

# PLASTICITY AND INTERACTIONS OF ARTICULAR CARTILAGE PROGENITOR CELLS

A thesis submitted to the University of Wales  
for the degree of Doctor of Philosophy

by

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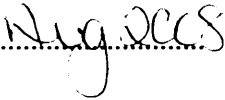


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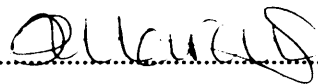
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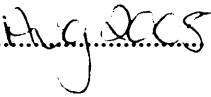
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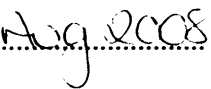
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## ABSTRACT

Articular cartilage is an avascular and aneural tissue and this is, in part, attributable to its low intrinsic capacity for repair after injury. Research is now focusing on alternate cell sources for tissue engineering of damaged cartilage, and recently a population of progenitor cells has been identified within the surface zone of bovine articular cartilage. These cells are capable of differentiating along a variety of mesenchymal lineages and are thought to be required for the appositional growth of the cartilage. The aims of this thesis were to further characterise these cells and determine factors affecting their differentiation. Prolonged growth of the clonal cells in culture was found to alter the ability of the cells to differentiate into a hyaline-like tissue, although these changes didn't always result in a decrease in the chondrogenic capacity. The rate of cell growth was also found to slightly affect the ability of the cells to differentiate, with more rapidly growing cells producing a matrix high in glycosaminoglycans. After short term culture, the cells also altered their expression of three different glycosaminoglycans sulphate epitopes 3B3(-), 4C3 and 7D4. When injected intramuscularly, the chondroprogenitor cells failed to form cartilage pellets despite expressing cartilage related genes. The progenitor cells also appeared unable to functionally engraft into the surrounding tissue, although one clonal cell line expressed the endothelial marker PECAM-1. Within this study we also assessed the ability of the chondroprogenitor cells to express connexins, and form functional gap junctions. The cells were found to fluctuate their connexin expression, although they maintained Cx43 expression throughout culture. Using a novel ultrasound standing wave trap, it was found that the cells failed to upregulate connexin after cell contact resulting in non-functional junctions, whilst the cells were able to form functional gap junctions with terminally differentiated chondrocytes. Treating the clonal cells with growth factors to enhance chondrogenesis also failed to cause the cells to functionally communicate. Finally we looked at the cellular organisation of the tissue to determine if paired cells within the surface zone of the cartilage may contain a progenitor population. These paired cells labelled positively for Notch-1, which is known to affect the clonality of the progenitor cells and could possibly signify the presence of the progenitor cell population. Cellular interactions are vital for controlling and coordinating cell differentiation, and manipulating cellular interactions could be an excellent way to enhance the chondrogenic differentiation of the cells and possibly improve tissue integration.

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## **ABBREVIATIONS**

ACI	Autologous chondrocyte implantation/transplantation
AER	Apical ectodermal ridge
ASC	Adult stem cells
BMP	Bone morphogenic protein
BrDU	Bromodeoxyuridine
CD	Cluster of differentiation
COMP	Cartilage oligomeric protein
Cx	Connexin
DMEM	Dulbecco's Modified Eagle Medium
DMMB	Dimethylmethylene blue
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ES	Embryonic stem cell
FCS	Fetal calf serum
FD	Full depth
FGF	Fibroblast growth factor
FITC	Fluorescein-5- isothiocyanate
GAG	Glycosaminoglycans
GDF	Growth and differentiation factor
HSC	Hematopoietic stem cell
IGF	Insulin-like growth factor
IL-1	Interleukin-1
ITS	Insulin transferring selenium
MAPC	Multipotent adult progenitor cells
MCP	Metacarpal-phalangeal
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MPC	Mesodermal progenitor cells
MSC	Mesenchymal stem cell
NBFS	Neutral buffered formal saline
NCAM	Neural cell adhesion molecule
NCID	Notch intracellular domain

NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD	Population doubling
PECAM-1	Platelet endothelial cell adhesion molecule-1
PG	Proteoglycan
PLGA	Polylactic-co glycolic acid
REU	Relative expression unit
SCID	Severe-combined immuno-deficiency
SEM	Standard error of the mean
SZ	Surface zone
SZP	Surface zone protein
TGF- $\beta$	Transforming growth factor-beta
TNF- $\alpha$	Tumor necrosis factor-alpha
USWT	Ultrasonic standing wave trap

**CHAPTER 1**  
**General Introduction**

## 1.1 Synovial Joints

The high level of mobility seen in vertebrates is due to the synovial joint (diarthrosis). This is located at a junction between two parts of the skeleton and is comprised of many different tissues including bone, meniscus, ligament, synovium, hyaline articular cartilage and the fibrous joint capsule (figure 1.1). Synovial joints vary in both size and range of motion throughout the body. The normal function of synovial joints is mediated by a smooth low friction surface provided by articular cartilage (Buckwalter and Hunziker, 1999).

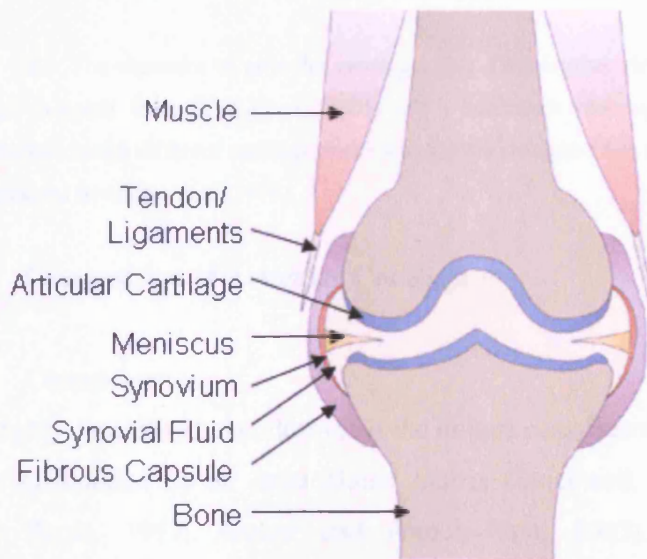


Figure 1.1 – Schematic of a synovial joint (adapted from Weiland et al. 2005)

## 1.2 Articular Cartilage

Articular cartilage is an avascular, aneural tissue that provides a low friction high load bearing surface to minimise stress on the bone beneath. The thickness, cell density and matrix composition of cartilage varies within the same joint. The depth of articular cartilage remains largely unchanged throughout adult life, providing normal joint movement for most of the population for about 80 years.

The surface of normal articular cartilage is smooth and white in appearance, and due to the avascular nature of the tissue, penetrating the surface does not cause pain or bleeding (Buckwalter et al., 1990). The tissues mechanical properties are provided for



by a well organised matrix that is able to distribute load (figure 1.2). The matrix is maintained and synthesised by the cellular component of cartilage, the chondrocyte.

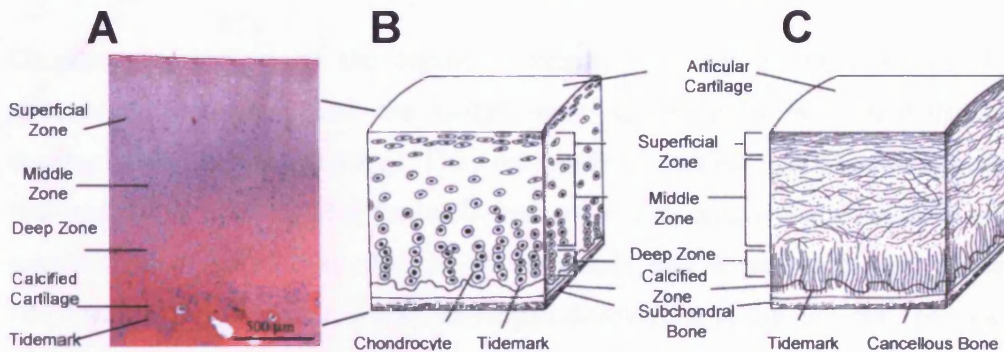


Figure 1.2 – The structure of articular cartilage. (A) Histological view of the structure of articular cartilage (adapted from (Yeh et al., 2005) (B) a schematic showing the cellular organisation of chondrocytes in the different cartilage zones and (C) the collagen fibre organisation within these zones (both adapted from Newman, 1998)

### 1.3 Composition of Articular Cartilage

#### 1.3.1 Chondrocytes

The highly specialised chondrocyte is the unique constituent cell of cartilage, where it exists surrounded by an extracellular matrix (Stockwell, 1978; Buckwalter et al., 1990; Poole, 1997; Archer and Francis-West, 2003). The chondrocytes are responsible for the maintenance of the matrix due to their ability to synthesise both major and minor components and to organise these components into an integrated, complex three-dimensional structure.

Chondrocytes form a sparse population and in many cases the cells remain cytoplasmically isolated from neighbouring chondrocytes. In human adult articular cartilage, chondrocytes make up less than 10% of the tissue's volume (Archer and Francis-West, 2003). The chondrocytes in articular cartilage differ in size, shape, metabolic activity and distribution depending on their location within the tissue.

The size and distribution of the chondrocytes through the matrix appear to increase as the distance from the articular surface increases. Cells located in the superficial zone are isolated and discoidal in shape, becoming spherical in the middle zone, where they are found both singly and in groups (figure 1.2B). The deep zone cells vary in

size and are arranged into columns perpendicular to the surface (Waldman et al., 2003).

Chondrocytes contain all the organelles required for matrix synthesis including endoplasmic reticulum and the Golgi complex, which is more numerous in developing and growing cartilage. They frequently contain intracytoplasmic filaments that are thought to stabilise the cells and some chondrocytes also possess short microvilli that extend from the cell to the matrix that sense mechanical changes (Buckwalter and Hunziker, 1999). Glycogen depositions in the cell may provide a store of raw material for matrix synthesis and growth as large amounts of lipids are found in the chondrocyte, with the fat globules increasing in size as the cartilage matures (Stockwell, 1979). Lysosomes are required for the removal of intracellular waste material and also have a role in extracellular matrix turnover. Due to the low respiratory activity of chondrocytes in adult tissue, the number of mitochondria decreases as the tissue ages. The mitochondria are also larger with more cristae in immature cartilage (Stockwell, 1978).

As a result of the avascular nature of the tissue and the large diffusion distance, chondrocyte metabolism is equipped to operate at very low oxygen tensions (ranging from <1% in the deep zones to 10% at the surface) and matrix synthesis is actually reduced by high oxygen tension (Grimshaw and Mason, 2001). Most of the cells energy requirements come from glycolysis, as all nutrients must pass through multiple barriers (synovial capillaries, synovial membrane, synovial fluid and then the matrix itself) to reach the chondrocyte.

In articular cartilage, the chondrocytes differ in activity and function as the tissue grows and matures. During development the chondrocytes are responsible for producing new tissue and altering the articular surface. It is at this point that the cells reach their highest rate of metabolic activity with rapid proliferation (Buckwalter and Hunziker, 1999). In skeletally maturing individuals, this proliferation rate decreases as the cells divide less frequently with less matrix synthesis and a lower rate of metabolic activity. In mature tissue, when skeletal growth is complete, the chondrocytes continue to produce matrix macromolecules to repair degradation (collagens, proteoglycans and non-collagenous proteins), even though they aren't

dividing. The cells respond to alterations in the composition of the matrix by detecting degraded matrix components and mechanical compression through calcium ions, cyclic AMP and signal transduction (Stockwell, 1978; Buckwalter and Mankin, 1998b; Fitzgerald et al., 2004).

Both appositional and interstitial growth phases are required to increase cartilage size. The mitotic rates of normal articular cartilage are about 1/20<sup>th</sup> of those in growth plate expansion (Stockwell, 1978). In the epiphyseal growth plate the mitotic rate is important for increasing the length of long bones and cell proliferation is also resumed after tissue damage (Stockwell, 1979). During maturation, the cellularity of human cartilage is decreased sevenfold and there is an inverse relationship in developing cartilage between tissue cellularity and cell size. This decline is due to the secretion and interposition of new matrix (Stockwell, 1978), with cell death also involved.

The distribution of cells varies through the tissue, with zoned differences detected. The number of cells in the superficial zone is greater than in the other zones, which may be due to the proximity to the synovial fluid and its nutrients. Cell density also differs between different adult cartilages. There is a twenty-five fold difference between cell density of the femoral condylar cartilage in a small species (mice) and man. This pattern is also seen in different size joints in the same species (Stockwell, 1979).

### **1.3.2 Extracellular Matrix**

The matrix of articular cartilage consists of two parts that give the tissue its mechanical properties, allowing movement, resilience and stiffness. The properties of the extracellular matrix (ECM) depend on the macromolecules physiochemical organisation.

### **1.3.3 Water**

The largest component of cartilage is water accounting for up to 80% of the wet weight of articular cartilage. The interactions between water and macromolecules are required to maintain the mechanical properties of the tissue. A portion of the water is able to move in and out of the tissue aiding joint lubrication and supplying nutrients

to the chondrocytes (Buckwalter et al., 1990; Buckwalter and Hunziker, 1999). The movement of the water depends on the interaction with structural macromolecules, primarily the large aggregating proteoglycans.

#### **1.3.4 Structural Macromolecules**

The structural macromolecules include collagens, proteoglycans and non-collagenous proteins synthesised by chondrocytes from amino acids and sugar. They contribute between 20 and 40% of the tissues wet weight and differ in concentration throughout the cartilage.

#### **1.3.5 Collagen**

About 60% of the cartilage dry weight is made up of multiple genetically distinct collagens (figure 1.2C). Although there are over 27 distinct types, articular chondrocytes have been shown to secrete only 10 of these, types I, II, III, V, VI, IX, X, XI, XII, and XIV. They form cross-banded fibrils that determine the tissues tensile strength and form. Collagen is synthesised by the articular chondrocytes and requires a series of complex steps. This initially takes place within the chondrocyte, with final assembly of the fibres occurring within the extracellular matrix (Eyre, 1991; Walchli et al., 1994). Collagen type II is the prominent collagen type, amounting for almost 90% of collagenous protein in cartilage and it is responsible for the fibrillar network.

Type II collagen is a class I fibril-forming collagen that is unique to cartilage. It is a homotrimer of three identical  $\alpha$  polypeptide chains that exists in two variants due to differential splicing. Type IIA collagen is the most frequent and is situated in adult tissues while type IIB is associated with immature chondrocytes (Sandell et al., 1991). Type II collagen is initially synthesised as a procollagen with N- and C-proterminal extensions which are required for the correct folding and assembly of the procollagen (Bulleid et al., 1997). As the cartilage develops and matures the collagen type II fibrils increase in diameter from under 20nm to between 50 and 100nm (Mendler et al., 1989; Eyre, 1991). Type XI collagen is thought to regulate this fibril diameter and it has been found that the fibril diameter differs between locations. The territorial and interterritorial matrices have been found to contain larger fibrils, with smaller fibrils located pericellularly (Sandell et al., 1999).

Like collagen type II, types IX and XI are also cartilage specific. These three molecules form a polymer by covalent interactions that result in a fibrillar matrix (figure 1.3). They also give the matrix mechanical stability (Mendler et al., 1989). Type XI collagen is a class I heterodimer comprising of three distinct  $\alpha$ -chains ( $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ). It is found within the fibres of the type II fibres allowing it to control the thickness of the fibre (Eyre et al., 2002). It is also thought that there is a small degree of cross-linking between the type II and XI molecules (Eyre, 1991).

Type IX collagen constitutes about 1% of the collagenous protein of adult mammalian cartilage, and almost 10% of fetal cartilage. The decrease in the amount of type IX collagen is relative to the increase in type II collagen that is seen with increasing age (Watton et al., 1988; Mendler et al., 1989). This relationship maybe due to the contribution collagen type IX has to the growth and fibre diameter of type II collagen.

Collagen type IX is also a heterotrimer of three  $\alpha$ -chains consisting of three triple helical domains (COL1, COL2 and COL3) that are separated by four non-collagenous domains (NC1-4). The non-collagenous region NC3 has a single site for attachment of a chondroitin sulphate chain (van der Rest and Mayne, 1988; Watton et al., 1988; Eyre et al., 2002). This results in collagen type IX being described as both a collagen and a proteoglycan.

Sites for cross-linking have also been found in the COL2 region, which allows for the covalent attachment of the telopeptides at the terminus of collagen type II. The three  $\alpha$ -chains in this domain have hydroxylysine residues to which the N-telopeptides can bind (Eyre et al., 2002). There is a further site in the  $\alpha 3$  (IX) chain where the C-telopeptide of the collagen type II can bind (van der Rest and Mayne, 1988; Buckwalter and Hunziker, 1999; Eyre et al., 2002). This cross-linking is similar to that seen between adjacent type II molecules and it is thought that it provides a link between the type II fibrils and the proteoglycans and that it provides interfibrillar links that enhance the stability of the network of fibrils (Wu and Eyre, 1989).

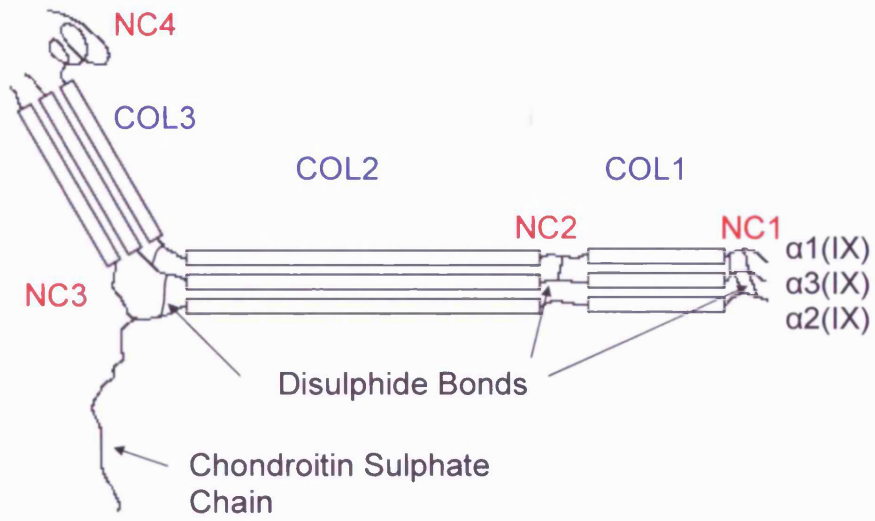
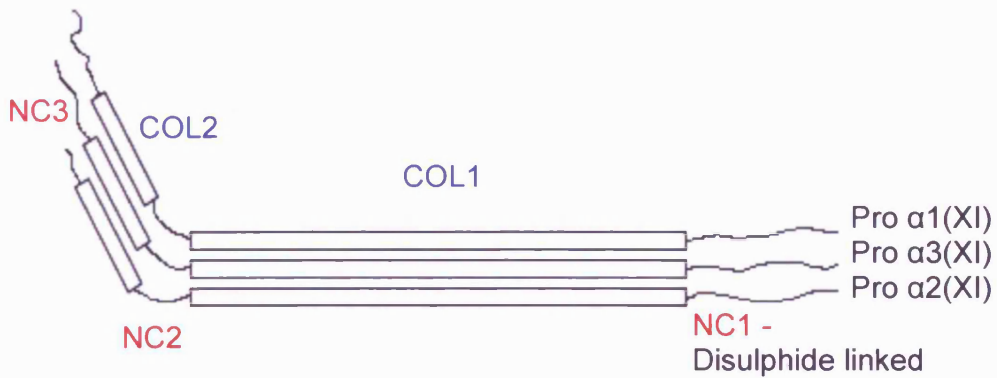
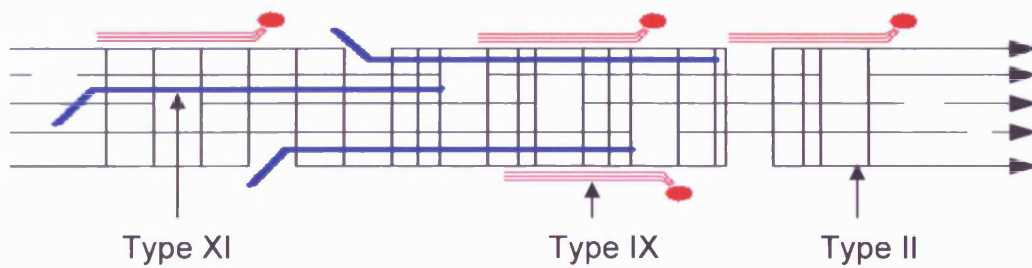
**A****B****C**

Figure 1.3 Collagens of articular cartilage. Diagram showing (A) collagen type IX (B) collagen type XI and (C) a heterotypic collagen fibril consisting of interwoven collagen types II, IX, and XI (adapted from Duance et al., 1999)

Type X collagen is a short-helix collagen consisting of three  $\alpha 1(X)$  chains and like type VI, it is not fibrillar. The chains contain a triple helical collagenous domain with a carboxy-terminal domain at one end and a short non-helical N terminus at the other (Eyre, 1991; Kwan et al., 1991; Shen, 2005). It is a cartilage specific collagen that is expressed by hypertrophic chondrocytes as levels of type II and IX collagen fall (Shen, 2005). The distribution of type X collagen indicates it could play a role in endochondrial ossification.

Type VI collagen is fundamentally a glycoprotein that consists of a short triple helix comprising three genetically distinct chains  $\alpha 1(VI)\alpha 2(VI)\alpha 3(VI)$ . As it doesn't undergo proteolytic cleavage there are large globular domains at each end of the chains (Bruns et al., 1986). Soon after synthesis, polymerisation occurs leading to the formation of disulfide-bonded dimers and tetramers that are not cross-linked (Doliana et al., 1990). Type VI collagen is able to interact with many different macromolecules and is found in most tissues and in the pericellular matrix of chondrocytes where it is thought to have a role in anchoring the cells to the ECM (Keene et al., 1988).

Type I collagen, like types II and III, is a fibrillar collagen and is present in porcine articular cartilage (Wardale and Duance, 1993), the surface of articular cartilage during development and in tissue undergoing repair (Duance et al., 1999). It is heterotrimer consisting of two  $\alpha 1(I)$  chains and a  $\alpha 2(I)$  chain (Benya et al., 1978).

Type III collagen is reported in both developing tissue and tissue undergoing repair. Although its function is unknown, it is found associated with type I collagen and forms cross-links with type II (Young et al., 2000).

Collagen types XII and XIV are structurally related FACIT (fibril-associated collagens with interrupted triple-helices) collagens that have homology to type IX collagen. In foetal bovine cartilage, both types XII and XIV have been found as a proteoglycan with chondroitin sulphate side chains (Watt et al., 1992). Both molecules form close associations with type I collagen and although they are unable to form fibrils, they are believed to influence the architecture of the fibrillar collagens. Type XIV collagen is expressed in all tissues expressing type-I whilst type XII is expressed mainly in embryos (Walchli et al., 1994).

### 1.3.6 Proteoglycans

Proteoglycans are a major component of the ECM and are involved in cell adhesion, proliferation, migration and the tissues ability to withstand compressive force (Wight et al., 1992). They consist of a central protein core linked covalently to glycosaminoglycans (GAGs) such as chondroitin sulphate and keratan sulphate (figure 1.4). The GAGs contain numerous negative charges, which produces two important physical properties. Adjacent chains repel each other allowing the molecule to remain expanded in solution. The negative charges also permit the binding of cations causing the proteoglycans to become hydrophilic, controlling the water content of the cartilage (Newman, 1998; Buckwalter and Hunziker, 1999).

Although there are multiple proteoglycan types, articular cartilage consists of two major forms: the large aggregating proteoglycans or aggrecans and smaller molecules such as decorin and fibromodulin. Aggrecan is the most abundant proteoglycan in articular cartilage (Mok et al., 1994) and it is located in the interfibrillar space in the ECM where it associates non-covalently with hyaluronan, link proteins and small non-collagenous proteins to form aggregates. The large aggregates enabling anchoring of the proteoglycans within the matrix and the collagen meshwork (Buckwalter and Hunziker, 1999)

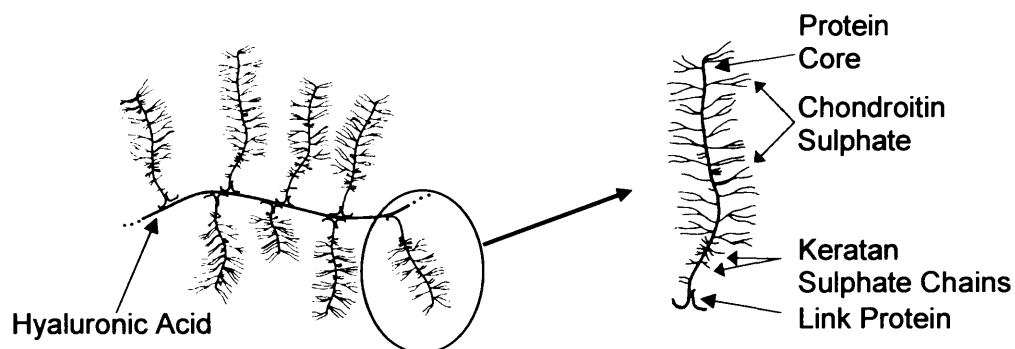


Figure 1.4 – The structure of the proteoglycan aggregate. Proteoglycan chains are able to bind to a hyaluonic acid backbone via small link proteins. Enlarged view of a single proteoglycan molecule (adapted from Newman, 1998)

A new proteoglycan has recently been identified that has unique biochemical properties compared to any other known proteoglycan. Surface zone protein/proteoglycan (SZP) or proteoglycan 4 (PRG4) is found in the matrix of chondrocytes on the surface of articular cartilage and can also be secreted by these



cells into the synovial cavity (Schumacher et al., 1994; Flannery et al., 1999; Rees et al., 2002; Schmidt et al., 2004). SZP is highly homologous to the proteoglycan lubricin that has been shown to protect the articular cartilage surface and aid in lubrication of the joint (Rhee et al., 2005). The composition of SZP allows for several biological functions including cell proliferation, cytoprotection and self-aggregation (Flannery et al., 1999).

### 1.3.7 Non-Collagenous Macromolecules

Normal adult articular cartilage has also been found to contain a variety of non-collagenous proteins and glycoproteins. Although many of their specific functions aren't well understood, it is believed they are involved in matrix organisation, maintenance and cell matrix interactions (Heinegard and Oldberg, 1989; Neame et al., 1999; Roughley, 2001).

#### Tenascin

Tenascin is a large extracellular matrix glycoprotein that is involved in embryonic development and wound healing (Chiquet-Ehrismann, 1990; Salmivirta et al., 1991; Erickson, 1993; Mackie and Murphy, 1998; Veje et al., 2003). The tenascin family consists of three members: tenascin-C, -X and -R (Erickson, 1993). They are a six armed oligomer, with the separate subunits connected by disulfide bridges into a hexabrachion structure (figure 1.5).

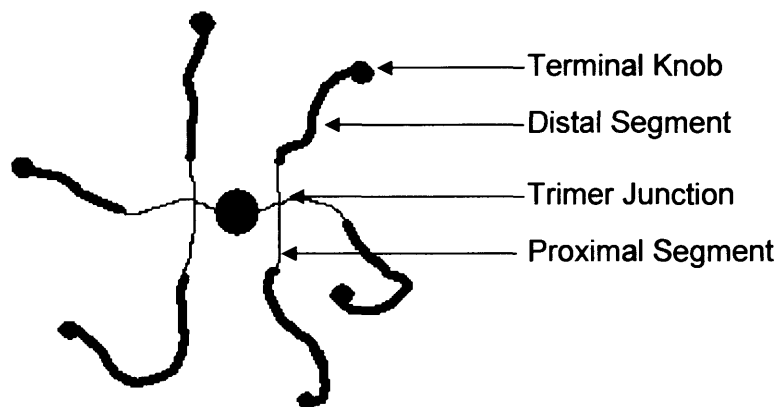


Figure 1.5 – Diagram showing the hexabrachion structure of tenascin. The molecule consists of six thin arms that are joined to form two trimers. These trimers are then linked by disulfide bonds around a central knob to give the hexabrachion structure (adapted from Erickson, 1989)

*Arms*

The protein consists of numerous differentially spliced fibronectin type III repeats sandwiched between a region of fibrinogen homology and a region of EGF like repeats (Chiquet-Ehrismann, 1990). Although the exact function of tenascin C is unknown, it has anti-adhesive and anti-spreading properties and adding it to cells in culture causes them to become rounded (Hsia and Schwarzbauer, 2005). Tenascin C is found in high levels during embryogenesis, where it is expressed in the dense mesenchyme of the developing cartilage and bone (Chiquet-Ehrismann and Tucker, 2004). Studies in adult tissue have shown it is found in the pericellular matrix of cartilage where it interacts with cell surface receptors including integrins and syndecans (Salmivirta et al., 1991). Tenascin C is not present in hyaline cartilage, whilst it is expressed in the surface zone and perichondrium of articular cartilage at low levels (Mackie et al., 1987), which maybe due to the load bearing properties of articular cartilage.

#### Fibronectin

Fibronectin is a structural glycoprotein found in serum and the ECM of many different tissues. It is a major adhesive protein that interacts with other extracellular molecules and has a role in cell attachment, migration (mainly during development and healing) and the regulation of cell growth and differentiation (Pierschbacher and Ruoslahti, 1984). Fibronectin is produced by chondrocytes and is also found at low concentrations in normal synovial fluids and its production is elevated during disease (Nishida et al., 1995). It has a major role in stabilising and organising the matrix and is involved in cell signalling interactions via  $\alpha 5 \beta 1$  integrin-mediated pathways (Burton-Wurster et al., 1997; Homandberg et al., 2002).

#### Cartilage Oligomeric Matrix Protein (COMP)

COMP is a pentameric cartilage matrix glycoprotein that is composed of five disulfide linked 1000kDa sub-units (Hedbom et al., 1992). Although the exact function of COMP is unclear, it has been suggested that it may be involved in chondrocyte-matrix attachment and formation of collagen fibrils due to its preferential localisation in the territorial matrix. The distribution of COMP has been found to differ after increased joint loading in equine cartilage (Murray et al., 2001) with this also affecting the zonal distribution of COMP.

## **1.4 Structure of Articular Cartilage**

Cartilage matrix differs in morphology, composition, and mechanical properties, as well as the synthetic ability of cells, as distance from the cartilage surface increases (figure 1.2). The tissue can be divided on the basis of chondrocyte and matrix organisation and depth into four zones: the superficial zone, the middle zone, the deep zone and the zone of calcified cartilage.

### **1.4.1 Superficial Zone**

The thinnest zone in articular cartilage is the superficial zone and it is believed that its structure and properties are responsible for the weight bearing and lubrication mechanisms that occur between the joint surfaces. It consists of two layers, an acellular layer comprising of a covering of amorphous material (Jurvelin et al., 1996) and a deeper cellular layer. The amorphous material is sometimes referred to as the 'lamina splendens' (MacConaill, 1951) and is a coating of fine non-collagenous fibrils. Below this are flattened, ellipsoid shaped chondrocytes that synthesise a high collagen, low proteoglycan matrix when compared with other zones (Aydelotte and Kuettner, 1988). The superficial matrix also contains a high water concentration with fibronectin that is thought to protect the joint and aid in lubrication by binding to the hyaluronan synovial fluid (Poole et al., 1987; Nishida et al., 1995).

The mechanical properties of the tissue are influenced by a dense mat of thin collagen fibrils that lie parallel to the surface of the superficial zone (Buckwalter and Hunziker, 1999). These fibrils control the passage of molecules through the zone and provide greater tensile strength to the tissue, enabling it to withstand the sheer force generated during joint movement.

It has been demonstrated by Setton *et al* (1993) that the compressive ability of articular cartilage is also partially influenced by the integrity of the superficial zone. Damage to the articular surface is believed to increase tissue permeability and disruption of the matrix. Alterations to this matrix in the superficial zone is thought to contribute to the development of osteoarthritis, as early osteoarthritis is marked by disruption and remodelling of the collagen fibrils (Guilak et al., 1994).

### **1.4.2 Middle Zone**

The cells and matrix composition of the middle, or transitional zone, is an intermediate between the morphology of the superficial and deep zones. The transitional zone has several times the volume of the superficial zone and consists of a matrix with an increased proteoglycan concentration and larger diameter, intersected collagen fibrils (Poole, 1997). The chondrocytes are spherical and contain more synthetic organelles, endoplasmic reticulum and Golgi complex than the superficial cells.

### **1.4.3 Deep Zone**

The cells in the deep or radial zone are rounded and aligned perpendicular to the articular surface. The collagen fibrils are thicker than in the other zones, with a higher concentration of proteoglycans and a low water concentration (Buckwalter and Hunziker, 1999). Fine collagen fibrils found at the 'tidemark' are able to form an anchoring system between the cartilage and the calcified cartilage (Redler et al., 1975).

### **1.4.4 Calcified Cartilage Zone**

The calcified cartilage zone is a thin layer (5-10 $\mu$ m) that separates the uncalcified cartilage of the deep zone with the subchondral bone. The chondrocytes are rounded in uncalcified lacunae, with few endoplasmic reticulum and Golgi complexes, indicating low levels of metabolic activity (Morris et al., 2002). A prominent feature of this layer is the articular tidemark which acts as an interface between calcified and non-calcified hyaline layers.

## **1.5 Matrix Regions**

Within the horizontal cartilage zones, variations in the matrix have been discovered from which three compartments can be distinguished (figure 1.6): the pericellular region, the territorial region and the interterritorial region (Buckwalter and Hunziker, 1999). Of these, the interterritorial matrix provides the tissues mechanical properties, whilst the pericellular and territorial regions support the chondrocyte. They allow binding of the cell membranes to matrix macromolecules, aiding in the transmission

of signals to the chondrocyte during deformation. They also provide a protective role, preventing damage during loading and tissue deformation (Buckwalter et al., 1990).

### **1.5.1 Pericellular Matrix**

Individual chondrocytes are surrounded by a pericellular matrix compartment that is free from fibrillar components but is rich in glycoproteins, non-collagenous macromolecules and non-fibrillar collagens including type VI (Poole et al., 1988; Poole, 1997) and IX (Wotton et al., 1991). These fine collagen fibres that are densely compacted and tightly woven contain cytoplasmic extensions that project from the chondrocyte to the territorial matrix. The pericellular matrix forms a thin film to which the chondrocyte cell membranes attach (Buckwalter and Hunziker, 1999). This is then surrounded by filamentous material that provides a 'felt-like' capsule around the chondrocyte and pericellular matrix, to form a chondron. The chondron forms a functional unit that is able to absorb loads and provide protection for the chondrocyte. The large quantity of proteoglycan present in the chondron allows for a greater swelling pressure (Poole, 1997).

### **1.5.2 Territorial Matrix**

The chondrocytes pericellular matrix is surrounded by an envelope of territorial matrix with thin collagen fibrils spanning both layers fusing them together. In the radial zone columns of cells are covered by one territorial matrix. As the distance from the cell increases, the fibrils form a fibrillar basket around that cell that is thought provide the chondrocyte with protection during joint loading. The fibrils then adopt a more parallel orientation, marking the boundary between the territorial and interterritorial matrices (Buckwalter and Hunziker, 1999).

### **1.5.3 Interterritorial Matrix**

The intercellular matrix contains the largest portion of the volume of articular cartilage. It contains the largest diameter collagen fibrils (Petit et al., 1996) that are not associated with the chondrocyte but change their orientation as the distance from the articular surface increases. In the superficial zone, the fibres lie parallel to the surface, with the orientation changing 90 degrees as they progress to the deep zone (Buckwalter and Hunziker, 1999).

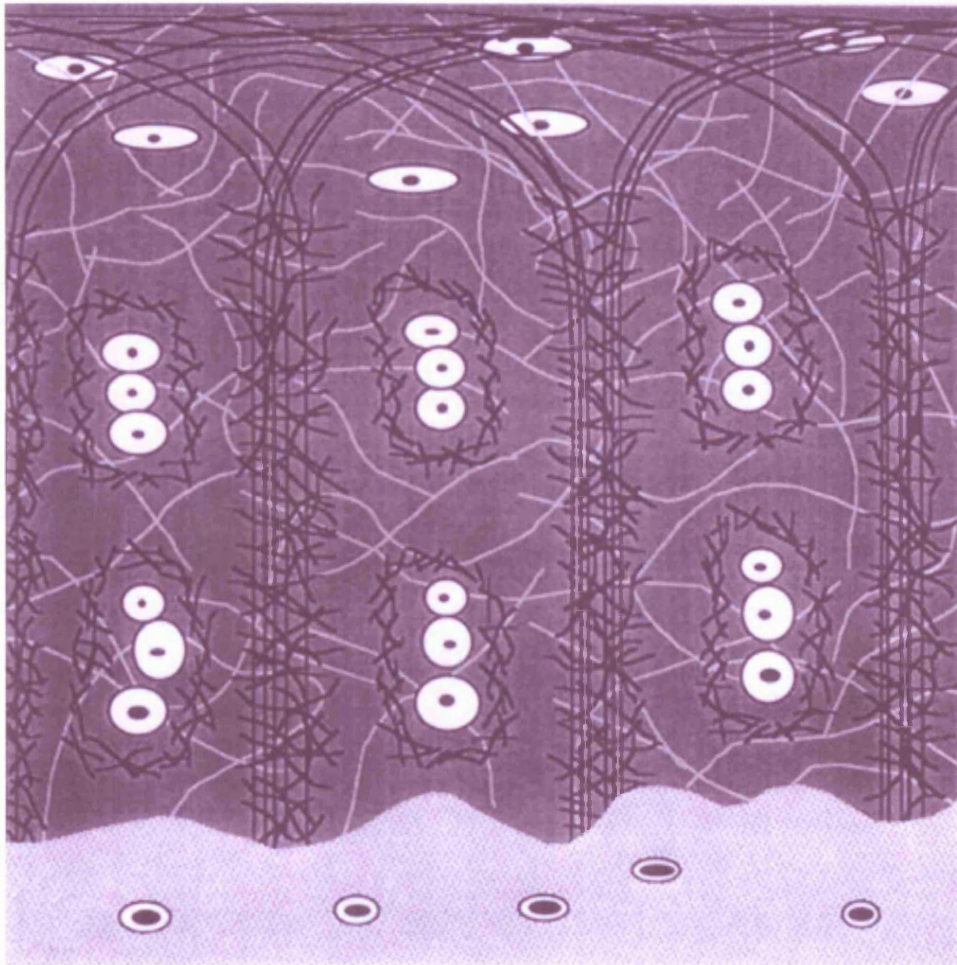


Figure 1.6 – Diagram showing the different regions of articular cartilage and the interactions of chondrocytes with the fibrillar matrix components. The pericellular matrix surrounds chondrocytes and is composed of cross-banded filaments, free from fibrillar components. The territorial matrix consists of a fibrillar basket of collagen fibres that encompass the cell and its pericellular matrix. Most of the tissue is formed by the interterritorial matrix and is composed of both fibrils and fibril bundles that are located in two different ways. The first type forms arcade-like structures and is highly orientated and organised whilst the second population is much more randomly orientated (adapted from Buckwalter and Hunziker, 1999).

## 1.6 Chondrocyte-Matrix Interactions

The matrix of articular cartilage is maintained throughout life by chondrocytes replacing degraded matrix molecules or increasing synthesis after cartilage injury or degeneration (Martin and Buckwalter, 2000). To maintain the integrity of the tissue there must be a mechanism in place for cells to monitor the adjacent matrix. This appears to be achieved by the integrin family of cell surface receptors that mediate cell-matrix interactions (Salter et al., 1992; Loeser, 2000).

Integrins are heterodimeric glycoproteins that result from a combination of one of 9 types of  $\beta$  subunit and 14 types of  $\alpha$  subunit. A large extracellular domain is able to bind to specific proteins in the ECM, whilst a short cytoplasmic domain is able to interact with cell signalling proteins and cytoskeletal proteins (Gumbiner, 1996). The link provided by integrins provide a means of transmitting signals between the ECM enabling alterations in cell function and gene expression (Loeser, 2000). Chondrocytes have been shown to express many different integrin subunits. The most common of these is the  $\beta 1$  subunit that has been found to be associated with various  $\alpha$  subunits to mediate interactions with fibronectin, collagen types II (Durr et al., 1993), VI, and laminin (Loeser, 2000). Both human and chicken chondrocytes have been found to express relatively high levels of the  $\alpha 5\beta 1$  subunit that functions as a fibronectin receptor influencing the spread and adhesion of cells (Salter et al., 1992; Enomoto-Iwamoto et al., 1997).

There are also other types of chondrocyte associated receptors capable of transmitting signals. The annexins are another subgroup of receptors that are able to bind calcium and phospholipids (Kirsch and Pfaffle, 1992). Annexin V (or anchorin CII) is able to bind to type II collagen and is located close to the surface of human chondrocytes (Mollenhauer, 1997). Levels of annexin V are elevated in diseased tissue, indicating it has a possible role in cartilage repair (Kurtis et al., 2001). CD44 is a membrane glycoprotein involved in the organisation and maintenance of the pericellular matrix through interactions with hyaluronan (Bosch et al., 2002).

## 1.7 Cartilage and Synovial Joint Development

Cartilage formation is one of the earliest obvious morphogenetic events in embryogenesis. The chondrocytes that form the limb and cartilage, are derived from mesenchymal cells within the mesoderm and these cells undergo a sequence of regulated steps to form mature cartilage (DeLise et al., 2000). These events are highly synchronized both environmentally and by the cell itself, although the exact mechanisms are unknown. It is thought that two adhesion molecules N-CAM and N-cadherin (Oberlender and Tuan, 1994; Archer et al., 2003), may have a role in chondrocyte condensation whereby the cells per unit area increases without an increase in cell proliferation, amplifying the amount of cell-cell contact (Rudnicki and Brown, 1997; DeLise et al., 2000). The transcription factor Sox9 is important during the process of condensation due to its role in the activation of collagen type II enhancer elements (Akiyama et al., 2002).

The joint develops from a pre-chondrogenic blastema, a densely packed cellular mesenchyme with high levels of cell-cell interactions (Oberlender and Tuan, 1994; Rudnicki and Brown, 1997). Cartilaginous nodules appear in the middle of this tissue and the regions between these nodules remain separate designating where the new joint will be. This area forms the interzone, a band of densely packed cellular blastema, which has the ability to differentiate into three layers: two chondrogenic layers covering the articulating surfaces like perichondrium, and one layer of loose cellular tissue (Mitrovic, 1978). Within this aggregate, cells differentiate into chondroblasts and begin producing the ECM. This leads to the expression of cartilage specific molecules such as collagen type II and the formation of a cartilaginous anlagen that specifies the position of the future skeletal elements (DeLise et al., 2000).

The mechanism by which a joint is specified is not well understood, although it is known to involve many different factors that have roles in blocking signals promoting chondrogenesis. Growth and differentiation factors (GDF) -5, -6 and -7, are a subgroup of secretory signalling molecules within the bone morphogenic protein (BMP) family that are found in the developing joint interzone and are amongst the earliest known markers of embryonic joint formation (Storm et al., 1994; Archer et



al., 2003). GDF-5 is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, consisting of a group of molecules normally associated with promotion of chondrogenesis (Archer et al., 2003). Mutations in this gene disrupt normal skeletal development resulting in a decrease in the length of long bones of the limbs and abnormal joint development (Francis-West et al., 1999; Storm and Kingsley, 1999).

Although GDF-5 is involved in joint development, it does not specify the joint type. It has been proposed that the induction of synovial joints is controlled by an upstream activator of GDF-5 and member of the Wnt family of genes (Hartmann and Tabin, 2001). Many of these genes have been implicated in various aspects of chondrogenesis and Wnt 14 has been found to be expressed at the sites of joint formation (Hartmann and Tabin, 2000; Hartmann and Tabin, 2001).

The expression of Wnt-14 has been shown to induce morphological changes in early joint-forming regions of the embryo by activating gene markers of joint development (i.e. chordin, CD44 and GDF-5) and inducing gaps in chondrogenic condensation and cavitation (Archer et al., 2003). Ectopic Wnt-14 expression leads to a repression of joint formation, and therefore each new joint region would block formation of an interzone, and therefore another joint, in the direct vicinity leading to joint spacing (Hartmann and Tabin, 2001). As the joint grows, this signal is lost allowing for joint induction to occur. BMPs have been shown to have a positive effect on the growth of interdigital areas, whilst Wnt-14 can activate the BMP antagonist chordin (Zou et al., 1997).

### **1.7.1 Articular Cartilage Development**

The mechanisms by which some mesenchymal cells acquire the ability to differentiate into articular cartilage, whilst others resist this chondrogenic pathway are not well understood (Pacifici, 1999). It is possible that this is due to intrinsic factors or the environment in which the cell is growing.

Tenascin-C is involved in the separation of epiphyseal ends of joints. It is located in the developing chick at tissue-tissue boundaries where it is able to interfere with cell attachment and spreading (Erickson and Bourdon, 1989). Exogenous tenascin-C

expression has also been found to stimulate prechondrogenic mesenchymal cells in micromass to undergo chondrogenic differentiation and it has been found to maintain a rounded cell phenotype (Mackie et al., 1987).

Another key macromolecule is syndecan-3, a cell surface glycoprotein that may have a role in regulating chondrocyte proliferation (Shimazu et al., 1996). In early development (day 6 in chick embryos) syndecan-3 is strongly expressed by perichondral cells around the diaphysis and epiphysis, whilst tenascin-C expression is much lower (Koyama et al., 1996). As the limb develops tenascin-C levels increase, especially around the epiphyses, as separation of the skeletal elements occurs, forming a non-adhesive barrier (Pacifci, 1995). At day 18, there is abundant expression of tenascin-C but it is not found in the growth plate and it is restricted to the articular chondrocytes in the epiphysis. Tenascin-C is able to retain the phenotype of these cells by competing with cell surface receptors (including syndecan) and decreasing the proliferative ability of these cells by maintaining the rounded phenotype. This allows for the maintenance of a normal cartilage phenotype during adult life (Pacifci, 1999).

The mechanism by which cartilage structure changes from an immature isotropic arrangement to an anisotropic structure is not yet known. Cartilage structure changes significantly throughout maturation, with reductions in cell density and tissue thickness (Stockwell, 1979). The thickness and cellular metabolism of cartilage has also been found to differ between species, individual joints and areas of a joint that endure different loads (Roughley and White, 1980; Hayes et al., 2001; Hayes AJ, 2001).

The idea that articular cartilage may grow by appositional growth was first described by Mankin (1962), when he concluded that development of cartilage was by a combination of appositional and interstitial growth. Studies in immature rabbit knees injected with tritiated thymidine, to determine chondrocyte proliferation found two bands of division. The first was at the articular surface and the second above the hypertrophic chondrocytes (Mankin, 1962).

The ability of cartilage to grow by apposition was also confirmed by the presence of insulin-like growth factor-1 (IGF-1) and 2 and proliferating cell nuclear antigen (PCNA) which are associated with chondrocyte proliferation. Prior to ossification these factors are found throughout the developing tissue. After formation of the secondary centre for ossification, the expression of these factors becomes localised to the upper layers of the presumptive articular cartilage and shortly before skeletal maturity they are found only in the upper most layers of the tissue (Archer et al., 1994). This suggests that tissue development is from the surface zone via appositional growth.

This observation was backed up by Hayes *et al* (2001) by treating the joint with bromodeoxyuridine (BrDU), an analogue of thymidine that is incorporated into the cell during cell division blocking chondrocyte proliferation. Unlike Mankin's study, only one region at the surface of the cartilage was positive for BrDU incorporation. It was also found that transitional chondrocytes were labelled after 4 days but only after 10 days were cells in the surface zone positive. After the injections were discontinued the number of labelled cells in the transitional zone began to fall. These data suggest that articular cartilage grows by apposition due to a small number of progenitor or stem cells located in the surface zone (figure 1.7) (Hayes et al., 2001; Hayes AJ, 2001; Archer et al., 2003). This combined with the number of growth factors and receptors present at the surface of articular cartilage suggests it is an important signalling centre (Archer et al., 1994).

One of the receptors seen at the surface of bovine articular cartilage is that of the Notch family (Hayes et al., 2003; Dowthwaite et al., 2004). The Notch molecules form a highly conserved family of cell surface signalling receptors that regulate cell fate decisions affecting asymmetric development and can be either inductive or inhibitory (Artavanis-Tsakonas et al., 1995; Lawson et al., 2001). Notch signalling is a complex process that allows for ossification and elongation of the growth plate. In cartilage, the Delta-Notch-2 pathway has been shown to regulate chondrocyte progression from prehypertrophic to hypertrophic during elongation of the growth plate in chicks (Crowe et al., 1999).

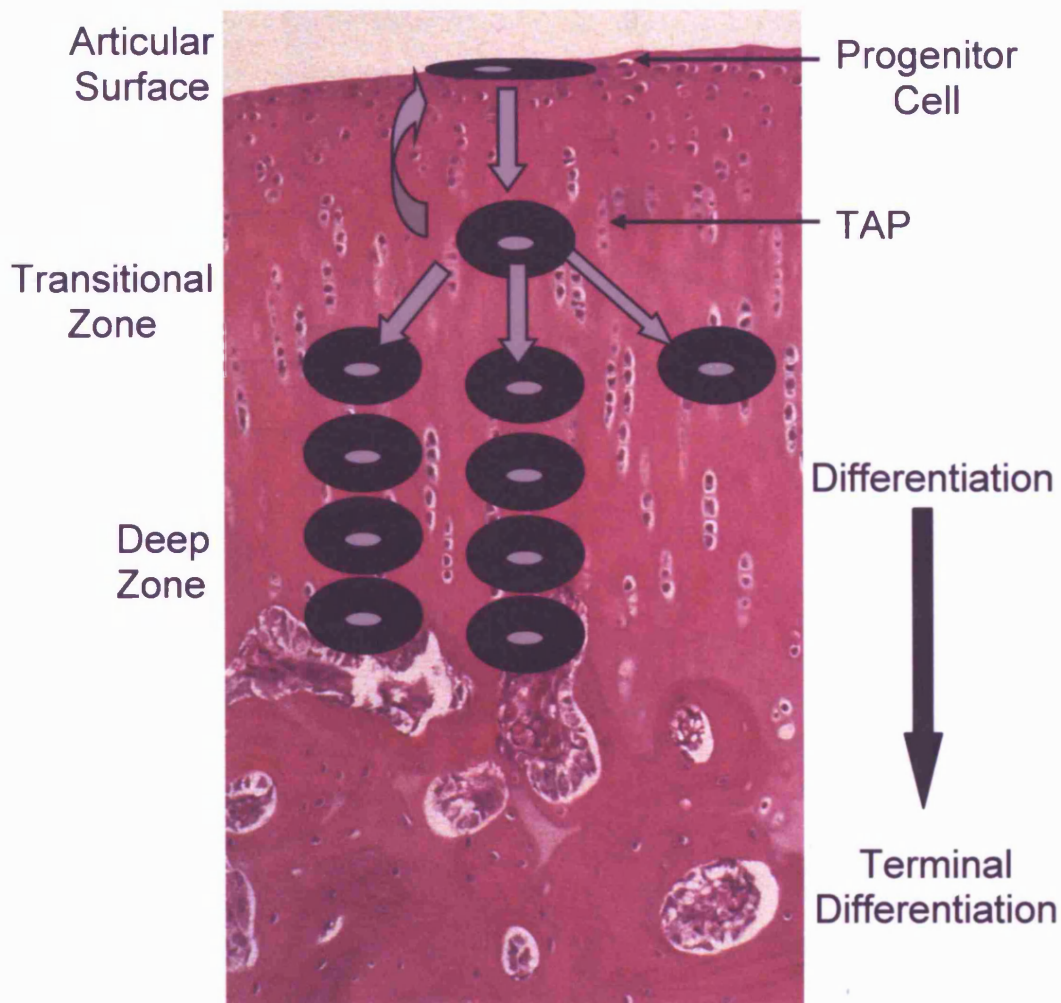


Figure 1.7 – Diagram to show the possible appositional growth mechanism of articular cartilage. Progenitor cells in surface zone divide to produce one progenitor cell and one transit-amplifying unit cell (TAP). The TAP forms the transitional zone and can continue dividing along chondrogenic lineage (adapted from Hayes et al. 2001)

The surface layer of cells in articular cartilage has been shown to express Notch-1, and these Notch-1 positive cells contain a chondrocyte progenitor population (Hayes et al., 2003; Dowthwaite et al., 2004). Inhibiting the Notch-1 signal in chondrocyte progenitor cells prevents the formation of colonies, indicating that Notch-1 has a role in controlling proliferation instead of differentiation (Dowthwaite et al., 2004).

## **1.8 Cartilage Degradation**

Articular cartilage structure is affected by many different factors including traumatic injury, age and also by a number of diseases including rheumatoid arthritis and osteoarthritis. Cartilage has a limited capacity for self repair beyond normal tissue maintenance, so any alteration to the composition of the cartilage has the potential to result in tissue degradation (Buckwalter and Mankin, 1998a; Lane and Buckwalter, 1999; Martin and Buckwalter, 2001; Martin and Buckwalter, 2002). Some form of age related fibrillation and degeneration is seen in most individuals, although this doesn't always lead to the joint disease osteoarthritis (Buckwalter, 1999b).

### **1.8.1 Osteoarthritis**

One of the most common degenerative joint diseases is osteoarthritis (OA), a non-inflammatory remodelling of movable joints (Wieland et al., 2005). OA can affect any diarthrodial joint, although the knee and hip are most commonly affected (Sharma, 2001). It is a progressive disease that begins with fibrillation of the cartilage surface which can lead to exposure of the subchondral bone and the formation of bone cysts and osteophytes (Buckwalter, 1999a; Buckwalter, 1999b). This eventually results in joint failure.

The cause for OA development is principally unknown, although many factors increase the possibility of developing this disease including excessive joint loading (Martin et al., 2004), aging and previous injury (Wieland et al., 2005). There are two main classifications of OA; primary (idiopathic) where the cause is unknown or secondary where OA develops due to infection, trauma, hereditary abnormalities or physiological disorders (Buckwalter, 1999b; Sharma, 2001).

The progression of the disease seems to follow three overlapping stages outlined by Buckwaler *et al.* (2001). One of the earliest events in OA is disruption or alteration to the ECM, which the chondrocytes respond to. The final stage is degradation of the tissue as the chondrocytes are no longer able to restore the tissue (figure 1.8) (Buckwaler *et al.*, 2001). Studies of early OA has shown that there is a decrease in the proteoglycan content of the matrix (Guilak *et al.*, 1994) and a decrease in GAG length. This decrease causes an increase in the permeability of the tissue causing hydration and reduction to the stiffness of the matrix (Roughley and White, 1980; Thonar *et al.*, 1986; Gibson *et al.*, 2001; Martin and Buckwaler, 2002).

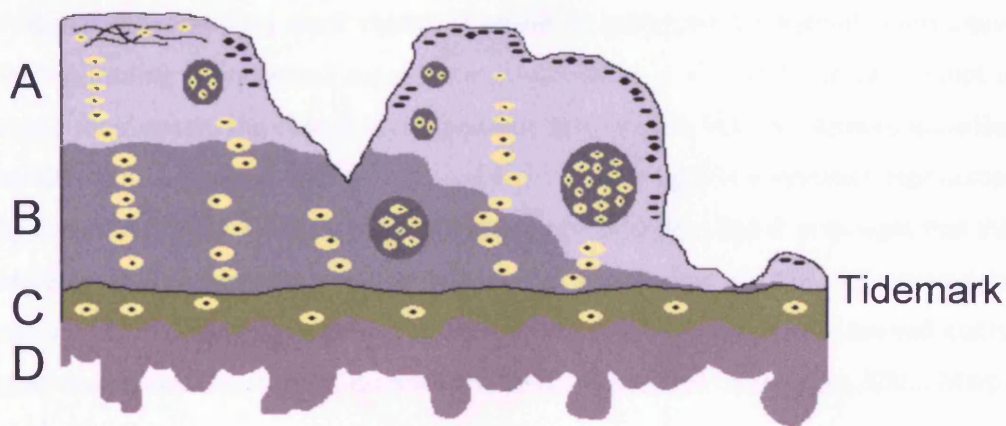


Figure 1.8 – Illustration of the histopathologic changes during the progression of osteoarthritis (left to right). Early stage of the disease is characterised by partial thickness defects and fibrillation of the articular surface. There is also the formation of cell clusters and dedifferentiation of the chondrocytes near to the clefts. The fissures then begin to deepen until they eventually penetrate the subchondral bone as the disease develops. The cell clusters become more prominent and the chondrocyte dedifferentiation more widespread. A, degradation of articular cartilage surface, B, remains of hyaline cartilage, C, calcified cartilage, D, subchondral bone (adapted from Buckwaler *et al.*, 2001)

The chondrocytes detect the changes to the matrix and respond to this by attempting to stabilise it. The chondrocytes increase synthesis of ECM components and express enzymes capable of degrading the matrix macromolecules. Clusters of proliferating cells are also visible in the superficial and transitional zones (Buckwaler *et al.*, 2001). Mechanical and chemical signals stimulate the chondrocytes to produce inflammatory cytokine interleukin-1 (IL-1) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). When combined, these cytokines produce a strong catabolic effect on the chondrocytes causing increased degradation, a reduction in the synthesis of

proteoglycans and type II collagen and in the synthesis of nitric oxide (NO) by the chondrocytes (Amin et al., 1995; Goldring et al., 2004). Chondrocyte-derived NO inhibits synthesis of aggrecan and offers resistance to the anabolic effect of insulin-like growth factor-1 (IGF-1). Increased NO also enhances the production of matrix metalloproteinases (MMPs) which disrupt the matrix by cleaving aggrecan and stimulating the production of collagenase. This process causes the release of many anabolic cytokines, resulting in an increase in chondrocyte proliferation (Goldring et al., 2004).

The collagenase degrades the minor collagens type IX and XI which destabilises the collagen network. This weak matrix is unable to withstand the normal compressive forces, leading to increased degradation (Buckwalter et al., 2001). In an attempt to repair the damage, the chondrocytes produce more matrix macromolecules including an altered proteoglycan with a decreased GAG chain length to counteract degradation by proteases. There is also a slight increase in proliferation and it is thought that this increase in proliferation may speed up the progression of OA by causing an accumulation of senescent cells that are no longer able to produce the normal matrix macromolecules (Martin and Buckwalter, 2001; Martin and Buckwalter, 2002; Martin et al., 2004).

Inability to restore or stabilise the tissue is the final stage of OA and is associated with increased pain and a decrease to joint mobility due to the insufficient repair response. The end result is a complete loss of cartilage on the surface of the bone. Changes to the bone also occur in advanced OA with thickening of the subchondral bone (Wieland et al., 2005) allowing for the development of subchondral bone sclerosis (Grynpas et al., 1991). An increase in type X collagen leads to endochondral ossification at the tidemark, resulting in vascular invasion into the cartilage and the production of osteophytes (Gibson et al., 2001).

There is very little treatment available for OA with only partial pain relief available in the form of non-steroidal anti-inflammatory drugs (NSAIDs). The main treatment for severe OA is total joint replacement as there are no existing drugs that help to stabilise the matrix or restore homeostasis (Wieland et al., 2005).

## 1.9 Cartilage Wounding

Articular cartilage has a very restricted capacity for repair. Defects that do not penetrate the subchondral bone show no signs of spontaneous healing and actually enlarge with time. This is due mainly to the avascular nature of the tissue as inflammatory and reparative aspects of the vascular system cannot reach the cartilage (Newman, 1998). Full-thickness defects penetrating the subchondral bone generate a repair response that produces a repair tissue that is an inadequate replacement for hyaline articular cartilage (Hunziker, 1999). The current methods for treatment of cartilage defects focus on the repair of full-thickness lesions due to their limited capacity of self-healing.

Articular cartilage defects are classified according to the depth of the lesion (Hunziker, 1999). Partial thickness defects remain within the confines of the cartilage tissue and do not penetrate the calcified cartilage into the subchondral bone. These lesions show similarities to the clefts and fissures seen in the initial stages of osteoarthritis (Hunziker, 1999; Redman et al., 2005).

Immediately after trauma, a limited repair response occurs, with cells undergoing apoptosis adjacent to the wound margin producing a zone of cell death (Tew et al., 2000). This is followed by a period of increased mitotic activity with an increase in matrix synthesis, cell proliferation and the formation of chondrocyte clusters (Redman et al., 2005). Although this is a short lived response that fails to repair the defect over time, it has been shown that these lesions remain relatively stable and only rarely progress to osteoarthritis (Newman, 1998). It has also been observed that growth factors can induce the migration of synovial cells across the articular surface where they fill the defect and produce a scar-like tissue (Hunziker and Rosenberg, 1996; Hunziker, 2001).

Full-thickness defects span the zone of calcified cartilage and penetrate the subchondral bone, allowing access to the bone marrow (Shapiro et al., 1993; Chuma et al., 2004). Blood cells, inflammatory molecules and mesenchymal cells that reside in the marrow space are therefore available and a repair response is elicited, resulting in the formation of a fibrocartilagenous tissue (Newman, 1998; Hunziker, 1999;



Redman et al., 2005) that is never completely integrated with the wound margins, which has been characterised in the rabbit (Shapiro et al., 1993). Artificially created defects initially fill with blood from the marrow and the formation of a fibrin clot allows for the trapping of inflammatory molecules from the blood and mesenchymal cells from the marrow that are able to differentiate into chondrocytes (Altman et al., 1992; Shapiro et al., 1993). The tissue shows transient similarities to hyaline cartilage, but degradation usually occurs between six and twelve months after formation of the defect (Kim et al., 1991; Altman et al., 1992; Shapiro et al., 1993).

## **1.10 Cartilage Repair**

Due to the limited repair capacity of cartilage, there has been much interest in the transplantation of cartilage tissue and chondrocytes from both allogenic and autologous sources. Whilst autologous tissue presents less chance of an immune response and an increase in cell viability, it still presents its own problems. In contrast there is only a limited supply of allogenic tissue and harvesting this may damage otherwise healthy joints (Newman, 1998).

### **1.10.1 Repair Methods – Grafts**

Perichondral and periosteal grafts have been used in animal models for many years and have produced varied results, with the production of hyaline-like tissue being reported (Newman, 1998; O'Driscoll, 1999; Redman et al., 2005). Autologous periosteum is readily available and has a chondrogenic potential. The healing response of the graft can be improved if the tissue is treated before transplantation with TGF- $\beta$  and by postoperative passive motion (Woo et al., 1987; O'Driscoll, 1999). Whilst some of the cells responsible for the repair of the defect are periosteal chondrocyte precursor cells, the majority are mesenchymal stem cells resulting from the debridement of the subchondral bone (Redman et al., 2005).

Osteochondral grafts have been used to treat large osteochondral defects and involve the allogenic or autogenic transplantation of a section of cartilage with the underlying bone, taking advantage of the bone to bone healing capabilities (Evans et al., 2004). Despite this there is still poor integration of the cartilaginous section and there are

many problems with low cell viability due to the pre-transplantation conditions (Newman, 1998; Hunziker, 1999).

### **1.10.2 Repair Methods – Cell based**

Autologous chondrocyte transplantation (ACI) involves the extraction of chondrocytes from a small healthy biopsy taken from a non-load bearing region of the cartilage. The cultured cells are then injected into the cartilage defect (Brittberg et al., 1994; Caplan et al., 1997; Hunziker, 1999; Redman et al., 2005) and a periosteal graft is sutured over the defect acting as a flap to keep the cells in place (Brittberg, 1999). Results obtained by Brittberg *et al* (1994) showed that there was hyaline-like repair tissue in 11 out of 15 patients with femoral defects and only 1 out of 7 patients with patellar defects although almost all patients did show improved joint function (Brittberg, 1999; Brittberg et al., 2003; Roberts et al., 2003).

Biological and synthetic matrices function as a scaffold to support the expansion of transplanted chondrocytes and induce the formation of hyaline cartilage. Although many different natural and synthetic materials have been used, hyaluronan and collagen based matrices are the most popular as they are substances naturally found in articular cartilage (Wakitani et al., 1989; Wakitani et al., 1994; Caplan et al., 1997; Hunziker, 1999; Pavesio et al., 2003; Redman et al., 2005). The use of scaffolds in cartilage repair is beneficial for two reasons. Firstly they can be used to fill a large full-thickness defect and encourage spontaneous healing by bridging the gap (Hunziker, 1999) and secondly it has been found that chondrocytes need a three-dimensional enclosed environment to maintain their phenotype (Wakitani et al., 1989).

Mesenchymal stem cells (MSC) ability to differentiate into a variety of connective tissue lineages has made them a good tissue source for the treatment of osteochondral defects (Nakahara et al., 1991; Caplan et al., 1997; Grove et al., 2004; Redman et al., 2005). The advantage of these cells is their ability to form the different layers of the cartilage, at the base they can progress to hypertrophic cartilage and eventually give rise to bone and at the surface the cells in contact with the synovial fluid will remain as chondrocytes (Caplan et al., 1997; Newman, 1998; Redman et al., 2005). The cells can be expanded in culture and seeded onto a collagen (Caplan et al., 1997) or

polylactic acid-co glycolic acid (PLGA) matrix for implantation into a defect (Lee et al., 2004). Although some regeneration occurred, with tissue resembling both bone and cartilage, there was still poor integration between the regenerated cartilage and the host tissue (Caplan et al., 1997). Cartilage (chondro) progenitor cells and dedifferentiated chondrocytes grown in monolayer culture (de la Fuente et al., 2004) may also be of use in cartilage repair for the same reasons as the MSCs .

Growth factors have been found to induce the repair of cartilage defects by both mesenchymal stem cells and chondroprogenitor cells. It is therefore possible that the administration of these factors could supplement other tissue engineering methods. Fibroblast growth factor (FGF)-2 is a mitogen for chondrocytes and has been found to activate an intrinsic repair response in large articular cartilage defects over 5mm in diameter (Hiraki et al., 2001; Chuma et al., 2004; Henson et al., 2005). Hiraki *et al* (2001) suggested that this was due to the ability of FGF-2 to stimulate the expansion of chondroprogenitor cells (Shida et al., 1996) and it is known to target connective tissue stem cells via extra-cellular signal-related pathways (Henson et al., 2005).

Another growth factor that may have a role in cartilage repair is TGF- $\beta$  which is involved in the regulation of chondrogenesis in MSCs and the formation of the ECM (Barry et al., 2001; Hunziker, 2001; Lee et al., 2004). TGF- $\beta$  can also stimulate chondrogenic differentiation in periosteal mesenchymal cells, and the rate of proliferation in these cells can also be increased with insulin-like growth factor (IGF)-1 (Fukumoto et al., 2003). Supplementation of growth media with growth factors allows for a rapid increase in cell numbers for tissue engineering procedures such as autologous chondrocyte transplantation.

### **1.11 Dedifferentiation of Chondrocytes in Culture**

It has been found that the expansion of chondrocytes in monolayer culture leads to their dedifferentiation and the cells lose their rounded morphology, becoming spindle-fibroblastic in shape (Benya and Shaffer, 1982; Kolettas et al., 1995; Chen et al., 2003). They regain their capacity for division but no longer express the specific articular cartilage proteins required for the creation of the ECM, switching their

collagen production from types II, IX and XI to types I, III and V. Proteoglycan production also decreases (de la Fuente et al., 2004).

It has also been hypothesised that dedifferentiated adult human articular cartilage chondrocytes (HAC) are actually a multipotent population of cells. De la Fuente *et al* (2004) compared the properties and marker profiles of these cells with MSCs. They expressed almost identical markers, most notably they both express CD105 and CD166 which have been used to define MSCs which have osteogenic potential, showing these cells have the ability to differentiate into more than just cartilaginous tissue. It is also known that CD105 is a marker of MSCs (Alsalameh et al., 2004). The HAC also expressed numerous markers found only on human embryonic stem, germinal stem and embryonic carcinoma cells. These properties were also seen when a single-cell derived colonies were used (de la Fuente et al., 2004).

## **1.12 Stem Cells**

By definition stem cells have the capacity for self-renewal and the potential to differentiate into one or more different progeny (Lajtha, 1979b; Lajtha, 1979a; Krause, 2002; Herzog et al., 2003; Cai et al., 2004). Signals are presented to the daughter cells to determine if they should remain stem cells or undergo terminal differentiation.

Stem cells are found throughout development and have been identified in most organs and tissues. They are classed according to the tissue of origin, differentiation potential and lifespan. Although they are present throughout development their number and ability to differentiate decreases with age (Reyes et al., 2001).

Embryonic stem (ES) cells are the most versatile stem cell. They are derived from blastocysts and are capable of differentiating into any cell type (Reyes et al., 2001; Herzog et al., 2003). Adult stem cells are found in permanently renewing tissue where they are able to undergo terminal differentiation to repair and regenerate adult tissue. They have a low capacity for self-renewal and a short life span compared with ES cells (Cai et al., 2004).

In the developing foetus there are a population of more committed pluripotent stem cells that are capable of developing into any of the three embryonic tissue types; endoderm, ectoderm and mesoderm. As development continues these cells become multipotent stem cells and their ability to differentiate is reduced. In postnatal life it is thought these cells disappear and are replaced by a population of adult stem cells capable of supporting almost all organ systems including skin, liver, central nervous system, hematopoietic system and connective tissue (Muschler et al., 2003).

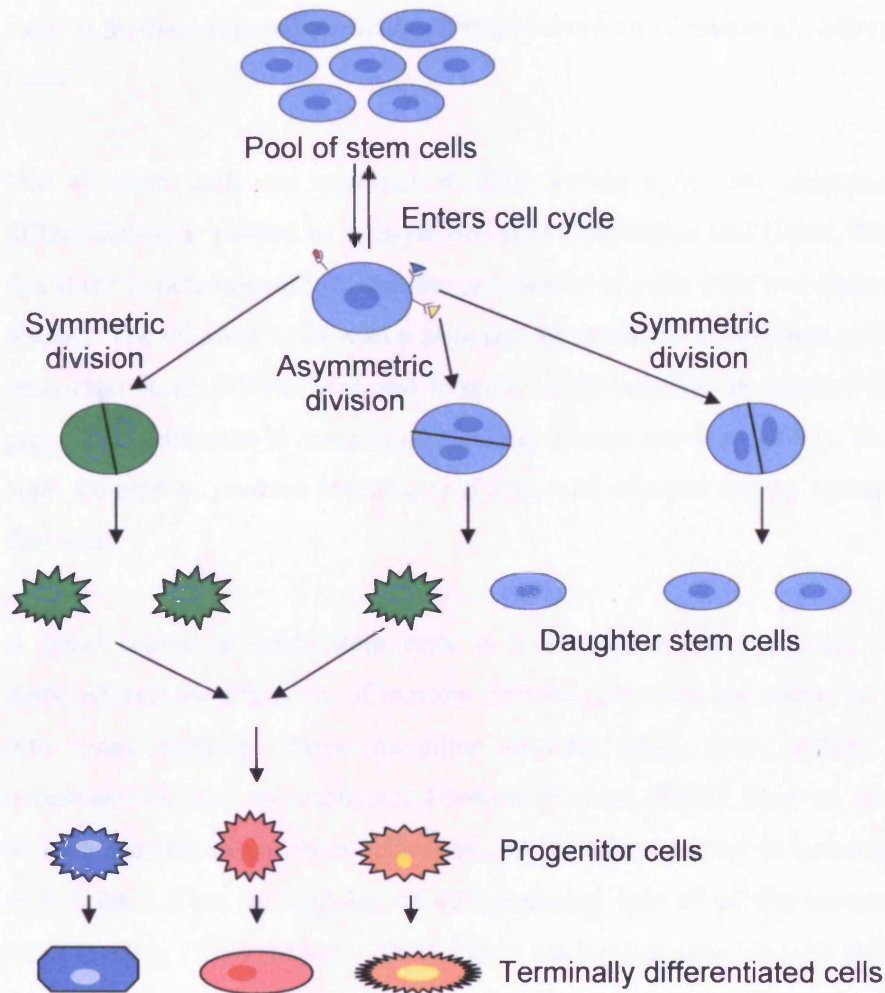


Figure 1.9 – Possible pathways of stem cell division. Stem cells are able to remain quiescent or enter the cell cycle after cell signalling by one of three mechanisms; asymmetric division producing one progenitor and one stem cell daughter, and symmetric division to either give two stem cell daughter cells or two precursor daughter cells (adapted from Cai et al., 2004)

Dividing adult stem cells are able to produce two non-identical daughter cells by asymmetric division (figure 1.9). One cell remains in the resting state of the parental cell, retaining the stem cell phenotype with the same properties as the parental cell. This process allows for self-renewal and prevents the depletion of the pool of stem cells. The second daughter cell proliferates symmetrically to produce a large number of multipotent progenitor cells (Muschler et al., 2003). These progenitor cells have a restricted differentiation potential and a reduced ability for proliferation compared with stem cells. Progenitor cells are capable of undergoing a series of differentiation steps to produce mature terminally differentiated cells (Grove et al., 2004) and mature tissue.

Not all stem cells are multipotent. Skin keratinocytes are unipotent and their differentiation is limited to a single cell type (Barrandon and Green, 1987). Despite this there is heterogeneity within the population of cells with two distinct cell types present. The dividing cells with a high rate of proliferation are stem cells, whilst the cells capable of self-renewal and terminal differentiation are similar to committed progenitor cells seen in other stem cell lines (Jones and Watt, 1993). The progenitor cells are able to produce transit-amplifying cells required for the upward growth of the tissue.

A good source of adult stem cells is bone marrow. Many recent reports have demonstrated the plasticity of marrow derived cells with the ability to differentiate into many different tissue including muscle, brain, liver, kidney, lungs and hematopoietic and mesenchymal lineages (Krause, 2002). One of the most well defined populations of stem cells found in the bone marrow is hematopoietic stem cells (HSC). They are capable of differentiating into all of the hematopoietic cell types (Lajtha, 1979a; Krause, 2002). HSCs are lineage-negative cells ( $LIN^-$ ) and lack the markers seen on differentiated cells. This phenotype allows for isolation by removing cells displaying lineage specific markers such as CD3 for T-cells and B220 for B-cells (Herzog et al., 2003; Cai et al., 2004).

The second bone marrow population are mesenchymal stem cells (MSC) that resemble adherent fibroblast-like cells *in vitro*. Like all stem cells they are  $LIN^-$  and this, combined with their adherence to plastic, allows for easy isolation of MSCs.

Bone marrow is not the only source of MSCs, they have also been found in adipose tissue (Lin et al., 2008), placenta (Zhang et al., 2006), skin and thymus (Musina et al., 2005), MSCs have the capacity to differentiate into osteoblasts, chondroblasts and adipocytes both *in vivo* and *in vitro* (Pittenger et al., 1999; Bosnakovski et al., 2005). MSCs have been found to express CD34, CD90, CD117, SH2, SH3 SH4 and major histocompatibility complex-1(MHC I), which are markers indicative of a stem cell phenotype (Pittenger et al., 1999; Rombouts and Ploemacher, 2003; Javason et al., 2004). However, variable expression of these markers has been found between species and populations.

Recently a new population of adult stem cells (ASC) has been isolated from bone marrow that is able to differentiate at the single cell level into not only mesenchymal tissue but tissue of all three germ layers (Reyes et al., 2001; Krause, 2002; Muschler et al., 2003). This has been shown by their contribution to the development of the three germ layers when transplanted into a blastocyst (Jiang et al., 2002a; Jiang et al., 2002b). They have been named multipotent adult progenitor cells (MAPC) or mesodermal progenitor cells (MPCs) and they share many properties with pluripotent stem cells found in the foetus. MAPCs also grow in culture as an adherent layer, but they can be grown in nutrient poor media and can be cultured indefinitely. Unlike MSCs they are negative for CD34 and CD117 expression and they do not express either MHC class I or II (Reyes et al., 2001).

There are many conflicting reports concerning the best type of stem cell to use for tissue engineering and clinical purposes. Whilst ES have the greater versatility due to their increased lifespan and differentiation potential, there are many ethical hurdles to overcome. For this reason a large amount of research is being performed to determine the possibility of using adult stem cells for tissue engineering.

One of the most explored uses of MSCs is in repairing defects of both bone and cartilage. The differentiation of MSCs can be controlled either *in vitro* by altering the conditions or *in vivo* due to environmental factors (Uematsu et al., 2005). A more effective transplantation method is being explored which involves seeding MSCs onto a suitable scaffold, such as PGLA, and grafting it into the defect (Johnstone et al., 1998; Lee et al., 2004; Uematsu et al., 2005). The results from grafting these

scaffolds have been positive with the scaffolds providing support for progenitor differentiation.

### **1.13 Articular Cartilage Progenitor Cells**

As discussed earlier, there is a convenient source of MSCs, but isolating the cells is a long and tedious process, even before they can be seeded onto scaffolds and implanted into the defect. It has been found that surface of adult cartilage contains a population of cells that show the properties of progenitor cells (de la Fuente et al., 2004; Dowthwaite et al., 2004; Hattori et al., 2007) which are required for the appositional growth of the tissue. When grown in culture the progenitor cells exhibit the same properties as those isolated from the bone marrow. They have an increased cell cycle time and increased ability to differentiate, although only connective tissue lineages. This population of progenitor cells express the cell surface signalling molecule Notch-1, which has been shown to control the clonality of these chondroprogenitor cells. Only a small proportion of the Notch-1 positive cells within the surface of the cartilage show the progenitor phenotype when grown in culture (Dowthwaite et al., 2004). Treatment with bromodeoxyuridine has shown that the surface zone cells have a longer cell cycle time than those in the transitional zone. This longer cell cycle time is typical of populations of chondroprogenitor cells and has been seen in other tissue such as gut and skin (Hayes et al., 2001). The progenitor cells maybe able to create cartilage that is both structurally and biochemically accurate and therefore provides a good repair method for cartilage lesions.



## **Aims**

The aims of this study are to further characterise the phenotype of a population of progenitor cells found within the surface zone of articular cartilage, more specifically the surface of 7-day-old bovine metacarpal-phalangeal joints. The human equivalent of these cells is an attractive alternative cell source for use during tissue engineering, but as yet the factors influencing their differentiation are not well understood.

This characterisation fits into a series of objectives;

- To characterise the effects of prolonged culture on the cells phenotype and their ability to undergo chondrogenic differentiation and to determine the expression pattern of a series of glycosaminoglycan epitopes after short-term culture
- To determine the plasticity of the cells when injected into a non-cartilaginous environment
- To assess the ability of the chondroprogenitor cells to express gap junctional proteins and form functional gap junctions
- To further investigate a series of recent studies suggesting that there are paired cells found within all three of the zones of articular cartilage after maturation of the tissue and to assess if the paired cells within the surface zone of the tissue could represent the stem cell population found within this zone of the tissue

## **CHAPTER 2**

### **Effect of Time in Culture on Cartilage Progenitor Cell Differentiation**

## 2.1 INTRODUCTION

Chondrocytes are considered to be cytoplasmically isolated (Archer and Francis-West, 2003) and this, combined with the avascular and aneural nature of articular cartilage, means that the tissue has a very poor capacity for repair after damage or injury. Prolonged growth of chondrocytes *in vitro* has also been shown to result in their dedifferentiation or, more correctly, their phenotypic modulation (Benya and Shaffer, 1982; Archer et al., 1990; Chen et al., 2003) suggesting that alternative cell sources may be required for cartilage repair of larger chondral lesions.

Dedifferentiated chondrocytes retain their ability to undergo chondrogenic differentiation for a limited time after isolation. This phenotypic instability is due to the change in shape to a flattened morphology which doesn't occur when cells are cultured in suspension, suspended in agarose (Benya and Shaffer, 1982), on a three dimensional porous scaffold or as a chondroid colony or *in vivo* (Chen et al., 2003). The switch in morphology can be reversed by placing the cells back into suspension, where they form clusters of rounded cells with an increase in ECM synthesis and a reduction in proliferation (Kolettas et al., 1995). Alternatively re-expression of the differentiated phenotype can be stimulated by the addition of certain growth factors such as transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1; (Chen et al., 2003).

The changes associated with the phenotypic modulation of the chondrocytes occur as the cells change to a fibroblast-like state, switching their collagen production from type II to type I, and downregulating proteoglycan production (de la Fuente et al., 2004). It is thought that by maintaining the cells original pericellular matrix it may be possible to maintain chondrogenicity (Villar-Suarez et al., 2004).

Chondrocytes are not the only cells that alter their ability to differentiate after long term culture (Akagi et al., 2003). Prolonged growth of MSCs in culture has been found to restrict their ability to differentiate along all mesenchymal lineages. Whilst they maintain the ability to differentiate into osteoblasts, they were no longer able to form adipocytes suggesting that extended growth reduces the plasticity of these cells (Digirolamo et al., 1999).

The ability of a cell population to undergo chondrogenic differentiation and form hyaline articular cartilage is determined by the matrix that the cells produce. Many cells appear able to produce a fibrocartilaginous matrix comprising of high levels of type I collagen in addition to type II collagen fibres. The matrix also consists of a much lower proteoglycan content when compared with hyaline cartilage. Whilst fibrocartilage is still a form of cartilage, its differing collagen composition leads to different properties. It appears less organised than articular cartilage, with collagen fibres arranged in layers surrounding cell lacunae. This spongy structure allows for the high levels of shock-absorption needed within the intervertebral discs.

The purpose of this study was to further analyse the characteristics of the cartilage produced by these clonal cell lines following monolayer expansion and determine the effect that long term culture has on these progenitor cells.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Cell Isolation and Differential Adhesion Assay**

Chondrocytes were isolated from the surface zone (SZ) of 7-day bovine metacarpal-phalangeal (MCP) joints (figure 2.1) by fine dissection as described by Dowthwaite et al. (2004), with a scalpel used to remove the top layer of the cartilage. The cartilage from 3 calves legs was collected in a sterile 50ml tube and the chondrocytes were released from their matrix by sequential enzyme digestion, initially in 3.17 units/ml pronase (Boehringer Mannheim) in Dulbecco's Modified Eagle Medium F12 (DMEM/F12; GIBCO BRL, Life technologies Ltd., UK) containing 5% foetal calf serum (FCS) and supplemented with 100µg/ml ascorbic acid (Sigma), 50µg/ml Gentamycin (GIBCO) and 5 mg/ml glucose for 3 hours on a roller at 37°C. The pronase was removed and replaced with 0.12 units/ml collagenase (type I (Sigma) in 5% DMEM/F12) and incubated overnight at 37°C on a roller. After digestion, the cells were passed through a 40µm mesh cell strainer (Falcon), and the suspension centrifuged at 2500 x g for 5 minutes. The supernatant was removed and the cell pellet resuspended in supplemented DMEM/F12 without FCS and counted on a haemocytometer.

Chondrocytes were resuspended at 4000 cells/ml in DMEM/F12 without FCS and seeded onto Petri-dishes (35mm) that had been coated overnight at 4°C with 10µg/ml bovine fibronectin (FN; Sigma, UK) in 0.1M phosphate buffered saline (PBS, pH 7.4) containing 1mM MgCl<sub>2</sub> and 1mM CaCl<sub>2</sub> (PBS+). Cells were incubated at 37°C for 20 minutes and after 20 minutes, the media and non-adherent cells was removed and dishes filled with 2mls DMEM/F12 plus 10% FCS. Surface zone chondrocytes were also resuspended in DMEM/F12 plus 10% FCS and plated onto FN coated 35mm dishes.

### **2.2.2 Isolation of Clones**

Colonies consisting of more than 32 cells derived from the differential adhesion assay were marked and media was removed from the dish. Sterile polystyrene 6.4mm cloning rings (Sigma) were dipped into Vaseline using sterile forceps. The ring was then placed over the marked colony with gentle pressure (figure 2.1) and 200µl trypsin-EDTA (0.05% trypsin, 0.53mM EDTA; GIBCO) added. The dish was then

incubated at 35°C for 2-5 minutes. Cells were then lifted by gentle pipetting and added to 1ml DMEM/F12 plus 10% FCS in a 24 well plate.

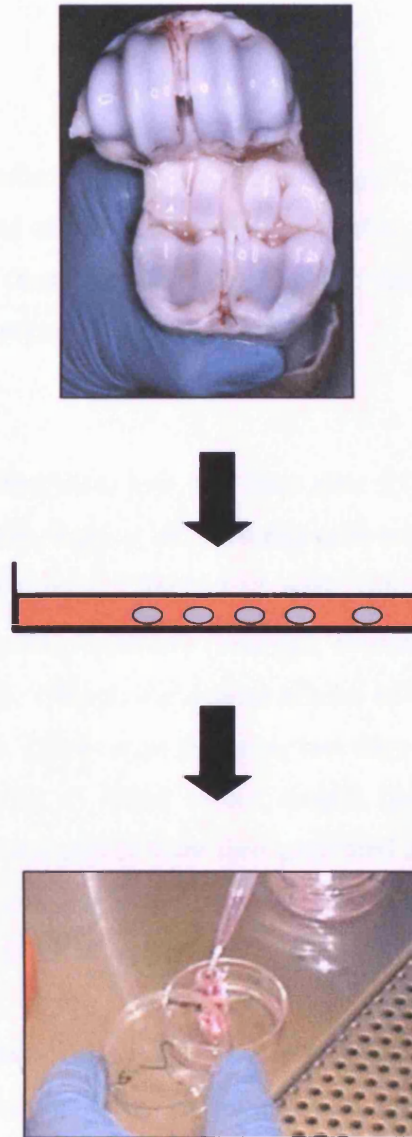


Figure 2.1 - Chondrocyte isolation and colony selection from 7 day bovine metacarpal-pharangeal joint

### 2.2.3 Enriched Populations and Expansion in Monolayer Culture

For comparison, some of the plates used for differential adhesion were allowed to grow until confluence. This produced a chondroprogenitor enriched population.

When the cells in the 35mm dishes were confluent, the cells were trypsinised, counted and placed into T75 flask (P=0). As cells approached confluence they were split again at 1:4 and the number of cells counted. The population doublings (PDs) could then be calculated using the formula below;

$$PD = \log (N/N_0)$$

Where N equals the number of cells at the beginning of the passage and  $N_0$  is the number of cells at the end of the passage (Cristofalo et al., 1998; Piera-Velazquez et al., 2002). The PD was then analysed using Excel to calculate the generation time (time required to form one population doubling).

#### **2.2.4 Pellet Culture**

Cell pellets were generated from four cell lines after 25 PDs (60 days in culture; figure 2.2). Confluent flasks were trypsinised and cells resuspended in chondrogenic media containing supplemented DMEM/F12 with 1 $\mu$ M dexamethasone (Sigma), 10ng/ml transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1; Peprotech, UK) and 1% insulin transferrin selenium (ITS; Gibco). Cells were diluted to 5x10<sup>6</sup> cells/ml and placed into 8 sterile Eppendorfs. Pellets were fed every two days and after two weeks were washed in PBS and stored in 500 $\mu$ l TRizol reagent (Invitrogen, UK). Cell lines remained in culture and cell pellets were then generated at 42-45 PDs (150 days in culture; late passage). A cell line with an increased rate of growth was also used for cell pellets at 42-45 PDs (55days in culture; figure 2.2).

#### **2.2.5 Quantitative Biochemical Analyse of Cell Pellets**

Three pellets for each cell line in the early clone, late clone and fast growing clone group were freeze-dried and their dried weights determined for the assay as described by Dickinson *et al* (2005). Freeze-dried samples were pooled for each cell line and solubilised with 2mg/ml TPCK-treated bovine pancreatic trypsin containing 1mM EDTA and 10 $\mu$ g/ml pepstatin A (all Sigma) at 37°C for 15 hours. A further 250 $\mu$ l of trypsin was added, and samples were then incubated at 65°C for 2hours before being boiled for 15 minutes to deactivate remaining enzyme. Analysis was carried out using an inhibition enzyme-linked immunosorbent assay (ELISA) with a monoclonal

antibody to collagen type II and an antipeptide antibody to collagen type I (both from Prof A. Hollander). Proteoglycan content of the digested pellets was calculated by the levels of sulphated glycosaminoglycans with the dimethylmethylene blue (DMMB) assay. Samples were placed in the wells of a 96-well plate with DMMB reagent (Aldrich, UK) was added to each well and plates were read at 575nm

### **2.2.6 RNA Extraction and cDNA Synthesis**

One pellet from each sample at the different time points was thawed and 500µl TRizol reagent (Invitrogen, UK) added. Pellets were then crushed using RNA-free disposable pestle (VWR International, UK) and another 500µl TRizol added. The samples were then incubated at room temperature for 5 minutes and 250µl of chloroform was added. The samples were then vortexed and left for a further 30 minutes. Tubes were centrifuged at 12000 x g for 15 minutes at 4°C resulting in the formation of an upper aqueous layer. This layer was removed and placed in a clean RNase-free tube. An equal volume of 2-propanol (Sigma) was added and the samples were precipitated at -20°C overnight.

After precipitation, samples were centrifuged at 12000 x g for 10 minutes at 4°C and the supernatant removed. The pellet was then washed in 1ml of 75% ethanol and centrifuged at 7500 x g for 5 minutes at 4°C. The supernatant was then removed and the pellet left to air dry. When dry, the pellet was re-suspended in 89µl Sigma molecular biology water. The samples were then DNase treated to remove genomic DNA using DNA-free kit (Ambion, UK) according to manufacturer's instructions (Appendix I). Reverse transcription (RT) was carried out using the SuperScript III reverse transcriptase kit (Invitrogen). The cDNA was transcribed from 10.5µl of RNA with 0.25µg Random primers and 0.8µM of each dNTP (both Promega).

### **2.2.7 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)**

In order to determine the relative expression of the cartilage matrix molecules aggrecan, collagen type I and II and the transcription factor Sox9 between the pellets from different cell lines real-time quantitative-PCR (RT-qPCR) was carried out. The RT q-PCR was performed with 0.2µmol of each primer in a total volume of 25µl using SYBR green JumpStart Taq ReadyMix reagents (Sigma) as per manufacturer's



guidelines, in a 24µl final volume. The master mix with primers added was placed into 96-well polypropene plates (Stratagene, UK) with 1µl cDNA. The RT-qPCR was carried out on a Stratagene MX3000P system (Stratagene) and data analysed using Mxpro.

	Forward Primer	Reverse Primer	Annealing Temp.	Accession No.
Aggrecan	CAG CCA GGC CAC CCT AGA G	GGG TGT AGC GCG TGG AGA T	60°C	U76615
Coll I	ACA TGC CGA GAC TTG AGA CTC A	GCA TCC ATA GTA CAT CCT TGG TTA GG	60°C	AB008683
Col II	AAC GGT GGC TTC CAC TTC	GCA GGA AGG TCA TCT GGA	60°C	X02420
Sox9	ACG CCG AGC TCA GCA AGA	CAC GAA CGG CCG CTT CT	60°C	AF278703

Table 2.1 Polymerase Chain Reaction Primers. All primers are shown 5'-3' with corresponding annealing temperature and accession number.

### 2.2.8 Immunocytochemistry on Monolayer Cells

A differential adhesion assay was carried out on 5 plates. At days 1, 3, 5, 7 and 9 a Monensin block was carried out using 0.1µM Monensin (Sigma) for 16 hours at 37°C. Plates were then fixed with 95% ethanol at 4°C and stored at -20°C, with control plates of SZ cells and clones that had reached 30 PDs were also established.

Plates were immunolabelled using monoclonal antibodies 3B3(-), 4C3 and 7D4 (gifts from Prof. B. Caterson), which recognise chondroitin sulphate (CS) motifs in the proteoglycans, and CIIC1 (DHSB, USA) which binds to collagen type II. Plates were washed with phosphate buffered saline (PBS; Sigma) containing 0.1% Tween (PBS/Tween; Sigma) for 5 minutes (x3) and each well circled with a DAKO pen (Dako, UK). Cells were blocked for 30 minutes with rabbit serum (Dako) diluted in PBS/Tween (serum used at 1:20) at room temperature. Antibodies were diluted either 1:100 (3B3) or 1:50 (4C3 and 7D4) in 0.1% PBS/Tween. CIIC1 was used at a

concentration of 2.5µg/ml. Appropriate isotype antibodies (Dako) were used as a negative control. The excess blocking serum was tipped off and the antibody added to the corresponding well. Plates were incubated overnight at 4°C. Sections were then washed in 0.1% PBS/Tween for 5 minutes (x3). Samples were incubated with swine anti-rabbit FITC (Dako) in 0.1% PBS/Tween at 10µl/ml for 1 hour at room temperature, which was washed off in 0.1% PBS/Tween for 5 minutes (x3). Finally, sections were mounted using one drop of 'Vectashield' with DAPI (Vector Laboratories, UK) and a coverslip placed over the top. Plates were then imaged using an Olympus BX61 fluorescent microscope.

### **2.2.9 Statistical Analyse on Pellet Weights**

Data for the average pellet weight was shown as mean±standard error of the mean (S.E.M), with each experiment having a minimum of three repeats. Analysis of the means was carried out using a two-tailed two sample t-test (Minitab 13.0). Where data was not normally distributed, or the variances were unequal, the Mann-Whitney test was carried out. Differences between data were considered significant at P values below 0.05.

## 2.3 RESULTS

### 2.3.1 Biochemical Pellet Analyse

Pellets were grown for cell lines at an early PD (60 days in culture; 25 PDs) and late PD (150 days in culture; 45 PDs) to determine the effect of time in culture on the cells ability to form a hyaline cartilage-like matrix (see Appendix III). Of the early PD clonal cell lines, one cell line (C92) produced a matrix that was found to consist predominantly of collagen type II and GAG (figure 2.3). The levels of collagen type I was over 50% lower than for collagen type II. The remaining three early PD cell lines produced greater percentage of matrix than compared to C92. In these clones, the levels of type I collagen were greater when compared with the percentage of type II collagen, with levels ranging from 2-2.5 times greater. The GAG production by these cell lines was almost the same as clone C92.

After a further 100 days in culture, the pellets generated from two of the clonal cell lines (C92 and C93) had an increased percentage of type I collagen in their matrix when compared to the pellets generated after 25 PDs. In the case of C92 these levels were similar to those of type II collagen, whilst in C93 they were more than double. In the remaining two cell lines, the levels of type I collagen fell greatly, to almost half the levels of type II collagen. In all cases the percentage GAG in the matrix halved when compared with early PD clones.

Pellets grown from two of the chondroprogenitor cell lines with an increased rate of growth (55 days in culture; 45 PDs) seemed to produce a matrix comprising of predominantly GAG, with much lower levels of collagen present when compared with the other cell lines (figure 2.3). This level of GAG was far greater than seen in any of other clonal cell lines (either 25 or 42 PDs). The final cell line failed to produce a competent cell matrix, with low levels of GAG as well as type I and II collagen.

The average dry weight of the pellets produced from younger clones was significantly greater than the digested matrix weight, yet the dry weight of the pellets from the older clones was almost the same as the digested weight, with no significant difference seen (figure 2.4). The clonal cell lines with an increased rate of growth also

showed this later trend, with very little difference seen between the pellet dry weight and the digested dry weight.

### **2.3.2 Real-Time Quantitative Polymerase Chain Reaction**

To determine gene expression levels of a variety of cartilage related markers, RT-qPCR was carried out (see Appendix II). The levels of collagen type I, type II and aggrecan varied greatly between clonal pellets, and the relative expression units (REU) for the mRNA levels corresponded with the biochemical analysis carried out on the cell pellets (figure 2.5). The exception to this trend was C90, where higher levels of collagen type I mRNA was observed in the late passaged cells, whilst expression of the protein in these cells was far lower than in the early passaged cells. In the fast growing clonal cells, the levels of collagen type II were greater than in any of the other pellets, and in the case of C94 the REU was almost ten times higher than in the other cell pellets (figure 2.5). This clonal cell line also expressed high levels of Sox9 mRNA (figure 2.6). Of the other cell lines, three increased the expression of Sox9 mRNA with increased time in culture (figure 2.6). The remaining cell line, C91, had higher relative expression of Sox9 mRNA at both early and late time points, and had higher expression than the remaining cell lines.

### **2.3.3 Immunocytochemistry on Monolayer Cells**

Cells were plated down and a differential adhesion assay carried out. The plates were then fixed at 2 day intervals and imaged for the presence of cell colonies. Colony formation first became apparent at 3 days (figure 2.7), and by 5 days colonies of over 32 cells could be detected. By day 7 individual colonies of cells were no longer visible as adjacent colonies had merged into a single semi-confluent layer of cells. After fixing the plates, immunocytochemistry was carried out on the cells to determine the presence of collagen type II. There was no collagen type II detected at any of the time points (figure 2.8), or in passaged clonal cells that had reached 30 PDs. The only cells that labelled positive for collagen type II were freshly isolated SZ cells.

Immunocytochemistry was then carried out to determine the presence of the CS proteoglycan side chains 3B3(-), 4C3 and 7D4. There was no 3B3(-) detected in the isolated cells seen 1 day after plating the cells (figure 2.9 A), although at day 3 some

small regions of positive labelling could be seen (B). By day 5 the regions of positive labelling had increased (figure 2.9 C and D), although not all cells expressed 3B3(-). This pattern was also seen at day 7 (figure 2.9 F) and by day 9, 3B3(-) was no longer detected (figure 2.9 G). Larger regions of positive labelling was found in the freshly isolated SZ cells (figure 2.9 J), but there was no 3B3(-) detected in passaged clonal cells (figure 2.9 K).

Isolated cells that expressed 4C3 were detected 1 day after the cells had been isolated (figure 2.10 A and B), with the expression occurring at regions around and the cell and via projections from the cell (figure 2.10 A). Not all cells at this time point were positive for expression of 4C3 (figure 2.10 C) and expression seemed to be reduced at day 3 (figure 2.10 D), although positive labelling could be seen. By day 5 (figure 2.10 E) and 7 (figure 2.10 F, G and H) positive labelling could be seen in regions between cells. At day 9, much larger regions of 4C3 expression could be seen (figure 2.10 I, J and K), with 4C3 making a mesh work linking cells. Confluent regions either appeared to have either high expression (figure 2.10 I), or low expression with isolated cells labelled (figure 2.10 L). Expression was also seen in SZ cells (figure 2.10 M, N and O), with isolated regions positive. No labelling was seen in passaged clonal cells (figure 2.10 P).

There was 7D4 detected in processes extending from isolated clones (figure 2.11 A) and in regions around clones (figure 2.11 B) only 1 day after plating, although not all cells were labelled (figure 2.11 C). By day 3 the processes extending from cells appeared to have linked up forming a network connecting cells (figure 2.11 D), with a similar pattern seen in day 5 (figure 2.11 E and F). By day 7 the cells were remained linked by 7D4 (figure 2.11 G), with large regions seen between cells (figure 2.11 H), although not all cells expressed 7D4 (figure 2.11 H). At day 9 the labelling was more restricted with large regions showing no positive labelling (figure 2.11 I) and only isolated cells still expressing 7D4 (figure 2.11 J). There was no positive labelling was seen in either SZ cells (figure 2.11 K) or passaged clonal cells (figure 2.11 L).

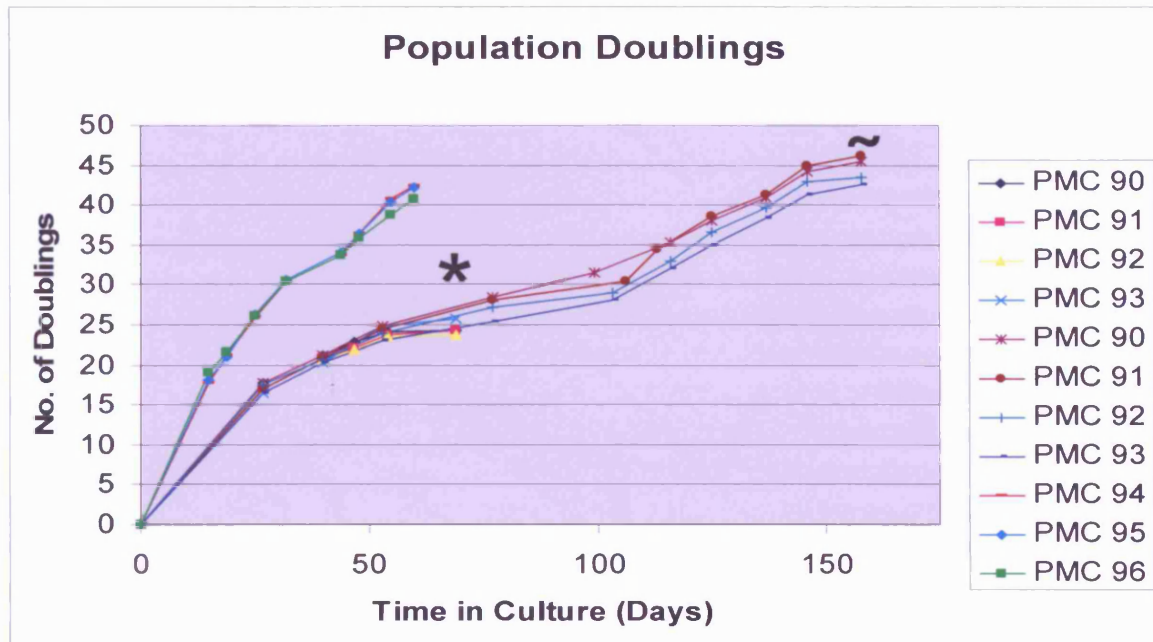
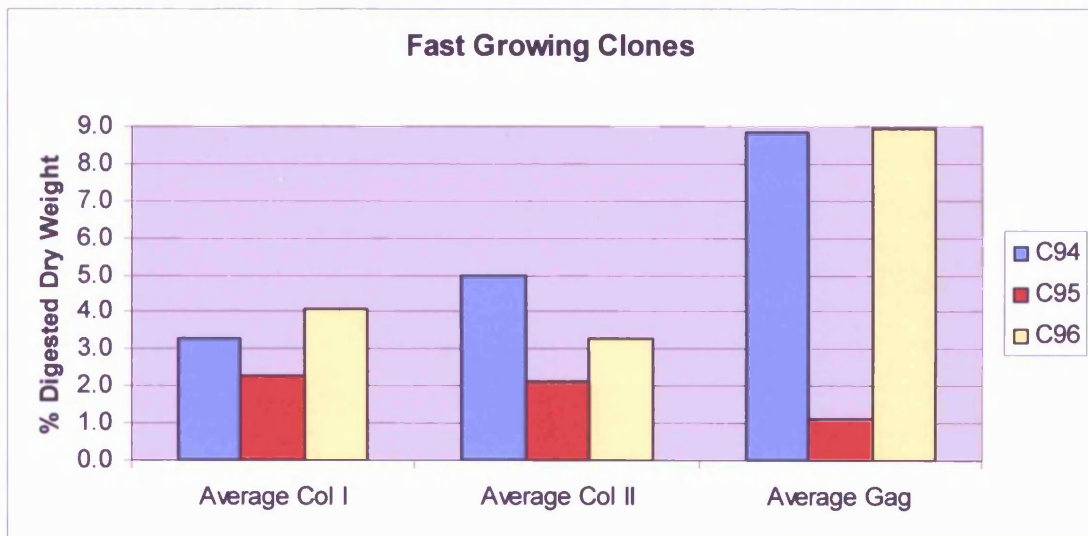
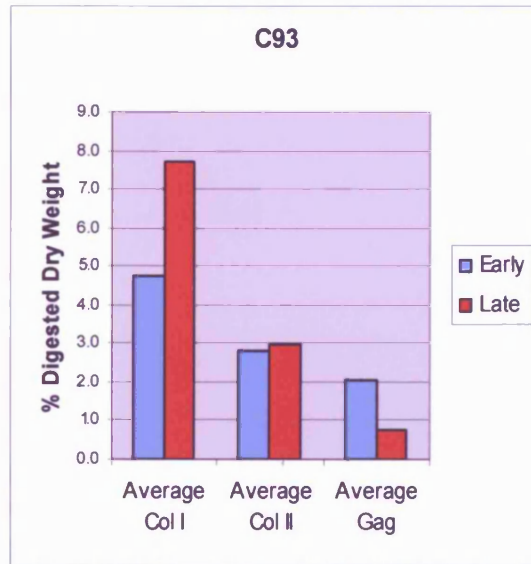
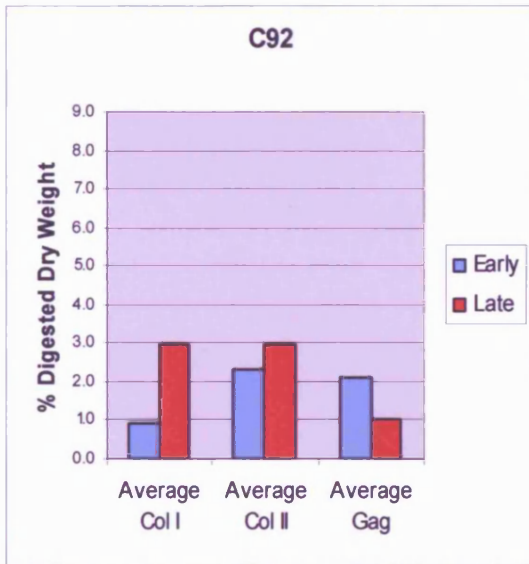
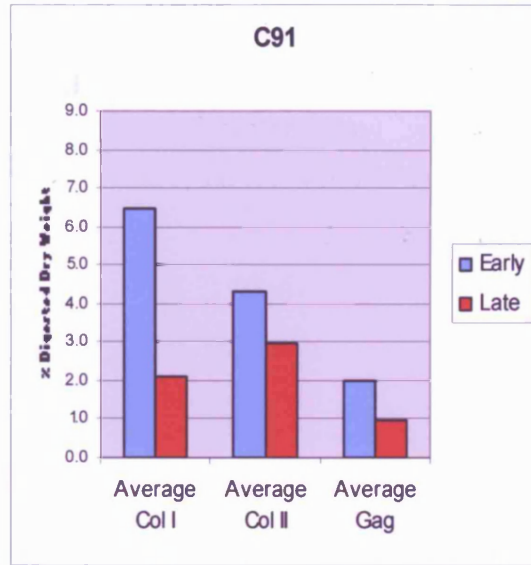
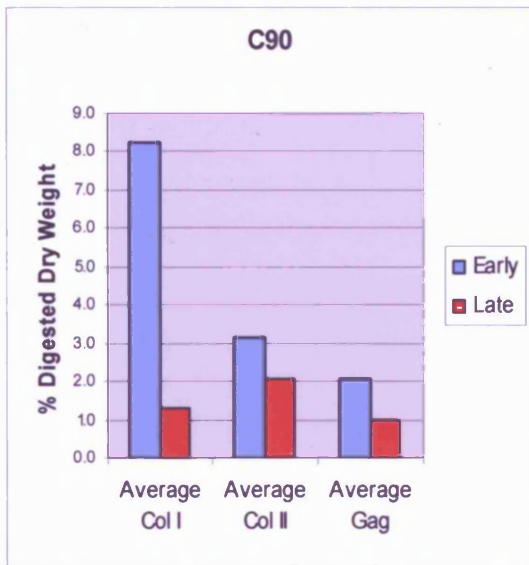


Figure 2.2 Graph showing population doublings and rate of growth

Graph showing the rate of growth and population doublings of chondroprogenitor cells grown for cell pellets. Cells were harvested after 25 (\*) and 42-45 (~) population doublings. A second population of cells were also used with an increased rate of growth. These cell populations reached 42 PD after only 60 days in culture, compared to 160 days in culture for the remaining cells.

**Figure 2.3 Graph showing ratio of collagen type I to type II and GAG**

Graphs showing the matrix composition of chondroprogenitor cell pellets grown from four different clonal cell lines, at either 25 PDs (early) or 45 PDs (late), as well as three populations of cells with an increased rate of growth. Pellets were freeze dried, and the percentage collagen type I, type II and GAG within each pellet calculated.





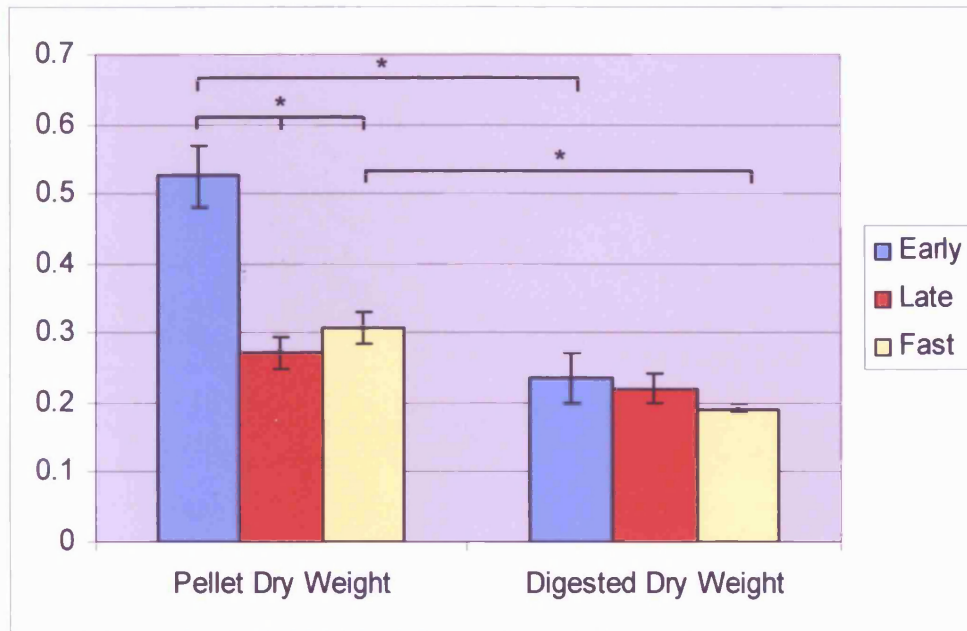
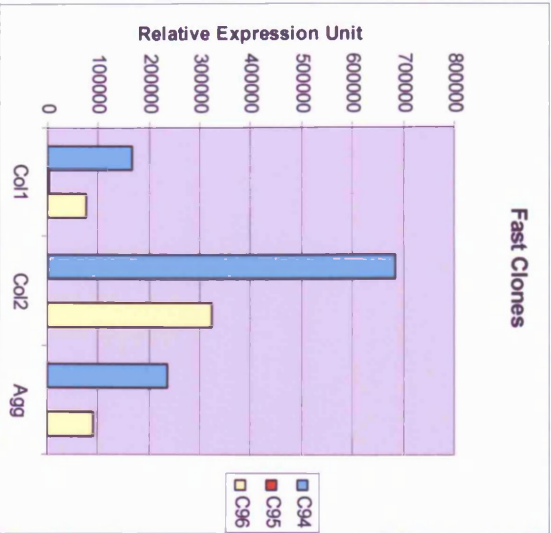
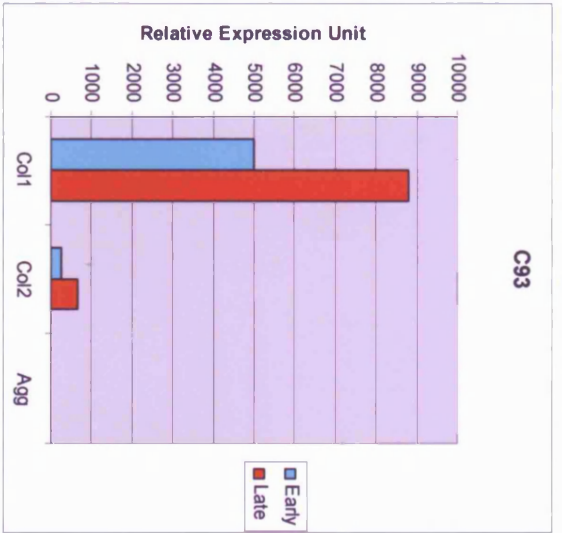
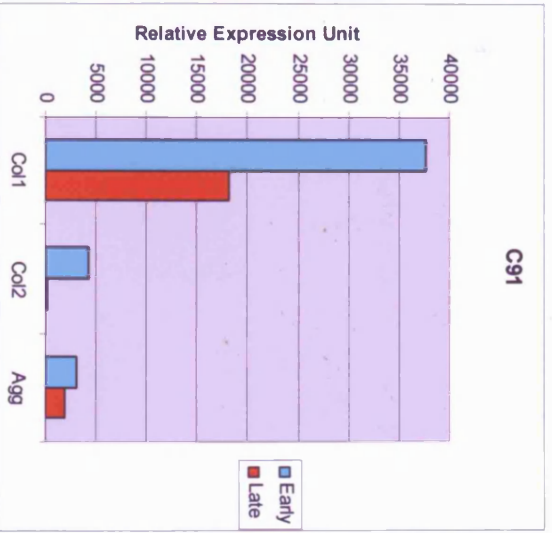
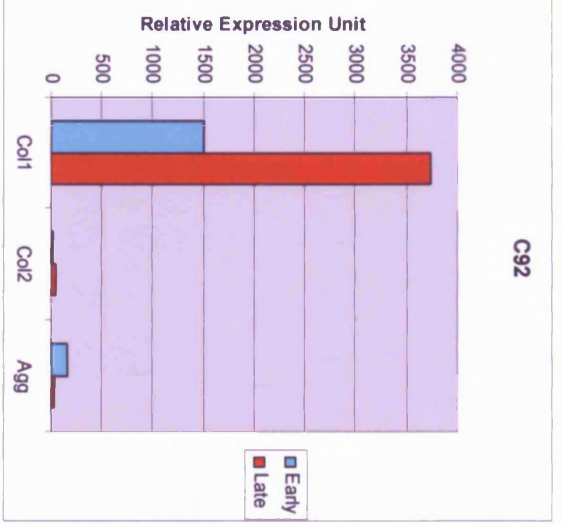
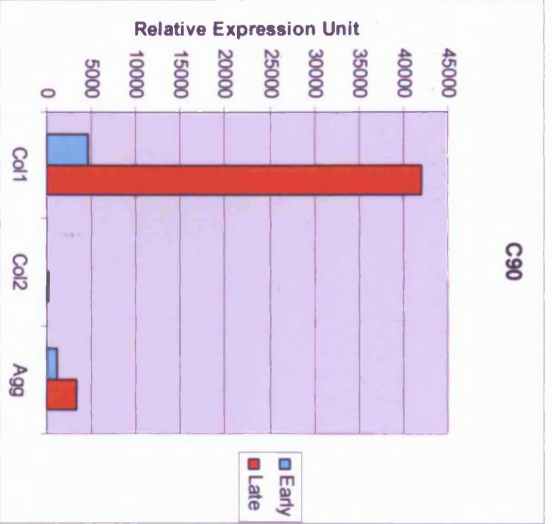


Figure 2.4 Graph showing average dry weight of pellets and digested samples

Chondrogenitor cell pellets were generated from four different clonal cell lines at either 25 PD (early) or 45 PD (late), as well as a three population of cells with an increased rate of growth. The average dry weight of the pellet and the dry weight of the digested sample were calculated. Significant differences ( $P < 0.05$ ) are indicated (\*) and error bars represent one standard error of the mean.

Figure 2.5 Graph showing relative expression levels of mRNA for cartilage associated genes

Graphs showing the levels of mRNA for the cartilage matrix components collagen type I, type II and aggrecan. Pellets were grown from chondroprogenitors at 25 PDs or 45 PDs, as well as three populations of cells with an increased rate of growth. Pellets were processed, the RNA extracted and RT-qPCR carried out. The axes differ between graphs.



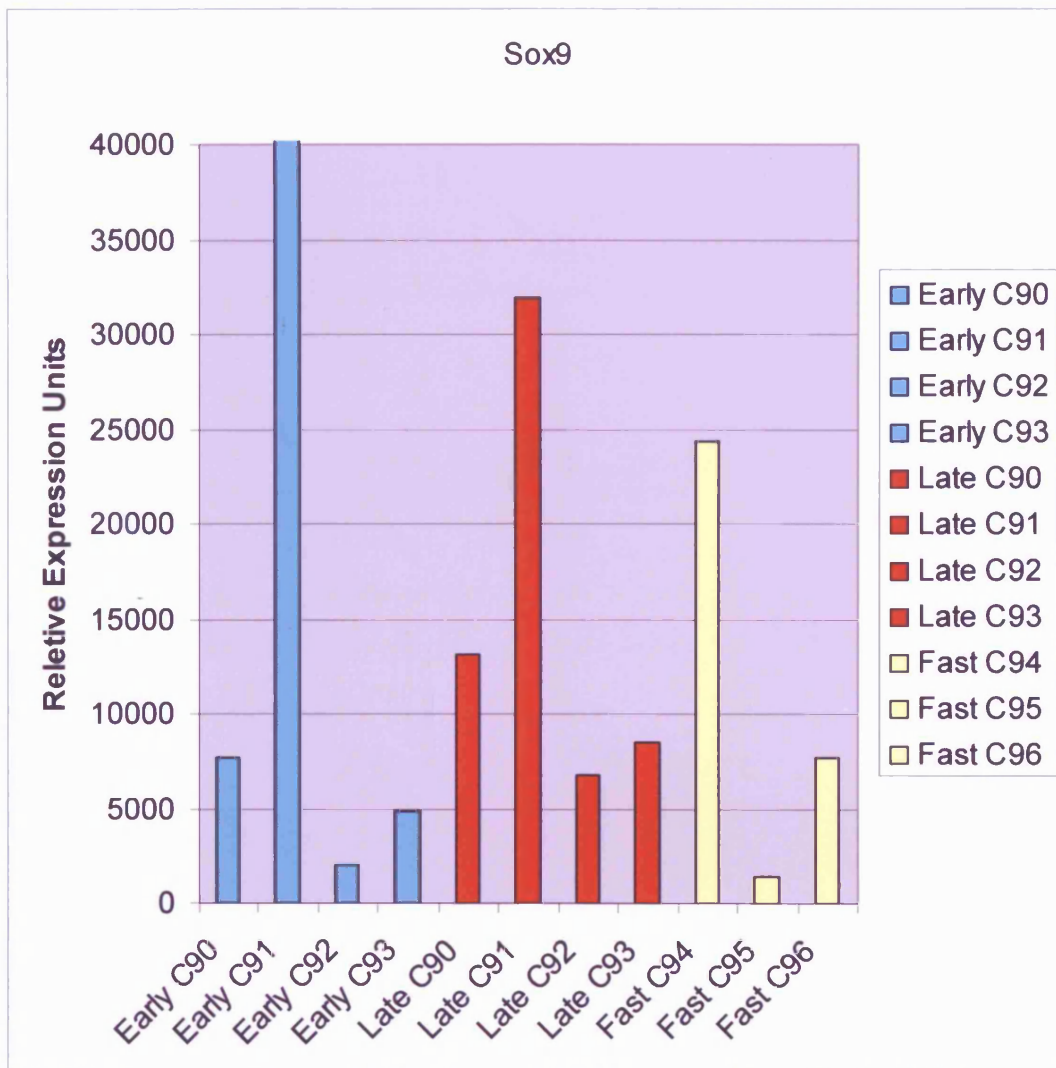


Figure 2.6 Graph showing relative expression levels of Sox9 mRNA

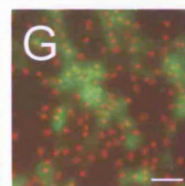
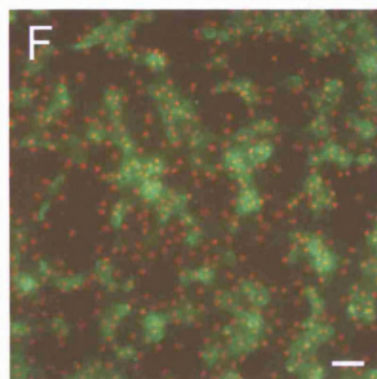
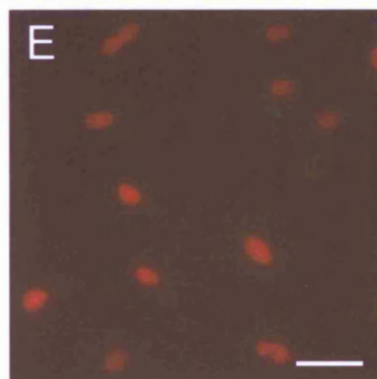
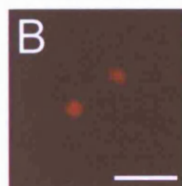
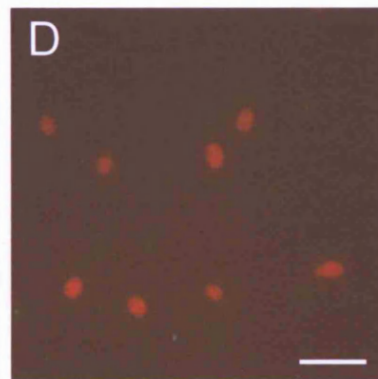
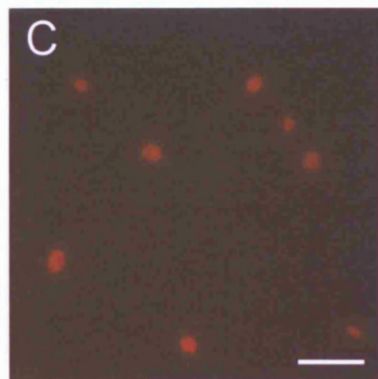
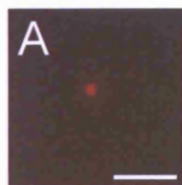
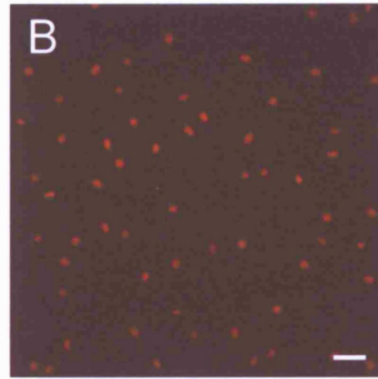
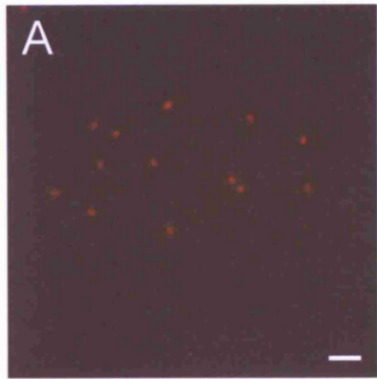
Graph showing the relative expression levels of the transcription factor Sox9 in pellets grown from chondroprogenitors at 25 PDs or 45 PDs, as well as three populations of cells with an increased rate of growth. Note early C91 value is beyond scale of graph.

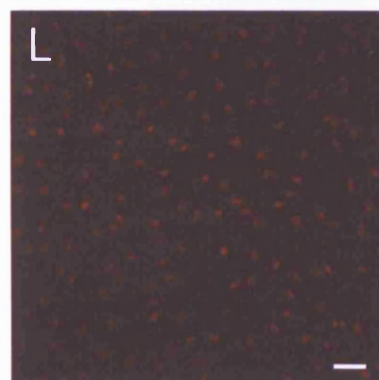
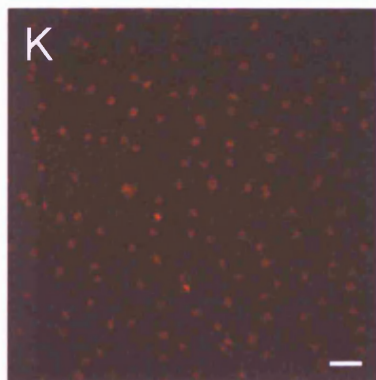
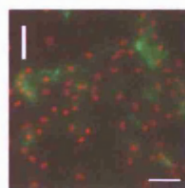
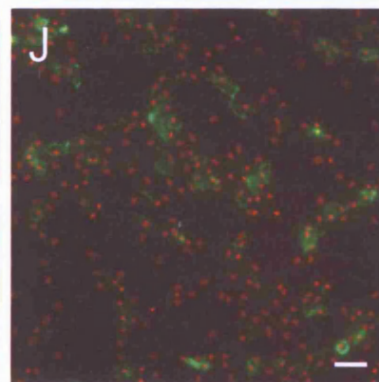
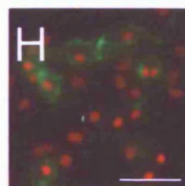
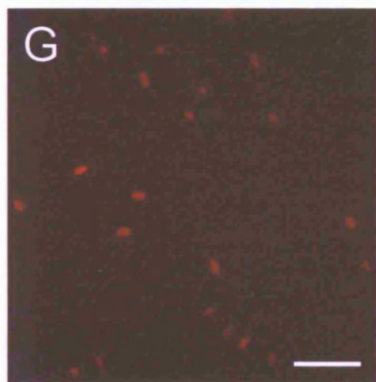
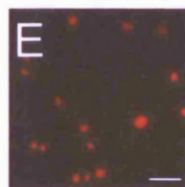
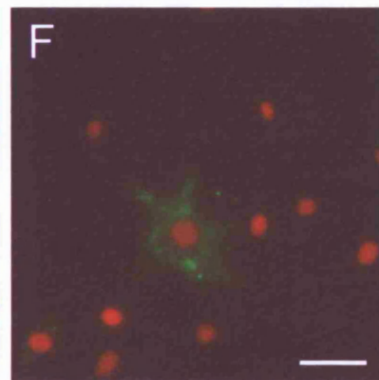
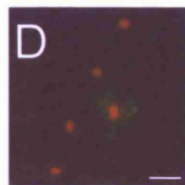
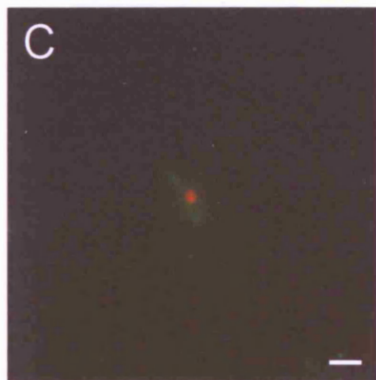
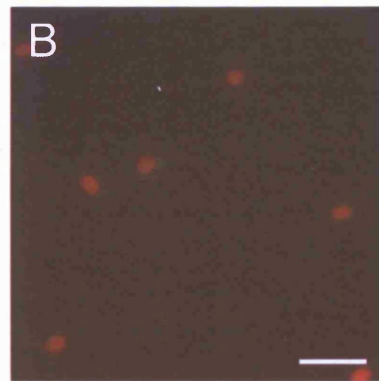
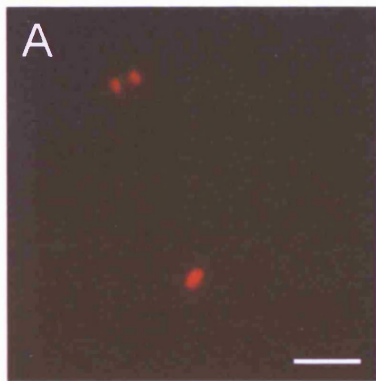
**Figure 2.7 Chondroprogenitor colonies after differential adhesion assay**

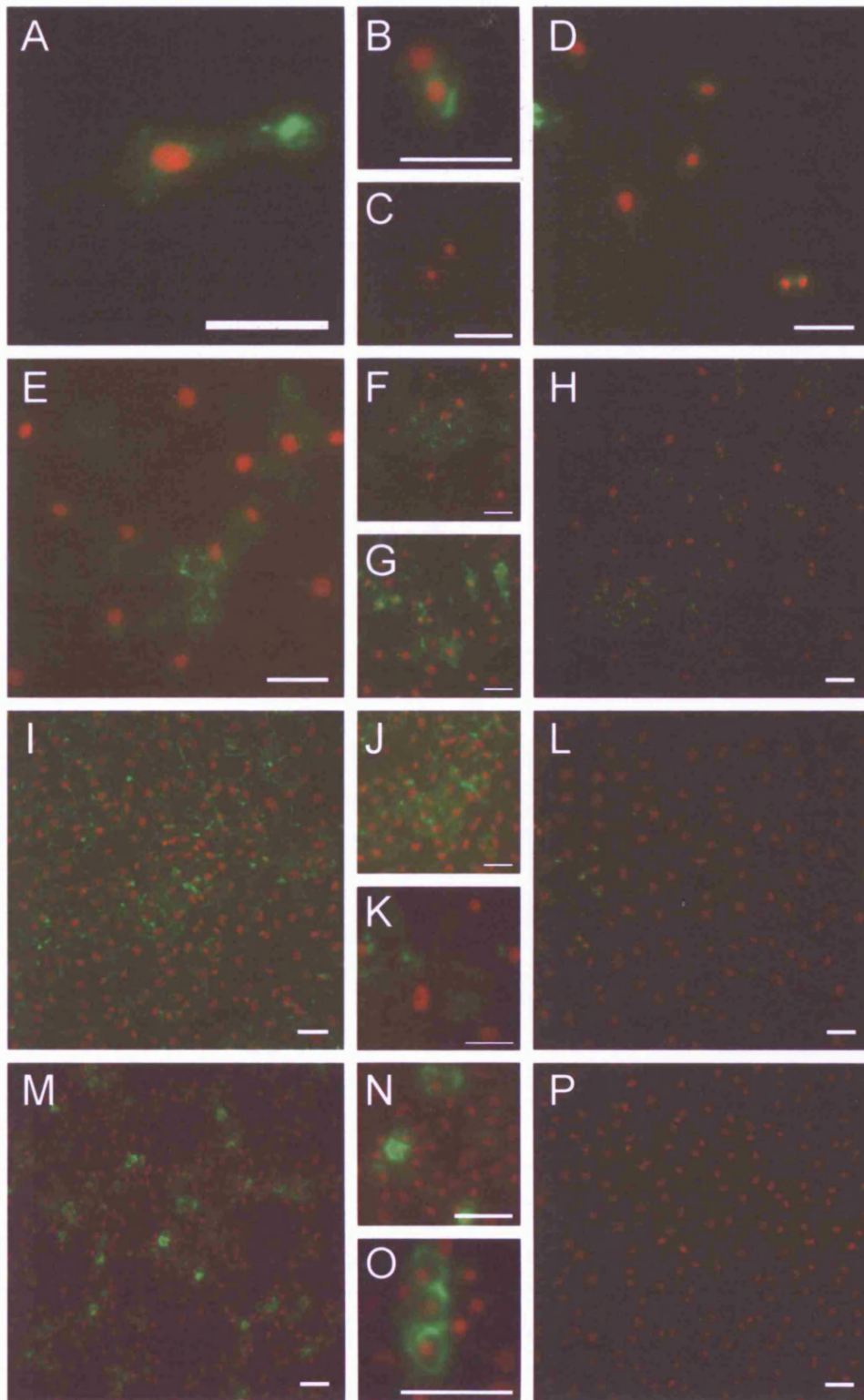
Colonies of chondroprogenitor cells 3 (A) and 7 (B) days after a differential adhesion assay was performed. Cells were labelled with a nuclear stain (red). Scale = 50µm

**Figure 2.8 Immunolabelling of surface zone cells and clones with collagen type II antibody**

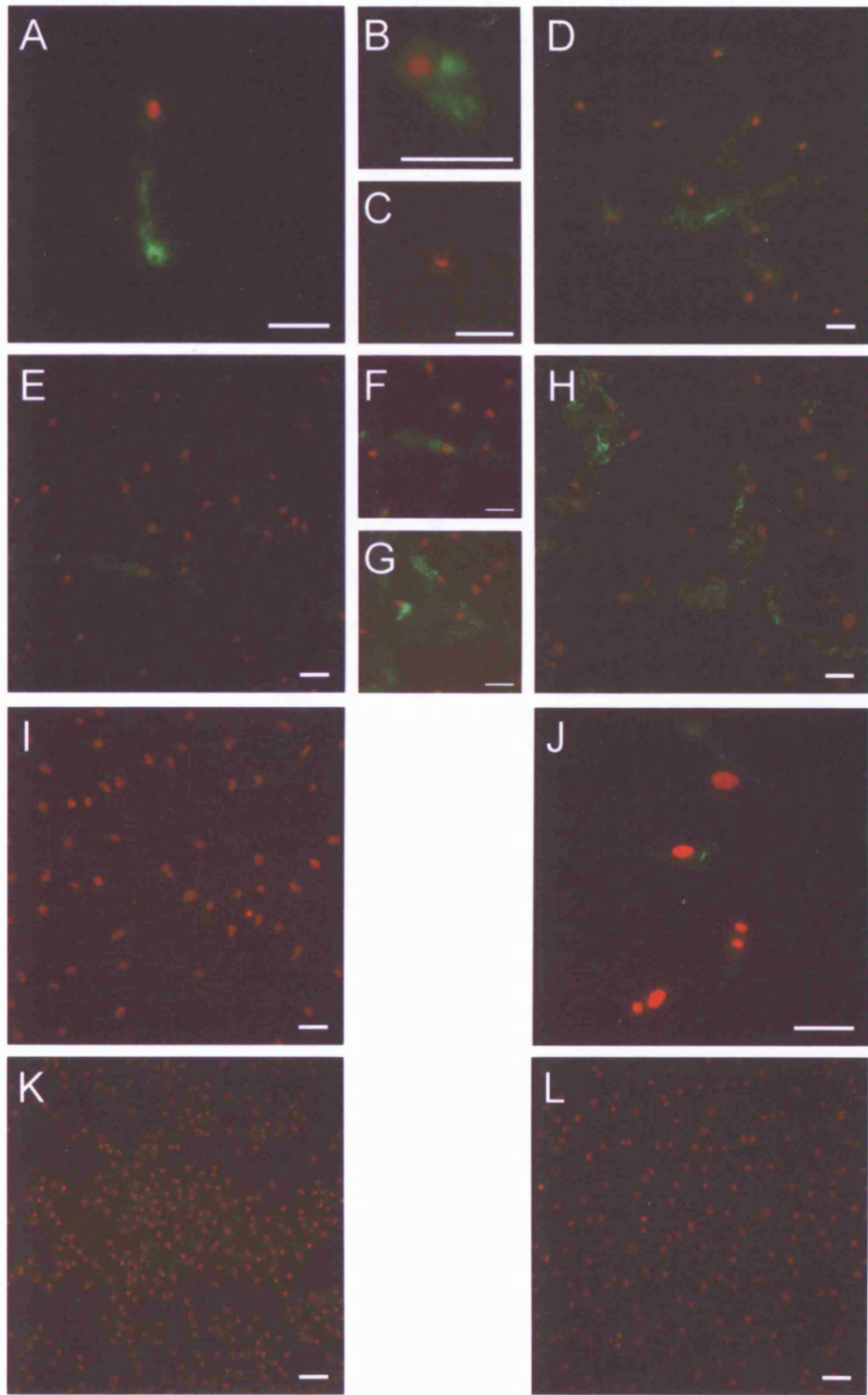
Plates were immunolabelled with type II collagen at days 1 (A), 3 (B), 5 (C), 7 (D) and 9 (E) after plating. No collagen labelling was seen in either colonies or isolated cells at any of the time points. Positive regions were detected in freshly isolated SZ cells (F and G), but there was also no labelling seen in passaged clonal cell lines (H). Cells were labelled with type II collagen (green) and nuclear stain (red). Scale = 50µm











## 2.4 DISCUSSION

It is already well established that long term culture of many cell types, including chondrocytes, results in changes to their phenotype. This study shows that it is not just long term culture that can affect the cells, and that similar structural changes and alterations to the cell phenotype that are observed in mature cells also occur in progenitor cells grown in monolayer culture.

The most well documented change that occurs when somatic cells are grown in culture is the progression to cell senescence, resulting in an increased generation time and cellular degradation (Hayflick, 1965). But not all changes that occur with long term culture result in cell death or are as obviously apparent. Long term culture of a clonal rat bone cell line has been found to result in the cells altering their collagen synthesis, possibly as a result of morphological changes to the cell shape (Limeback et al., 1984). Human fibroblasts also show similar changes after long term culture with cells changing their shape, size and rate of growth with increased time in culture (Pourreya et al., 2003).

In the case of chondrocytes, the mechanisms responsible for the changes that occur in culture are well established. Within the tissue, the cells are naturally rounded, and growing the cells in monolayer culture results in changes to not only their morphology, but their phenotype (Benya and Shaffer, 1982). These changes result in the cells adopting a fibroblastic morphology as a result of changes to gene expression (Stokes et al., 2002), and can be reversed by growing the cells in 3D culture.

Differences in the phenotype of clonal cell lines isolated from the superficial zone of bovine articular cartilage have already been found (Bishop, 2004), and this current study found that the ability of these clonal cell lines to produce pellets varied. After 25 PDs, one of the cell lines produced a cell matrix with levels of collagen type I over 50% lower than for collagen type II suggesting a more hyaline-like matrix was formed (although levels of collagen type I were still high). The remaining three young cell lines produced greater percentage of matrix than compared to C92, although this matrix appeared to be a fibrocartilage-like matrix. The levels of type I collagen were higher when compared with the percentage of type II collagen, with

levels ranging from 2-2.5 times greater. The GAG content of the pellets was the same for all the cell lines. The differences seen between cell lines may be due to the clonal cells having differing levels of maturity when isolated from the cartilage. After maintaining the cells in culture for a prolonged period, two of the cell lines reduced collagen type I content of the pellet to below 2%, resulting in a pellet consisting of predominantly collagen type II, and the production of a more hyaline-like cell matrix. The remaining two cell lines increased collagen type I production, whilst maintaining type II. The levels of GAG in all the pellets decreased when compared with the 25 PD pellets.

Interestingly, the pellet produced from clonal cell lines with an increased rate of growth produced pellets similar to those after 45 PDs, with lower levels of collagen found than in the younger clonal pellets. The one difference seen was a considerable increase in the percentage of GAG in the pellet matrix in two of the faster growing clones. It is possible that the increase in GAG produced by the faster growing cells is involved in enhancing the differentiation of these cells (Wight et al., 1992; Kresse and Schonherr, 2001).

The dry weight of the pellets also differed significantly between the different cell populations. The cells at 25 PDs produced pellets with a significantly higher dry weight than the 45 PDs or fast growing cell lines, however, the dry weight of the digested pellet was also significantly lower suggesting that, although the younger cells are able to make larger pellets, these pellets consist of less matrix. There was no difference in the weight of the pellets in either the 45 PDs or fast growing clonal cell lines, and although the digested weight of the fast growing cell pellets was significantly lower than the whole pellet weight, the difference was smaller than for the 25 PDs clones. The relative levels of mRNA for collagen type I, type II and aggrecan all followed a similar trend, with the levels of these genes varying greatly between different cell lines. The two cell lines that produced the highest levels of collagen type II (C91 and C94) also had higher relative expression of Sox9 mRNA, whilst the pellet with the lowest matrix synthesis also had the lowest levels of Sox9 mRNA. These data suggest that high levels of Sox9 may influence the cells ability to produce a hyaline-like matrix consisting of predominantly collagen type II (figure 2.6).

In short term culture, it is possible to explain some of the changes seen in cell marker expression by progression through the cell cycle. It was found that the phenotype of certain progenitor cell types not only fluctuates depending on where in the cell cycle the cells are, but the ability of the cells to differentiate also changes depending on where the cell is in the cell-cycle (Habibian et al., 1998; Dooner et al., 2008). The markers expressed by cells can, therefore, change after relatively short periods in culture.

It has already been found that proteoglycans have a varied and important role in cell signalling and cell behaviour. A series of monoclonal antibodies have been developed that recognise specific epitopes within the chondroitin sulphate glycosaminoglycans. The antibodies to these epitopes (3B3(-), 4C3 and 7D4) have been found in bovine and human articular cartilage (Sorrell et al., 1990). More specifically, these antibodies bind to sulphation motifs associated with aggrecan, the key proteoglycan found in articular cartilage (Hayes et al., 2008). They have also been identified within the cell clusters found in osteoarthritic tissue suggesting a role in cell proliferation (Slater et al., 1995). These clusters may contain progenitor-like cells (Hiraoka et al., 2006).

The sulphated motifs may have a key role in controlling the development of the chondroprogenitor cells, possibly by regulating chondrogenesis. The fact that the cells switch and alter the production of these motifs when grown in culture may support this. As 7D4 is only expressed by isolated cells after differential adhesion, and not in the isolated SZ cells suggest that this motif may be expressed solely by only a small number of cells in the tissue after isolation, or by changes in cell shape by a small cohort of cells. After an initial increase in the expression of 7D4, the levels began decreasing with increased time in culture, disappearing by day 9.

These sulphation motifs are not only expressed in OA tissue, but also within the surface zone of articular cartilage, possibly in association with the progenitor cell population associated with this region (Hayes et al., 2008), although the frequency of labelling would suggest this is not specific as approximately a third of the cells label positively at the surface for 7D4 and almost all label positively for 4C3. Results from this study support the work by Hayes *et al.* that the three markers 3B3(-), 4C3 and 7D4 are found within the cartilage and possibly associated with cells from the SZ of

the tissue. Whilst 3B3(-) and 4C3 were expressed by the isolated SZ cells, 7D4 was absent and it is possible that culturing and isolating the cells resulted in alterations to the cells GAG profile. It is highly unlikely that 3B3(-) is associated with a progenitor cell population after isolation, as there were no cells expressing this sulphate motif in the first 3 days after the differential adhesion assay had been performed, despite previous data showing that between 1 and 3 clonal cells are isolated after the assay (Bishop, 2004).

When any cell is taken out of its natural microenvironment, changes to the morphology of the cell are always likely to have downstream effects on its differentiation ability. Results from this study suggest that long-term culture of chondroprogenitors isolated from the SZ of bovine cartilage has no negative effect on the ability of these cells to differentiate, and in the case of some cell lines growth in culture actually enhanced the hyaline-like cartilage that the cells produced.

## **CHAPTER 3**

### ***In Vivo* Plasticity of Cartilage Progenitor Cells**

### 3.1 INTRODUCTION

Chondrocytes are thought to be cytoplasmically isolated (Archer and Francis-West, 2003) and this, combined with the avascular and aneural nature of articular cartilage, means that the tissue has a very poor capacity for repair after damage or injury. Prolonged growth of chondrocytes *in vitro* has also been shown to result in their dedifferentiation or, more correctly, their phenotypic modulation (Benya and Shaffer, 1982; Chen et al., 2003) suggesting that alternative cell sources may be required for cartilage repair of larger chondral lesions.

The *in vivo* plasticity of mesenchymal progenitor and stem cells isolated from a variety of tissue sources has already been established. These cells have been shown to maintain the ability to differentiate into many different tissue types including myocardium, skeletal muscle, cartilage, tendon and neural cell lineages (Barry and Murphy, 2004). Many of the advances in the use of MSCs for tissue engineering and repair have occurred recently. MSCs isolated from the human bone marrow have been found to possess the ability to differentiate into both skeletal and cardiac muscle *in vivo*. Injecting bone marrow cells into the tail of *mdx* mice, a mouse model of muscular dystrophy, results in partial restoration of haematopoietic compartment and expression of dystrophin (Gussoni et al., 1999; Barry and Murphy, 2004). Bone marrow isolated cells also have a use in orthopaedic research (Barry and Murphy, 2004) with MSCs showing both osteogenic and chondrogenic potential *in vivo*. Krebsbach et al (1998) demonstrated that the bone marrow contained a source of osteoprogenitor cells capable of repairing defects in the cranium of immunocompromised mice.

Human bone marrow cells can also be induced to form chondrocytes via growth on a perchloroethylene scaffold (Barry and Murphy, 2004). When implanted under the dorsal skin in nude mice, the constructs formed cartilage (Jiang et al., 2003). A similar result has been shown by Cui et al., (2006) who isolated MSCs from rabbit bone marrow and seeded them onto a polyglycolic scaffold. The MSC seeded scaffold was then transplanted into subcutaneous tissue where it formed cartilage. The cartilage forming ability could be enhanced by stimulation with low-intensity ultrasound (Cui et al., 2006; Lee et al., 2007). Implantation on a scaffold is not the

only method for inducing chondrogenic differentiation of MSCs. Direct injection of the cells into the joint of a caprine model of osteoarthritis resulted in partial cell engraftment with some degree of regeneration of the meniscal tissue and the presence of injected cells within the meniscus, fat pad and synovium (Murphy et al., 2003).

A population of MSCs has been identified in the synovial membrane of human knee joints that are capable of chondrogenic differentiation *in vitro* (De Bari et al., 2001). Although the cells show a large differentiation potential *in vitro*, this phenotype has since been shown to remain unstable and the cells fail to form cartilage *in vivo*. Micromass cultures cultured in the presence of TGF- $\beta$ 1 were either implanted into the back of nude mice or injected intramuscularly. Mice were sacrificed at intervals of up to 40 days but human cells were not detectable after 15 days *in vivo* (De Bari et al., 2004). Despite this result, the cells were able to engraft into muscle and transcribe muscle markers and dystrophin when injected into the *mdx* mouse (De Bari et al., 2003).

It is not only stem cells that are capable of undergoing chondrogenic differentiation after the correct stimulation. Recently, progenitor cells have been isolated from chorionic villi of human placenta and these cells have been shown to have the ability to differentiate into chondrocytes. Isolated cells were seeded onto a collagen sponge and maintained in chondrogenic media for 2 weeks. The cell sponge was then implanted into subcutaneous pockets on the backs of nude mice. Histological analysis showed the presence of chondrocyte-like cells with the formation of extracellular matrix (Zhang et al., 2006).

The ability of chondroprogenitor cells isolated from the surface of articular cartilage to differentiate into adipogenic, osteogenic and chondrogenic tissue *in vitro* has already been demonstrated (Thornemo et al., 2005) as well as *in ovo* (Dowthwaite et al., 2004). Clonally derived labelled progenitor cells were injected into the distal and proximal limb of 3-day-old chick embryos (stage 19-21) and tracked *in ovo* for a minimum of 1 week (Dowthwaite et al., 2004). Lac-z positive cells were detectable around the injection site after 1 day. After 1 week, cells could be detected in several different tissue types including cartilage, bone, tendon and muscle. By using an antibody for bovine type I collagen that was species specific, it was possible to



determine that these cells had formed articular fibrocartilage, perimysium, tendon and bone, thus suggesting functional engraftment. However, differentiation *in vitro*, or in a developmental system, isn't always a reliable indicator of differentiation capacity in mature tissue *in vivo*.

The use of progenitor/stem cells in cartilage repair procedures may avoid some of the constraints of expanded mature chondrocytes such as dedifferentiation and variable cell quality. In addition, using progenitor/stem cells from the host tissue may improve the structural characteristics of the repair tissue as these cells may have the appropriate developmental repertoire to recapitulate this morphogenesis, particularly in younger patients.

Most of the current repair methods available for cartilage lesions do not provide reliable integration between host and repair tissue (Archer et al., 2006). The progenitor cells may be able to create cartilage that is both structurally and biochemically accurate and, therefore, may provide an alternative cell source for treating cartilage lesions. The aims of this study were to ascertain if bovine articular cartilage progenitor cells can survive *in vivo* in a mammalian model and determine their ability to differentiate into mesenchymal tissue.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Cell Labelling and *in vivo* Implantation**

Chondrocytes were isolated from immature bovine MCP joints as previously described, with three different cell populations used for this experiment (3 full-depth, 6 clonal and 3 enriched). Progenitor cells were isolated at two week intervals (from a pool of cells collected from 3 bovine MCP joints at each point) grown to between 30 and 40 population doublings ( $5^8 - 5^{11}$  cells), whilst full depth cells were isolated 24 hours before injection. Cells were trypsinised and resuspended at  $2 \times 10^7$  cells  $\text{ml}^{-1}$  in a polypropylene tube. They were then washed in serum-free media and centrifuged at  $400 \times g$  for 5 minutes. Cells were then labelled with PKH26 red fluorescent cell marker (Sigma) as per the manufacturer's guidelines (Appendix IV). Freshly isolated bovine chondrocytes were used as a positive control with unlabelled human chondrocytes as a negative control.

### **3.2.2 Muscle Injections**

Eight-week-old severe-combined immuno-deficiency (SCID) mice were used for this study. Labelled cells were transferred to an Eppendorf tube and re-suspended in 50 $\mu$ l phosphate buffered saline (PBS; Sigma). Finally, 25  $\mu$ l ( $5 \times 10^5$  cells) were injected intramuscularly into the thighs of eight mice. Different cell lines were injected into each leg. After 2 weeks, the mice were sacrificed by cervical dislocation and the muscles were dissected to determine the presence of a cartilage pellet. If no pellet was found, the muscle fibers were pooled together and frozen for cryosectioning and PCR analysis. Once the samples were sectioned, they were examined using an Olympus BX61 fluorescent microscope to determine the presence of fluorescently labelled cells.

### **3.2.3 Safranin O Staining**

Sections were stained for glycosaminoglycans using Safranin O (see Appendix V). Samples were fixed in 10% normal buffered formalin solution (10%; Sigma) for 10 minutes before being rinsed in running water for 2 minutes. Sections were then placed in haematoxylin (RA Lamb, UK) for 2 minutes and then washed again in water for 5 minutes. They were then placed into 0.1% Safranin O solution (BDH, UK) for a further 2 minutes before being washed for 30 seconds in water. Finally, the

sections were passed through a series of increasing industrial methylated spirit concentrations (IMS; Fisher scientific; 70-100%) for 2 minutes at each concentration. The slides were then cleared in xylene for 2 minutes (x2). Coverslips were placed over the sample and mounted with DPX (RA Lamb). Slides were then viewed and images then taken using a Leica DMRB light microscope.

### **3.2.4 Immunohistochemistry**

**Sox9** - To perform immunolabelling for the chondrogenic transcription factor sox9, sections were circled with a DAKO pen (Dako, UK) fixed in 2% paraformaldehyde/PBS (both Sigma) for 2 minutes and blocked with swine serum (Dako) for 20 minutes at room temperature (serum used at 1:20). The anti-Sox9 rabbit polyclonal antibody (Abcam, UK) was diluted to 10µg/ml in 0.1% PBS/Tween.. The excess blocking serum was tipped off and the antibody added to the slides. An appropriate isotype control (Dako), and a slide with 0.1% PBS/Tween instead of a primary antibody were used as controls. The sections were then incubated overnight at 4°C. Mouse limb sections were used as a positive control. Sections were then washed in 0.1% PBS/Tween for 5 minutes (x3). Samples were then incubated with swine anti-rabbit FITC (Dako) in 0.1% PBS/Tween at 10µl/ml for 1 hour at room temperature, which was washed off in 0.1% PBS/Tween for 5 minutes (x3). Finally, sections were mounted using one drop of 'Vectashield' with DAPI (Vector Laboratories, UK) and a coverslip placed over the top. Slides were then imaged using an Olympus BX61 fluorescent microscope.

**Collagen Type IIa** - Sections were circled with a Dako pen, fixed and washed as described for the Sox9 immunolabelling. The endogenous hydrogen peroxidase activity was blocked with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes. Sections were then blocked with 2mls 0.1% PBS/Tween plus 1 drop of blocking serum (Universal quick kit; Vector Labs) for 10 minutes at ambient temperature. The antibody diluent was prepared and this was then used to dilute the mouse CIICI antibody (DSHB, USA) and isotype control (Dako) to 2.5µg/ml. Excess blocking serum was tipped off and slides were incubated with the primary antibody overnight at 4°C. A negative control was also set up using antibody diluent only. Sections were washed in 0.1% PBS/Tween before the biotinylated secondary antibody (universal horse anti-rabbit/goat; Vector Laboratories) was added, and a control was set up with no

secondary antibody. Slides were then washed in 0.1% PBS/Tween (x3 for 5 minutes). After washing, the slides were incubated with Streptavidin complex (as per maker's instructions) for 5 minutes at room temperature. The peroxidase substrate solution (Nova Red Peroxidase Quick Kit; Vecta Laboratories) was prepared as per the manufacturer's guidelines (see Appendix VI). The slides were then passed through a round of increasing concentration of IMS (70%, 95% and 100% twice) for 2 minutes at each concentration to re-hydrate the samples. The slides were then cleared in xylene for 4 minutes before mounting with DPX. Images were then taken of the sections using a Leica DMRB light microscope.

### **3.2.5 RNA Extraction and cDNA Synthesis**

Small biopsies (between 2mm and 5mm in diameter) were taken from each sample and the tissue was powdered by dismembration (Braun Bioteck, Germany). Samples were placed in the dismembrator for 90 seconds at 2000rpm x g with 200µl TRizol reagent (Invitrogen). After three cycles in the dismembrator, the powder was collected and placed in a 1.5ml RNase free tube (Alphalabs, UK) with 800µl TRizol. The RNA was then extracted from the samples and cDNA synthesised (see chapter 2.2.6).

### **3.2.6 Polymerase Chain Reaction (PCR)**

Primers for PCR were either designed using the Primer 3 software (see table 3.2) or known sequences used (see table 3.2). All primers were synthesised by MWG (UK). Each 12.5µl PCR reaction contained 1µl cDNA, 200µM of each dNTP, 0.24µM of each primer (0.2µM of β-actin primers), 10µl 5x buffer (GoTaq Flexi DNA polymerase kit; Promega) 1.25–1.5µM MgCl<sub>2</sub> and 4 units GoTaq DNA polymerase (Promega). Initial denaturation occurred for 1 minute at 94°C, with amplification comprising of 35–40 cycles of 30s at 94°C, 30s at 58–60°C (see tables 3.1 and 3.2) and 30s at 72°C. A final extension step of 5 minutes at 72°C then occurred. Amplification was carried out in a Techne FTGENE2D thermocycler (Techne, UK). The products were then run on a 1% agarose gel stained with ethidium bromide (both Promega). No PCR product was detected when either RNA was replaced with water at the RT reaction, or the cDNA was replaced with water at the PCR stage. To

determine if RNA extraction had been successful, PCR was carried out using primers for  $\beta$ -actin.

Bovine Specific	Forward Primer	Reverse Primer	Annealing Temp.	Product Size
Coll Type II	ACG TCC AGA TGA CCT TCC TG	TGG ATG AGC AGA GCC TTC TT	60°C	127bp
PECAM-1	CTG GAG TCT TCA GCC ACA CA	TAT AAC CCG CTG TCC CAC TC	60°C	164bp
MyoD	GCT ACT TCG CGA CCA GGA C	GGC GCC TCG CTG TAG TAA GT	60°C	219bp

Table 3.1 Polymerase Chain Reaction Bovine Specific Primers. All primers are shown 5'-3' with corresponding annealing temperature and product size.

Standard Primers	Forward Primer	Reverse Primer	Annealing Temp.	Product Size
$\beta$ -Actin	TGT ATG CCT CTG GTC GTA CCA C	GAG GAC TCG CGT TCA TGA GAC	58°C	596
Sox9	CTC AAG GGC TAC GAC TGG AC	CGT TCT TCA CCG ACT TCC TC	60°C	313
Coll Type II (Valcourt et al., 1999)	GCC TCG CGG TGA GCC ATG ATC	CTC CAT CTC TGC CAC GGG GT	60°C	IIA: 472 IIB: 268

Table 3.2 Polymerase Chain Reaction Standard Primers. All primers are shown 5'-3' with corresponding annealing temperature and product size.

### 3.2.7 Cloning and Sequencing PCR Products

A ligation reaction was set up using 2.5 $\mu$ l of 2x ligation buffer, 50ng pGEM-T vector, 3 weiss units  $\mu$ l<sup>-1</sup> (Promega) and 1.5 $\mu$ l of PCR reaction. Reactions were mixed by gentle inversion and placed at 4°C overnight. Frozen JM109 cells (Promega) were thawed, and 2 $\mu$ l of the ligation reaction was placed in the bottom of sterile 15ml tube

with 25µl of the JM109 cells. Tubes were mixed and placed on ice for 20 minutes. Cells were then heat shocked for 45secs in a 42°C water bath before being placed back in ice for 2 minutes. Tubes were taken off ice and 475µl SOC media (Invitrogen) was added to each tube. Cells were then incubated for 90 minutes at 37°C on a Innova 4300 incubator shaker (New Brunswick Scientific, USA) at 150rpm. After incubation, 100µl of transformation was plated onto LB/Amp/IPTG/X-gal plate (see Appendix VII) and plates were incubated overnight at 4°C.

After 24 hours white colonies were selected from plates using sterile pipette tips. Cell numbers were increased by placing tips in 20ml of LB broth (see Appendix VIII) overnight at 37°C on the cell shaker at 220rpm. The DNA was then isolated using the Wizard Plus SV Miniprep DNA Purification System (Promega) as per the manufacturers guidelines (see Appendix IX), and isolated DNA was sent for sequencing.

### 3.3 RESULTS

Chondroprogenitor cells were injected into SCID mice intramuscularly to determine differentiation capacity *in vivo* in a mammalian model. Specifically, we tested the stability of the progenitor phenotype within a myogenic milieu. After two weeks, the muscles were dissected to determine if the progenitors would still express the chondrogenic phenotype or if they had differentiated according to their surrounding tissue. Fluorescent PKH26 labelled cells were detected in all of the samples analysed after two weeks (Figure 3.1A, C, E, G) suggesting the chondroprogenitor cells and full- depth chondrocytes are able to survive within the muscle tissue *in vivo*.

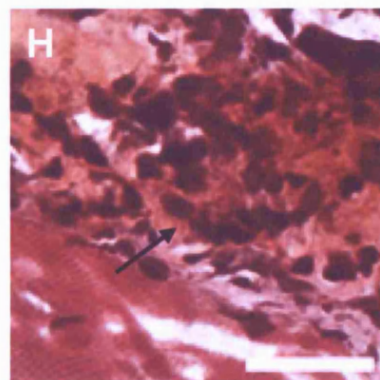
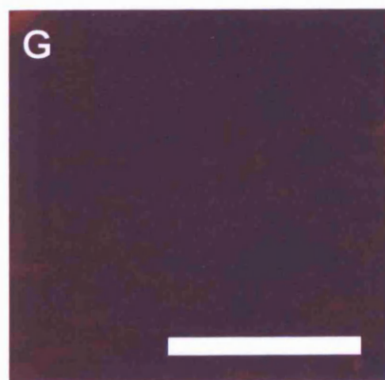
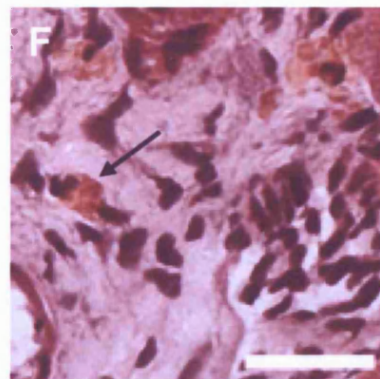
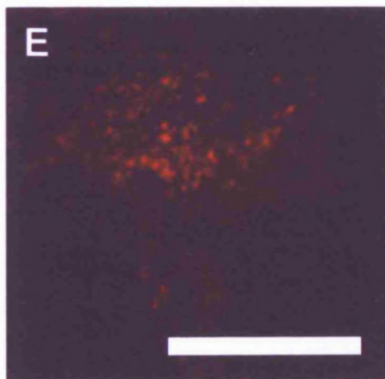
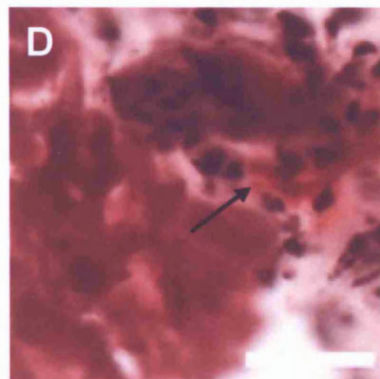
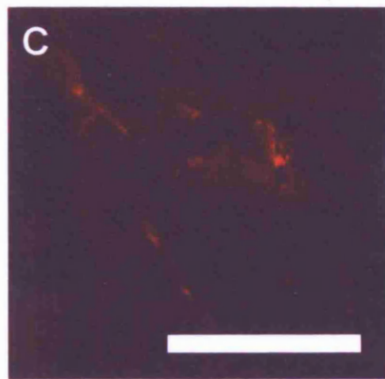
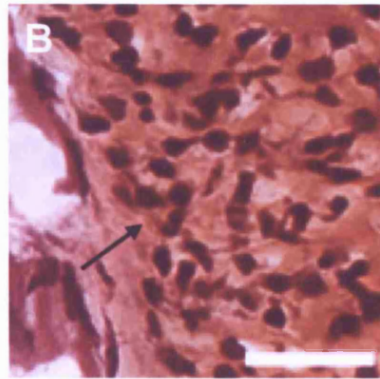
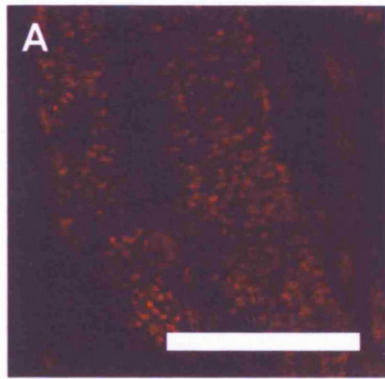
Whilst a distinct cartilage pellet was present after intramuscular injection of freshly isolated full-depth chondrocytes, there was no pellet present in the muscle of the mice injected with either the clonal or enriched cells. The full depth cell pellet was cryo-sectioned for analysis and sections stained with Safranin O to determine the presence of glycosaminoglycans, a core component of a cartilage matrix. The pellet stained intensely with Safranin O (Figure 3.1B) and the regions of intense staining corresponded to areas with a high proportion PKH26 labelled cells (Figure 3.1A). Cells within this pellet were rounded and compact, typical of a chondrogenic phenotype during cartilage development. Although there was no detectable pellet in the sample containing human unlabelled chondrocytes, some regions of positive Safranin O staining were found suggesting the presence of GAG nodules within the muscle fibres (Figure 3.1G and H), whilst fluorescently labelled cells were not detected in any sections. In both the clonal and enriched progenitor populations, small cartilage nodules were found that were positive for Safranin O (Figure 3.1D and F). These regions corresponded to areas with fluorescently labelled cells (Figure 3.1C and E), although it appeared that a more fibrocartilagenous matrix was formed by these cells. The levels of staining observed after injection with chondroprogenitor cells were much weaker than for the full-depth cell lines. Although the cells appeared slightly rounded, they were not as condensed as those observed in the full-depth cell pellet. Some of the labelled progenitor cells had a more elongated morphology, but there was no apparent difference in the morphological appearance of the cells from both the clonal and enriched populations.

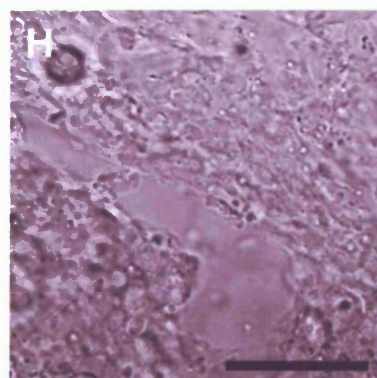
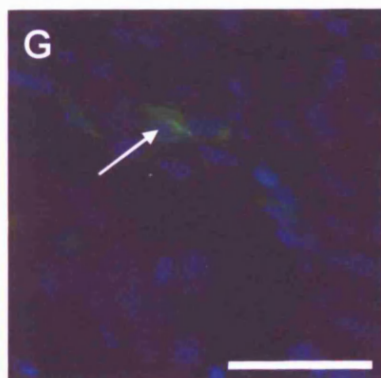
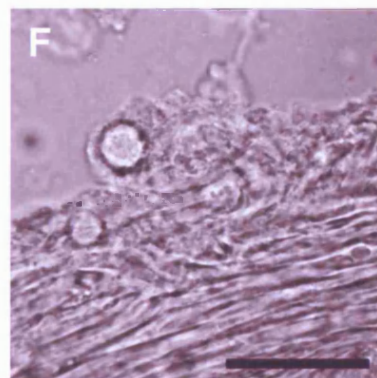
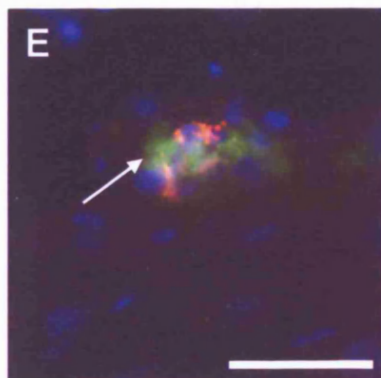
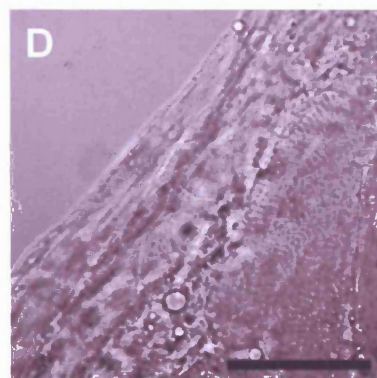
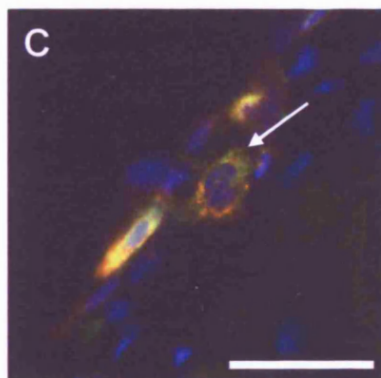
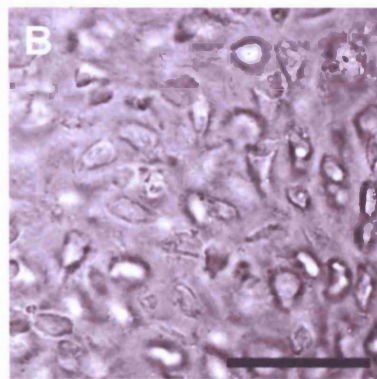
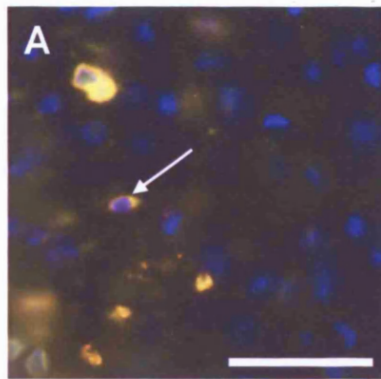
Sections were then labelled for the transcription factor Sox9 and collagen type II. Full-depth chondrocytes were found to express high levels of Sox9, with intense labelling seen within the pellet (Figure 3.2A). This pellet also labelled positively for type II collagen (Figure 3.3A). The human full-depth chondrocytes were also positive for both Sox9 and type II collagen (Figure 3.2D). After injection with bovine clonal and enriched populations, no type II collagen could be detected by immunocytochemistry in any of the sections analysed (Figure 3.3B and C). Despite this absence, Sox9 was detected in some of the PKH26 positive cells, whilst other cells appeared negative (Figure 3.2C and E).

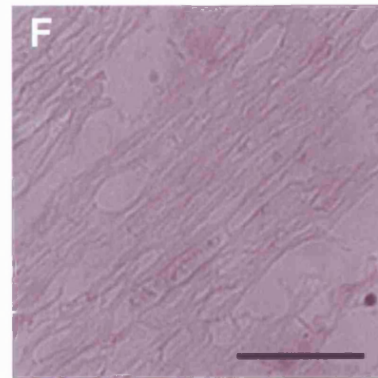
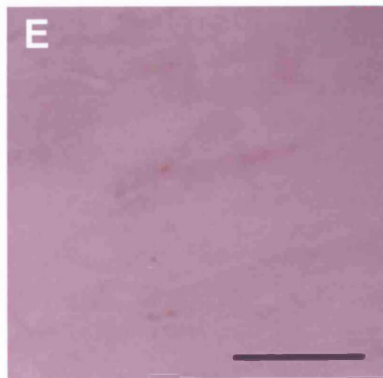
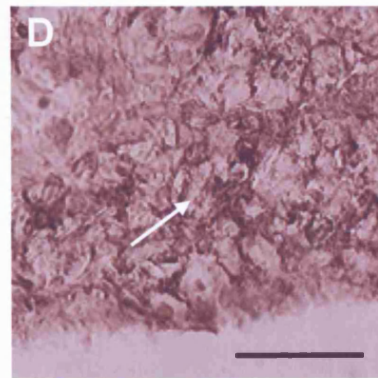
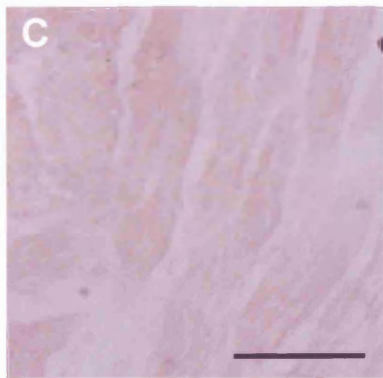
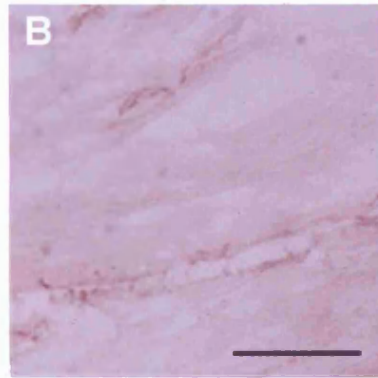
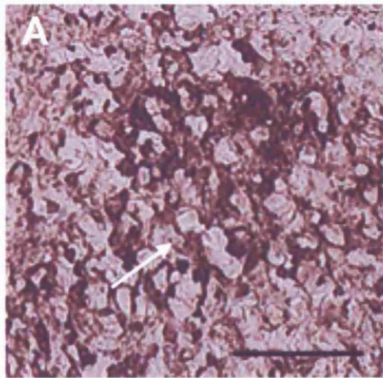
Both *Sox9* and type II collagen mRNA was present in all samples analysed by PCR (Figure 3.4). In the case of type II collagen, only the mature form of type II collagen, type IIB was found in the full-depth samples, whilst type IIB and the immature form type IIA were present in some of the enriched and clonal populations. To ensure that this collagen was from the progenitor cells and not the mouse tissue, primers were designed specifically for bovine type II collagen. Bovine collagen was found in all the treated samples but was not present in a mouse control sample.

*In vitro*, and when the cells were implanted into an embryonic *in vivo* model (chick), the progenitor cells showed plasticity (Dowthwaite et al., 2004). To ascertain if the cells were capable of differentiating into muscle or blood vessels, bovine specific primers were designed. To determine if the cells were forming muscle, primers to the muscle marker *MyoD* were used. There was no *MyoD* mRNA detected in either the full-depth, clonal or enriched populations (Figure 3.4). It was, however, detected in bovine muscle controls, but was absent in mouse tissue. The marker used to assess for differentiation into endothelial cells was platelet-endothelial cell adhesion molecule (PECAM-1; CD31). There was also no *PECAM-1* detected in the full-depth and enriched populations, but it was present in one of the clonal populations of cells examined (Figure 3.4). As with *MyoD*, *PECAM-1* was present in bovine muscle samples but not detected in mouse controls.











### 3.4 DISCUSSION

The current experiments show for the first time that bovine articular cartilage progenitor cells are able to survive within the muscle mass after injection into a SCID mouse. The presence of the fluorescently labelled cells, but no cartilage matrix elaboration, after 2 weeks *in vivo* suggests that the progenitor cells are unable to fully differentiate within this inappropriate environment and may require further signals to undergo complete differentiation. In contrast, mature chondrocytes were able to elaborate a cartilaginous matrix within the muscle over the same time span.

Human chondrocytes have been shown to have a limited growth potential *in vitro*, with growth arrest and senescence reached after approximately 30-35 population doublings (Martin et al., 2002). The bovine cells have been shown to undergo over fifty population doublings and maintain their chondrogenic potential throughout monolayer expansion (Khan et al., *submitted*). However, they senesce at around this point which is telomere dependent hence we consider them a progenitor population similar to MSCs (Tropel et al., 2004) where over 60 PDs have been recorded.

The *in vitro* plasticity of these cells has already been demonstrated (Bishop, 2004), but this is the first study assessing their *in vivo* differentiation potential within a mammalian model. To determine the ability of these cells to form cartilage-like tissue *in vivo*, immunohistochemical analysis was carried out for type II collagen and the chondrogenic transcription factor Sox9. Sox9 is a key regulator of chondrogenic differentiation that is expressed constitutively in differentiated chondrocytes, where it has been shown to have a role in the initiation of chondrogenesis (Bi et al., 1999). Type II collagen is a key component of the matrix of articular cartilage and Sox9 is known to bind to the collagen type II promoter (Bell et al., 1997). Samples were also stained for the presence of another key matrix component, glycosaminoglycans, via Safranin O staining. The freshly isolated full-depth chondrocytes appeared to be able to maintain a chondrogenic phenotype when injected intramuscularly as shown by the presence of a defined cell pellet with an extracellular matrix. The cells within the pellet had a slightly rounded morphology similar to mature chondrocytes (Archer and Francis-West, 2003) and showed high expression of the transcription factor Sox9. The pellet also labelled positively for type II collagen and showed intense staining

with Safranin O indicating the presence of sulphated glycosaminoglycans. Interestingly, PCR analysis only detected the mature type IIB (Ryan and Sandell, 1990) transcripts within the full-depth pellets, even though the cell had been isolated from their original matrix. In contrast, both mature and immature transcripts (type IIA) (Ryan and Sandell, 1990) were detected in some clonal and all enriched populations in the absence of a palpable extracellular matrix. Similarly, although the human unlabelled chondrocytes were unable to create a defined large pellet *in vivo*, condensed areas of the cells were still visible. These regions showed high levels of sulphated glycosaminoglycans and labelled positively with Sox9 after immunohistochemistry. The presence of mRNA for both immature and mature type II collagen was confirmed with PCR. Whether these cells are incapable of forming a complete matrix, or if any matrix that is elaborated is rapidly turned over is not known.

In relation to the above, it has already been shown that Sox9 expression alone is not enough to generate stable cartilage formation and expression of this transcription factor does not always result in cartilage matrix components such as type II collagen. Sox9 interacts with two other Sox transcription factors, L-Sox5 and Sox6 and all three factors working together results in a high expression of collagen type II (Lefebvre and Crombrughe, 1998; Lefebvre et al., 2001). Sox9 is required for the initiation of cartilage formation, whilst Sox5 and 6 are required for the maintenance of the phenotype (Lefebvre et al., 1998; Stokes et al., 2001; Ikeda et al., 2004).

The chondroprogenitor cell's morphology was also not consistent with that normally observed in a chondrogenic cell pellet being much more elongated which is consistent with a fibrocartilage phenotype and probably reflects the fact that the cells were not freshly isolated. Despite the absence of a cell pellet, there was positive Safranin O staining seen in all of the samples analysed. This staining appeared to correspond to areas of the section with high numbers of PKH26 labelled cells and these regions also labelled positively for Sox9. The presence of Sox9 and sulphated glycosaminoglycans alone is not enough to state that the cells are producing a cartilaginous matrix. The absence of collagen type II in both enriched and clonal samples suggests that the cells aren't completely capable of maintaining a chondrogenic phenotype. However during

normal chondrogenic differentiation *in vivo*, it is known that aggrecan is expressed before type II collagen (Fukada et al., 1999).

Previous work by Dell'Accio (2003) showed that when human articular chondrocytes were expanded up to 6 population doublings *in vitro* and similarly implanted into mice muscle, cartilage was generated (Dell'Accio et al., 2003). However, if the chondrocytes had been expanded extensively (de-differentiated) then some cells were reprogrammed to express human muscle markers. Interestingly, if early passage cells were implanted into regenerating muscle, then these too expressed muscle markers demonstrating the importance of the type of milieu the cells experience that can override an existing phenotype, particularly when there may be high levels of growth factors coupled with extensive cell signalling. It was also found that after prolonged expansion in culture collagen type IIa could not be detected by PCR. It is possible that expansion of a pure population of clonal cells may aid in the preservation of chondrogenic potential.

Skeletal muscle differentiation is associated with a group of transcription factors including MyoD, the major transcription factor (Yanagisawa et al., 2007). This transcription factor was found in none of the clonal cell lines analysed, indicating that none of the populations of cells are capable of undergoing myogenic differentiation under the conditions experienced. It was also possible that the cells were engrafting into blood vessels within the muscle. Consequently, we used the expression of PECAM-1 as a marker of endothelial differentiation (Bogdanov et al., 2007). Interestingly, PECAM-1 was detected in a single clonal population of progenitor cells suggesting that this cell line is capable of forming endothelial cells (figure 3.4), and this is the first time the progenitor cells have been found to differentiate along a non-mesenchymal lineage. We do not know why only one clonal cell line should switch lineage. However, we do know that the chondrogenic capacity of these clonal cell lines is not equivalent with some lines showing strong capacity whilst others weak capacity, the latter perhaps indicating a more immature status with greater plasticity.

The progenitor cells inability to produce a cartilage-like tissue shows that the cells do not spontaneously produce cartilage *in vivo* in a mature system. When injected into a developing chick embryo it has been shown that reprogramming of the cell is possible

(Dowthwaite et al., 2004) presumably due to extensive cell signalling and abundance of growth factors occurring during development. It is possible that these environmental cues are required to encourage the progenitors to differentiate along specific lineages. This has also been identified in MSCs, where differing environmental and growth factors have been shown to have an affect on differentiation both *in vitro* (Fukumoto et al., 2003; Bosnakovski et al., 2005) and *in vivo* (Cassiede et al., 1996; De Bari et al., 2006). It is possible that the cells either do not have or are unable to express all of the factors required for true cartilage formation. Our data show that all of the bovine cell lines and human chondrocytes survive *in vivo* and maintain chondrogenic potential, expressing mRNA for several cartilage related factors. This chondrogenic ability would suggest that chondrogenesis is the default differentiation pathway of the progenitors when injected intramuscularly but fail to elaborate a functional matrix. It is predicted that the injection of these cells into a more favourable environment would lead to true chondrogenesis.

The rapid growth rate of these cells and their ability to undergo many population doublings makes them an ideal cell source to investigate for tissue engineering. They can be easily grown in culture, maintain chondrogenic potential and have been shown to retain the ability to differentiate along multiple cell lineages even after extensive growth in culture. Current efforts are directed at identifying similar cell types in human articular cartilage. With view for treating larger defects that currently cannot be treated by autologous chondrocytes implantation due to the constraint of generating sufficient cell numbers that possess chondrogenic potential.



## **CHAPTER 4**

# **Connexin Expression and Its Role in Cartilage Progenitor Cell Differentiation**

## 4.1 INTRODUCTION

Cell interactions are known to enable the coordination of many different cellular processes including embryogenesis, tissue remodelling and repair. These associations between neighbouring cells are mediated through assorted adhesion molecules and gap junction communication (Caspar et al., 1977). Communication through gap junctions occurs via the formation of channels on adjacent cells allowing for the direct transfer of intercellular signals and small molecules under about 1 kiloDalton in size (Naus et al., 1997; Martinez et al., 2002; Stains and Civitelli, 2005b).

Gap junction channels are formed by the joining of two connexon hemichannels on adjacent cells (Caspar et al., 1977). Each hemichannel is composed of protein hexamers encoded by a family of proteins, the connexins, of which there are over twenty known members in the human genome and seventeen in the mouse (Caspar et al., 1977; Martinez et al., 2002; Willecke et al., 2002).

Connexin monomers consist of four transmembrane domains that make up the channel and two extracellular loops that are thought to be involved in the docking process (Kumar and Gilula, 1996). Connexin molecules have a rapid turnover due to their relatively short half-life (for review see Berthoud et al., 2004) and this may act as a way of regulating cell communication. The connexin proteins have been designated according to their molecular weight in kiloDaltons and each different connexin molecule has different biophysical properties (Berthoud et al., 2004; Tsuchiya et al., 2004; Stains and Civitelli, 2005a). The connexin expressed will, therefore, influence the particle size able to pass through the channel.

Connexins are ubiquitously expressed in cells and are thought to be a vital component of a multicellular organism (Sosinsky and Nicholson, 2005; Stains and Civitelli, 2005b). There is a distinct cellular distribution of connexin molecules, with many cells expressing more than one connexin protein on their surface. This variety permits the formation of heteromeric channels between different connexin units (figure 4.1) allowing for the transfer of a signal even when cells do not express the same connexin on their surface (Martinez et al., 2002). Of the different connexin proteins known, the most widely expressed is connexin43 (Cx43) (Lampe and Lau, 2004; Laird, 2005),

and this is also the predominant connexin found in mesenchymal cell types (Schirmacher et al., 1992; Paul, 1995; Schwab et al., 1998; Stains and Civitelli, 2005b). Of the other known connexin proteins, connexin32 has been shown to be expressed in tenocytes (Waggett et al., 2006) and in the developing limb (Makarenkova et al., 1997), whilst connexin45 is expressed by osteoblasts and osteocytes (Stains and Civitelli, 2005a).

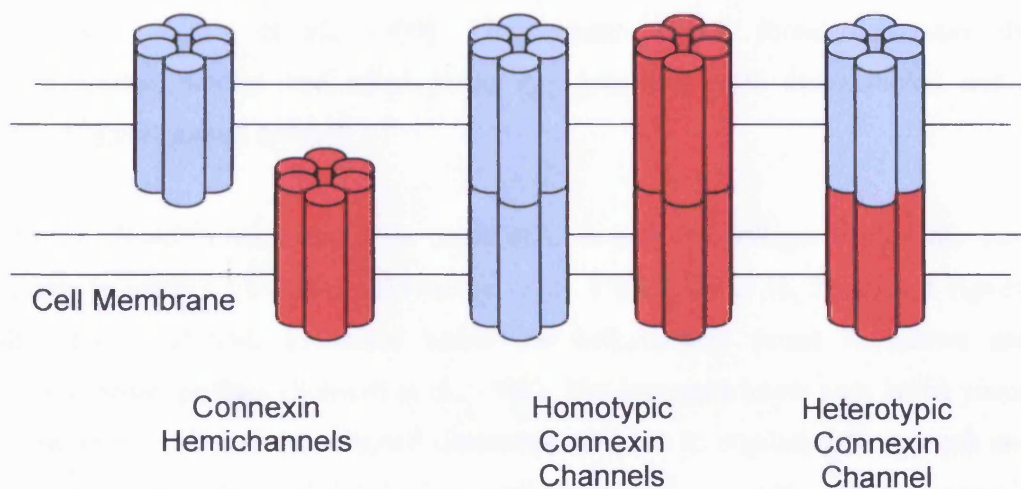


Figure 4.1 – Schematic showing different connexin channel formations. Channels can be both homotypic (same connexons on each cell) or heterotypic (where connexon subunits are different). The formation of hemichannels is also possible (adapted from Kumar and Gilula, 1996).

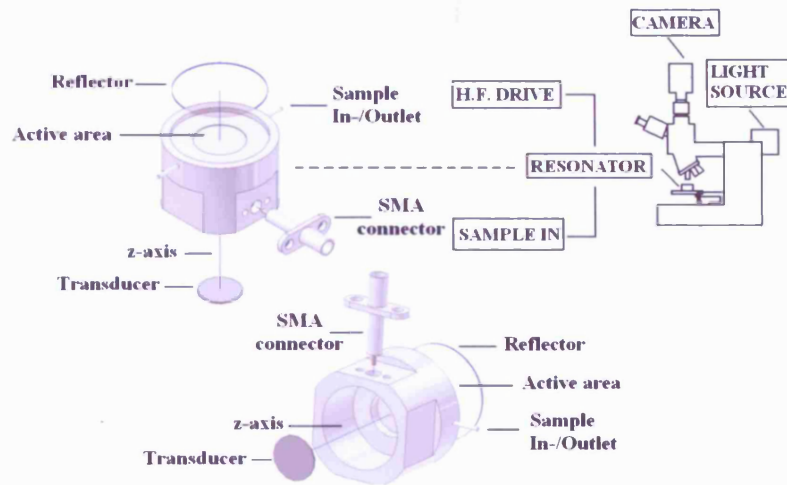
During limb development, one of the earliest morphological events is the formation of a dense mesenchymal cell condensation that will give rise to skeletal elements (Hall and Miyake, 2000). This condensation is associated with an increase in cell-cell contact and an up-regulation of connexins (especially Cx43) on the cells surface (Zimmermann, 1984). It is thought that the formation of gap junctions during condensation enables the passing of signals and morphogens between cells coordinating the further development of the tissue (Zimmermann, 1984; Allen et al., 1990). Adding a tumour promoting phorbol ester to limb mesenchymal cells reduces the formation of gap junctions and prevents chondrogenesis (Ferrari et al., 1994). At this stage in development, cells have been found to express Cx32 as well as Cx43 (Makarenkova et al., 1997).

Patterning of the limb is controlled from a polarising region of mesenchyme located at the posterior aspect of the distal limb bud. Allen *et al* (1990) found that blocking gap junctions formed between this polarising region and the adjacent cells resulted in a decrease in digit duplication. The polarising region has also been shown to express higher levels of connexin when compared with other regions of the developing limb bud (Makarenkova *et al.*, 1997). This gradient is thought to be maintained by the apical ectodermal ridge (AER) and removal of this region results in decreased Cx43 expression (Green *et al.*, 1994). Zimmermann (1983) found that after the condensation process had taken place, gap junctions were disassembled and a chondrogenic matrix formed.

Despite chondrocytes existing as single cells in mature cartilage *in vivo*, the cells appear to retain Cx43 mRNA (Donahue *et al.*, 1995; Loty *et al.*, 2000) and express the protein in both immature tissue and cell clusters found in mature and osteoarthritic cartilage (Schwab *et al.*, 1998). The increased levels seen in the young tissue may be due to the role cell communication has in regulating the growth and differentiation of the still developing cartilage. It is also possible that the connexin molecules are functioning as hemichannels allowing for the release of small signalling molecules such as calcium (Evans *et al.*, 2006).

The channels may have a role in mechanotransduction, eliciting a response within the cartilage to mechanical stress. Connexin hemichannels have already been found to have a role in sensing mechanical strain placed on the tissue, possibly via ATP-release (Romanello *et al.*, 2001; Bao *et al.*, 2004). This strain causes the channel to open (Bao *et al.*, 2004) allowing ATP secretion, and due to the size of ATP molecules they are able to pass through the channel resulting in the initiation of a calcium wave (Cotrina *et al.*, 1998). In relation to chondrocytes, Cx43 channels have been found to exist in hemichannel form (Li *et al.*, 1996), although they failed to form hemichannels under hypoosmotic stress (Bao *et al.*, 2004). Chondrocytes have been shown to maintain the ability to express Cx43 after isolation (Loty *et al.*, 2000) and the cells are also able to reform functional gap junctions upon aggregation *in vitro* (Donahue *et al.*, 1995; Loty *et al.*, 2000; Chi *et al.*, 2004). Whether Cx43 is capable of forming open hemichannels within the chondrocyte membrane is unclear.

A)



B)

*Sound off*      *1sec after "on"*      *after some seconds*

i)	REFLECTOR	ii)	REFLECTOR	iii)	REFLECTOR
Pressure					
node					
	TRANSDUCER	TRANSDUCER	TRANSDUCER	TRANSDUCER	TRANSDUCER

Monitoring single cells in suspension allows for insights into the processes involved in cell aggregation and communication. A new method has recently been developed that enables the direct observation of 2D cell aggregates in suspension using an ultrasound standing-wave trap (USWT) (Spengler and Coakley, 2003). This technique is a non-invasive means for observing cell interactions without the cells being adhered to a solid surface (Bazou et al., 2006). The trap contains an ultrasound resonator that is able to drive the cells to a single node plane (figure 4.2 A). The cells then gather at the centre of this field due to the pressure caused by the wave field (figure 4.2 B). Increasing the field causes an increase in pressure resulting in the cells moving at an increased rate (Coakley et al., 2004; Bazou et al., 2005a). When placed in the USWT, surface zone chondrocytes have been shown to maintain their connexin 43 expression and form functional gap junctions within one hour (Bazou et al., 2006). It is possible that communication between cells in the superficial zone of articular cartilage may be required for maintaining the surface zone boundary of cartilage, as Notch-1 is also expressed in this region and Notch-1 is known to be involved in tissue boundary specification (Hayes et al., 2003).

Understanding the interactions that occur between cells may aid in the understanding of cartilage repair and ways to improve the tissues capacity for repair. In the present study, we investigated the expression pattern of Cx43 in populations of bovine articular cartilage progenitor cells and the effect cell aggregation had on this expression and the cells ability to functionally communicate with each other and with terminally differentiated chondrocytes.



## 4.2 MATERIALS AND METHODS

### 4.2.1 RNA Extraction and cDNA Synthesis

Aliquots of  $0.5 \times 10^6$  progenitor and enriched cell populations were collected at approximately 20, 25 and 30 PDs and stored at  $-80^\circ\text{C}$  in TRizol reagent (Invitrogen). Cells were also isolated from cartilage explants as previously described (see 2.2.6). RNA was extracted using the manufacturers recommended conditions and RT carried out using the SuperScript III reverse transcriptase kit (Invitrogen). The cDNA was transcribed from  $10.5\mu\text{l}$  of RNA with  $0.25\mu\text{g}$  random primers and  $0.8\mu\text{M}$  of each dNTP (both Promega).

### 4.2.2 Polymerase Chain Reaction (PCR)

Primers for PCR were designed using the Primer 3 software and synthesised by MWG (Germany). Each  $12.5\mu\text{l}$  PCR reaction contained  $1\mu\text{l}$  cDNA,  $200\mu\text{M}$  of each dNTP,  $0.24\mu\text{M}$  of each primer ( $0.2\mu\text{M}$  of  $\beta$ -actin primers),  $10\mu\text{l}$  5x buffer (GoTaq Flexi DNA polymerase kit; Promega)  $1.25\text{--}1.5\mu\text{M}$   $\text{MgCl}_2$  and 4 units GoTaq DNA polymerase (Promega). Amplification process consisted of 35 cycles (see table 4.1 for annealing temperatures and 3.2.6 for method). No PCR product was detected when either RNA was replaced with water at the RT reaction, or the cDNA was replaced with water at the PCR stage.

	Forward Primer	Reverse Primer	Annealing Temp.	Product Size
$\beta$ -Actin	TGT ATG CCT CTG GTC GTA CCA C	GAG GAC TCG CGT TCA TGA GAC	$58^\circ\text{C}$	596bp
Cx32	CCA GCA GCA CAT TGA AAA GA	AGG GGT AGG CAT CAC ACT TG	$60^\circ\text{C}$	227bp
Cx43	GGA CAT GCA CTT GAA GCA GA	TGT AAA CGG CAC TCA AGC TG	$58^\circ\text{C}$	188bp
Cx45	TCC TGG TTG GGC AGT ATT TC	GCA AAG GCC TGT AAC ACC AT	$58^\circ\text{C}$	155bp

Table 4.1 Polymerase Chain Reaction Standard Primers. All primers are shown 5'-3' with corresponding annealing temperature and product size.

### 4.2.3 Ultrasound Trap

The ultrasound trap has three essential features: a transducer (Ferropem, Kvistgard, Denmark) in a housing of radial symmetry, an aqueous phase and a reflector that provided optical access from above. The disc transducer (12 mm diameter) had a nominal resonance of 3 MHz (figure 4.2 A), i.e. its thickness was half of a wavelength ( $\lambda_{\text{trans}}/2$ ). Its back electrode was etched to a 6 mm diameter circle with the objective of producing a single central aggregate in a single half-wavelength chamber (Bazou et al., 2005a). The steel coupling-layer on which the transducer was mounted had a thickness of  $3\lambda_{\text{steel}}/4$ . The trap, driven at 2.2 MHz, had an acoustic pathlength of one half wavelength in water (0.25 mm) at the driving frequency. Finally, the quartz glass reflector had a thickness of 0.5 mm ( $\lambda_{\text{glass}}/4$ ) so that the single pressure node plane in the sample volume was located half way through that volume (Bazou et al., 2005a). The trap was driven with a function generator (Hewlett Packard 33120A, UK). The acoustic pressure amplitude ( $P_0$ ) estimated as described by Khanna et al. (2003), was 0.85 MPa for the first 1 minute of ultrasound initiation and subsequently reduced to 0.09 MPa (the minimal pressure at which aggregates remained levitated in suspension) for the remaining 4 minute of aggregate levitation in the trap. The sample was introduced into the ultrasound trap at room temperature with a 2 ml sterile syringe (Plastipak, Becton Dickinson, UK). Once the cells move towards the pressure node, there is no external force on the cells and any effect seen is a result of cell interactions alone.

### 4.2.4 Optical System

A fast, high-resolution CCD F-view II camera (12-bit depth) (Soft Imaging System, SIS, GmbH) mounted on an Olympus BX41M reflection epi-fluorescence microscope allowed observation in the direction of sound propagation (negative z-axis) (figure 4.2 A). The microscope was pre-focused on the trap's pressure nodal plane. Sonication started shortly after the sample was introduced into the trap. Images were captured by a standard PC equipped with the Cell-P image acquisition and processing software (Soft Imaging System, SIS, GmbH).

### 4.2.5 Immunolabelling of Cultures

Cells were prepared as described previously and were diluted to  $3 \times 10^6$  cells/ml. An aggregate of significant size (i.e. 1 mm diameter and approximately 7500 cells) was



formed within 30 secs of ultrasound exposure. Aggregates remained levitated in the trap for 1 and 30 minutes They were then slowly recovered from the trap using a 2 ml sterile syringe, placed on a 'HistoBond' microscope slide (RA Lamb, UK) and fixed with 90 % ethanol. Slides were then rinsed with saline and serum blocked (goat serum, DAKO, UK) at room temperature at 1:20 dilution in PBS for 20 minutes, followed by an overnight incubation at 4°C with the primary antibody (monoclonal anti-connexin 43, 5 µg/ml, Chemicon, UK). After washing in PBS, samples were incubated with AlexaFluor-594 conjugated anti-mouse IgG (5 µg/ml, Molecular Probes, Inc. Eugene, OR, USA). Finally, samples were rinsed in PBS and mounted with DAPI-Vectasheild (Vector Labs, UK). Immunolabelling of non-sonicated chondrocytes was performed on cells (approximately  $3 \times 10^4$  cells/ml) immediately after their isolation and preparation. A 100 µl cell suspension aliquot was placed on a HistoBond slide, fixed and labelled for Cx43 as described above. Clonal cells were also placed onto coated slides and immunolabelled for Cx43. A cytoplasmic label, CMFDA was used to show the outer membrane of the cells. Slides were imaged using a Leica DM2500 confocal microscope. Vertical cartilage sections were taken and labelled for Cx32 (Chemicon).

#### **4.2.6 Measurements of Integral Intensity of Cx43 staining at the Cell Interface**

The integral intensity of the Cx43 labelling at the cell interface was quantified by placing a rectangle provided by the Cell P software, to contain the interface of each of 50 randomly selected pairs of cells. The integral intensity of an image  $I$  defined for this purpose as the sum of all the intensities within the defined area multiplied by the pixel area.

#### **4.2.7 Gap Junction Function Dye Transfer Experiments**

Isolated surface zone and clonal cell populations were diluted to  $1 \times 10^6$  cells/ml and labelled with the cell tracker probe 5-chloromethylfluorescein diacetate (CMFDA) as per manufacturer's guidelines (20µM; Invitrogen, UK). Gap junctions are permeable to the reaction product of Green CMFDA Cell Tracker Probe (Barhoumi et al., 1993). Populations of cells were then mixed as follows: i) clone:clone, ii) surface zone:surface zone and iii) clone:surface zone all at a ratio of 1:3 labelled to unlabelled cells. A sample was then introduced into the trap and the aggregates formed at the pressure node were retained there for 30-60 minutes. Gap junction functionality was

assessed by continuous video fluorescence microscopy. In separate experiments, to demonstrate that cell to cell dye transfer occurs via gap junctions, the gap junctional blocker 1-octanol (4 $\mu$ M, Sigma-Aldrich) was included in the above cell mixtures. An aggregate was formed in the trap and the microscope (objective magnification  $\times 50$ ) was focussed on a randomly selected field of view containing approximately 100-200 cells (the total number of cells in an aggregate is approximately  $7 \times 10^4$ ). This particular set of cells was monitored over a period of 30-60 minutes. Images were captured and processed using the Cell P software during the 30-60 minutes period, as dye transfer occurs after initial cell contact. Each experiment was repeated at least three times and the number of fluorescent cells after 60 minutes of levitation in the trap was compared to the initial number of fluorescent cells in the same field of view (i.e. at 5 min following activation of the sound field).

#### **4.2.8 TGF Treatment**

Clonal cell populations were then treated in monolayer for 5 days with 20ng/ml transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1; Peprotech, UK) with 1% insulin transferrin selenium (ITS; Gibco) to induce differentiation. The cells were then trypsinised and labelled with CMFDA before being placed in the ultrasound trap as previously described.

#### **4.2.9 Micromass Culture**

Micromass cultures were set up using mixed populations of surface zone and 3 clonal cells. Cells were trypsinised and resuspended at a density of  $3 \times 10^5$  cells/10 $\mu$ l containing i) clonal cells only, ii) 95% clonal and 5% surface zone cells, iii) 90% clonal and 10% surface zone cells, iv) 80% clonal and 20% surface zone cells, or v) 100% surface zone cells. The 10 $\mu$ l droplets were seeded onto 12-well cell culture plates and allowed to form aggregates for 5hrs at 37°C. Wells were then carefully flooded with supplemented DMEM/F12 with 10% FCS. Cultures were fixed after 1, 2, 3 and 7 days in culture with normal buffered formalin solution (Sigma) and stained with 1% alcian blue to assess for the chondrogenic differentiation of the cells. To quantify micromass differentiation, the average intensity of staining in each micromass culture was calculated using Image J software.

## **4.3 RESULTS**

### **4.3.1 PCR analysis**

PCR was carried out to determine the presence of mRNA for the three different connexin molecules: connexin 32, 43 and 45. Cx43 and 45 mRNA was expressed by enriched and clonal cells at each passage point (figure 4.3). Connexin32 mRNA was expressed by progenitor cells initially but this expression decreased in two of the clonal populations with increased time in culture.

### **4.3.2 Immunohistochemistry**

Immunohistochemistry was carried out to determine the presence of Cx32 in cartilage sections (figure 4.4). Cx32 showed a highly organised expression pattern, with high levels seen in both the surface and deep zones of the tissue, and within these regions almost every cell appeared positively labelled. The levels of labelling seen in the mid-zone of the tissue were much lower, with isolated cells expressing Cx32. Immunocytochemistry was also carried out to determine if Cx43 protein was expressed by clonal progenitors. Labelling showed that cells not only expressed the protein, but that they expressed it at regions of cell contact around the periphery of the cell (figure 4.5). Single cell (non-sonicated) populations of clonal, enriched and surface zone cells expressed Cx43 as indicated by fine punctuate labelling on the cell surface (figure 4.6). All cell lines were initially positive for Cx43 as indicated by the fine punctuate staining on the cell surface. After 1 or 30mins in the trap, the levels of expression appeared to increase around the periphery of the cell (figure 4.6). The level of labelling at the interface of adjacent cells was quantified using Cell P imaging software. After 1min in the trap, levels of Cx43 expression was similar in the three cells line (figure 4.7). After 30mins the levels of Cx43 expressed at the interface between SZ cells significantly increased, whilst both clonal and enriched cell populations showed no significant upregulation.

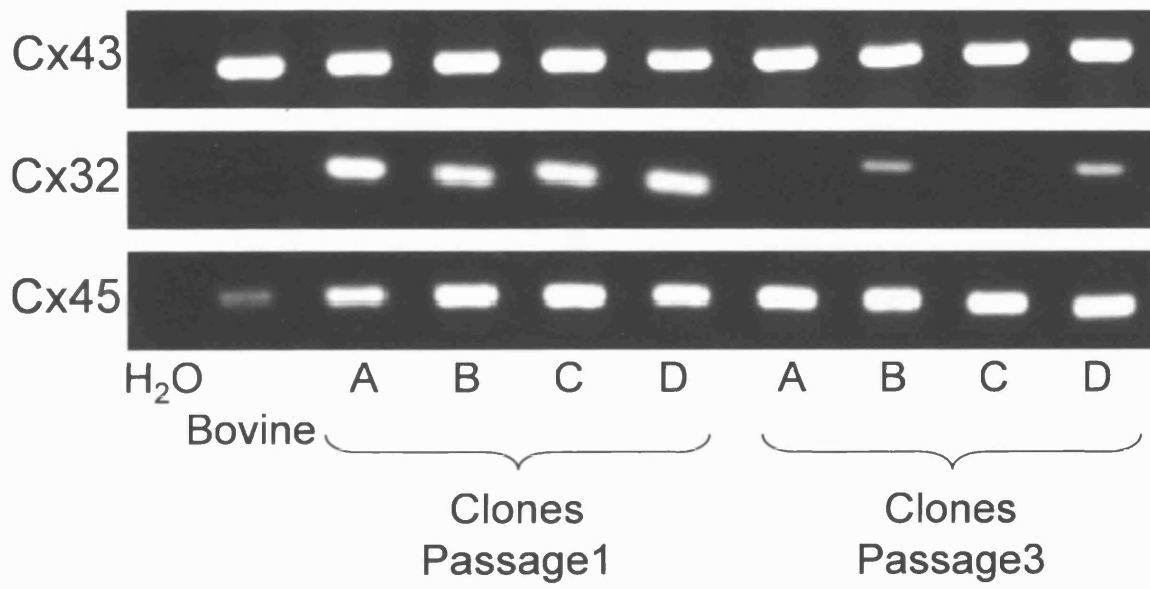
### **4.3.3 Functional Gap Junction Analysis**

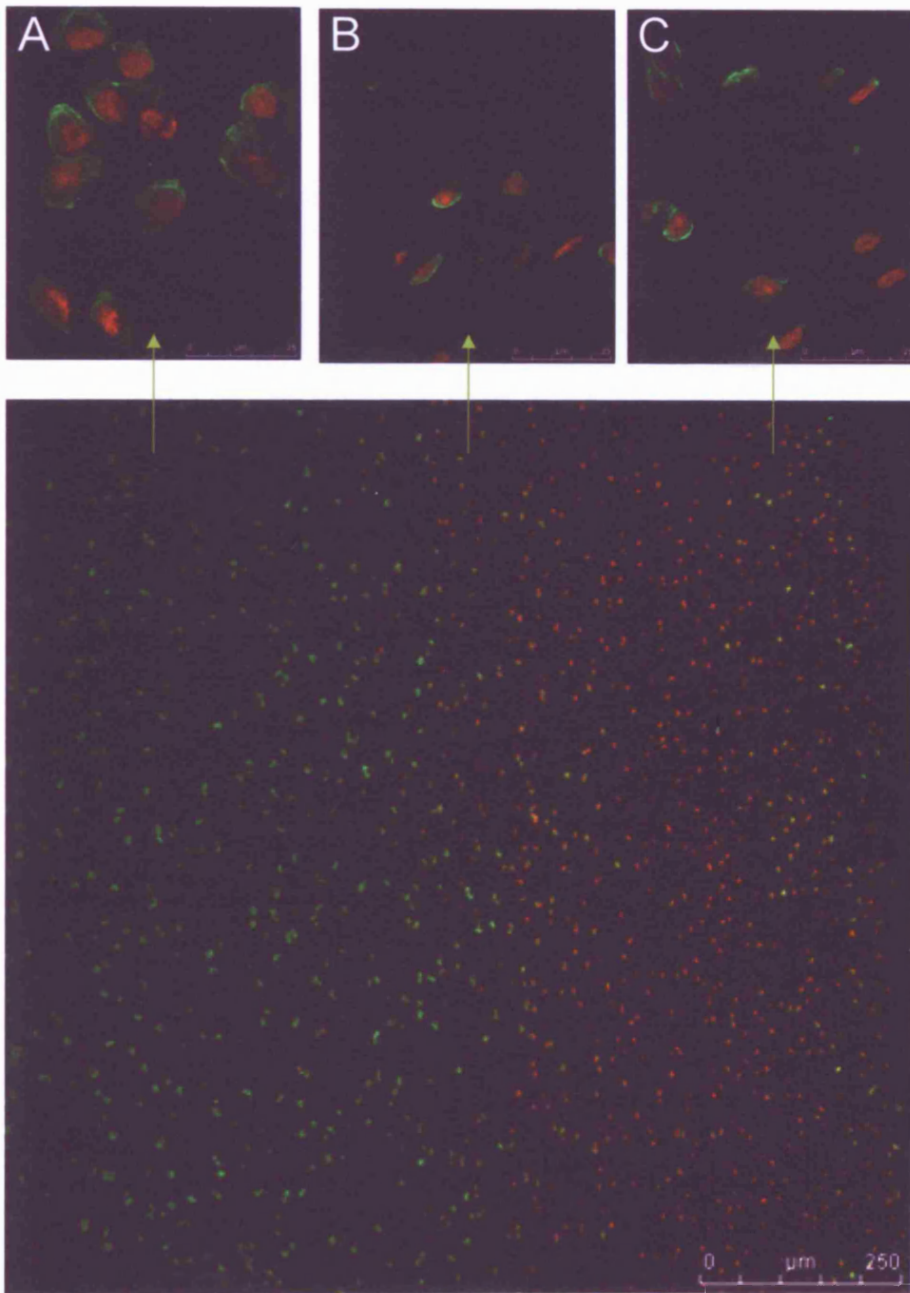
Transfer of CMFDA between cells was used to determine the ability of cells to form functional gap junctions (figure 4.8). In freshly isolated surface zone cells (used as a positive control), dye transfer occurred between labelled and unlabelled cells soon

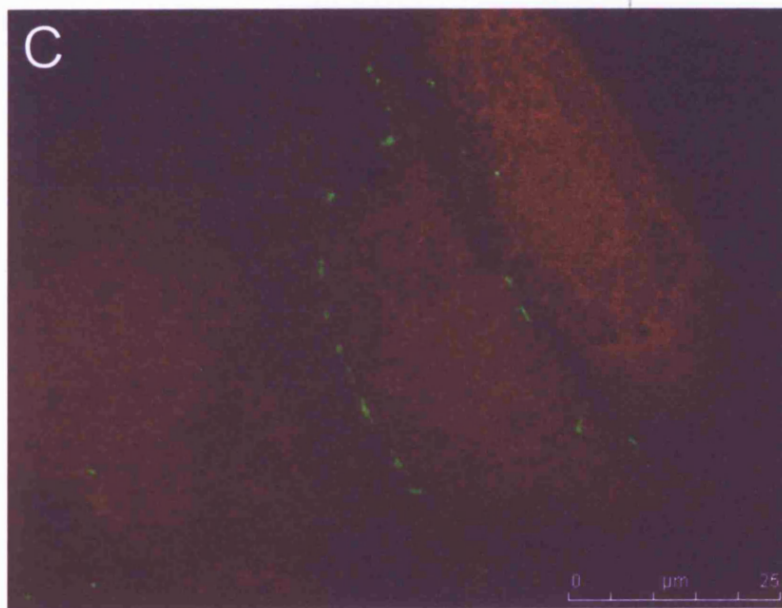
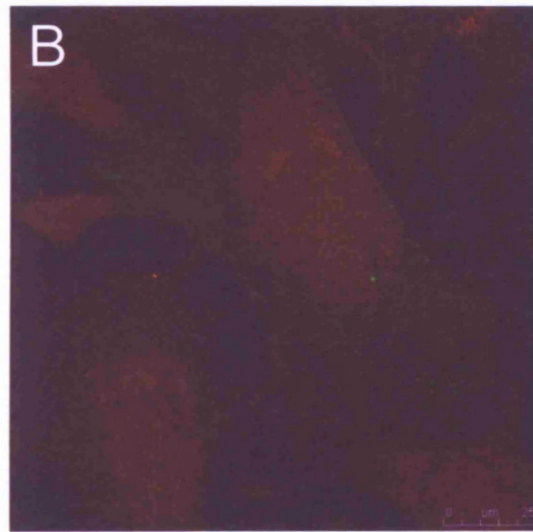
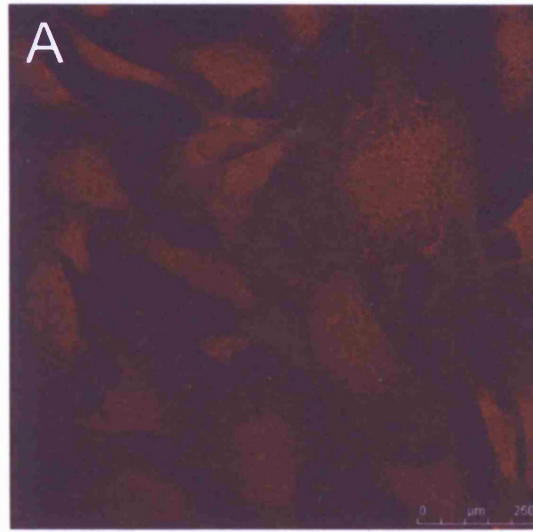
after aggregate formation (i.e. after 5 minutes). Addition of the gap junction blocker 1-octanol either blocked or greatly reduced dye transfer between cells. Clonal populations of cells appeared unable to successfully couple with adjacent cells and no dye transfer was seen in any of the clonal cell lines utilised after 30mins in the trap despite a high level of cell contact. Progenitor cells were then treated with media consisting of TGF- $\beta$  and ITS before being placed in the trap, but no dye transfer was seen in any of the treated cell lines (figure 4.9). Treatment with differentiation media induces dye transfer in the clonal cell populations that can be blocked with the addition of 1-octanol. By mixing labelled surface zone and unlabelled clonal cells it was possible to initiate dye transfer towards the clonal cells (figure 4.10). Transfer was also possible between labelled clones and unlabelled surface zone cells showing the communication was bi-directional (data not shown). In all cases, this transfer could be blocked or greatly reduced by addition of 1-octanol.

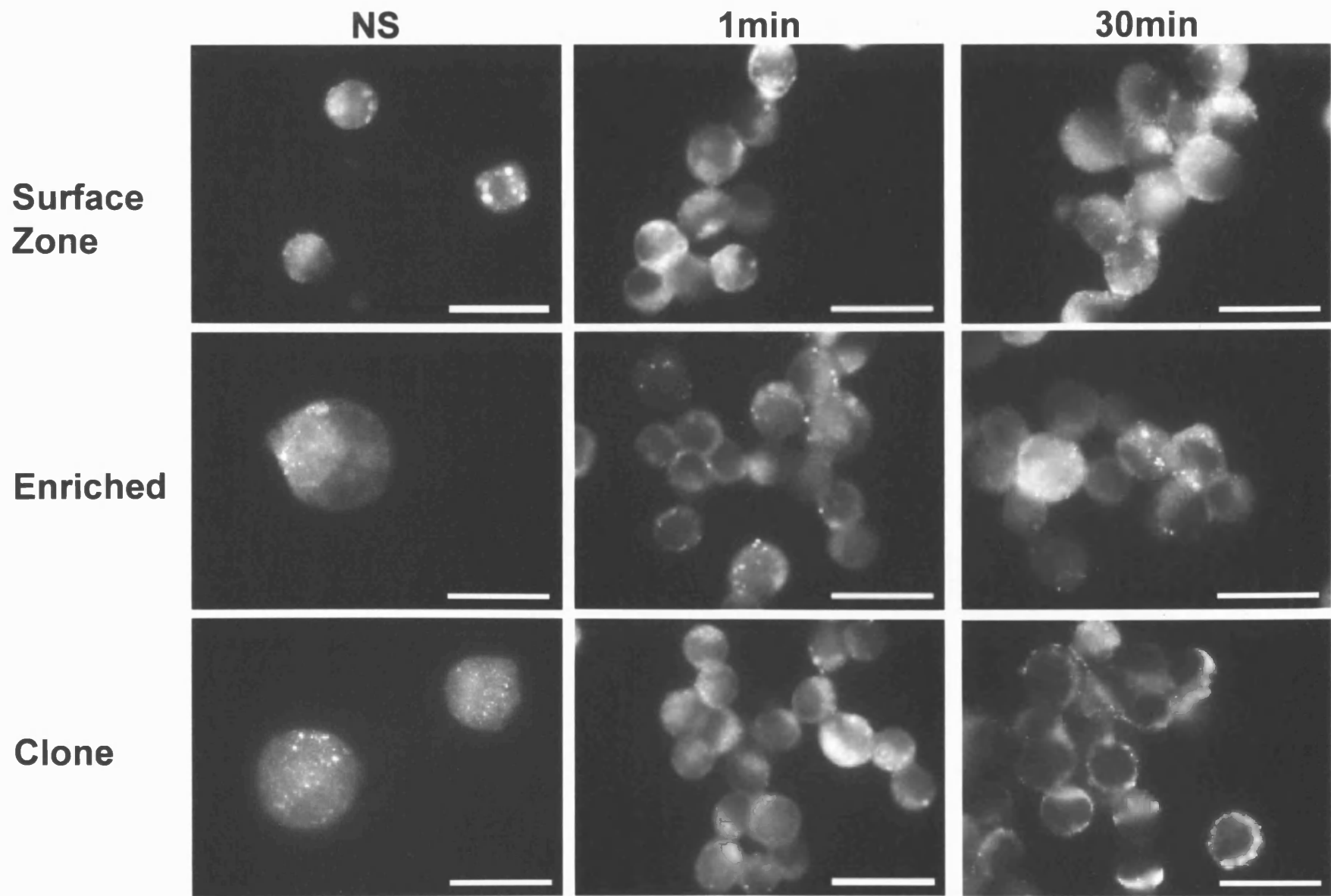
#### **4.3.4 Micromass Cultures**

Micromass cultures were generated by mixing clonal cell populations with a small portion of freshly isolated surface zone chondrocytes. After 1, 2, 3 and 7 days in culture the cultures were fixed and stained with alcian blue. After three days in culture only the 100% surface zone cultures formed chondrogenic nodules that stained with alcian blue (figure 4.11). The staining seen in all of the micromasses containing surface zone cells was more uniform. The intensity of the alcian blue staining was used as a guide of the extent of chondrogenic differentiation. The level of alcian blue staining varied between different clonal cell lines with two clear patterns seen. The first pattern was found in two of the clonal cell lines. When only surface zone cells were used, the levels of staining increased with prolonged time in culture, whilst the levels of staining decreased in cultures containing only clonal progenitor cells (figure 4.12). When either 5% or 10% surface zone cells were used the alcian blue staining of the micromass cultures was enhanced, while increasing the number of surface zone cells to 20% did not alter the chondrogenic differentiation further. The second pattern was observed in the remaining clonal cell line and showed little difference between the levels of alcian blue staining in the 100% clone micromasses and those containing surface zone cells (figure 4.13).









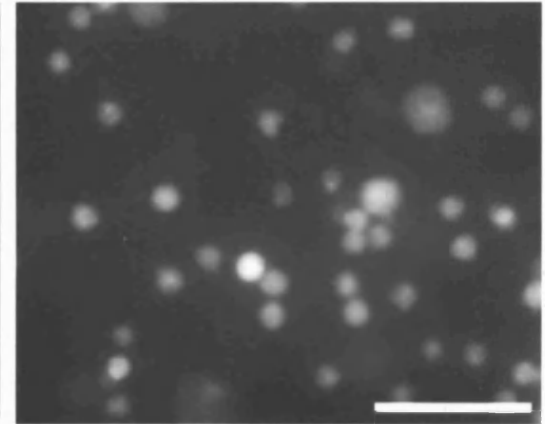
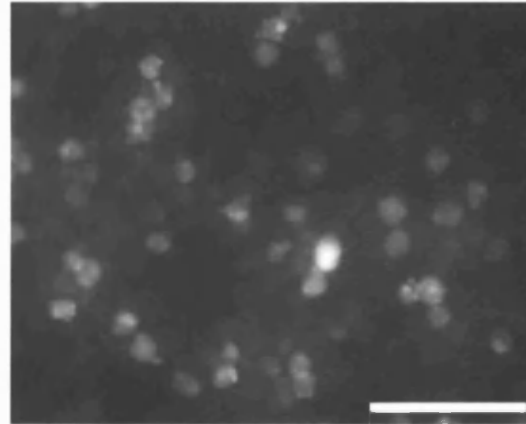
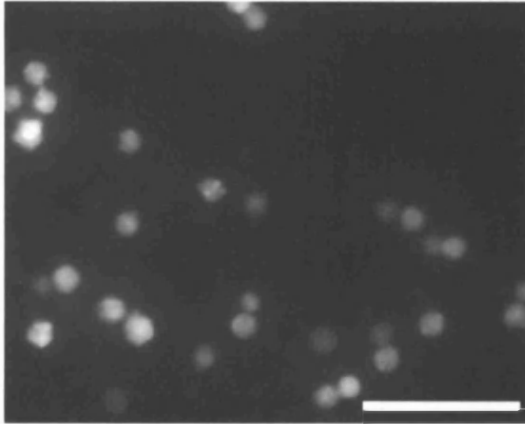


**0min**

**30min**

**30min + Block**

**Surface  
Zone**

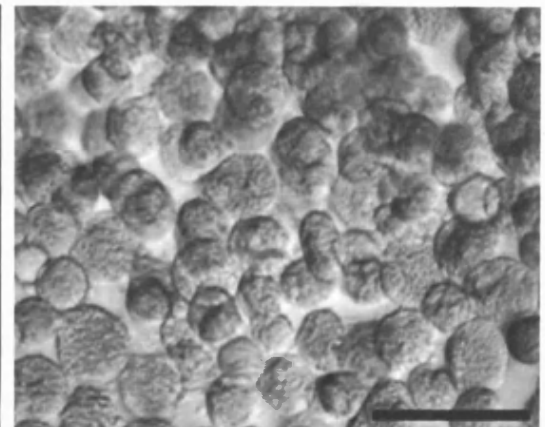
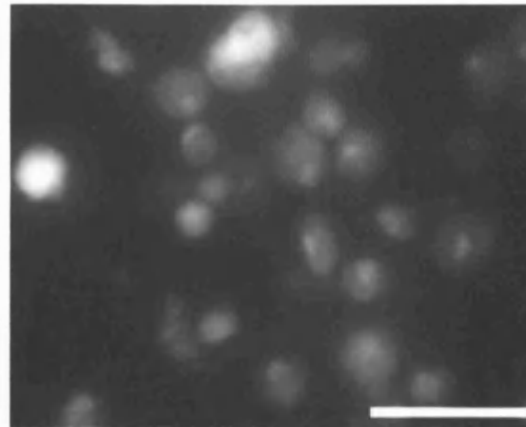
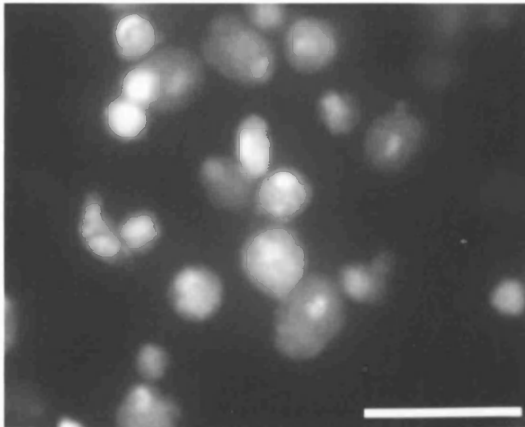


**0min**

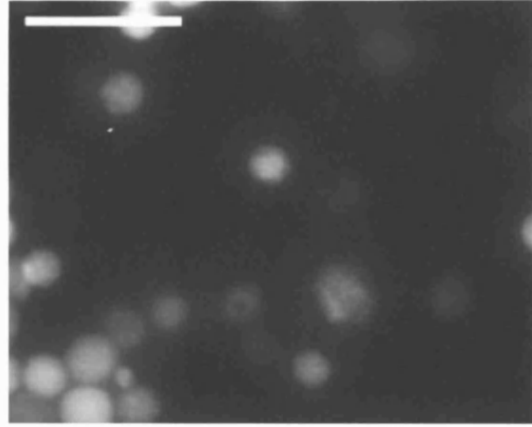
**30min**

**30min**

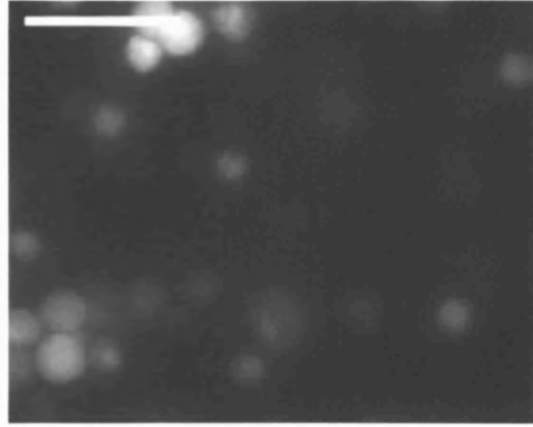
**Clone**



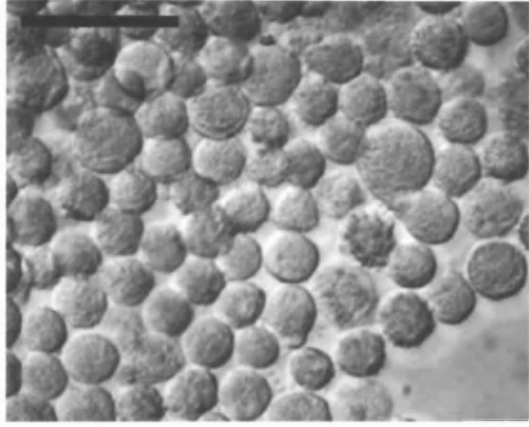
Clone +  
TGF- $\beta$



0min



60min



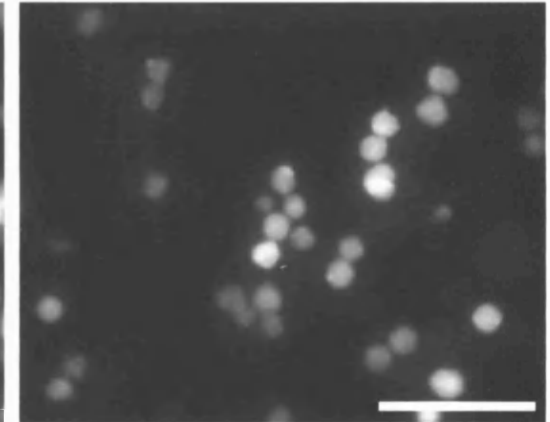
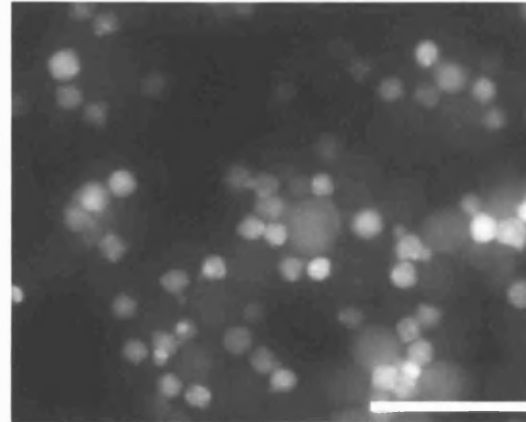
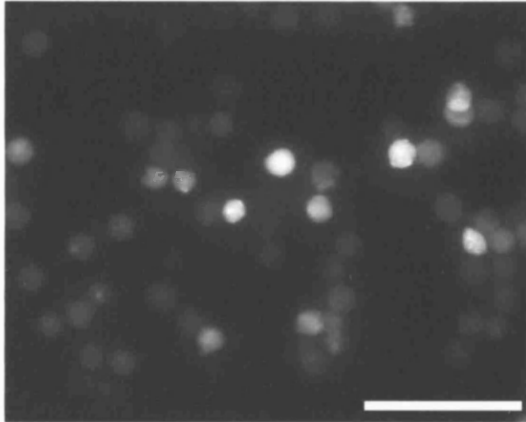
60min

**0min**

**30min**

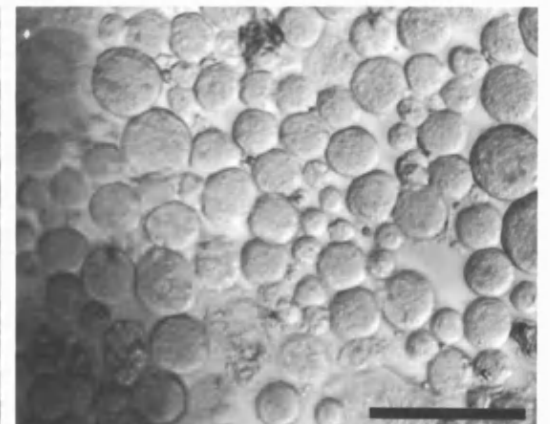
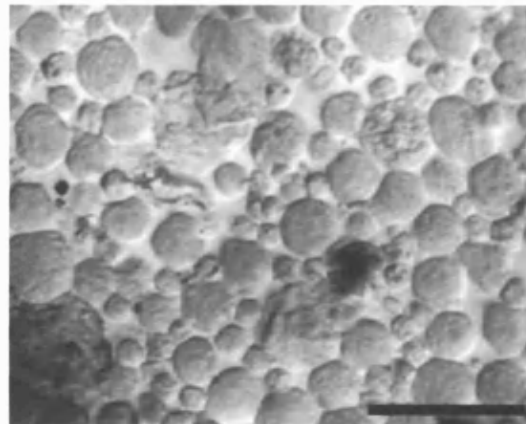
**30min + Block**

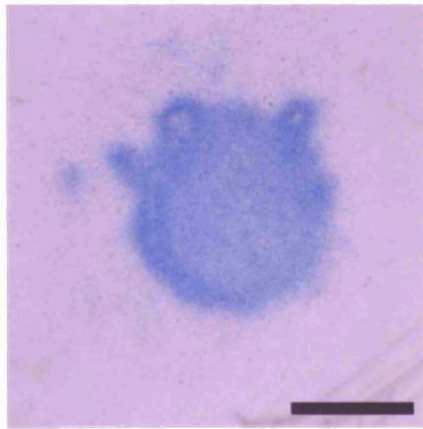
**Surface  
Zone +  
Clone**



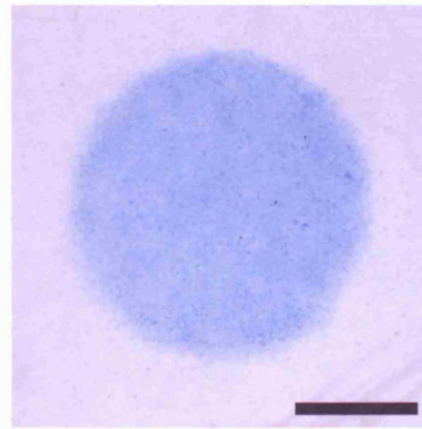
**30min**

**30min + Block**

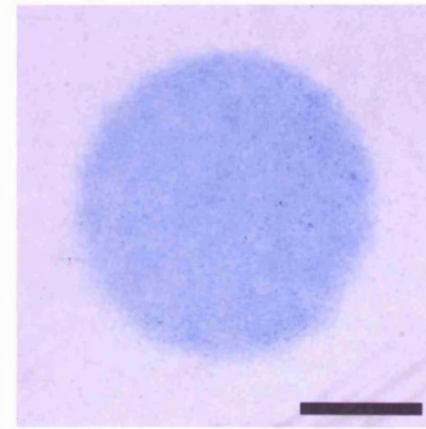




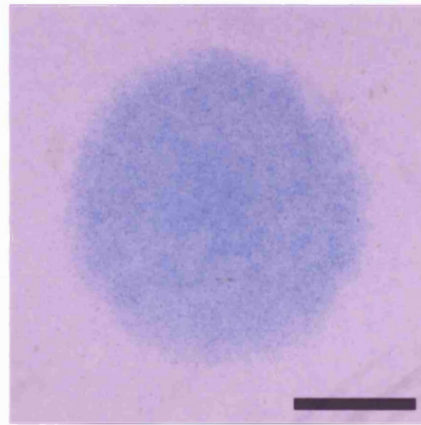
Clone 100%  
SZ 0%



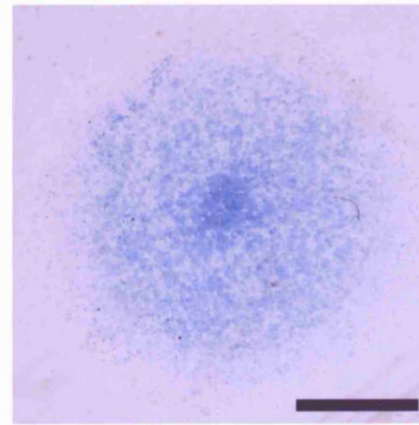
Clone 95%  
SZ 5%



Clone 90%  
SZ 10%



Clone 80%  
SZ 20%



Clone 0%  
SZ 100%

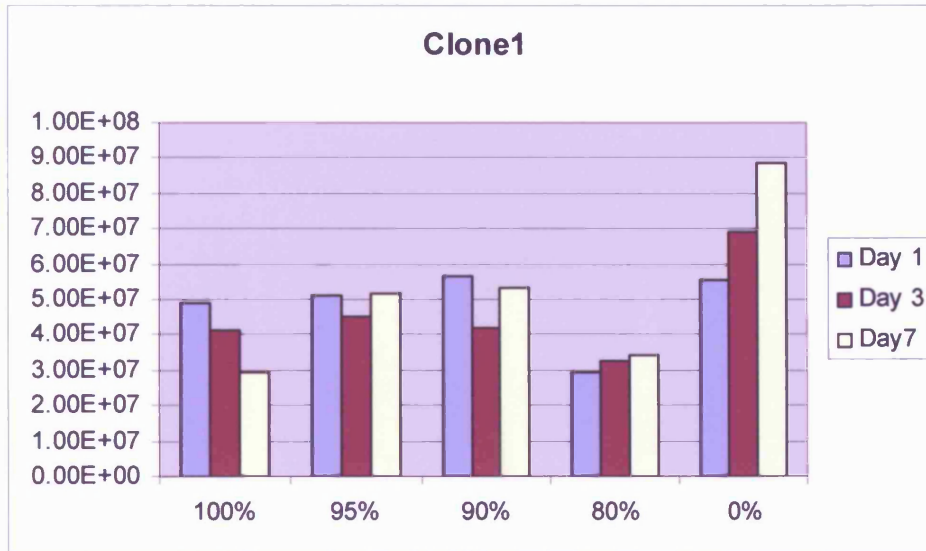


Figure 4.12 Graph showing levels of alcian blue staining in central region of micromass cultures for clonal cell line 1

The intensity of alcian blue staining was calculated on micromass co-cultures set up for 1, 3 and 7 days. The levels of staining in cultures consisting of 100% surface zone cells increased with increased time in culture, whilst the levels for the clonal progenitor cells decreased. Co-culturing clonal and surface zone cells resulted in an increase in the intensity of alcian blue staining observed.

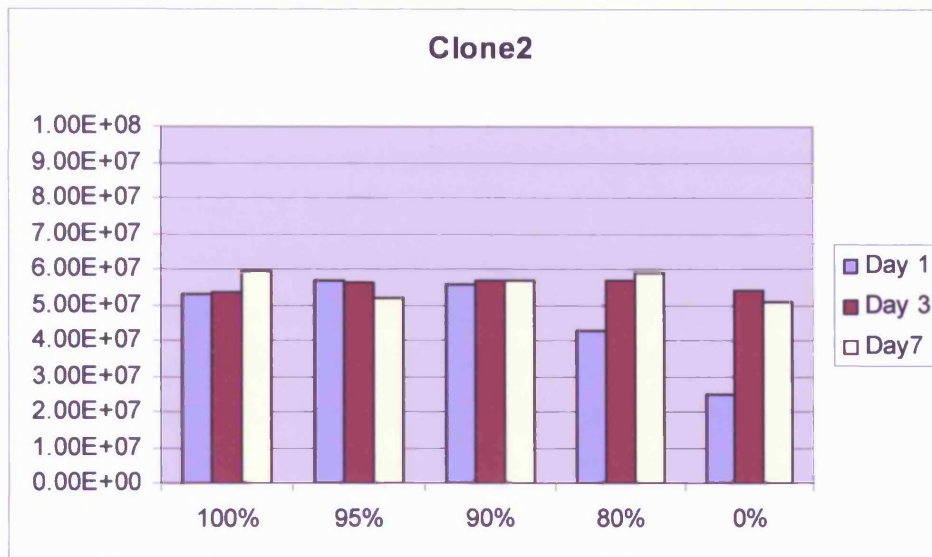


Figure 4.13 Graph showing levels of alcian blue staining in central region of micromass cultures for clonal cell line 2

The intensity of alcian blue staining was calculated on micromass co-cultures set up for 1, 3 and 7 days. There was no difference seen in the intensity of staining between cultures consisting of 100% clonal cell lines and those with surface zone cells added.

## 4.5 DISCUSSION

Connexin expression and gap junction formation are already known to be vital for the development of articular cartilage (Allen et al., 1990). Whilst it has been shown that mature chondrocytes express Cx43, this study shows that populations of progenitor cells isolated from the surface of bovine articular cartilage are also capable of Cx43 expression in culture.

All clones examined expressed the mesenchymal associated connexins, Cx32, 43 and 45 mRNA between 20 and 30 PDs, although Cx32 levels varied with repeated passaging. Whilst Cx32 is found in immature chondrocytes, it is also associated with tenocytes (Ralphs et al., 1998) and expression of this molecule could be required to control differentiation of the cells. Cx45 is one of three main connexin molecules expressed by both osteoblasts and osteocytes (Stains and Civitelli, 2005b) and it is possible that by altering the expression of different connexin molecules, progenitor cells are able to control their differentiation towards certain cell types.

Changes in connexin expression have already been noted during differentiation of certain stem and progenitor cells types. The expression of Cx43 appears to be required for the development of both cardio and skeletal myocytes (Oyamada et al., 1996; Araya et al., 2005). It has been shown that embryonic stem cells express both Cx43 and Cx45, but cardiomyocytic differentiation of the cells results in a gradual increase in Cx40 expression. This switch coincides with the expression of cardiac-specific genes and may cause the cells to become uncoupled (Oyamada et al., 1996). Although skeletal muscle cells do not express Cx40, both Cx43 and Cx45 are present, with Cx43 shown to be required for myogenesis to occur (Araya et al., 2003; Araya et al., 2005). This switching effect has also been observed during the differentiation of hippocampal progenitor cells into neurons, with cells altering their phenotype as a result of cell uncoupling (Rozental et al., 1998).

Despite mature chondrocytes ostensibly existing in an isolated state, connexin expression appears to be maintained throughout the depth of the tissue. In the knee joints of both rats and mice, Cx43 was expressed between chondrocytes in both the surface and deep zones of the tissue (Schwab et al., 1998). Whilst the expression

profile of Cx43 has already been established, there appears to be little data on the expression of other connexin molecules within articular cartilage. Immunolabelling bovine cartilage for the presence of Cx32 resulted in an organised pattern similar to that seen for Cx43 by Schwab et al. (1998,) where expression was seen throughout the depth of the tissue, but with levels highest in the surface and deep zones.

Confocal microscopy confirmed that not only do the progenitor cells express Cx43, but they are capable of expressing it within the plasma membrane around the periphery of the cell. Despite expressing Cx43 at the cell membrane, clonal and enriched progenitor cell populations failed to significantly increase expression of Cx43 at the cell-cell contact interface after time in the trap, suggesting the cells are incapable of up-regulating connexins after cell contact. Expression of connexin mRNA and protein is not enough to assume that the cells are capable of forming functional gap junctions. Despite expressing Cx43, the chondroprogenitors were only capable of forming functional gap junctions with differentiated chondrocytes. It is possible that the mature cells provide signals required to initiate terminal differentiation of the transient amplifying cells. These signals may be soluble factors that are naturally produced by the chondrocytes, which could be taken up through connexin hemichannels on the chondroprogenitors surface, resulting in the cells being able to form functional gap junctions, or be able to correctly join channels with the mature cells.

The formation of gap junctions is one way to direct the differentiation of cells and it has already been shown that co-culture of mature populations of cells with stem cells affects the differentiation of these cells. Growing embryonic stem cells (ESC) with primary bone derived cells resulted in osteogenic differentiation of the stem cells. It is thought that this is due to both the presence of cellular communication and the release of osteogenic inducing factors (Ahn et al., 2006). This effect has also been shown in chondrocytes that have dedifferentiated after prolonged periods in culture. It is possible to enhance the redifferentiation of these cells by co-culture with early passage (Gan and Kandel, 2007). The work by Gan and Kandel (2007) suggests that mature chondrocytes alone may be able to promote chondrogenic differentiation of progenitor cells. It is not clear if the mature cells directly enhance the differentiation of the undifferentiated cells via cell contact, or the release of soluble factors.



It has also been shown that co-culturing MSCs with mature bovine chondrocytes resulted in improved differentiation of the MSCs along a chondrogenic lineage. Decreasing in monolayer the amount of chondrocytes used caused an increase in the ratio of type II to type I collagen demonstrating that the MSCs were able to reduce the dedifferentiation effect usually seen on mature chondrocytes (Tsuchiya et al., 2004). The major difference observed in this study though was the number of cells used. In each case they used ratios of 1:2 or 2:1 suggesting that they still required large numbers of mature cells. It would be interesting to see if the same effect was found using only a small number of chondrocytes.

Without the addition of growth factors or cytokines, clonal chondroprogenitor cells vary in their ability to maintain a chondrogenic phenotype when grown in micromass culture. In some of the clonal cell lines the levels of alcian blue staining decreasing with prolonged time in culture. With the addition of 5% or 10% surface zone cells, the levels of alcian blue staining increased to above the clone alone levels by day seven, suggesting that the surface zone cells are enhancing the chondrogenic differentiation of the cells. In the remaining clonal cell lines there was no detectable difference in the intensity of alcian blue staining observed. In the cell lines with increased chondrogenic ability, co-culturing the cells was found to have no negative effect on the clonal cells ability to undergo chondrogenic differentiation.

This study demonstrates that progenitor cells isolated from the surface of bovine articular cartilage express connexin molecules on their surface. Despite this expression it appears the cells are only capable of forming functional gap junctions with terminally differentiated chondrocytes. It is possible that signals transferred from the mature surface zone chondrocytes to the progenitor cells are required for the initiation of differentiation. Co-culture of the cells with mature cells maybe a way of enhancing the differentiation of progenitor cells whilst still utilising their potential for generating large number of cells with chondrogenic potential.

## **CHAPTER 5**

### **Cell Localisation and Notch Expression in Paired Cells Within the Surface of the Cartilage**

## 5.1 INTRODUCTION

The differences that exist between the structure of developing and mature articular cartilage are well documented. In early development the cells are found as part of a dense cell mesenchyme with a high amount of cell contact and high expression of the gap junction protein connexin43 (Stains and Civitelli, 2005b). In mature tissue, the cells are thought to occur almost in isolation, surrounded instead by a pericellular matrix comprising interwoven collagen fibres. Despite this isolation, mature chondrocytes have still been shown to express connexin43 on their surface and when grown in culture the cells are capable of forming functional junctions (Chi et al., 2004). If chondrocytes do exist in isolation, is there really a need to continue expressing a molecule required for cell communication between adjacent cells?

There is the possibility that the expression of connexin is required in the form of hemichannels allowing the movement of molecules into and out of the cell (Evans et al., 2006). Equally, they may play a role in mechanosensitivity, initiating calcium waves (Bao et al., 2004). Many of the ideas and observations of the structure of articular cartilage have focussed on vertical tissue sections perpendicular to the surface, which illustrates the localisation of cells in the different zones that are known to exist in the cartilage. Studying the organisation of cells in this orientation has led to the belief that the cells within the surface and middle zones are found primarily in isolation, or in columns in the deep zone. Work by Chi *et al.* (2004) demonstrated that by taking sections that were both parallel and perpendicular to the surface of the tissue it is possible to identify paired cells within the surface zone. The number of paired cells differs between different synovial joints and may affect the development of osteoarthritis in these joints (Schumacher et al., 2002).

More recently, a series of papers have been published examining *en face* sections taken throughout the depth of the tissue. In this orientation, the cells appear to be in contact with adjacent cells (Jadin et al., 2005). In the surface zone, the cells are ordered into horizontal pairs that it would be difficult to observe using vertical sections. This study also confirmed columns of cells within the deep zone. It is therefore possible that connexins may have a role in mature cartilage, passing signals between cells that could be responsible for maintaining the tissue and maintaining

differentiation. It is also possible to gain insight into the way in which cartilage is organised by studying the cellularity of the tissue. Using 3-dimensional imaging, Jadin *et al.* (2007) were able to show that the cellularity of bovine articular cartilage decreased with tissue depth. Since there are increased cell numbers towards the surface of the cartilage (within the smallest zone) it is more likely that these cells will be in contact with each other.

Chondrocytes, in conjunction with the cells pericellular matrix make up the chondron. Chondrons can consist of multiple cells, with the chondrocytes surrounded by a type VI collagen meshwork (Poole, 1997). It has also been found that the number of cells within a chondron differs between either different tissues (Schumacher *et al.*, 2002) or different zones within the same tissue (Youn *et al.*, 2006).

Whilst cell contact is associated with early development, there is another period in the life of a joint where there are increased levels of contact between cells. It has already been found that chondrocyte cluster formation occurs in osteoarthritic cartilage (Buckwalter and Mankin, 1998a). These clusters have been found to be positive for the cell signalling molecule Notch-1 and its ligands (Hiraoka *et al.*, 2006). Members of the Notch family of transmembrane proteins are known to be involved in cell communication and regulating cell fate decisions (Gridley, 1997). There are two forms of Notch signalling; canonical and non-canonical. In canonical Notch signalling, interactions between Notch and one of its ligands causing the proteolytic cleavage of Notch via a presenilin/ $\gamma$ -secretase complex resulting in the release of the Notch intracellular domain (NICD) (Tandon and Fraser, 2002; Nelson *et al.*, 2007). The NICD is then translocated to the cell nucleus where it is able to regulate gene expression and transcription of downstream elements (Nelson *et al.*, 2007).

The ligands that bind to Notch belong to the Delta, Serrate, Lag-2 (DSL) family of proteins. In *Drosophila*, the closely related ligands Delta and Serrate show slight structural variation, with a cysteine-rich region present in Serrate that is absent in Delta. It is thought that this region may be required for preventing aggregate formation (Weinmaster, 1997). In vertebrates, there are two Serrate-like ligands (Jagged1 and Jagged2) and two Delta-like ligands (Delta1 and Delta2). In the case of

cartilage, the Notch-2 and Delta signalling pathway has been shown to control the switch from pre-hypertrophic to hypertrophic chondrocytes (Crowe et al., 1999).

If some of the cells within the surface of the tissue are in contact with adjacent cells, it is possible that they may be able to transmit signals required for either maintaining a progenitor phenotype or to begin undergoing differentiation. Whilst Notch-1 is not a marker for the cartilage progenitor cells, it is a requirement for the clonality of the cells and preservation of progenitor status. Determining if the paired cells within the surface are positive for Notch-1 could help to establish if these cells are part of the progenitor cell population.

If connexin expression does have a role in co-ordinating the differentiation of progenitor cells, then the cells within the tissue must exist with some level of cell contact. The aims of this study were to further characterise the organisation of bovine articular cartilage to determine if cell contact could occur between cells in the surface of the tissue, and if these paired cells may be part of the progenitor cell population.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Cartilage Isolation**

Full-depth explants approximately  $0.25\text{cm}^2$  were taken from the metacarpal-phalangeal joint of 7-day bovine joints. Explants were snap-frozen in n-hexane (Sigma; UK) placed in industrial methylated spirit (IMS; Fisher Scientific, UK) and dry ice bath. Samples were stored at  $-20^\circ\text{C}$ . Samples were embedded in OCT embedding medium (RA Lamb, UK) and then cryosectioned from the deep zone through to the surface with  $40\mu\text{m}$  sections cut through the full thickness of the section. A selection of explants were also processed for histology and fixed for 24 hours in 10% neutral buffered formalin solution (NBFS, Sigma) and processed for histology (see Appendix X).

### **5.2.2 Histology**

After processing,  $8\mu\text{m}$  sections were taken through the full-depth of explants. Sections were split according to zone and stained with Safranin O (BPH) and counterstained with Mayer's haematoxylin (RA Lamb; see Appendix V). Stained sections were imaged using a Leitz DMRB light microscope.

### **5.2.3 Immunohistochemistry**

Sections were dissociated from slides with PBS/Tween and placed into separate wells of a 24-well plate and fixed for 10mins with 2% paraformaldehyde. After fixation, sections were treated with 0.25units/ml chondroitinase ABC (Sigma) combined with either 1unit/ml or 2units/ml hyaluronidase (Sigma) for 30 minutes on a shaker. Samples were then blocked with rabbit serum (1:20) and incubated with anti-collagen type II (CIICI, DSHB; USA) at  $4^\circ\text{C}$  overnight (for method see 2.2). Appropriate isotype controls (Dako) were used as a negative control. Sections were then incubated with rabbit anti-mouse Alexa Fluor 488 (Invitrogen) for either 1 hour or 2 hours on a shaker. After washing, each section was removed from the 24-well plate, placed onto a slide and mounted with Vectashield with Propidium Iodide (Vector Labs). Slides were then imaged using a Leica TCS SP2 AOBS confocal scanning microscope. Depth of antibody penetration was determined using Leica LCS Lite software.

After optimal concentrations were obtained sections were immunolabelled for surface zone protein (SZP, Lubricin, PRG4; gift from Dr S. Gilbert) to ensure the surface zone had been sectioned. Immunohistochemistry was then carried out using antibodies to Notch-1 ( $\beta$ tan-20, DSHB), type VI collagen, Delta-1 and Jagged-1 (Santa Cruz, UK).

## 5.3 RESULTS

### 5.3.1 Histology

In order to determine if paired cells were found in the surface zone of bovine cartilage, *en face* sections were taken throughout the depth of the tissue. A section from each of the three zones (surface, middle and deep) was stained with Safranin O and haematoxylin. Paired cells were visible within both the surface and middle zones of the tissue (figure 5.1 D and E). In the deep zone, cells appeared in pairs, but it is also possible that the cells were arranged into columns and this was not detected with *en face* sections (figure 5.1 F).

### 5.3.2 Antibody Penetration

To test the depth of antibody penetration, immunocytochemistry was carried out for type II collagen. Due to the thickness of the sections, different concentrations of hyaluronidase and chondroitinase were used to break down the matrix and ensure that the primary and secondary antibodies were able to diffuse through the section. This procedure was combined with increasing the duration that the samples were exposed to the secondary antibody. Serial optical sections taken using laser confocal microscopy were then used to detect the full-depth of the labelling.

A concentration of 1unit ml<sup>-1</sup> hyaluronidase and 0.25units ml<sup>-1</sup> chondroitinase ABC resulted in the labelling being detected up to 10µm from the surface of the section (figure 5.2A) and increasing the time the secondary antibody was applied resulted in slightly stronger labelling (figure 5.2 B). Increasing the concentration of hyaluronidase to 2units ml<sup>-1</sup> (figure 5.2 C) and using the secondary antibody for 2hrs (figure 5.2D) resulted in the antibody being detected 18µm from either surface. This procedure not only increased the depth of the antibody penetration but also the intensity of the labelling.

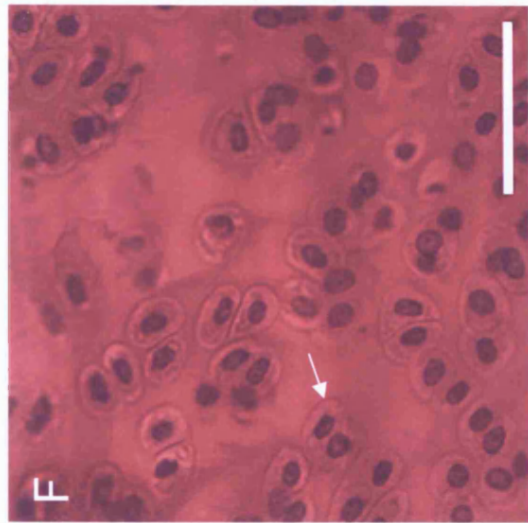
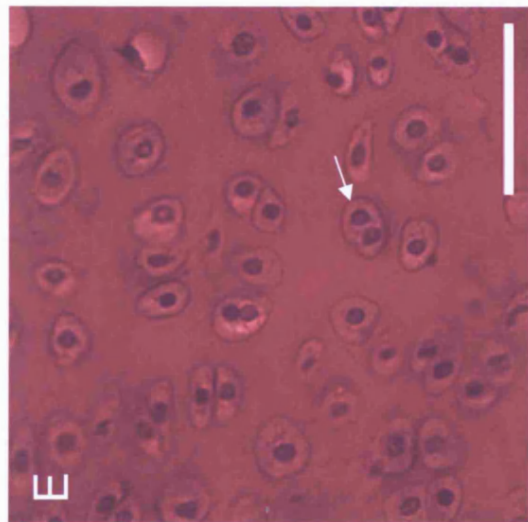
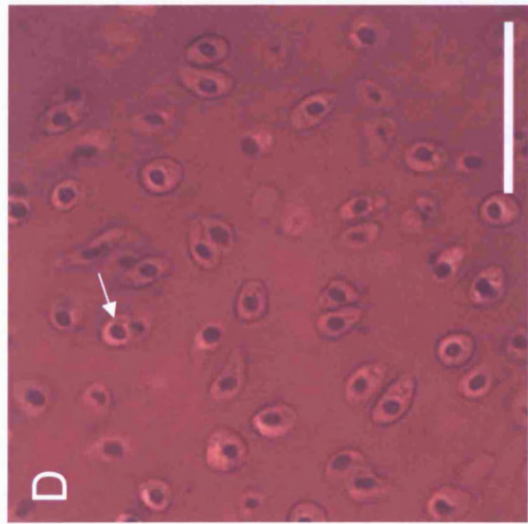
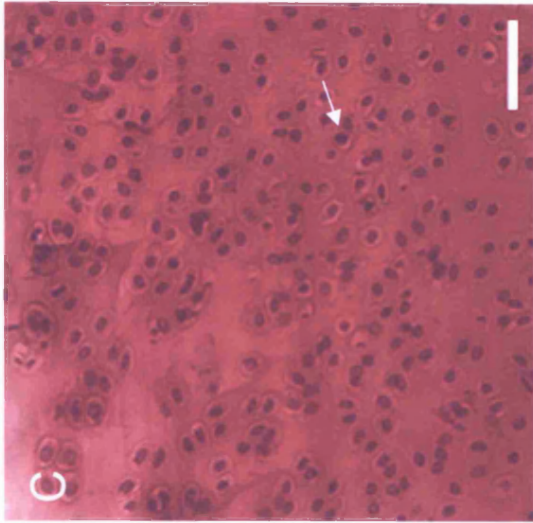
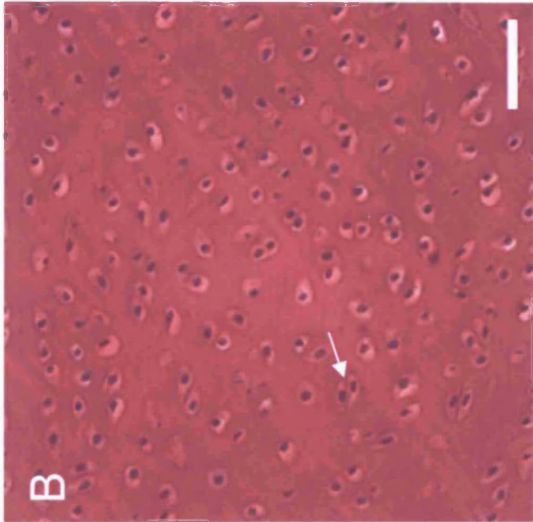
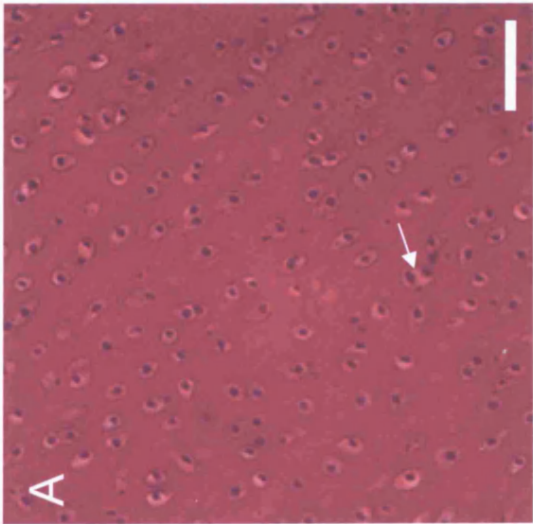
### 5.3.3 Immunohistochemistry

To ensure that the 40µm sections were large enough to contain the entire surface zone, immunohistochemistry was carried out for SZP. Labelling for SZP was only detected in the section representing the surface of the tissue, and was not found in any



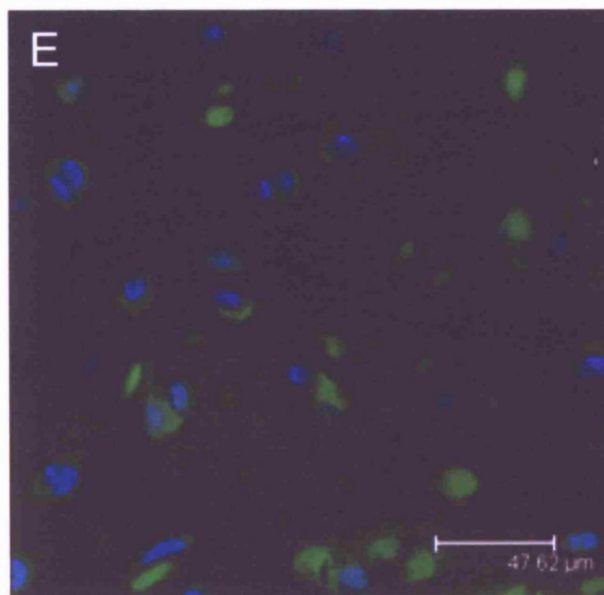
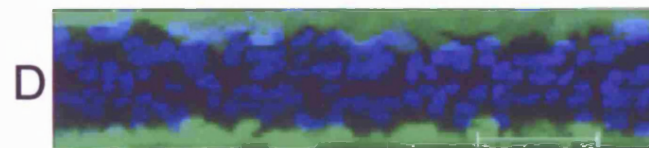
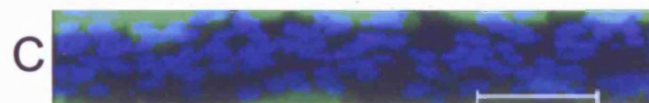
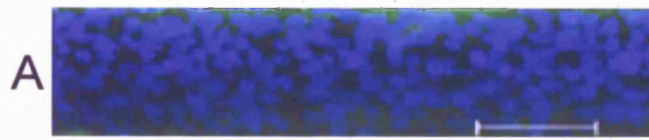
of the other sections (figure 5.3). Cell chondrons were identified via positive labelling with type VI collagen (figure 5.4).

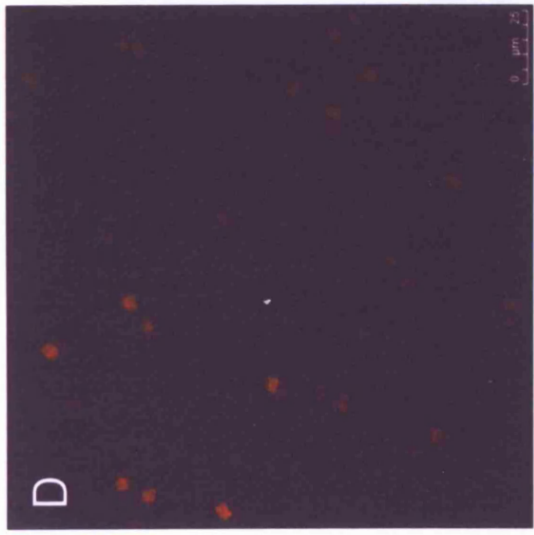
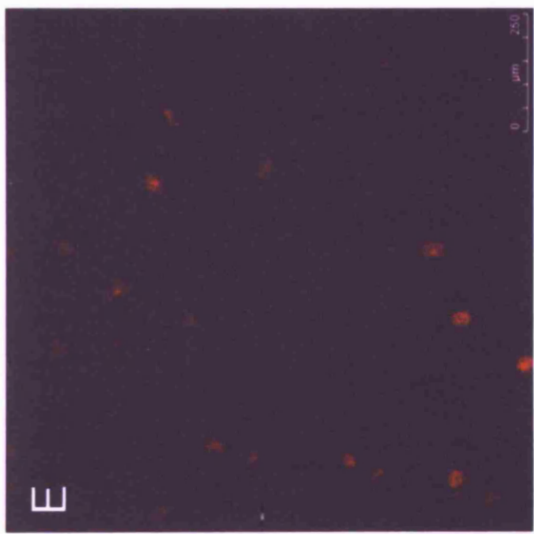
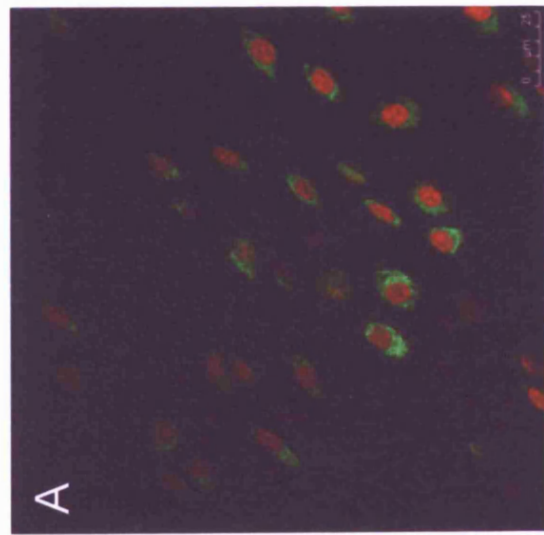
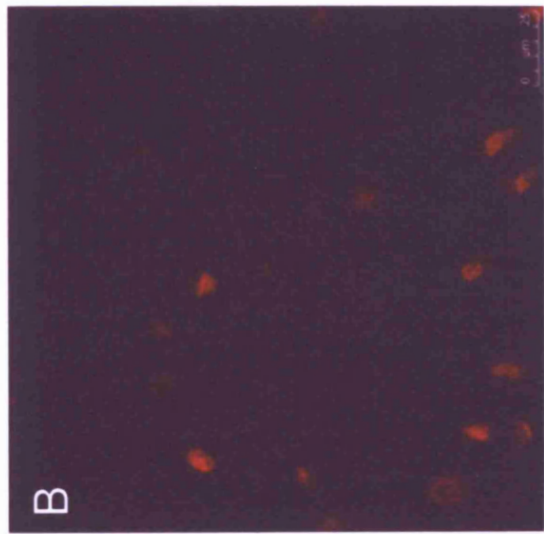
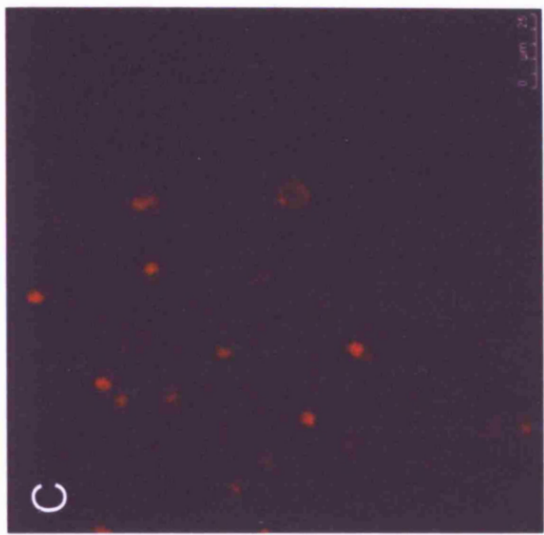
Cells positive for Notch-1 were present in both the surface and deep zone of the tissue with expression much lower in the mid-zone where only a small number of cells were positively labelled (figure 5.5). This expression pattern was similar to that of Delta-1 (figure 5.6), although the levels in the mid zone of the tissue were higher than those seen in the samples labelled for Notch-1. Jagged-1 was detected throughout the depth of the tissue with little difference seen between the different zones (figure 5.7). It was also apparent that paired cells found within the surface do express Notch-1 and its ligands.

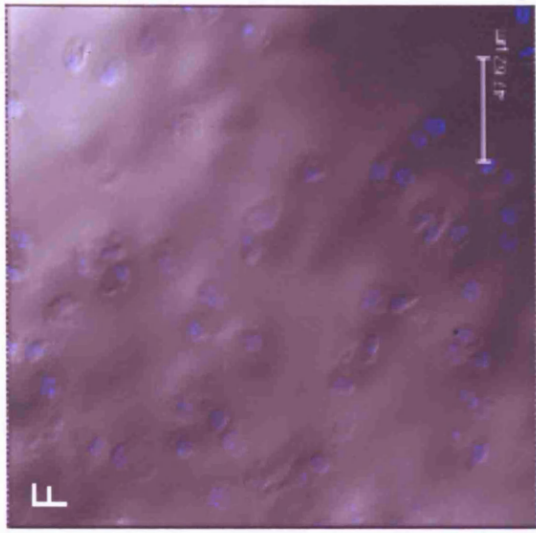
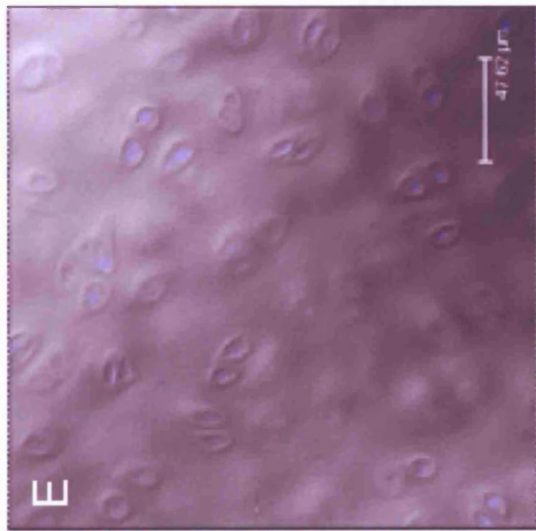
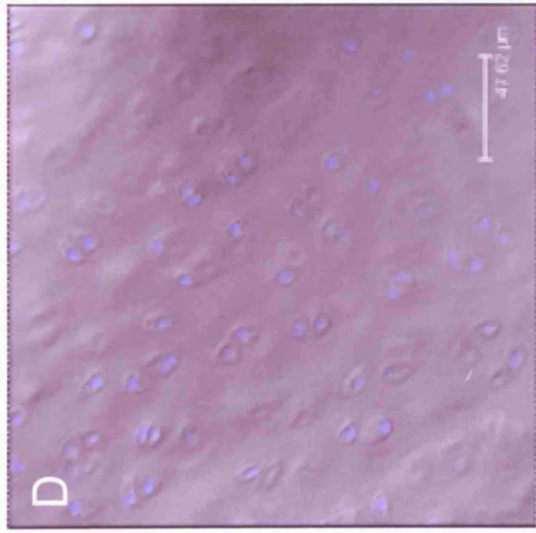
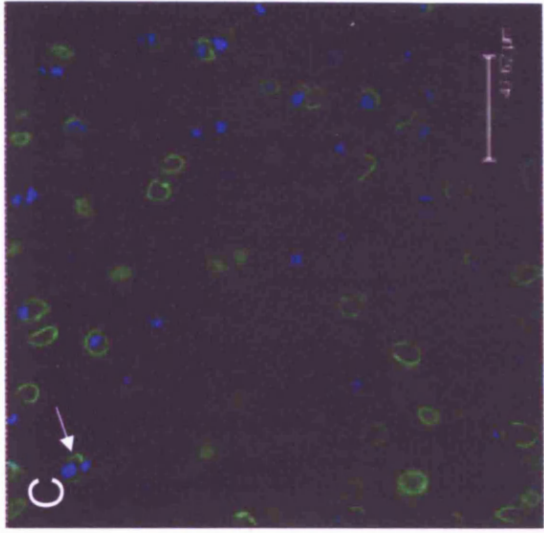
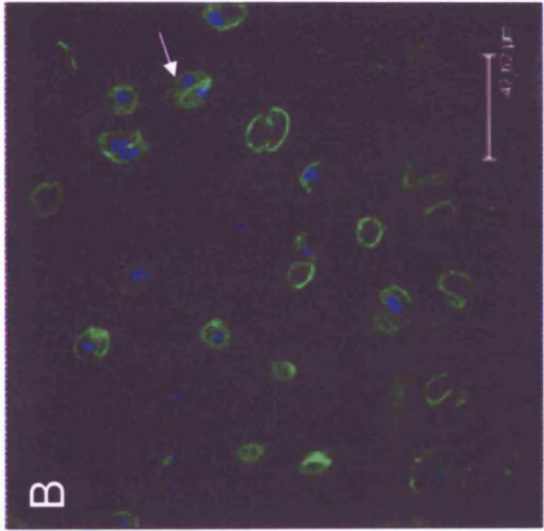
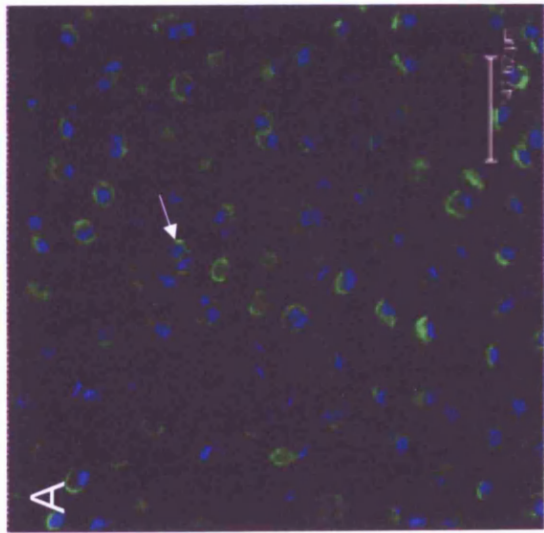


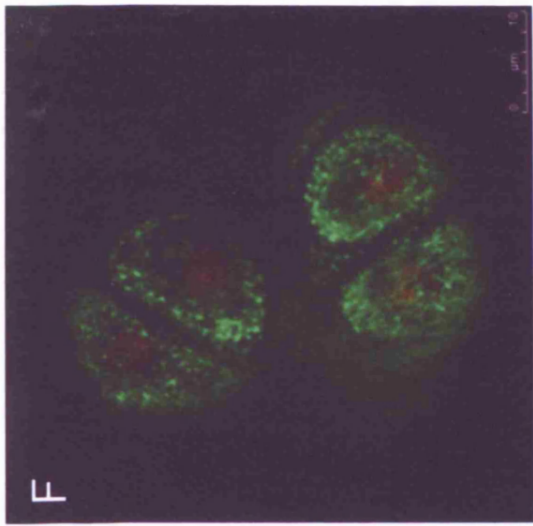
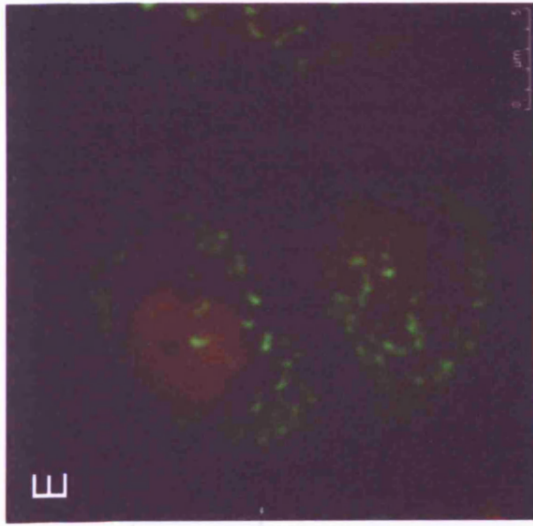
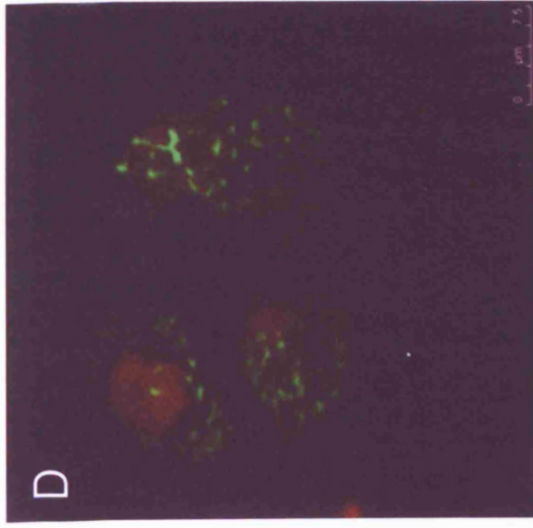
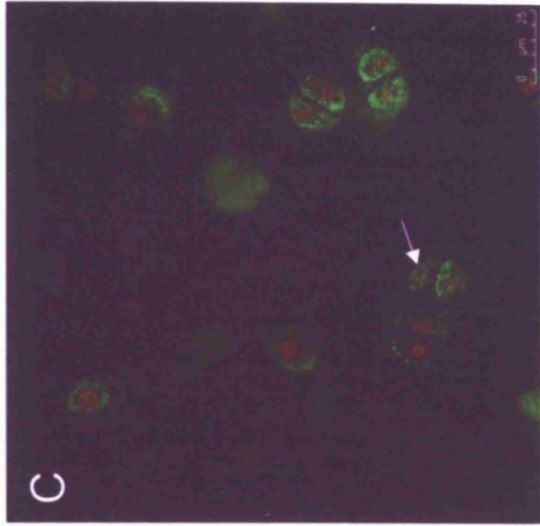
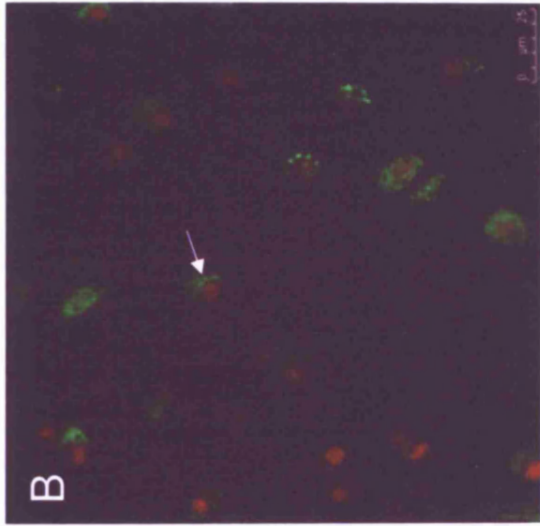
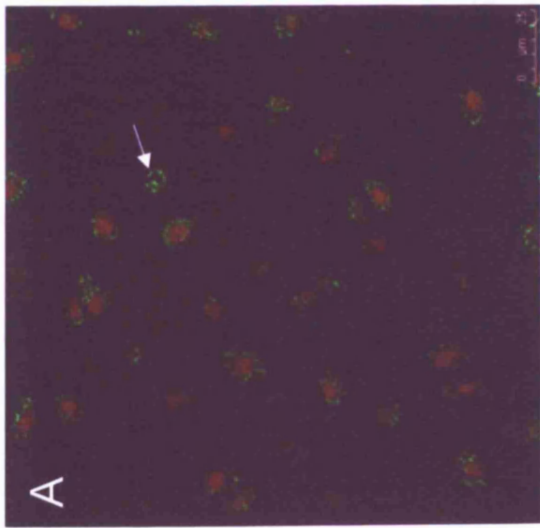
# Figure 1. Immunofluorescence of the rat retina

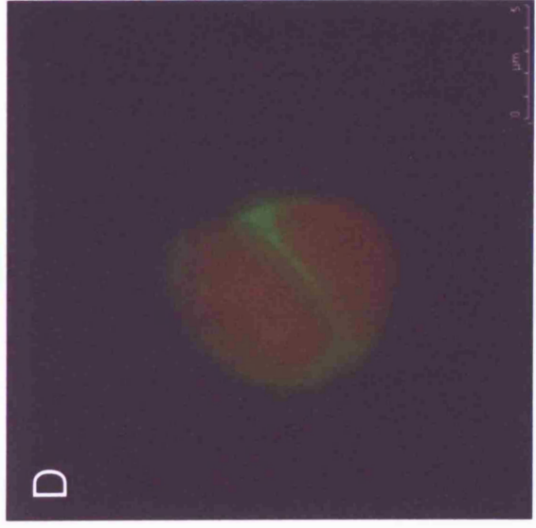
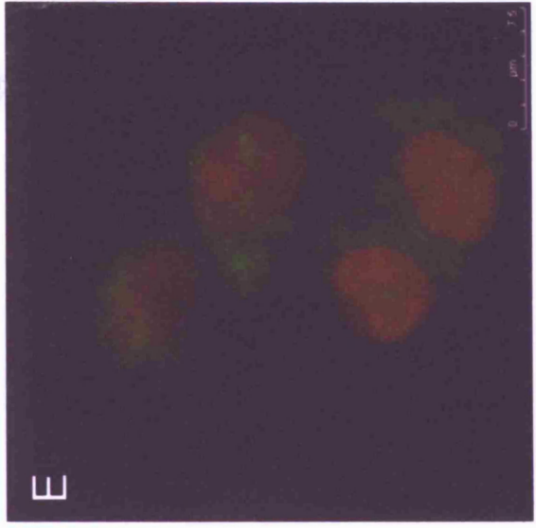
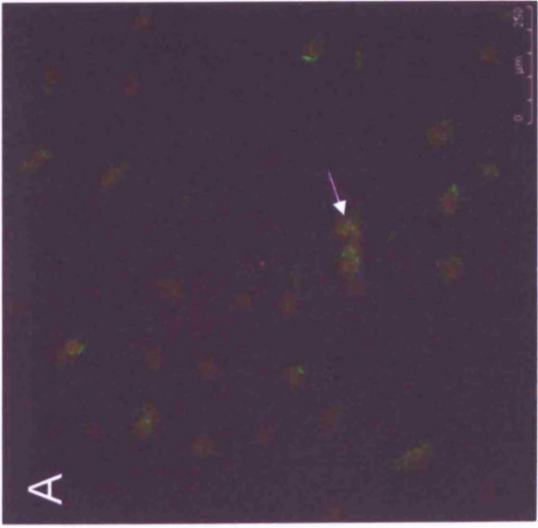
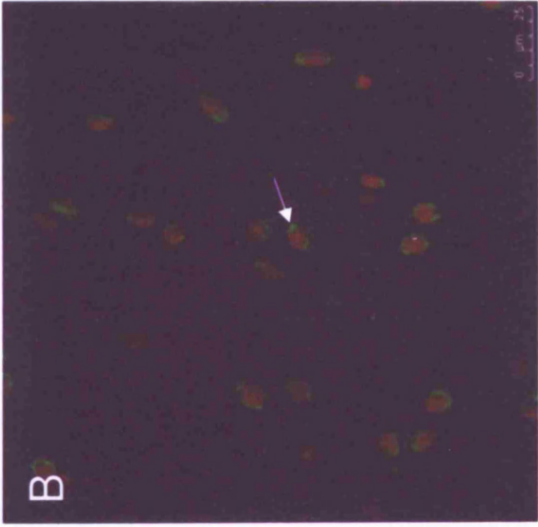
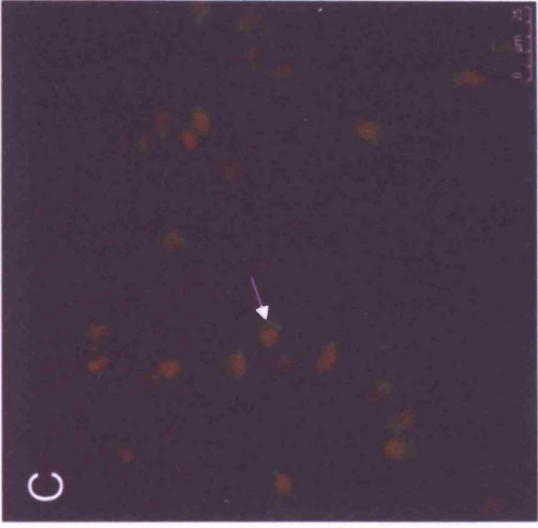
Figure 1 shows immunofluorescence images of the rat retina. The images are arranged in a vertical column, labeled A through E. Panels A, B, C, and D show cross-sections of the retina, while panel E shows a higher magnification of individual cells.

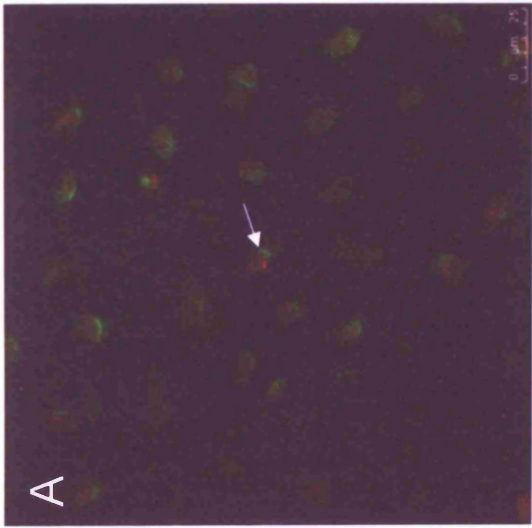
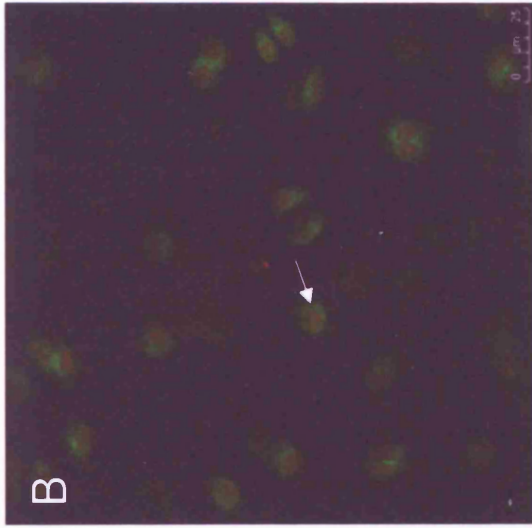
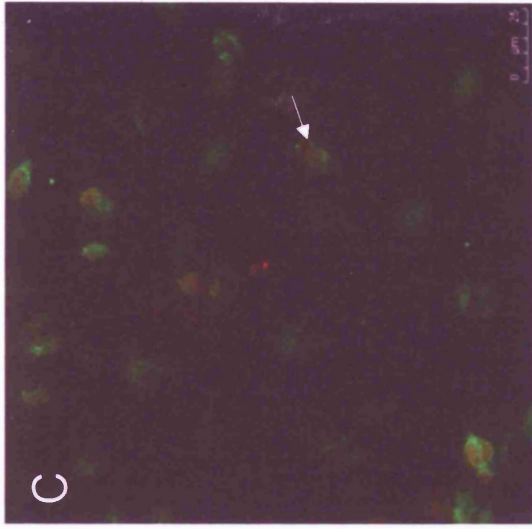




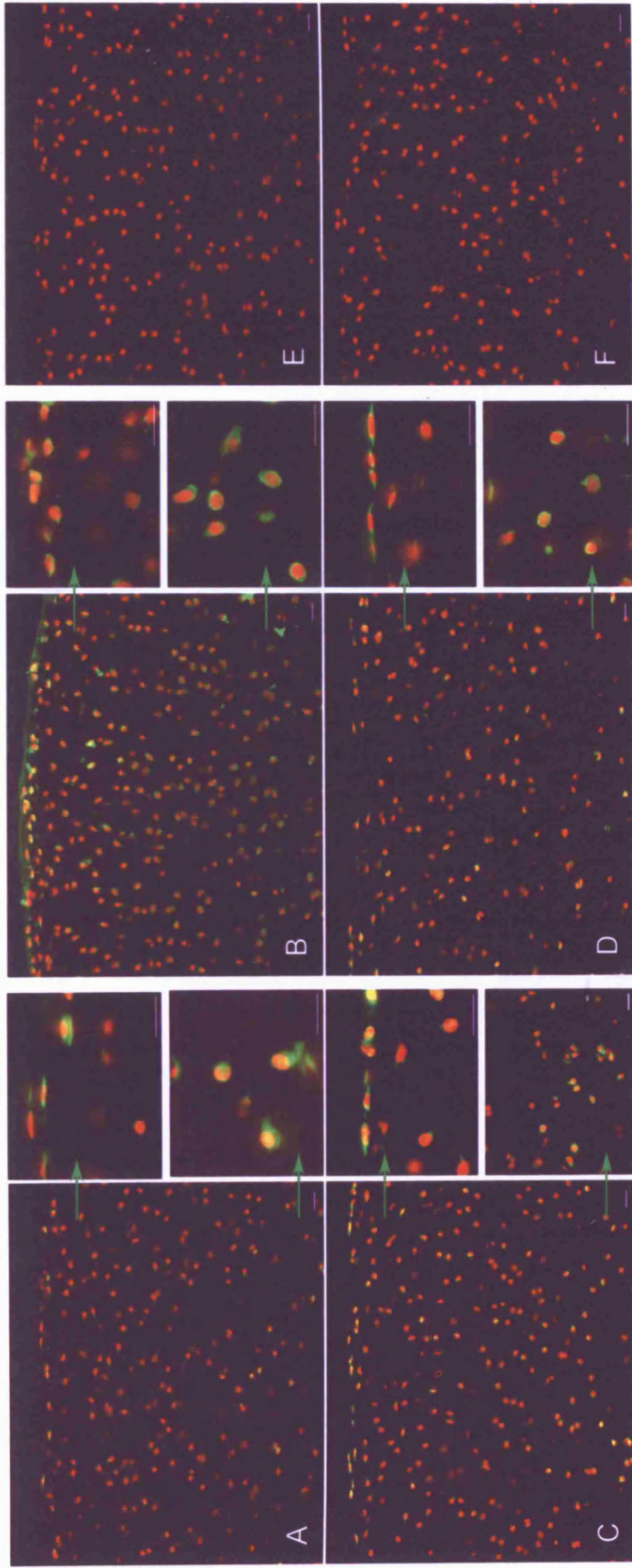












## 5.4 DISCUSSION

Our results support previous data suggesting that cells throughout the depth of the cartilage are not completely isolated but many are situated in pairs or small clusters (Schumacher et al., 2002; Chi et al., 2004). This leads to the possibility that connexin expression may be maintained by the cells to allow communication to occur between adjacent cells within the tissue.

Previous studies have already shown that cells within cartilage may not be in complete isolation within the tissue after maturation (Schumacher et al., 2002; Jadin et al., 2005; Jadin et al., 2007). Staining *en face* cartilage sections with Safranin O clearly demonstrated the paired cells Chi *et al.* (2004) found existed in mature tissue. The organisation of cells within the tissue is closely related to the orientation of collagen fibres. Within the superficial zone of the cartilage, the fibres lie horizontal to the surface allowing for paired cells to exist between the fibres (Jadin et al., 2007). It is also possible that the progenitor cells use the collagen fibres to keep the cells in a 'stem-like' state by separating them from neighbouring cells, thus representing part of a 'niche'. In the deep zone the collagen fibres are orientated at right angles to the surface of the tissue, allowing for vertical columns of cells to form (Jadin et al., 2007).

Due to the thickness of the sections, they were initially treated with differing concentrations of hyaluronidase and chondroitinase to increase the penetration of the antibody. These enzymes cleave bonds within the GAGs that make up the matrix of the cartilage making it easier for the antibody to diffuse through the section without damaging the structure of the Notch or any of its ligands. The secondary antibody was also left on for two different time points to ensure maximum signal strength was achieved. An optimum concentration of 2u ml<sup>-1</sup> hyaluronidase with 0.25u ml<sup>-1</sup> chondroitinase ABC was found, with the secondary antibody used for 2hrs.

To ensure that the upper 30µm of the cartilage explants contained the surface zone, immunohistochemistry was carried out for superficial zone protein (SZP). This is a glycoprotein secreted by the cells that make up the superficial zone of articular cartilage due to its requirement for joint lubrication. As it is not produced by any

other cells, it is an excellent marker for the chondrocytes that are found in the surface zone of the cartilage. The superficial zone of the cartilage is known to be under 30µm thick, so positive labelling for SZP shows that the final sections contain only the uppermost layer of the cartilage.

Paired cells were found within the surface of the tissue, supporting work previously carried out (Schumacher et al., 2002; Youn et al., 2006). The cells existed in the form of a single chondron, as indicated by positive labelling for collagen type VI. This form of collagen is known to be associated with the pericellular matrix found around a cell chondron (Poole et al., 1988). The chondrons within the tissue may be a result of cell division, and in the case of a progenitor cell asymmetrically dividing, it is possible that channels formed between the two cells may aid in determining which daughter cell remains in the progenitor state, and which undergoes terminal differentiation.

Notch-1 was present in the deep and superficial zones, and it was also found in paired cells located within the surface of the tissue. It is already known that Notch-1 has a role in cell fate decisions in progenitor and stem cell populations and also in setting tissue boundaries. This affects its expression pattern with Notch-1 initially expressed at the newly formed articular surface during murine development, possibly controlling the growth of the tissue. As the tissue matures, this expression becomes restricted to the deep zone and the hypertrophic chondrocytes situated in the growth plate (Hayes et al., 2003). In 7-day-old bovine tissue sections, expression of Notch-1 remains high in the surface zone, possibly signifying the difference in developmental stage between the two species. By 18-months the expression is more restricted, with higher levels still seen in the surface of the tissue and around pairs of cells or cell clusters in the deep zone, whilst there are still some cells labelling positive for Notch-1 in the mid-zone (unpublished data).

The expression pattern for Delta-1 was similar to Notch-1, with expression highest in the surface and deep zones and low levels detected in the mid-zone. Delta-1 has already been shown to have a key role in cartilage differentiation by inhibiting prehypertrophic chondrocytes from progressing to hypertrophic chondrocytes, and therefore it would be expected that levels would be highest at the top of the deep

zone. This supports previous work carried out by Richardson *et al* (2006) looking at the expression of Notch-1 and its ligands in the different zones of the cartilage. Using vertical sections, it was found that Notch-1, Delta and Jagged-1 were all expressed by cells within the surface and deep zones of the tissue (figure 5.8). Expression of Delta was lower than both Notch-1 and Jagged-1, with these two molecules showing a similar expression pattern (Richardson *et al.* 2006).

There is growing evidence that cells within the surface of articular cartilage are not in complete isolation, and this work supports that theory. The presence of paired cells within all zones of bovine articular cartilage could be a plausible explanation why the cells maintain the expression of connexin molecules even after the tissue is fully developed. In early development, connexin channels are required for co-ordinating the differentiation of the tissue, and it may have a similar role in controlling the terminal differentiation of progenitor cells within the surface of the cartilage. The expression of the signalling molecule Notch-1 and its ligands by the paired cells further supports the possibility that a progenitor cell population maybe the reason for some of the paired cells seen within the surface of the tissue.

## **CHAPTER 6**

### **General Discussion**

## 6.0 GENERAL DISCUSSION

It has already been established that articular cartilage has a low intrinsic capacity for repair after injury, and due to phenotypic modulation, it is difficult to expand sufficient cell numbers in culture to treat larger defects. This has led to research focusing on alternative cell sources for repair of these defects. One such cell source is a population of chondroprogenitor cells found within the surface zone of the tissue, and work within this thesis aims to further characterise and understand the phenotype of these cells; albeit from a bovine source.

Previous work has shown that the surface zone of the tissue contains a population of progenitor cells capable of undergoing differentiation along a variety of mesenchymal lineages (Dowthwaite et al., 2004) but, as yet, the effect on time in culture has not been investigated. Growing cells in culture for prolonged periods can have a variety of different effects on cells, mainly resulting in changes to the morphology or phenotype of the cells (Limeback et al., 1984; Pourreyron et al., 2003). In the case of stem or progenitor cells, these changes can then alter the cells ability to differentiate into specific cell types.

One of the main advantages in using progenitor cells over terminally differentiated cells is the increased proliferative ability of progenitor cells (Cai et al., 2004). But, before a population of cells can be considered for tissue engineering, it is important to determine the effect that long term culture may have on the cells. Work in this study found that there was no negative effect on the ability of the clonal progenitor cells to differentiate along a chondrogenic lineage after prolonged growth in culture and the most significant differences in pellet matrix composition were found between pellets from different clonal cell lines. This variance suggests that slight differences seen between clonal cell lines (such as rate of growth or morphological observations) may actually have an affect on the cells ability to differentiate.

It was found that the collagen type I and II ratio of the pellet was possibly not a result of the time the cells had been in culture. Instead, it was more likely caused by the number of population doublings the cells had undergone, as there was no distinguishable difference seen between the collagen ratio of a fast growing cell line

and those that had been maintained in culture for 100 days more. The number of PDs that the cells had undergone in culture also resulted in producing denser cell pellets, and this alone may suggest that prolonged growth in culture provides slightly improved differentiation along a chondrogenic lineage. Interestingly, the amount of GAG in the pellet was affected by time in culture as opposed to number of PDs the cells had undergone, as the fast growing clonal pellets comprised approximately 8% more GAG than the pellets generated after 25 or 45 PDs. This result may be due to the effect that GAG is known to have on the proliferation of the cells due to the GAG molecules ability to bind a variety of molecules including growth factors, cytokines and chemokines. Binding these molecules can protect them from degradation or allow them to activate cell receptors, initiating cell signal pathways (Hayes et al., 2008). There is also the possibility that low sulphated chondroitin sulphate chains (including 3B3(-) and 7D4) may actually act as a boundary, helping to maintain the progenitor cells in this state by acting as both a physical and biochemical barrier preventing growth factors binding with the cells (Hayes et al., 2008).

It is also thought that the presence of differing sulphate motifs that are not found throughout the tissue may represent part of the stem cell niche, responsible for maintaining the progenitor cells in their naive state. Even though the stem cell niche has long been hypothesised (Williams et al., 1992), it has only recently become a focal point for further understanding the maintenance of a stem cell population within many tissue types (Whetton and Graham, 1999; Mills and Gordon, 2001).

Individual cells labelled positive for both 4C3 and 7D4 one day after isolation, and expression of these PG motifs remained in cell colonies for up to 7 days in culture. At these earlier time points expression occurred mainly between cells, in some cases forming a meshwork surrounding and linking neighbouring cells. By day 9, the cells had formed confluent sheets in many regions of the plate, and some of these regions remained positive for both 4C3 and 7D4 expression. This expression was not seen in any of the areas where cells were sparse and suggests that these motifs were only expressed by the more proliferative cells that made up the more confluent regions of the plate. It was also worth noting that the cells were still able to produce these sulphated motifs even when there was reduced space between the cells, as at earlier

time points the cells were sparse and producing large cytoplasmic processes that labelled positive for 4C3 or 7D4.

The levels of 3B3(-) observed were much lower at all time points than both 4C3 and 7D4, with no single cells labelling positive for 3B3 (-) at day 1. Although some cells were positive for 3B3(-) at days 3, 5 and 7, the levels seen were much lower than for the other two sulphate motifs, and by day 9 labelling was absent. There were high levels of expression in the freshly isolated surface zone cells, although this increased expression in SZ cells may be a result of the cells flattening out after isolation from the tissue. The levels of 3B3(-) were much lower in tissue sections, with expression restricted to the region around a few cells within the cartilage surface zone (Hayes et al., 2008). The observed changes in expression of these motifs might be due to increased time in culture as whenever cells are taken out of their natural environment it is inevitable that some changes in their phenotype will occur. Alternatively, it is also possible that these changes in the expression of the sulphate motifs may be a result of differentiation or maturation of the cells.

The ability of cells to differentiate along certain lineages *in vitro* does not necessarily mean that the cells will be capable of differentiating *in vivo* (De Bari et al., 2004). When grown *in vitro*, it is possible to have strict control over the type of tissue the cells form, either via the addition of growth factors, or by growing the cells on scaffolds designed to enhance specific differentiation. When the cells are placed *in vivo*, the level of control over native signals acting on these cells is minimal, and this has already proved a major obstacle in terms of tissue engineering, especially when using ES cells. It is known that ES cells have a greatly increased plasticity, but controlling the differentiation of these cells, and inhibiting tumour development is limiting their use for tissue engineering (Humpherys et al., 2001; Odorico et al., 2001; Chi et al., 2004; Yang et al., 2008). Adult stem cells, with much more restricted plasticity, are more favourable cell sources (Prockop, 2003), but it is still important to understand the default pathway the cells will undergo when placed *in vivo* with multiple different signals acting on these cells.

When examining the ability of progenitor cells to differentiate along multiple lineages, it is usual to inject the cells into the tissue that it has already been found the



cells are capable of forming *in vitro*, with factors to aid in their differentiation. What is less well defined is what occurs when cells are injected into foreign environments with no growth aids. More importantly, it is vital to understand just how ‘plastic’ the cells are. After intramuscular injection into SCID mice the chondroprogenitor cells failed to form a cartilage like tissue, despite producing key components of a cartilage like-matrix, such as glycosaminoglycans. They also maintained expression of the key transcription factor Sox9, which is required for the initiation chondrogenic differentiation.

It is possible that chondrogenesis is the default pathway for the cells, but their inability to produce cartilage suggests that the cells may not express all the factors required to undergo complete differentiation. Surprisingly all but one of the cell lines failed to differentiate along any other lineage, despite previous studies *in ovo* showing the cells were capable of forming multiple mesenchymal tissue types (Dowthwaite et al., 2004). It is possible that multiple signals are required to reprogram the cells, such as the abundance of growth factors released during development. However, surprisingly, one cell line expressed an endothelial marker PECAM-1 and this is the first time that these cells have expressed components found outside the mesenchymal lineage. There were also no muscle differentiation markers detected (i.e. MyoD).

Signals from the surrounding tissue are not the only factor responsible for inducing or controlling cartilage development, and it is sometimes possible to overlook the impact cell-cell contact can have on chondrogenic differentiation (DeLise et al., 2000; Khan et al., 2007). During early cartilage development, cell communication is known to be vital for tissue formation, with an increase in connexin expression seen in the cells that make up the mesenchymal condensations (DeLise et al., 2000; Hall and Miyake, 2000; Stains and Civitelli, 2005b). It has already been established that expression of Cx43 is maintained in mature chondrocytes, with cells retaining the ability in the correct conditions to communicate with adjacent cells. Retaining Cx43 in mature cells, when communication may not be required for any known cellular process, may actually be vital due to the role of connexins in mechanotransduction via hemichannels (Bao et al., 2004).

The chondroprogenitor cells appear able to alter their connexin expression when grown in culture, with fluctuations seen in Cx32 levels with increasing culture time. As yet it is unclear what stimulus may be responsible for these alterations, or if it is intrinsically programmed into the cells. Their ability to express more than one connexin type allows molecules of differing sizes to pass between cells and could be a way for the cells to control their differentiation. Surprisingly, despite expressing connexin at the cell surface, the chondroprogenitors are incapable of forming functional channels with other progenitor cells. This may be a result of the cells being unable to get close enough to form gap junctions with other cells (which is very unlikely), or it is also possible that the channels formed remain shut. As mature chondrocytes were capable of forming functional junctions there is also the possibility that cell communication was differentiation specific, with the progenitor cells keeping their channels closed to prevent their differentiation, as alterations in connexin expression are known to have an effect on cell phenotype (Zhang and Thorgeirsson, 1994; Oyamada et al., 1996; Leung et al., 2002; Araya et al., 2005). Treating the clonal cells with TGF- $\beta$  to initiate chondrogenesis failed to enhance cell communication, although it is possible that treating the high density monolayer cultures was not enough to really promote chondrogenic differentiation.

Even though the chondroprogenitor cells failed to communicate with other progenitor cells, they were able to form functional gap junctions with terminally differentiated chondrocytes and this communication was shown to be connexin channel specific. There is still the possibility that this communication is differentiation-specific and signals released from SZ cells initiated chondrogenesis in a manner that TGF- $\beta$  alone could not. When *in vivo* it is possible that signals released from mature chondrocytes pass through connexin hemichannels, stimulating the progenitor cells within the surface of the tissue to begin undergoing chondrogenic differentiation after division, opening the channels to allow direct cell-cell contact and the transmission of signals to further promote chondrogenesis. Alternatively, it is equally likely that direct cell contact is required to stimulate the cells.

If the surface zone cells are able to communicate with the progenitor cells resulting in initiating the differentiation of these cells, then co-culture of the cells is a possible way to combine the increased proliferative ability of the clonal cells with the

enhanced differentiation provided by the mature chondrocytes. Co-culturing the cells did increase the chondrogenic differentiation in some of the cell lines. As has been found with intramuscular injection of clonal cells, the biggest differences seen were almost certainly caused by the differences between clonal cell lines, causing very varied results. Increasing the levels of surface zone cells in the micromass cultures above 5% had little extra effect on the level of differentiation seen, suggesting that only a small number of cells are required to enhance chondrogenesis. It is worth noting however, that in the clonal cell lines that were poorly chondrogenic, co-culture did actually improve the levels of staining seen, whilst it had no negative effect in those cell lines that made micromasses with higher levels of alcian blue staining. This resulted in all micromasses having similar levels of staining after 7 days in culture, and, therefore, co-culture could be a tool used to standardise the levels of chondrogenic differentiation seen in clonal cell lines.

It is widely known that structure and cellular orientation of articular cartilage follows a long accepted model; with flattened cells found within the surface of the tissue in near isolation, progressing to spherical cells found in isolation or small groups in the mid-zone, and finally, the large rounded cells forming columns in the deep zone (Newman, 1998). More recently, it has been found that cells within the three distinct cartilage zones may be in contact with neighbouring cells (Schumacher et al., 2002; Chi et al., 2004). If the cells maintain contact after development, it could be another reason for retaining connexin expression and if there are paired cells within the surface of the tissue, it may be a way of coordinating the progenitor cells differentiation.

Work in this thesis confirmed previous studies that found that cells within both the surface and mid-zones of the tissue are in close contact, whilst those in the deep-zone are either in small clusters or found in columns which are clearly visible when viewing horizontal sections (figure 6.1). In understanding tissue structure, it is important to view it as a 3D entity as apposed to 2D sections, as has been done for many years. The structure of the tissue is highly organised on all planes and viewing it in this way gives a greater insight into interactions that may occur between cells.

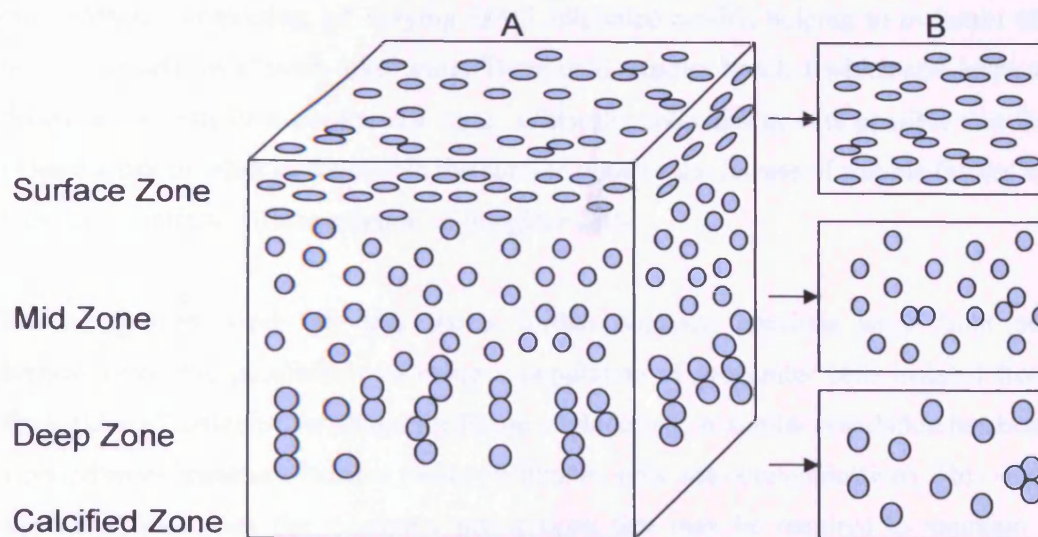


Figure 6.1 Representation of the organisation of articular cartilage showing chondrocyte localisation through the different zones. Within the surface the cells appear isolated (A) until viewed using vertical sections, paired cells are clearly visible (B). This is also seen within the mid-zone, with fewer cells evident. In the deep zone the clear columns of cells are seen in horizontal sections (A), yet they appear as either single cells or clusters when viewed using *en face* sections.

Notch-1 is known to be associated with both stem and progenitor cells where it has a role in regulating the clonality of the cells, influencing cell fate decisions (Gridley, 1997). Expression of Notch-1 has already been found to be important during the development of articular cartilage (Hayes et al., 2003) and all progenitor cells isolated from the surface of the tissue are Notch-1 positive (Dowthwaite et al., 2004). Most of the cells in the surface and deep zones were Notch-1 positive, and these are thought to be the regions containing progenitor cell populations. However, in the base of the tissue it is likely to be related to cell fate decisions involving terminal differentiation (Hayes et al., 2003). A similar pattern of labelling was also seen for both Notch ligands Jagged-1 and Delta-1, although in both cases, slightly more labelling was seen in the mid-zone of the tissue. Expression of Notch-1 and its ligands was also seen between paired cells in all zones of the tissue, especially within the surface of the cartilage, which may signify the presence of progenitor cells.

The presences of Notch-1 and its ligands within the surface zone of the cartilage further supports the presence of a stem/progenitor cell niche within the surface zone of the tissue. It is possible that the cells within this zone exist in a tightly controlled

environment comprising of varying GAG sulphated motifs, helping to maintain the progenitor cells in a 'stem-like' state. These cells express Notch-1 which also helps to preserve the cells in a progenitor state. When the cells divide, it is possible that the close contact of other cells within the surface zone or the release of soluble factors by these cells, initiate differentiation in daughter cells.

Taken together, work in this thesis further supports previous work from our laboratory on the possibility of using a population of progenitor cells isolated from the surface of articular cartilage for tissue engineering. A similar population has been isolated from immature human tissue (Williams, personal communication). This work further characterises the complex interactions that may be required to maintain a progenitor cell population within the surface of articular cartilage and looks at some of the considerations being made when growing these cells in culture, as well as ways to possibly improve the chondrogenic differentiation of these cells.

## **CHAPTER 7**

### **Future Work**

## **7.0 FUTURE WORK**

### **7.1 Chondroprogenitor classification**

From this study the main unanswered question is what differences are there between differing clonal cell lines, and this is something that needs to be further examined. If these cells are going to be an alternative chondrocyte cell source, it is important to fully understand how individual clones differ either in age or just phenotype and their chondrogenic potential. In this respect, it might also be useful to try and better understand the existence of the stem cell niche and how this may maintain the progenitor cell population within the surface. It is also important to be able to easily identify the progenitor cells soon after adhesion, and it is possible that the cells expressing 7D4, an epitope not present in passaged clonal cells or surface zone cells, are the earliest form of progenitor cells. Isolating cells based on expression of 7D4 and growing the cells in culture should be able to confirm this possibility.

Work in this thesis demonstrated that the cells are able to survive *in vivo* in a mammalian model and show limited plasticity. If chondrogenesis is the default pathway for the progenitor cells, it may be important to determine if the cells are capable of undergoing complete chondrogenic differentiation *in vivo* after injection into the cartilage. Alternatively, injecting the cells into a foreign environment with the addition of several growth factors known to initiate chondrogenesis could also demonstrate the requirement for the cells to undergo chondrogenic differentiation *in vivo*.

### **7.2 Connexin Expression**

There is currently only limited data characterising connexin expression in chondrocytes, and the roles connexins play in mature cells should be further explored. Currently, the interesting expression pattern seen by Cx32 in full-depth tissue sections is not understood. With expression decreasing in a region just below the surface of the tissue, it is possible that Cx32 may have a role in differentiation of the cells as they enter the transitional zone. It is also unclear why there are fluctuations seen in the expression of Cx32 when clonal cell lines are grown in culture. These fluctuations may be a result of differing levels of cell confluence *in vitro*, and again this needs to be further examined.

The inability of progenitor cells to communicate with each other could be a key factor in fully understanding how the cells may differentiate. It may be the inability of the cells to communicate is due to failure to express some of the cell adhesion molecules required for bringing the cells together such as neural cell adhesion molecule (N-cam) and N-cadherin, which may be expressed by mature chondrocytes. Immunolabelling to determine if these molecules are expressed by the progenitor cells, or upregulated after cell contact, may help in understanding the cell coupling that is required to bring the cells close enough for communication to occur. It is still likely that progenitor communication is differentiation specific, but the addition of TGF- $\beta$  alone was not enough to stimulate the cells to undergo chondrogenesis whilst in monolayer culture. Altering the culture method may be enough to further enhance chondrogenesis and determine if the initiation of communication really is a result of the cells differentiating. It may also be beneficial to attempt mixing clonal cells with surface zone cells in pellet culture to try and continue assessing the effect co-culture has on progenitor differentiation.



## **CHAPTER 8**

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## **CHAPTER 9**

### **Appendices**

## APPENDICES

### Appendix I

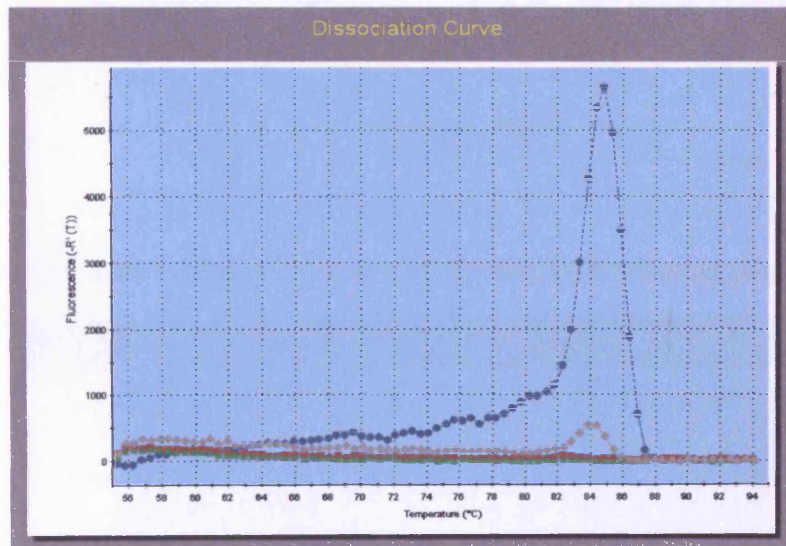
#### DNA-Free Kit

1. 89 $\mu$ l RNA + 1 $\mu$ l DNase + 9 $\mu$ l 10x buffer
2. Leave for 30mins at 37°C
3. Add 10 $\mu$ l of inactivation reagent and leave for 2mins at room temperature – vortex before use
4. Centrifuge for 1min at 10,000g
5. Remove RNA to new tube
  - a. Store RNA at -80°C

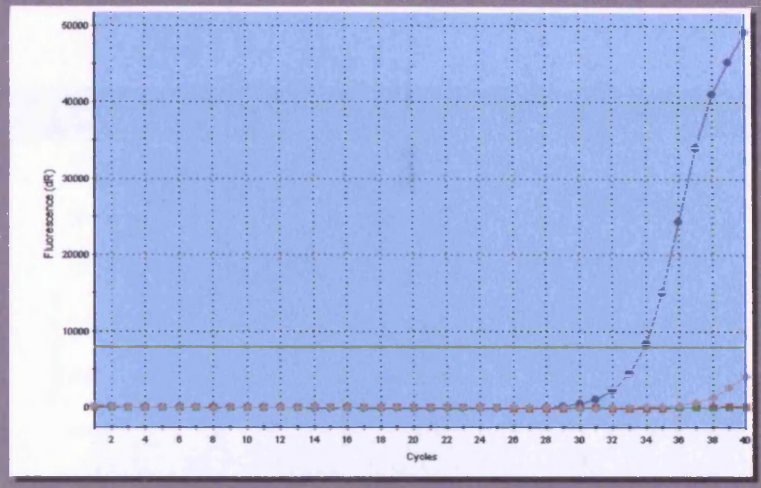
### Appendix II

#### Dissociation Curves

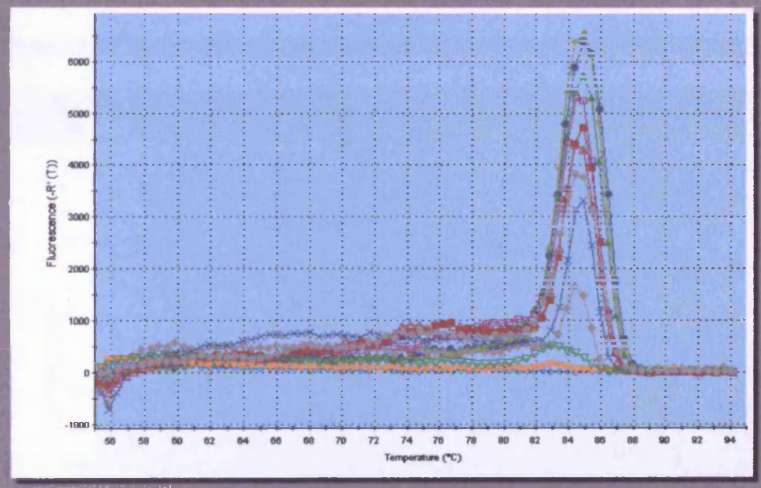
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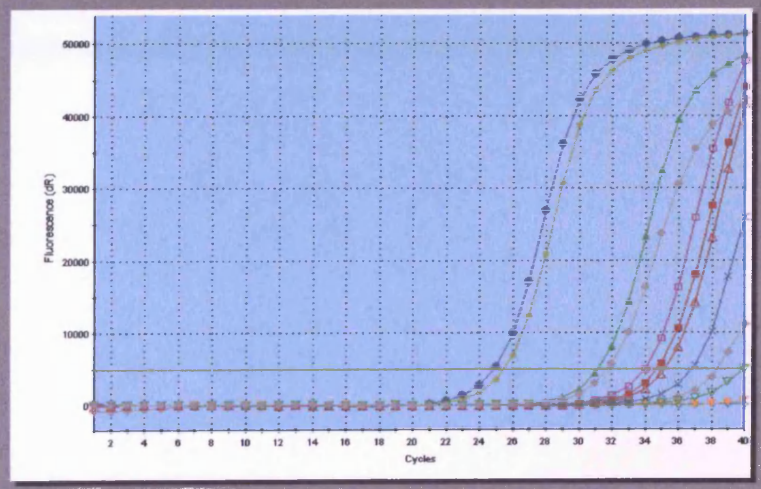
Amplification Plots



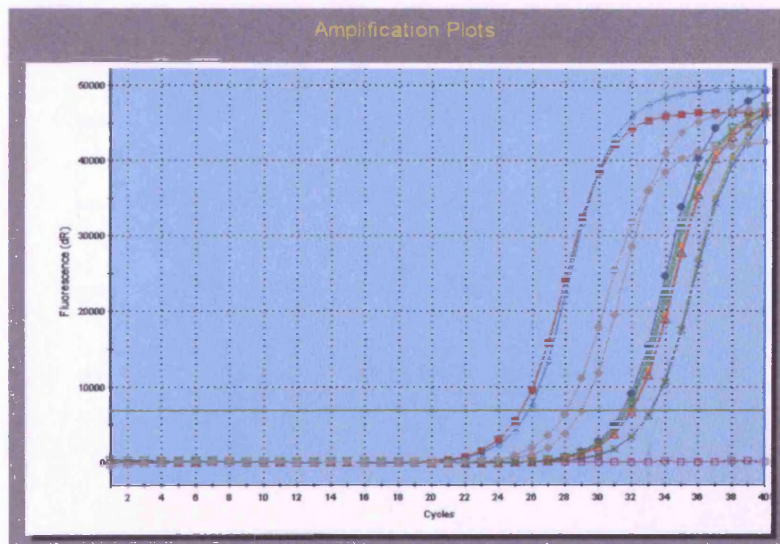
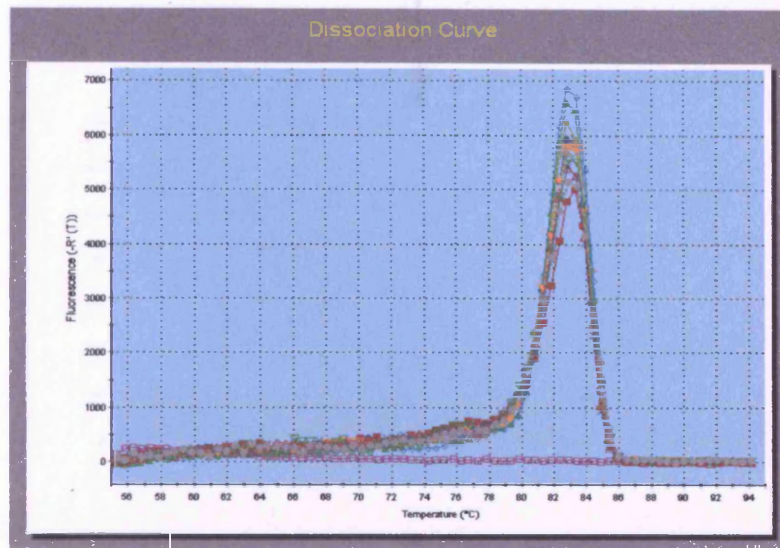
Dissociation Curve



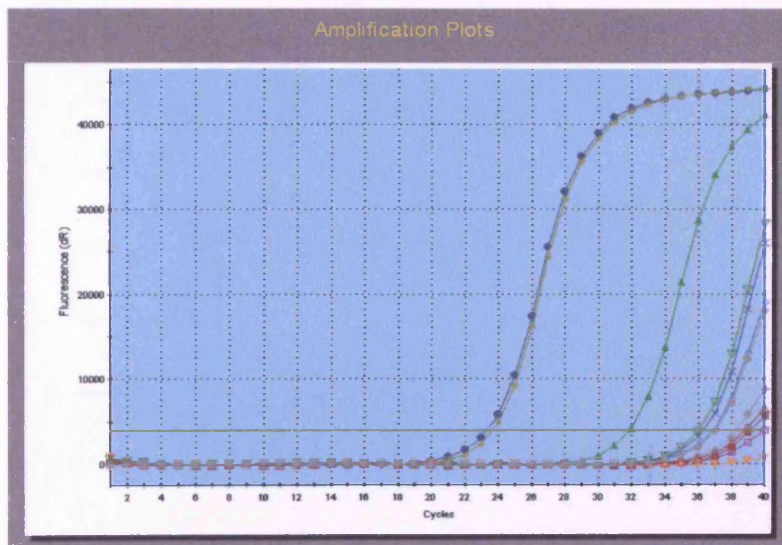
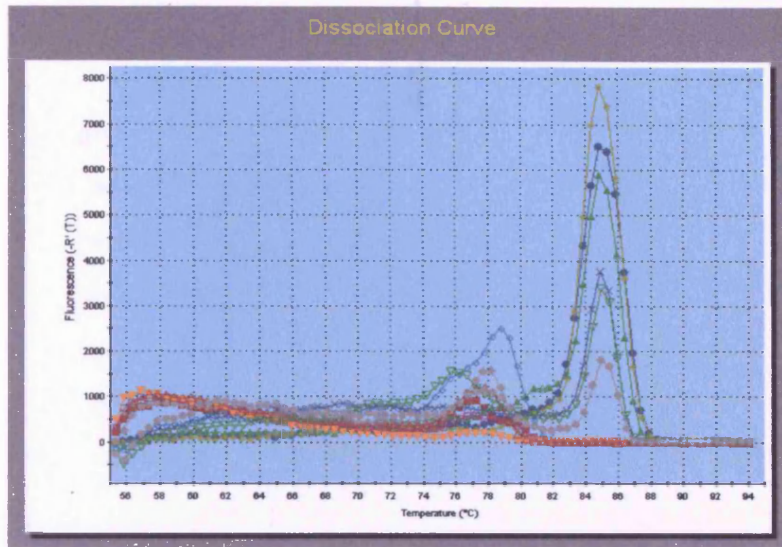
Amplification Plots



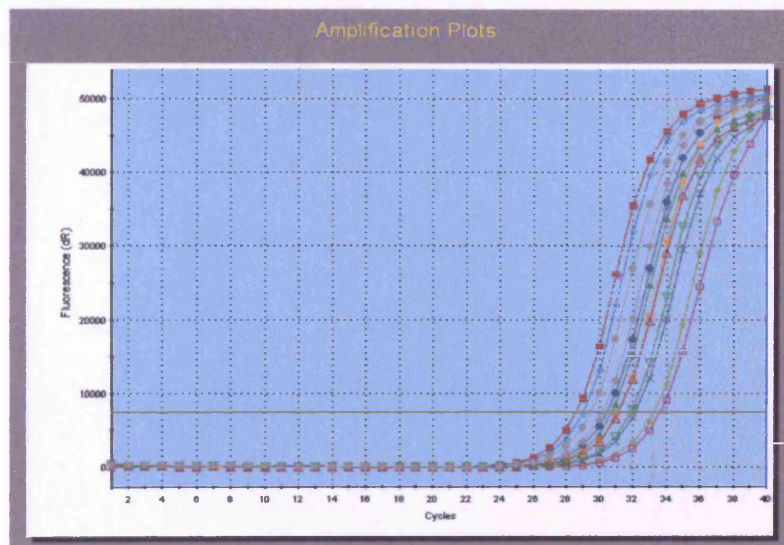
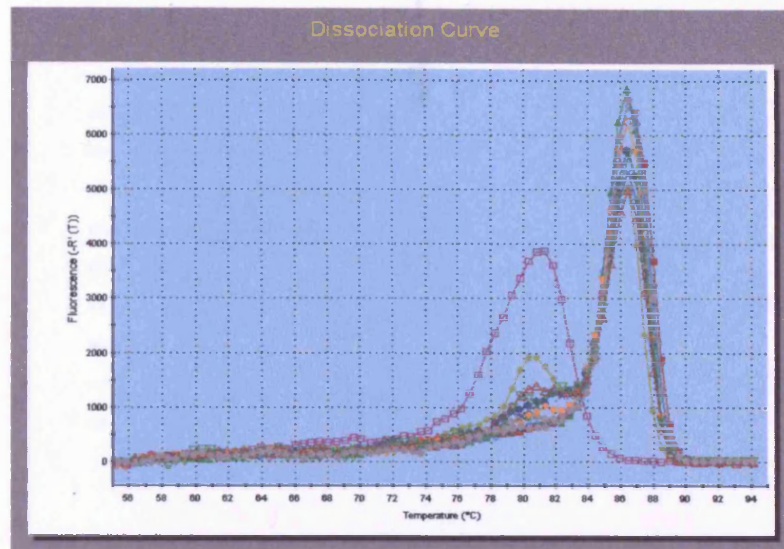
# Collagen Type I



# Collagen Type II

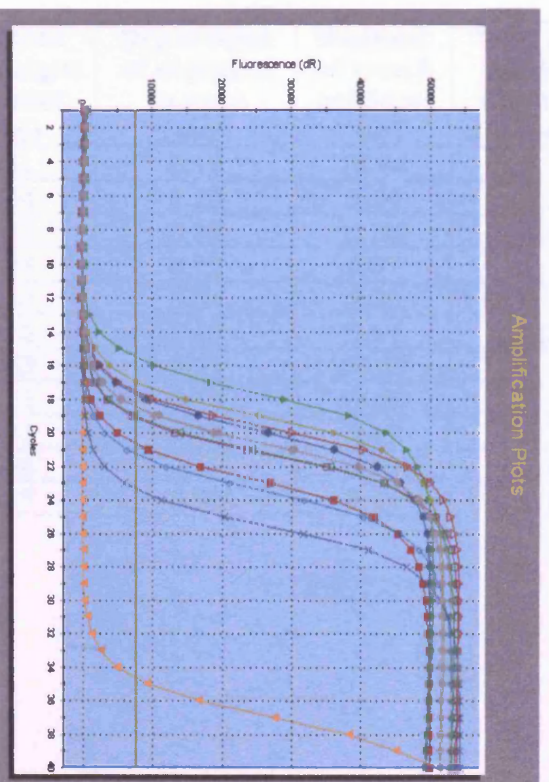
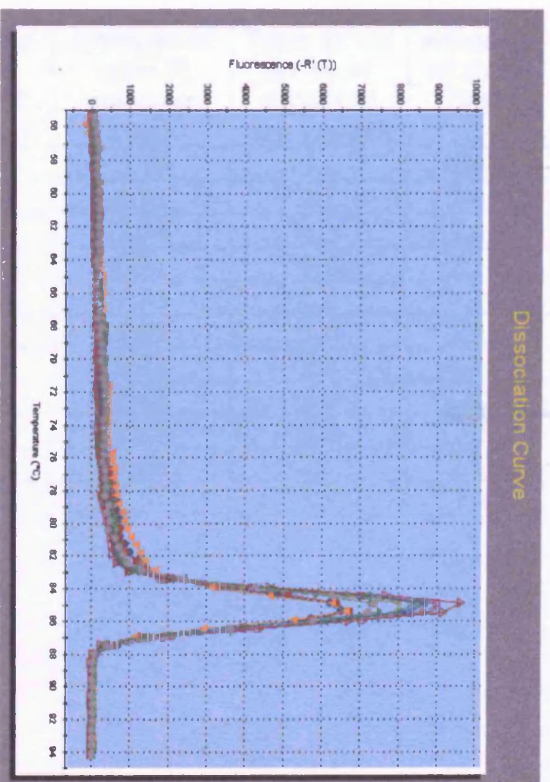


Sox9





# 18s Control



### Appendix III

Table of Biochemical Pellet Analysis

Sample Number	Sample Code	Sample Date	Original dry weight of pellet (mg)	Dry weight of digested sample (mg)	Amount of type I collagen (ug)	Type I col as % of digested dry weight	Amount of type II collagen (ug)	Type II col as % of digested dry weight	Amount of GAG (ug)	GAG as % of digested dry weight
1	C90	Early	0.51	0.19	15.64	8.2	5.98	3.1	3.91	2.1
2	C90	Late	0.34	0.28	3.64	1.3	5.81	2.1	2.69	1.0
3	C91	Early	0.42	0.18	11.66	6.5	7.74	4.3	3.63	2.0
4	C91	Late	0.24	0.22	4.67	2.1	6.51	3.0	2.19	1.0
5	C92	Early	0.64	0.34	3.06	0.9	7.83	2.3	7.22	2.1
6	C92	Late	0.26	0.19	5.61	3.0	5.63	3.0	1.91	1.0
7	C93	Early	0.53	0.23	10.85	4.7	6.42	2.8	4.75	2.1
8	C93	Late	0.25	0.19	14.68	7.7	5.63	3.0	1.42	0.7
9	C94	Fast	0.31	0.18	5.87	3.3	8.98	5.0	15.87	8.8
10	C95	Fast	0.26	0.19	4.33	2.3	4.05	2.1	2.13	1.1
11	C96	Fast	0.35	0.20	8.19	4.1	6.51	3.3	17.84	8.9

## **Appendix IV**

### **PKH26 Red Fluorescent Cell Marker Protocol**

1. Trypsinise cells and put into a single cell suspension.
2. Resuspend cells at  $2 \times 10^7$  and place in a single polypropylene tube. Wash once using serum free media and centrifuge cells for 5mins at 400 x g.
3. Aspirate supernatant leaving no more than 25 $\mu$ l of supernatant. Add 1ml of Diluents C and resuspend by pipetting.
4. Immediately prior to staining prepare  $4 \times 10^{-6}$  molar PKH26 dye (forming 2x stock) using Diluents C. The amount of dye added should be less than 1% of individual sample volume. Preparation must be kept at room temperature.
5. Rapidly add 1ml of 2x cells to 1ml of 2x dye and mix by pipetting immediately as staining is almost instant.
6. Incubate at 25°C for 2-5 minutes periodically inverting the tube to mix.
7. Stop the reaction with 2mls FCS and dilute sample with 4mls of complete media.
8. Centrifuge cells at 400 x g for 10 minutes to remove staining solution.
9. Remove supernatant, transfer pellet to a new tube and re-wash a minimum of 3 times.
10. Add 10ml of complete media to wash cells. Spin again and fill tube with complete media + 10% HEPES buffer.
11. Examine the cells under microscope to determine if staining has been successful and is uniform. Remove 8 x 10 $\mu$ l samples and place onto 2 slides. Freeze down for later observations and testing.

## **Appendix V**

### **Haematoxylin and Safranin O Staining**

Slides were

1. Fixed in NBFS for 2mins
2. Placed in Mayer's haematoxylin for 5mins
3. Washed in running tap water for 1min
4. Counter-stained with Safranin O for 30secs
5. Washed in running tap water for 30secs

6. Dehydrated in graded series of alcohols
  - a. 70% ethanol for 30secs
  - b. 90% alcohol for 1min
  - c. 100% alcohol for 1min
  - d. 100% alcohol for 2mins
7. Cleared in xylene for 2mins (x2)
8. Mounted in DPX

## **Appendix VI**

### **Nova Red Peroxidase**

5mls dH<sub>2</sub>O

3 drops Reagent 1 and mix

2 drops Reagent 2 and mix

2 drops Reagent 3 and mix

2 drops Hydrogen Peroxidase and mix

Filter through 0.22micron filter

## **Appendix VII**

### **LB Agar (Amp/IPTG/X-gal)**

1 tablet per 50mls (Sigma)

100µg/ml (

50mg/ml X-gal (Sigma)

500µM IPTG (Sigma)

## **Appendix VIII**

### **LB Broth**

1 tablet per 50mls (Sigma, UK)

100µg/ml ampicillin

## **Appendix IX**

### **Miniprep DNA Purification System**

1. Pellet cells – centrifuge max speed for 5 minutes.
2. Resuspend pellet in 250µl Cell Resuspension Solution.
3. Add 250µl Cell Lysis Solution and invert 4 times to mix.
4. Add 10µl Alkaline Protease Solution. Invert 4 times to mix and incubate at room temp for 5 minutes.
5. Add 350µl Neutralization Solution and invert 4 times to mix.
6. Centrifuge top speed for 10 minutes at room temp.
7. Insert spin column into collection tube and label both.
8. Remove cleared lysate and place into spin column.
9. Centrifuge at top speed for 1 minute and discard flow through.
10. Add 750µl Wash Solution and centrifuge at top speed for 1 minute and discard flow through.
11. Add 250µl Wash Solution and centrifuge at top speed for 2 minute and discard flow through.
12. Transfer column to sterile 1.5ml Eppendorf, add 100µl Nuclease free Water and centrifuge at top speed for 1 minute and store at -20°C.

## **Appendix X**

### **Explant Processing for Routine Histology**

Explants were

1. fixed in NBFS overnight
2. Dehydrated in graded series of alcohols
  - a. 70% ethanol for 30secs
  - b. 90% alcohol for 1min
  - c. 100% alcohol for 1min
  - d. 100% alcohol for 2mins
3. Cleared in xylene for 2mins (x2)
4. Placed in molten paraffin wax (VWR International) at 59°C for 1hour
5. Embedded in moulds and placed at 4°C

## Appendix XI

### Publications arising from this work

Marcus, P., De Bari, C., Dell'Accio, F., Dowthwaite, G. P., Archer, C. W. (2006) The *in vivo* plasticity of bovine chondroprogenitor cells in SCID mice. *Journal of Anatomy* **209**:pp561-79 (Poster presentation)

Marcus, P., De Bari, C., Dell'Accio, F., Dowthwaite, G. P., Archer, C. W. (2006) The *in vivo* fate of implanted chondroprogenitor cells in SCID mice. *European Cells and Materials* **12(suppl. 1.)**, 64 (Poster presentation)

Marcus, P., Bazou, D., Howard, M., Archer, C. W. (2008) Connexin43 expression in cartilage progenitor cells and its possible role in cell differentiation. *Journal of Anatomy* **212**:1pp80-96 (Poster presentation)

Marcus, P., Bazou, D., Archer, C. W. (2008) Connexin43 expression in cartilage progenitor cells and its possible role in cell differentiation. *European Cells and Materials* **16(suppl. 2.)**, 48 (Oral presentation)

Winner of award for best student oral presentation

embryonic tooth primordia from cultured stem cells. In animal studies we have shown that stem cells that are easily cultured such as embryonic stem cells, embryonic neural stem cells and adult bone marrow cells can all form tooth primordia that are able to develop into complete teeth in adults. Similarly studies are now ongoing with human stem cells.

34

#### Control of chondrogenesis and the assembly of cartilage matrix

T. Hardingham, S. Tew, R. Oldershaw and A. Murdoch

UK Centre for Tissue Engineering, Faculty of Life Sciences,  
University of Manchester

In the development of strategies for the repair of articular cartilage by tissue engineering, there is a need for cells with the potential to produce a mechanically competent cartilage matrix. We have investigated primary human articular chondrocytes isolated from OA cartilage removed at joint replacement. Results show that chondrocytes within 2–3 passages from isolation retain matrix forming ability, but that this is progressively lost with longer culture. However, retroviral transduction of the cells with the transcription factor SOX9 enables even late passage cells to reform matrix. The SOX9 transduced cells show improved response to chondrogenic signals provided by (a) culture in cell aggregates (b) growth factors and (c) hypoxic conditions. Chondrocytes from OA tissue were thus shown to have retained key elements of their phenotype and the expression of SOX9 was sufficient to re-activate their matrix forming potential.

Human adult bone marrow stem cells provide an alternative source of cells and we have investigated their differentiation into chondrocytes. In cell aggregate cultures there is evidence of early but transient Notch signalling. This appears to be necessary for chondrogenesis to proceed, but constituent activation of Notch signalling was found to block chondrogenesis. Notch signalling thus appears to be switched on to initiate chondrogenesis, but must be switched off for it to proceed. Gaining control of Notch signalling may therefore be helpful in directing stem cell differentiation into chondrocytes.

## POSTERS

P1

#### The *in vivo* plasticity of bovine chondroprogenitor cells in SCID mice

P. Marcus,<sup>1</sup> C. De Bari,<sup>2</sup> F. Dell'Accio,<sup>2</sup> G. Dowthwaite<sup>1</sup>  
and C. Archer<sup>1</sup>

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Articular cartilage is a complex tissue comprising phenotypically distinct zones with a low capacity for repair. Recent research has identified the presence of a progenitor cell population found in the surface zone of articular cartilage based on differential adhesion to fibronectin. These cells were shown to possess a long cell cycle time. Form colonies from a single cell and shared many of the properties found in mesenchymal stem cells.

The aim of the present study was to determine the *in vivo* plasticity of articular cartilage progenitor cells isolated from the surface of 7-day-old bovine metacarpal-phalangeal joints with anticipation of using them these cells to repair articular cartilage defects.

Chondrocytes were collected from the bovine metacarpal-phalangeal joints by fine dissection and progenitor cells were isolated by differential adhesion to fibronectin. To determine progenitor cell plasticity *in vivo*,  $2 \times 10^7$  cells ml<sup>-1</sup> were labelled with PKH26 and infected into the rear thigh muscle of 8-week-old, skeletally mature severe-combined immunodeficient (SCID) mice. Freshly isolated chondrocytes were used as a positive control and the negative control used were unlabelled human cells. After 2 weeks, the muscles were dissected to determine the presence of a cartilage pellet. If no pellet was present, the muscles were frozen for cryo-sectioning. Once the samples were sectioned they were examined under a fluorescent microscope and stained with toluidine blue.

Fluorescent cells were detected after 2 weeks in all the samples analysed. A cartilage pellet was only found in the positive control and no cartilage was evident with toluidine blue staining indicating that the cells are not producing a cartilage matrix in the muscle. The cells appeared to be forming muscle structures including perimysium, blood vessels and tendon.

The findings of this study indicate that the progenitor cell shows plasticity *in vivo* in a mammalian model. The presence of the fluorescent cells also suggested that the progenitor cell were able to engraft into muscle and the cells are able to survive *in vivo* for at least 2 weeks.

P2

#### Comparative study of elastic fibre gene expression *in vitro* and in a murine wound healing model

L. Bennett, R. A. Black, M. W. J. Ferguson and S. E. Herrick

UK Centre for Tissue Engineering, University of Manchester and  
University of Liverpool, UK

The ability of the skin to extend and recoil is mediated by an elastic fibre network comprising elastin molecules deposited on a microfibrillar scaffold. Studies have demonstrated reduced tensile strength in scar tissue following cutaneous wounding and application of skin substitutes, possibly due to decreased amounts of elastic fibres. This study aimed to document the expression of elastic fibre components using both an *in vitro* fibroblast populated fibrin lattice model and a murine excisional wound model. Adult human dermal fibroblasts were seeded in 3-dimensional fibrin gels, containing TGFβ1 and plasmin inhibitor, α-aminocaproic acid, to mimic the provisional wound matrix, and harvested at 48 h to 4 weeks. Excisional wounds (6 mm punch biopsy) were made on the dorsum of male Balb/c mice, and harvested 5 d to 6 month post-wounding in accordance with UK Home Office legislation. Samples were assessed for expression of fibrillin-1 and tropoelastin mRNA by real time reverse transcription PCR, and protein levels by immunolocalisation. Gene expression of fibrillin-1 by dermal fibroblasts in fibrin lattices peaked at 48 h, decreased three-fold by 1 week and remained at this level for 4 weeks. However tropoelastin mRNA levels increased 10-fold between 48 h and 2 weeks, then decreased by half between 2 and 4 weeks. Following excisional wounding fibrillin-1 and tropoelastin expression levels increased 3-fold between days 5 and 7 and remained elevated at 3 weeks post-wounding. Gene expression for fibrillin-1

## The in vivo Fate of Implanted Chondroprogenitor Cells in SCID Mice

P Marcus<sup>1</sup>, C. De Bari<sup>2</sup>, F. Dell'Accio<sup>2</sup>, G. Dowthwaite<sup>1</sup>, C. W. Archer<sup>1</sup>

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**INTRODUCTION:** Articular cartilage is a complex tissue comprising phenotypically distinct zones with a low capacity for repair. Recent research has identified the presence of a progenitor cell population found in the surface zone of articular cartilage based on differential adhesion to fibronectin<sup>1</sup>. These cells were shown to possess an extended cell cycle time, form colonies from a single cell and shared many of the properties of mesenchymal stem cells.

The aim of the present study was to determine the in vivo plasticity of articular cartilage progenitor cells isolated from the surface of 7 day old bovine metacarpal-phalangeal joints.

**METHODS:** Chondrocytes were collected from the bovine metacarpal-phalangeal joints by fine dissection and progenitor cells were isolated by differential adhesion to fibronectin and expanded in supplemented DMEM-F12 with 10% fetal calf serum<sup>1</sup>. To determine progenitor cell plasticity in vivo,  $2 \times 10^7$  cells  $\text{ml}^{-1}$  were labelled with PKH26 and injected into the rear thigh muscle of 8-week-old, skeletally mature severe-combined immunodeficient (SCID) mice. After 2 weeks, the muscles were dissected to determine the presence of a cartilage pellet. If no pellet was present, the muscle fibres from each sample were pooled and frozen for cryosectioning. Sections were then examined under a fluorescent microscope and stained with safranin O. Immunohistochemical analysis was carried out looking for sox9 and collagen type II.

**RESULTS:** Fluorescent PKH26 labelled cells were detected after two weeks in all the samples analysed. A cartilage pellet was present after intramuscular injection of freshly isolated full depth chondrocytes. After injection with a clonal population of chondroprogenitors, a distinct pellet was not present although regions of intense safranin O stain and sox9 expression corresponded to areas containing fluorescently labelled cells. Low levels of collagen type II were also detected in these areas. The intensity

of the staining was weaker than the staining seen after injection of full depth chondrocytes.



Fig. 1: Immunohistochemical analysis of chondroprogenitors in vivo labelled with PKH26 (red) and sox9 (green) (left image) and the same image with a nuclear stain (blue) (right).

**DISCUSSION & CONCLUSIONS:** The plasticity of bovine chondroprogenitors isolated from the surface zone of articular cartilage has already been demonstrated in vitro and in ovo<sup>1</sup>. Here we demonstrate that full depth chondrocytes maintain the chondrocytic phenotype when injected intramuscularly into the SCID mouse as shown by the presence of an intensely stained safranin O pellet and the expression of sox9 after 2 weeks in vivo. The injection of pure chondroprogenitor populations resulted in the absence of a distinct pellet but the presence of sox9 and PKH26 positive cells distributed throughout the muscle fibres. These results suggest that these progenitor cells can express and maintain a chondrogenic-like phenotype within a foreign tissue albeit at a low level. Current work is concentrated on analysing the expression of homotypic genes such as MyoD.

**REFERENCES:** <sup>1</sup> G. P. Dowthwaite, et al. (2004) The surface of articular cartilage contains a progenitor cell population *J Cell Sci* 117:889-97 <sup>2</sup> W. Bi, et al. (1999) Sox9 is required for cartilage development *Nat Genet* 22:85-9

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differentiation of human stem cells and produce specific neuron subtypes *in vitro*. Furthermore, this system acts as a useful model to investigate the molecular mechanisms and signalling pathways that control neural differentiation in man.

#### P24

##### **Presentation of extracellular matrix motifs by biomimetic substrates to control cellular attachment and differentiation**

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Many different factors influence cellular behaviour. *In situ* cells are subject to several different signalling mechanisms, including; cell-cell interactions, soluble molecules and the local environmental geometry. *In vitro* model systems have been developed to recreate the *in vivo* environment and hence control and regulate cellular behaviour using known signalling molecules. *In vivo* the extracellular matrix (ECM) is composed of many such signalling molecules and these have been extensively studied. For example culture substrates can be coated with ECM components such as laminin, collagen and fibronectin to enhance cell adhesion and differentiation. Coating substrates with these molecules has proved invaluable in the growth and maintenance of cell types that do not readily attach to uncoated substrates. Furthermore substrates coated with these molecules have been shown to enhance neuritogenesis and increase process outgrowth. However the process of coating substrates in this manner is limiting in that the procedure is time consuming, has poor control and reproducibility. In this study, we in collaboration with Orla Protein Technologies, are developing novel biomimetic substrates to control cell behaviour *in vitro*. The effects elicited by molecules can be attributed to motifs within the molecules. We present different motifs derived from the ECM molecules laminin, collagen and fibronectin using a biomimetic substrate. In this way we mimic the interaction between cells and their local environment experienced in tissues. We have evaluated the application of this technology on cell adhesion and differentiation using the well established rat pheochromocytoma (PC12) cell line. Our data indicate that this technology can be used to control cell attachment to the substrates. We also demonstrate that the biomimetic substrates can be used to control process outgrowth of PC12 cells following nerve growth factor induced differentiation. We propose this system as an alternative method for consistent attachment and differentiation of cells.

#### P25

##### **Connexin43 expression in cartilage progenitor cells and its possible role in cell differentiation**

P. Marcus,<sup>1</sup> D. Bazou,<sup>1</sup> M. Howard<sup>2</sup> and C. W. Archer<sup>1</sup>

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Recent research has identified the presence of a progenitor cell population in the surface zone of articular cartilage that possesses an extended cell cycle capable of forming large colonies from a single cell and can engraft functionally into a variety of connective tissues. There has been a relationship noted in some cell types between the expression levels of connexins and the initiation of differentiation in progenitor cells. In this study, the ability of the progenitor cells to express connexin43 and form functional gap junctions was examined. Initially cells were suspended in an ultrasound trap to create the formation of cell aggregates which were immunolabelled for connexin43. To determine if these connexin molecules were capable of forming functional gap junctions, cells were labelled with the gap junction permeable cell tracker CMFDA. Three groups of cells were used: surface zone cells (containing approximately 0.5% progenitors and 99.5% differentiated chondrocytes), a clonal progenitor population and surface/progenitor mix. In each case a 1:3 ratio of labelled:unlabelled cells was used. Although the progenitor cells do express connexin on their surface, especially at regions of cell-cell contact, they do not appear to form functional gap junctions. Non-progenitor cells isolated from the surface zone of articular cartilage are able to form functional gap junctions almost immediately after aggregate formation. When these cells are mixed with unlabelled progenitor cells, dye transfer also occurs from the non-progenitor surface zone cells toward the progenitor cells suggesting that even though the progenitor cells cannot form gap junctions together, they are capable of communicating with differentiated cells types. It is possible that signals transferred by more mature cells in the surface could be a signal for the progenitor cells to undergo differentiation.

#### P26

##### **Proteomic identification of neural stem cell biomarkers**

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Stem cell research suffers from a lack of protein markers which allow precise definition of cellular identity. Of the few antigens widely used to define neural stem/progenitor cells none are exclusive in these populations. Consequently accurate description of cellular status relies upon the measurement of multiple markers in combination. Comparative protein profiling offers rapid detection of multiple biomarkers making possible the high-throughput determination of cellular identity. Protein profiling via time-of-flight (TOF) mass spectrometry provides sample-specific peptide/protein mass signatures which can discriminate cell types without recourse to individual protein identification. Previously, surface-enhanced laser desorption ionization (SELDI)-TOF biomarker profiling has been successfully used in our laboratory to distinguish between control and differentiated cultures of a model stem cell system. Currently we are investigating the use of high accuracy matrix-assisted laser desorption ionization (MALDI)-TOF profiling to detect neural stem cell specific markers and to investigate proteins of neurogenic potential in conditioned media. Preliminary data indicate the potential to allow rapid evaluation of sample status, and to provide a lead into statistically significant protein candidates requiring further characterization. Findings may form the basis for the development of diagnostic immunological tools and novel growth supplements.

## Connexin43 expression in cartilage progenitor cells and its possible role in cell differentiation.

P. Marcus, D. Bazou, C.Archer

*Connective Tissue Biology Labs, School of Biosciences, Cardiff University, Wales, GB*

**INTRODUCTION:** Recent research has identified the presence of a progenitor cell population in the surface zone of articular cartilage<sup>1</sup>. These cells have been shown to possess an extended cell cycle, be capable of forming large colonies from a single cell and can engraft functionally into a variety of connective tissues. It is thought these cells are required for the appositional growth of the tissue. During normal skeletal development, one of the earliest events seen is the formation of a dense cell mesenchymal cell condensation. It is thought that an increase in gap junction formation within this cell condensation is responsible for passing signals between cells and directing differentiation<sup>2</sup>. A novel non-invasive method for observing cell interactions *in vitro* has recently been developed using an ultrasound wave. When placed in the ultrasound wave trap cells have been shown to form 2D aggregates without altering the cells in any way<sup>3</sup>. In this study, the ability of the progenitor cells to express connexin43 and form functional gap junctions was examined.

**METHODS:** Initially cells were suspended in an ultrasound trap to create the formation of cell aggregates which were immunolabelled for connexin43. To determine if these connexin molecules were capable of forming functional gap junctions, cells were labelled with the gap junction permeable cell tracker CMFDA. Three groups of cells were used: surface zone cells (containing approximately 0.5% progenitors and 99.5% differentiated chondrocytes), a clonal progenitor population and surface/progenitor mix. In each case a 1:3 ratio of labelled:unlabelled cells was used.

**RESULTS:** Although the progenitor cells do express connexin on their surface, they are incapable of transferring dye between cells. Non-progenitor cells isolated from the surface zone of articular cartilage are able to form functional gap junctions almost immediately after aggregate formation. When these cells are mixed with unlabelled progenitor cells, dye transfer also occurs from the non-progenitor surface zone cells toward the progenitor cells

**DISCUSSION & CONCLUSIONS:** It has already been demonstrated that connexin expression and gap junction formation are vital for articular cartilage development. This study shows that populations of progenitor cells isolated from the surface of bovine articular cartilage express connexin43 in culture. After aggregate formation, connexin43 expression occurs predominantly at regions of cell-cell contact. Despite this the cells are incapable of forming functional gap junctions with other progenitor cell populations after one hour in the ultrasound trap. Mixing the progenitor cells with terminally differentiated cells does however result in dye transfer, suggesting that they are capable of communicating with more differentiated cells types. It is possible that signals transferred by mature cells within the surface of cartilage could be a signal for the progenitor cells to undergo differentiation.

**REFERENCES:** <sup>1</sup>G.P. Dowthwaite, J.C. Bishop, S.N. Redman, I.M. Khan, P. Rooney, D.J. Evans, L. Haughton, Z. Bayram, S. Boyer, B. Thomson, M.S. Wolfe and C.W. Archer (2004) *J Cell Sci* 117:889-97 <sup>2</sup>B. Zimmermann, (1984) *J Anat* 138(2):351-63. <sup>3</sup>W.T. Coakley, D. Bazou, J. Morgan, G.A. Foster, C.W. Archer, K. Powell, K.A. Borthwick, C. Twomey, and J. Bishop (2004) *Colloids Surf B Biointerfaces* 34: 221-30.

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