich proteoglycans (decorin and biglycan) to immature native articular cartilage. However, increasing the Mwt of HA used in graft generation, resulted with less Type II collagen labelling within the 'mid region' of grafts, and an increased (more diffuse) labelling of Type I collagen, decorin and biglycan within the surface region of grafts.

Similar to the results found in the previous chapter, neocartilage grafts produced in the presence of an extremely low molecular weight (5kDa) HA caused increased levels of s-GAG (i.e. presumably proteoglycan (aggrecan)) to be released into the Transwell culture media (inner and outer wells) and less s-GAG to be retained within the graft. The measurements obtained for graft weight and thickness corroborated with the s-GAG results, as did the histological findings that grafts produced in the presence of 5kDa HA were the least alcianophilic. A potential explanation is that the 5kDa HA oligomers compete for aggrecan binding, resulting in the release of non-link protein stabilised aggrecan from graft extracellular matrices.

The presence of a very high molecular weight (3000^{+}kDa) HA resulted in the production of a more enriched graft, of increased weight and thickness compared to

all other treatments, with less s-GAG being released to media (inner and outer well) and more s-GAG being retained within grafts (as in previous chapter). The addition of the very high molecular weight HA appears to cause retention of aggrecan within the tissue. This is suggestive that the high molecular weight HA is able to diffuse into graft extracellular matrices and entrap the aggrecan monomer in very large aggrecan aggregates. Whether or not these larger aggregates were link protein stabilised was not determined in this experiment.

Interestingly, grafts produced in the presence of a hyaluronan (i.e. 490kDa) with a similar molecular weight to endogenous HA were similar in weight and thickness to Control treated grafts, but resulted in a x3.7-fold increase in the total s-GAG synthesised over a 4 weeks culture period. This particular molecular weight HA appeared to increase the anabolic process of neocartilage grafts and its inclusion in chondrocyte transplantation protocols may significantly improve cartilage repair in tissue engineering procedures. This study needs to be repeated with a larger range of different molecular weight HA that are less than and greater than the 490kDa HA.

As discussed previously in chapter 5, injections of high molecular weight HA are used clinically as an accepted visco-supplementation procedure to treat degenerative joint diseases (e.g. OA) to eliminate pain and reduce the rate of cartilage degradation. However, the mechanisms of action of this HA visco-supplementation treatment are at present unclear but potential anti-inflammatory and 'anabolic' effects of HA on chondrocyte / cartilage metabolism have been implied in the literature. Collectively, this data suggest that HA visco-supplementation procedures should incorporate the use of a mixture of HA Mwt sizes in the range of 490kDa - 3000^{+}kDa HA in order to provide optimal benefits of aggrecan retention and biosynthesis to OA patients receiving this treatment modality.

6.4 Chapter Summary

- Neocartilage grafts produced over a 4 week culture period in the presence of different molecular weight hyaluronan, support the previous conclusions obtained in chapter 5, where grafts were treated after they were generated with different molecular weight hyaluronan.

- Neocartilage grafts subjected to exogenous hyaluronan of a smaller Mwt (i.e. 5kDa) than the typical *Mr* of endogenous HA (500kDa–1000kDa range) found in native aggrecan aggregates, showed an increased release of s-GAG to Transwell media and decreased s-GAG retention within neocartilage grafts supported by graft weight thickness and histological findings. This is suggestive that the 'smaller' molecular weight hyaluronan compete for newly synthesised and existing aggrecan that was either free or present in non-link protein stabilised aggregates, thereby facilitating the 'release' of these aggrecan / smaller HA aggregates from graft extracellular matrices into the media of the Transwell cultures.

- In contrast, addition of HA to neocartilage grafts of a significantly higher molecular weight (i.e. 3000^{+}kDa HA) than the typical molecular weight of endogenous HA found in aggrecan aggregates, showed decreased s-GAG release to media, and increased s-GAG retention within graft extracellular matrices implying that the larger molecular weight hyaluronan can diffuse into the graft matrices and more efficiently 'trap' newly synthesised and endogenous aggrecan in HA-aggrecan aggregates preventing the diffusion of this pool of aggrecan out of the cartilage graft ECM.

- Interestingly and additionally, this data provides evidence that chondrocytes exposed to exogenous hyaluronan of similar molecular weight (i.e. 490kDa) to the typical *Mr* of endogenous HA (500kDa–1000kDa range) found in native aggrecan aggregates resulted in a marked effect on the rate and amount of aggrecan synthesised (3.7 fold greater) compared to Control grafts.

- Collectively, the data obtained from both chapters 5 and 6 suggest that a potential improvement upon HA visco-supplementation procedures would be to incorporate a mixture of Mwt HA ranging between 490kDa-3000^{+}kDa as

to provide optimal benefits of aggrecan retention and biosynthesis to OA patients receiving this treatment modality.

7.0 GENERAL DISCUSSION

There has been recent interest in cartilage repair strategies with a particular focus on cell based, tissue engineering approaches (Kim *et al.*, 2000, Risbud and Sittinger., 2002 and Kuo *et al.*, 2006). This involves the delivery of culture expanded autologous chondrocytes to a cartilage lesion site either; (1) in suspension under a sutured covering i.e. autologous chondrocyte implantation (ACI) (Brittberg *et al.*, 1994) or (2) utilising a biodegradable scaffold or carrier that provides a three-dimensional biomimetic environment that facilitates cell delivery (Hutmacher 2000, Hunziker 2001, Mahmoudifar and Doran., 2005, Geffre *et al.*, 2008). The implanted chondrocytes within the defect synthesise and lay down an extracellular matrix, generating tissue that to a certain extent resurfaces the site of injury and restores joint functionality (Brittberg *et al.*, 1994).

These cell based therapies are an improvement upon preceding cartilage repair strategies, such as microfracture (Steadman *et al.*, 2001) and mosaicplasty (Hangody *et al.*, 1997 & 1998). However, it has been reported that sutured membrane coverings plus the injected chondrocytes in some circumstances are lost to the synovial cavity during the recovery period (Driesang and Hunziker 2000). There are also concerns that cartilage resurfacing is limited and unsatisfactory integration occurs between native cartilage and the repair tissue. The reparative tissue generated, remains problematic, as it consists of mixed morphologies ranging between hyaline-like and fibro-like cartilage (Henderson *et al.*, 2007, Moriya *et al.*, 2007).

In 1995, Kandel *et al* reported a potentially improved approach over traditional cell based therapies, that involved engineering neo-cartilage grafts *ex vivo* (*from high density primary chondrocytes seeded onto either porous, collagen coated synthetic membranes or as biphasic constructs consisting of cartilaginous tissue generated upon a calcium polyphosphate substrate*) using a Transwell culture system and implanting the developed graft within a defect site (Kandel *et al.*, 1995). This strategy has potential advantages as; (1) there would be no requirement of a periosteal/collagen covering or a scaffold carrier as the graft would exist as a fully differentiated matrix; (2) the initial generation of cartilage grafts would be highly regulated in culture, as most non-gaseous graft metabolites produced throughout the culture duration can be monitored, which is impossible to achieve in a diseased or

damaged joint; and (3) potentially a larger degenerate area of the joint could be repaired and resurfaced, arthroscopically reducing a patients rehabilitation time.

The results of this study add to previous data (*e.g. chiefly arising from the Kandel laboratory*) showing that neo-cartilage grafts generated *ex vivo* from immature chondrocytes seeded at high cell densities in Transwell cultures produced opaque, hyaline tissues with potential to treat articular cartilage defects. All tissues generated from a primary immature chondrogenic source following just two weeks of culture were stratified into distinct zones approximating to the superficial, middle and deep regions. This morphological stratification became more apparent with increased time and cell seeding density.

In our laboratory, cell tracker studies eliminated the possibility that zone specific cell sorting contributed to zonal formation in neo-cartilage grafts as minimal inter-zonal chondrocyte movement occurred (Hayes *et al.*, 2007). The formation of the distinct strata in Transwell cultured grafts could potentially be explained by: (1) the effects subjected on the grafts due to the environment of the culture system, or (2) the generation of a metabolic / morphological gradient (*i.e. nutrient, oxygen or pH*) occurring between the graft's tissue surface and the basal region.

Although distinct cartilaginous zones were observed within the neo-cartilage grafts, no identifiable calcified zone was observed. In the literature, methodologies have been reported that encourage calcification in engineered constructs, such methods include the addition of phosphoethanolamine (PEA), β-glycerophosphate (β-GP) or adenosine triphosphate (ATP) to deep zone chondrocytes (Kandel *et al.*, 1997). This is suggestive that the addition of PEA, β-GP or ATP to outer Transwell culture media during graft generation could potentially promote the formation of a calcified zone similar to that observed in native articular cartilage. More recent advances involve the generation of neo-cartilage tissue on bone substitute calcium polyphosphate (CPP); this has been found to improve graft integration with bone within a cartilage defect (Waldman *et al.*, 2007, Pilliar *et al.*, 2007). The presence of a calcified zone in reparative tissues has been reported to increase both tissue stiffness and interfacial shear properties at the cartilage-CPP interface (Allan *et al.*, 2007).

At both histological and ultrastructural levels chondrocyte morphology and collagen fibril organisation within neocartilage zones, showed a resemblance to mature native articular cartilage. In the superficial region chondrocytes were discoidal in appearance surrounded by a sparse, meshwork of collagen fibrils that had a prominent

horizontal organisation. Chondrocytes located in the mid region were small, spheroidal, pre-hypertrophic cells that were widely spaced within an extracellular matrix that was comprised of well defined pericellular, territorial and interterritorial compartments. In the deep zone chondrocytes were large, hypertrophic cells that were densely packed together and appeared to acquire a more columnar organisation with an increased culture period. Collagen fibrils within the deep zone were vertically arranged with respect to the grafts superficial region. Similar to native articular cartilage, neo-cartilage grafts became progressively more alcianophilic with tissue depth from the superficial region to the basal portions, indicating increased proteoglycan content.

During this study, using a Transwell system, it appeared that an increased cell seeding density and culture duration generated grafts of increased cellularity and thickness, respectively. The optimal culture conditions required for neo-cartilage graft production for analyses throughout this project were determined histologically with respect to mature native articular cartilage, they were found to be a cell seeding density of $4–6x10^6$ cells over 4 weeks culture duration. Potentially, this study could have been improved if optimum culture conditions for graft production were not determined from histological data alone, but also from intensive biochemical analysis showing how both cell density and culture duration affected graft: (1) mRNA expression and protein levels (*identifying if any vital extracellular matrix (ECM) components are missing that may jeopardise the integrity of the neo-cartilage graft matrix*) (2) proteoglycan concentrations and (3) collagen concentrations compared to native articular cartilage.

At optimum culture conditions neo-cartilage graft tissues generated from an immature chondrogenic source were more synthetically active compared to those produced from a mature chondrogenic source, generating significantly higher amounts of both total sulphated glycosaminoglycan (s-GAG) and collagen. A higher content of both s-GAG and collagen were found to be retained within grafts produced from an immature chondrogenic source, in comparison to a mature chondrogenic source. Clinically, it is more likely that a mature cell source would be utilised in cell therapy treatments, therefore it would be desirable to identify culture conditions that potentially could improve the synthetic capacity of mature chondrocytes. It would have been interesting to have compared how both s-GAG and collagen concentrations in both immature and mature cartilaginous grafts, compared with the s-GAG and

collagen concentrations of the native cartilage tissues to which the chondrogenic sources were originally isolated. An interesting characteristic of the Transwell culture system was that more s-GAG was found to be released to the inner insert media in comparison to the outside media, and the opposite was observed for collagen release. Interestingly, immunohistochemical (IHC) labelling assessing the presence of commonly found cartilage extracellular matrix molecules revealed that graft tissue composition closely resembled immature native articular cartilage, unlike the histological findings that resembled mature native articular cartilage. Type I collagen labelling was predominant at the surface of the grafts and both aggrecan and type II collagen labelling were present throughout the whole depth of the graft tissue, as found in native articular cartilage. However, the immaturity of the tissue was reflected by the fact both collagen type X and chondroitin-6-sulphate (C-6-S) 'stubs' were not detectable in neo-cartilage tissues following a 4 week culture period. Extension of the graft culture period to more than 12 weeks resulted with weak association of type X collagen within the pericellular matrix of hypertrophic chondrocytes within the basal zone. These findings are not particularly surprising, as grafts were generated over a four week time frame using an immature chondrogenic source.

The mechanical strength of neo-cartilage tissue generated during this study was not quantified, but graft pliability during handling indicated that they lacked the mechanical stiffness of mature articular cartilage. In the literature, it has been reported that applying a force in Transwell culture greatly improved the mechanical properties and stimulated the synthesis of extracellular component proteins affecting the overall structure of the engineered tissues (Waldman *et al.*, 2003, 2006 & 2007). The application of mechanical forces during tissue engineering have been found to have many effects such as; altering sulphation patterns on chondroitin sulphate GAG chains (Sauderland & Steinmeyer 2007), increasing decorin concentrations suggestive that decorin through interactions with type II collagen may aid cartilage adaptation to increased loading (Visser *et al.*, 1994), induces the down regulation of BMP-3 an inhibitor of cartilage differentiation enabling the induction of cartilage (Aspenburg *et al.*, 2000), increases levels of chondrogenic markers such as aggrecan, Type II collagen and SOX 9 and decreases levels of more fibroblastic markers such as type I collagen (Miyanishi *et al.*, 2006), Kawanishi *et al.*, 2007). The application of surface motion, but not axial motion to chondrocytes has been found to stimulate the

production of superficial zone protein (SZP), aiding the generation of normal articular cartilage (Grad *et al.*, 2005). It is known that load bearing tissue in the absence of mechanical forces will atrophy. Therefore, applying a force to Transwell cultures during graft generation could potentially optimise the functional value of the neo-cartilage tissue for the repair process. Alternatively, the engineered constructs could be modified post-implantation by physiotherapy.

One potential problem for the use of grafts in the repair process is the integration of neo-cartilage repair tissue to adjacent native articular cartilage within defects. Kandel *et al* in 1995 found that the absence of an adhesive was more productive than the presence of an adhesive, enhancing grafts to remain in position within full thickness defects. However, maintaining graft positioning within intra-chondral defects was poor and required further analysis. There is potential to improve integration between repair tissue and native cartilage at a defect site, as proteoglycans carrying dermatan sulphate GAG chains are thought to inhibit cell attachment. Following the debridement of a defect site, and prior to implantation of repair tissue to the defect site, dermatan sulphate could be removed from the surfaces of the defect by enzymatic treatment (using Chondroitinase ABC) having the effect of encouraging cell attachment (Brittberg 1999). A more recent suggestion is to use a biopolymer to develop a novel bio-adhesive that enables integration between both native cartilage and repair tissue. One proposed bio-adhesive is a chemically modified chondroitin sulphate that has both methacrylate and aldehyde groups on its polysaccharide backbone to chemically bridge biomaterials and tissue proteins via 2 fold covalent cross-links (Wang *et al.*, 2007).

All neo-cartilage graft experimentation within this study utilised animal chondrogenic sources that were of plentiful supply. However, the use of neo-cartilage grafts for clinical application would require human chondrogenic sources to minimise recipient rejection; these are of a more limited supply. Neo-cartilage grafts are produced *ex vivo* from high cell seeding densities and for clinical use an unavoidable necessary step would be to increase chondrocyte cell numbers using monolayer culture. Cell culture expansion results in the undesirable de-differentiation of chondrocytes to a more fibroblastic state, accompanied by the up-regulation of type I collagen and the down regulation of aggrecan, type II collagen and matrix specific genes SOX 5, 6, and 9 (Binette *et al.*, 1998). It has been reported that re-differentiation of chondrocytes can be stimulated by placing them into 3-dimensional (*alginate or*

agarose) or high cell seeding density cultures (*Transwell or pellet*). Although, re-differentiation potentially may encourage hypertrophic differentiation, dramatically diminishing chondrocyte ability to produce hyaline tissue (Benya *et al.*, 1978, Stokes *et al.*, 2001, Yang *et al.*, 2006).

In this study, chondrocytes were isolated from both immature and mature bovine full depth cartilage and were culture expanded (*passaged*) from 0 (P0) to 5 (P5) times, then each chondrogenic source following each passage was seeded into high cell density Transwell cultures to find out if the culture system encouraged re-differentiation of de-differentiated chondrocytes. It was shown that with an increasing passage number of the chondrogenic source, the grafts generated increasingly lacked the distinct appearance of native articular cartilage. The immature cell source is excellent at producing large amounts of extracellular matrix, but is not clinically relevant or even practical. The mature chondrogenic source has poor synthetic abilities in comparison to the immature cell source, and they produce grafts lacking a zonal architecture, with little matrix, appearing extremely cellular. One possible explanation for this could be that *in vivo* the natural repair process as well as environmental factors change the phenotype of the mature chondrocyte to a more fibroblastic state. Therefore mature chondrocytes de-differentiate prior to the cell isolation, expansion and seeding onto filter inserts for graft production, de-differentiated cells lose their zonal differences.

After both the immature and mature chondrogenic sources had been passaged three times (P3) they lost the ability to re-differentiate. No grafts were produced from the immature cell source and grafts produced from the mature source were very thin, suggesting that prior to this point the chondrocytes had undergone a phenotypic change and de-differentiated. The capacity of the chondrogenic sources to lay down an extracellular matrix was restored following successive passages of the cells after P3, although the zonal organisation of hyaline cartilage was not apparent, appearing more fibrocartilaginous in nature. This was supported by IHC labelling showing that with an increased passage number neo-cartilage grafts showed decreased staining intensity for aggrecan labelling and increased labelling for type I collagen. Histological analysis of neo-cartilage grafts generated from an immature source that had been passaged 4 and 5 times revealed that engineered grafts contained discrete cell clusters within the basal portion, that IHC labelled spatiotemporally for versican suggestive that P4 and P5 grafts resembled a more fibrocartilaginous tissue. Parallel

results of other studies in the literature support these findings, showing that phenotypic changes occur in culture expanded chondrocytes between P0 (*primary chondrogenic source*) and P4 (*chondrogenic source that had been passaged 4 times*) (De Haart *et al.*, 1999, Honda *et al.*, 2004, Chua *et al.*, 2005).

Therefore, there is still a need to discover ways of bulking out chondrocyte numbers preventing the detrimental effects of cellular de-differentiation. Ideally, the culture conditions utilised need to encourage chondrocyte differentiation (*if working with a cell source that is not committed to the chondrogenic lineage*) and suppress chondrocyte hypertrophy. The transcription factor SOX-9 has been found to be an essential factor in chondrocyte differentiation functioning as a potent inhibitor of chondrocyte hypertrophy. It has been reported that passaged human osteoarthritic chondrocytes show an increased collagen type II expression in monolayer culture when transduced using adenoviral or retroviral vectors of SOX 9 (Tew *et al.*, 2005).

It would be advantageous if a detailed understanding of the effects various culture methodologies (*i.e. following chondrocyte isolation, during monolayer culture and re-differentiation in Transwell culture*) have on chondrocytes and the extracellular matrix in which they generate were established. Changes in cartilage matrix mRNA expression levels, protein levels and paracrine signalling molecules could be used as markers to determine the chondrocytic state at each culture stage; i.e. differentiated (*CDMP-1, Wnt 5a &b, BMP-13 and BMP-3 down regulation, Type II collagen and aggrecan expression*), de-differentiated (*Monoclonal antibody II-fibrau, type I collagen up-regulation*) or hypertrophic to terminally differentiated (*BMP-6, Type X collagen, alkaline phosphatase, osteopontin, Wnt 5a and b*). An improved basic knowledge in this area would help clinicians intervene in attempt to prevent bio-artificial cartilage from losing its stability.

The optimisation of culture conditions as to prevent the de-differentiation of chondrocytes will play an imperative role in future advancement of Transwell cultured neo-cartilage grafts. It has been reported that low oxygen conditions in culture help maintain the stability of a chondrocytes phenotype and stimulates the re-differentiation of de-differentiated chondrocytes (Brittberg 1999). Recently, it has been reported that the amount of collagen cross-links present within neo-cartilage grafts are affected by oxygen concentrations. During this study, there were indications that neo-cartilage graft matrices were loosely packed together, as quite high amounts of s-GAG were released to culture media in Control treated grafts. The

lack of collagen cross-linking in neo-cartilage grafts may be a consequence of the highly potent mitotic TGF-β_2 being present in Transwell media, an agent known to decrease the number of collagen cross-links within extracellular matrices (Jenniskens *et al.*, 2006). Therefore, it would be interesting to investigate the effects varying oxygen tensions in Transwell culture have on graft generation.

The presence of serum in culture media has been found beneficial for increasing cell numbers, but consequentially serum containing media decreases cartilage matrix gene expression (Malpeli *et al.*, 2004), altering chondrocyte morphology encouraging them to become more fibroblastic (Chua *et al.*, 2005). The presence of serum within culture media could potentially impede culture analyses, as serum contains exogenous growth factors that hinder the identification and quantification of the growth factors generated from cultured chondrocytes (Kita *et al.*, 2006). In future analyses, decreasing serum concentrations used in Transwell culture systems could potentially prevent any recipient immune reactions against engineered neo-cartilage grafts due to the presence of foreign bodies or viral proteins (Chua *et al.*, 2005). One potential step towards a serum free environment would be to develop chemically defined serum supplements (Heng *et al.*, 2004). Some cells are unable to proliferate in serum free environments and require supplementation with Insulin-Transferrin-Selenium (ITS), FGF or TGF-β in the presence of a small percentage of serum.

It is important to add the right factor at the right moment during the various culture stages as to optimise culture media. In the literature, it has been reported that addition of FGF-2 to culture media stimulates chondrocyte differentiation, proliferation (Takahashi *et al*, 2005, Jingushi *et al.*, 2006 & Miot *et al.*, 2006), re-differentiation of expanded chondrocytes (Miot *et al.*, 2006) and inhibits the hypertrophic state (Tchetina *et al.*, 2006). Parathyroid hormone / parathyroid hormone related protein (PTH/PTHrP) have also been found to inhibit the hypertrophic state, whereas insulin sends chondrocytes to hypertrophy (Liu *et al.*, 2007). In the future, investigating the effects addition of various factors to Transwell culture media have on neo-cartilage grafts could potentially improve tissue quality.

There are alternate cell sources other than chondrocytes that show potential for future generation of Transwell cultured grafts these include; stem cells, progenitor cells and spore-like cells. Both adult stem cells and spore-like cells have been reported to be activated in normal tissue turnover, by injury or disease, possibly acting as a body's natural repair mechanism. If these particular cells were found to be located in

articular cartilage (*i.e. to date a progenitor cell has been located in the surface zone of articular cartilage* (Dowthwaite *et al.*, 2004)), then future cartilage repair therapies may minimise patient operations excluding the need for autologous chondrocyte implantations or the generation of tissues *ex vivo*, alternatively activating these cells *in situ* inducing a cartilage repair response. There are three types of stem cells i.e. totipotent (*obtained from the earliest stages of development*), pluripotent (*embryonic stem cells (ESC) and embryonic germ cells*) and multipotent (*Adult stem cells found in specialised organs after birth, such as: bone marrow, blood, skin, GI tract, dental pulp, retina of the eye, skeletal muscle, liver, pancreas and the brain*), all of which are found at various stages throughout development. Stem cells that are obtained in the later stages of development are more functionally inept in comparison to stem cells found in earlier developmental stages.

There are both moral and ethical considerations that arise surrounding the use of pluripotent embryonic stem cells. They are an immortal cell source with the ability to provide an unlimited supply of chondrocytes (Heng *et al.*, 2004). However, there is no evidence that ESCs can consistently be driven down the correct lineage resulting in the generation of a mixed population of cells. Adult stem cells are limited to produce one or more lineages of specialised cells depending on the potential of the source, where it originated and environmental cues to which the source is subjected. They have the ability to self renew and last throughout a lifetime of an organism, this proving advantageous in medical transplantations, as adult stem cells would supply an unlimited donor material. However, the ability of adult stem cells to self replicate and proliferate diminishes with age (Redman 2005).

Mesenchymal stem cells are a multipotent stem cell source that has been of recent interest in the literature for future tissue engineering applications. They originate from the mesodermal layer of a blastocyst in a developing embryo and have the capacity to develop into cartilage, bone, tendon, adipose tissue and muscle (Redman 2005). Transdifferentiation to a chondrogenic lineage from another tissue type (i.e. bone, tendon, adipose tissue or muscle) have been reported, but is a rare and sporadic event proving difficult to cross boundaries and maintain a desired phenotype. Specific environmental cues acting upon the cell source are responsible for the maintenance of a desired cell lineage, changes in the environmental cues in which a cell source is subjected would drive the cell source down an undesired lineage as would occur at the transplantation site.

Progenitor cells occur in foetal and adult tissues and are committed to a cell lineage (Vacanti 2004); they are generated by condensations of stem cells and are unable to self renew. They are parent cells that give rise to a distinct cell lineage by a series of cell divisions that are coupled with cell maturation of the specialised cell. Progenitor cells have similar properties to stem cells with an undifferentiated nature and a capacity to proliferate. An articular cartilage progenitor has been isolated from the superficial zone of bovine articular cartilage (Dowthwaite *et al.*, 2004). These surface zone progenitor cells are thought to allow appositional growth of articular cartilage from the surface (Hayes *et al.*, 2001).

Spore-like cells are ubiquitous and are found in a dormant form dispersed throughout the parenchyma of most tissues. They are small cells ranging less than 5μm, there are concerns as to whether 5μm diameter cells could contain the whole genome and the concept of a minimal genome is beginning to emerge (Vacanti 2004). Spore-like cells are small undifferentiated cells that contain a nucleus, a small amount of cytoplasm, a few mitochondria and a coating of glycolipid and mucopolysaccharide. They also have a mitotic cleft which is supportive of cell division. These cells have the ability to withstand hostile conditions such as a low oxygen environment and extreme temperature, still maintaining the capacity to proliferate and differentiate. Spore-like cells have the ability to differentiate into mature cells of the tissue from which they were isolated (Vacanti *et al.*, 2001). As the cells possess the ability to replicate, differentiate and survive extreme temperatures and hypoxic conditions, they may have many potential clinical uses in the future (Vacanti *et al.*, 2001).

Therefore, it can be concluded that there is significant potential in the continued development of autologous chondrocyte grafts for the repair of human articular cartilage defects. However, there is still immense room for improvement of the Transwell culture system by: optimising both Transwell culture medium and methodologies, utilising gene therapy and investigating the potential of new cell sources as to minimise the deleterious effects of cellular de-differentiation.

The latter half of this study utilised the Transwell system as a culture model to determine how the addition of exogenously added hyaluronan (*of varying molecular weights (Mwt) ranging from 5–3000^{+}kDa*), during and post neo-cartilage graft generation affected cartilage / chondrocyte metabolism. The size of endogenous hyaluronan (HA) produced by chondrocytes to form aggrecan aggregates in hyaline articular cartilage is in the range of 500kDa to 1000kDa (Hardingham & Fosang,

1992). Interestingly, when neocartilage grafts were subjected to exogenous hyaluronan of a smaller Mwt (*i.e. 5kDa, 100kDa & 250kDa HA*) than the typical *Mr* of endogenous HA (*500kDa–1000kDa range*) found in native aggrecan aggregates, an increased release of s-GAG to Transwell media and decreased s-GAG retention within neocartilage grafts was observed. This is suggestive that the 'smaller' molecular weight hyaluronan diffuse into the graft extracellular matrix and compete for newly synthesised and existing aggrecan that was either free or present in non-link protein stabilised aggregates, thereby facilitating the 'extraction' or 'release' of these aggrecan / smaller HA aggregates from graft extracellular matrices into the media of the Transwell cultures.

However, HA of Mwt 1600kDa (*slightly larger than endogenously produced HA*) had no effect on increasing the s-GAG content of the Transwell culture media. In contrast, addition of HA to neocartilage grafts of a significantly higher molecular weight (*i.e. 2200kDa and 3000^{+}kDa HA*) than the typical molecular weight of endogenous HA found in aggrecan aggregates, showed decreased s-GAG release to media, and increased s-GAG retention within graft extracellular matrices implying that the larger molecular weight hyaluronan can diffuse into the graft matrices and more efficiently 'trap' newly synthesised and endogenous aggrecan in HA-aggrecan aggregates preventing the diffusion of this pool of aggrecan out of the cartilage graft ECM.

Interestingly and additionally, it was found that chondrocytes exposed to exogenous hyaluronan of similar molecular weight (*i.e. 490kDa*) to the typical *Mr* of endogenous HA (*500kDa–1000kDa range*) found in native aggrecan aggregates resulted with a marked effect on the rate and amount of aggrecan synthesised (3.78 fold greater) compared to Control grafts. This is suggestive that the inclusion of 490kDa HA within Transwell cultures greatly enhances proteoglycan biosynthesis. Overall, these results show similarities to recent publications, in that low molecular weight hyaluronan are more likely to have detrimental effects on extracellular matrices (Solursh *et al.*, 1980, Stern *et al.*, 2006, Iacob & Knudson, 2006, Sugahara *et al.*, 2006) and high molecular weight HA tend to have beneficial effects (Kang *et al.*, 1999, Julovi *et al.*, 2004, Monfort *et al.*, 2005).

High molecular weight HA is used clinically as an accepted viscosupplementation procedure in the treatment of degenerative joint diseases (i.e. Osteoartritis-OA). This procedure has been found to improve the visco-elastic properties of the synovial fluid

encouraging lubrication, shock absorbance, the formation of a semi-permeable barrier, decreasing both inflammatory and proliferative responses that occur within diseased joints. It has been found that pain reduction lasts considerably longer than the half life of injected HA, suggestive that the high molecular weight HA injections potentially may have disease modifying activity (Goldberg & Bulkwater, 2005). The mechanisms of action of exogenous high molecular weight hyaluronan in viscosupplementation at present are unclear, but data obtained in this study suggest that high molecular weight hyaluronan may be facilitating the retention of newly synthesised 'repair aggrecan' in the pathological cartilage and thereby slowing down the rate of cartilage destruction through loss of proteoglycan from the tissue. A potential improvement upon HA visco-supplementation procedures would be to incorporate a mixture of Mwt HA ranging between 490kDa-3000^{+}kDa as to provide optimal benefits of aggrecan retention and biosynthesis to OA patients receiving this treatment modality.

For future experimentation, it would be of interest to determine the endogenous levels of hyaluronan present within neo-cartilage grafts in comparison to native articular cartilage. It would also be beneficial to fluorescently trace the penetration of exogenously added HA (*of varying molecular weights*) into neo-cartilage grafts, and also determine if the major proteoglycan present in cartilage (aggrecan) is in an aggregated or non-aggregated form within graft tissue or Transwell media, as to determine the modes of action and mechanisms involved with the addition of each Mwt HA. Overall, it can be concluded that the Transwell culture system does not only show significant potential in the continued development of autologous chondrocyte grafts for the repair of human articular cartilage defects, but also provides an ideal culture model for scientific investigations as it enables high regulation and monitoring of most non-gaseous graft metabolites produced throughout the culture duration.

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APPENDIX

Papers

Hayes, A,J., **Hall, A**., Brown, L., Tubo, R., Caterson, B. (2007) Macromolecular Organisation of Scaffold-free Neocartilage Grafts. Journal of Histochemistry and Cytochemistry. 55(8): 853-866

Abstracts

September 2004	British Society for Matrix Biology (Autumn meeting) joint with the UK Tissue and Cell Engineering Society. 'Cell-based therapies' University of Bristol, UK Tissue-engineered cartilage grafts for use in cartilage repair technologies Won poster prize
Spring meeting	British Society for Matrix Biology (BSMB) Tissue engineered cartilage grafts
September 2005	Cardiff Institute of Tissue Engineering Research (CITER) Carmarthen, UK Tissue engineered cartilage grafts
September 2006	Cardiff Institute of Tissue Engineering Research (CITER) Abergavenny, UK Effects of different molecular weight hyaluronan on cartilage grafts cultured for tissue engineering applications
October 2006	Tissue Engineering International Regenerative Medicine Society (TERMIS) Rotterdam, NL

Tissue engineered cartilage grafts for use in cartilage repair technologies

Oral Presentaions

September 2004	British Society for Matrix Biology (BSMB) 'Cell based therapies' University of Bristol, UK
September 2005	Cardiff Institute of Tissue Engineering Research (CITER) Carmarthen, UK
September 2006	Cardiff Institute of Tissue Engineering Research (CITER) Abergavenny, UK
October 2006	Tissue Engineering International Regenerative Medicine Society (TERMIS) Rotterdam, NL

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ARTICLE

Macromolecular Organization and In Vitro Growth Characteristics of Scaffold-free Neocartilage Grafts

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SUMMARY Recent advances in tissue engineering offer considerable promise for the repair of focal lesions in articular cartilage. Here we describe (1) the macromolecular organization of tissue-engineered neocartilage grafts at light and electron microscopic levels, (2) their in vitro development, and (3) the effect of chondrocyte dedifferentiation, induced by monolayer expansion, on their resultant structure. We show that grafts produced from primary cultures of chondrocytes are hyaline in appearance with identifiable zonal strata as evidenced by cell morphology, matrix organization, and immunohistochemical composition. Like native articular cartilage, their surface zone contains type I collagen, surface zone proteoglycan, biglycan and decorin with type II collagen, aggrecan, chondroitin sulfate, chondroitin-4-sulfate, and keratan sulfate, becoming more prominent with depth. Assessment of cell viability by Live/Dead staining and cell-cycle analysis with BrDU suggest that the in vitro tissue has a high cellular turnover and develops through both appositional and interstitial growth mechanisms. Meanwhile, cell-tracker studies with CMFDA (5-chloromethyl-fluorescein diacetate) demonstrate that cell sorting in vitro is not involved in their zonal organization. Finally, passage expansion of chondrocytes in monolayer culture causes progressive reductions in graft thickness, loss of zonal architecture, and a more fibrocartilaginous tissue histology, consistent with a dedifferentiating chondrocyte phenotype.

(J Histochem Cytochem 55:853–866, 2007)

KEY WORDS
cartilage repair
tissue engineering
neocartilage
zonal organization
immunohistochemistry
extracellular matrix
in vitro development
chondrocyte dedifferentiation

RECENTLY, there has been considerable interest in the application of novel, cell-based tissue-engineering strategies to articular cartilage repair (Kuo et al. 2006). In essence, utilizing this technology, culture-expanded autologous chondrocytes can be delivered to a cartilage lesion site either (1) in suspension, beneath a periosteal patch or biodegradable membrane sutured to the wound margins, i.e., autologous chondrocyte transplantation (ACT) (Carticel; Genzyme, Cambridge, MA) (Brittberg et al. 1994,2003) or (2) immobilized within a biodegradable, biomimetic, three-dimensional carrier or scaffold (Hutmacher 2000). Following implantation, and under favorable rehabilitative conditions, implanted chondrocytes have the potential to synthesize and secrete new extracellular matrix (ECM) material, thereby resurfacing the lesion and restoring functionality to the joint (Brittberg et al. 1994).

Although this type of therapy has shown significant progress (Brittberg et al. 2003), it has still not fully lived up to expectations (Wood et al. 2006). The reported loss of periosteal/collagen membranes, together with their underlying cells, into the synovial cavity remains an issue; and the development of a fibrocartilaginous repair tissue, which lacks the appropriate organization and material properties to the original hyaline tissue, remains problematic. Additional concerns yet to be resolved include imperfect cartilage resurfacing, unsatisfactory integration of the new tissue with the surrounding matrix, and inadequate assimilation/remodeling of the carrier or scaffold (reviewed in Hunziker 2002; Wood et al. 2006).

Considering some of these problems, an alternative and potentially improved approach would be to

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the long term, rigorous evaluation of their macromolecular composition and in vitro growth characteristics is clearly essential. Furthermore, to generate sufficient quantities of cells for an autologous human application, harvested chondrocytes would require proliferation in monolayer growth conditions. Crucially, this type of culture induces their dedifferentiation (Benya et al. 1978), characterized by a change in their cellular morphology, a downregulation of cartilage-specific genes (e.g., Sox 5, 6, and 9, aggrecan, and type II collagen), and a reduced ability to produce hyaline cartilage (Stokes et al. 2001; Yang et al. 2006). Whereas chondrocytes can be partially redifferentiated by their transferral to a three-dimensional culture model more conducive toward chondrogenesis, e.g., high density or agarose (Benya and Shaffer 1982; Watt 1988); the subsequent ability of these cells to synthesize genuine hyaline tissue remains ambiguous. Thus, in the present study, we (1) describe the macromolecular composition and organization of scaffold-free neocartilage tissues grown on synthetic filter membranes at light- and electron-microscopic levels, (2) explore their in vitro growth characteristics, and (3) examine the effect of passage expansion of chondrocytes, in monolayer culture, on resultant tissue architecture.

Materials and Methods

Cell Culture

Full-depth slices of articular cartilage were obtained from the hock joints of 7-day-old bovines under sterile conditions. Cartilage slices were digested in 7 U/ml pronase (Roche; Hertfordfshire, UK) for 3 hr at 37C followed by overnight digestion in 100 U/ml type II collagenase (Worthington Biochemical Co.; Berkshire, UK). To obtain enriched subpopulations of cells from each cartilage zone for tracker studies, the tissue was first subdissected into surface, middle, and deep layers and corresponding layers pooled prior to the above digestion steps. The resultant cell suspensions were filtered through a 40-μm cell strainer (BD Falcon; Erembodegem, Belgium), washed in DMEM containing 4500 mg/liter glucose and L-glutamine (DMEM; Gibco BRL, Paisley, UK), and seeded at high density in 0.5-ml volumes (see below) onto type II collagen-coated (0.5 mg/ml; Sigma-Aldrich, Dorset, UK) 0.6 cm^2 Millipore filter inserts (0.4-μm pores; Millipore, Billerica, MA) (Kandel et al. 1995). Initially, to investigate the effect of cell-seeding density on graft histology, chondrocytes were seeded at a range of cell densities: 2, 4, 6, 8, 10, and 12 × 10^6 cells/insert (i.e., 4, 8, 12, 16, 20, and 24 × 10^6 cells/ml). To assess the effect of culture time on graft thickness, cells were cultured for 2-, 4-, 6-, and 8-week periods. Subsequently, grafts were made with 6 × 10^6 cells per insert and typically cultured for 4 weeks. Cultures were fed three times weekly on DMEM containing 20% heat-inactivated FBS, TGF β2 (5 ng/ml; PeproTech, London, UK), and ascorbate (100 μg/ml) and were maintained at 37C in a humidified atmosphere containing 5% CO_2 as described by Kandel et al. (1995). To study the effect of dedifferentiation on the histological organization of the graft tissue, chondrocytes were serially passage expanded in monolayer culture in DMEM containing 10% FBS. Briefly, cells were grown to confluency in aerated 75-cm^2 flasks before passage with trypsin–EDTA (Gibco BRL). Harvested chondrocytes were washed, counted, and seeded at a density of 6 × 10^6 cells per filter insert and grown for 4 weeks in culture as described above. Meanwhile, the remaining cells were returned to monolayer culture at low density for further rounds of expansion. This procedure was repeated until passage number five, by which time the growth rate in monolayer was insufficient to sustain sufficient cell numbers for further graft production.

Histology

Cartilage grafts were fixed in 2% paraformaldehyde and processed into paraffin wax using standard histological methods. Six-μm transverse sections were cut through the diameter of the graft and mounted on HistoBond (R.A. Lamb; Eastbourne, UK) slides. Sections were stained for proteoglycan (PG) content in 1% (w/v) alcian blue 8GX (pH 2.5) and Mayers hematoxylin/eosin or for collagen fiber organization by staining in 0.1% (w/v) sirius red F3B in saturated aqueous picric acid. Tissue organization was evaluated using bright-field, differential interference contrast (DIC), and polarizing optics. Graft thickness was measured at 10 arbitrary points across the diameter of the tissue on three different tissue sections, i.e., 30 measurements per graft.

Electron Microscopy

For ultrastructural investigation, grafts were processed for both scanning and transmission electron microscopy. For transmission electron microscopy, tissue was fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, containing 0.7% ruthenium hexamine trichloride (RHT; Sigma-Aldrich) for 2 hr, followed by postfixation in 1% OsO4 in 0.1 M cacodylate, pH 7.4, containing 0.7% RHT for 2 hr. Samples were subsequently processed into Spurr's resin (Agar Scientific; Essex, UK) using routine methodology. Ultrathin sections were cut and then stained with uranyl acetate and lead citrate before examination in a Philips EM 208 transmission electron microscope (Philips Electron Optics; Eindhoven, The Netherlands). For scanning electron microscopy, RHT was omitted from the fixatives to prevent PG retention within the tissue. After the primary fixation step, samples were macerated in 10% NaOH for 1 week at 4C to facilitate an appraisal of the collagenous network. After maceration, the tissue was processed for scanning electron microscopy using routine methodology and viewed in a Philips XL-20 scanning electron microscope (Philips Electron Optics).

Immunohistochemical (IHC) Analysis of Graft ECM

Cartilage grafts were fixed in cold 75% ethanol, cryoprotected in PBS containing 5% sucrose, and snap frozen onto cryostat chucks in Cryo-M-Bed tissue mountant (Bright Instruments; Cambridgeshire, UK). Grafts were cryosectioned at 10 μm and frozen sections collected onto Histobond (R.A. Lamb) slides for immunostaining. Sections were labeled by standard indirect immunofluorescence procedures using primary antibodies to a wide range of ECM components (see Table 1). All immunoreagents were diluted in 0.05 M PBS

between unlabeled deep- and surface-zone fractions, allowing 3 hr between the addition of successive cell subpopulations to minimize the extent of initial cell mixing. Additionally, to identify the potential for zonal cell sorting during the culture period, labeled surface-, middle- or deep-zone cells were first mixed with their unlabeled counterparts before seeding into filter inserts at a density of 6×10^6 cells per insert (i.e., 2×10^6 cells from each layer). After 4 weeks of culture the grafts were harvested, fixed, and processed into paraffin wax, as described previously. Wax sections were cut and counterstained with propidium iodide (0.5 μg/ml; Molecular Probes, Invitrogen) to impart nuclear context to the tissue or were immunofluorescently labeled with a monoclonal antibody toward chondroitin sulfate to allow visualization of the ECM.

Results

Primary Culture

Macroscopically, all neocartilage graft tissues produced in this study had a glassy, hyaline appearance (Figure 2A). Based upon histological, ultrastructural, and IHC criteria, the grafts were stratified into well-defined cartilaginous zones that approximated with the superficial, middle, and deep layers of mature articular cartilage, although no calcified zone was identifiable (see below). In primary culture, this histological zonal organization was apparent at all cell-seeding densities and all culture periods studied (Figure 2, Figure 3, and Figure 4).

Cell Density and Culture Duration

Cell-seeding density and culture period were important factors in determining the thickness of the resultant neocartilage tissues. Increasing cell number from 2 to 4×10^6 yielded a highly significant increase ($p<0.001$) in the thickness of neocartilage grafts produced at all culture periods examined. Increasing cell number $>4 \times 10^6$ cells per insert provided subtle increases of variable significance in resultant graft thickness (Figures 2B and 2C). Consistent with an increase in cell-seeding density was a concomitant increase in graft tissue cellularity; thus, seeding $>6 \times 10^6$ cells per insert yielded a tissue with a high cell-to-matrix ratio characterized by an excessively hypercellular appearance. The effect of increasing the duration of culture from 2 to 8 weeks was a steady increase in the thickness of the cartilage grafts produced at all cell-seeding densities (Figures 2B and 2C). This appeared to result from a combination of cell proliferation, hypertrophy, and progressive accumulation of ECM material (see below). Interestingly, at each time point examined, the zonal proportions of the graft appeared relatively constant, suggesting that in vitro growth occurred uniformly throughout the tissue during culture. Furthermore, with advancing time the cells in the basal part of the tissue (i.e., those closest to the filter membrane) appeared to acquire a more columnar organization (Figure 2C; see below).

Graft Organization

Light and electron microscopic examination of neocartilaginous grafts showed the surface zone to be weakly alcianophilic (Figure 3A), consisting of discoidal cells surrounded by a sparse, fibrillar meshwork of collagen (Figures 3A, 3B, and 3E). In this zone, both cells and matrix had a prominent horizontal organization that was clearly apparent under Nomarski DIC and polarizing optics (Figures 3H and 3I). The underlying mid-zone was strongly alcianophilic, indicating a higher PG content than the surface zone, and contained spheroidal chondrocytes that were separated by significant amounts of ECM (Figures 3A, 3C, and 3F). Ultrastructural observation of this zone showed that its ECM comprised pericellular, territorial, and interterritorial matrix compartments, each with distinct collagen fiber densities and organizations, similar to that of articular cartilage (Figures 3C and 3F). The subjacent deep zone of the tissue was more cellular in appearance than the overlying cartilage. Chondrocytes in this zone had an ovoid, hypertrophic morphology and were densely packed together, with some cells occurring in columnar arrays separated by thin septa of alcianophilic ECM (Figure 2C, Figures 3A, 3D, and 3H). The interterritorial matrix of these septa, evident at the ultrastructural level, consisted of prominent longitudinal fibrils that were arranged vertically with respect to the graft surface (Figures 3D and 3G). Observation with both Nomarski DIC and polarizing optics indicated that tissue organization in the deep zone (Figures 3H and 3J) was perpendicular to that occurring at the graft surface (Figures 3H and 3I).

Composition of Graft ECM

Negative controls showed no nonspecific labeling with either primary or secondary antibodies (data not shown). Immunofluorescent labeling patterns obtained with antibodies toward the chief ECM components suggested that after 4 weeks of primary culture the graft tissue was immunohistochemically similar to immature native articular cartilage (refer to Figure 1).

Collagens. Type I collagen was strongly detectable at the surface of the graft but was absent from the underlying connective tissue (Figure 4A) as occurs within the superficial zone of articular cartilage, whereas type II collagen was identifiable throughout the graft matrix (Figure 4B), confirmatory of the tissue's hyaline nature. Type X collagen was not detectable in grafts after 4 weeks of culture, reflecting the immaturity of the ECM; however, it became weakly associated with the pericellular matrix of hypertrophic chondrocytes in the basal zone after extended culture periods (i.e., >12 weeks; data not shown); its location corresponding to that occurring in articular cartilage.

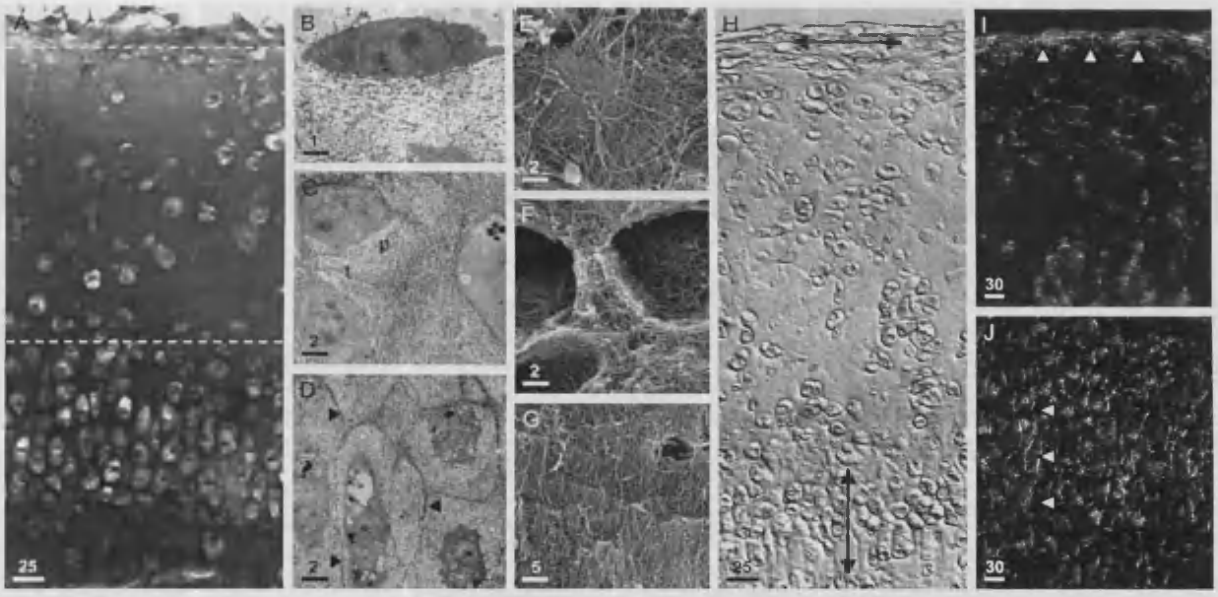

Figure 3 Histological and ultrastructural organization of neocartilage tissue. **(A)** Wax histology. The graft is composed of three histologically distinct zones that approximate to the surface, middle, and deep zones of native adult cartilage (dashed lines demarcate boundaries between each cartilage zone). Alcian blue and hematoxylin/eosin. **(B–D)** Transmission electron micrographs demonstrating cell morphology and matrix organization associated with each graft zone (top to bottom). p, pericellular matrix; t, territorial matrix; i, interterritorial matrix; arrowheads in **D** denote position of interterritorial matrix. **(E–G)** Scanning electron micrographs showing topographic detail of collagenous organization in graft zones (top to bottom). **(H)** Nomarski differential interference contrast image showing distinct tissue organizations associated with each zonal strata (arrows denote predominant orientation of cells and matrix). Picrosirius red. **(I,J)** Polarized light microscope images showing **(I)** horizontal organization of collagen fiber bundles at the graft surface and **(J)** vertical organization of collagen associated with the interterritorial matrix of deep-zone chondrocytes. Bars represented in μm.

(>12 weeks) it became more prominent at the surface of the graft (Figure 4H), having a similar organization to that of articular cartilage.

Glycosaminoglycans (GAGs). Unsulfated chondroitin (C-0-S) and C-4-S "stubs" (i.e., after chondroitinase digestion) were prominent throughout the graft ECM and had a similar distribution to aggrecan, suggestive of their association with this PG (Figures 4I and 4J, respectively). Chondroitin-6-sulfated "stubs" were not detectable in the graft ECM (Figure 4K), indicative of the immaturity of the engineered tissue. Keratan sulfate was weakly present in the pericellular matrix compartment, becoming more prominent with depth (Figure 4L) as occurs in articular cartilage, where it is associated with aggrecan. In contrast, dermatan sulfate was present predominantly in the surface zone of the graft tissue (Figure 4M), suggesting that this molecule was associated with decorin that was similarly located and to which it has known association in articular cartilage.

In Vitro Growth Characteristics

Assessment of cell viability with a fluorescent Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen) showed the majority of cells in thin (~0.25 mm) living sections of graft tissue were viable, i.e., had stained green after 4 weeks of culture (Figure 5A). During the same time period, dead cells (both necrotic and apoptotic) evident by their red nuclei had accumulated throughout the graft tissue; however, there was no distinct zone of cell death per se. Cell-cycle analysis with BrDU revealed strong incorporation of this thymidine analog in proliferating cells both at the graft surface and in the underlying cartilage tissue (Figure 5B), the high levels of BrDU incorporation probably reflecting the known effect transforming growth factor has on chondrocyte proliferation. Comparison with control sections incubated with naive immunoglobulin or antibody diluent instead of BrDU antibody confirmed that there was no nonspecific labeling (data not shown). Cell-tracker studies with CMFDA (5-chloromethyl-fluorescein diacetate) showed that after 4 weeks of culture the labeled mid-zone subpopulation largely retained its intermediate position in tri-layered composites, indicating there was little interzonal movement of chondrocytes (Figure 5C). When grafts were made by mixing a single fluorescently labeled subpopulation with its unlabeled counterparts from the adjacent zones, in all cases the labeled chondrocytes were randomly distributed throughout the tissue depth (Figures 5D–5F). Although there was no compelling evidence of zonal cell sorting mechanism per se, occasionally there appeared to be slightly more labeled deep zone cells in the basal part of the tissue (Figure 5F). This was possibly due to the hypertrophic cells of the deep zone being larger (mean cell volume ± SEM of 1689 ± 145 μm^3) and therefore settling more readily during the initial cell-

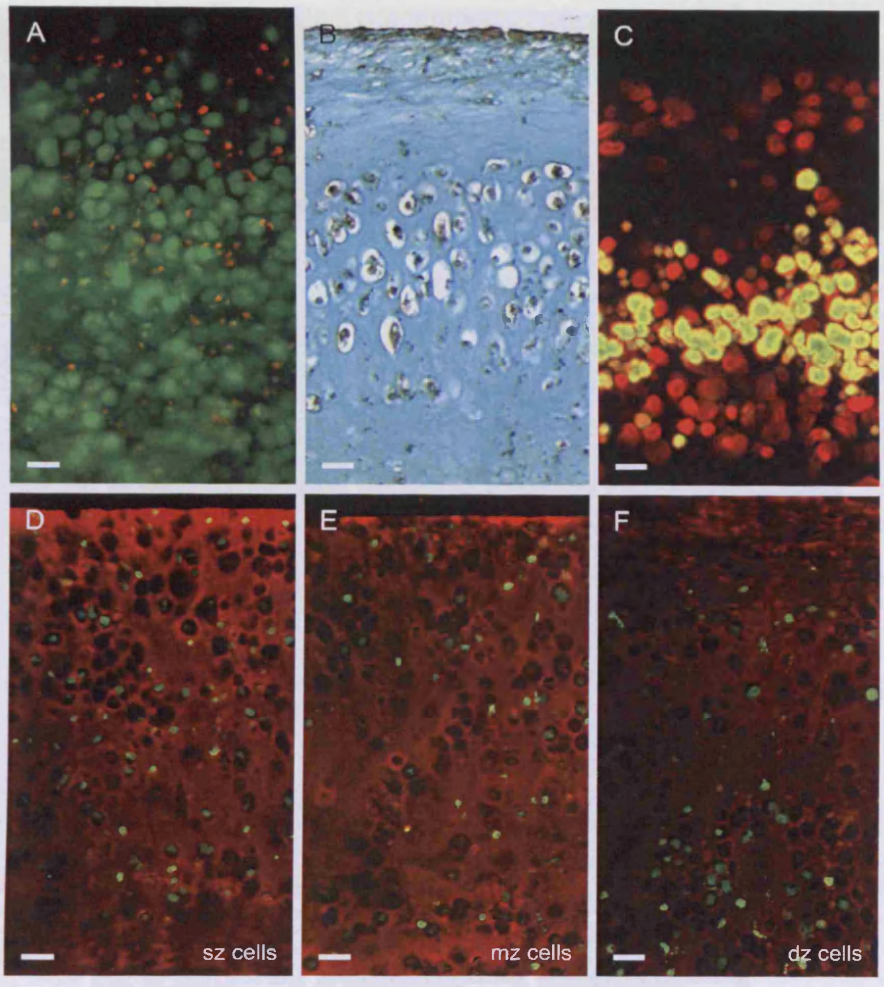

Figure 5 In vitro growth characteristics of neocartilaginous grafts. (**A**) Viability staining with calcein AM (live cells; green) and ethidium homodimer (dead cells; red) in living sections of graft tissue. The majority of chondrocytes in the graft are viable after 4 weeks of culture; however, dead cells (both apoptotic and necrotic) are observable throughout the tissue depth. (**B**) Immunolocalization of bromodeoxyuridine (90-min exposure) shows strong incorporation of this thymidine analog in proliferating cells both at the surface and within the subjacent cartilaginous tissue (brown staining). Note that the matrix has been counterstained with alcian blue for tissue context. (**C**) Cell-tracker studies with CFDA (5-chloromethyl-fluorescein diacetate) show that the labeled mid-zone subpopulation of chondrocytes (yellow) largely retains its intermediate position in tri-layered constructs. Note that cell nuclei have been counterstained red for cellular context. (**D–F**) Fluorescently tagged subpopulations of surface- (sz) (**D**), middle- (mz) (**E**), and deep-zone (dz) chondrocytes (**F**). Green cells when mixed with their unlabeled counterparts show no compelling evidence of zone-dependent cell sorting in vitro. Matrix has been immunofluorescently labeled red for tissue context. Bar = 25 μm.

zonally stratified neocartilage grafts potentially useful for the biological repair of intrachondral lesions of articular cartilage. All tissues engineered from a primary chondrocyte source in this study showed histological, ultrastructural, and IHC similarities to native articular cartilage. The neocartilage tissues comprised three histologically distinct zones that resembled the superficial, middle, and deep zones of native articular cartilage, and within these zones there were striking similarities in cell morphology, collagen fiber organization, and matrix composition to that of native articular cartilage. Interestingly, whereas the histological organization of the tissue closely resembled that of mature articular cartilage, the composition of its ECM suggested closer similarities with immature, fetal-like hyaline cartilage: there was no detectable chondroitin-6-sulfate; type X collagen was initially absent, and there was no calcified cartilage matrix. This was not altogether surprising, however, given that the matrix was synthesized de novo over a short, 4-week period. Furthermore, although we did not specifically quantify the mechanical strength of the tissue, the pliability of the neocartilage evident during handling indicated that it lacked the mechanical stiffness of mature articular cartilage.

Previous studies, chiefly by Waldman and colleagues, indicate that media supplementation and/or mechanical stimulation have the potential to add functional value to neocartilage grafts. Media supplementation has been successfully used to improve the quality of tissue-engineered cartilage, resulting in a more mature tissue phenotype. β-glycerophosphate, for example, induces deep zone chondrocytes to calcify their cartilage matrix (Kandel et al. 1997; Waldman et al. 2002), and $NaHCO_3$ supplementation has been shown to improve the histological quality of neocartilage, resulting in more flattened cells resembling superficial zone chondrocytes at the graft surface (Waldman et al. 2004a). Biomechanical loading regimes, both compressive and shear (long and short term) have also been used to increase matrix deposition, improve matrix composition, and increase the thickness and biomechanical properties of the engineered cartilage (Waldman et al. 2003b,2004b,2005). Indeed, the importance of utilizing appropriate biomechanical kinematics in articular

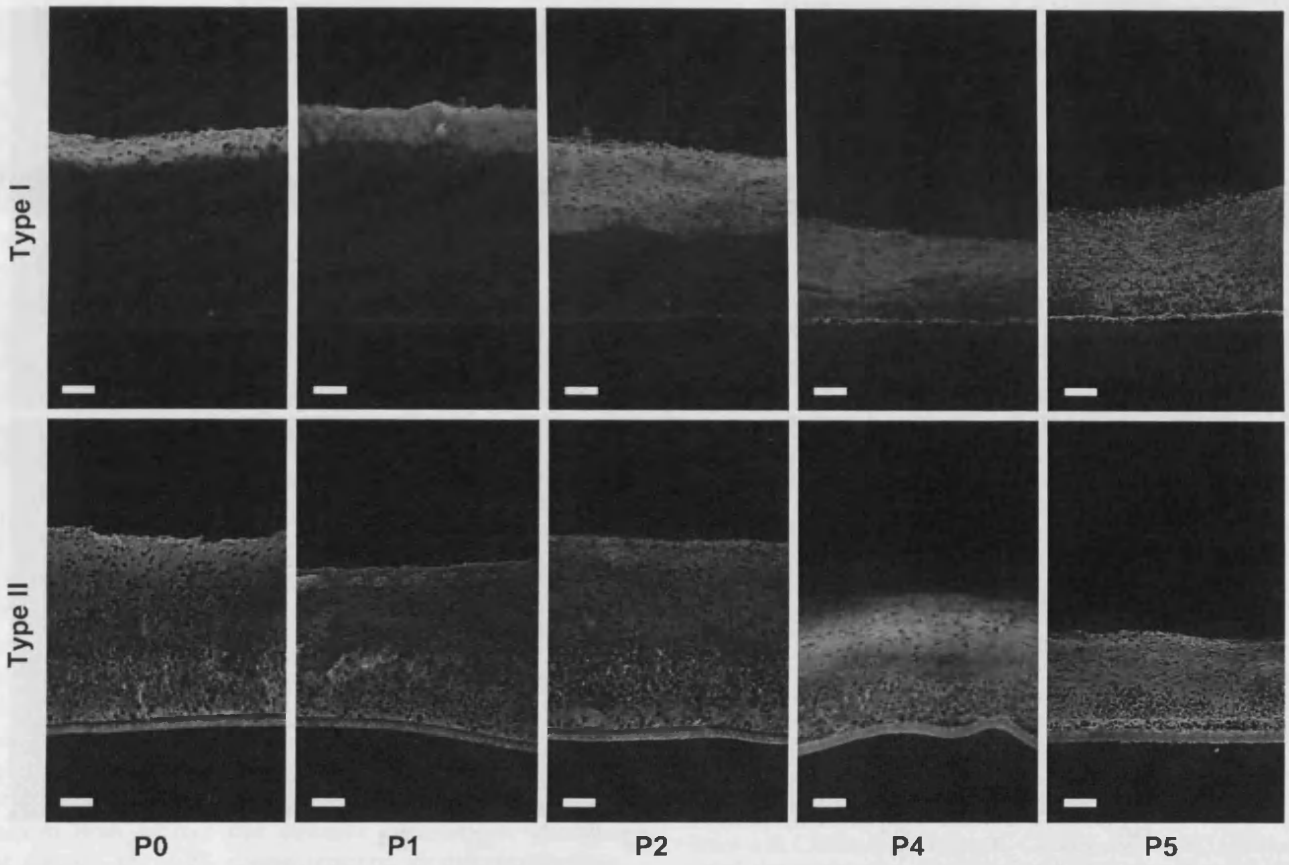

Figure 7 Effect of monolayer expansion upon the distribution of collagen types I and II in neocartilaginous tissue. Note progressive increase in immunolabel for type I collagen with successive passage (P). Qualitative changes in the distribution of type II collagen were not apparent. Bar = 80 μm.

largely by the culture environment. At the surface of the graft, chondrocytes would begin to flatten out, proliferate, and undergo dedifferentiation, as occurs in monolayer culture (Benya et al. 1978; Stokes et al. 2001). These changes might not only explain the change in cell morphology from spheroid to discoid, but also account for the high levels of BrDU incorporation in cells at the surface of the tissue. It would also drive the expression of molecules typically associated with the superficial zone, such as collagen type I (Benya et al. 1978). Additionally, as the cells spread and assume a more fibroblastic morphology, collagen fibrils would be secreted more across the surface of the graft tissue, as occurs in monolayer culture. Deeper within the tissue the spheroid/ovoid chondrocyte morphology would be preserved as the cells are constrained at high density in three dimensions by neighboring cells. This environment would be conducive toward chondrogenesis (Watt 1988) as reflected in the presence of increased amounts of sulfated PG, type II collagen, and aggrecan deeper within the tissue, potentiated further by the presence of transforming growth factor in the culture medium. During the initial period of growth, effete chondrocytes are probably crushed by the growing tissue and undergo necrosis/apoptosis as evidenced by the red nuclear staining seen with ethidium homodimer-1. Progressive deposition and accumulation of ECM between stacks of densely packed cells at the base of the culture might also generate the interstitial septa seen in this zone, as occurs in the developing growth plate (Eggli et al. 1985). Also, because the tissue is constrained laterally by the walls of the culture vessel, growth would proceed in a predominantly vertical direction, made manifest as an increase in tissue thickness as seen in this study.

It is conceivable that the distinct strata might also arise partly through metabolic/morphogenic gradients occurring within the developing tissue. These might arise spontaneously as a result of a differential between the metabolic conditions cells experience at the surface of the developing tissue and its base. For instance, at the base of the culture competition for metabolites would probably be greatest because, in this region, cells are most densely packed and depend upon diffusion of metabolites through the underlying filter membrane and the overlying cells and matrix. It is known that oxygen

cartilage repair purposes, the deleterious effects of chondrocyte dedifferentiation resulting from multiple passages still remain a major obstacle. Chondrocytes differentiated from stem cells would clearly obviate the necessity to perform multiple expansions of primary cells; thus, stem cell technology currently offers considerable promise. A number of recent studies, for example, have identified chondroprogenitor/stem cell populations within the superficial zone of articular cartilage (e.g., Dowthwaite et al. 2004). These cells have been shown to be highly proliferative, exhibit phenotypic plasticity in their differentiation pathway, and can undergo multiple rounds of passage without loss of chondrogenic phenotype (Dowthwaite et al. 2004; Martin et al. 2005), showing significant potential for future cartilage-engineering applications.

In summary, results of this study add to previous data demonstrating that high-density primary cultures of full-depth chondrocytes grown on Millipore filter membranes produce a zonally stratified hyaline cartilage tissue with histological, ultrastructural, and IHC features of immature articular cartilage. These features arise spontaneously in primary culture and are progressively lost after chondrocytes have been passage expanded in monolayer growth conditions. The resultant changes in graft thickness, organization, and composition thus reflect the cellular phenotypic changes that occur through chondrocyte dedifferentiation. Thus, significant potential exists in the continued development of autologous chondrocyte grafts for the repair of intrachondral lesions of human articular cartilage. There is, however, a pressing need to accommodate recent advancements in cartilage stem cell technology (Dowthwaite et al. 2004), new defined growth medium (Jakob et al. 2001; Chua et al. 2005), cell culture methodologies (Murphy and Polak 2004; Barbero et al. 2006), and gene therapy (Tew et al. 2005) that will allow bulking of chondrocyte numbers without the deleterious effects of cellular dedifferentiation.

Acknowledgments

Monoclonal antibodies CIICI (Holmdahl et al. 1986) and 12C5 (Asher et al. 1991) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. Antibodies toward type X collagen and decorin were generously provided by Professor G. Gibson and D. Bidanset, respectively.We would also like to acknowledge Dr. Mari Nowell for help with data analysis.

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engineer a neocartilaginous repair tissue completely ex vivo and then implant the mature construct within the intrachondral lesion site (Kandel et al. 1995). The potential advantages of this approach would be 3-fold: (1) growth conditions would be subject to a high degree of regulation in vitro, otherwise impossible within a diseased or damaged joint; (2) there would be no requirement for a periosteal/collagen flap or carrier because the engineered repair tissue would already possess a mature, fully differentiated cartilage matrix; and (3) this would make the repair and resurfacing of greater and more degenerative lesions a possibility, potentially without the requirement for open knee surgery. Thus, providing the appropriate technologies for implant fixation were in place (Kandel et al. 1995), these potential advantages would translate to an improvement in the scope and success of the repair and, significantly, a reduction in patient rehabilitation time.

Articular cartilage would appear well suited to an ex vivo regeneration strategy of the type described: it is a relatively simple tissue with a low cell-to-matrix volume ratio; it contains a single cell type, the chondrocyte, and it has no innervation, blood, or lymphatic supplies (reviewed by Poole et al. 2001). Yet the superficial simplicity of articular cartilage belies its underlying complexity. The mature tissue is both structurally and functionally heterogeneous and consists of four distinct zones: superficial/tangential, middle/transitional/intermediate, deep/radial, and a zone of calcified cartilage. Each of these zones has striking differences in chondrocyte morphology, gene expression, biochemical composition, and collagen fiber organization (refer to Figure 1). Collectively, these attributes allow the tissue to resist and dissipate biomechanical load while providing smooth, low-friction joint articulation for movement.

Over the last decade there have been a number of interesting publications, chiefly from the Kandel laboratory, describing neocartilaginous tissues engineered, scaffold-free, from high-density primary chondrocytes (Boyle et al. 1995; Kandel et al. 1995,1997,1999; Peel et al. 1998; Sun and Kandel 1999; Adkisson et al. 2001; Waldman et al. 2002,2003a,b,2004a,b,2005; Park et al. 2006; Allan et al. 2007). Primarily, cartilage tissues have been fabricated either upon porous, collagen-coated synthetic membranes or as biphasic constructs consisting of a cartilaginous tissue grown upon a calcium polyphosphate substrate. Interestingly, the engineered tissues have been shown to possess broad similarities with native articular cartilage and have been used for allogeneic transplantation in animal models with variable success rates (Kandel et al. 1995,2006; Peel et al. 1998; Park et al. 2006; Allan et al. 2007).

Significant potential exists in the further development of these types of neocartilaginous tissues for the repair of human intrachondral lesions. However, to ensure their successful integration and functioning in

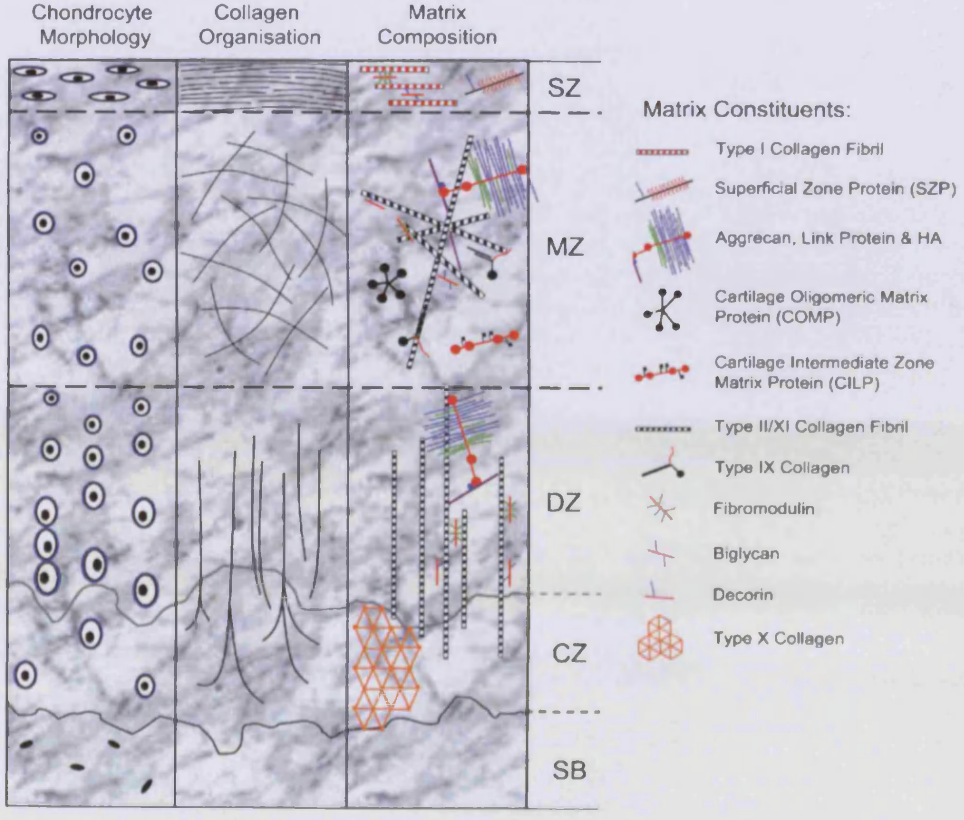

Figure 1 Schematic summarizing the macromolecular organization of mature articular cartilage. The tissue consists of four distinct zones: superficial (SZ); middle (MZ), deep (DZ), and a zone of calcified cartilage matrix (CZ), below which is the subchondral bone (SB). Each zone is distinct in terms of its cell morphology (left panel), collagen fiber organization (middle panel), and the biochemical composition of its extracellular matrix (ECM) (right panel). Matrix constituents of each cartilage zone are presented as molecular schematics.

Table 1 Antibodies used for immunohistochemistry

Antibody (dilution)	Clone (isotype)	Pretreatment	Specificity	Source/Reference
#70-XR90 (1:200)	P	ABC/K	Type I collagen	Fitzgerald Industries International; Concord, MA
CIICI (1:5)	M (IgG2a,κ)	ABC/K	Type II collagen	DSHB/Holmdahl et al. (1986)
αType X (1:100)	P	ABC/K	Type X collagen	Gibson et al. (1996)
6B4 (1:20)	M (IgG1)	ABC/K	Aggrecan (interglobular domain)	Caterson et al. (2000)
12C5 (1:5)	M (IgG1)	ABC/K	Versican (HA-binding region)	DSHB/Asher et al. (1991)
28.4 (1:20)	M (IgG1)	ABC/K	Decorin (core protein)	Bidanset et al. (1992)
PR8A4 (1:20)	M (IgG)	ABC/K	Biglycan (core protein)	Roughley et al. (1993)
1B5 (1:500)	M (IgG1κ)	ABC/K	Unsulfated chondroitin	Couchman et al. (1984) Caterson et al. (1985)
2B6 (1:20)	M (IgG1)	ABC/K	Chondroitin-4-sulfate and dermatan sulfate	Couchman et al. (1984)
		B/K	Dermatan sulfate	Caterson et al. (1985)
3B3 (1:20)	M (IgM)	ABC/K	Chondroitin-6-sulfate	Caterson et al. (1985)
5D4 (1:20)	M (IgG1)	None	Keratan sulfate	Caterson et al. (1983)
MCA2060 (1:200)	M (IgG2a)	HCl	Bromodeoxyuridine (BrDu)	AbD Serotec

ABC, chondroitinase ABC; B, chondroitinase B; K, keratanase; DSHB, Developmental Studies Hybridoma Bank; HA, hyaluronic acid.

containing 0.1% Tween 20 (Sigma-Aldrich), which was also used for each washing step. Where necessary, either to generate a neo-epitope or to improve antibody penetration, sections were enzymatically deglycosylated by pretreatment with either 0.5 U/ml chondroitinase ABC or chondroitinase B (both from Sigma-Aldrich) and 0.5 U/ml keratanase (AMS Biotechnology; Oxon, UK) in 100 mM Tris–acetate buffer (pH 7.4) for 1 hr at 37C (see Table 1). After washing, sections were treated with blocking serum (Dakopatts Ltd.; High Wycombe, UK) at 1:20 dilution for 30 min at room temperature before overnight incubation at 4C with primary antibody (see Table 1). After a second wash, sections were fluorescently labeled with FITC-conjugated goat anti-mouse or swine anti-rabbit Fab fragments (Dakopatts Ltd.), recognizing either mouse or rabbit species, for 30 min at room temperature. Sections were counterstained with propidium iodide (0.5 μg/ml; Molecular Probes, Invitrogen, Paisley, UK) for 5 min to impart nuclear context and then mounted in Vectashield fade-retarding mountant (Vector Laboratories Ltd.; Peterborough, UK). Control sections were incubated with PBS, 10 μg/ml mouse immunoglobulins (Sigma-Aldrich), or non-immune rabbit serum (Sigma-Aldrich) instead of primary antibody, with or without the enzymatic digestion step. Sections were viewed and photographed using a Leitz fluorescent microscope (Leica; Wetzlar, Germany) equipped with digital image acquisition.

Cell Viability Assay

To determine cell viability and identify the presence and distribution of dead cells within the neocartilage tissue, grafts were incubated in the presence of 2 μm calcein AM and 4 μM ethidium homodimer solution (Live/Dead Viability/Cytotoxicity Kit; Molecular Probes, Invitrogen) for 30 min. After incubation with the above reagents, grafts were washed in PBS, cut transversely into thin (~0.25 mm) slices using a sharp razor blade to minimize cell death, and mounted under coverslips in PBS. Sections were photographed immediately under epifluorescence optics as described previously.

Cell Proliferation

To identify proliferating cells and study the role of cell proliferation on tissue reorganization, neocartilage grafts were incubated with bromodeoxyuridine (BrDU, 1:1000; Amersham, Buckinghamshire, UK) in complete tissue culture medium for 90 min at 37C. Grafts were then fixed and processed for paraffin wax histology as described previously. A rat monoclonal antibody toward BrDU (AbD Serotec; Oxford, UK) (see Table 1) was used to localize proliferating cells using immunoperoxidase labeling in conjunction with DAB staining. This procedure was, in essence, similar to that described previously with the following modifications. After sections had been dewaxed and rehydrated they were incubated in 1 M HCl at 60C for 10 min to denature the DNA and improve antibody access. Endogenous peroxidase labeling was then irreversibly inhibited using 1.5% hydrogen peroxide (Sigma-Aldrich) in 100% methanol for 1 hr, followed by washing in PBS prior to the antibody blocking stage using rabbit serum (1:20). Sections were incubated with the anti-BrDU antibody (1:100) for 1 hr, washed in PBS, and then treated with a rabbit anti-rat biotinylated secondary antibody (1:200; Vector Laboratories Ltd.) for 30 min at room temperature. To check for nonspecific labeling with primary and secondary antibodies, respectively, tissue sections were incubated with either naive rat immunoglobulin or antibody diluent (i.e., PBS) instead of the anti-BrDU antibody. After washing in PBS, sections were incubated with avidin/biotin/peroxidase complex (ABC; 20 μl avidin DH, 20 μl biotinylated enzyme, and 1 μl PBS) for 30 min. The ABC complex was localized using a DAB kit (Vector Laboratories Ltd.) including nickel chloride in the reaction substrate to produce a black/brown reaction product. As a further check for nonspecific staining, the ABC complex or DAB staining steps were omitted. Sections were counterstained with 1% (w/v) alcian blue 8GX (pH 2.5) to impart tissue context and then photographed using bright-field optics.

Cell-Tracker Studies

To follow the in vitro kinetics of graft development, enriched chondrocyte subpopulations from each cartilage zone were labeled with a green fluorescent cell-tracker probe (Vybrant CFDA cell tracer kit; Molecular Probes, Invitrogen). To determine the extent of interzonal cell movement occurring during culture, a labeled mid-zone subpopulation was layered

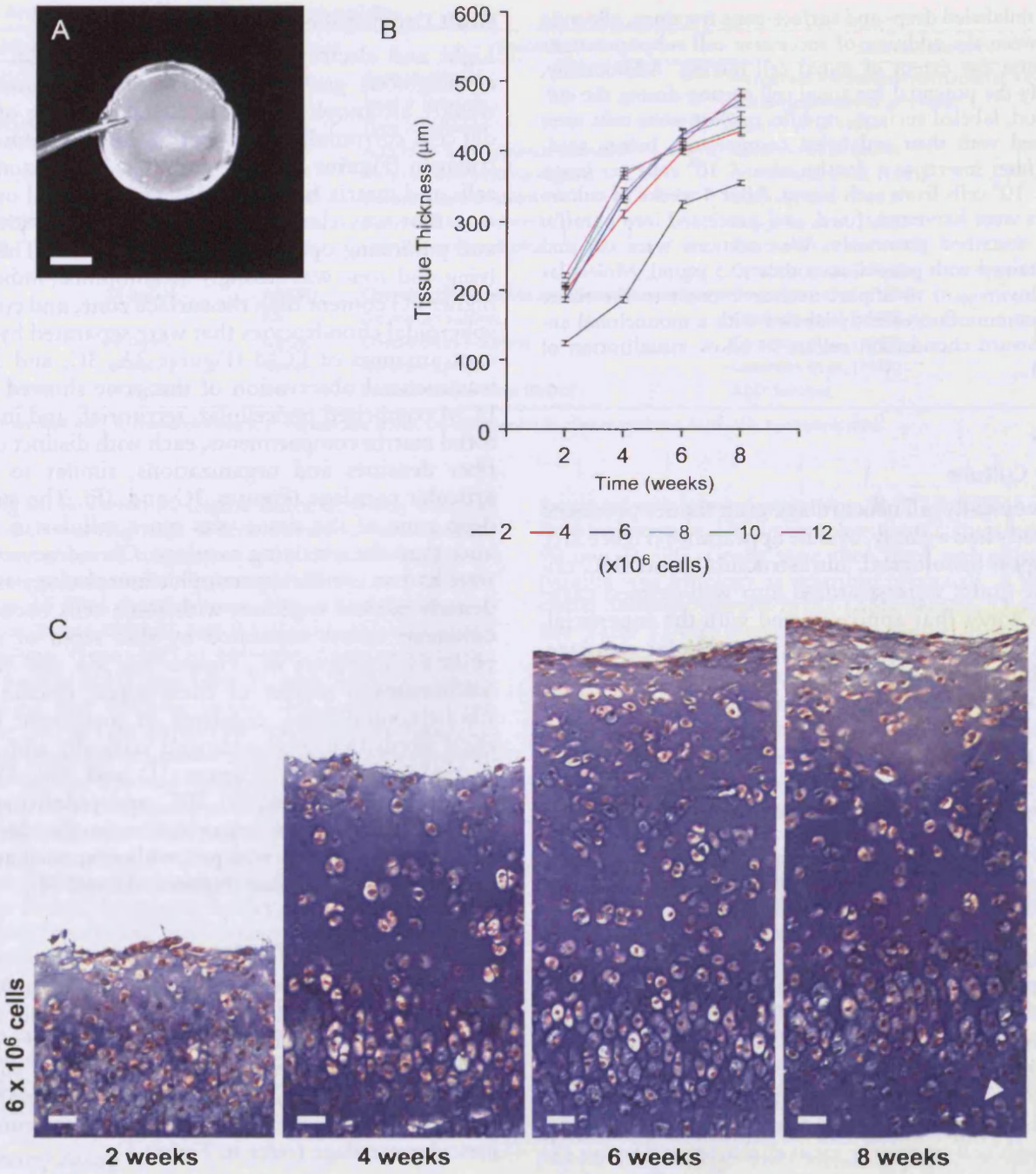

Figure 2 Effect of culture time and cell-seeding density on neocartilage tissue histology (primary culture). **(A)** Macroscopic appearance of a hyaline cartilage graft after 4 weeks of primary culture (6×10^6 cells). **(B)** Graph showing increase in cartilage tissue thickness over time associated with each cell-seeding densities (10^6 cells). Note highly significant increase in graft thickness ($p<0.001$) between seeding densities of two and four million cells per insert at all timepoints. **(C)** Histology of grafts after 2-, 4-, 6-, and 8-week periods (6×10^6 cells). Arrowhead indicates columnar organization of cells in basal zone. Alcian blue and hematoxylin/eosin. Bars: **A** = 2 mm; **C** = 25 µm.

PGs. Aggrecan, the large aggregating PG of cartilage, was detectable throughout the ECM of the graft tissue (Figure 4C); however, versican was not evident (Figure 4D). The small leucine-rich PGs biglycan and decorin were also detectable throughout the graft tissue (Figures 4E and 4F, respectively), but decorin, in particular, was more prominent in the surface zone of the tissue and diminished with depth, as occurs in articular cartilage. Superficial zone proteoglycan (SZP/lubricin/PRG4/MSF precursor protein) was weakly immunolocated to cells of the graft surface after 4 weeks of culture (Figure 4G); however, with advancing culture time

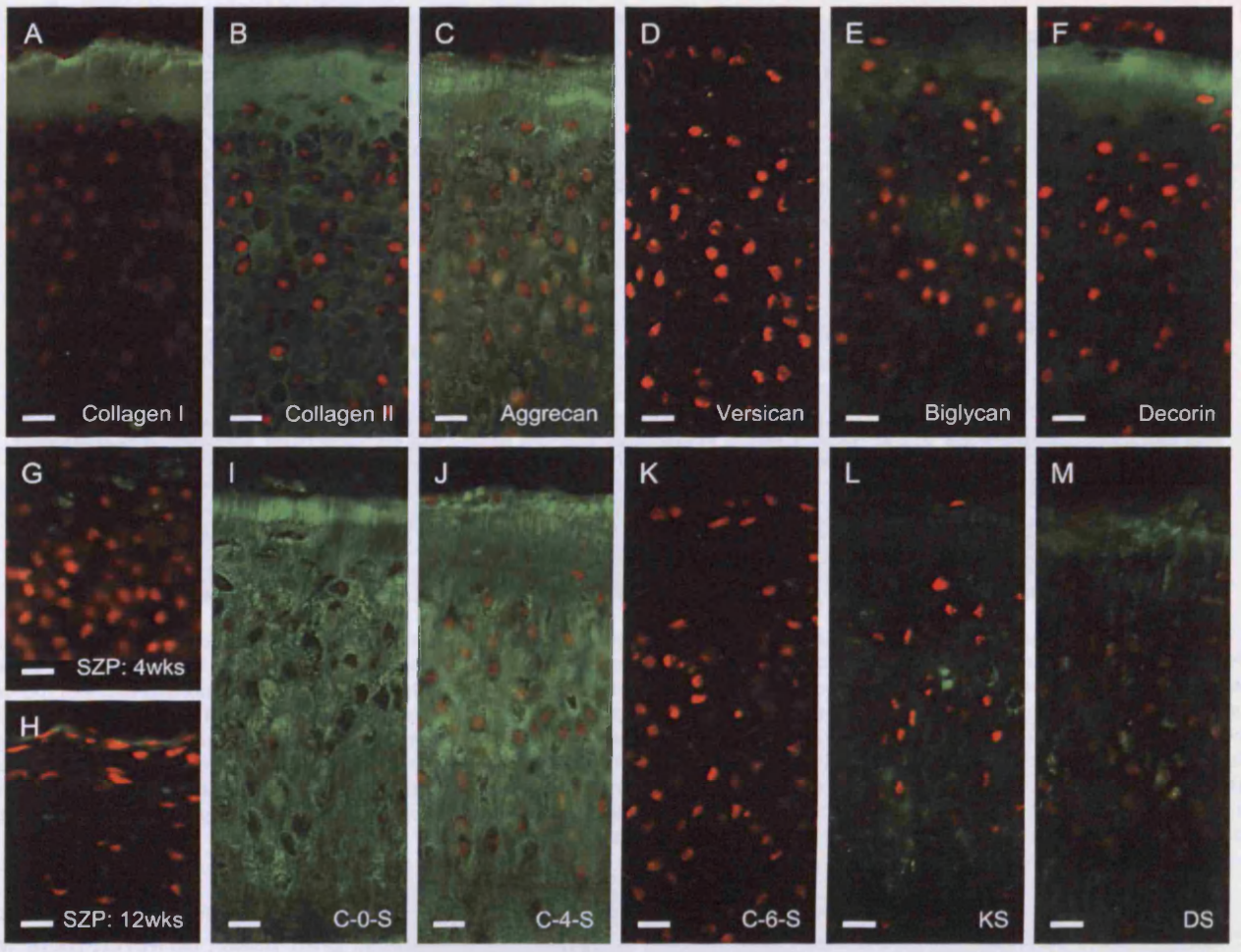

Figure 4 Immunohistochemical composition of neocartilaginous graft ECM. Sections have been labeled (green) with a panel of antibodies toward the chief structural components of the extracellular matrix, i.e., collagens (**A,B**), proteoglycans (**C–H**), and glycosaminoglycans (**I–M**). Cell nuclei have been counterstained red with propidium iodide. SZP, superficial zone proteoglycan; C-0-S, unsulfated chondroitin; C-4-S, chondroitin-4-sulfate; C-6-S, chondroitin-6-sulfate; KS, keratan sulfate; DS, dermatan sulfate. Bar = 25 μm.

seeding step than the comparatively smaller cells of the middle and surface zones (mean cell volumes ± SEM of 567 ± 14 μm^3 and 467 ± 8 μm^3, respectively).

Effect of Monolayer Expansion

Successive passage expansion of chondrocytes in monolayer conditions led to striking changes in tissue depth, histological organization, and IHC composition of the resultant tissue (Figure 6–Figure 8). There were significant deceases in graft thickness from >500 μm at P0 to <300 μm at P5 and a steady reduction in the extent of alcian blue staining after successive passage, indicative of a reduced sulfated GAG content (Figures 6A and 6B). Furthermore, histological organization of the tissue became less apparent with a progressive loss of zonal architecture (Figure 6B). At P3, chondrocytes had a profoundly reduced ability to lay down ECM, resulting in a progressive loss of cells to the medium and premature termination of the cultures at this stage. Interestingly, this capacity was restored following successive passage of cells, although the resultant grafts lacked any semblance of zonal organization, contained discrete clusters of cells in the basal portion of the tissue, and appeared histologically similar to fibrocartilage (Figure 6B). IHC labeling of the grafts also showed a qualitative increase in the extent of labeling for type I collagen and versican and an overall decrease in the extent of aggrecan immunolabeling with increasing passage number (Figure 7 and Figure 8), also indicative of a switch to a more fibrous cartilage phenotype. The labeling pattern for versican was particularly interesting because it coincided spatiotemporally with the cell clusters at the base of the tissue after P3, clearly suggestive of a fibrochondrocytic cell phenotype in this tissue zone.

Discussion

Tissue Organization

This study adds to previous data (e.g., Kandel et al. 1995) demonstrating that chondrocytes grown at high density upon porous filter membranes can produce

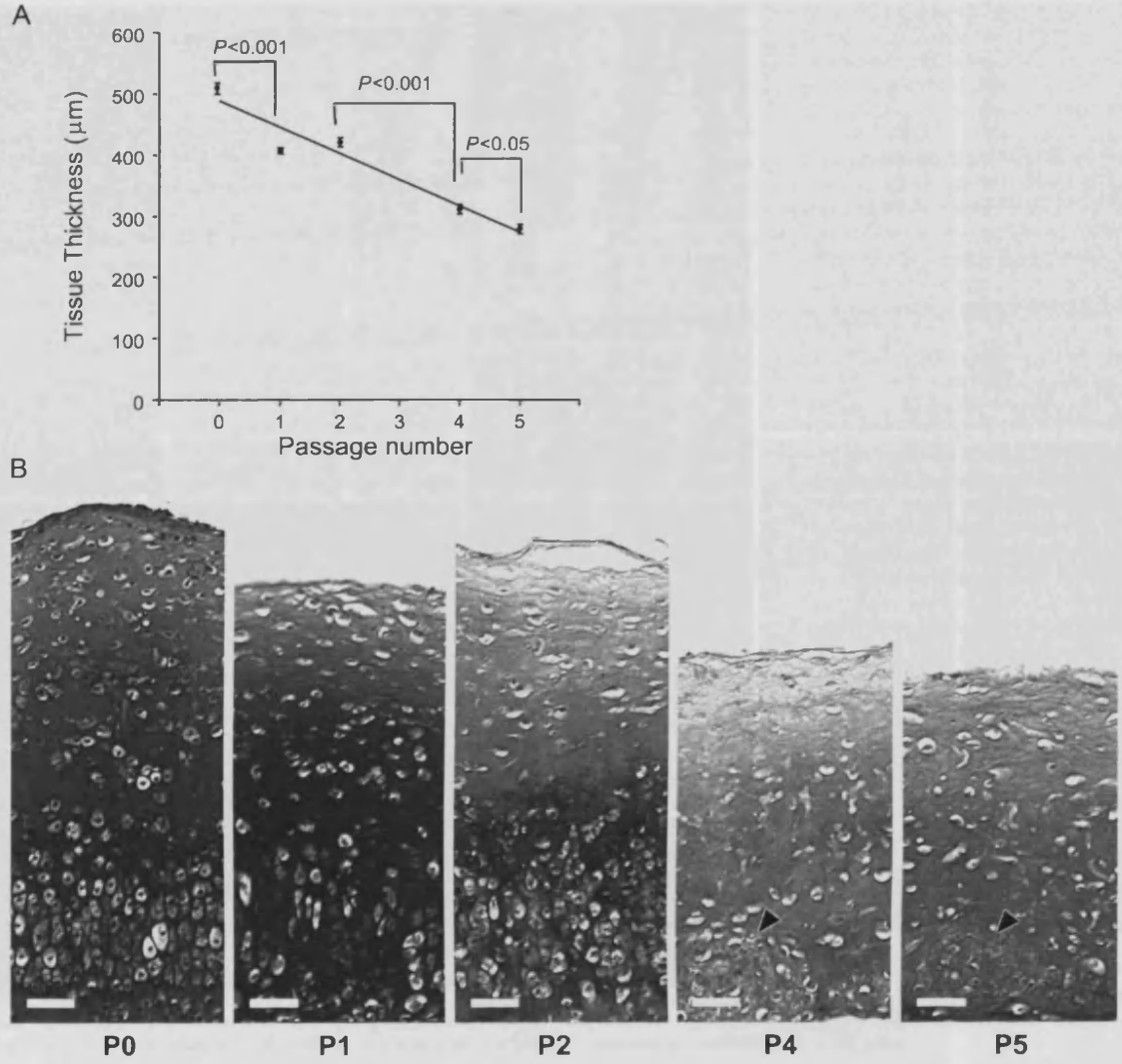

Figure 6 Effect of monolayer expansion upon the histological organization of engineered constructs. (**A**) Graph showing reductions in tissue thickness associated with increasing passage number. No data are presented at P3 as the cells transiently fail to elaborate an ECM, resulting in loss of tissue integrity. (**B**) Neo-cartilaginous tissues made from chondrocytes proliferated in monolayer culture. Note reductions in tissue thickness, organization, and extent of alcian blue staining with increasing passage number (P0-5). Cell clusters become apparent in the basal part of the tissue after P3 (arrowheads). Bar = 40 μm.

cartilage engineering cannot be overstated. For example, recent data show that chondrocytes require stimulation by applied surface motion, but not axial compression, to upregulate production of SZP and to generate a normal articular surface (Grad et al. 2005). Provision for these factors may thus be important in optimizing the functional value of neocartilage tissues generated for repair purposes. It is worthwhile considering, however, that although an engineered construct with similar mechanical properties to mature articular cartilage may be desirable from a biomechanical standpoint, an immature tissue phenotype may facilitate better integration with the host tissue and could be mechanically conditioned in situ, i.e., postimplantation, via a prudent exercise regimen.

Cellular Growth Mechanisms

Together our data suggest that the engineered tissues have a high cellular turnover and develop through a combination of appositional and interstitial growth mechanisms. Appositional growth would appear to occur principally at the graft surface, as evidenced by prominent BrDU incorporation at this site, and interstitial growth in the underlying cartilage tissue via a combination of cell proliferation and matrix accumulation. Our cell-tracker studies strongly suggest that zone-specific cell sorting does not play a role in the establishment of the distinct strata in vitro. Instead, our data suggest that chondrocytes divide or differentiate according to their potential and position in the developing tissue and that these behaviors are dictated

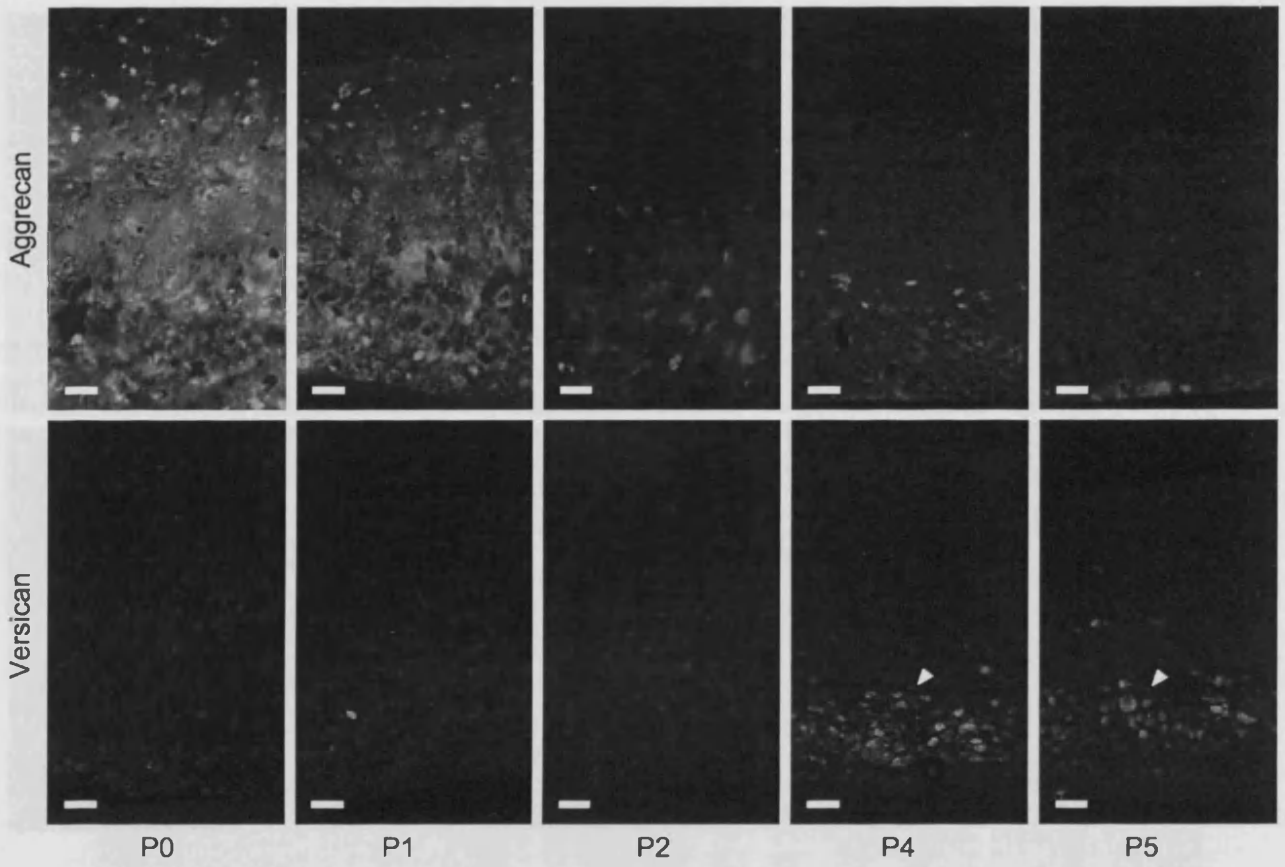

Figure 8 Effect of monolayer expansion upon the distribution of aggrecan and versican in neocartilaginous tissue. Higher magnifications showing progressive decrease in immunolabel for aggrecan with successive passage and corresponding increase in versican immunolabel, particularly associated with cell clusters in the basal part of the tissue after P3 (arrowheads). Bar = 40 μm.

tension, for example, plays an important regulatory role during cartilage growth by promoting chondrocyte differentiation and cartilage matrix synthesis while suppressing terminal chondrocyte differentiation (Hirao et al. 2006). Oxygen tension may play a similar role in the current context: its physiological effects manifesting in the zonal organization of cells and matrix within the in vitro-generated tissue.

Effect of Passage Expansion

Although chondrocytes are readily obtainable from animal models to generate allogeneic grafts for experimental purposes, to obtain sufficient cell numbers for a clinical application would necessitate multiple expansions of autologous chondrocytes in monolayer culture. It is well established that this type of culture causes their cellular dedifferentiation to a more fibroblastic phenotype: downregulation of cartilage-specific genes such as Sox-5, 6, and 9, aggrecan, and type II collagen and upregulation of type I collagen (Benya et al. 1978; Stokes et al. 2001; Yang et al. 2006). Although there is some evidence that passage-expanded chondrocytes can partially redifferentiate when transferred to culture models more conducive to chondrogenesis (e.g., agarose or high-density cultures) (Benya and Shaffer 1982; Watt 1988), in this study we have shown that their ability to produce true hyaline tissue resembling articular cartilage becomes dramatically reduced.

Other studies have shown similar phenotypic changes to occur in human articular chondrocytes between P0 and P4 in monolayer culture, with profound changes identifiable at the mRNA level identifiable as early as P1 (Darling and Athanasiou 2005). Chondrocytes isolated from human nasal septum cartilage have been shown to exhibit a similar impairment in their ability to proliferate at P3, whereupon they begin to acquire a fibroblastic dedifferentiation morphology (Chua et al. 2005). Binette et al. (1998) have also noted a similar downregulation of mRNA for aggrecan and type II collagen and an upregulation of versican and collagen type I by human articular chondrocytes after monolayer expansion, consistent with the IHC data presented in this study.

Thus, at present, although significant potential exists for the use of autologous chondrocyte grafts for

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