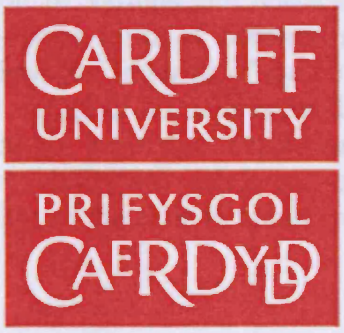
Characterisation of Articular Cartilage Progenitor Cells: Potential Use in Tissue Engineering

A thesis submitted in candidature for the degree of Doctor of Philosophy of the University of Wales



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ABSTRACT

Articular cartilage is a resilient and load bearing material that provides diarthrodial joints with excellent friction, lubrication and wear characteristics required for continuous motion. However, articular cartilage has a poor regenerative capacity and its degeneration is a common cause of morbidity in terms of loss of joint function and osteoarthritis, frequently resulting in the need for total knee replacement.

Articular cartilage has a distinct zonal architecture with biochemical and cellular variations existing from the surface zone to the deeper calcified layers. Thus, the development of the tissue must be stringently controlled, both spatially and temporally in order for the complex structure to be established. Importantly, the surface zone is believed to be responsible for the appositional growth of articular cartilage during development and this growth is believed to be driven by a population of slow cycling progenitor cells within the surface zone itself.

The focus of this thesis is the isolation and characterisation of articular cartilage progenitor cells together with an exploration of the cells capabilities in potential cartilage repair therapies. The cells were identified on the basis of differential adhesion assays and colony forming ability. Subsequent experiments were carried out to show the differential expression of various cell surface markers eg Notch 1 receptors and the role of the onco-foetal form of fibronectin, known as fibronectin-EDA on the modulation of cell behaviour. In terms of the potential of the cells for use in tissue engineering, a promising feature of the cells is the discovery that enriched populations of the cells can undergo extensive expansion in simple monolayer cultures and yet retain their ability to undergo chondrogenic differentiation. This property may enable the use of the cells in commercial cartilage repair and/or tissue engineering strategies.

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ABBREVIATIONS

ACI	Autologous chondrocyte implantation
bFGF or FGF-2	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine
CFE	Colony forming efficiency
DMEM	Dulbecco's modified eagle medium
ECM	Extra-cellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
FCS	Foetal calf serum
FITC and TRITC	Fluorescein and tetramethylrhodamine isothiocyanate
FN	Fibronectin
FTCs	Full thickness chondrocytes
GAG	Glycosaminoglycan
HS	Human serum
IGF-I and –II	Insulin-like growth factor –I and II
IgG	Immunoglobulin G
IL-1β	Interleukin-1 beta
IMS	Industrial methylated spirits
MMP	Matrix metalloproteinase
MSCs	Mesenchymal stem cells
OA	Osteoarthritis
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDs	Population doublings
SZCs	Superficial zone cell populations enriched in articular cartilage progenitor cells
$TGF\beta_{1-3}$	Transforming growth factor beta 1-3
TIMP	Tissue inhibitors of matrix metalloproteinase
TNF-α	Tumour necrosis factor alpha

CHAPTER 1

INTRODUCTION

1.1 ARTICULAR CARTILAGE STRUCTURE, FUNCTION AND DEVELOPMENT

1.1.1 Introduction

Articular cartilage is a resilient and load-bearing material that provides diarthrodial joints with excellent friction, lubrication and wear characteristics required for continuous gliding motion and also acts as a shock absorber to distribute applied loads over the subchondral bone (Mow et al., 1992; Mankin et al., 1994). Although articular cartilage varies in thickness, cell density, matrix composition, and mechanical properties within the same joint, among joints, among species and with age, in all synovial joints it consists of the same components, the same general structure and performs the same functions (Stockwell, 1971; Athanasiou et al, 1991). Despite articular cartilage being in the order of only a few millimetres in thickness, its unique structure and composition allow it to withstand considerable biomechanical forces over many decades of walking, running and jumping (Buckwalter and Mankin, 1997; Mow et al., 1992).

The uniqueness of articular cartilage arises from the tissue being primarily composed of extracellular matrix with relatively few cells, known as chondrocytes. There is no direct blood supply, relying on diffusion for nutrition. Articular cartilage lacks nerves and a lymphatic system (Mankin et al., 1994). Although at first glance the tissue may appear to be of a simple nature, detailed examination reveals it to possess a distinct, highly ordered structure (Jeffery et al., 1991; Poole et al., 2001) that facilitates a complex interaction between the extracellular matrix and the chondrocyte (Benjamin et al., 1994; Buckwalter and Mankin, 1997) which serves to actively maintain the tissue throughout the life of the organism.

Articular cartilage has a limited capacity for repair (Campbell, 1969), a consequence of the unique properties described above. The chondrocytes have a low mitotic ability and are restricted in their ability to migrate to the site of injury due to the cells being individually enclosed in extracellular matrix. In addition, the lack of a blood supply eliminates the prospect of a repair response initiated and orchestrated by the process of inflammation (Newman, 1998). The lack of an effective reparative response means

that limited damage to articular cartilage due to trauma or from degenerative diseases will remain unhealed. Such lesions, over time, may progress to more severe and progressive disabilities of the joint, culminating in total erosion of the articular cartilage and loss of joint function. Total or partial joint replacement procedures are routinely used to restore pain free motion as a last resort in the treatment of end-stage degenerative joint disease (Buckwalter and Lohmander, 1994; Buckwalter and Mankin, 1997a; Gilbert, 1988; Newman, 1998; Hunziker, 2001a).

This section presents an overview of the current understanding of articular cartilage structure, function and development in order to provide a basis for understanding its degeneration, current repair techniques and the prospects for more efficacious future therapies.

1.1.2 The chondrocyte

Articular cartilage is composed of a single cell type known as the chondrocyte which occupy around only 10% of the total tissue volume (Archer and Francis-West, 2003). This figure varies highly, primarily depending upon factors such as age and species (Buckwalter and Mankin, 1997). Chondrocytes differ in size, shape and metabolic activity throughout the thickness of the tissue (Zanetti et al, 1985; Aydelotte and Kuettner, 1988; Aydelotte et al., 1988; Archer et al., 1990) and these differences in chondrocyte phenotype are responsible for subtle differences in matrix composition throughout the depth of the tissue (Poole et al., 2001) (this aspect will be covered in greater detail in section 1.1.3). The structure of articular cartilage is illustrated in figure 1.1. Despite these location specific differences, chondrocytes share a number of features which together distinguish them from other cell types. The chondrocytes are metabolically active and are responsible for the synthesis and maintenance of a unique and stable extracellular matrix with which the chondrocytes surround themselves preventing the formation of cell-cell contacts. The chondrocytes are primarily spherical in shape, synthesise type II collagen and aggrecan (a large aggregating proteoglycan) (Buckwalter and Mankin, 1997) and various other minor collagens, proteoglycans and non-collagenous proteins (Neame et al., 1999; Poole et al., 2001). These matrix components are assembled by the chondrocyte into the complex structure that gives articular cartilage its characteristic properties. This complex

structure needs to be constantly maintained by the cell, involving replacement of degraded matrix components and alterations in the macromolecular composition and framework in response to loading of the joint during use (Buckwalter and Mankin, 1997). Thus the cells must sense these changes in the extracellular matrix composition due to degradation and loading and respond accordingly (Buschmann et al., 1995; Quinn et al., 1998; Durrant et al., 1999; Salter et al., 2001; Smith et al., 2004).

In the adult organism articular cartilage is avascular, thus chondrocytes derive most of their nutrition from the synovial fluid. The nature of this system results in a low oxygen concentration in the tissue relative to other tissues as nutrients must first diffuse through the synovial fluid and then through the cartilage matrix which is also restrictive with respect to molecular size and charge. Therefore, the chondrocyte depends primarily on glycolytic metabolism (Buckwalter and Mankin, 1997).

Chondrocyte activity and density within articular cartilage differs significantly between the phase of skeletal growth during foetal development and in the early stages of life and the phase when skeletal growth has ceased in the adult (Mankin et al., 1994). Cartilage is formed from undifferentiated mesenchymal condensations in the foetus (Archer et al., 1994; Archer and Francis-West, 2003) and subsequent matrix synthesis leads to the separation of cells which assume a spherical morphology. At these early stages in the growth of articular cartilage the cell density and metabolic activity is high as the chondrocytes proliferate rapidly and synthesise large quantities of matrix, thus driving growth. With skeletal maturity, metabolic activity, matrix synthesis and cell division declines and hence cell density declines (Stockwell, 1967; Leutert, 1980; Buckwalter and Mankin 1997). In the mature skeleton, articular chondrocytes rarely undergo mitosis under normal physiological conditions, although still synthesise collagens, proteoglycans and other matrix components in an ongoing maintenance of the macromolecular framework. With ageing, the capacity of the cells to synthesise certain components of the matrix (Thonar et al., 1986; Bolton et al., 1999) and respond to stimuli, such as growth factors, decreases (Guerne et al., 1995; Loeser et al., 2000), thus limiting the ability of the cell to maintain the tissue. These age-related changes may ultimately contribute to the development of degenerative joint disease (Roughley, 2001).

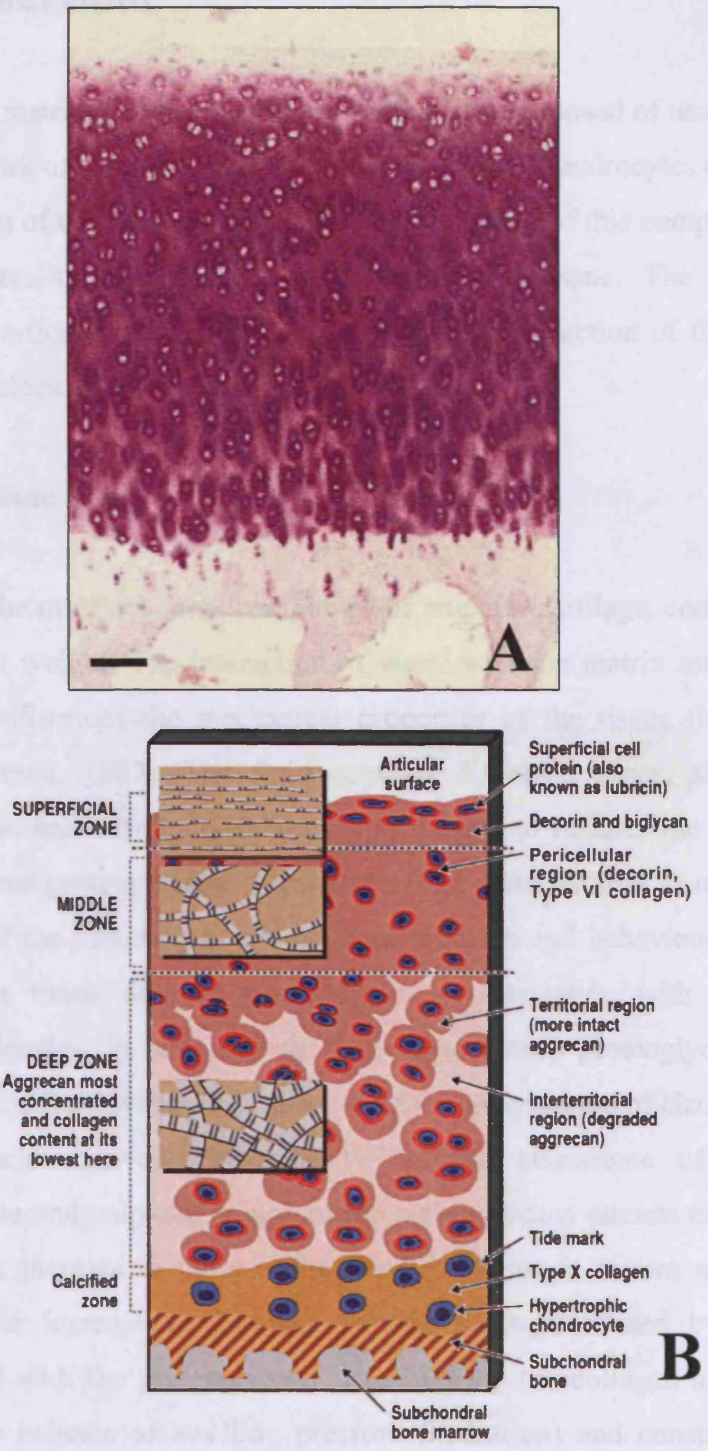


Figure 1.1: Histological section of articular cartilage (canine) stained with haematoxylin and eosin (Bar = 100μm) (A) and schematic representation of human articular cartilage illustrating the different zones and diameter and orientation of collagen macrofibrils (inset) (B). Taken from Breinan et al., (2001) and Poole et al., (2001) respectively.

1.1.3 Extracellular matrix

The extracellular matrix (ECM) of articular cartilage is composed of tissue fluid and a complex framework of structural macromolecules. As the chondrocytes comprise only a small proportion of the total volume, the chemical nature of this complex molecular framework dictates the mechanical properties of the tissue. The stiffness and resilience of the articular cartilage is provided by the interaction of the tissue fluid with the macromolecular framework.

1.1.3.1 Tissue fluid

Water is the most abundant component of articular cartilage, contributing 80% of the wet weight. The interaction of water with the matrix macromolecules strongly influences the mechanical properties of the tissue (Maroudas and Schneiderman, 1987). The fluid contains dissolved gases, small proteins, metabolites and a high concentration of cations to balance the abundance of poly-anionic proteoglycans. A portion of the water is able to move freely in and out of the tissue. The volume, concentration and behaviour of the water within the tissue depends primarily on its interaction with the structural macromolecules, in particular the large aggregating proteoglycans that help maintain the fluid within the matrix and the concentration of electrolytes in the fluid (Buckwalter and Mankin, 1997). The abundance of poly-anionic carboxylate and sulphate groups of the proteoglycans attracts cations such as Na+. This increase in the osmolarity of the tissue is known as the Donnan effect. The increase in Donnan osmotic pressure caused by the cations associated with the proteoglycans is resisted by the collagen network. Thus, there is a balance of swelling pressure (hydration) and constraining forces (collagen network) leading to the formation of a cohesive and strong solid gel.

1.1.3.2 Structural macromolecules

Collagens, proteoglycans and non-collagenous proteins comprise the structural macromolecules of the tissue. Collagens contribute about 60% of the dry weight; proteoglycans 25-35%; non-collagenous proteins and glycoproteins

15-20%. Collagens are distributed evenly throughout the thickness of the cartilage (except for the collagen rich superficial zone) and the collagen fibrillar network provides the tensile strength. Proteoglycans bind to the collagenous meshwork or become mechanically entrapped within it. The framework is organised and stabilised by non-collagenous proteins (Buckwalter and Mankin, 1997).

1.1.3.3 Collagens

More than 20 different collagen types have been identified so far (Gelse et al., 2003), with types II, VI, IX, X and XI being present in articular cartilage (Mankin et al., 1994). Collagens contain a characteristic triple-helical structure composed of three polypeptide α-chains. The α-chains of collagen types II and XI aggregate together to form cross-banded fibrils that can be seen with electron microscopy. The fibrils are organised into a tight meshwork, providing tensile stiffness and mechanically entraps large proteoglycans (figure 1.2). Collagen types VI, IX and X do not form fibrils and are termed non-fibrillar collagens.

The major cartilage collagen which represents 90-95% of the total, is type II (Mankin et al., 1994; Buckwalter and Mankin, 1997). Type IX collagen molecules bind to the surfaces of the type II fibrils and project into the matrix and may also bind covalently to other collagen type IX molecules. Type XI collagen molecules bind covalently to type II collagen molecules and may form part of the interior structure of the cross-banded fibrils. Although the precise function of type IX and XI is uncertain, they probably help in the formation and stabilisation of the fibrils and stabilise both the collagenous meshwork and the collagen-proteoglycan interaction (Bruckner et al., 1988; Mankin et al., 1994). Type VI collagen forms an important part of the matrix immediately surrounding the chondrocyte and may be involved with matrix stabilisation and chondrocyte attachment to the matrix (Marcelino and McDevitt, 1995; Keene et al., 1988). Type X collagen is located only in the calcified cartilage zone at the junction of the articular cartilage with the

subchondral bone, suggesting a role in mineralisation (Schmid and Linsenmayer, 1985).

1.1.3.4 Proteoglycans

Proteoglycans are complex macromolecules that consist of a protein core to which are linked extend glycosaminoglycan (GAG) chains. Approximately 80-90% of all proteoglycans in articular cartilage are of the large aggregating type called aggrecan (Mankin et al., 1994; Hardingham and Fosang, 1995) the structure of which is illustrated in figure 1.3. These proteoglycans consist of a large extended protein core to which are attached up to 100 chondroitin sulphate (CS) and 50 keratan sulphate (KS) glycosaminoglycans chains. The protein core is large and complex with several distinct globular and extended domains. One extended domain contains the majority of KS GAG chains which is adjacent to a large extended domain rich in attached CS GAG chains. A G1 globular domain at the N-terminal end of the protein core functions to non-covalently bind the proteoglycan to hyaluronate, and this binding is stabilised by link protein. As the GAG hyaluronate chain may be long and unbranching, many proteoglycans chains (up to 300) may bind, forming a large proteoglycan aggregate (Hardingham and Fosang, 1995). The formation of the large aggregates helps anchor the proteoglycans within the matrix preventing their displacement during deformation of the tissue. Versican is another hyaluronate binding proteoglycan from the same family as aggrecan, that also includes neurocan and brevican (Margolis and Margolis, 1994). Versican is present in articular cartilage to a lesser extent than aggrecan and may function (together with aggrecan) to stabilise the ECM and cell-matrix interactions (Chen et al., 2003).

Besides the large aggregating proteoglycans in cartilage there are small, leucine-rich proteoglycans including decorin, biglycan, fibromodulin, perlecan and lumican (Roughly and Lee, 1994; Poole et al., 1996a and 2001). Type IX collagen is also considered a proteoglycan as it contains a CS chain (Mankin et al., 1994). Decorin has one dermatan sulphate chain, biglycan has two dermatan sulphate chains and fibromodulin has several KS chains. Perlecan

contains heparan sulphate whereas lumican contains KS. These small non-aggregating proteoglycans have shorter protein cores than do aggrecan molecules and do not fill a large volume of the tissue or contribute directly to the mechanical behaviour. Instead these proteoglycans may bind to other macromolecules and/or influence cell behaviour. Decorin and fibromodulin bind type II collagen and may be involved with fibrillogenesis, organising and stabilising the type II meshwork (Hedbom and Heinegard, 1993; Mankin et al., 1994; Roughly and Lee, 1994; Poole et al., 2001). Biglycan is localised in the pericellular matrix and may interact with type VI collagen (Roughly and Lee, 1994).

1.1.3.5 Non-collagenous and non-proteoglycan components

Other matrix components include anchorin CII (a collagen binding chondrocyte surface protein involved in anchoring the chondrocyte to the collagen fibrils of the matrix), COMP (cartilage oligomeric protein), matrillin-I, CILP (cartilage intermediate layer protein), fibronectin and tenascin. The functions of the components is not clearly understood but may be involved in organisation and maintenance of the macromolecular structure of the matrix and cell-matrix interactions (Mollenhauer et al., 1984; Hedbom et al., 1992; DiCesare et al., 1994; Chevalier et al., 1994 and 1996; Lorenzo et al., 1998; Neame et al., 1999; Poole et al., 2001).

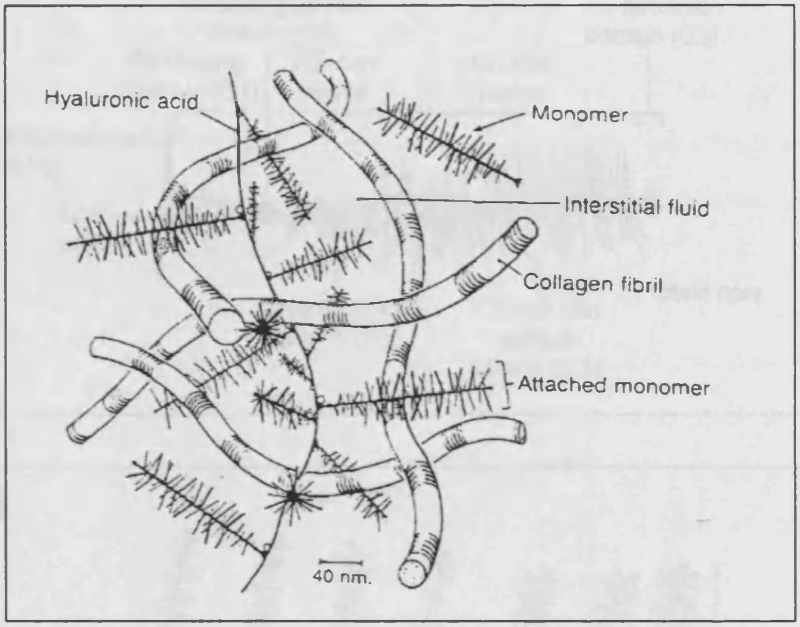


Figure 1.2: Molecular organisation of the solid matrix of articular cartilage as a fibre-reinforced composite solid matrix. The swelling pressure exerted by the proteoglycan keeps the collagen network inflated. Taken from Mankin et al., 1994.

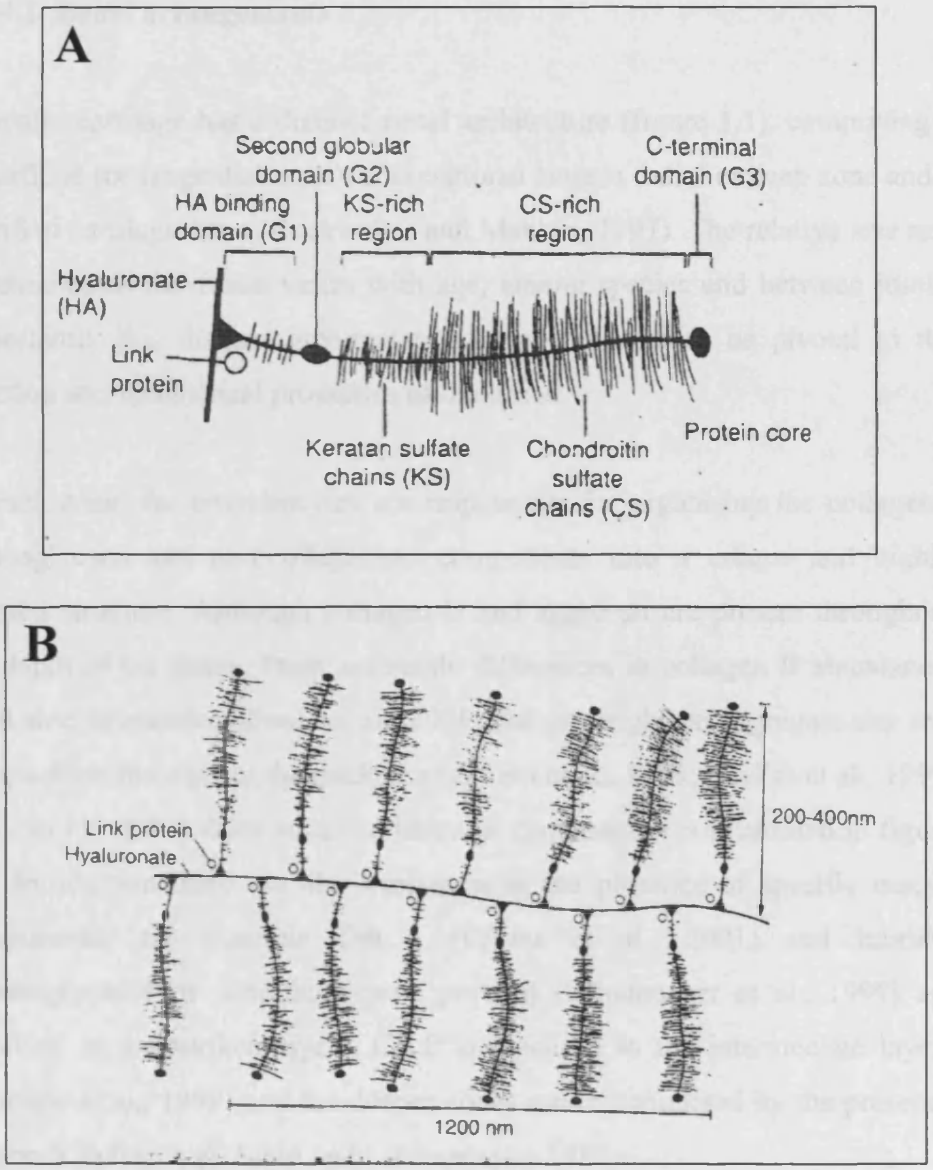


Figure 1.3: (A) Schematic diagram illustrating the structure of the aggrecan molecule and its binding to hyaluronate. The protein core has several globular domains (G1, G2, and G3), with other regions containing the keratan sulphate and chondroitin sulphate glycosaminoglycan chains. The N-terminal G1 domain is able to bind specifically to hyaluronate. This binding is stabilised by link protein. (B) Schematic diagram illustrating arrangement of aggrecan monomers as an aggregate which would be immobilised within the collagen network. Taken from Mankin et al., 1994.

1.1.4 Architecture of articular cartilage

1.1.4.1 Zonal arrangements

Articular cartilage has a distinct zonal architecture (figure 1.1), comprising a superficial (or tangential zone), a transitional zone, a radial or deep zone and a calcified cartilage zone (Buckwalter and Mankin, 1997). The relative size and appearance of the zones varies with age, among species and between joints. Importantly this distinct architecture is now believed to be pivotal to the function and mechanical properties of the tissue.

In each zone, the chondrocytes are responsible for organising the collagens, proteoglycans and non-collagenous components into a unique and highly ordered structure. Although collagen II and aggrecan are present throughout the depth of the tissue, there are subtle differences in collagen II abundance, fibril size, orientation (Poole et al., 2001) and proteoglycan aggregate size and composition throughout the thickness (Zanetti et al., 1985; Bayliss et al., 1999; Poole et al., 2001). This zonal variation in composition is illustrated in figure 1.1. In addition there are also variations in the presence of specific matrix components, for example Del 1 (Pfister et al., 2001) and lubricin (Proteoglycan-4 or superficial zone protein) (Schumacher et al., 1999) are localised in the surface layers. CILP is localised to the intermediate layers (Lorenzo et al., 1998) and the deeper zones are characterised by the presence of type X collagen (Schmid and Linsenmayer, 1985).

This biochemical heterogeneity is paralleled by variations in chondrocyte cellularity, morphology and metabolic activity in each of the different zones (Zanetti et al, 1985; Aydelotte and Kuettner, 1988; Aydelotte et al., 1988; Archer et al., 1990). In the superficial zone, the cells are ellipsoid-shaped and arranged parallel to the articular surface. In the transitional zone the cells assume a spheroidal shape in the deeper zone the cells lie in columns which are arranged perpendicular to the joint surface (Buckwalter and Mankin, 1994). Superficial zone cells in culture are known to secrete less proteoglycan than cells lying deeper in the tissue (Aydelotte and Kuettner, 1988) and this

proteoglycan contains a lower level of KS GAG chains (Zanetti et al., 1985). These properties of cultured chondrocytes isolated from the specific zones correspond to the known biochemical composition throughout the thickness of the tissue. Hence the biochemical composition and consequently biophysical properties of the matrix at different depths is a reflection of the specific metabolic activities of the cells within the particular zone.

1.1.4.2 Regional variations

Within the zones there are variations or regions in the cartilage matrix components, illustrated in figure 1B. These are known as the pericellular, territorial and inter-territorial regions (Mankin et al., 1994; Buckwalter and Mankin, 1997; Poole et al., 2001). In the pericellular and territorial regions the chondrocytes bind to matrix macromolecules and protect the cells from damage during loading of the tissue. These regions may also act as a system whereby signals are transmitted to the chondrocyte following loading and deformation of the cartilage, thus allowing the chondrocyte to sense mechanical stimuli and respond appropriately (Buschmann et al., 1995; Quinn et al., 1998; Durrant et al., 1999; Salter et al., 2001). The primary function of the inter-territorial region is to provide the mechanical properties of the tissue (Mankin et al., 1994).

Pericellular region: The pericellular matrix covers the cell surface, is rich in proteoglycans and contains little collagen. Also present is the cell membrane-associated molecule Anchorin CII (Mollenhauer et al., 1984) and the non-fibrillar type-VI collagen (Marcelino and McDevitt, 1995). Cytoplasmic extensions project from the chondrocyte through the pericellular matrix and into the territorial matrix.

Territorial region: The territorial matrix envelopes the pericellular matrix. The thin collagen fibrils of the territorial matrix closest to the cell appears to bind to the pericellular matrix whereas more distant to the cell the fibrils bisect at various angles to form a fibrillar basket around the cells. This basket structure

is thought to provide protection for the chondrocyte from mechanical loading (Mankin et al., 1994; Buckwalter and Mankin, 1997).

Inter-territorial region: This region makes up most of the volume of the tissue of adult, mature articular cartilage and contains the largest diameter collagen fibrils. In the superficial zone the fibrils are thinner and arranged parallel with the joint surface. In the transitional zone the fibrils lie at oblique angles relative to the joint surface and in the radial zone the fibrils are arranged perpendicular to the surface (Mankin et al., 1994; Buckwalter and Mankin, 1997).

1.1.5 Changes in structure with age

The development of articular cartilage in the embryo and foetus will be discussed in the section 1.1.6. Following birth there is a shift from the immature to the mature form of articular cartilage (Mankin et al., 1994). Articular cartilage from neonatal or immature animals is relatively thick and blue-white in colour. The thickness of the articular cartilage reduces as ossification from the secondary centre of ossification progresses. In addition, immature articular cartilage is considerably more cellular than mature adult articular cartilage (Leutert, 1980) and there are also differences in the thickness of the various articular cartilage zones between immature and mature cartilage.

Cell replication in immature articular cartilage occurs at two distinct regions: one region occurs subjacent to the articular surface and probably accounts for the growth of the tissue during development. The second region occurs above the subchondral plate (Mankin, 1962). As the cartilage matures, proliferation becomes confined to the region above the subchondral plate and proliferation at the articular surface ceases. All mitotic activity ceases in the adult, coinciding with the development of a well-defined tidemark.

In addition to changes in cellularity there are also changes in matrix composition with age. Water content is higher in immature tissue and slowly reduces to a static level that remains constant throughout adulthood. In addition, the collagen content of foetal

articular cartilage is lower than that of mature cartilage, with collagen concentration rising after birth and maintained throughout life. The major changes that take place in the cartilage matrix occur with the proteoglycans. Proteoglycan content is highest at birth and diminishes slowly throughout life. In addition, the length of the protein core and GAG chains reduce with age, together with the level of proteoglycan aggregation. There is also a shift towards reduction in the level of chondroitin sulphate relative to keratan sulphate (Thonar et al., 1986; Mankin et al., 1994; Bolton et al., 1999). There are subtle differences in the composition of GAG chains of minor proteoglycans such as decorin, fibromodulin, biglycan and lumican with age. Some of the changes are due to variations in synthesis whereas others are due to variations in degradation (Roughly, 2001). Although the functional consequences of these changes remains unclear it is likely some of the alterations in composition may predispose the tissue to degenerative changes.

1.1.6 Development of articular cartilage

Embryonic skeletal development involves the recruitment, commitment, differentiation and maturation of mesenchymal cells into cartilage and bone along the intramembranous and endochondral ossification pathways. The process of endochondral ossification begins during the sixth week of human embryonic development when mesenchymal cells differentiate, condense, and transform into chondrocytes which form a cartilaginous model of the early skeleton (Archer, 1994; DeLise et al., 2000; Vortkamp, 2001; Tuan, 2004). In the central region of the cartilaginous anlage, the chondrocytes hypertrophy and the matrix begins to calcify. Subsequently a periosteal sleeve of bone forms around the periphery of the anlage in the central portion. By the eighth week, capillary buds invade this central portion of the hypertrophied and calcified cartilaginous anlage, which brings mesenchymal cells to the region which subsequently differentiate into osteoblasts and osteoclasts (Iannotti, 1990). The osteoblasts lay down an osteoid matrix on the spicules of calcified cartilage forming trabecular bone. At this stage, the primitive long bone element now consists of a central diaphyseal region of trabecular bone with cartilaginous regions at both epiphyseal ends. At both interfaces between the epiphyseal cartilaginous regions and the central bone portion the growth plates form. Longitudinal bone growth at the growth plates is driven by proliferation of pre-

chondrocytes, with subsequent chondrogenic differentiation and hypertrophy, terminal differentiation and mineralisation. At around birth, the epiphyseal cartilaginous regions ossify through secondary centres of ossification which form in the epiphyseal ends (Iannotti, 1990).

The mechanism underlying chondrocytic differentiation during endochondral ossification at epiphyseal growth plates has been the focus of much research in recent years (Sandell and Adler, 1999; DeLise et al., 2000; Vorthamp, 2001). Cartilage growth at epiphyseal plates is regulated by a complex series of molecular signals such as bFGF, IGF-I, Indian Hedgehog, parathyroid hormone related peptide, GDF-5, bone morphogenetic proteins and wingless signalling systems (Francis-West et al., 1999; Sandell and Adler, 1999; Volk and Leboy, 1999; DeLise et al., 2000; Vorthamp, 2001; Buxton et al., 2003; Tuan, 2004). The factors driving the growth of articular cartilage during development have received comparatively less attention, possibly a result of a presumption that articular cartilage represents the remnants of the embryonic epiphysis that fails to be replaced by bone during endochondral ossification (Archer et al., 2003). Although this may still be case, it is now becoming clear that the development of articular cartilage is a complex process involving numerous signalling mechanisms regulated in a temporal and spatial manner (Archer, 1994; Archer et al., 1994; Hayes et al., 2001; Hayes et al., 2003; Archer et al., 2003; Dowthwaite et al., 2004). In addition, complex temporal and spatial changes in patterns of matrix components of articular cartilage during growth have also been described (Morrison et al., 1996; Archer et al., 1996). In view of the well documented limited repair capacity of articular defects (Campbell, 1969; Hunziker, 2001a), understanding the mechanisms underlying the development of articular cartilage and the cellular and molecular processes involved in tissue patterning and morphogenesis may greatly facilitate the development of regenerative approaches to cartilage repair.

Of particular interest is the manner by which, during development, articular cartilage shifts from an immature, highly cellular and predominantly isotropic tissue with collagen orientation running parallel to the articular surface, to a mature, anisotropic tissue with a distinct zonal architecture with decreased cellularity (Meachim, 1969; Leutert, 1980; Mankin et al., 1994) and with collagen fibres predominantly perpendicular to the articular surface in the basal regions which then arch over to

become parallel at the surface (Jeffrey et al., 2001). Does this change in matrix structure occur through remodelling of the existing matrix or by replacement of the existing matrix (Hayes et al., 2001; Archer et al., 2003)?

How this shift occurs is likely to be explained by understanding the mechanism by which articular cartilage develops following joint cavitation. As described earlier, early in development, condensation of pre-chondrogenic mesenchyme, via N-cadherin, N-CAM and CD44 interactions, begins the process of the formation of the cartilaginous skeleton (Archer, 1994; DeLise et al., 2000; Vortkamp, 2001; Tuan, 2004). Initially in the location of the prospective joint there is cartilaginous continuity which then segments secondarily through the formation of a non-cartilagenous region known as the interzone (Craig et al., 1987; Archer et al., 1994; Archer et al., 2003). The interzone comprises a thin layer of elongated cells closely compacted between the developing articular surfaces. The interzone becomes an important signalling centre (Hartman and Tabin, 2000) as the elements begin to oppose, and joint cavitation occurs, driven by the selective high-level synthesis of hyaluronan (Dowthwaite et al., 1998; Ward et al., 1999) by interzone cells and presumptive synovial cells. The interzone subsequently disperses during cavity enlargement as the articular surfaces further develop.

In immature rabbit articular cartilage, two zones of proliferation are evident, one above the subchondral plate, one just beneath the articular surface. When skeletal maturity is reached, no zones of proliferation can be observed (Mankin, 1962 and 1964). This observation is suggestive that articular cartilage growth occurs by apposition from the articular surface rather than by interstitial mechanisms. Subsequent studies using BrdU labelling in the articular cartilage of *Monodelphis domestica* (a marsupial born with the hind limbs at an early stage of development) has indeed demonstrated that subsequent to the formation of the secondary centre of ossification, the surface zone is responsible for the appositional growth of articular cartilage during development (Hayes et al., 2001). In addition, other studies have shown that the surface region has a central role in joint cavitation (Ward et al., 1999) and that many growth factors such as IGF-I and –II, and $TGF\beta_{1-3}$ and their receptors are also highly expressed in this region (Archer et al., 1994; Hayes et al., 2001).

Although the above evidence illustrates that the surface region plays a key role in the development and growth of articular cartilage, such a growth mechanism would require the presence of a population of progenitor cells in the surface of the tissue. These cells would generate new progeny which would then go onto expand within the transitional zone and progress to terminal differentiation in the upper and lower radial zones (figure 1.4). The extensive hypertrophy that accompanies terminal differentiation during endochondral ossification need not occur in the case of articular cartilage as there is no requirement for longitudinal growth. The deeper zones would be remodelled and resorbed during endochondral ossification (Hayes et al., 2001).

It is possible, the identification and characterisation of the progenitor cells present within the surface regions would further knowledge of cartilage developmental mechanisms and may provide insights into potential novel tissue repair routes.

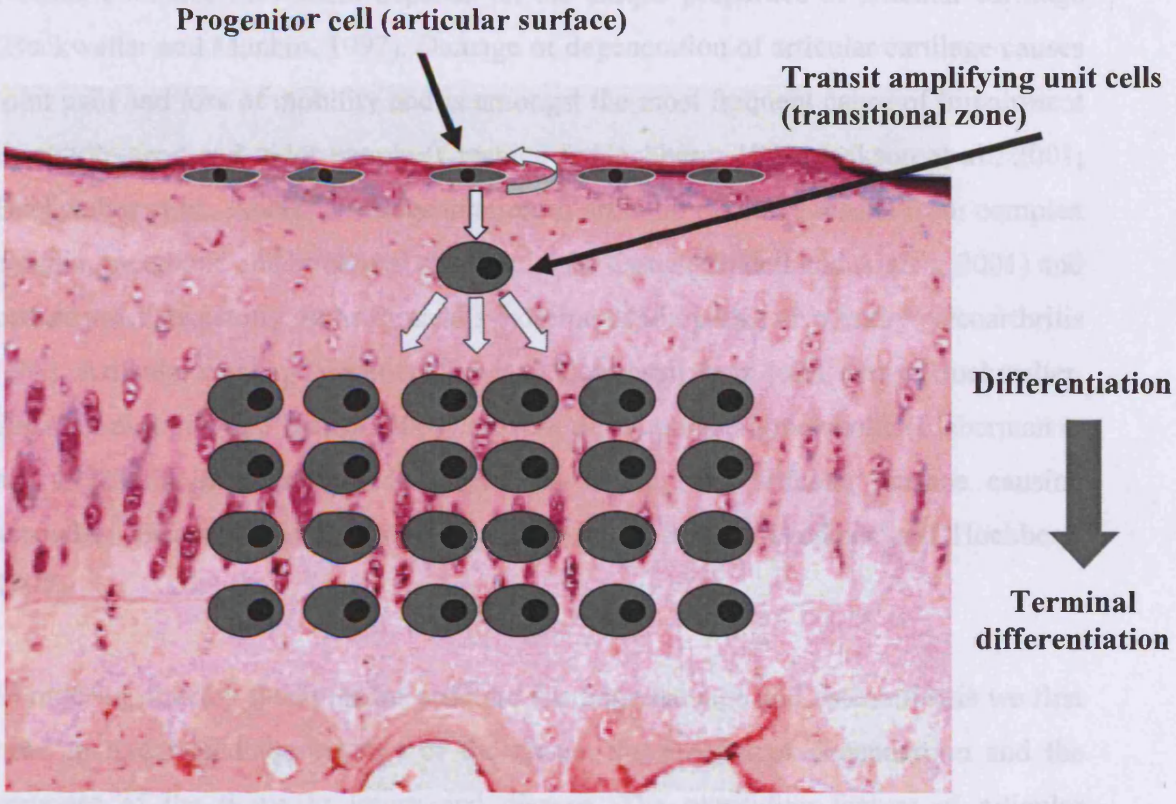


Figure 1.4: Diagram summarising proposed cell lineage of articular cartilage. Progenitor cells in the articular surface divide to give to daughter cells, one being another progenitor cell, the other being a transit-amplifying cell within the transitional zone. The transit amplifying unit cell can then undergo further cell divisions along the chondrocyte differentiation pathway. Note that the maturing chondrocytes do not migrate through the matrix. Rather, as the articular cartilage grows through apposition, the relative position of the original transit amplifying cells moves relative to the original progenitor cells, which remain at the articular surface. Adapted from Hayes et al., 2001.

1.2 ARTICULAR CARTILAGE INJURY AND OSTEOARTHRITIS

1.2.1 Introduction

Normal pain free movement depends on the unique properties of articular cartilage (Buckwalter and Mankin, 1997). Damage or degeneration of articular cartilage causes joint pain and loss of mobility and is amongst the most frequent cause of impairment in middle-aged and older people (Creamer & Hochberg, 1997; Jackson et al., 2001; Buckwalter et al., 2004). The degeneration of articular cartilage results from complex cellular, metabolic and structural changes in the tissue (Sandell and Aigner, 2001) and occurs most frequently in the clinical syndrome of idiopathic or primary osteoarthritis (OA). Articular cartilage degeneration may also result from joint injury (Buckwalter, 2002; Buckwalter and Brown, 2004) or from developmental, metabolic (Lieberman et al., 1992) or inflammatory disorders that destroy the articular surface causing secondary osteoarthritis (Buckwalter and Mankin, 1997a; Creamer and Hochberg, 1997).

In order to develop therapies for articular cartilage damage and osteoarthritis we first need to understand the biology of the tissue, the process of degeneration and the response of the tissue to injury and disease. The overriding feature of articular cartilage that pre-disposes the tissue to degeneration is the intrinsic lack of a reparative capacity of the tissue following injury and disease (Campbell, 1969; Newman, 1998) and was first described in the scientific literature centuries ago (Hunter, 1743). Although a limited attempted repair response is evident following injury (Campbell, 1969) the repair is ineffectual. This limited repair response challenges earlier views that the tissue is an inert material that simply degrades with wear and joint use. It is now evident that the degenerative process that occurs during idiopathic or secondary OA following trauma or other pathological disturbance is a highly complex cycle of events including cell changes, cell signalling and matrix changes (Sandell and Aigner, 2001). In addition, besides the articular cartilage, OA involves the entire synovial joint including the synovium and underlying bone (Buckwalter and Mankin 1997a; Buckwalter et al., 2000).

This section presents an overview of the current understanding of articular cartilage injury and its degeneration, including an introduction to the disease of osteoarthritis. Changes that occur at the cellular and biochemical level will be described in order to provide a basis for understanding current and future repair strategies.

1.2.2 Impact of diseases of articular cartilage: prevalence and costs

OA affects people of all ethnic groups in all geographic locations. It develops in both men and women, although it occurs most commonly in women and it is the most common cause of long-term disability in most populations of people over 65. It is estimated that more than 20 million Americans have OA; the World Health Organisation estimates that 10% of the world's people over the age of 60 years suffer from OA, and that 80% of people with OA have limitation of movement and 25% cannot perform major daily activities (Jackson et al., 2001; Woolf and Pfleger, 2003; Buckwalter et al., 2004). Interestingly, in areas of China, OA of the hip is reported to be 80-90% less frequent than in white persons in the US (Nevitt, et al., 2002).

The prevalence of OA in all joints increases with age. More than a third of people over 45 years report symptoms that vary from a sensation of occasional joint stiffness and intermittent aching associated with activity, to permanent loss of motion and constant deep pain. In some populations, more than 75% of people over the age of 65 have OA that involves more than one joint (Buckwalter et al., 2004).

After the age of 40, the incidence of OA increases rapidly with each passing decade in all joints and in most joints the incidence is greater in women than in men. Because of the strong correlation between age and the incidence of OA, the total number of people suffering from this disease is rising rapidly as the proportion of the population over age 40 increases.

It is reported that the total economic burden of arthritis is 1 to 2.5% of the gross national product of western nations and that OA accounts for a major share of this burden. OA is estimated to cost more than $60 million per year in the US. OA is currently one of the most prevalent chronic conditions in the US and is second to heart disease in causing work disability in men over 50 years. These estimates of costs

and economic impact do not include pain and suffering, adverse psychosocial effects, lost opportunities for increased productivity, decreased ability to participate in regular exercise that could improve general health and the costs to family members who help provide care for patients with OA (Jackson et al., 2001; Buckwalter et al., 2004; Heijbel et al., 2005).

1.2.3 Risk factors

In addition to the disorders responsible for the multiple forms of secondary OA, genetic pre-disposition, obesity, female gender, greater bone density and joint laxity have been identified as risk factors. Although these factors may increase the risk of OA in selected populations, the most important risk factor in all populations is age. Repetitive joint use over decades, joint injury, post-traumatic joint incongruity, instability or malalignment and joint dysplasia all can create mechanical demands that damage articular surfaces (Buckwalter and Brown, 2004; Buckwalter et al., 2004; Felson, 2004).

1.2.3.1 Age

Age is the overriding risk factor for OA. The percent of people with evidence of OA in one or more joints increases from less than 5% of people between 15 and 44 years, to 25-30% of the people between 45 to 64 years of age and to more than 60% (may be as high as 90% in some populations) of the people over 65 years of age (Buckwalter et al., 2004; Gorevic, 2004).

The composition of the articular cartilage matrix changes with increasing age and this is linked to alterations in the activity of the chondrocyte (see section 1.1.5). Importantly, with increasing age, chondrocyte senescence increases and the capacity of the chondrocyte to synthesise matrix components and respond to stimuli decreases (Thonar et al., 1986; Mankin et al., 1994; Guerne et al., 1995; Bolton et al., 1999; Loeser et al., 2000; Martin & Buckwalter, 2002; Aigner et al., 2004; Loeser, 2004; Martin et al., 2004) (see section 1.1.2). Thus, there is an age-related decrease in the ability of the tissue to repair itself.

Some of the changes in matrix composition are due to variations in synthesis whereas others are due to variations in degradation (Roughley, 2001). Chondrocyte senescence is considered to result in phenotypic changes within the cell altering the expression of mediators of cartilage destruction such as the matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) (Price et al., 2002). Although the functional consequences of these age-related changes remains unclear, it is likely some of the alterations in composition may predispose the tissue to degenerative changes (Roughley, 2001).

1.2.3.2 Joint injury

Joint injuries and intra-articular fractures frequently lead to progressive joint degeneration that causes the clinical syndrome of post-traumatic osteoarthritis (Buckwalter and Brown, 2004). The pathophysiology of post-traumatic OA has not been fully explained, and it is not simply the magnitude and type of injury that determines whether an injured articular surface will repair and remodel or undergo progressive degeneration. Joint dislocations, direct and indirect joint impact loading, cruciate and collateral ligament, joint capsule and meniscal tears all lead to increased risk of OA (Lohmander and Loos, 1994; Buckwalter and Mankin, 1997a; Buckwalter et al., 2000; Englund et al., 2003; Buckwalter and Brown, 2004; Felson, 2004). Studies have found men with a history of knee injury were at a 5-6-fold increased risk of developing osteoarthritis (Felson et al., 1995). This usually occurs in a younger age group and can lead to prolonged disability and unemployment. Articular cartilage injuries will be covered in greater detail in section 1.2.6.

1.2.3.3 Overuse

Lifelong normal daily activities and regular recreational running have not been shown to cause increase the risk of joint degeneration (Buckwalter et al., 2004). However, repetitive loading of joints over a decade or more, for example by individuals in physically demanding occupations such as farmers, construction workers, metal workers, miners and pneumatic drill operators

increases the risk of joint degeneration (Sandmark et al., 2000; Holmberg et al., 2004). The risks are highest in jobs that entail both knee bending and mechanical loading (Cooper et al., 1994). The strongest association with occupational activity has been shown with OA of the knee in men. It is thought that up to 30% of all knee OA is attributable to occupational activity that involves repeated knee bending, kneeling, squatting or climbing (Felson & Zhang, 1998). Sports that subject joints to repetitive high levels of impact and torsional loading have also been demonstrated to increase the risk of joint degeneration, particularly in association with injuries (Buckwalter and Martin, 2004). The continuous stress that physical activity places on the joints can result in microtrauma and degeneration of the articular cartilage. The onset of OA appears to be dependent upon the frequency, intensity and duration of the physical activity (Saxon et al, 1999).

1.2.3.4 Genetics

Epidemiological studies have demonstrated a major genetic component to OA. These studies have also revealed differences in risk between males and females and for different skeletal sites. Twin pair and family risk studies have highlighted the surprisingly large genetic component of OA. Linkage analysis studies have highlighted that chromosomes 2, 4, 6, 7, 11, 16 and the X may each harbour an OA susceptibility gene. Chromosomes 2, 4 and 16 were identified in multiple genome scans and are, therefore, the most likely to encode susceptibility. Association analysis of candidates suggests that the syntenic genes for collagen II and the vitamin D receptor (12q12—q13.1 may also encode for OA susceptibility (Loughlin, 2001; Loughlin, 2002; Spector and MacGregor, 2004; Peach et al., 2005; Spencer et al., 2005).

1.2.3.5 Obesity

Obesity is a well accepted risk factor for osteoarthritis and this has been demonstrated in numerous studies (Coggon et al, 2001; Nevitt, 2002; Eaton, 2004; Felson, 2004a; March & Bagga, 2004). Although it is believed the increased risk of OA associated with obesity results from both mechanical and

metabolic factors (Eaton, 2004), other studies (Davis et al., 1990; Sturmer et al., 2000) have indicated the risk is associated with the mechanical aspects only, with little evidence for the metabolic or systemic effects of obesity. Obesity is also associated with the development of disability and the radiological progression of OA once established (March & Bagga, 2004).

1.2.3.6 Sex

Although it appears there is a risk factor associated with gender in OA, reports vary on the actual prevalence. Data from the U.S. has shown there is a greater incidence of hand and knee OA in females, particularly beyond the age of 50, with incidences of hip OA being similar (with the exception of women in their 80's, where there seems to be a sharp increase in incidence compared to men) (Oliveria et al., 1995).

Other studies (March and Bagga, 2004) have suggested that prior to the age of 50, men have more radiological OA than women, presumed to be largely due to secondary OA of the knee following trauma. After the age of 50, the prevalence among women increases dramatically. In the hip, the prevalence is higher in men aged 55-64. After the age of 65, the prevalence becomes greater in women. In the hand, women have a 2.6 times greater risk of developing OA. The figures described here are a good estimate of the ratio of OA in men and women and it is clear that women are generally at a higher risk of developing OA than men.

1.2.4 The degeneration of articular cartilage and osteoarthritis

OA develops most commonly in the absence of a known cause (primary or idiopathic OA). Less frequently it may develop as a result of joint injury, joint infection or from one of a variety of developmental, metabolic, inflammatory or neurological disorders when it is known as secondary or post-traumatic OA (Buckwalter and Mankin, 1997a).

OA is characterised pathologically by focal areas of damage to the articular cartilage, centred on load bearing areas, associated with remodelling and sclerosis of subchondral bone and in many cases the formation of subchondral bone cysts and marginal osteophytes (Reimann et al., 1977; Sobokbar et al., 2000; Salaffi et al., 2003; Durr at al., 2004) (figure 1.5). There will also be varying degrees of mild synovitis and thickening of the joint capsule. OA is characterised clinically by the presence of symptoms and signs that may include joint pain, restriction of motion, crepitus with motion, joint effusions and deformity (Buckwalter and Mankin, 1997a). Although OA occurs most frequently in the foot, knee, hip, hands and spine it may occur in any synovial joint (Buckwalter, 2004).

Although there is a strong association between age and OA and a widespread view that OA develops as a result of "normal wear and tear," the relationship between joint-use, aging and joint degeneration is uncertain. What is clear however is that OA is not simply a result of aging and mechanical wear from joint use (Aigner et al., 2004). Nor is primary OA caused by inflammation, although inflammatory episodes are common. OA consists of a sequence of changes in the cells and matrix that results in the loss of structure and function of articular cartilage accompanied by cartilage repair and bone remodelling reactions (Sandell and Aigner, 2001). Because of the repair and remodelling reactions, the degeneration of the articular surface is not uniformly progressive and the rate of degeneration varies among individuals and among joints. Occasionally, degeneration occurs rapidly, but in most cases it progresses slowly over many years, although it may stabilise or even decrease spontaneously, with at least partial restoration of the articular surface and a decrease in symptoms (Buckwalter and Mankin, 1997a).

Although OA is primarily characterised by loss of articular cartilage and remodelling of subchondral bone and formation of osteophytes, it usually additionally involves all of the tissues that form the synovial joint, including synovial tissue, ligaments, joint capsule and muscle. The earliest histological changes seen in OA include fraying or fibrillation of the superficial zone of articular cartilage, extending into the transitional zone (Buckwalter and Mankin, 1997a) (figure 1.6). As the disease progresses, these surface irregularities become clefts, more of the articular surface becomes roughened and the fibrillation extends deeper into the subchondral bone. There is decreased

staining for proteoglycan in the superficial and transitional zones, breaching of the tidemark by blood vessels from subchondral bone and remodelling of subchondral bone (Reimann et al., 1977; Hamerman and Stanley, 1996). Chondrocytes are present as small clones, which arise from an attempted repair response involving limited chondrocyte proliferation. It is unclear at present whether the subchondral bone remodelling occurs initially and leads to the degeneration of the articular cartilage or whether loss of articular cartilage increases stresses on the subchondral bone leading to remodelling (Radin and Rose, 1986). Interestingly, in the Dunkin-Hartley guinea pig model of spontaneous OA, subchondral bone changes occur prior to the onset of changes in the articular cartilage (Anderson-Mackenzie et al., 2005). As the disease progresses and the cartilage fissures deepen, fragments of cartilage tear and are released into the joint space, decreasing the thickness of the cartilage. Eventually, the progressive loss of articular cartilage leaves only dense and often necrotic eburnated bone.

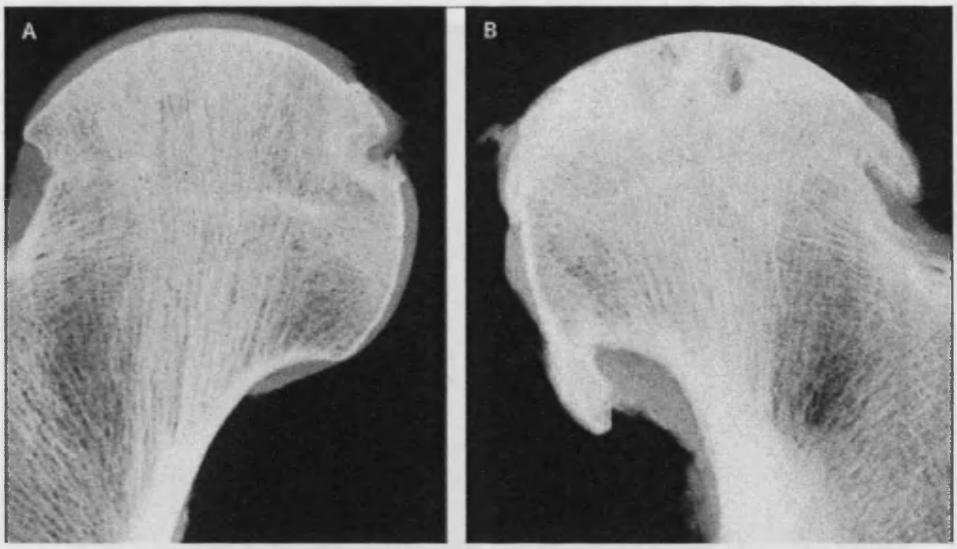


Figure 1.5: Slab radiograph of (A) normal and (B) osteoarthritic femoral head. Osteoarthritic joint shows marginal osteophytes, change in shape of bone, subchondral cysts and focal areas of extensive loss of articular cartilage. Taken from Dieppe and Lohmander, 2005.

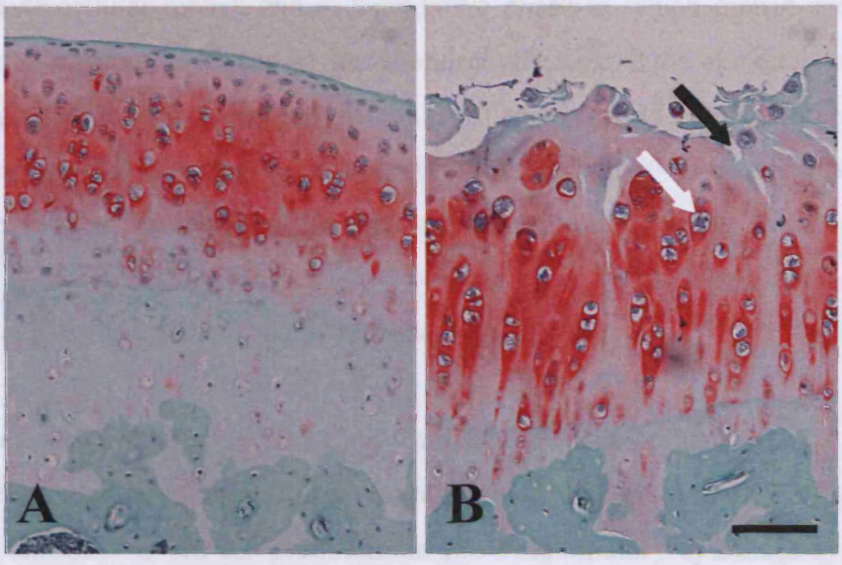


Figure 1.6: Comparison of normal (A) and osteoarthritic (B) articular cartilage. Coronal sections taken from the knee of a Dunkin-Hartley Guinea pig, stained with Safranin O. Loss of proteoglycan staining in the matrix is clearly evident together with superficial fibrillation and clefts (black arrow) and chondrocyte cloning (white arrow). Scale bar = 250μm.

1.2.5 Cell biology of osteoarthritis

It is generally believed that degradation of cartilage in OA is characterised by two phases: a biosynthetic phase, during which the chondrocytes attempt to repair the damaged extracellular matrix and a degradative phase in which matrix synthesis is inhibited and enzymes produced by the chondrocytes digests the extracellular matrix. In the degradative phase, erosion of the cartilage is accelerated (Howell, 1986; Hamerman, 1989). It is during OA that the biosynthetic activity is unable to keep pace with the degradative catabolic activity, and degeneration of the tissue results.

1.2.5.1 Cytokines in the pathophysiology of OA

The presence of inflammation during the development of OA is well documented and believed to be involved in the progression of the disease. IL-1β and TNF-α are the predominant pro-inflammatory cytokines synthesised during the OA process. In addition to IL-1β and TNF-α there are a number of other cytokines that may modulate OA progression by having pro-inflammatory and anti-inflammatory properties (van de Loo et al., 1995; Martel-Pelletier et al., 1999).

The pathogenesis of OA involves the disturbance of the balance of degradation and repair of articular cartilage. In addition to changes in the articular cartilage, there are also distinct changes that occur in the synovial membrane and subchondral bone. Cytokines and growth factors appear to be first produced by the synovial membrane, and diffuse into the cartilage through the synovial fluid. They activate the chondrocytes, which in turn are able to produce catabolic factors such as proteases and pro-inflammatory cytokines. In the OA synovial membrane, the synovial lining cells are key inflammatory effectors (Martel-Pelletier et al., 1999).

1.2.5.2 Pro-inflammatory cytokines

IL-1β and TNF-α are believed to play key roles in the development of the disease process. IL-1β stimulates cartilage degradation and TNF-α drives the

inflammatory process (van de Loo et al., 1995). IL-1β and TNF-α up regulate chondrocyte and synovial cell synthesis of IL-8, IL-6 and LIF in addition to increasing protease and prostaglandin production.

Although IL-1β and TNF-α are believed to be the key inflammatory mediators in OA, other pro-inflammatory cytokines including IL-8, LIF, IL-11, IL-6 and IL-17 have been shown to be expressed in OA. IL-8 is a potent chemotactic cytokine for neutrophils and also stimulates them to synthesise reactive oxygen intermediates. In OA patients, IL-8 has been detected in the synovial fluid, the lining cell layers of the synovium and the chondrocytes and can enhance the release of inflammatory cytokines such as IL-1β, IL-6 and TNF-α in human mononuclear cells, which may further modulate the inflammatory process (Guerne et al., 1989; Maier et al., 1993; Villigier et al., 1993; Dechanet et al., 1994; Deleuran et al., 1994; Martel-Pelletier et al., 1999).

Leukemia inhibitory factor (LIF) has been detected in the synovial fluid of OA patients (Dechanet et al., 1994) and has been shown to enhance IL-1β and IL-8 expression in chondrocytes and IL-1β and TNF-α in synovial fibroblasts (Villigier et al., 1993). In addition, LIF stimulates the expression of collagenase and stromolysin by human articular chondrocytes without affecting production of specific tissue inhibitor of metalloproteinases (TIMPs).

1.2.5.3 Anti-inflammatory cytokines, antagonists and growth factors

The anti-inflammatory cytokines IL-4, IL-10 and IL-13 have been found in increased levels in the synovial fluid of OA patients (Martel-Pelletier et al., 1999). These cytokines are considered to decrease production of IL-1β, TNF-α and MMP, up regulate IL-1Ra and TIMP-1 and inhibit PGE_2 release in various cell types. In human OA synovial fibroblasts, IL-10 down regulated TNF receptor density. IL-13 also has anti-inflammatory effects in human OA synovial tissue. IL-1Ra blocks many of the pathological effects of OA including PGE_2 production in synovial cells, collagenase production by

chondrocytes and cartilage matrix degradation. Although IL-1Ra is elevated in OA it is thought that this level may not be enough to inhibit the action of IL-1.

1.2.5.4 Cellular response in OA cartilage

The cellular responses seen in OA cartilage can be summarised into 3 categories: (1) proliferation and cell death, (2) changes in extracellular matrix synthetic activity and degradation, (3) alterations in chondrocyte phenotype.

Proliferation and cell death

Although normal chondrocytes have a limited mitotic potential (depending on species), chondrocytes in OA cartilage have a proliferative capacity, albeit low (Hulth et al., 1972). The reason for this is unclear and is probably the activity that causes characteristic chondrocyte clustering in osteoarthritic cartilage (figure 1.6). There have been various reports on the extent of cell death in OA cartilage. Although cell death and apoptosis is believed to occur in OA cartilage the true extent of the actual amount remains undetermined due to varying reports in the literature (Meachim et al., 1965; Vignon et al., 1976; Blanco et al., 1998; Kim et al., 2000; Sandell and Aigner, 2001). Figures range from 5-11% cell death in normal cartilage to 22 to 51% in OA cartilage.

Changes in extra-cellular matrix synthetic activity and degradation

During OA there is an enhanced synthesis of extracellular matrix components. However, loss of proteoglycan from the matrix is a key feature of OA cartilage and can be clearly seen in histological sections (figure 1.6). Although some researchers have assumed this simply to be due to increased overall degradation, the true picture may be more complex as differences in matrix loss and chondrocyte activation occur in the different zones of the cartilage (Sandell and Aigner, 2001).

In normal cartilage, the overall rate of synthesis of matrix components by the chondrocytes matches the level of degradation. However, in OA the balance is

altered such that the rate of proteoglycan degradation is increased through increased action of aggrecanases (Little et al., 1999; Sabatini et al., 2005). In addition, there is gross tissue reduction in proteoglycan synthesis although there are focal increases in proteoglycan around individual chondrocytes or groups of chondrocytes. The inhibition of proteoglycan synthesis and increased MMP production is believed to result from altered growth factor/cytokine signalling from pro-inflammatory cytokines such as IL-1 and TNFα.

Whereas the loss of proteoglycan is believed to be reversible, the degradation of collagen is considered to result in irreversible disruption of the collagen network (Shingleton et al., 1996). The loss of the proteoglycan is believed to be a key factor in the degradation of articular cartilage, resulting in decreased mechanical properties, predisposing the collagen network to mechanical disruption resulting in tissue fibrillation and fissuring. MMP-13 (collagenase 3) and aggrecanase are the principle enzymes responsible for collagen and proteoglycan degradation in OA. Other MMPs are known to be involved in the catalytic process, by either activating pro-MMP13, secondary cleavage of collagen helices or directly degrading fibrilar collagen (i.e. MMP-3 (stromolysin) and MT1-MMP) (Murphy et al., 1987; Ohuchi et al., 1997; Cowell et al., 1998).

The elevated MMP activity observed in OA is not only attributed to the increased synthesis and activation of MMP but also to a decrease in the production of the inhibitors of MMPs, the TIMPs and α2Macroglobulin. Thus, there is believed to be a shift in the balance in the regulation of MMP activity favouring excessive matrix degradation.

Alterations in chondrocyte phenotype

During OA there is a shift in the phenotype of the chondrocytes which is dependant on zone. The chondrocytes in the middle zone begin to express collagen IIA, typically associated with chondroprogenitor cells, in addition to

aggrecan and collagen type II (Sandell and Aigner, 2001). In the upper middle zone there is a shift towards expression of collagen III. In the deeper zones the cells begin to re-express collagen X, a specific marker for hypertrophy of growth-plate chondrocytes.

1.2.6 Articular cartilage injuries

Despite the well known relationship between joint damage and the subsequent development of OA (section 1.2.3.5), the natural history of articular surface injuries is poorly understood (Lohmander, 1998; Newman, 1998; Buckwalter, 2002; Shelbourne et al., 2003). Difficulties in diagnosis and limited awareness of these injuries make it difficult to determine their incidence or their relationship to the development of OA. Difficulties in understanding the natural history of chondral lesions or surface defects also arise as such injuries often occur in association with injuries to other tissues of the synovial joint such as the menisci, ligaments, joint capsule and synovium (Lohmander and Roos, 1994). In these cases, it is difficult to distinguish the effects of the cartilage injury from the effects of the injuries to the other tissues. In addition, the cartilage injury may be overlooked in such cases. The natural history of the lesion will also be dependant on whether the fracture is purely chondral or osteochondral or may not even result in visible disruption to the articular surface. Such latter injuries probably occur with greater frequency than chondral or osteochondral fractures but are more difficult to detect (Buckwalter, 2002).

1.2.6.1 Types of articular surface mechanical injury

Mechanical injuries to articular surfaces can be classified into 3 types: (1) Damage to cells and matrix of articular cartilage and subchondral bone not associated with visible disruption of the joint surface. (2) Visible disruption of articular cartilage limited to the articular cartilage eg. chondral flap tears or chondral defects. (3) Visible mechanical disruption of articular cartilage and bone known as intra-articular fractures. Each type of tissue damage stimulates a different repair response (Buckwalter and Mankin, 1997a; Newman, 1998; Buckwalter and Brown, 2004). Each of these types of injury will be dealt with in turn.

1.2.6.2 Cell and Matrix Damage

The intensity and type of joint loading that cause chondral and subchondral damage without visible articular surface disruption has not been well defined. Impacts above normal physiological levels of joint loading generated during activities such as walking or lifting can produce disruption to the cartilage matrix, damage or kill chondrocytes, decrease proteoglycan synthesis, disruption of the collagen fibril framework and alter the hydration status of the tissue (Buckwalter, 2002). All of this can occur without visible disruption to the cartilage surface. The ability of chondrocytes to sense changes in matrix composition and synthesise new molecules makes it possible for them to repair damage to the macro-molecular framework (Buckwalter and Mankin, 1997). It is unclear as to at what point this type of injury becomes irreversible and leads to progressive loss of articular cartilage. Chondrocytes will be able to restore the matrix as long as the loss of proteoglycan does not exceed the capabilities of re-synthesis and provided the fibrillar framework remains intact and enough chondrocytes remain capable of responding in the local area.

Hence when the balance of factors results in an inability to repair the damaged tissue, then the chondrocyte will be exposed to excessive stresses due to the altered mechanical properties of the tissue caused by the matrix disruption and will increase risk of subsequent degeneration.

1.2.6.3 Cartilage disruption

Following injury to cartilage that does not extend into the subchondral bone, the chondrocytes proliferate and increase synthesis of matrix molecules near the site of the injury. However, the newly synthesised matrix and proliferating cells do not fill the tissue defect, and soon after the injury, the increased proliferative and synthetic activity ceases (Campbell, 1969; Mankin, 1982; Newman, 1998; Buckwalter and Brown, 2004). The cellular proliferation results in small clusters of chondrocytes (chondrones), and is seen only in injury and osteoarthritis. Although this results in a permanent articular surface

defect, it is a matter of debate as to whether these lesions progress to OA (Newman, 1998; Shelbourne et al., 2003).

1.2.6.4 Intra-articular fractures

Injuries that extend into subchondral bone cause haemorrhage and fibrin clot formation and activate an inflammatory response. Thus, the sequence of events following intra-articular fractures differs from injuries that cause only cell and matrix injury or damage limited to the articular cartilage surface only. Studies show that soon after injury, blood escaping from the damaged bone blood vessels forms a haematoma that temporarily fills the defects site. Fibrin forms within the haematoma, creating a fibrin clot that fills the bone defect and partially fills the cartilage defect and becomes invaded by undifferentiated mesenchymal cells from the marrow (Shapiro et al., 1993). At around 2 weeks, a portion of the mesenchymal cells assume the form of rounded chondrocytes, synthesising a matrix containing collagen II and proteoglycans, producing a hyaline-like matrix in portions of the defect. Later in the process, there are significant amounts of type I collagen present (20-35%) in the repair tissue and the proteoglycan content decreases significantly (Furukawa et al., 1980). In the bony portion of the defect, the cells produce immature bone, fibrous tissue and hyaline like cartilage and becomes vascularised. This is in contrast to the chondral portion of the defect which is rarely entered by blood vessels.

This fibrocartilagenous repair tissue that fills the osteochondral defect is less stiff and more permeable than normal articular cartilage with a composition intermediate between hyaline cartilage and fibrocartilage. In addition, the orientation and organisation of the collagen fibrils does not resemble that seen in normal articular cartilage (Mitchell and Shepard, 1976; Buckwalter and Mankin, 1997, Buckwalter and Brown, 2004) and there will be poor integration of the collagen fibrils of the repair tissue with that of the residual cartilage at the defect edge (Shapiro et al., 1993). By 12 months the matrix and cells become more typical of fibrocartilage. Evidence suggests that the chondral repair tissue begins to degenerate within 1 year or less (Buckwalter and Mankin, 1997a; Buckwalter, 2002). The fibrous tissue usually fragments

and often disintegrates, leaving areas of exposed bone. The inferior mechanical properties of chondral repair tissue are thought to be responsible for its frequent deterioration (Furukawa et al., 1980; Buckwalter and Mankin, 1997a).

It can be seen from this section that damage to articular cartilage occurs frequently in the population and the responses to that damage by the tissue is not only complex but can also be debilitating. The focus of the next section is to overview the current treatment options available today in order to understand where the prospects for therapies to be developed in the future may lie.

1.3 CARTILAGE REPAIR

1.3.1 Introduction

The aims of operative treatment for cartilage defects or osteoarthritis (OA) are to decrease or eliminate pain and to improve function through increased range of motion (Buckwalter and Lohmander, 1994). Given the multitude of possible origins of these lesions, treatment attempts have involved symptomatic measures which are useful only if the patient gains relief, joint functionality can be significantly restored and if the progression to severe joint degeneration can be prevented or slowed down (Hunziker, 2001a).

There exists a wide variety of treatment options available to the clinician or surgeon depending on the patient, the nature of the lesion and the extent of the degeneration. These options range from relatively simple arthroscopic interventions, such as lavage and debridement at one extreme, to total joint replacement for late stage OA. Although these treatments tend to involve either the resection (eg. debridement, shaving) or replacement (eg. allograft, total joint replacement) of damaged tissue, or the relief of load and stresses on the tissue (eg. high tibial osteotomy), the ultimate goal is the restoration or regeneration of the joint surface, including hyaline cartilage and subchondral bone, which remains elusive (Buckwalter and Lohmander, 1994; Hunziker, 2001a). Whereas the techniques involved in resection, relief and replacement of damaged tissue have been around for some time and are current practice, the techniques for the regeneration of hyaline cartilage are only just being developed and are some years away from fruition in a commercial product or accepted surgical technique. Current techniques aimed at repair such as microfracture and, possibly, autologous chondrocyte implantation (ACI) fall short of regenerating true hyaline articular cartilage. In addition, as future techniques tend to involve biologic approaches (eg. cell-based therapies), as more studies are done the complexities and hurdles that need to be overcome along the way are becoming ever more apparent (Hunziker, 1999). This section will focus on current techniques for the treatment of articular cartilage defects, prior to a description of tissue engineering based approaches in the next section.

1.3.2 Lavage and arthroscopy

The irrigation or lavage of a joint with solutions of saline is believed to be beneficial in osteoarthritic patients with painful knee joints. Some investigators have reported the beneficial effects to persist for greater than 12 months (Livesley et al., 1991), whereas others have reported no beneficial effect (Gibson et al., 1992). Other investigators have suggested the beneficial effects of lavage may be related to a placebo effect of the surgical intervention. There is no direct scientific evidence for the reported effects of lavage and indeed no biological basis for the beneficial effects seen. It is possible the effects may be due to the removal of debris or the rinsing removing some of the catabolic cytokines and other mediators involved in the generation of pain (Gilbert, 1988; Moseley et al., 2002).

1.3.3 Chondral shaving and debridement

Arthroscopic chondral shaving removes diseased or disrupted cartilage tissue using specially designed surgical tools. Although now only performed infrequently it is mainly used for the treatment of patello-femoral pain or chondromalacia patellae (Ogilvie-Harris and Jackson, 1984). The objective of the shaving is to remove the fibrillated cartilage to provide a smoother surface with reduced friction.

Debridement of the joint involves the arthroscopic shaving of severely degenerated cartilage combined with lavage, removal of loose bodies, meniscectomy and limited excision of osteophytes (McLaren et al., 1991; Gibson et al., 1992). Debridement used in combination with lavage has been shown to alleviate pain and provide short-term benefit in the treatment of osteoarthritis (Chang et al., 1993; Shannon et al., 2001).

Reports have indicated that debridement decreases the symptoms of OA in most patients (Baumgaertner et al., 1990; Ogilvie-Harrie and Fitsialos, 1991). As with lavage, the procedures do not initiate a repair response in the cartilage and the effect may be due to placebo or a decrease in pain stimulus due to the removal of cartilage particulates and/or inflammatory mediators from the joint space (Moseley et al., 2002).

1.3.4 Penetration of subchondral bone (abrasion chondroplasty, Pridie drilling and microfracture)

These techniques involve surgical penetration of the subchondral bone in regions of full thickness cartilage loss, leading to bleeding from the marrow spaces into the defect and the formation of a fibrin clot (Hunziker, 2001a). Undifferentiated mesenchymal cells from the marrow present in the fibrin clot are responsible for forming a fibrocartilagenous repair tissue of limited durability (Kim et al., 1991; Shapiro et al., 1993; Menche et al., 1996).

Surgeons have developed various methods for the penetration of subchondral bone to stimulate cartilage repair such as drilling (Pridie drilling), abrasion (abrasion chondroplasty), use of sharp picks (microfracture) or resection (spongialisation) (Insall, 1974; Ficat et al., 1979; Moseley et al., 1996; Akizuki et al., 1997; Sledge, 2001).

Abrasion arthroplasty involves superficially abrading the subchondral bone so that bleeding and fibrin clot are produced. Subchondral drilling (Pridie drilling) is similar to abrasion arthroplasty except that the subchondral bone is penetrated in a more precise fashion using small drills. Microfracture (figure 1.7) involves the debridement of the damaged tissue down to the subchondral bone which is then perforated repeatedly with the use of a small pick (Gilbert, 1998; Sledge, 2001). It is currently not clear as to which of these methods produces the better results with few clinical studies directly comparing the different techniques. Although these procedures can lead to positive outcomes, the improvements can be variable which results from the unpredictable nature and limited durability of the fibrocartilagenous repair tissue itself and the age of the patient (Johnson, 2001; Steadman et al, 2003). One study compared abrasion with drilling for the treatment of experimental defects in rabbits and showed drilling to give better long term results (Menche et al., 1996). One clinical study compared abrasion chondroplasty and osteotomy to osteotomy alone and reported that individuals who received the combined approach had improved hyaline repair tissue formation after 12 months compared to osteotomy alone (Akizuki et al.,1997). There was no difference in clinical outcome observed after 2 years.

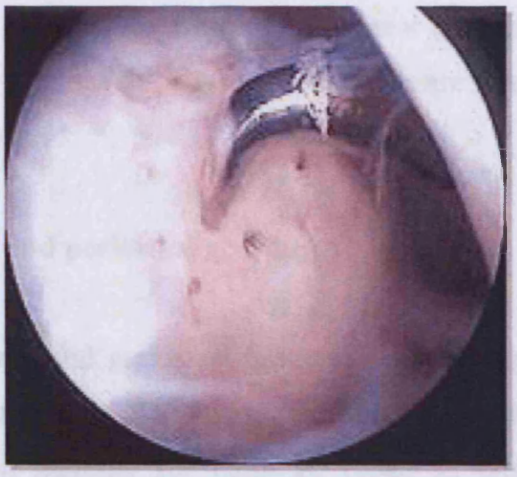


Figure 1.7: Arthroscopic microfracture.

Taken from http://www.kneeclinic.info/problems_articular_cartilage.php

1.3.5 Osteotomy

High tibial osteotomy is a palliative surgical treatment frequently adopted for the treatment of painful OA (Wright et al., 2005). It is also undertaken to correct large extra-articular deformities such as those affecting the valgus or varus which can occur in association with OA. Osteotomy is thus undertaken with a view to not inducing cartilage repair but to relieve pain, improve alignment as well as biomechanical load transfer in knee joints. In cases of painful OA, patients are usually relieved of pain for some considerable time.

1.3.6 Perichondrial and periosteal grafting

The use of perichondrial and periosteal tissue to promote repair of cartilage lesions was first recognised approximately 50 years ago and has subsequently been utilised as an auto-transplantation material for cartilage repair in both rabbits and humans (Cohen, 1955; Homminga et al., 1990; Coutts et al., 1992; Kreder et al., 1994).

The basis underlying these studies is that the cambial layer of the perichondrium or periosteum maintains chondrogenic activity throughout life (Cohen, 1955; Nakahara et al., 1991) and this is considered to be due to a population of adult stem cells within this layer that can be driven down a chondrocytic lineage given the correct stimuli (De Bari et al., 2001). During the transplantation process, the tissue is oriented such that the cambial layer is uppermost on the floor of the full thickness cartilage defect. This allows the adult stem cells to proliferate, differentiate and hence repair the defect.

Studies examining the efficacy of periosteal and perichondral grafting in both humans and animals have yielded mixed results (Hunziker, 2001a), consequently some studies have attempted to improve outcomes by incorporating growth factors such as TGF-β, using continuous passive motion or polylactic acid matrices. All have met with limited success.

1.3.7 Osteochondral grafting

The transplantation of multiple autologous osteochondral plugs is often referred to as Mosaicplasty™ (Smith &Nephew) or OATS™ (Arthrex) and is a widely used technique for the treatment of large osteochondral defects (figure 1.8). The procedures can be carried out on an open-joint or by arthroscopy. The procedures involve the removal of small cylindrical osteochondral plugs from non-weight bearing portions of the joint surface at the periphery of the joint using specialised surgical tools. The plugs are then transplanted into the previously prepared and debrided defect area on the weight bearing portion of the knee (Hangody et al., 2001; Jakob et al., 2002). Although the osseous portion of the plug is considered to become stably integrated into the subchondral bone, it is not currently known as to the longevity of the cartilage layer (Hunziker, 2001a). Clinical outcomes are reported to be favourable, showing reduced pain and improved joint function (Hangody et al., 2001; Jakob et al., 2002).

Allogeneic osteochondral grafting has been in practice for many years and uses fresh or cryopreserved tissue derived from cadaveric donors (Meyers et al., 1983; Czitrom et al., 1986; Garrett, 1986) and has yielded good results in patients with large osteochondral defects, such as those resulting from osteochondritis dissecans, extensive trauma, tumour resection or osteonecrosis. The results have been positive despite concerns over possible immunological problems or loss of tissue viability following cryopreservation (Langer et al., 1978; Stevenson et al., 1989; Sirlin et al., 2001). However, a disadvantage of the technique is the low availability of suitable donor tissue, either fresh or cryopreserved.

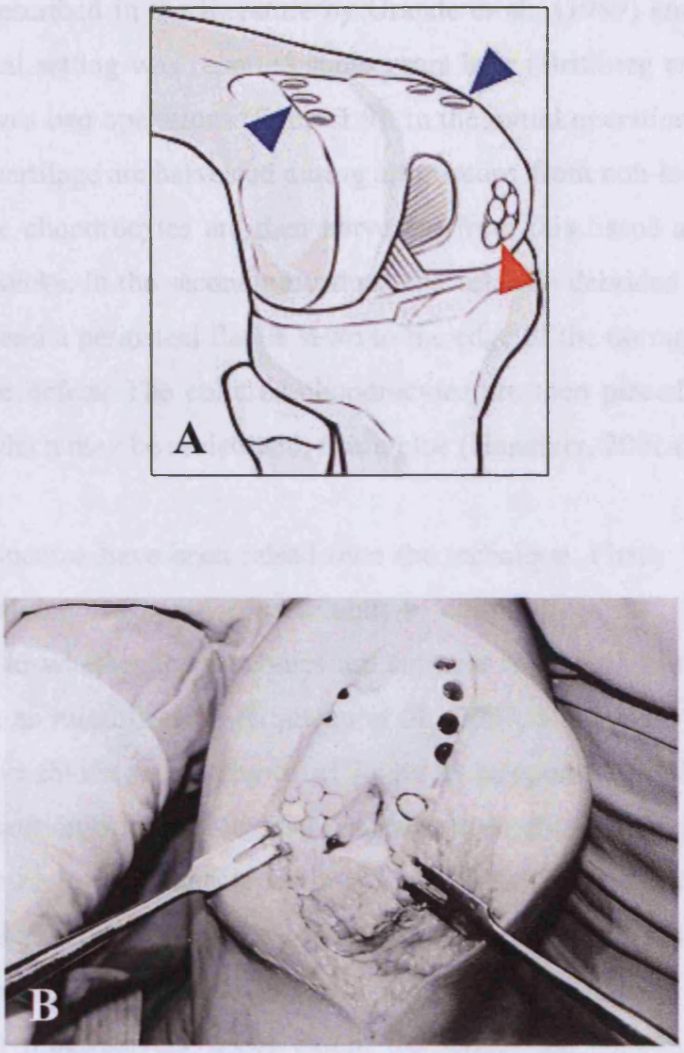


Figure 1.8: (A) Schematic diagram of osteochondral transplantation using mosaicplasty. Cylindrical grafts have been removed from the donor site (blue arrows) and implanted at the recipient site (red arrow). Taken from http://www.pathology.unibe.ch/Forschung/osteoart/osteoart.htm). (B) Open mosaicplasty on the medial and lateral femoral condyles. Taken from Hangody et al., 2001.

1.3.8 Autologous chondrocyte implantation (ACI)

ACI was first described in the literature by Grande et al., (1989) and its subsequent use in the clinical setting was reported some years later (Brittberg et al., 1994). The procedure involves two operations (figure 1.9). In the initial operation, small portions of the patient's cartilage are harvested during arthroscopy from non-load bearing areas of the joint. The chondrocytes are then harvested from this tissue and expanded in culture for 3-4 weeks. In the second procedure, the defect is debrided of all fibrous or damaged tissue and a periosteal flap is sewn to the edge of the normal tissue forming a cover over the defect. The cultured chondrocytes are then placed underneath the periosteal flap which may be sealed with fibrin glue (Hunziker, 2001a).

A number of concerns have been raised over the technique. Firstly the technique is expensive, involving two procedures and a culturing phase. Secondly it is questionable as to whether the outcomes are superior to that of marrow stimulating techniques such as microfracture (Knutson et al., 2004; Clar et al., 2005) although other studies have shown superiority of ACI over mosaicplasty (Bentley et al., 2003). Thirdly, the expansion of chondrocytes in monolayer conditions rapidly leads to their dedifferentiation or loss of phenotype (Benya and Schaffer, 1982; Von der Mark et al., 1997; Binette et al., 1998). The reversal of this process can occur in 3-dimensional culture and using combinations of growth factors but the reversal is rarely complete and occurs to a progressively lesser extent the longer the chondrocyte is kept in monolayer culture (Benja and Schaffer, 1982; Bonaventure et al., 1994). Thus one would expect the cartilage forming capabilities of the transplanted cells to be low.

A study has demonstrated an inability to reproduce in a canine model the results that were obtained in rabbits earlier (Grande et al., 1989; Breinan et al., 1997) and showed that the transplanted cells did not contribute to the repair response. Other studies have shown that the majority of the periosteal flaps are frequently lost from the defect soon after implantation (Hunziker, 2001a) unless suitable measures are taken to immobilise the joint.

Despite these issues, ACI is widely used in clinical practice with reportedly good to excellent results (Brittberg et al., 1994; Peterson et al., 2000). However, a recent

report of a randomised clinical trial comparing ACI and the less expensive technique of microfracture suggests little difference in clinical outcomes and repair responses between the two techniques at two years post surgery (Knutson et al., 2004).

A recent development has been the replacement of the periosteal flap with a collagen membrane (Haddo et al., 2004). The use of the periosteal flap has been associated with complications such as hypertrophy of the graft and decalcification and delamination. The harvesting of the periosteum increases operating time and may lead to increased pain for the patient. Another development has been the introduction of three dimensional scaffolds or matrices in combination with the autologous chondrocyte transplantation. In these studies the chondrocytes are seeded onto a matrix such as a collagen membrane (MACI™) or hyaluronan (Hyalograft C™) (Pavesio et al., 2003; Ronga et al., 2004). The procedures have the advantage that the matrix can be secured using fibrin glue without a cover and so are suture-free – sutures have been associated with cell death in the healthy cartilage surrounding the defect (Breinan et al., 1997). Because no periosteal coverage is required to keep the graft in place, surgical time and morbidity are reduced, and handling of the graft is much simpler than currently available autologous chondrocyte implantation techniques. However, a recent study has shown that although MACI™ is technically attractive, clinical, arthroscopic and histological outcomes were similar for both ACI and MACI™ (Bartlett et al., 2005).

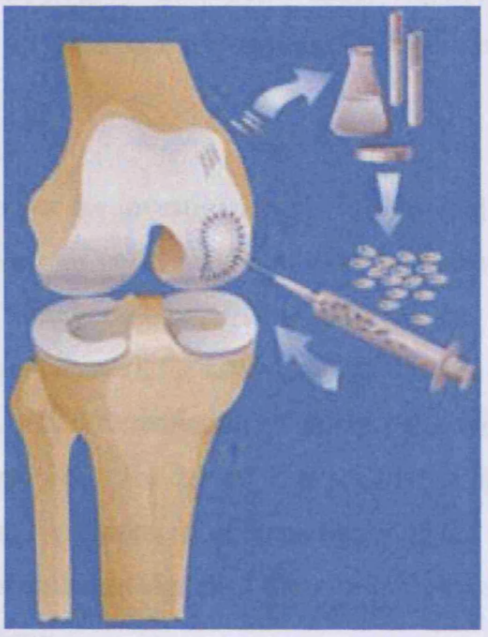


Figure 1.9: Diagram illustrating the technique of autologous chondrocyte transplantation.

Taken from

http://www.alphaklinik.de/de/toft/diagnose_therapie/arthrose/bioprothese-detail

1.4 TISSUE ENGINEERING

1.4.1 Introduction

It is clear from the previous section that functional restoration of articular cartilage remains a challenge and none of the existing treatment regimens gives a consistently good outcome (Hunziker, 2001a). Orthopedic tissue engineering is a growing field which has the potential to develop novel approaches to the regeneration of cartilage defects.

Tissue engineering involves the investigation of how to repair and regenerate organs and tissues by the delivery of cells, maybe in combination with growth factors and biomimetic scaffolds (Sittinger et al., 2004). Key to the development of tissue engineering has been an increase in the knowledge of the role of growth factors in the control of proliferation and differentiation of many cells types, combined with the development of novel protocols for the rapid expansion of specifically selected cell populations. In addition, development of knowledge of the biology and culture of adult mesenchymal stem cells has signalled these cells may have an important role to play in regenerative biology.

Initial interest in tissue engineering began with the regeneration of skin and epidermis (Potten and Booth, 2002) and has since been applied to a whole range of tissue types including blood vessels, cardiac tissue, cornea, liver, kidney, bladder and pancreas (Atala and Lanza, 2002). Interest in the tissue engineering of cartilage has grown for a number of reasons. Firstly, the high incidences and high level of morbidity linked with cartilage degeneration and costs associated with it (Jackson et al., 2001; Buckwalter et al., 2004). Secondly, articular cartilage is aneural, avascular, and is considered to be composed of 1 functional cell type, the chondrocyte (Buckwalter and Mankin, 1997), thus negating the need for complex arrangements of multiple cell types (although this may be true that cartilage is composed of only 1 cell type, it is clear that chondrocytes are very different throughout the thickness of the cartilage (Zanetti et al, 1985; Aydelotte and Kuettner, 1988; Aydelotte et al., 1988; Archer et al., 1990)). As articular cartilage is avascular, its nutrition is derived from diffusion

from the synovial fluid and thus chondrocytes may be ideally suited to the growth of tissue constructs *in vitro* in bioreactor conditions.

This section will overview current progress in the area of tissue engineering of articular cartilage and the potential use of mesenchymal stem cells in these applications. The important components of a tissue engineering system such as scaffold and cell type will each be reviewed followed by differing approaches to articular cartilage tissue engineering: (1) implantation of mature of semi-mature tissue or (2) *in situ* regeneration.

1.4.2 Scaffolds

Scaffolds or matrices in tissue engineering are intended to act as a 3-dimensional template to guide the regeneration of the tissue. Requirements that need to be borne in mind in the design of a tissue engineering scaffold are summarised in table 1.1. Scaffolds can be of natural or synthetic origin, each having their advantages and disadvantages.

1.4.2.1 Collagen

Scaffolds composed of collagen sponges or gels have been evaluated for tissue repair for many years (Speer et al., 1979). They have been evaluated in combination with cells such as chondrocytes (Kawamura et al., 1998) and bone marrow derived mesenchymal stem cells (Wakitani et al., 1994), sometimes with the inclusion of growth factors (Toolan et al., 1996).

1.4.2.2 Fibrin

Fibrin is a natural choice for a scaffold in articular cartilage tissue engineering due to its role in normal wound healing and its part in the repair of full thickness articular cartilage defects (Shapiro et al., 1993). Fibrin is pro-inflammatory with numerous roles involving cell adhesion, migration and binding of growth factors (Clark, 2001). In addition, the degradation products also have pro-inflammatory effects. Fibrin has been used in combination with

Matrix properties	Biological basis
Porosity	Cell migration, ingress and egress of nutrients and cell waste products
Carrier	Lodgement and release of signalling substances
Adhesion	Cell attachment
Biodegradability	Physiological remodelling
Volume stability	Smooth surface contour of repair tissue flush with that of native articular cartilage
Biocompatibility	Good contact with the native tissue compartment
Bonding	Enhances interfacial integration between collagen fibrils in repair and native tissue compartments
Internal cohesiveness	Prevention of matrix outflow
Elasticity	Resiliency during and following dynamic or static deformation
Structural anisotropy	Promotion of native anisotropic tissue organsiation

Table 1.1: Requirements of a scaffold for tissue engineering of articular cartilage. Adapted from Hunziker, 2001a.

chondrocytes (Hendrickson et al., 1994) and with combinations of chondrocytes and growth factors (Fortier et al., 1999) or with growth factors alone (Nixon et al., 1999).

1.4.2.3 Agarose and Alginate

Agarose and alginate are polysaccharide hydrogels derived from seaweed (Frenkel and Di Cesare, 2004). Although both agarose and alginate have been used extensively in *in vitro* studies of chondrocyte differentiation (Benya and Shaffer, 1982; Bonaventure et al., 1994; Yaeger et al., 1997), studies *in vivo* have yielded poor results in cartilage repair studies. Alginate and agarose have slow resorption rates, and elicit a foreign body reaction (Rahforth et al., 1998; Diduch et al., 2000; Fragonas et al., 2000).

1.4.2.4 Chitosan

Chitosan is a co-polymer of glucosamine and N-acetylglucosamine and may be modified to form a hydrogel. Although *in vitro* it has been evaluated as to its ability to support chondrocyte growth and differentiation (Lahiji et al., 2000) it has not yet been used in *in vivo* cartilage repair studies.

1.4.2.5 Hyaluronan (HA)

Hyaluronan (Hyaluronic acid) is a major component of the cartilage matrix. Scaffolds composed of cross-linked HA have been used in conjunction with chondrocytes (Brun et al., 1999) and bone-marrow-derived mesenchymal stem cells (Radice et al., 2000) and favourable results have been obtained *in vivo* (Solchaga et al., 1999 and 2000). HA is also currently used clinically in autologous chondrocyte implantation techniques (Pavesio et al., 2003).

1.4.2.6 Polylactic acid/polyglycolic acid (PLA/PGA)

These polymers have been utilised in cartilage repair applications for over 10 years (Vacanti et al., 1988; Freed et al., 1993; Vacanti et al., 1994; Cao et al.,

1997) in the form of foams and fibrous mats. The scaffolds have been used chiefly to serve as a 3dimensional scaffold for *in vitro* culture of chondrocytes to generate semi-mature or mature tissue prior to implantation.

1.4.3 Cell type

The choice of cell type for tissue engineering is key. Obviously the cell must be able to form a cartilage-like matrix. Ideally the cell type must support extensive expansion while retaining its ability to form a cartilaginous matrix in the appropriate conditions. In addition the cell type should be abundant in a readily accessible source.

1.4.3.1 Chondrocytes

Not surprisingly chondrocytes have been extensively studied in cartilage tissue engineering protocols. Both autologous (Grande et al., 1989) and allogeneic chondrocytes (Kawamura et al., 1998) have been investigated on fibrin (Hendrickson et al., 1994), agarose (Sittinger et al., 1994) or PGA scaffolds (Vacanti et al., 1988; Freed et al., 1993; Vacanti et al., 1994; Cao et al., 1997).

Although in general, positive results have been obtained, questions have been raised about the immunologic properties of allogeneic chondrocytes (Kawabe and Yoshinao, 1991) and the potential of chondrocytes to re-express the chondrocytic phenotype following expansion in monolayer culture (Benya and Shaffer, 1982; Bonaventure et al., 1994).

1.4.3.2 Perichondrial/periosteal cells

The chondrogenic potential of perichondrial and periosteal tissue has been known for some time (Cohen, 1955) and cells derived from these tissues have been investigated in tissue engineering applications (Chu et al., 1997; Dounchis et al., 2000). Despite the advantage that these cells may represent a precursor cell population with a high proliferative and differentiative capacity irrespective of donor age (De Bari et al., 2001) these cells have not proceeded past pre-clinical studies.

1.4.3.3 Synovial cells

The chondrogenic capacity of synovial cells and tissue is well documented (Nishimura et al., 1999; De Bari et al., 2001a) and is believed to result from a common pool of chondrogenic precursor cells that has important functions during the development of the joint (Dowthwaite et al., 1998; Archer et al., 2003). Although isolated synovial cells have not yet been used in tissue engineering studies for cartilage repair, studies have been attempted to use growth factors such as TGF-β to recruit synovial cells *in situ* into partial thickness defects (Hunziker and Rosenberg, 1996).

1.4.3.4 Stromal cells of the bone marrow

Mesenchymal stem cells (MSCs) were first identified in the bone marrow stroma by Owen and Friedenstein (1988) who isolated bone forming progenitor cells from the rat marrow. The cells have the capacity to differentiate into cells of connective tissue lineages, including bone, fat, cartilage and muscle. In addition, they play a role in providing the stromal support system for haematopoietic stem cells in the marrow. MSCs represent a very small fraction, 0.001-0.01% of the total population of nucleated cells (Pittenger et al., 1999). However, they can be isolated and expanded with high efficiency, and induced to differentiate to multiple lineages under defined culture conditions. The cells have generated a great deal of interest because of their potential use in tissue engineering and regenerative medicine (Deans and Moseley, 2000; Tuan et al., 2003; Barry and Murphy, 2004).

The strong chondrogenic potential of the cells derived from both rabbit (Johnstone et al., 1998) and human bone marrow (Mackay et al., 1998) led to suggestions as to the possibilities of the use of the cells in articular cartilage tissue engineering (Johnstone and Yoo, 2001). Indeed, the cells have been used in *in vivo* cartilage repair studies (Wakitani et al., 1994; Im et al., 2001) and in a human study for articular cartilage repair using ACI-like protocols (Wakitani et al., 2002).

1.4.4 Tissue engineering approaches

One approach to the functional tissue engineering of cartilage involves the *in vitro* cultivation of cartilaginous constructs that would have a capacity to further develop structurally and biomechanically following implantation, and to integrate completely with the adjacent bone and cartilage. In these approaches, chondrogenic cells are seeded at high density onto biodegradable scaffolds and cultured in bioreactors (specialised environments designed to promote chondrogenesis) (Freed et al., 1999; Vunjak-Novakovic, 2003). This process is designed to generate immature but functional constructs which would subsequently remodel and mature following *in vivo* implantation.

However, these approaches have encountered problems with cell source, mechanical fixation and tissue integration and little success has been achieved to date *in vivo*. This has led many groups to attempt to induce chondrogenesis *in situ* using exogenous growth factors on suitable carrier matrices. For example studies have utilised IGF-I in fibrin matrices or TGF-β in a variety of matrices in partial and full thickness defects (Hunziker and Rosenberg, 1996; Nixon et al., 1999; Hunziker, 2001; Hunziker et al., 2001). Some studies have combined these scaffolds with cells, for example chondrocytes in fibrin and IGF-I (Fortier et al., 1999), chondrocytes in agarose and FGF-2 (Weisser et al., 2001) and perichondrial cells in a polylactide matrix containing TGF-β (Dounchis et al., 1997).

It is unclear as to which of these approaches may prove to be successful in the future. Little success has been achieved to date over many decades of research (Hunziker, 2001a). However, our increasing knowledge of cell signalling and developmental biology, combined with further advances in biomaterial development may lead to efficacious therapies in the future.

1.5 AIMS OF THIS THESIS

As described in this introduction, articular cartilage is a complex tissue, reliant on a complex framework of extracellular matrix that dictates the mechanical properties of the tissue. The extracellular matrix is maintained by the chondrocyte, which constantly synthesises and remodels the matrix in response to changes in its composition and alterations in patterns of loading and other factors (Buckwalter and Mankin, 1997).

Current transplantation and tissue engineering strategies such as ACI (Brittberg et al., 1994) have employed *in vitro* expanded chondrocytes from the full depth of the cartilage. However it is clear that chondrocytes differ in size, shape and metabolic activity throughout the thickness of the tissue (Zanetti et al, 1985; Aydelotte and Kuettner, 1988; Aydelotte et al., 1988; Archer et al., 1990) and these differences in chondrocyte phenotype are responsible for subtle differences in matrix composition throughout the depth of the tissue. Thus, in ACI-like procedures, these sub-populations of chondrocytes will not be re-implanted in the zones they are present in the native tissue. In light of this, some studies have been carried out to assess the functional importance of the various sub-populations of cells in tissue engineering protocols (Waldman et al., 2003). Furthermore, section 1.1.6 introduced the developmental biology of the synovial joint and the importance of a putative population of chondroprogenitor cells in the surface region of the articular cartilage which drive the appositional growth of the tissue during development. In view of the possible role of these cells in driving development they could also potentially be applied to new tissue repair or tissue engineering therapies for the treatment of defects in articular cartilage and may also further our knowledge of cartilage developmental biology. In addition, in terms of cartilage repair, the re-establishment of the specific zones after injury or matrix degradation due to OA would be beneficial from a functional aspect since most repair strategies generate a fibrocartilagenous matrix and even when a hyaline matrix is achieved, there is little evidence of zonal variation (Kim et al., 1991; Shapiro et al., 1993; Menche et al., 1996). The matrix of this repair tissue does not have the same biochemical and hence biomechanical features of the normal articular cartilage and thus is prone to further injury or incomplete restoration of function.

The isolation and characterisation of these articular cartilage progenitor cells from the superficial zone (SZCs) is the focus of this thesis, together with an exploration of the cells capabilities in potential repair therapies.

Chapter 2: General materials and methods:

General protocols used throughout the thesis.

Chapter 3: Characterisation:

This chapter describes the methods used to demonstrate the presence of the cells in the superficial zone of immature bovine articular cartilage and the methods to produce enriched cultures of the cells. It also describes the immunolabelling of the cells with antibodies to a number of cell surface markers.

Chapter 4: Fibronectin–EDA+:

Fibronectin-EDA+ is a form of fibronectin expressed during development, wound healing and tumourigenesis. This chapter describes the demonstration of the expression of fibronectin-EDA+ by articular cartilage progenitor cells in the superficial zone and the effect of fibronectin-EDA+ on articular cartilage progenitor cell behaviour.

Chapter 5: Articular cartilage progenitor cell expansion and chondrogenic ability:

The ability of a cell to undergo extensive *in vitro* expansion while still retaining its chondrogenic ability would be a desirable characteristic for tissue engineering purposes. This chapter describes the extensive subculture of enriched populations of bovine articular cartilage progenitor cells and an evaluation of their chondrogenic potential during the expansion process.

Chapter 6: Surgical cartilage cutting techniques and *ex vivo* cell transplantation defect creation:

The objective of this chapter is to determine whether the implantation of enriched populations of superficial zone articular cartilage progenitor cells into experimental defects in articular cartilage can reduce the cell death and matrix loss at the wound margin associated with surgical cutting techniques.

Chapter 7: Human studies:

Previous studies describing evidence for the existence of a population of articular cartilage progenitor cells within the superficial zone of articular cartilage have investigated developing or neonatal oppossum, murine or bovine tissue. The prospect of isolating articular cartilage progenitor cells from human cartilage would be of interest as the cells may replicate the extensive expansion and chondrogenesis properties exhibited by their bovine counterparts (Chapter 5). This chapter describes attempts to generate cultures enriched in human articular cartilage progenitor cells and examine the extent of retention of chondrogenic capacity during the expansion process.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 TISSUE DISSECTION

For studies on bovine tissue, metatarsophalangeal joints of 7 day-old calves were obtained from an abattoir. The joints were washed thoroughly using Virkon and transferred to a laminar flow hood. The skin was removed and the joint opened aseptically to expose the articular cartilage (figure 2.1).

For cartilage cutting studies, strips of cartilage, approximately 30mm in length, 4mm wide and 3mm deep were removed and transferred to PBS (Oxoid) until used (figure 2.1). For histological staining and immunostaining, tissue was further chopped into smaller pieces as required prior to embedding.

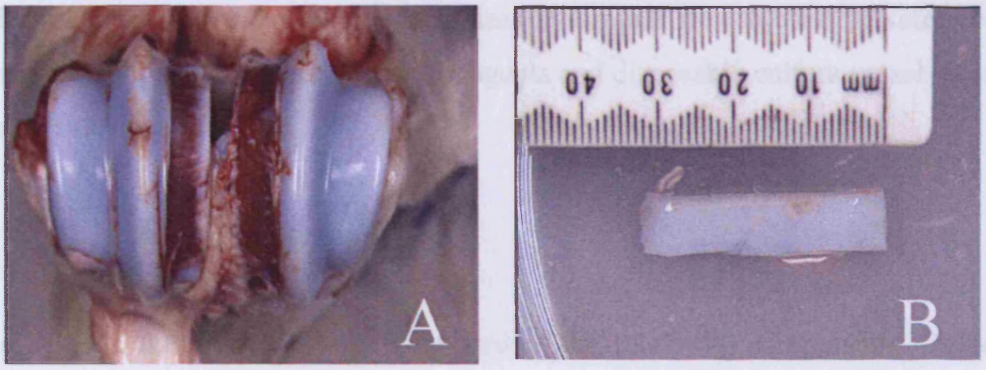


Figure 2.1 Photograph of 7 day-old bovine metatarsophalangeal joints with cartilage strips removed (A) and a photograph of the removed cartilage strip (B).

2.2 CELL CULTURE

2.2.1 Materials

All cell culture materials were obtained from Sigma unless otherwise stated. Heat inactivated foetal calf serum was obtained from Helena Biosciences.

2.2.2 Methods

All procedures were carried out in a class II laminar flow biological safety cabinet using standard aseptic technique. All reagents and disposable culture vessels were of a tissue culture grade.

2.2.2.1 Media components

Cell culture media was composed of the following components: Dulbecco's Modified Eagles Medium (DMEM) with 4500mg/l glucose. DMEM was supplemented with the following components: Foetal calf serum (FCS) (10%), L-Glutamine (2mM), Penicillin (50IU/ml)/Streptomycin (50ug/ml) and Non-essential amino acids (1%). Tissue culture media was stored at 4°C.

2.2.2.2 Passaging and general cell culture maintenance

All cells were maintained at 37°C in a 5% CO_2 atmosphere. When cells reached confluence the spent tissue culture media was removed and the monolayer was washed with PBS (Oxoid). Sufficient pre-warmed trypsin/EDTA (0.05% w/v/0.02% w/v) was added to the flasks to cover the monolayer. The cells were incubated in trypsin/EDTA at 37°C until they had started to round up and lift from the plastic. The cells were finally dislodged by gently tapping the flask and sufficient DMEM + 10% FCS was added to bring the final volume to 10mls. The cell suspension was centrifuged at 300g for 5 minutes at 4°C. Following centrifugation the supernatant was removed and the pellet resuspended in fresh DMEM + 10% FCS. Aliquots of the

suspension were transferred to fresh flasks as appropriate to obtain the desired split ratio.

2.3 HISTOLOGY AND IMMUNOSTAINING

2.3.1 Materials

All solvents, phosphate buffered formaldehyde and acetic acid were obtained from Fisher Scientific. Paraffin wax, DPX mountant and Weigert's haematoxylin were supplied by Surgipath. Gill's and Mayer's haematoxylin were supplied by TAAB. All other materials and reagents were obtained from Sigma unless otherwise stated.

2.3.2 Paraffin wax embedding

For wax embedding all tissue samples were washed once with PBS and fixed overnight in phosphate buffered formaldehyde. Samples were chopped into small portions and transferred to embedding cassettes and infiltrated with wax using a Tissue-Tek VIP processor according to the protocol shown in table 2.1.

2.3.3 Sectioning

Paraffin wax blocks were sectioned at a thickness of 5μm using a Reichert-Jung microtome. Sections were floated onto water and transferred onto 'Superfrost' glass slides (BDH). Sections were dried overnight on the slides at 40°C and then stored at room temperature.

2.3.4 Histological staining

Paraffin wax sections were dewaxed and stained using a Leica Autostainer XL. Staining protocol for Haematoxylin and Eosin is shown in table 2.2. Staining protocol for Safranin O/haematoxylin is shown in table 2.3. Staining protocol for Picrosirius-red is shown in table 2.4. Following staining, sections were mounted using DPX mountant and coverslipped.

	Reagent	Concentration	Time (hours:mins)
1	IMS	50%	1:00
2	IMS	70%	1:00
3	IMS	90%	1:00
4	IMS	100%	1:30
5	IMS	100%	1:30
6	IMS	100%	1:30
7	Chloroform	100%	0:30
8	Chloroform	100%	0:30
9	Chloroform	100%	0:30
10	Wax	-	0:30
11	Wax	-	0:30
12	Wax	-	1:00

Table 2.1: Protocol for paraffin wax embedding using the Tissue-Tek VIP tissue processor.

IMS – Industrial methylated spirits

Reagent	Time (min)
Xylene	5
Xylene	5
100% IMS	2
100% IMS	2
Distilled water	2
Gills haematoxylin	2-4
Gills haematoxylin	2-4
Tap water	2
Tap water	2
Scott's tap water	2
Tap water	2
70% IMS v/v	1.5
90% IMS v/v	1.5
Alcoholic eosin	4
100% IMS	2.5
100% IMS	2
Xylene	2
Xylene	2
Xylene	Mount

Table 2.2: Staining protocol for Haematoxylin and Eosin (H&E)

Reagent	Time (min)
Xylene	5
Xylene	5
100% IMS	2
100% IMS	2
95% IMS v/v	20 secs
70% IMS v/v	20 secs
Distilled water	1
Weigerts iron haematoxylin	6
Tap water	2
1% acidified alcohol	1 sec
Tap water	3
0.02% Fast green FCF (aqueous w/v)	4
1% acetic acid	3 secs
0.1% Safranin O	6
95% IMS v/v	1
100% IMS	2
100% IMS	2
Xylene	2
Xylene	2
Xylene	Mount

Table 2.3: Staining protocol for Safranin O/Haematoxylin

Reagent	Time (min)
Xylene	5
Xylene	5
100% IMS	2
100% IMS	2
Distilled water	2
Celestine blue	14
Distilled water	0.5
Mayer's haematoxylin	14
Tap water	2
Scott's tap water	2
Tap water	1
Picro-sirius red	60
Acidified water	2
Acidified water	2
100% IMS	2
100% IMS	2
100% IMS	2
Xylene	2
Xylene	2
Xylene	Mount

Table 2.4: Staining protocol for Picro-sirius red

2.3.5 Reagents for histological staining

Scott's tap water

2g Sodium hydrogen carbonate

10g Magnesium sulphate

1000ml Tap water

Acidified water

5ml glacial acetic acid

1000ml Water (tap or distilled)

1% acidified alcohol

5ml Hydrochloric acid (conc)

500ml IMS

Celestine blue

2.5g Celestine blue B

25g Ferric ammonium sulphate

500ml Distilled water

70ml Glycerin

Picro-sirius red

500ml Saturated aqueous picric acid

0.5g Sirius red F3BA

2.3.6 Frozen embedding and sectioning

For frozen embedding, tissue samples were chopped into small portions (approximately 4mm x 4mm), immersed in OCT (Gurr) and then frozen using a liquid nitrogen cooled iso-pentane slush. 7μm sections were cut from the specimens using a Leica Jung CM3000 cryostat and transferred onto poly-lysine coated slides (Sigma). Sections were fixed by immersion in acetone for 10 minutes at room temperature and air-dried. Slides were stored at -20°C until used.

CHAPTER 3

CHARACTERISATION

3.1 INTRODUCTION

The introduction (section 1.1) described the complex, zonal architecture of articular cartilage. The chapter described how although the main components of the articular cartilage extracellular matrix such as collagen II and the large aggregating proteoglycan aggrecan, are present throughout the thickness of the tissue, there are subtle differences in minor collagens and non-collagenous proteins from the surface zone to the deeper calcified layer (Zanetti et al., 1985; Bayliss et al., 1999; Poole et al., 2001). This biochemical heterogeneity results from different chondrocyte morphology and phenotypes at different depths in the tissue (Zanetti et al, 1985; Aydelotte and Kuettner, 1988; Aydelotte et al., 1988; Archer et al., 1990). For example, the surface zone is characterised by flattened, discoid cells that express lubricin (superficial zone protein, proteoglycan-4) and Del 1 (Schumacher et al., 1999; Pfister et al., 2001). The mid zone is composed of rounder cells that express cartilage intermediate layer protein (CILP) (Lorenzo et al., 1998) and the deeper zones and calcified layers express collagen type X and alkaline phosphatase (Schmid and Linsenmyer, 1985).

In order for such complex tissue architecture to be established, the differentiation and proliferation of the chondrocytes during development of the tissue must be stringently controlled both temporally and spatially. It is now becoming clear that the surface zone is centrally involved in the regulation of the development of articular cartilage. The surface zone is an important signalling centre during development, where many growth factors are expressed (Archer et al., 1994; Hayes et al., 2001) and also plays a major role in the morphogenesis of the joint via differential matrix synthesis (Ward et al., 1999). Studies using BrdU labelling in *Monodelphis domestica*, a marsupial born at an early stage of development, have illustrated that subsequent to the formation of the secondary centre of ossification, articular cartilage growth is appositional in nature (Hayes et al., 2001), a mechanism which requires the presence of population of stem/progenitor cells in the surface zone.

Stem cells have previously been identified in the epidermis on the basis of high cell surface expression of $\alpha_5\beta_1$ integrins and rapid adhesion to extracellular matrix

proteins (Jones and Watt, 1993). The cells that adhered most rapidly to substrates such as fibronectin possessed the ability to form large colonies from an initial low seeding density. Fibronectin is a component of the cartilage extracellular matrix, and articular cartilage chondrocytes are known to express the $\alpha_5\beta_1$ integrin homodimers which are known to bind to fibronectin (Salter et al., 1995).

This chapter describes the identification of a population of articular chondrocytes within the superficial zone of developing articular cartilage with a high affinity for fibronectin and the ability to form large numbers of colonies from a low seeding density as described in Dowthwaite et al., (2004). These cells are believed to represent the stem/progenitor cell population hypothesised in Hayes et al., (2001). This chapter also describes a limited study of the expression of a number of cell surface proteins by superficial zone cells *in vivo* as well as an examination of the relative differences in cell size throughout the thickness of the tissue. These latter aspects have been investigated with a view to identifying possible routes to more rapid or more efficient means of isolation/enrichment of articular cartilage progenitor cells.

3.2 MATERIALS AND METHODS

3.2.1 Differential adhesion and colony forming assays

3.2.1.1 Fibronectin coating of plates

Bovine plasma fibronectin (Sigma) was reconstituted to 2mg/ml with 2ml sterile deionised water and incubated at 37°C for 30 minutes to dissolve. This was diluted to 10μg/ml using Dulbecco's PBS with 1mM $MgCl_2$ and 1mM $CaCl_2$ (Sigma). This solution was added to each well of 24 well plates and incubated overnight at 4°C for coating. The fibronectin solution was then aspirated and the plates were blocked with 1% BSA prior to addition of chondrocytes. Control plates were treated with Dulbecco's PBS with 1mM $MgCl_2$ and 1mM $CaCl_2$ containing 1% BSA (uncoated wells).

3.2.1.2 Cell isolation

Chondrocytes were isolated from the surface, middle and deep zone articular cartilage of 2-3 week old bovine metatarsophalangeal joints by fine dissection. The portions of cartilage were placed directly into Dulbecco's Modified Eagle Medium (DMEM) containing 5% foetal calf serum (FCS) and 0.1% pronase (Merck, $4x10^6$ units/g) and incubated at 37°C for 3 hours. Cartilage was then washed once with PBS and incubated in DMEM containing 5% FCS and 0.04% collagenase (Worthington, 237U/mg) and incubated overnight at 37°C with gentle shaking.

Tissue digests were strained through a 70μm cell strainer (Falcon) to remove debris. The resultant filtrate was centrifuged at 300g for 5 minutes to pellet cells. Cell pellet was resuspended in 10ml of serum free DMEM, centrifuged at 300g for 5 minutes and the pellet resuspended in 10ml serum free DMEM. Cell number was then counted according using a Neubauer haemocytometer and resuspended to a final concentration of 4000 cells/ml.

3.2.1.3 Differential adhesion and colony forming efficiency assays

After isolation, 750 chondrocytes in serum free DMEM were seeded into wells of coated and uncoated 24 well plates and incubated at 37°C for 20 minutes. After 20 minutes, the media was gently swirled and transferred to a second well and incubated for a further 20 minutes at 37°C. After 20 minutes the media was gently swirled and discarded. After removal of media at both time points, fresh DMEM containing 5% FCS was added to each well. Plates were incubated at 37°C in a 5% CO_2 atmosphere.

Within 3 hours after initial plating, chondrocyte adhesion was assayed by counting the total number of cells adhering to the bottom of the dish using an inverted microscope equipped with phase contrast optics. Adhesion was expressed as a percentage of the initial seeding density.

After 7 days, media was removed from the wells and washed with PBS. Cells were then fixed for 10 minutes with ice-cold methanol and allowed to air dry. Wells were washed once with distilled water to remove the salt residue. Numbers of colonies (>32 cells) per well were either counted using darkfield illumination or the cells were stained using 0.1% crystal violet for 30 minutes and then washed extensively in distilled water prior to viewing using phase contrast microscopy. Colony forming efficiency (CFE) was calculated by dividing the number of colonies by the initial number of adherent cells.

Data was analysed using Student's t-test to determine where significant differences exist. Differences were considered significant at the 0.05 confidence limit.

3.2.2 Immunolabelling: Notch 1, BST-1, CD106 and CD166

Strips of cartilage were removed from 2-3 week old bovine metatarsophalangeal joints were frozen, embedded in OCT and sectioned as described in section 2.1.

For immunolabelling, sections were washed twice for 10 minutes in PBS (Oxoid) prior to blocking with 2.5% normal rabbit serum (Dako) in PBS for 20 minutes at room temperature. Blocking solution was wicked off using a tissue and sections were incubated with primary antibodies in PBS overnight at 4°C as described in table 3.1.

Sections were then washed three times for 10 minutes with PBS and incubated with secondary antibodies for 45 minutes at room temperature followed by washing again three times for 10 minutes. Sections labelled with biotinylated secondary antibodies were subsequently incubated in a 1:100 dilution of streptavidin-FITC (Dako) followed by washing three times for 10 minutes in PBS. Sections were then mounted with Vectashield (Vector) containing propidium iodide, coverslipped and viewed using a Leica confocal microscope and ArKr laser exciting at 488nm and 568nm.

Controls comprised of sections incubated with non-specific IgG raised in the same species as the corresponding primary antibody.

Primary antibody		Final concentration	Secondary antibody	Dilution
Notch 1 (200μg/ml)	Goat anti Notch 1 (Santa Cruz)	20μg/ml	Rabbit anti goat IgG-FITC conjugated (Sigma)	1 in 100
BST 1 (1mg/ml)	Mouse anti-human BST 1 (MBL)	20μg/ml	Rabbit anti mouse IgG, FITC-conjugated (Sigma)	1 in 100
CD105 (0.25mg/ml)	Goat anti-human TGF-βRIII (R&D)	12.5μg/ml	Rabbit anti goat IgG, biotin-conjugated (Sigma)	1 in 100
CD166 (0.2mg/ml)	Rabbit anti-human ALCAM (R&D)	10μg/ml	Rabbit anti goat IgG, biotin-conjugated (Sigma)	1 in 100

Table 3.1: Details of antibodies used in immunolabelling studies.

3.2.3 Cell size analysis

Superficial, middle and deep zone chondrocytes were isolated from the articular cartilage of 2-3 week old bovine metatarsophalangeal joints as described in section 3.2.1.2.

Aliquots of the isolated cell suspensions were pipetted onto a haemocytometer and coverslipped. Cells were viewed using an inverted Olympus CK40 microscope and photographed at x100 magnification ensuring sufficient cells were present in the image (n>120). Image analysis using Image-Pro plus software calibrated with a graticule was carried out to determine the average diameter of cells present in the image. Statistical analysis was carried out using the Student's t-test.

3.3 RESULTS

3.3.1 Differential adhesion

Table 3.2 shows the initial cell adhesion over the two 20 minute time points for superficial, middle and deep zone chondrocytes cultured on fibronectin coated and un-coated wells (BSA coated).

Significant differences in adhesion were evident between cells from all zones grown on fibronectin for 20 minutes and those grown on uncoated dishes for 20 minutes. Although there was no significant difference in adhesion between the first 20 minute time point and second 20 minute point for the superficial zone cells grown on fibronectin, there was a significant difference for the middle and deep zone cells between the time points when grown on fibronectin ($p<0.05$ and $p<0.005$ respectively). Significant differences in adhesion were noted between superficial zones cells and middle zone cells ($p<0.005$), superficial zone cells and deep zone cells ($p<0.05$) and middle and deep zone cells ($p<0.05$) when cultured for 20 minutes on fibronectin.

3.3.2 Colony forming assays

Figure 3.2 shows the colony forming efficiency (CFE) of superficial, middle and deep zone chondrocytes cultured on fibronectin coated and uncoated wells for 7 days after the differential adhesion assay. CFE of the cohort of superficial zone cells that adhered to fibronectin within the first 20 minutes was significantly greater than the cohort of superficial zones cells than bound to fibronectin during the second 20 minute incubation and all other middle and deep zone cell cohorts ($p<0.01$).

	Percentage cell adhesion					
	SFZ20	**SFZ40**	**MZ20**	**MZ40**	**DZ20**	**DZ40**
Fibronectin coated	6.01±1.23	5.01±0.52	13.17±1.88	9.18±1.37	7.22±0.32	4.02±0.21
Uncoated (BSA coated)	1.21±0.10	2.22±0.34	4.59±1.18	3.00±1.00	4.00±1.98	3.05±2.02

Table 3.2: Initial adhesion to fibronectin coated and uncoated wells. SFZ = superficial zone cells; MZ = middle zone cells; DZ = deep zone cells; 20 = first 20 minute time point; 40 = second 20 minute time point. n = 4

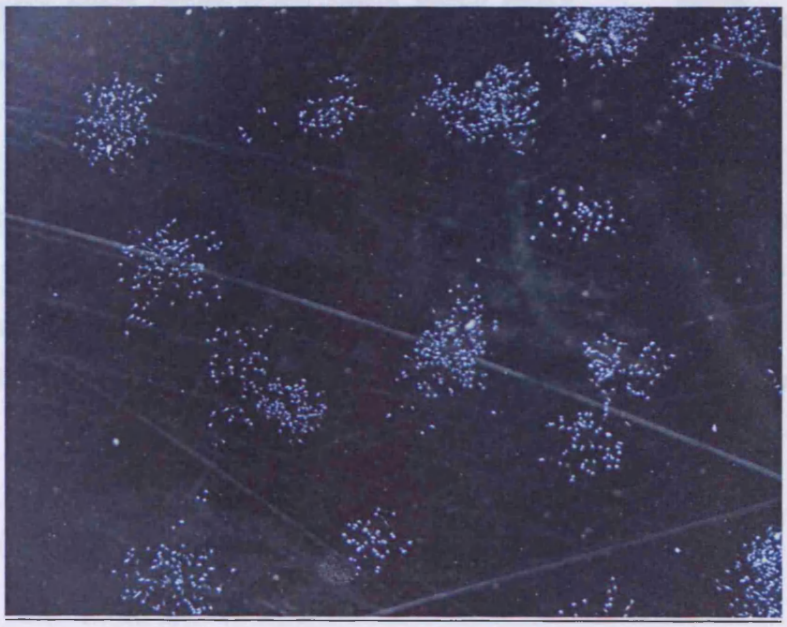


Figure 3.1: Image illustrating colony formation by the population of superficial zone cells that binds to plasma fibronectin within 20 minutes. The adherent cells were cultured for 7 days in 5% FCS. Viewed using darkfield illumination on a stereomicroscope with x4 magnification.

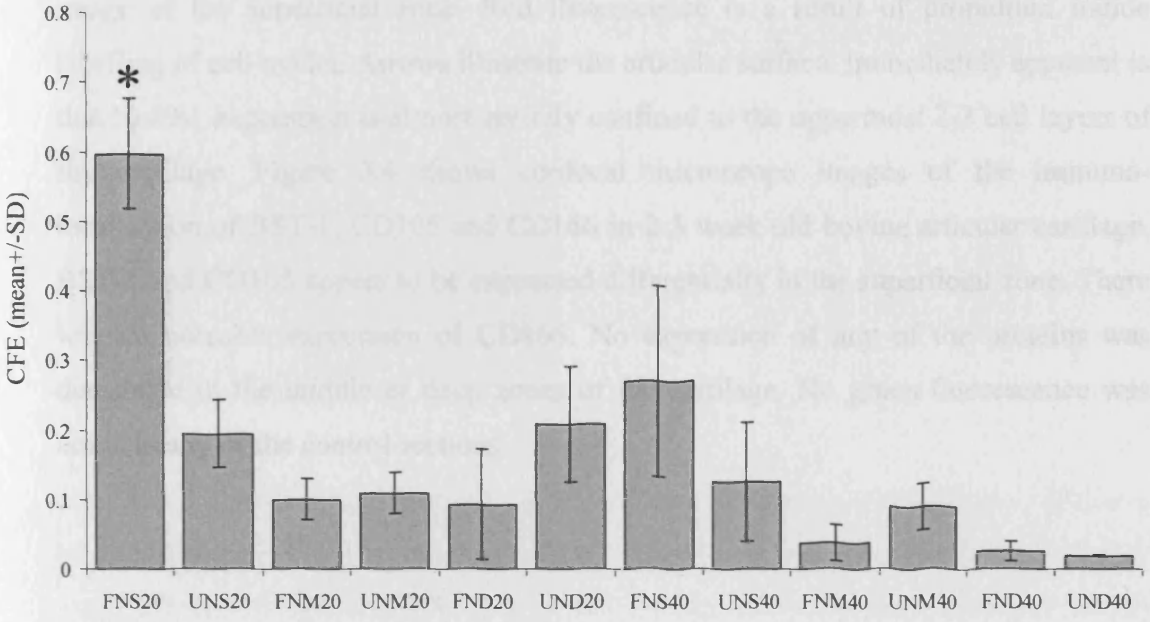


Figure 3.2: Colony forming efficiency of surface, middle and deep zone chondrocytes plated onto fibronectin coated and uncoated plates and cultured for 7 days in 5% FCS. FN = fibronectin coated; UN = uncoated; S= superficial cells; M = middle zone cells; D = deep zone cells; 20 = first 20 minute time point; 40 = second 20 minute time point. *p<0.01 compared to all other cohorts.

3.3.3 Immunolabelling: Notch 1, BST-1, CD106 and CD166

Figure 3.3A and B are confocal microscope images showing the immuno-localisation of Notch1 in 2-3 week old bovine cartilage. Figure 3.3B is a higher magnification image of the superficial zone. Red fluorescence is a result of propidium iodide labelling of cell nuclei. Arrows illustrate the articular surface. Immediately apparent is that Notch1 expression is almost entirely confined to the uppermost 2-3 cell layers of the cartilage. Figure 3.4 shows confocal microscope images of the immuno-localisation of BST-1, CD105 and CD166 in 2-3 week old bovine articular cartilage. BST-1 and CD105 appear to be expressed differentially in the superficial zone. There was no noteable expression of CD166. No expression of any of the proteins was detectable in the middle or deep zones of the cartilage. No green fluorescence was noted in any of the control sections.

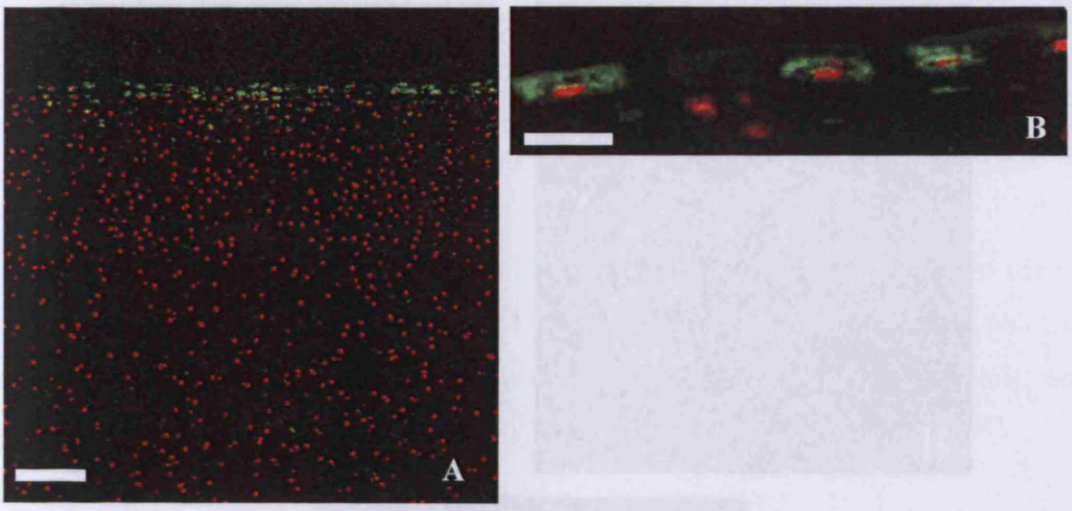


Figure 3.3: Confocal microscope images showing immunolocalisation of Notch 1 in 2-3 week old bovine articular cartilage at low magnification (A) and higher magnification of the superficial zone (B). Scale bars = 100μm and 10μm in (A) and (B) respectively.

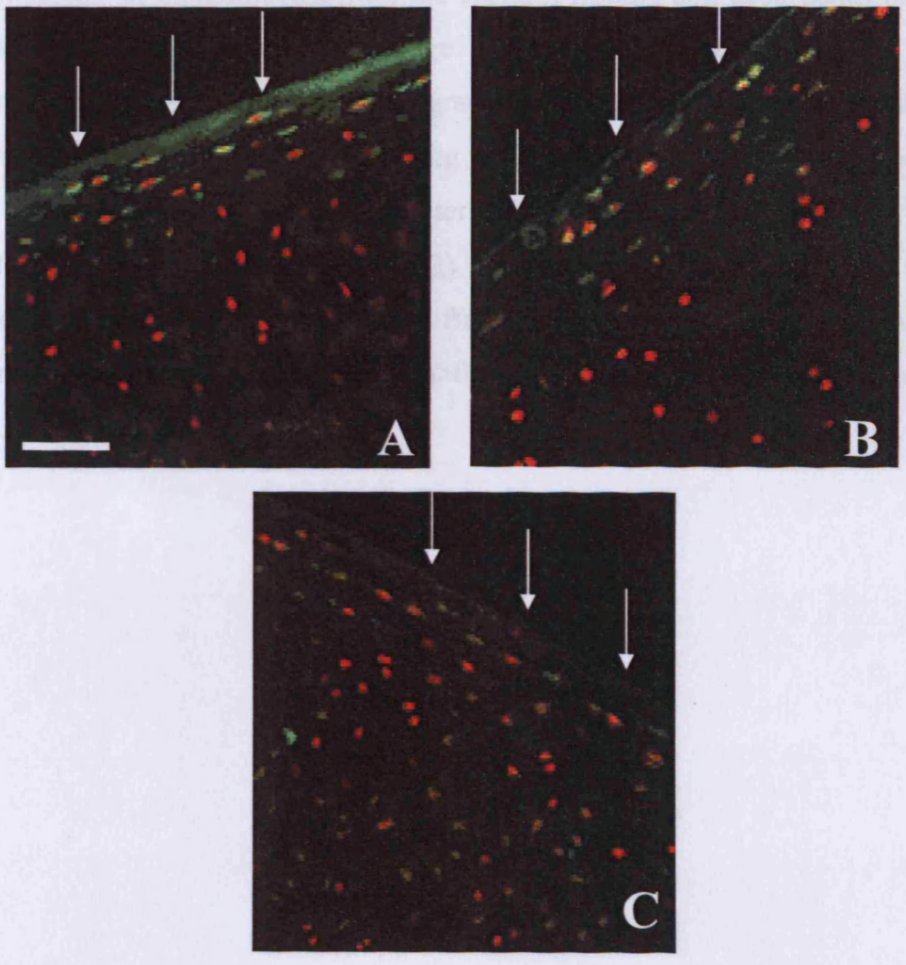


Figure 3.4: Images showing immunolocalisation of BST-1 (A), CD105 (B) and CD166 (C). Arrows indicate articular cartilage surface. Scale bar = 50μm

3.3.4 Cell size analysis

The diameter of chondrocytes from the surface, middle and deep zones of articular cartilage was determined using image analysis immediately following isolation. Figure 3.5 shows representative photographs of a superficial, middle, and deep zone chondrocytes respectively, viewed using phase contrast optics. Images analysis was used to determine the average diameter of large numbers of superficial (n=124), middle (n=275) and deep zone (n=182) chondrocytes and the results are shown in figure 3.6. There was a significant difference in average diameters between both superficial zone and middle zone ($p>0.05$) and superficial and deep zone cells ($p>0.005$).

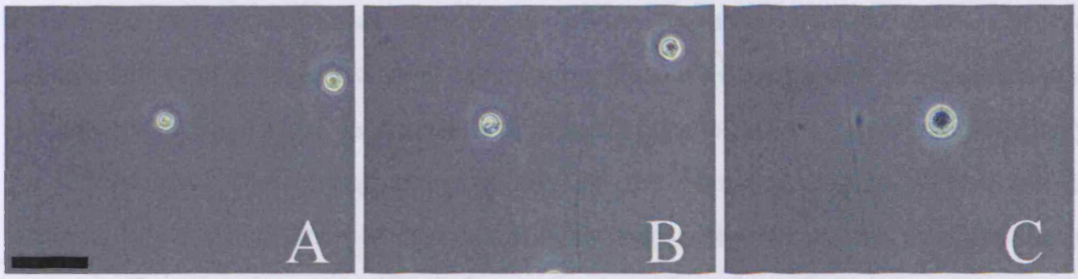


Figure 3.5: Phase contrast images of superficial (A), middle (B) and deep (C) zone articular chondrocytes immediately following isolation. Scale bar = 50μm

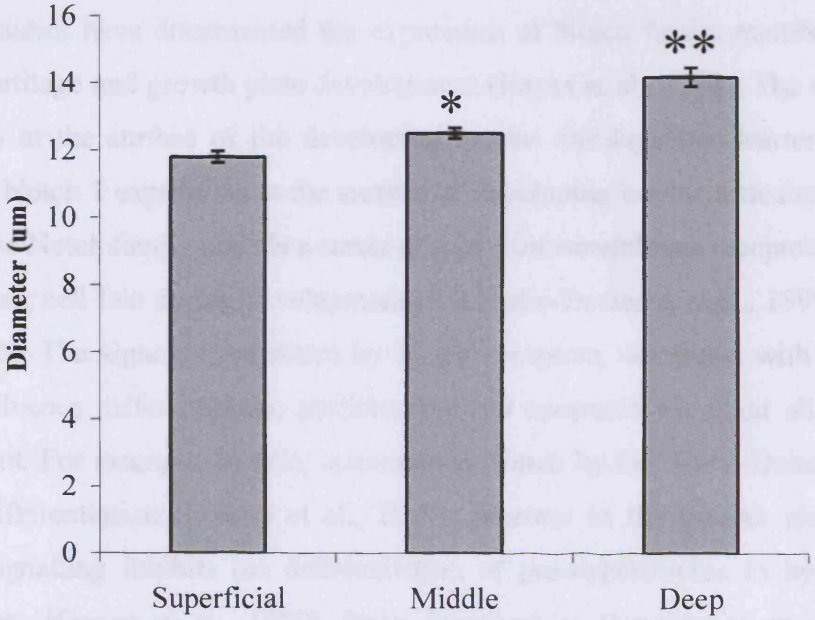


Figure 3.6: Average cell diameter of superficial, mid and deep zone chondrocytes (means +/- SEM). *p < 0.05, **p < 0.005 compared to superficial zone.

3.4 DISCUSSION

This chapter and published data (Dowthwaite et al., 2004) has described the use of differential adhesion to serum fibronectin to identify a population of progenitor cells within the surface layers of developing bovine articular cartilage. The cells are capable of forming large numbers of colonies from an initially low seeding density, unlike cells isolated from the middle and deeper zones which also have a high affinity for fibronectin. The strong adhesiveness of the chondrocytes to fibronectin is explained by the high level of expression of the $\alpha_5\beta_1$ integrin subunits, the fibronectin receptor (Dowthwaite et al., 2004). Interestingly, although surface zone cells express higher levels of the $\alpha_5\beta_1$ subunits than middle and deep zone cells, this does not correspond with increased adhesiveness to the fibronectin substrate, rather middle zone cells exhibited a higher affinity for fibronectin but lack colony forming ability and may signify a transit amplifying cell population.

Previous studies have documented the expression of Notch family members during articular cartilage and growth plate development (Hayes et al., 2003). The expression of Notch 1 at the surface of the developing murine cartilage was mirrored in this chapter by Notch 1 expression at the surface of developing bovine articular cartilage. Genes of the Notch family encode a series of type I transmembrane receptors involved in controlling cell fate during development (Artavanis-Tsakonas et al., 1999; Bianchi et al., 2006). The signals transmitted by Notch receptors, combined with other cell factors, influence differentiation, proliferation and apoptotic events at all stages of development. For example, in skin, activation of Notch by the ligand Delta promotes terminal differentiation (Lowell et al., 2000), whereas in the growth plate, Delta-Notch 2 signalling inhibits the differentiation of pre-hypertrophic to hypertrophic chondrocytes (Crowe et al., 1999). Data described in Dowthwaite et al., (2004) suggests that Notch 1 plays a significant role in the signalling mechanisms controlling the clonality of surface zone chondrocytes, although its precise role in this regard is unclear, data suggests Notch 1 signalling maintains clonality, promoting proliferation and inhibiting differentiation.

In addition to Notch 1, data described here shows further proteins that are differentially expressed in the surface layers of developing bovine articular cartilage,

bone marrow stromal cell antigen-1 (BST-1; CD157) and CD105 (endoglin). BST-1 is a highly glycosylated, glycosylphosphatidylinositol (GPI)-anchored membrane protein with a molecular weight of 43 kDa. which displays ADP ribosyl cyclase activity and facilitates pre-B cell growth. It is expressed in the bone marrow stroma and is thought to be involved in the regulation of haematopoiesis (Podesta et al., 2005). Studies using gene chip analysis to compare gene expression between surface zone and middle zone chondrocytes highlighted the differential expression of BST-1 by surface zone cells (B Thomson, personal communication). Immunolabelling studies described here have confirmed this finding at the protein level. The role of differential expression of BST-1 by the surface zone cells is unclear at present. CD105, also known as endoglin is a homodimeric membrane glycoprotein and is thought to play a key role in cellular interactions with transforming growth factor-β (TGF- β). The precise role of endoglin in the surface layers is unknown. High levels of are known to be expressed in this region (Hayes et al., 2001) and thus, CD105 may play a role in the regulation of TGF- β signalling in the surface regions during development.

Although Notch 1, BST-1 and CD105 appear to be differentially expressed by the superficial zone chondrocytes, an examination of the data reveals that expression of these proteins is not restricted to progenitor cells. From the data it is clear that only 1-2% of the cells in the superficial zone are capable of forming colonies whereas the majority of the cells in the superficial zone appear to express these proteins. In the case of Notch 1, 75% of the superficial zone cells have been determined to express Notch 1 (Dowthwaite et al., 2004). Thus, these proteins could not be considered markers of the progenitor cells.

Chondrocytes are known to exhibit different phenotypes and morphologies throughout the depth of the tissue (Zanetti et al, 1985; Aydelotte and Kuettner, 1988; Aydelotte et al., 1988; Archer et al., 1990). In order to compare cell phenotype throughout the depth of the tissue, a protocol is needed for optimal separation of the different zones. Various methods of dissecting articular cartilage into separate zones of cartilage have been reported (Archer et al., 1990; Sun and Kandel, 1999), although it is unlikely these techniques yield pure preparations of the different types of chondrocytes, nor do they give cell populations that correspond exactly with the

different zones of the tissue. The finding in this chapter that the middle and deep zones possess different sizes compared to the surface zone cells raises the possibility of the use of density gradient centrifugation to prepare more uniform and more pure fractions of chondrocytes (Min et al., 2002) that also correspond more accurately to the different zones of the tissue.

In conclusion, the surface zone of immature bovine articular cartilage contains a progenitor cell population that is responsible for the appositional growth of articular cartilage. The focus of the following chapters is to further characterise the cells and to investigate the potential of the cells in human cartilage repair therapies.

CHAPTER 4

FIBRONECTIN-EDA+

4.1 INTRODUCTION

It is well accepted there is a close relationship between cellular phenotype and specialised extra-cellular (ECM) matrix formation. Cells synthesise an ECM according to their state of maturation and function and matrix components are able to modulate fundamental cellular properties, for example proliferation and differentiation (Adams and Watt, 1993). Tissue modulation processes in embryonal and immature tissues require a supporting and connecting extracellular matrix. In this respect, the ECM protein fibronectin has been extensively studied and found to be a nearly ubiquitous cell-cell and cell-matrix adhesion molecule.

Fibronectins (FNs) are multifunctional adhesive glycoproteins present as an insoluble form in the ECM and as a soluble form in various body fluids such as plasma. FNs are involved in many cellular processes, acting as substrates for cell migration and adhesion during embryogenesis, tumour progression, tissue repair and blood clotting (Hynes, 1990). FNs are disulphide-bonded dimers of two closely related subunits, each consisting of three types of homologous repeating units termed types I, II and III (Kosmehl et al., 1996). The domain structure of fibronectin is illustrated in figure 4.1. These repeats are organised into a series of functional domains that bind to integrins, collagens, heparin and heparan sulphate, fibrin, and FNs themselves.

FNs also interact with cells at three distinct regions: the central cell binding domain (CCBD), the COOH-terminal heparin-binding domain (Hep2), and the type III-connecting segment (IIICS) including the CS1 region. CCBD is the major cell-adhesive domain of FN and contains the Arg-Gly-Asp (RGD) motif and the Pro-His-Ser-Arg-Asn (PHSRN) sequence that are recognised by members of the integrin family of cell adhesion receptors, including $\alpha5\beta1$, the primary FN receptor in many cell types (Manabe et al., 1997). Interaction of $\alpha5\beta1$ with CCBD has been shown to transduce signals that regulate cell proliferation, differentiation, and apoptosis (Giancotti and Ruoslahti, 1990; Meredith et al., 1993).

FNs purified from different sources have been determined to possess different subunit sizes. This heterogeneity of FN subunits arises mainly from alternative splicing of a

primary transcript at three distinct places termed EDA, EDB and IIICS (Kornbliht et al., 1985). The EDA and EDB segments are each encoded by single exons and comprise intact type III repeats, whereas the IIICS segment consists of five distinct variants due to exon subdivision. Up to 20 different FN subunits may result from alternative splicing at these regions.

Although there is substantial evidence that alternative splicing at these regions is regulated in a tissue-specific and onco-developmental manner, little is known about the specific functions of these various isoforms. Soluble plasma FN produced by adult hepatocytes contains neither EDA or EDB segments in both subunits and lacks the entire IIICS segment in one of the subunits (Yamada et al., 1985), whereas insoluble cellular FNs expressed during wound healing and in fetal and tumour tissues contain a greater percentage of EDA and EDB segments than those expressed in normal adult tissues (Carnemolla et al., 1989; ffrench-Constant et al., 1989; ffrench-Constant and Hynes, 1989). Correspondingly, fibroblastic, tumourigenic and fetal cell lines express greater quantities of FNs with EDA and/or EDB segments. The presence of the EDA segment within the FN molecule has been shown to promote cell spreading and migration (Manabe et al., 1997) and regulate cell cycle progression and mitotic signal transduction through up-regulation of integrin-mediated mitotic signal transduction (Manabe et al., 1999). Thus EDA containing FN appears to be associated with cellular processes requiring high levels of cell proliferation and migration as occurs in tumourigenesis, embryogenesis and wound healing.

Specific temporal and spatial changes in the content of the extracellular matrix or in expression of cell adhesion molecules is likely to be involved in the regulation of cartilage differentiation and chondrocyte function (Dessau et al., 1980; Adams and Watt, 1993; Tuan, 2004). In addition, cartilage homeostasis is partly regulated by chondrocyte-extracellular matrix interactions and FN is known to play a role in chondrocyte adhesion and mechano-transduction signalling events (Millward-Sadler et al., 2000). Interestingly, although FN-EDA has been demonstrated to be absent from mature articular cartilage (Chevalier et al., 1996; MacLeod et al., 1996) its expression has been demonstrated in the surface layers of human fetal articular cartilage (Salter et al., 1995) and in chick embryonic limb pre-cartilage mesenchyme (Kuo et al., 2002; Peters et al., 2002; White et al., 2003), thus suggesting a specific

role for the isoform during chondrocyte differentiation. Furthermore, antibodies specific for the EDA region inhibited chondrogenesis of limb micro-mass cultures *in vitro* and when injected into chick limb buds *in vivo*, caused moderate to severe skeletal malformations (Gehris et al., 1996 and 1997). Thus, the function of the EDA region appears to be the regulation of mesenchymal cell spreading (White et al., 2003) therefore permitting and/or promoting adequate cell-cell interaction to take place during the condensation form of chondrogenesis. The tissue localisation of FN-EDA during development appears to overlap with the predicted location of the articular cartilage progenitor cells (Salter et al., 1995). The enhanced adhesiveness of FN-EDA and its ability to promote cell spreading, migration and proliferation via increased binding to the $\alpha 5\beta 1$ integrin (Manabe et al., 1999) may be important to the maintenance of phenotype of the progenitor cells and/or necessary for supporting the high proliferative potential of the cells in this region.

The aims of this study were three-fold:

[1] Determine the expression of FN-EDA in immature bovine articular cartilage.

[2] Evaluate the expression of FN-EDA by fibroblast, tumour and fetal cell lines and purify FN-EDA from media conditioned by a selected cell line using immuno-affinity chromatography.

[3] Compare the difference between plasma fibronectin and purified FN-EDA on superficial zone and middle zone chondrocyte attachment and colony forming efficiency.

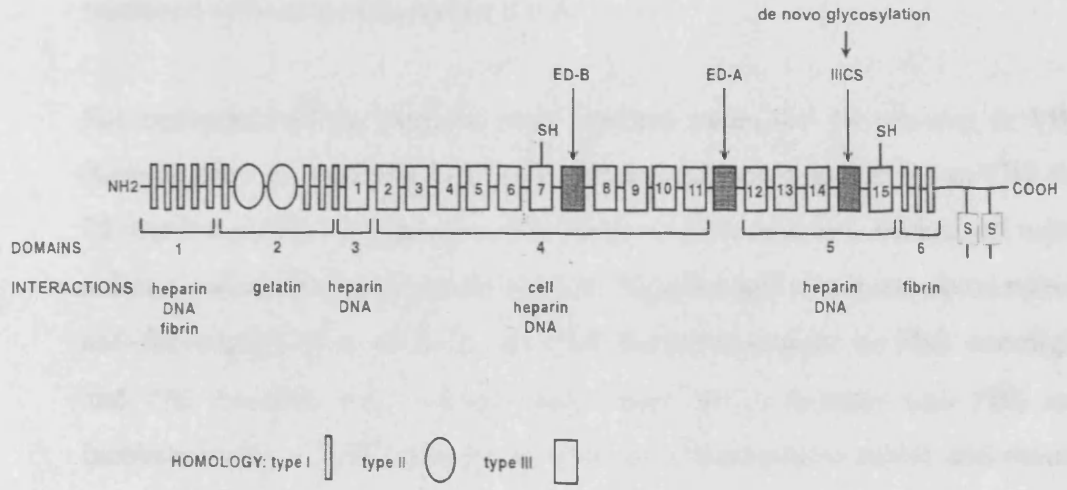


Figure 4.1: Model of the domain structure of a fibronectin subunit. Taken from Kosmehl et al., 1996.

4.2 MATERIALS AND METHODS

4.2.1 Immuno-localisation of Fibronectin-EDA in immature articular cartilage

4.2.1.1 Fibronectin-EDA immunolabelling

Strips of cartilage were removed from 7 day-old bovine metatarsophalangeal joints as described in section 2.1. The cartilage was embedded in OCT and sectioned as described in section 2.3.6.

For immunolabelling, sections were washed twice for 10 minutes in PBS (Oxoid) prior to blocking with 2.5% normal rabbit serum (Dako) in PBS for 20 minutes at room temperature. Blocking solution was then wicked off using a tissue and sections were incubated with 50μg/ml IgG of a monoclonal mouse anti-fibronectin-EDA antibody (Oxford Biotechnologies) in PBS overnight and 4°C. Sections were washed three times for 10 minutes with PBS and incubated with a 1:100 dilution in PBS of a biotinylated rabbit anti-mouse secondary antibody (Dako) for 45 minutes at room temperature followed by washing three times for 10 minutes. Sections were then incubated with a 1:100 dilution in PBS of a Streptavidin-FITC conjugate (Amersham Pharmacia) for 1 hour at room temperature followed by washing for three times for 10 minutes. Sections were finally mounted with Vectashield (Vector, Peterborough) containing propidium iodide for visualisation of cell nuclei, coverslipped and viewed using a Leica confocal microscope with an ArKr laser exciting at 488nm and 568nm.

4.2.1.2 Fibronectin-EDA/Notch1 double labelling

Protocol was repeated as described above with the addition of 20μg/ml of the polyclonal goat anti-human Notch1 (C-terminal) antibody (Santa Cruz) together with the anti-fibronectin-EDA antibody. Detection was carried out using a rabbit anti-goat-FITC conjugated secondary antibody (Dako) for Notch1 and a biotinylated rabbit anti-mouse secondary antibody followed by a streptavidin-Texas Red conjugate (Amersham Pharmacia) for fibronectin-EDA. The use of Texas Red provides a greater separation of emission spectra from FITC than TRITC and also excites to a greater degree on the 568nm spectral line of the ArKr laser than TRITC. Sections were mounted with Vectashield (Vector, Peterborough) without propidium iodide. Special care was taken to minimise crossover of fluorescence between the two photo-multipliers due to emission spectra overlap by reduction of laser power at the specific excitation values.

4.2.1.3 Controls

Control sections were incubated with the same concentration of non-immune mouse IgG1 and/or goat IgG.

4.2.2 Purification of Fibronectin-EDA from conditioned media

In these studies, fibroblastic, tumourigenic and fetal cell lines were used as these would be expected to produce greater quantities of FNs with the EDA segment (see section 4.1).

4.2.2.1 Fibronectin-EDA immunocytochemistry on cell lines

Human dermal fibroblasts (passage 4), MRC-5 cells (human fetal lung fibroblasts) (passage 22) and MG-63 cells (human osteosarcoma) (passage 107) were passaged and maintained as described in section 2.2. Cells were

counted using a haemocytometer and seeded into 4-well chamber slides at 100,000 cells/well and grown to confluence.

Monolayers were washed three times with PBS and fixed for 10 minutes with -20°C methanol. Monolayers were then washed three times for 10 minutes with PBS and blocked with 2.5% normal rabbit serum in PBS for 20 minutes. Monolayers were washed once with PBS and incubated with 50μg/ml IgG of a mouse monoclonal anti-FN-EDA antibody (Oxford Biotechnologies) in PBS for 90 minutes. Monolayers were again washed three times for 10 minutes with PBS, incubated with a 1:100 dilution of a biotinylated rabbit anti-mouse secondary antibody (Dako) for 1 hour, washed three times for 10 minutes with PBS and incubated with a 1:100 dilution of a streptavidin-FITC conjugate (Amersham Pharmacia) in PBS for 1 hour. Monolayers were then washed three times for 10 minutes with PBS, mounted with Vectashield (Vector, Peterborough) containing propidium iodide, coverslipped and viewed using a Leica confocal microscope with an ArKr laser exciting at 488nm and 568nm.

4.2.2.2 Fibronectin-EDA ELISA on conditioned media

ELISAs were used semi-quantitatively as a guide to the efficiency of production of fibronectin-EDA by the cell lines.

For coating of plates, media conditioned by various cell lines for various amounts of time was pipetted into the wells of 96 well plates at 200μl/well. Cell lines used are illustrated in table 4.1. MG-63 and MRC-5 cells were used to condition media over a 6 day period. In addition, MG-63 cells were allowed to condition media in the absence of serum. The use of media without serum would be preferable as serum contains large quantities of plasma fibronectin. Samples of this treatment were also taken over time to look at the extent of accumulation of FN-EDA in the media over the conditioning period. Human dermal fibroblasts and RCM-1 cells were allowed to condition media for a greater length of time as these cells took longer to reach confluence. Dermagraft conditioned media was also used (Supplied by Advanced Tissue

$CaCl_2$ and $MgCl_2$) was used for coating. Coating was allowed to take place overnight at 4°C.

PBS-T was prepared by adding 0.04g sodium azide and 0.1ml Tween-20 to 200ml PBS. After coating, wells were washed five times with PBS-T and then blocked with 5% low fat milk powder in PBS-T for 2 hours at 37°C. Plates were washed five times with PBS-T and incubated with 100µl of 10µg/ml anti-FN-EDA antibody (Oxford Biotechnologies) in PBS-T containing 1% low fat milk powder overnight at 4°C. Plates were then washed five times with PBS-T and incubated with 100µl of an alkaline phosphatase conjugated rabbit anti-mouse secondary antibody (Sigma, Poole) diluted 1:3000 in PBS-T containing 1% low fat milk powder for 90 minutes at 37°C. Plates were then washed five times and the substrate added (10mg p-nitrophenyl phosphate in 10mM diethanolamine, 1mM $MgCl_2$, pH 9.8) and development of yellow colouration monitored and photographed.

In addition to the above, wells coated with plasma fibronectin were detected using a pan-fibronectin antibody (rabbit anti-human FN; Dako) diluted 1:100 and detected using a horseradish peroxidase conjugated goat anti-rabbit secondary antibody at 1:100 dilution (Dako). The substrate for this consisted of 200µl of 10mg/ml tetramethyl benzidine in DMSO and 3µl of hydrogen peroxide added to 20ml of 0.1M sodium acetate buffer pH 6.0. Development of blue colouration monitored and photographed.

Cell line	Description	Passage number	Length of time conditioned (days)
MG-63 in serum-free media	Human osteosarcoma	116	1
MG-63 in serum-free media	Human osteosarcoma	116	2
MG-63 in serum-free media	Human osteosarcoma	116	6
MG-63	Human osteosarcoma	117	6
MRC-5	Human fetal lung fibroblast	43	6
Human dermal fibroblasts	Human fibroblast	30	8
RCM-1	Human adenocarcinoma	70	9

Table 4.1: Cell lines used to condition media for ELISA to evaluate FN-EDA content.

4.2.2.3 Production of fibronectin-EDA immuno-affinity column

Anti-FN-EDA antibody (2mg) was dialysed twice against coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH8.3) for 90 mins and overnight respectively at 4°C using 10,000 MWCO dialysis tubing. CNBr activated Sepharose 4B (Amersham Pharmacia Biotech) (1g) was swollen in 1mM HCl and washed with >200ml 1mM HCl on a scinter funnel. The dialysed antibody was mixed with the gel and rotated at room temperature for 2 hours before the remaining active sites were blocked by incubation in 0.1M Tris-HCl for 2 hours at room temperature. The gel was then washed with 3 cycles of high and low pH buffer to ensure removal of unbound material, transferred to PBS containing 0.02% sodium azide and packed into a column and equilibrated at 10ml/h.

Aliquots of antibody (2 x 20µl) were removed prior to mixing with gel. In addition, aliquots of supernatant (2 x 20µl) were removed after incubation with gel. These aliquots were removed for protein estimation using the BCA assay (Pierce) according to the manufacturer's instructions to evaluate degree of antibody coupling to the gel.

4.2.2.4 Affinity purification of fibronectin-EDA from conditioned media

Immuno-affinity chromatography methods used were similar to that described by Miyashita et al., (1998). Two confluent flasks of MG-63 cells were transferred to serum-free media and the media collected after two days. (ρ-amidino-phenyl)methanesulphonyl fluoride (PMSF; Sigma, Poole) was made up by dissolving 34.8mg in 95% ethanol. This was added at a ratio of 1:100 to the collected media to bring the final concentration to 2mM PMSF. Media was stored at –70°C until used.

All chromatographic steps were carried out at 4°C. Forty ml of the conditioned media was applied to column at 10ml/h and re-circulated. Unbound material was washed from the column using PBS, fractions were collected every 10 minutes and the absorbance at 280nm determined using a Perkin Elmer

Lambda 2 spectrophotometer. When the absorbance reached a stable baseline, FN-EDA was eluted from the column with elution buffer (20mM CAPS, 150mM NaCl, 10mM EDTA, pH11). Eluted fractions with peak absorbance at 280nm were pooled and dialysed against 4l of 10mM CAPS, 150mM NaCl, 1mM $CaCl_2$, pH11 overnight at 4°C using 10,000 MWCO dialysis tubing. Aliquots were taken for protein estimation, SDS-PAGE and ELISA and the remainder stored at -70°C.

ELISA of the un-fractionated conditioned media, the unbound material from the column and eluted fractions were carried out as described above. Media and solutions from the column were added to the wells of the 96-well plates at 200µl/well and coating was allowed to take place overnight at 4°C.

For SDS-PAGE, aliquots were freeze-dried and reconstituted to 100µg/ml protein with deionised water. Ten µl aliquots of 100µg/ml of purified FN-EDA, plasma fibronectin (Sigma, Poole) and cellular fibronectin (Sigma, Poole) were prepared. Three µl of sample buffer (see below) was added to each of the samples. The total sample (1µg) of plasma fibronectin, cellular fibronectin and purified FN-EDA from the column was applied to a 4-12% bis-acrylamide gel (Nupage-Novex) and run at 200V for 2 hours in a running buffer of 25mM Tris, 190mM glycine, 0.1%SDS, pH8.3. The gel was stained with Simply Blue Safestain (Invitrogen, Paisley) for 1 hour at room temperature then washed twice for 45 minutes in distilled water. Rainbow recombinant protein markers (Amersham) with a molecular weight range of 10,000 to 250,000 were used for comparison according to the manufacturers instructions.

Sample buffer: 1mg bromophenol blue; 800µl 2M Tris-HCl pH8.8; 50ml 60% sucrose; 3.5ml 20% w/w SDS; 500µl 2-Mercaptoethanol; 200µl UHQ water.

4.2.2.5 Cell adhesion to purified FN-EDA

Pooled FN-EDA from the column (100µl) was added to 6 wells of a 96 well plate and incubated overnight at 4°C. Control wells were coated with 1% BSA. The wells were then washed 3 times with PBS, blocked for 1 hour at 37°C with 1 % BSA and washed a further 3 times with PBS. MG-63 cells were trypsinised as described in section 2.2.2.2, counted using a haemocytometer, resuspended in serum-free DMEM and 30,000 cells added to the wells of the 96-well plate and the cultures incubated for 30 minutes at 37°C. The wells were then washed and live cell stain added (2.5µl calcein AM in 5ml PBS – Molecular Probes), and viewed using a Leica DM-IRBE inverted confocal microscope using an ArKr laser exciting at 488nm.

4.2.3 Evaluation of articular cartilage progenitor cell behaviour on Fibronectin-EDA

4.2.3.1 Differential adhesion and colony forming efficiency assays using purified Fibronectin-EDA

Differential adhesion and colony forming assays were carried out as described in section 3.2.1. Plates were coated with purified FN-EDA, bovine plasma fibronectin (pFN) (Sigma) at 10µgml^{-1} using Dulbecco's PBS with 1mM $MgCl_2$ and 1mM $CaCl_2$ (Sigma).

Colonies were counted after 7 days. Media was removed from the wells and washed with PBS. Cells were then fixed for 10 minutes with ice-cold methanol and allowed to air dry. In some cases, fixed cells were stained with 0.1% crystal violet for 30 minutes and washed with distilled water. Colonies were counted using phase contrast optics and colony forming efficiency (CFE) was calculated by dividing the number of colonies by the initial number of adherent cells. Data was analysed using a Student's T Test and considered significant at the 0.05 confidence limit.

4.3 RESULTS

4.3.1 Fibronectin-EDA immunohistochemistry

Figure 4.2A is a confocal microscope image showing the immuno-localisation of FN-EDA in the articular surface of 2-3 week old bovine cartilage. Figure 4.2B is an image taken from the middle zone and figure 4.2C shows the control image incubated with an equivalent concentration of non-immune mouse IgG1. Red fluorescence is a result of propidium iodide labelling of cell nuclei. Arrows illustrate the articular surface. Immediately apparent is that FN-EDA expression is cell-associated and is almost entirely confined to the superficial zone of the cartilage. Small amounts of fluorescence can be seen in the lower regions, however this was also seen on the control sections.

Figure 4.3 is a confocal microscope image of the articular surface showing Notch1 and FN-EDA co-localisation in the cells of the superficial zone of immature bovine articular cartilage. Arrows illustrate the articular surface. Control images showed no fluorescence.

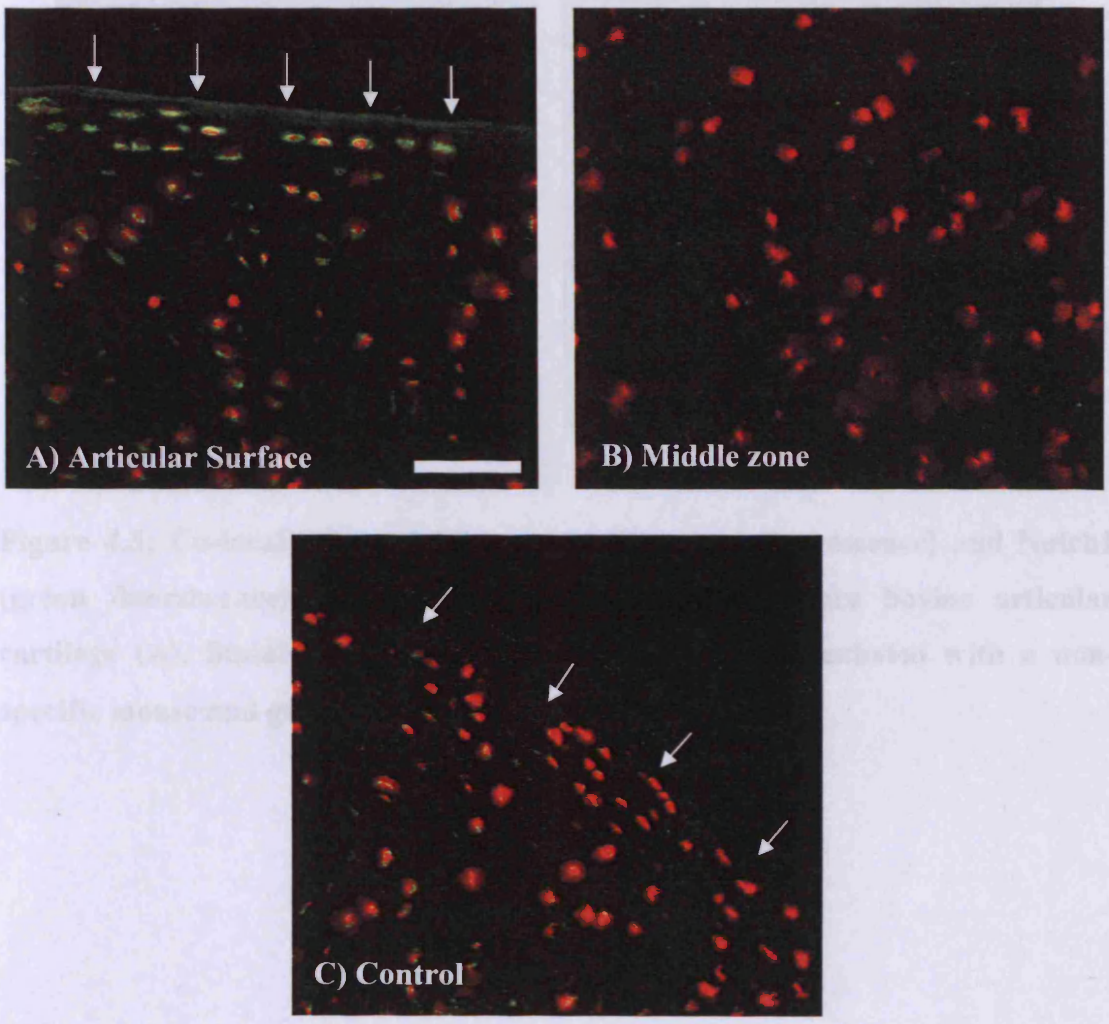


Figure 4.2: Immunolocalisation of Fibronectin-EDA in 2-3 week old bovine articular cartilage. Fibronectin was localised pericellularly around superficial zone chondrocytes (A) and absent from chondrocytes deeper into the tissue (B). Staining was absent in control sections incubated with a non-specific mouse IgG1 (C). Scale bar = 50μm

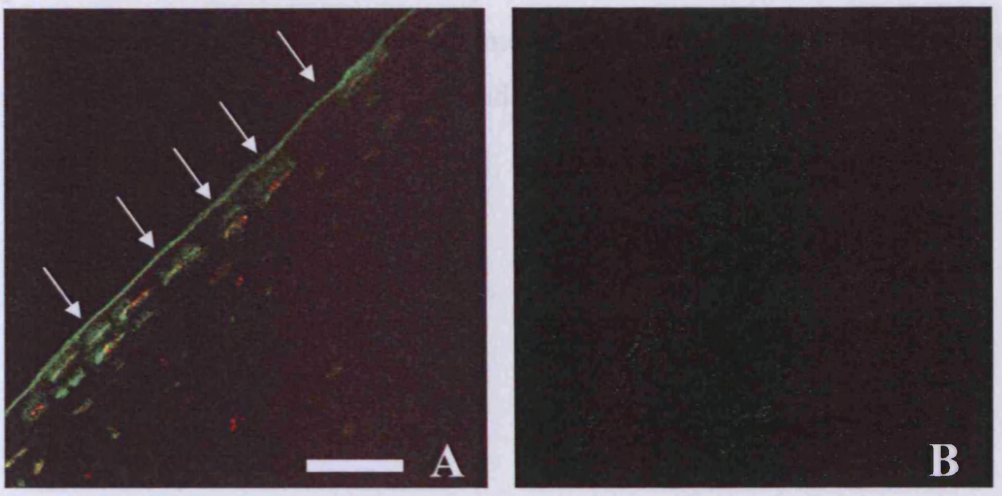


Figure 4.3: Co-localisation of Fibronectin-EDA (red fluorescence) and Notch1 (green fluorescence) in the superficial zone of immature bovine articular cartilage (A). Staining was absent in control sections incubated with a non-specific mouse and goat IgG (B). Scale bar = 50μm

4.3.2 Fibronectin-EDA immunocytochemistry on cell lines

Figures 4.4 (A), (B) and (C) are confocal microscope images of anti-FN-EDA immunocytochemistry on the three cell lines. Figure 4.4 (D) is a confocal microscope image of MG-63 cells stained with an equivalent concentration of non-immune mouse IgG1 as a control. FN-EDA in the cell monolayer is clearly visible in all three cell lines. Red fluorescence is a result of propidium iodide labelling of cell nuclei.

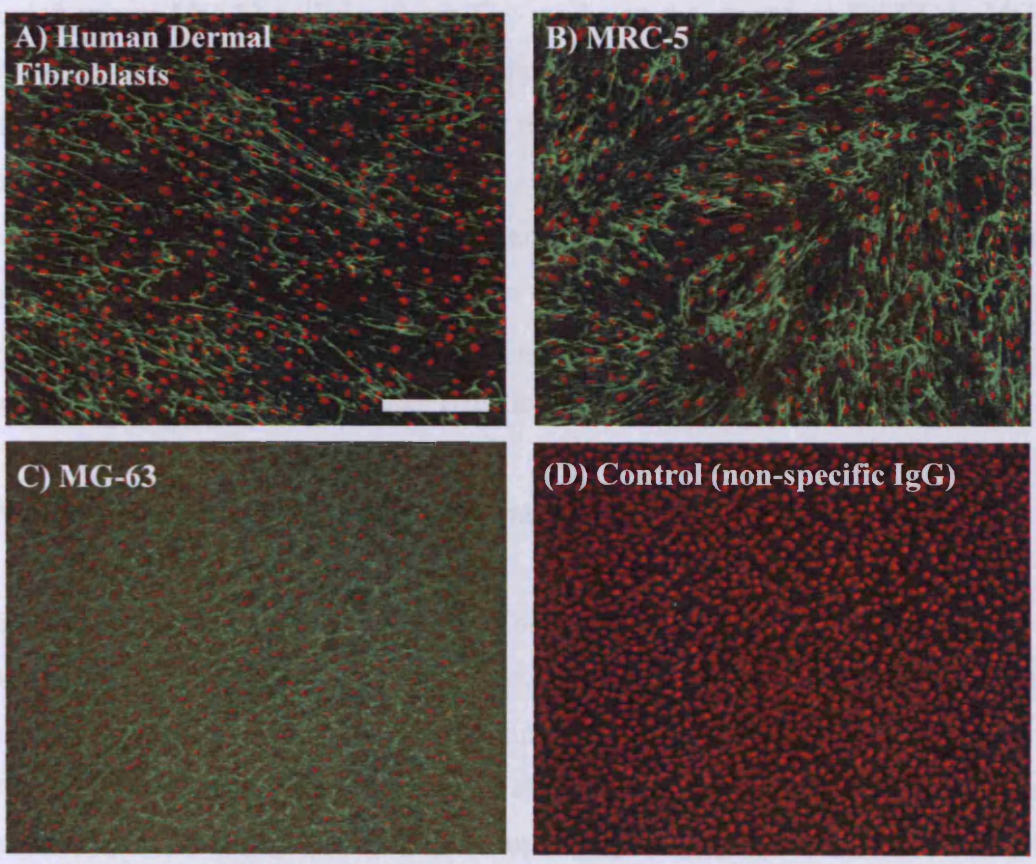


Figure 4.4: Confocal microscope images of three cell lines immunostained for FN-EDA. Scale bar = 100μm.

4.3.3 Fibronectin-EDA ELISA on conditioned media

Figure 4.5 illustrates the ELISA results from the conditioned media. The colouration that developed was not quantified using spectrophotometry. Instead, visual comparisons were made between the different treatment groups. There appeared to be little difference between any of the samples from the MG-63 cells (with or without serum), MRC-5 cells or the human dermal fibroblasts. From these results it was decided to use MG-63 cells in serum-free media as the source of FN-EDA. MG-63 cells were chosen as the cells not only appear to secrete large amounts of FN-EDA into the media, but they also grow rapidly in culture. It was decided to use serum-free media for conditioning as serum contains a high level of plasma fibronectin that may interfere with the purification process and also non-specific proteases that may degrade the column. The absence of serum does not appear to affect the production of FN-EDA by the MG-63 cells. Figure 4.5 also shows that the anti-FN-EDA antibody does not cross-react with plasma fibronectin.

4.3.4 Production of fibronectin-EDA immuno-affinity column

Aliquots of antibody solution were taken before and after mixing with the Sepharose 4B gel for protein estimation to estimate the degree of antibody coupling. Protein concentration in the antibody solution during the coupling step should decrease as the antibody binds to the Sepharose 4B gel. Table 4.2 shows the protein concentrations of the antibody solutions before and after coupling and suggests that all the antibody has bound to the gel. The reason for the negative protein value after coupling is unclear and may be due to the presence of material from the coupling gel interfering with the assay.

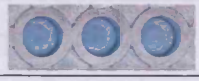
Cell line	Anti-FN-EDA	Anti-total FN
MG-63		-
MG-63 in serum-free media 1 day		-
MG-63 in serum-free media 2 days		-
MG-63 in serum-free media 6 days		-
MRC-5		-
Human dermal fibroblasts		-
RCM-1		-
Dermagraft CM		-
Normal Tissue Culture Media		-
Plasma fibronectin		
Cellular fibronectin		-

Figure 4.5 Digital photographs of colour change during FN-EDA ELISA on various conditioned media and forms of fibronectin.

	Protein concentration (μg/ml)
Before Coupling	188.6
After Coupling	-29.4

Table 4.2: Protein concentrations of antibody solutions before and after coupling to the sepharose 4B gel.

4.3.5 Affinity purification of fibronectin-EDA from conditioned media

Figure 4.6 shows the elution profile of proteins exiting the column. Fractions with peak absorbance after the addition of elution buffer were pooled and dialysed, then analysed by ELISA and SDS-PAGE to confirm the identity of the protein.

Figure 4.7 illustrates anti-FN-EDA and anti-total FN ELISA of various fractions from the column. This identifies the protein in the pooled fractions as being FN-EDA. The results also indicate that all the FN-EDA is not being fully removed from the conditioned media during the affinity purification as there is a level of FN-EDA present in the unbound material (ie. media which has passed through the column).

The pooled fractions were also subjected to SDS-PAGE in order to check the purity of the FN-EDA. Cellular fibronectin and plasma fibronectin was also added to the gel as a comparison. The results are shown in figure 4.8. The FN-EDA appears at approximately 270 kDa and runs as a tight band in comparison to cellular fibronectin and plasma fibronectin, thus suggesting its relative purity. FN-EDA and cellular fibronectin appear at a higher molecular weight than plasma fibronectin due to presence of extra polypeptide domains that are absent in plasma fibronectin.

4.3.6 Biological activity of FN-EDA

Figures 4.9 (A) and (B) show the attachment and spreading of MG-63 cells to non-coated and purified FN-EDA coated cell culture plates in serum-free media. Whereas few cells adhered to the non-coated wells, MG-63 cells rapidly adhered to the FN-EDA coated wells, suggesting the purified FN-EDA has retained some of its biological properties.

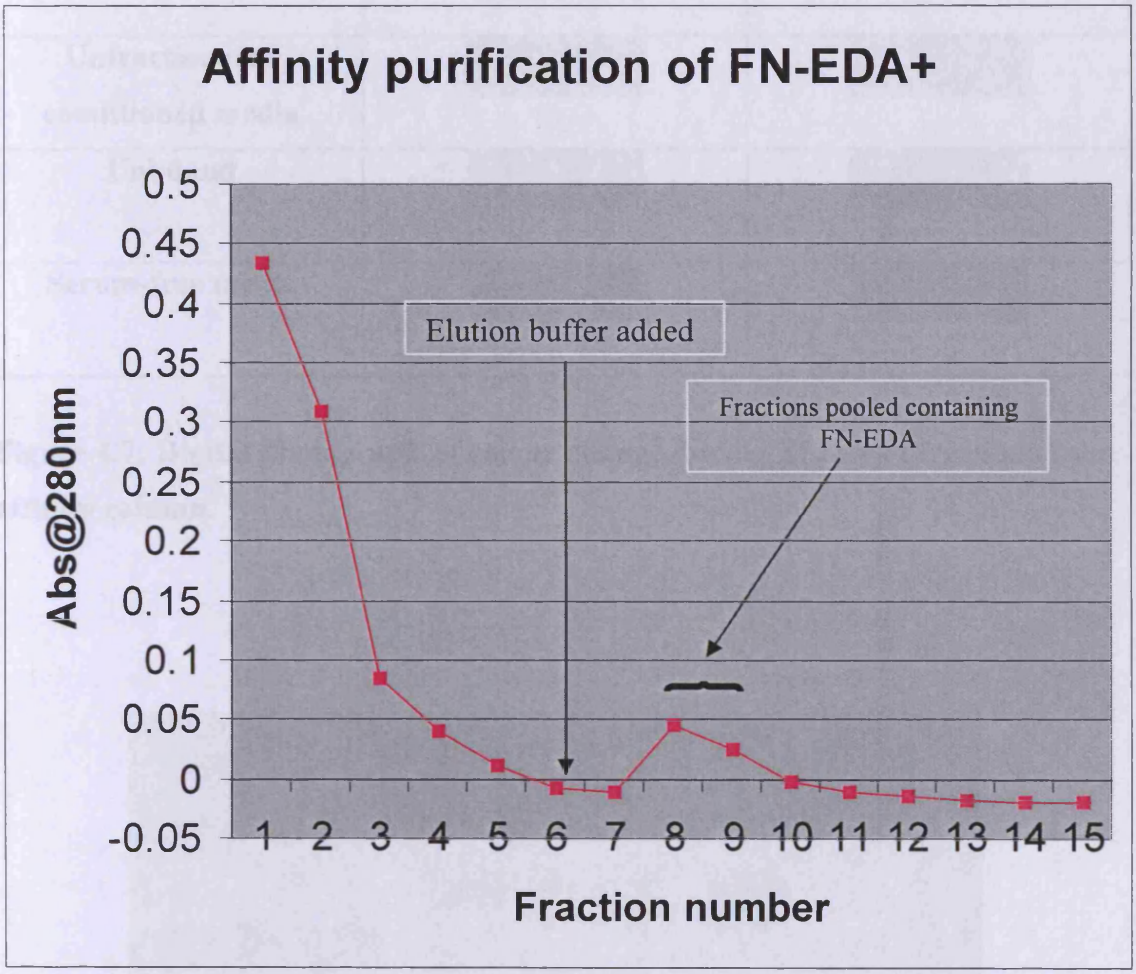


Figure 4.6: Elution profile during immuno-affinity purification of FN-EDA.

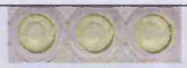
	Anti-FN-EDA	Anti-total-FN
Pooled eluted fractions		Not Done
Unfractionated conditioned media		
Unbound		
Serum-free media		

Figure 4.7: Digital photograph of colour change during ELISA of fractions from affinity column.

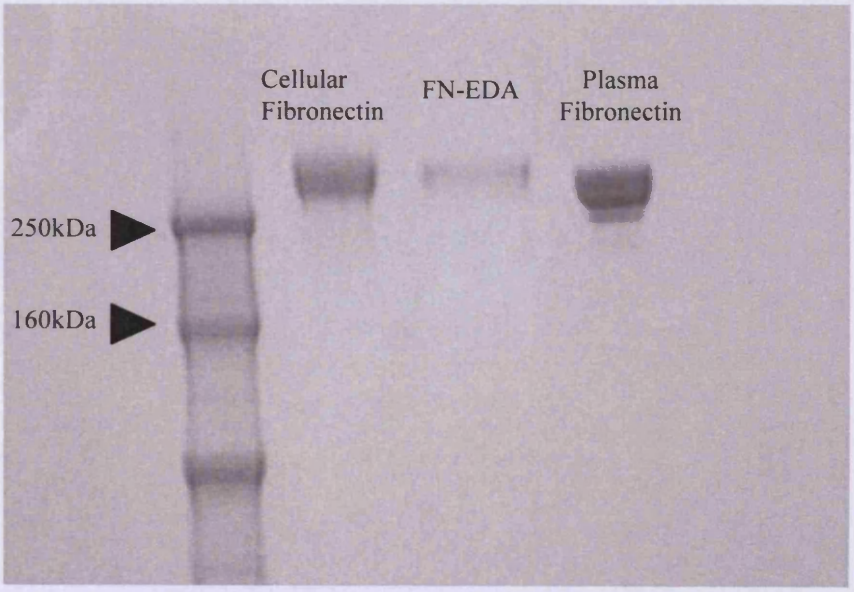


Figure 4.8: SDS-PAGE of cellular and plasma fibronectin in comparison to purified FN-EDA.

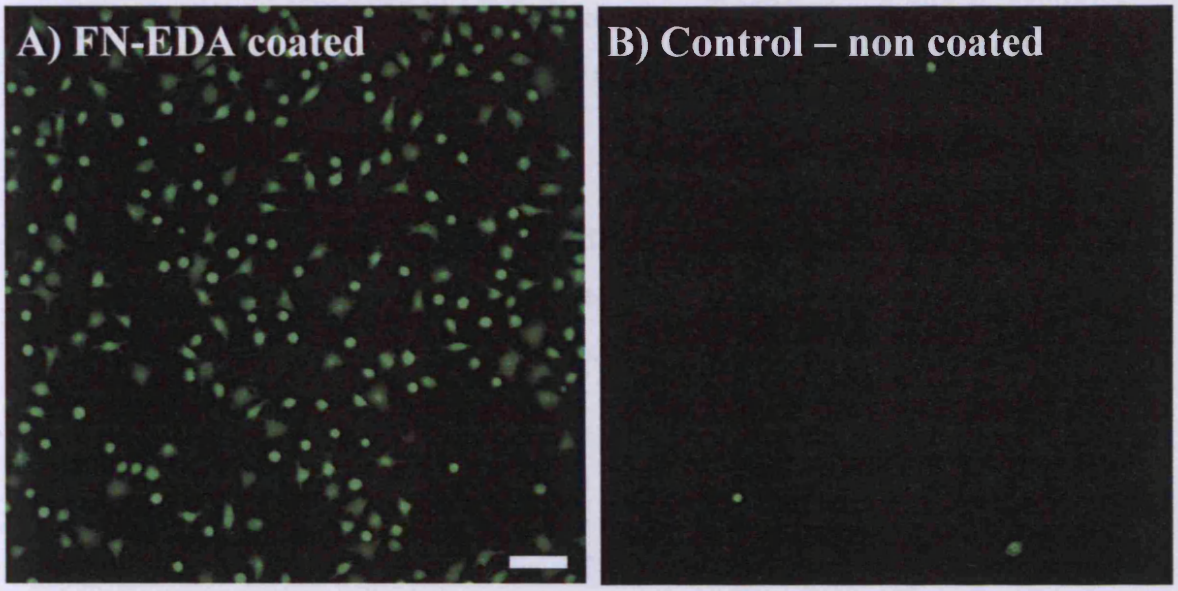


Figure 4.9: Adhesion of MG-63 cells to purified fibronectin-EDA (A) in comparison to a non-coated surface (B). Scale bar = 100μm.

4.3.7 Differential adhesion and colony forming efficiency of articular cartilage progenitor cells on FN-EDA in comparison to plasma fibronectin

4.3.7.1 Differential adhesion

Table 4.3 shows the initial cell adhesion over the two 20 minute time points for superficial and middle zone chondrocytes cultured on FN-EDA and pFN coated wells.

The data illustrates that although middle zone chondrocytes have a higher affinity to the fibronectins than the superficial zone chondrocytes, there is no statistically significant difference between adhesion of the chondrocytes to pFN and FN-EDA at either time point.

4.3.7.2 Colony forming efficiency

Figure 4.10 shows the colony forming efficiency of superficial and middle zone chondrocytes cultured on FN-EDA and pFN coated wells for 6 days after the differential adhesion assay. The only detectable difference between the two substrates could be noticed in the superficial zone cells over the first 20 minute adhesion; Superficial zone cells that adhere to FN-EDA over the first 20 minutes appear to have a lower CFE than those that adhere to pFN ($p<0.005$). There no obvious differences in colony size.

	Percentage cell adhesion			
	SFZ20	**MZ20**	**SFZ40**	**MZ40**
FN-EDA coated	9.83±1.7	15.13±2.7	3.8±1.3	8.45±2.8
pFN coated	12.5±3.1	15.6±2.5	4.24±0.9	7.6±0.79

Table 4.3: Initial adhesion to FN-EDA and plasma fibronectin (pFN) coated wells. SFZ = superficial zone cells; MZ = middle zone cells. N = 4

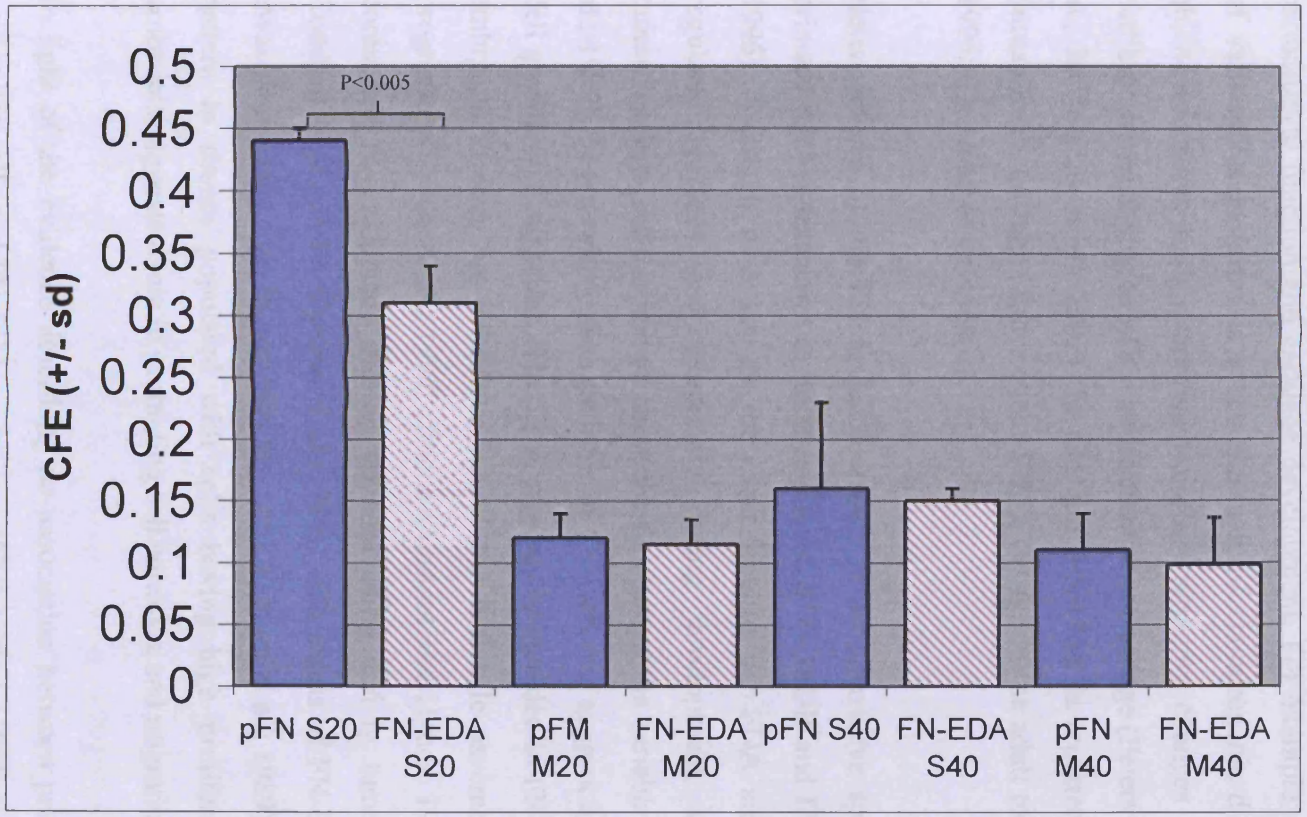


Figure 4.10: Colony forming efficiency of superficial and middle zone chondrocytes on plasma-FN (pFN) and FN-EDA following 20 minute and 40 minute adhesion.

4.4 DISCUSSION

There is substantial evidence that extracellular matrix regulates development and cell differentiation (Adams and Watt, 1993). Controlled production of matrix proteins and their isoforms in a spatial and temporal manner will have important roles in chondrocyte function and cartilage development. For example, regulated expression of various FN isoforms is a key mediator of chondrogenic differentiation. During chick embryonic limb chondrogenesis, FN structure changes from EDA+ in pre-cartilage mesenchyme to EDA- in differentiated cartilage (Peters et al., 2002; White et al., 2003; Kuo et al., 2002). In addition, FN-EDA is reported to be expressed in human fetal cartilage (Salter et al., 1995), but not in the adult tissue (MacLeod et al., 1996; Chevalier et al., 1996).

Heterogeneity of the FN molecule results from alternative splicing of a common primary mRNA transcript at three segments, EDA, EDB and IIICS (Kosmehl et al., 1996). Although it is known the expression of the EDA and EDB segments is regulated spatially and temporally during development, wound healing and tumourigenesis, little is known about the function of the variable domains (Manabe et al., 1997). *In vivo* expression patterns of FN isoforms suggest a role for FN-EDA in cell growth and migration. The EDA segment is included in FN species expressed in embryonic tissues but is spliced out of the molecule as embryonic development progresses (Vartio et al., 1997; ffrench-Constant and Hynes, 1989). In adults, EDA containing FN reappears during wound healing and in tumour tissues (ffrench-Constant et al., 1989; Oyama et al, 1989), with levels of FN-EDA being higher in invasive tumours than in non-invasive ones (Oyama et al., 1989). Such an expression pattern in tissues populated with cells having high proliferative and migratory potentials suggests a role in promoting cell adhesion and migration *in vivo*.

In light of the evidence describing an association between primitive mesenchymal progenitor cells and FN-EDA expression (Gehris et al., 1996 and 1997; Peters et al., 2002; White et al., 2003; Kuo et al., 2002) this study has sought to determine whether the isoform is also associated with the articular cartilage progenitor cells of the superficial zone, and to examine whether the isoform is important in maintaining or promoting the function/phenotype of the cells.

Firstly, EDA containing FN was determined to be associated in a pericellular fashion with the superficial 2-3 cell layers of immature bovine articular cartilage and was seen to be co-expressed with Notch 1 in double labelling immunostaining experiments. As described in section 3.3.3 and discussed in section 3.4, Notch 1 is a cell fate determination receptor (Artavanis-Tsakonas et al., 1999) expressed non-exclusively on the surface of articular cartilage progenitor cells in the superficial zone of developing articular cartilage (Hayes et al., 2003; Dowthwaite et al., 2004). Thus, the differential expression of EDA containing FN by the progenitor cells suggests it may be an important component of the progenitor cell pericellular environment and may act to influence cell behaviour.

Secondly, experiments were carried out to determine whether isolated superficial zone cells displayed increased adhesiveness to purified EDA containing FN in comparison to plasma FN, and to examine the effect of growth of the progenitor cells on a substrate of EDA containing FN in comparison to plasma FN in terms of colony forming efficiency. Although no effect on adhesion could be determined, subsequent culture of the cells that had adhered to the substrates demonstrated a reduction in colony forming efficiency from 0.44 with plasma fibronectin to 0.33 ($p<0.005$) with FN-EDA in the cohort of superficial zone cells that binds in the 20 minute adhesion step ie. the cohort that contains the progenitor cell population.

Previous studies have described contradictory results in terms of cellular adhesiveness to FN-EDA. Guan et al., (1990) showed no difference between recombinant EDA+ and EDA- in terms of cell adhesion whereas Manabe et al., (1997) and (1999) showed increased adhesion of human HT1080 fibrosarcoma cells to EDA+FN. White et al., (2003) also found no difference in adhesion of chick limb-bud mesenchymal cells to various combinations of EDA and EDB containing recombinant FNs although spreading was decreased on EDA+/EDB+ FN. These discrepancies may, in part, be due to the nature of the EDA containing FN used in the studies. The recombinant EDA+ FN used in the studies of Guan et al., (1990) did not contain the IIICS segment whereas the IIICS segment was included in the isoforms evaluated in the studies of Manabe et al., (1997) and (1999) and this segment may be required for the EDA-dependant potentiation of the cell-adhesive activity of FNs. In the studies described

here, the nature of the EDA+ containing FN is unclear. For example, the FN-EDA was purified from media conditioned by the human MG-63 osteosarcoma cell line solely on the basis of the presence of the EDA segment using immuno-affinity chromatography. The EDA containing FN may be heterogeneous in composition as it is unknown as to the extent of the presence of the EDB segment or other segments in the EDA containing FN. In addition, EDA containing FN occurs naturally *in vivo* as an insoluble aggregate of dimers of FN molecules (Yamada and Akiyama, 1984) whereas the EDA containing FN in the conditioned media in this study must have possessed a high degree of solubility and thus may differ structurally from the EDA containing FN shown to be laid down in the cell monolayers (figure 5.4) and that which is present in extracellular matrices. In addition, novel cartilage-specific variants of fibronectin have been demonstrated in human (Parker et al., 2002) and animal tissues (Macleod et al., 1996). Although the precise physiological roles for these variants is yet to be determined, the abundance of these isoforms in the tissue (50-80% of the total FN transcripts in cartilage) suggests they may play a key regulatory role in chondrocyte interactions with the ECM. It is unlikely EDA containing FN purified from MG-63 cell conditioned media will contain a high level of these variants and, thus, may not provide the correct signals to the chondrocytes and/or progenitors.

The reduction in CFE of the cohort of superficial zone cells that binds in the 20 minute adhesion step (the cohort that contains the progenitor cell population) on FN-EDA was surprising, given the putative role of the isoform during development, and the significance of this is not clear. The reduced proliferation could either be due to alterations in integrin-mediated mitotic signal transduction induced by the presence of the EDA segment or the EDA segment could have resulted in the adhesion of a subtly different population of cells in the cohort that binds in the first 20 minutes. At this point it is unclear as to whether the FN-EDA remained bound to the tissue culture plastic throughout the experiment or whether it was competed from the surface after the adhesion step by the abundance of plasma fibronectin in the bovine serum of the culture media. In addition, the chondrocytes are likely to have secreted their own fibronectin rich ECM in culture (Dessau et al., 1978 and 1981) which would be the predominant substrate to which the progenitor cells would be attached. These data suggest altered integrin-mediated mitotic signal transduction may not be responsible

for the reduced CFE as the influence of the FN-EDA on the behaviour of the progenitor cells may reduce with time.

In conclusion, this study has demonstrated the purification of EDA containing FN and an *in vitro* examination as to its effect on cell behaviour. Initial data suggests EDA containing FN may play a role in modulation of articular cartilage progenitor cell proliferation *in vivo*. Further studies using a more characterised FN-EDA substrate would be required to confirm the precise nature of the role of the EDA segment in articular cartilage progenitor cell behaviour.

CHAPTER 5

ARTICULAR CARTILAGE PROGENITOR CELL EXPANSION AND CHONDROGENIC ABILITY

5.1 INTRODUCTION

The poor regenerative capacity of articular cartilage is well characterised (Hunziker, 2001a) and has been attributed to a lack of proliferative activity of the chondrocytes surrounding the cartilage lesion (Campbell, 1969). Such lesions are frequently linked to joint pain, reduced function and often progress to secondary osteoarthritis (Buckwalter and Mankin, 1997a). Therapeutic intervention in the degenerative process includes debridement, various abrasion strategies, microfracture, mosaicplasty and arthroscopy (Gilbert, 1998), thus imposing a significant economic burden on healthcare providers (Jackson et al., 2001).

Recent studies have highlighted the promise of tissue engineering or cell transplantation for cartilage repair (Freed et al., 1993; Sittinger et al., 1994; Cao et al., 1998). Such studies have employed cells frequently seeded onto synthetic (Freed et al., 1993; Cao et al., 1998) or natural scaffolds such as collagen (Kawamura et al., 1998) or fibrin (Hendrickson et al., 1994). In addition to providing mechanical support, the scaffold acts as a provisional extracellular matrix and is supposed to support the cells in an optimal 3-D environment that supports cell proliferation and differentiation (Freed et al., 1999). The identification of a suitable cell source for cartilage tissue engineering has proved elusive. Whilst attention has mainly focussed on the use of autologous (Grande et al., 1989) or allogeneic (Kawamura et al., 1998; Grande et al., 1987) chondrocytes and mesenchymal stem cells derived from bone marrow (Wakitani et al., 1994; Kadiyala et al., 1997; Im et al., 2001; Johnstone and Yoo, 2001; Wakitani et al., 2002), other cell sources such as periosteum (De Bari et al., 2001), perichondrium (Chu et al., 1997) and synovial cells (Nishimura et al., 1999; De Bari et al., 2001a;) have also been investigated.

Difficulties arise in the use of chondrocytes in tissue engineering processes due to the limited number of cell divisions a mature chondrocyte will undergo *in vitro* prior to the onset of senescence coupled with a loss of ability to form cartilage induced by the cell culture environment (Benya and Shaffer, 1982; Evans and Georgescu, 1983; Binette et al., 1998). The proliferative potential is dependent on species and inversely proportional to the age of the donor. The importance of this becomes apparent considering the limited number of chondrocytes that can be harvested from a patient

and the large numbers of cells required for cell transplantation procedures and tissue engineering scaffold seeding protocols. The limited growth potential of chondrocytes in culture is further complicated by the rapid loss of phenotype, known as dedifferentiation, of chondrocytes in monolayer culture. Dedifferentiation is characterised by a loss of the normal spherical cell morphology with concomitant decrease in collagen II and aggrecan synthesis to a fibroblastic-like morphology with up-regulation of collagen I, III and versican (Benya and Shaffer, 1982; Von der Mark et al., 1997; Binette et al., 1998). This process is illustrated in figure 5.1. Possible solutions to enable the rapid expansion of chondrocytes whilst retaining their phenotype have been suggested and usually involve the use of growth factors such as basic-FGF or TGF-β1 (Froger-Gaillard et al., 1989; van der Kraan et al., 1992; Pujol et al., 1994; de Haart et al., 1999; Martin et al., 1999; Jakob et al., 2001; Barbero et al., 2004). The reversal of dedifferentiation, known as redifferentiation, by transfer of the cells into a culture format that supports a spherical cell morphology such as agarose, alginate or pellet culture systems (Benya and Shaffer, 1982; Bonaventure et al., 1994; Yaeger et al., 1997; Jakob et al., 2001) has also been described. However, redifferentiation is rarely complete and occurs to a progressively lesser extent the longer the chondrocyte is kept in monolayer culture (Benya and Shaffer, 1982; Bonaventure et al., 1994). Although these methods at first glance appear promising, the inclusion of growth factors or the need to utilise 3-D culture environments to retain articular chondrocyte phenotype during expansion significantly increases the cost and complexity of the tissue engineering process.

The identification of a cell with extensive growth potential and which retains its chondrogenic ability after extensive growth in culture would be a major step forward in addressing these issues. Mesenchymal stem cells (MSCs) from the bone marrow stroma have a high capacity for self-renewal (Bruder et al., 1997) and therefore thought to be of great value for cartilage repair and other tissue engineering applications (Johnstone and Yoo, 2001). The chondrogenic potential of MSCs is well characterised (Johnstone et al., 1998; Mackay et al., 1998; Pittenger et al., 1999) and the effect of various growth factors on the proliferation and differentiation of MSCs have also been studied (van den Bos et al., 1997; Mastrogiacomo et al., 2001; Worster et al., 2001; Solchaga et al., 2005). However, MSCs have been shown to generate

repair tissues with varying degrees of success in *in vivo* cartilage repair studies (Wakitani et al., 1994; Im et al., 2001).

Although we have demonstrated the ability of superficial zone articular cartilage progenitor cells to form colonies *in vitro*, it remained unclear as to whether this growth could be sustained to enable long-term cultures of phenotypically stable articular cartilage progenitor cells to be established. Furthermore, the extent to which the chondrogenic potential of these cells is retained during extensive growth and passaging in culture is unknown. The chondrogenic ability of progenitor cell populations can be studied by culture in pellet format in the presence of a serum-free media containing insulin and TGF-β_1, a system widely used by other workers to study chondrogenic differentiation and maturation (Kato et al., 1988; Ballock and Reddi, 1994).

The objective of this study was to prepare enriched populations of bovine superficial zone articular cartilage progenitor cells (SZCs) and to evaluate their chondrogenic potential during expansion by transfer into pellet culture. Chondrogenesis was determined using safranin O staining and collagen I and II immunostaining.

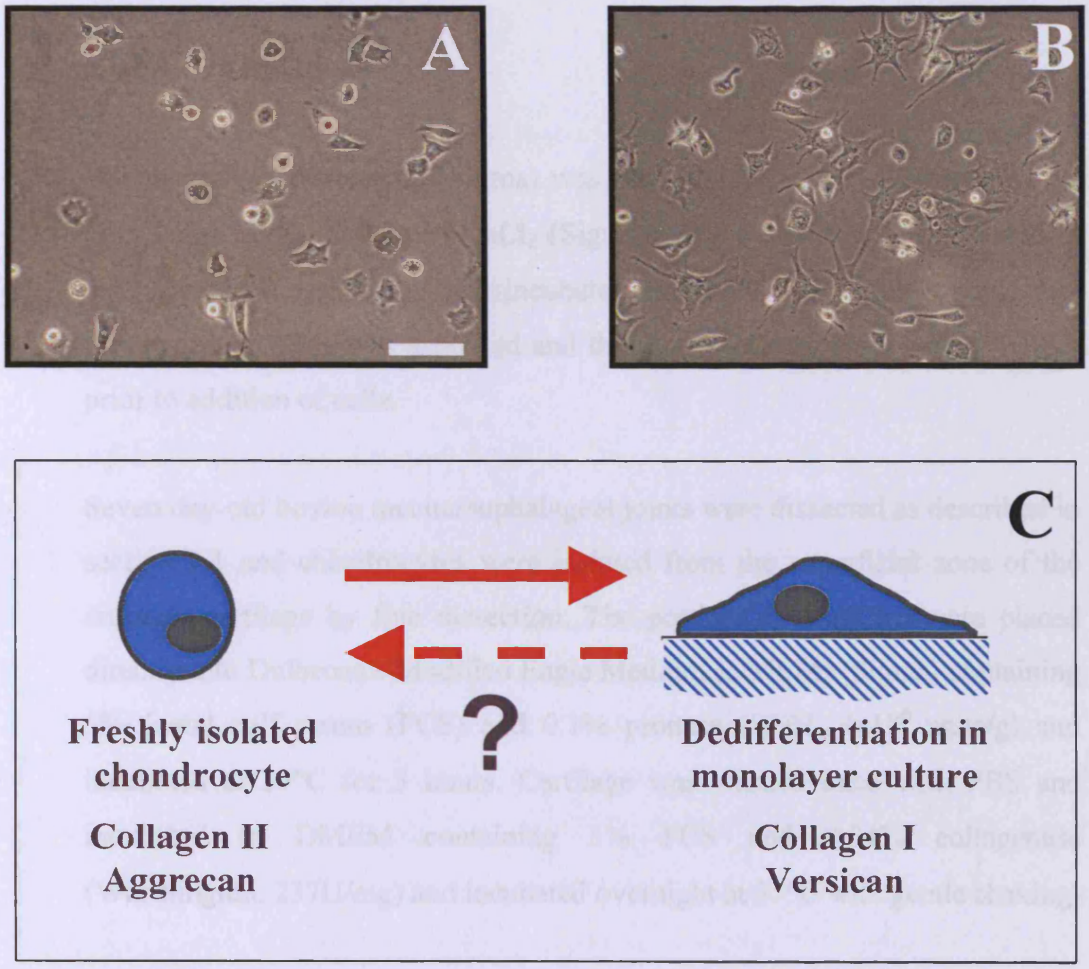


Figure 5.1: De-differentiation of adult human articular chondrocytes. Freshly isolated chondrocytes at day 1 of culture (A) and after 10 days in culture (B). Diagram illustrating the process is shown in (C). Bar = 100μm.

5.2 MATERIALS AND METHODS

5.2.1 Isolation and expansion of populations of cells enriched in articular cartilage progenitor cells

5.2.1.1 Cell isolation

Bovine plasma fibronectin (Sigma) was used at 10μgml^{-1} in Dulbecco's PBS with 1mM $MgCl_2$ and 1mM $CaCl_2$ (Sigma). This solution was then added to each well of 6 well plates and incubated overnight at 4°C for coating. The fibronectin solution was aspirated and the plates were blocked with 1% BSA prior to addition of cells.

Seven day-old bovine metatarsophalageal joints were dissected as described in section 2.1 and chondrocytes were isolated from the superficial zone of the articular cartilage by fine dissection. The portions of cartilage were placed directly into Dulbecco's Modified Eagle Medium (DMEM) (Sigma) containing 5% foetal calf serum (FCS) and 0.1% pronase (BDH, 4x10^6 units/g) and incubated at 37°C for 3 hours. Cartilage was washed once with PBS and incubated in DMEM containing 5% FCS and 0.04% collagenase (Worthington, 237U/mg) and incubated overnight at 37°C with gentle shaking.

Tissue digests were strained through a 70μm cell strainer (Falcon) to remove debris. The resultant filtrate was centrifuged at 300g for 10 minutes to pellet cells and then resuspended in 10ml of serum free DMEM followed by centrifuging again at 300g for 5 minutes. The cell pellet was resuspended in 10ml serum free DMEM, cell number counted and resuspended to a final concentration of 4000 cells ml^{-1}.

5.2.1.2 Differential adhesion

Following isolation, 4000 superficial zone cells in 1ml serum free DMEM was seeded into each of the wells of fibronectin coated 6-well plates and incubated

at 37°C for 20 minutes. After 20 minutes, the media was gently swirled and discarded. Fresh DMEM supplemented with 10% FCS, 2mM L-glutamine, 1% non-essential amino acids, 100IU/ml penicillin and 100μg/ml streptomycin was added to each well. The plates were incubated at 37°C in a 5% CO_2 atmosphere.

Within 3 hours after initial plating, chondrocyte adhesion was assayed by counting the total number of cells adhering to the bottom of the dish using an inverted microscope equipped with phase contrast optics. This was carried out on 6 wells to estimate the percentage of cells attached and used for calculation of the initial number of doublings that occur in the 6-well plate.

5.2.1.3 Cell expansion

When cells in the 6-well plate were seen to be approaching confluency, media was removed and the monolayer washed with PBS. Cells were removed by the addition of 1.5 ml typsin/EDTA (0.05% w/v/0.02% w/v) to each well for 10 minutes. DMEM containing 10% FCS was then added and the media aspirated and pooled. The cell suspension was centrifuged at 1000rpm for 5 minutes and the resultant pellet resuspended in 10mls DMEM/10% FCS and the cells were transferred to a 75cm^2 culture flask (P1). When these cells were approaching confluency, the cells were removed from the flask by trypsinisation and transferred to a 175cm^2 culture flask (P2). Cells were maintained and passaged during the expansion as described in section 2.2. Subsequent growth in 175cm^2 culture flasks was carried out by continual passage at a ratio of 1:3. Passaging occurred prior to the cells reaching full confluence to maintain the cells in the log phase of growth. Detailed accounts of cell numbers harvested and seeded at each stage were kept throughout the process to allow the number of population doublings at each stage to be calculated.

Number of cell doublings at each stage was calculated using the following equation:

$$\text{N}^{\text{o}}\text{ of doublings} = \frac{\log\left(\frac{\text{N}^{\text{o}}\text{ cells harvested}}{\text{N}^{\text{o}}\text{ cells seeded}}\right)}{\log 2}$$

Aliquots of cells at P1, P3, P6, P9, P12 and P23, were removed for pellet culture.

5.2.1.4 Controls

Normal chondrocytes were isolated from the full thickness of 2-3 week old bovine metatarsophalangeal joints by sequential digestion in pronase and collagenase as described above. These cells were maintained in culture and passaged at a ratio of 1:3. Pellet cultures were set up immediately following isolation and at P9. P9 was chosen as the cells would have been expected to have fully dedifferentiated by this point.

5.2.2 Chondrogenic differentiation: pellet culture

At P1, P3, P6, P9, P12 and P23, cells were harvested, counted using a haemocytometer and aliquots of 250,000 cells were resuspended in chondrogenesis media (DMEM supplemented with 50IU/ml penicillin and 50μg/ml streptomycin, ITS premix (Becton Dickinson), ascorbate 2-phosphate (100μM; Sigma), dexamethasone (10^{-7}M; Sigma) and TGFβ-1 (10ng/ml; R & D Systems). One ml aliquots of this suspension were transferred into 15ml polypropylene Falcon tubes and the cells pelleted by centrifugation at 1000rpm for 5 minutes. Pellets were incubated with the lids of the tubes loosened at 37°C in a 5% CO_2 atmosphere for 14 days with media changes carried out every 2-3 days.

5.2.2.1 Histology and immunolabelling

After 14 days in culture, pellets were fixed overnight in phosphate buffered formaldehyde and embedded in paraffin wax as described in section 2.3. Sections of 5μm thickness were cut and stained with Safranin O/haematoxylin as described in sections 2.3.3 and 2.3.4 and immunostained for the presence of collagens I and II.

5.2.2.2 Collagen II immunolabelling

Sections were dewaxed in xylene and transferred to 100% Industrial Methylated Spirits (IMS) (1 minute), followed by distilled water (1 minute) and finally into PBS. Antigen retrieval was carried out using Pepsin (Dako) in 0.2N HCl for 15 minutes at 37°C and washed in PBS. Sections were blocked with 2.5% normal rabbit serum for 20 minutes at room temperature followed by incubation with 2μg/ml mouse anti-collagen II antibody (Neomarkers clone 2B1.5) overnight at 4°C. Some sections were also incubated with an equivalent concentration of non-immune mouse IgG as a control. Sections were washed again three times for 5 minutes with PBS and incubated with a 1:100 dilution of biotinylated rabbit anti-mouse secondary antibody (Dako) in PBS for 1 hour at room temperature. Finally, sections were washed three times for 5 minutes with PBS and incubated with a 1:100 dilution of Streptavidin-FITC (Amersham) for 1 hour at room temperature. Following washing, slides were mounted with Vectashield (Vector) and coverslipped. Sections were viewed using fluorescence microscopy and photographed.

5.2.2.3 Collagen I immunolabelling

Method was repeated as described in section 6.2.2.2 with the following exceptions: Sections were blocked with 2.5% normal goat serum prior to incubation with 10ug/ml of a polyclonal rabbit anti-bovine type I collagen primary antibody (Biogenesis) and detected using a biotinylated goat anti-rabbit secondary antibody (Dako). Some sections were also incubated with an equivalent concentration of non-immune mouse IgG in place of the primary antibody as a control.

5.2.3 Osteogenic differentiation

A 175cm^2 flask of P5 SZCs was passaged using trypsin/EDTA (0.05% w/v/0.02% w/v), centrifuged for 10 minutes at 1000rpm and resuspended in DMEM/10% FCS. Cells were counted haemocytometer and resuspended to 50,000 cells/ml. One ml of

this suspension was then transferred to each of 6 wells of 2 x 24 well plates. Cells were incubated overnight at 37°C in a 5% CO_2 atmosphere. Media in the wells of one of the 24-well plates was then replaced with osteogenic media (see below). Media in the other plate was replaced with DMEM/10% FCS. Plates were incubated for 12 days at 37°C in a 5% CO_2 atmosphere with media changes every 2-3 days.

Osteogenic media: DMEM/10%FCS supplemented with 50IU/ml penicillin and 50μg/ml streptomycin, 2mM L-glutamine, 1% non-essential amino acids, ascorbate 2-phosphate (50μM) (Sigma), dexamethasone (10^{-7}M) (Sigma); and β-glycerophosphate (1mM) (Sigma).

Cell monolayers were examined visually for the presence of nodules and stained for the presence of alkaline phosphatase using Sigma Histochemical staining kit (86-R) according to the manufacturers instructions.

5.3 RESULTS

5.3.1 Cell growth

Populations of cells enriched in bovine superficial zone articular cartilage progenitor (SZCs) cells underwent approximately 61 population doublings over 162 days in culture. At P0 the cells had a doubling time of approximately 24 hours where after the cells maintained a moderately constant rate of growth, requiring subculture every 3-4 days. This was maintained until P28 when the rate of growth declined with subculture being required every 7-8 days. After P35 cell growth had virtually stopped. Figure 5.2 (A) and (B) illustrate the cumulative population doublings of SZCs plotted as a function of time and passage number respectively. The bars represent an average of two flasks from one experiment. Normal chondrocytes isolated from the full thickness of bovine articular cartilage were expanded in culture to P9, equating to 13 population doublings over 34 days. These cells were used as controls for the pellet culture experiments.

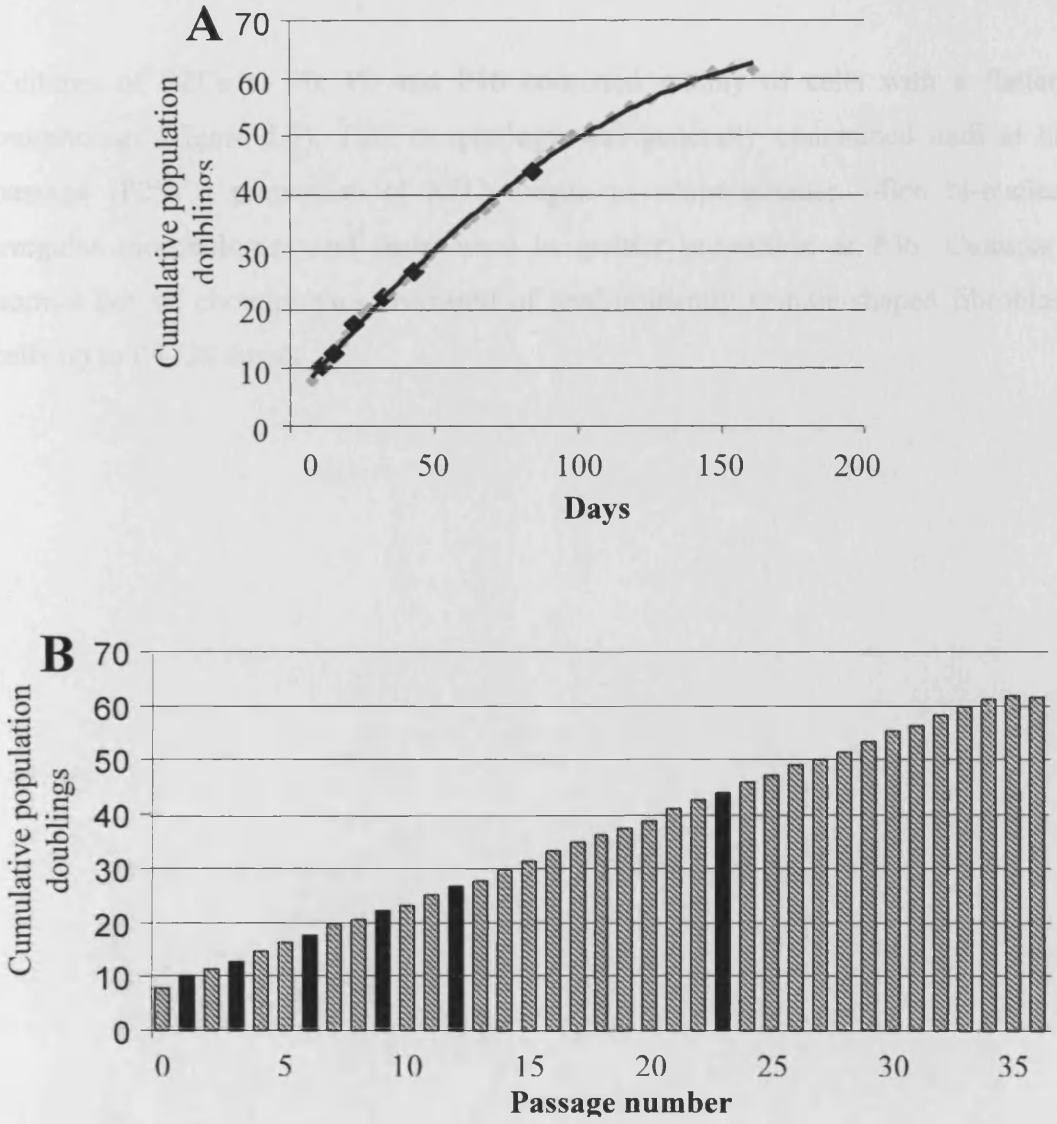


Figure 5.2: Population doubling potential of bovine SZCs as a function of time (A) and passage number (B). Points and bars in black indicate stages at which pellet cultures were set up.

5.3.2 Cellular Morphology

Cultures of SZCs at P0, P2 and P16 consisted mainly of cells with a flattened morphology (figure 5.3). This morphology was generally maintained until at high passage (P25) a proportion of SZCs began to adopt broader, often bi-nucleate, irregular morphologies and these were in greater proportion at P36. Cultures of normal bovine chondrocytes consisted of predominantly spindle-shaped fibroblastic cells up to P9 (34 days).

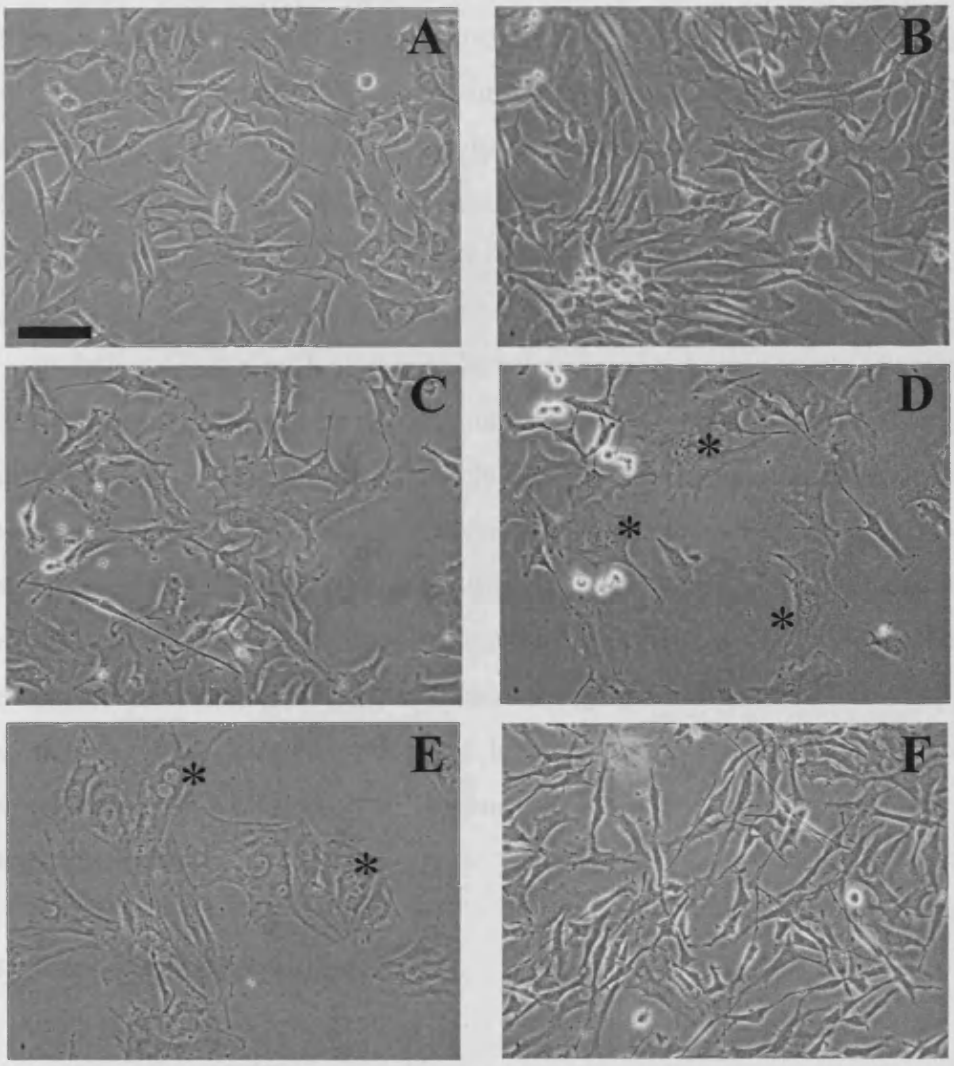


Figure 5.3: Morphology of bovine SZCs and chondrocytes cells throughout expansion. Cells are shown at P0 (A), P2 (B), P16 (C), P25 (D) and P36 (E). Normal chondrocytes isolated from the full thickness of bovine articular cartilage at P5 are shown in (F). * - denotes binucleate cells. Bar = 100μm.

5.3.3 Chondrogenic differentiation: pellet culture

Enriched populations of bovine superficial zone articular cartilage progenitor (SZCs) expanded in culture and subsequently grown in pellets synthesised a cartilage–like matrix that stained strongly with Safranin O, indicating an abundance of sulphated proteoglycans (figure 5.4). The periphery of the pellets at all time points stained weakly with Safranin O. In addition, flattened cells were seen to be present on the surface of the pellet. P1, P3, P6, P9 and P12 (P12 = 25 population doublings) SZC cell pellets were rich in collagen II (figure 5.5) whereas pellets derived from P23 (P23 = 42 population doublings) SZCs appeared to contain only low levels of collagen II. Control sections incubated with non-immune mouse IgG were negative for collagen II (data not shown). Pellet cultures of freshly isolated normal chondrocytes appeared to be smaller in size and histologically have less matrix and rounder cells than SZC pellets (figure 5.6). The matrix stained strongly for collagen II. In comparison, pellet culture of expanded normal chondrocytes at P9 (13 population doublings) synthesised a greater amount of matrix but this contained negligible collagen II. A low level of collagen I was consistently noted to be located around the periphery of all pellet cultures from both SZCs and full thickness chondrocytes. An example of this is illustrated in figure 5.7.

5.3.4 Osteogenic differentiation

When monolayers of SZCs were cultured in conditions known to promote osteogenic differentiation of mesenchymal stem cells (Pittenger et al., 1999), no osteogenic differentiation was noted. No nodules were seen to form and no red staining indicative of alkaline phosphatase was noted in the cells cultured in osteogenic media (figure 5.8).

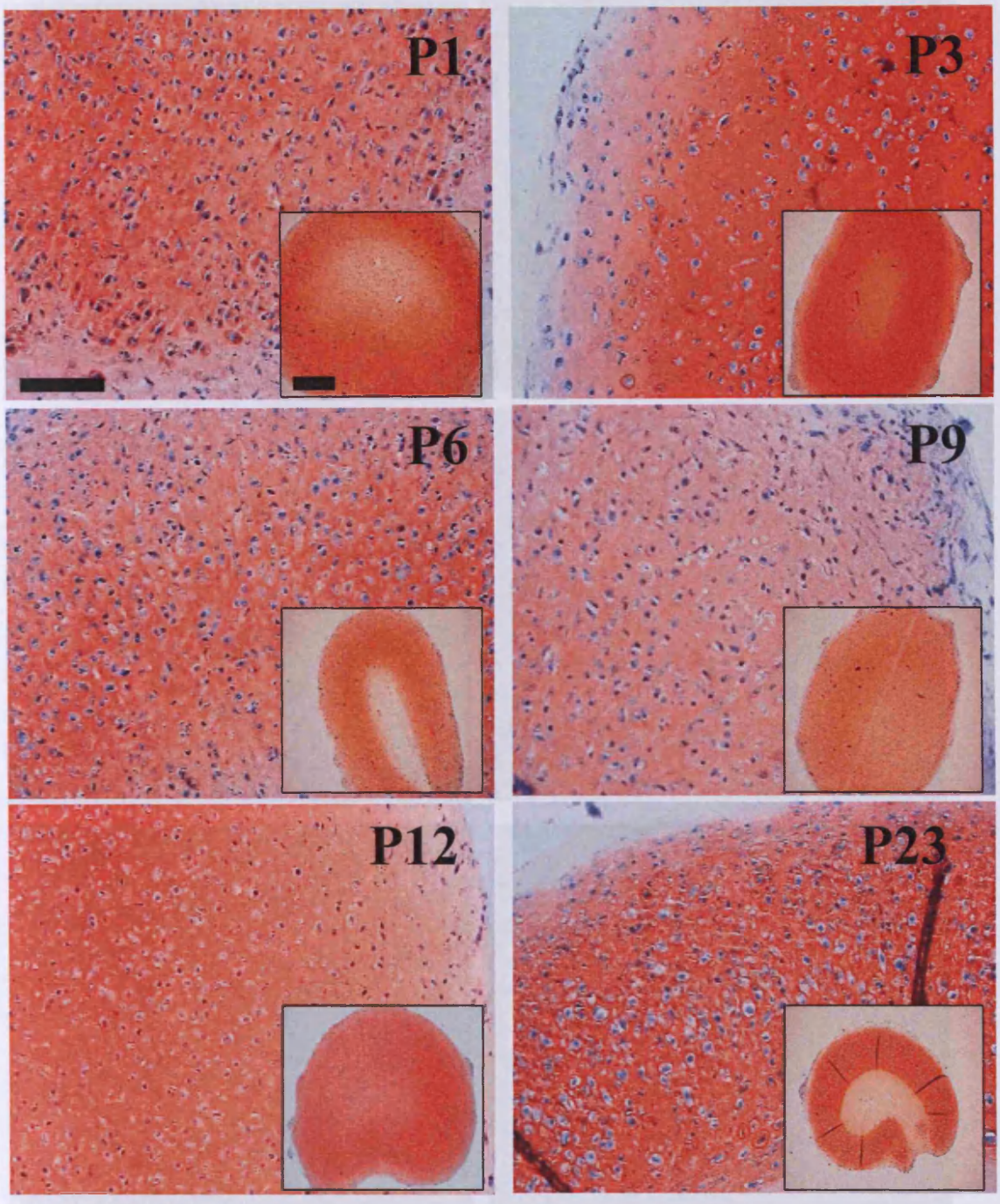


Figure 5.4: Pellet culture of culture expanded SZCs. Safranin O staining (Bar = 200μm). Low magnification image inset (Bar = 500μm).

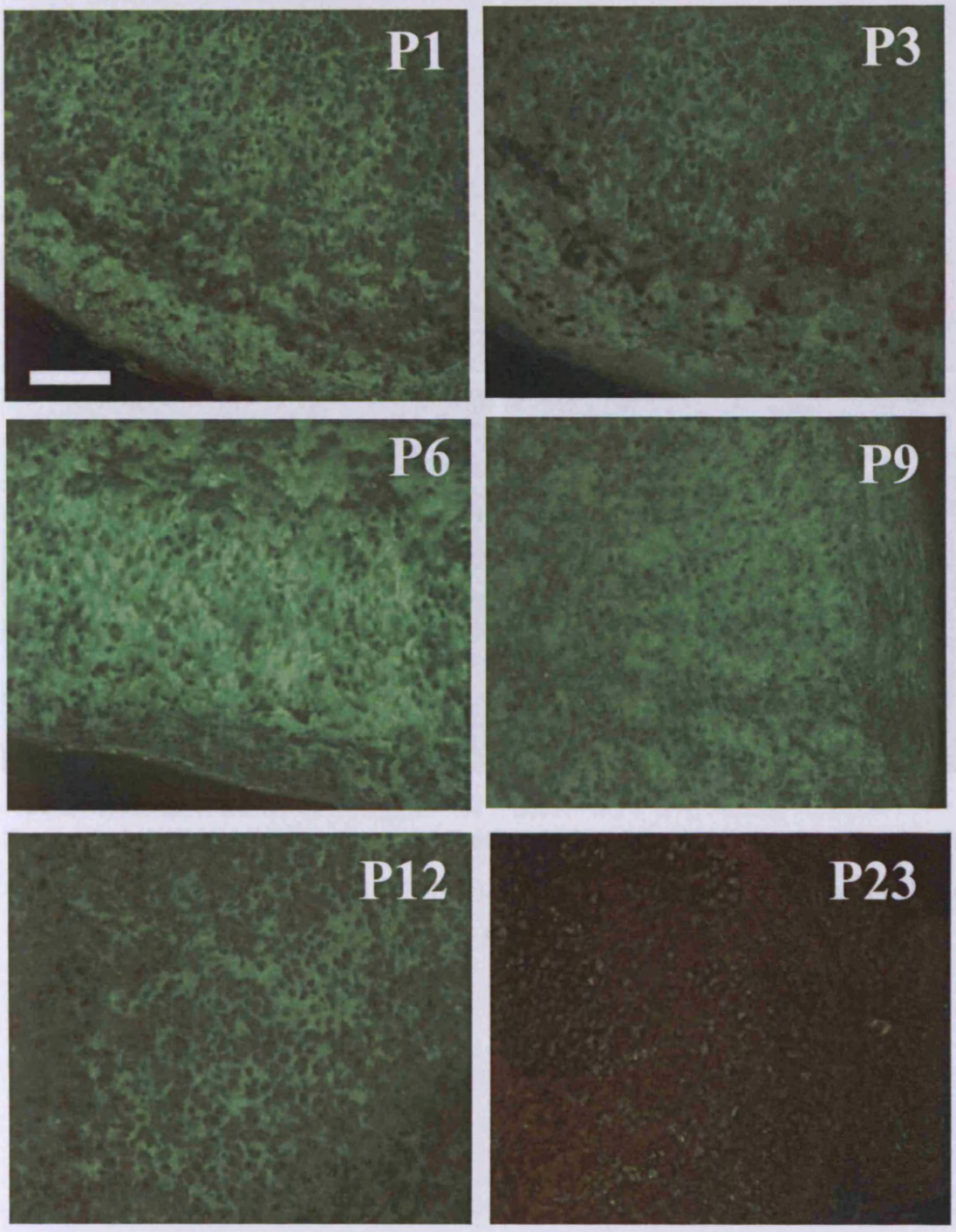


Figure 5.5: Collagen II immunolabelling of pellet cultures of culture expanded SZCs. Note decrease in staining in pellets derived from bovine SZCs at P23 (Bar = 200μm).

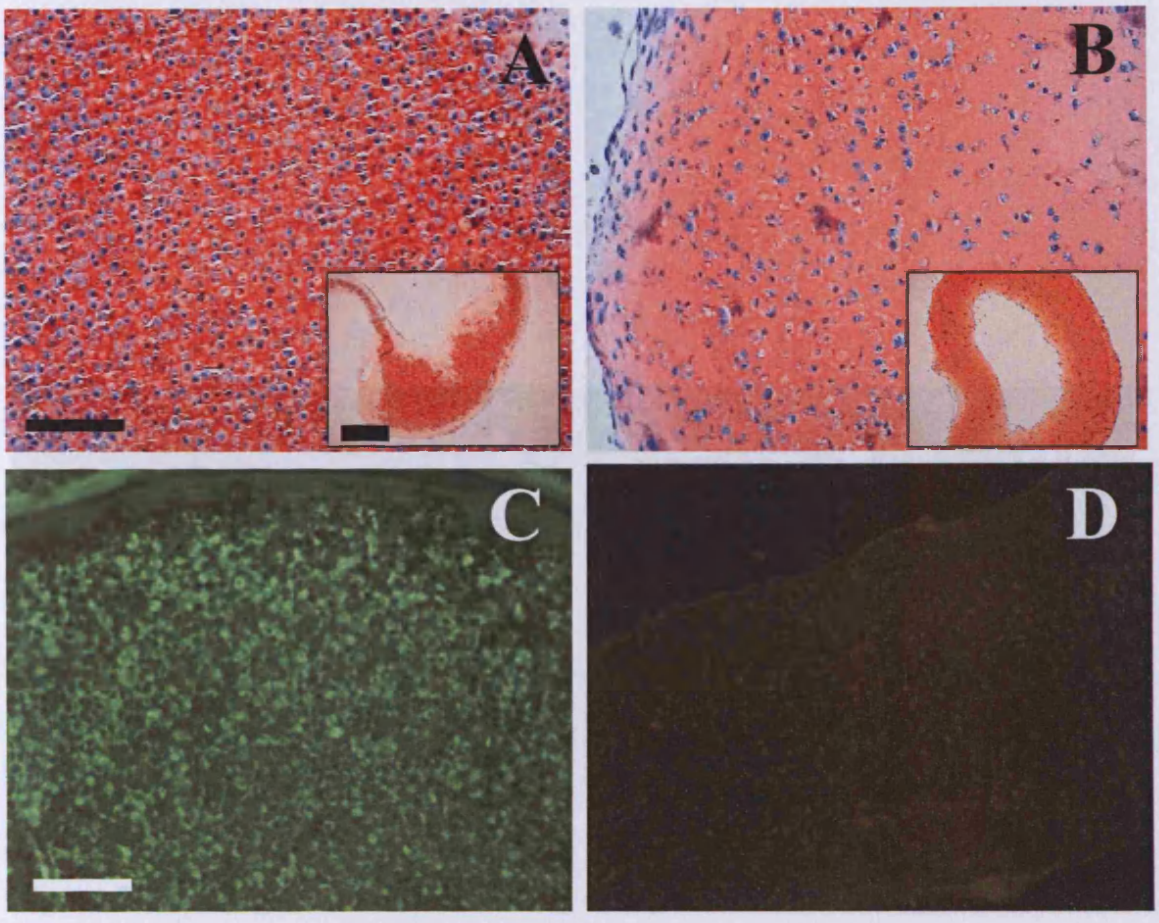


Figure 5.6: Pellet culture of control freshly isolated (A,C) and culture expanded full thickness chondrocytes at P9 (B,D). Safranin O staining (A,B) and collagen II staining (C,D). Bar = 200μm (Inset = 500μm).

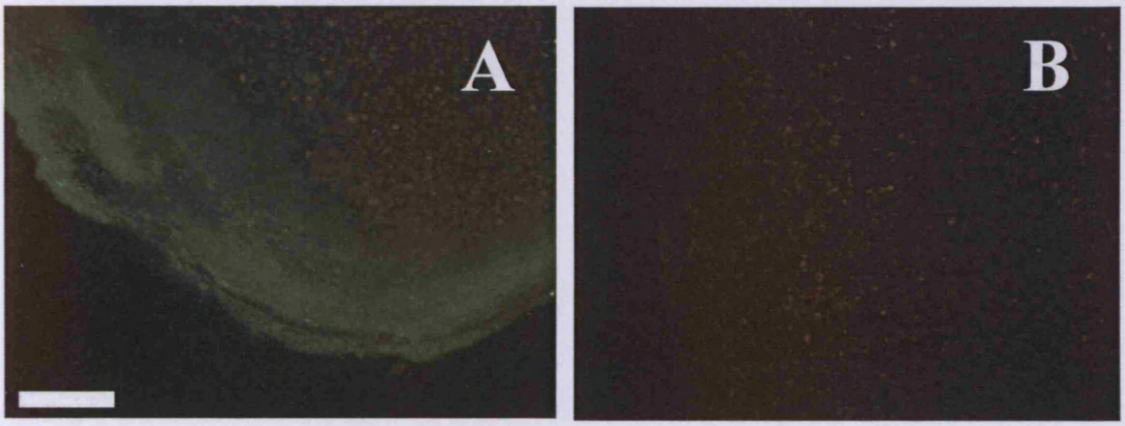


Figure 5.7: Collagen I immunolabelling of a pellet culture of expanded SZCs at P6 (A) and a control section incubated with an equivalent concentration of non-immune mouse IgG in place of the primary antibody (Bar = 200μm).

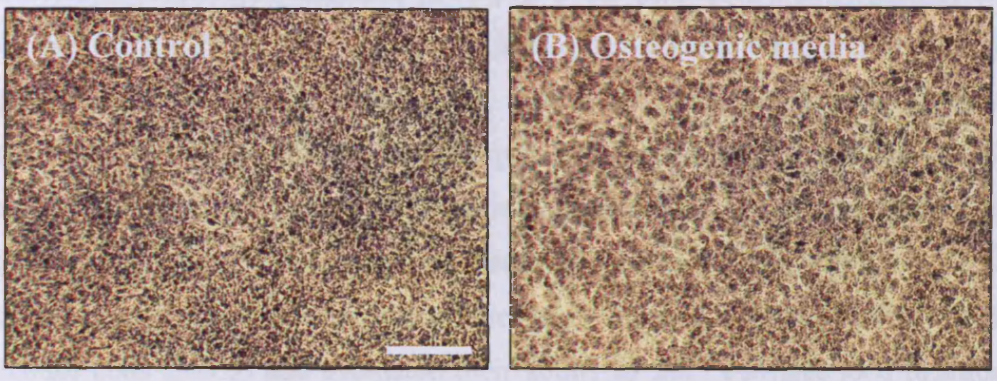


Figure 5.8: Osteogenic differentiation assay. Expanded bovine SZCs (P5) culture in control (A) and osteogenic media (B). Note lack of alkaline phosphatase activity (red staining) of cells cultured in osteogenic media. Bar = 200μm.

5.4 DISCUSSION

Previous studies have indicated that articular cartilage growth during development occurs by apposition from the articular surface (Hayes et al., 2001) rather than by interstitial mechanisms. This growth appears to be driven by a slowly dividing population of cells in the superficial zone of articular cartilage and a more rapidly dividing population of cells in the transitional zone. These observations suggest the presence of a specific articular cartilage precursor or chondroprogenitor cell population in the superficial zone and a population of transit amplifying cells in the transitional zone. Studies described in chapter 3 and published studies (Archer et al., 2002; Dowthwaite et al., 2004) have confirmed that a population of articular cartilage progenitor cells do indeed exist in the superficial zone that exhibit differential adhesion to fibronectin, differential integrin expression, and the ability to form colonies from an initially low seeding density; properties that are common to known progenitor cell populations of other tissues (Jones and Watt, 1993). The study described here has produced cultures enriched in superficial zone articular cartilage progenitor cells (SZCs) and evaluated their ability to form cartilage as a function of expansion in culture.

This work has demonstrated the high expansion potential of SZCs. SZCs maintained a stable cellular morphology even after extensive expansion in monolayer culture. The polygonal morphology of SZCs contrasted with the more fibroblastic normal chondrocytes isolated from the full thickness of bovine articular cartilage. At high passage the growth rate of SZCs declined, and this was accompanied by a gradual increase in the proportion of SZCs adopting a broader, irregular, often binucleate morphology. Similar features have been described of chick sternal chondrocytes after a prolonged time in culture (Von der Mark et al., 1997), although SZCs in this study underwent this shift in morphology at a significantly later stage and is clearly age and species dependant.

Other studies have described the cloning of bovine SZC colonies and subsequent expansion and chondrogenic differentiation in comparison to full thickness chondrocytes with similar results to that described here ie reduction in collagen II in pellets derived from SZCs at around 40 population doublings. (Bishop et al., 2004).

Interestingly these studies also showed the retention of Sox-9 expression by SZCs during expansion whereas Sox-9 expression was rapidly down-regulated in full thickness chondrocytes during expansion. Sox-9 is a transcription factor involved in the regulation of genes expressed during chondrogenesis. This clonal expansion results in a more homogeneous initial cell population which may have benefits in terms of characterisation of the cells and would be more advantageous in terms of transplantation.

Importantly, SZCs can be expanded using standard monolayer conditions and standard media components, whilst retaining their ability to differentiate into chondrocytes, synthesising a hyaline-like cartilage matrix following high levels of expansion. This expansion occurs without the need to incorporate growth factors in addition to the FCS during the expansion phase as has been suggested for bovine (Martin et al., 1999) and human (Jakob et al., 2001; Barbero et al., 2004) chondrocytes. SZCs retain the ability to synthesise a hyaline-like cartilage matrix rich in collagen II and sulphated proteoglycans, with low levels of collagen I even after 12 passages (25 population doublings). Thus, SZCs can undergo chondrogenesis after a $33x10^6$-fold expansion in monolayer culture. Low levels of immuno-detectable collagen II were also evident at P23, where the cells had undergone 42 population doublings, a $4x10^{12}$-fold increase in cell number. As expected in this study, normal chondrocytes isolated from the full thickness of immature bovine articular cartilage dedifferentiated relatively rapidly in monolayer culture and completely lost the ability to redifferentiate in pellet culture at P9, corresponding to 13 population doublings. Thus, in contrast to the SZCs, normal chondrocytes lost the ability to undergo chondrogenesis after only an 8000-fold increase in cell number. Further studies are required to determine whether the differentiation of SZCs produces a stable chondrocytic phenotype, without progression to terminal differentiation characterised by expression of alkaline phosphatase and Type X collagen (Kato et al., 1988), a property exhibited by bone marrow derived MSCs. Lack of progression towards terminal differentiation would be a desirable property of a cell for articular cartilage tissue engineering, as terminal differentiation may subsequently promote vascular invasion and calcification of the implanted cells.

In this study, monolayers of SZCs exhibited an inability to differentiate down an osteogenic lineage in vitro. This data may illustrate the SZCs are committed to the chondrocytic lineage and lack multipotentiality. However, it contrasts with other studies showing plasticity of bovine articular cartilage progenitor cells in an embryonic chick trafficking system (Dowthwaite et al., 2004), where the cells engrafted into a variety of tissues including bone. This suggests component(s) of the embryonic environment that enable and drive osteogenic differentiation are absent from the *in vitro* culture conditions described here.

The reason for the increased phenotypic stability of SZCs in comparison to differentiated chondrocytes is unclear. Previous authors have speculated whether phenotypic stability in chondrocyte culture populations is related to their degree of maturity (Von der Mark et al., 1997), with younger or more undifferentiated cells (eg. from regions immediately beneath the perichondrium or in this case, the superficial zone of articular cartilage) requiring more doublings to dedifferentiate than older or more differentiated chondrocytes.

Similar data to that reported here has been obtained with other mesenchymal stem/progenitor cell populations. De Bari et al., (2001) and (2001a) have described the isolation of a population of cells from human periosteum and synovium that maintain a stable phenotype and retain a chondrogenic potential throughout the expansion phase. Although Bruder et al., (1997) have described the extensive subculture of bone marrow derived human MSCs and the retention of osteogenic potential even after extensive subculture, other groups have shown that the retention of chondrogenic potential of MSCs requires the addition of growth factors such as basic fibroblast growth factor (bFGF) in the culture media during the expansion phase (Mastrogiacomo et al., 2001; Solchaga et al., 2005).

The findings from this study could provide significant benefits in their application to cell based-strategies for cartilage repair. Current cartilage repair strategies have focused on Autologous Chondrocyte Implantation (ACI) (Brittberg et al., 1994) and tissue engineering (Freed et al., 1993; Hendrickson et al., 1994; Sittinger et al., 1994; Cao et al., 1997; Kawamura et al., 1998; Freed et al., 1999) approaches. ACI involves arthroscopically harvesting an area of the patient's own cartilage from a non-weight

bearing location for cell isolation. Chondrocytes are then isolated in a designated laboratory facility, expanded in culture and then, in a second procedure, the cells are returned to the defect site either by injection beneath a periosteal flap or via a biodegradable scaffold. There are a number of drawbacks to autologous cell approaches. The initial biopsy creates a second defect site with associated issues of donor site morbidity. Furthermore, there may be only a limited amount of healthy cartilage available for biopsy. As the ACI procedure requires two operations it is expensive and requires a specialised laboratory service for cell isolation and expansion. The extent of dedifferentiation of the chondrocytes during the expansion phase on the overall clinical outcome of the procedure is also unknown. The use of allogeneic progenitor cells such as SZCs has the potential to reduce this process to a one step procedure, preventing donor site morbidity and negating the need for a costly specialised laboratory service. While it appears that the SZC cell type would have sufficient expansion potential to meet the commercial need, it is currently not known if the cell type would be tolerated in the joint by the host immune system. Further studies are therefore required in order to determine the feasibility of such an approach.

An alternative approach to ACI for cartilage repair is that of tissue engineering routes where allogeneic cells are seeded onto a 3-D scaffold acting as a provisional extracellular matrix (Freed et al., 1999). These cell-seeded constructs may either be implanted immediately or cultured in bioreactor conditions in order to generate a functional tissue engineered neo-cartilage for subsequent implantation (Freed et al., 1993). Whilst this process may have the advantage in that the matrix deposited around the cells may lower their immunogenicity, the fixation of such mature grafts into a joint site may prove problematic.

In conclusion, the development and application of both ACI and tissue engineering approaches in the clinical environment has been hampered due a restricted availability of suitable cartilage producing cells caused by limited chondrocyte growth potentials and culture-induced loss of phenotype (Benya and Shaffer, 1982; Evans and Georgescu, 1983; Von Der Mark et al., 1997; Binette et al., 1998). The enhanced potential of these SZCs to retain the ability to form cartilage after extensive expansion in culture may enable the generation of large cell banks for use in allogeneic tissue engineering applications.

CHAPTER 6

SURGICAL CARTILAGE CUTTING TECHNIQUES AND *EX VIVO* CELL TRANSPLANTATION

6.1 INTRODUCTION

Lesions in articular cartilage generally do not repair or repair only partially under certain conditions. Such defects are often associated with disability, joint pain, locking and reduced range of motion. Left untreated these lesions may progress to osteoarthritis, requiring extensive surgical interventions such as osteochondral transplantation (mosaicplasty), high tibial osteotomy and joint replacement (Campbell, 1969; Gilbert, 1988; Buckwalter and Lohmander, 1994; Buckwalter and Mankin, 1997a; Newman, 1998; Hunziker, 1999; Hunziker, 2001a).

Surgical removal of articular cartilage is commonplace in the treatment of osteoarthritic diseases (McLaren et al., 1991). Osteochondral transplantation techniques such as mosaicplasty involves the incision of healthy articular cartilage, both to create the tissue plugs and prepare the lesion site for implantation of the plugs (Hunziker, 1999; Hangody et al., 2001;). In addition, early surgical interventions in the degenerative process such as lavage and arthroscopy, chondral shaving and debridement is carried out on the principle such options provide symptomatic relief from pain, restore joint functionality and delay the need for total joint replacement (Buckwalter and Lohmander, 1994; Newman, 1998; Gilbert, 1998; Hunziker, 2001a). Chondral shaving and debridement involves mechanical removal of loose cartilage fragments and smoothing of fibrillated areas using surgical cutting tools and instruments (Mitchell and Shepard, 1987; Kim et al., 1991; McLaren et al., 1991). Arthroscopic chondral shaving is also commonly used for the treatment of chondromalacia patellae (Ogilvie-Harris and Jackson, 1984). However, the beneficial effects of techniques such as chondral shaving and debridement remain controversial, with a lack of experimental or clinical evidence to justify the procedures (Buckwalter and Lohmander, 1994; Newman, 1998; Hunziker, 2001a). For example, chondral shaving has been shown to stimulate neither a degenerative nor reparative response in one study (Mitchell and Shepard, 1987) and induce degeneration in the remaining cartilage in other studies (Schmid and Schmid, 1987; Kim et al., 1991).

Experimental mechanical wounding of cartilage tissue using cutting tools has been demonstrated to initiate a deleterious response in the cartilage tissue immediately adjacent to the wound edge. This response is characterised by cartilage degeneration

and cell loss (Shapiro et al., 1993; Hunziker and Quinn, 2002; Tew et al., 2000; Redman et al., 2004), probably due to both necrosis and apoptosis, and altered matrix metabolism characterised histologically by empty lacunae and loss of metachromasia from the extracellular matrix at the lesion edge (Bennett et al., 1932; Tew et al., 2000; Walker et al., 2000). In contrast, deeper into the tissue, adjacent to this 'zone of cell death' there is a zone of cell proliferation and up-regulation of matrix synthesis evidenced by increased ^{3}H-thymidine and ^{35}S incorporation in the territorial matrix of this region (Calandruccio and Gilmer, 1962; Mankin, 1962; Buckwalter, 2002; Redman et al., 2004). The use of sharper cutting instruments, such as scalpels, diminishes the extent of the 'zone of necrosis' thus causing cell proliferation and up-regulation of matrix synthesis to occur at the wound edge (Redman et al., 2004).

Current research into mechanisms of improving cartilage repair encompasses a variety of combinations of approaches such as incorporating growth factors into the defect (Hunziker, 2001), synthetic or natural scaffolds either alone (Solchaga et al., 2002) or seeded with chondrocytes or mesenchymal stem cells (Freed et al., 1993; Hendrickson et al., 1994; Wakitani et al., 1994; Cao et al., 1997; Kawamura et al., 1998). Cell-based approaches using mesenchymal stem cells (Im et al., 2001; Wakitani et al., 2002), and in the technique of autologous chondrocyte transplantation (Brittberg et al., 1994) have also incorporated cells in the defect without a scaffold. Studies such as these have frequently been hampered by poor integration of the repair tissue with the native articular cartilage (Shapiro et al., 1993; Wakitani et al., 1994; Hunziker, 2001). This phenomenon may be partly due to the cell and matrix changes described above, as the lesion sites are most likely to undergo some form of preparation prior to the implantation procedure using surgical cutting tools and instruments. Thus, repair tissue may be attempting to integrate into 'dead cartilage' and may eventually separate in time. Therefore, the use of sharper, more precise cutting instruments may reduce this effect, allowing cell proliferation and matrix synthesis to occur immediately at the would edge and promote a more successful integration of the repair tissue with the host.

The superficial zone articular cartilage progenitor cells are believed to be responsible for the appositional growth of articular cartilage during development (Hayes et al., 2001; Dowthwaite et al., 2004). As the cells appear to play a pivotal role in the

growth of articular cartilage, we hypothesised they also may play an important role in repair. They may either be directly responsible for the laying down of the new repair tissue or may orchestrate a repair response by the secretion of growth factors or cytokines that may modulate the synthetic activity of the chondrocytes surrounding the defect site or may prevent entry of the cells into the apoptotic pathway. If this is the case then it may be that the cells could reduce the deleterious effects described above that occur at cartilage wound margins when implanted into the defect site.

Therefore the objectives of this study were two-fold:

[1] To develop a model to study the extent of cellular necrosis that occurs in cartilage following wounding and to use the model to evaluate the extent of cell death caused by several different surgical cutting techniques.

[2] To determine whether the implantation of populations of cells enriched in superficial zone articular cartilage progenitor cells (SZCs) into an experimental defect in articular cartilage can reduce the cell death and matrix loss at the wound margin in an *ex vivo* wound model.

6.2 MATERIALS AND METHODS

6.2.1 Effects of surgical cutting techniques on cell viability at the cartilage wound margin.

6.2.1.1 Cartilage Cutting

Strips of cartilage were removed from 7 day-old bovine metatarsophalangeal joints as described in section 2.1 and kept in PBS (Oxoid) until used for cutting (1-2 hours).

Cartilage strips were then cut using the following techniques:

[A] Scalpel blade (fresh blade for each cut).

[B] Duckling elevator Acufex punch (014765 L194).

[C] 4.0mm Full radius Dyonics powered cutter (7205306 L397678)

[D] 4.5mm Mosaicplasty tubular chisel (7207097 L395249)

[E] 6.5mm drilled trephine (7205515 L362684)

Cutting technique [A] served as a minimally traumatic negative control. Cutting technique [C] is an arthroscopic shaving tool illustrated in figure 6.1A. For this technique, the strip of cartilage was clamped in a specially crafted aluminium cutting jig (figure 6.1B) using two crocodile clips. This was to allow accurate, reproducible cutting. The jig with attached cartilage strip was submerged in PBS for cutting. For cutting technique [D], the procedure was carried out prior to removal of the cartilage from the bone. Once the cartilage had been cut it was removed as previously described.

Following the cutting procedure the cartilage strips were cut into small pieces (approx. 5mm in length) and transferred to 6-well tissue culture plates containing 6ml DMEM +10% FCS. Plates were incubated overnight in a RS Biotech incubator at 37°C in humidified 5% CO_2 atmosphere.

6.2.1.2 Visualisation of live and dead cells at cut cartilage surfaces

After the culture period, cartilage portions were processed by either of two methods:

[1] Live/dead staining of cryostat sections

Cartilage portions were washed briefly with PBS and then immersed in PBS containing 2µl/ml ethidium homodimer-1 (2mM, Molecular Probes) for 3 hours to label the dead cells. Longer incubations, up to overnight, were also tried.

Cartilage portions were washed for 4 hours in 20ml PBS containing 0.04mg/ml salmon sperm DNA (Sigma) and then frozen in OCT (Gurr) using a liquid nitrogen cooled iso-pentane slush. 7µm sections were cut from the centre of the portion using a Leica Jung CM3000 cryostat, placed on poly-lysine coated slides (Sigma) and counterstained using 2µl/ml Syto10 (2mM Molecular Probes). Syto10 is a green fluorescent stain that will bind to the DNA of all cells present, live or dead. Sections were viewed using a Leica confocal microscope with an ArKr laser exciting at 488nm and 568nm.

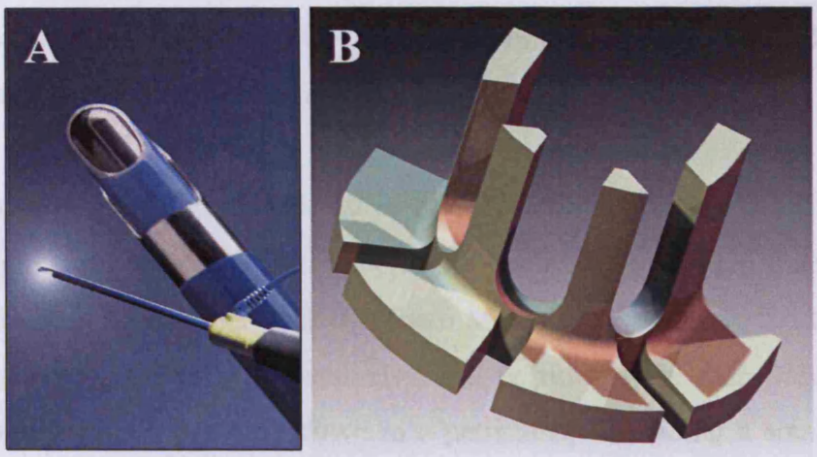


Figure 6.1: Illustration of Dyonics powered cutter (A) and cutting jig (B)

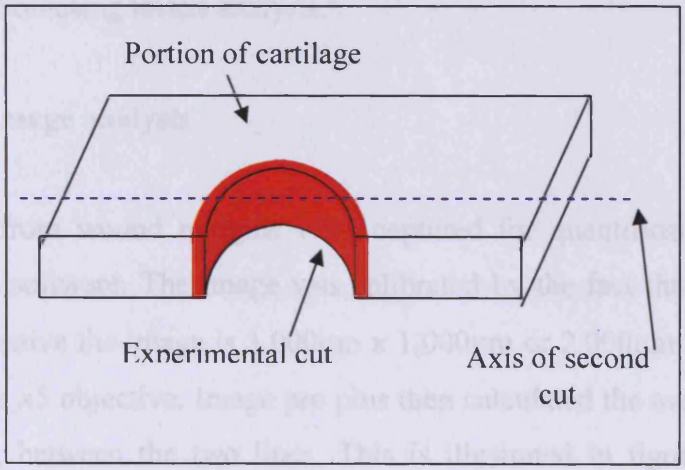


Figure 6.2: Diagram illustrating axis of second cut perpendicular to the axis of the first experimental cut surface (red) for visualisation of wound margin using optical sectioning technique (Section 6.2.1.2).

[2] Optical sectioning of live/dead stained specimens

Portions were washed briefly with PBS and the cut across the centre of the portion, using a fresh scalpel blade each time, perpendicular to the axis of the first experimental cut surface (figure 6.2).

Samples were then immersed in 5ml PBS containing the 2.5µl of the green fluorescent probe Calcein AM (1mg/ml) and 10µl of the red fluorescent probe Ethidium homodimer-1 (2mM) (both Molecular Probes) for 1.5 hours to label live and dead cells respectively. The portions were then viewed with the second cut surface face down in a petri-dish, containing a small quantity of PBS, using the inverted Leica confocal microscope with an ArKr laser exciting at 488nm and 568nm. Images from 6 different wound margins were captured with the wound margin parallel to the edge of the image for quantitation using image analysis.

6.2.1.3 Image analysis

Images from wound margins were captured for quantification using Image pro-plus software. The image was calibrated by the fact that when using the x10 objective the image is 1,000µm x 1,000µm or 2,000µm x 2,000µm when using the x5 objective. Image pro plus then calculated the average distance, in microns, between the two lines. This is illustrated in figure 6.3. Statistical significance was determined using one way ANOVA test followed by Tukey's post hoc testing to determine where significant differences exist between treatment groups. Differences were considered significant at the 0.05 confidence level.

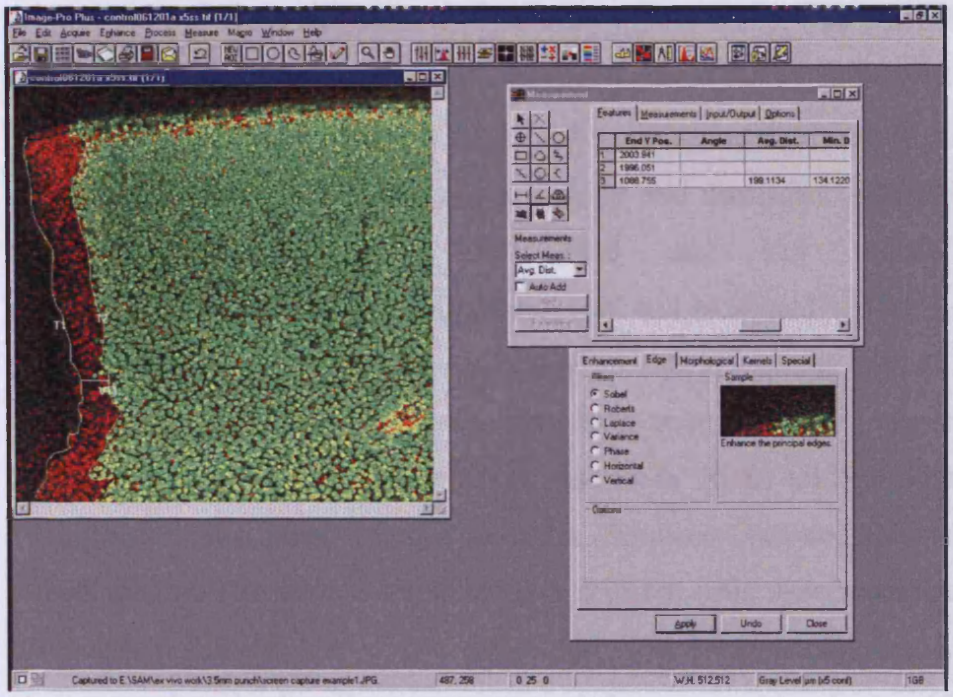


Figure 6.3: Illustration of image analysis technique to estimate the depth of the wound margin.

6.2.2 Cell Transplantation

6.2.2.1 Cell isolation

Chondrocytes were isolated from the superficial and middle zone of articular cartilage of 7 day-old bovine metatarsophalangeal joints as described in section 3.2.1.2. After isolation, 2 x 10^6 cells in 1ml serum free DMEM were seeded into each of the wells of the fibronectin coated (10 μg/ml) 6 well plates (coated as described in section 3.2.1.1) and incubated at 37°C for 20 minutes. After this time, the media was gently swirled and discarded. Adherent cells were washed once with 5ml PBS and 1ml trypsin/EDTA (0.05%w/v/0.02%w/v) was added to each well and incubated at 37°C in a 5% CO_2 atmosphere for 10 minutes. One ml of DMEM containing 10% FCS was then added and media from wells containing each cell type were pooled, centrifuged at 300g to pellet and resuspended in 10mls DMEM containing 10% FCS. Cells were counted using a Neubeuer haemocytometer. For transplantation into defects made using a drill bit, cells were resuspended to give approximately 1.5 x 10^6 cells/3.5μl. For transplantation using the 'Agarose gel system', cells were resuspended to approximately 1 x 10^6 cells /150μl.

6.2.2.2 Cartilage wounding using a drill bit and cell transplantation

Strips of articular cartilage were dissected from 7 day-old bovine metatarsophalangeal joints as described in section 2.1 and chopped into portions 5mm in length. Cartilage explants were placed into DMEM containing 10% FCS and incubated at 37°C in a 5% CO_2 atmosphere overnight while the above cell isolation was taking place.

The next day, a defect a 2mm deep was made in the cartilage portions using a 2mm drill bit. 3.5μl of cell suspension was carefully pipetted into the defect and cells were allowed to settle. The cartilage explant was very slowly covered

with DMEM containing 10% FCS and incubated at 37°C in a 5% CO_2 atmosphere for 6 days. Replicates of 6 were used for each cell type.

6.2.2.3 Agarose gel-based system

Strips of cartilage were isolated as described above, chopped into 5mm x 5mm portions and placed in DMEM containing 10% FCS. The next day, a fresh wound margin was created on one side of the portion using either a scalpel or 3.5mm Acufex punch.

Low melting temperature agarose (Sigma; A-9045) was made up to 4.6% in 50ml deionised water and autoclaved. Agarose was remelted by briefly microwaving and kept molten in a 50°C water bath. From this solution, 6.5 ml was removed to leave 43.5 ml. To this remaining solution, 5ml 10x DMEM (sigma – D-2554), 0.5ml 5000IU/ml penicillin and 5mg/ml streptomycin, 0.5ml 200mM L-Glutamine and 0.5ml non essential amino-acids was added. The resulting solution was 4% agarose. It was also necessary to adjust the pH of the molten gel solution using several drops of 1M NaOH to return to the cherry red colouration. A small amount of this solution was then pipetted into 5ml bijou tubes and a gel former was then placed into the agarose and allowed to solidify. This procedure forms a gel with a narrow slot that allows a portion of cartilage to be placed in it (figure 6.4). The cartilage portions were then transferred to the agarose slot with the wound margin facing upwards (figure 6.4 B) and DMEM containing 10% FCS was then used to cover the agarose and cartilage. Cells were introduced onto the wound margin by carefully inserting the pipette tip down into the agarose slot and pipetting 150µl of cell suspension just above the wound margin. A modified screw cap that had been drilled to accommodate a luer lock fitting and a 0.2µm filter was then placed onto the bijou tube to allow gas exchange and maintain sterility (figure 6.4 C). Explants were incubated at 37°C in a 5% CO_2 atmosphere for 6 days. Replicates of 6 were used for each cell type.

6.2.2.4 Histology and confocal microscopy

Cartilage explants were bisected perpendicular to the wound margin. One half was embedded in paraffin wax, sectioned and stained with Safranin O/ haematoxylin as described in section 2.3. The other half was used to evaluate cell viability at the wound margin using confocal microscopy as described in section 6.2.1.2 method [2]. Image analysis of confocal images was carried out as described in section 6.2.1.3.

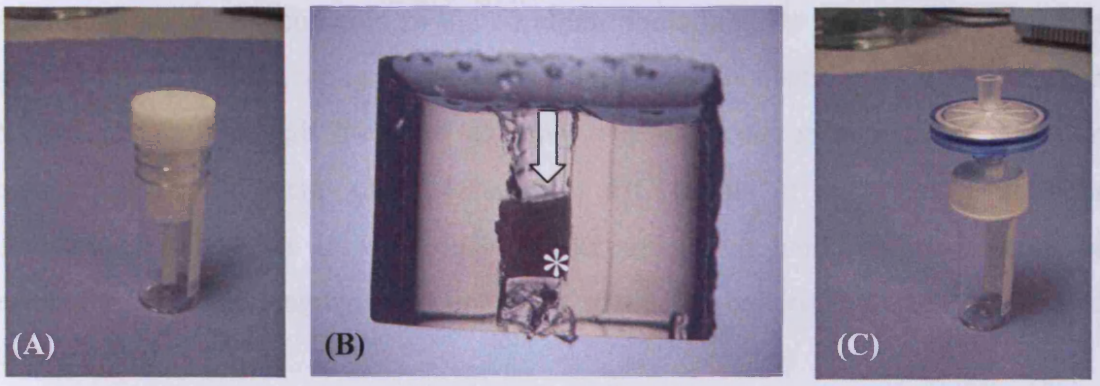


Figure 6.4. Photographs illustrating agarose gel based system. (A) Gel former in bijou tube. (B) Cartilage explant (*) embedded in agarose with wound margin facing upwards (solid white arrow). (C) Modified bijou tubes for culture of explants (section 6.2.2.3).

6.3 RESULTS

6.3.1 Effect of surgical cutting technique on cell viability at the cartilage wound margin.

The first processing technique (Live/dead staining of cryostat sections) was discontinued from use due to the ethidium homodimer-1 only penetrating approx 100-200μm into the tissue. This was demonstrated using pieces of cartilage freeze-thawed repeatedly prior to incubation in the ethidium homodimer-1. Penetration depth was not increased significantly even after overnight incubation in the ethidium homodimer-1. Although literature evidence suggests full penetration of ethidium homodimer-1 will occur after 24 hours at 4°C (Poole et al., 1996) a 24 hour incubation may result in additional cellular effects. In addition, this length of incubation plus the lengthy, work intensive sectioning results in a long turn around time for results.

By using the second processing technique (Optical sectioning of live/dead stained specimens) the problem of dye penetration could be avoided. Provided penetration of the ethidium homodimer-1 is more than a few cells deep and the laser power of the confocal microscope is kept high, the extent of cell death at the wound margin could be visualised.

Using this technique, images were captured of cell death at the wound margins caused by the five cutting techniques. Representative examples of the images captured for each cutting technique are shown in figure 6.5 and results of the quantification of the cell death using image analysis shown in table 6.1. Cartilage cutting using a scalpel produced minimal cell death whereas using an Acufex punch and the Dyonics powered cutter produced relatively low levels of cell death (54.7μm and 32.4μm respectively) although there was no significant difference from that achieved using the scalpel. Cartilage cutting using the 4.0mm Tubular Chisel and the drilled 6.5mm Trephine produced extensive cell death. However this effect may be due more to the instruments being blunt after extensive use than the cutting technique itself.

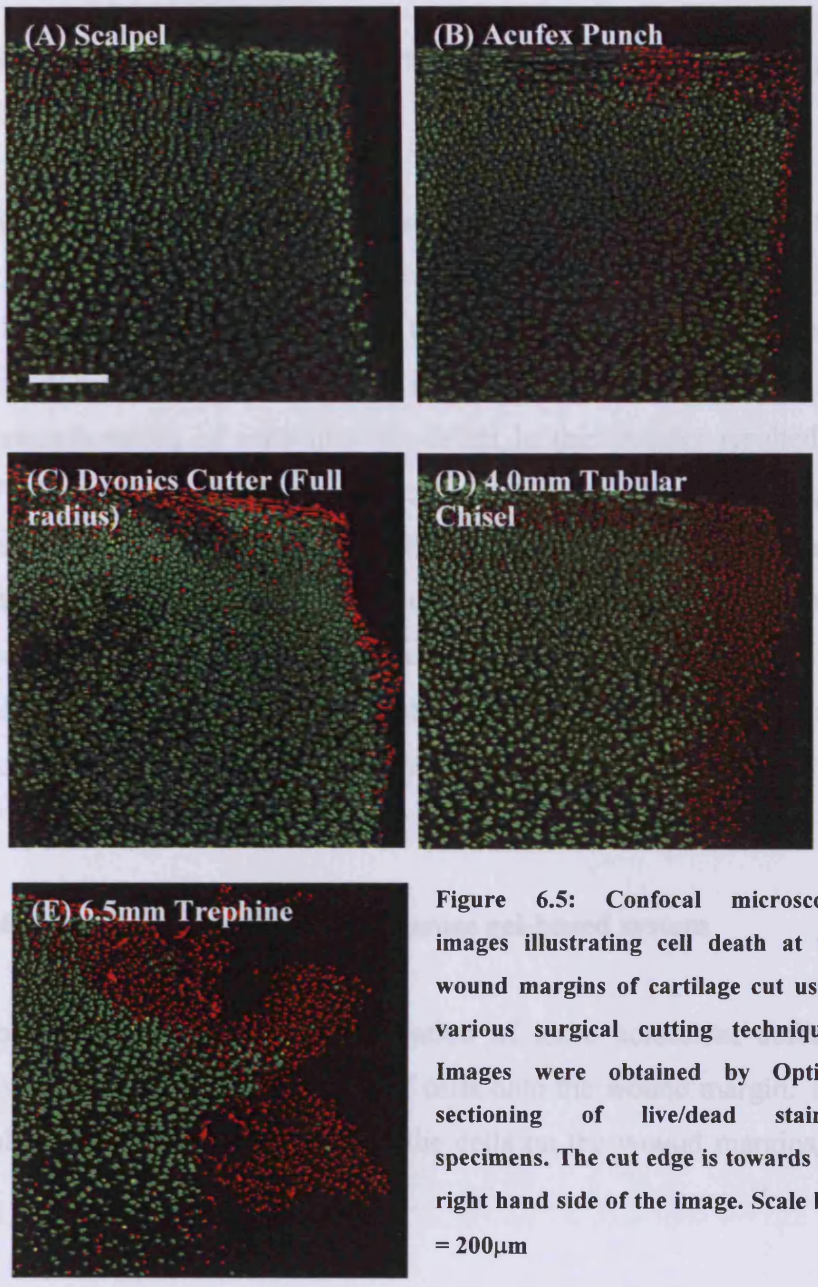


Figure 6.5: Confocal microscope images illustrating cell death at the wound margins of cartilage cut using various surgical cutting techniques. Images were obtained by Optical sectioning of live/dead stained specimens. The cut edge is towards the right hand side of the image. Scale bar = 200μm

Cutting technique	A	B	C	D	E
Depth of cell death (μm ± s.d.)	9.2 ± 2.2	54.7 ± 3.1	32.4 ± 7.3	215.0 ± 54.1*	456.6 ± 232.0**

Table 6.1: Table showing average wound margin thickness (depth of cell death) using various cutting techniques calculated using image analysis as described in section 6.2.1.3. Statistical significance was determined using one way ANOVA and Tukeys' post hoc testing. *p<0.05; **p<0.01 compared to scalpel blade (A).

6.3.2 Cell transplantation: effect on articular cartilage wounded using a drill bit.

It was evident from the histology and confocal microscopy (figures 6.7 and 6.8) that wounding the cartilage explants using a drill bit produced extremely variable defects, often involving quite extensive trauma to the cartilage. This variability and extensive trauma would make the quantification of the effect very difficult. In addition, although transplantation of cells into the defect in this manner resulted in a large number of cells being present in the defect, this also appeared variable. Apparent from the images is that the transplanted cells from the superficial or mid zone had little effect on the wound margin in terms of cell viability or matrix glycosaminoglycan (GAG) loss. The effect of the cells may have been masked by the variability inherent in the model. Therefore, a method of creating more constant defects was required, in addition to a means of introducing a more uniform number of cells onto the wound margin.

6.3.3 Cell transplantation using the agarose gel-based system

The use of this system allowed the creation of more consistent defects and the introduction of a more uniform number of cells onto the wound margin. Therefore, a more reliable evaluation of the effect of the cells on the wound margins could take place.

The use of a scalpel to wound the cartilage resulted in a very narrow wound margin of approximately 1-3 cells deep. This resulted in difficulties in determining whether the transplanted cells had any effect on the wound margin. It was, therefore, decided to use a 3.5mm Acufex punch to create the defect. The use of this tool resulted in a wound margin of approximately 9-11 cells deep. Results of transplanting the cells into the defects are illustrated in figures 6.9 and 6.10 and the results from the image analysis of the confocal images are shown in table 6.2. Apparent from the images and the data is that the transplantation of cells from the superficial or mid zone that have a high affinity for fibronectin had little effect on the wound margin in terms of cell viability. There was also no visible effect on matrix (GAG) loss, although this was not quantified.

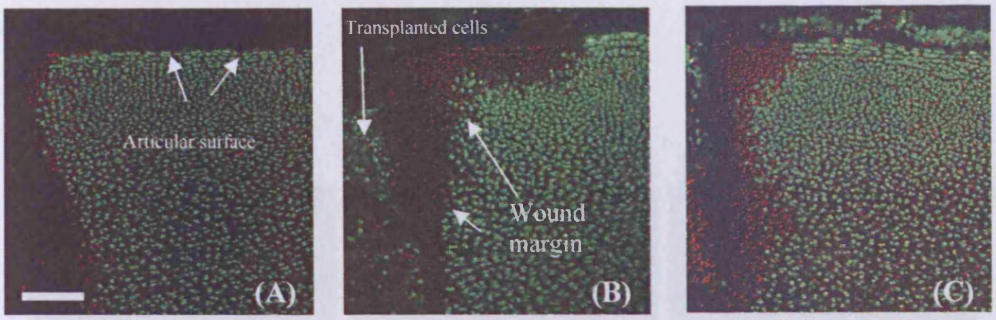


Figure 6.7: Confocal microscope images showing cell viability at wound margins created using a drill bit and left empty (A), transplanted with cells with a high affinity for fibronectin from superficial zone (B) and middle zone (C). Scale bar = 200μm.

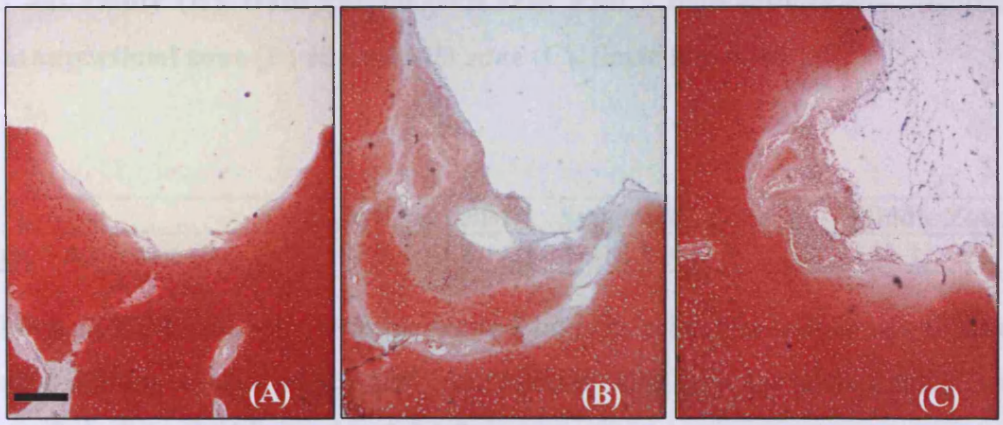


Figure 6.8: Images showing Safranin O staining of defects created using a drill bit and left empty (A), transplanted with cells with a high affinity for fibronectin from superficial zone (B) and middle zone (C). Scale bar = 400μm.

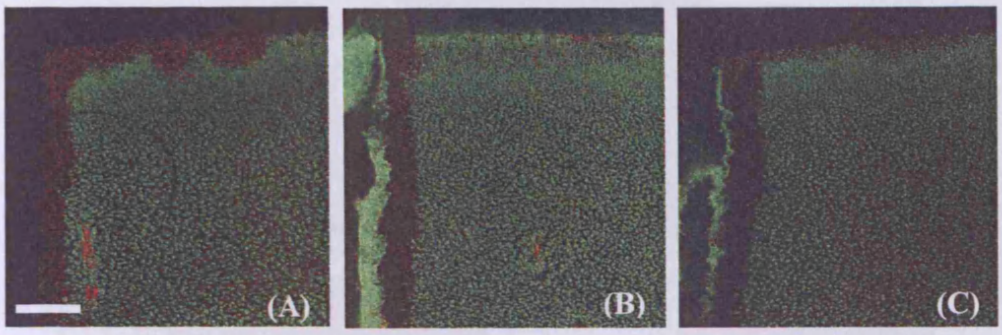


Figure 6.9: Confocal microscope images from the agarose gel based system showing cell viability at wound margins created using a 3.5mm Acufex punch and left empty (A), transplanted with cells with a high affinity for fibronectin from superficial zone (B) and middle zone (C). Scale bar = 400μm.

	Control (no cells)	Superficial Zone	Middle Zone
Thickness (μm ± SD)	180.3 ± 13.8	183.9 ± 12.1	185.3 ± 23.3

Table 6.2: Table showing average wound margin thickness (depth of cell death) using a 3.5mm Acufex punch and transplanted with cells calculated using image analysis as described in section 6.2.1.3.

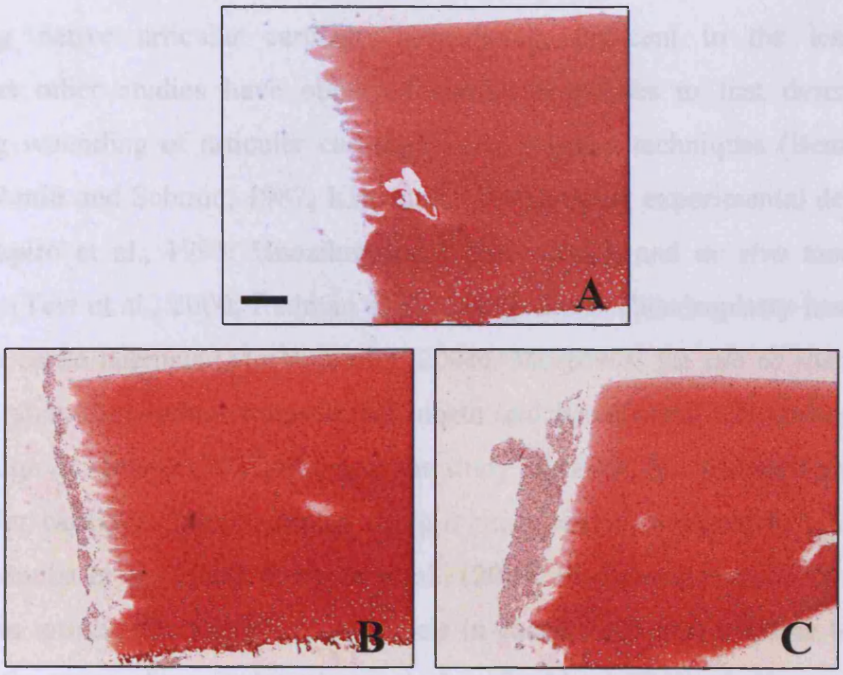


Figure 6.10: Images showing Safranin O staining of defects created using a 3.5mm Acufex punch, cultured using the agarose gel based system and left empty (A), transplanted with cells with a high affinity for fibronectin from superficial zone (B) and middle zone (C). Scale bar = 400μm.

6.4 DISCUSSION

This study has demonstrated the capacity of various different cutting tools used for the surgical management of lesions in articular cartilage to induce cell death in the remaining native articular cartilage immediately adjacent to the lesion edge. Numerous other studies have observed similar responses to that described here following wounding of articular cartilage using surgical techniques (Bennett et al., 1932; Schmid and Schmid, 1987; Kim et al., 1991), using experimental defects in *in vivo* (Shapiro et al., 1993; Hunziker and Quinn, 2002), and *ex vivo* models using trephines (Tew et al., 2000; Redman et al., 2004). Laser chondroplasty has also been shown to cause necrosis (Mainil-Varlet, 2001). Moreover, the use of sharp scalpels has been shown to induce minimal cell death and leave viable chondrocytes at the wound edge (Redman et al., 2004) and one study has even demonstrated total healing of articular cartilage defects created using a sharp scalpel to occur in a foetal lamb model (Namba et al., 1998). Redman et al., (2004) demonstrated minimal cell death induced in articular cartilage using scalpels in comparison to a trephine but did not compare the range of surgical cutting tools described here. These studies together with data generated here demonstrate that the extent of cell death induced is dependant on the type of cutting technique used with drill-powered trephines creating extensive cell death in comparison to the minimal or low levels of cell death caused by sharp scalpels, Acufex punches and the Dyonics powered cutter. Thus, the use of sharper, less traumatic and more precise cutting tools would reduce the mechanical stresses at the lesion edge and reduce cell death.

The confocal microscope technique used here utilises the properties of two fluorescent cell viability probes, calcein AM and ethidium homodimer-1 that detect live and dead cells respectively. Calcein AM is a cell membrane-permeant probe that is metabolised within living cells to a green fluorescent product that is excited by the 488nm line of the Krypton-Argon laser. Ethidium homodimer-1 can only cross the compromised membranes of dead cells where it binds nucleic acids and emits a red fluorescence when excited by the 568nm line of the Krypton-Argon laser. The use of these probes together allows the simultaneous visualisation of live and dead cells within the cartilage explants. However, previous studies have demonstrated that the cell death induced by wounding of cartilage with a trephine comprises a combination of necrosis

and apoptosis (Tew et al., 2000; Walker et al., 2000). The initial necrotic response is proposed to be caused by the mechanical insult that occurs as a consequence of the wounding. This necrosis is followed by a 'wave' of apoptosis that occurs over the subsequent days following injury, extending deeper into the tissue and believed to be caused by mechanical disruption of the chondrocyte-extracellular matrix interaction (Tew et al., 2000; Buckwalter, 2002; Kuhn et al., 2004). Although the ethidium homodimer-1 does not discriminate between apoptotic and necrotic mechanisms of cell death the extent of the apoptotic response is likely to be proportional to the magnitude of the initial mechanical insult and extent of disruption of the chondrocyte-extracellular matrix interaction (Redman et al., 2004). In addition, the cell death in this study was measured 1 day after wounding whereas other studies have demonstrated the apoptotic cell death response to continue up to 10 days following wounding (Tew et al., 2000). Thus the degree of cell death shown here may be an underestimation of the total response that will occur over time.

The results described here were obtained from immature cartilage tissue and it is not known how this correlates with the responses that will occur in adult or more mature cartilage tissue. Previous studies have demonstrated immature and mature cartilage to respond to wounding in a similar manner (Tew et al., 2001) whereas another study has shown injurious compression to induce a greater apoptotic response in immature cartilage compared to more mature cartilage (Kurz et al., 2004).

Immediately evident from the above discussion is that understanding the events that occur in articular cartilage in response to wounding is of prime importance if future cartilage repair approaches are to prove successful. A feature frequently described of attempts to repair defects in articular cartilage is the poor integration between the repair tissue and the native articular cartilage (Shapiro et al., 1993; Wakitani et al., 1994; Hunziker, 2001). The extent of cell death induced by the surgical cutting technique is likely to have a major bearing on whether or not the repair tissue that forms within the defect integrates successfully with the host tissue i.e. the use of sharper cutting techniques would reduce the size of the zone of cell death between repair tissue and host tissue and may promote a more successful integration (Tew et al., 2000; Redman et al., 2004). Wounding with a trephine has been shown to induce a zone of cell death and stimulate cell proliferation and up-regulation of matrix

synthesis deeper in the tissue and adjacent to the 'zone of cell death' (Redman et al., 2004). Interestingly, although wounding with a sharp scalpel induces minimal cell death and matrix disruption, presumably a result of minimising the stresses that occur in the tissue, there is still an up-regulation of matrix synthesis and cell proliferation immediately adjacent to the wound edge in response to the wounding (Redman et al., 2004). Understanding the factors responsible for this shift in the attempted repair response to the lesion edge when mechanical stresses and hence cell death and matrix loss has been kept to a minimum is an example of how future approaches to cartilage repair may be improved. Alternatively, approaches to inhibit the apoptotic response may be of benefit. For example, such approaches could include the localised use of molecular inhibitors of apoptosis or the prevention of the loss of survival factors such as IGF-I from the extracellular matrix using hydrogels (Kuhn et al., 2004).

The potential ability of a chondrogenic cell type to prevent the detrimental apoptosis and loss of glycosaminoglycans that occurs in cartilage following wounding would be of great interest for tissue engineering based approaches to cartilage repair. However, data generated in this study showed there to be little effect of transplanting populations of cells enriched in superficial zone articular cartilage progenitor cells onto cartilage wound margins. No effect was observed on the wounding induced necrosis or loss of glycosaminoglycans from the wound edge. These results are in contrast to data generated in other studies (Bishop et al., 2002) that indicate that transplanting similar cells into defects in articular cartilage reduces the extent of cell death and matrix loss at the wound margin. The reasons for the contrasting results are unclear although may be due to the fact that the studies of Bishop et al., (2002) used cells transplanted in the form of pellets that were created several days prior to transplantation. Thus, the cells may have been in a different state of differentiation at the time of implantation in the defect to the cells used in the studies presented here. It would also be interesting to look at the effect on wound margins of purer populations of progenitor cells using clonal lines derived from individual colonies following differential adhesion. The populations used in these studies were only enriched and thus any beneficial effect of the progenitor cells may be being diluted by non-progenitor cells in the populations.

In conclusion, the surgical removal of articular cartilage has a detrimental effect on the remaining articular cartilage and this detrimental effect may, in part, lead to the poor integration of repair tissue in the defect. The extent of the detrimental response is dependant on the cutting technique used. Although the implantation of populations of cells enriched in articular cartilage progenitor cells failed to influence events at the wound margin, the model developed may be useful for evaluating the effects and integration of other cells or tissue engineered cartilage constructs on cartilage wound margins. The model may therefore provide a useful tool for the design of cell-based strategies for cartilage repair prior to *in vivo* evaluations.

CHAPTER 7

HUMAN STUDIES

7.1 INTRODUCTION

Previous studies describing evidence for the existence of a population of articular cartilage progenitor cells within the superficial zone of articular cartilage and their role in development have investigated developing or neonatal opossum, murine or bovine tissue (Hayes et al., 2001; Hayes et al., 2003; Dowthwaite et al., 2004 respectively). Little is known about the role of the cells in the development of human articular cartilage and the fate of the cells during maturation of the cartilage through to the adult organism. The prospect of isolating articular cartilage progenitor cells from human cartilage would be of interest as the cells may replicate the extensive expansion and chondrogenesis properties exhibited by their bovine counterparts (Chapter 5). The large expansion potential may allow the development and commercialisation of cartilage repair therapies through the establishment of allogeneic cell banks.

In order to study the properties of the human cells a tissue source is needed. As little is known about the existence of human articular cartilage progenitor cells or their fate during the ageing process, the optimal age of donor for their isolation is unknown. Bearing in mind their role in cartilage development they are likely to be more abundant in human neonatal or paediatric cartilage, however a demonstration of their existence in adult human tissue would be of benefit as it would be relatively easier to obtain suitable adult donor tissue compared with human neonatal or paediatric tissue. With this in mind, together with previous data suggesting their existence in developing neonatal animal articular cartilage (Hayes et al., 2001; Hayes et al., 2003; Dowthwaite et al., 2004) it was decided to attempt to prepare enriched populations of human articular cartilage progenitor cells from both adult and developing human tissue.

Little is known about the effect of aging on human chondrocyte proliferation and redifferentiation ability (Barbero et al., 2004). Previous studies have investigated age-related changes in proliferation of chondrocytes derived from human (Guerne et al., 1995; Barbero et al., 2004) and animal donors (Adolphe et al., 1983; Evans and Georgescu, 1983). These studies have shown a species dependant proliferative potential with an inverse relationship between proliferative potential and age of the

donor (Evans and Georgescu, 1983). The proliferative and chondrogenic ability of adult human chondrocytes during culture is low, preventing the widespread use of the cells in cartilage repair therapies or in the generation of allogeneic cell banks. The large numbers of cells required for these applications suggest the requirement for a stem cell or cartilage progenitor cell with extensive and rapid expansion capabilities. However, it is now becoming apparent that even mesenchymal stem cells derived from human bone-marrow lose their chondrogenic ability during expansion (Mastrogiacomo et al., 2001) thus prompting research into specific expansion conditions to prevent this. Studies have evaluated the effect of culture conditions or various serum lots (Lennon et al., 1996; Caterson et al., 2002; Sekiya et al., 2002) or the addition of growth factor supplements such as FGF-2 to the culture media on the cartilage forming potential of human marrow derived stem cells (Mastrogiacomo et al., 2001; Solchaga et al., 2005). These studies have shown it to be possible to enhance the retention of chondrogenic ability of human cartilage forming progenitor cells during expansion by careful optimisation of the culture conditions.

The objective of this study was to prepare enriched populations of human superficial zone articular cartilage progenitor cells (SZCs) and expand under different conditions and evaluate their chondrogenic potential at various passages by transfer into pellet culture. Chondrogenesis was determined using safranin O staining and collagen II immunolabelling. Neonatal, paediatric and adult human cartilage were examined. A secondary objective was to examine the structure of human articular cartilage throughout development using histological techniques.

7.2 MATERIALS AND METHODS

7.2.1 Human Tissue

Human knee joints of neonatal, paediatric and adult donors were obtained using informed consent. Summary of the donors are shown in table 7.1. The knee joints were opened aseptically and the articular cartilage surfaces exposed. Tissue was removed for histology and cell culture.

7.2.2 Histology and immunolabelling

Articular cartilage was excised from condyle and in some cases the patellar groove surface of the joint. The cartilage was washed once in PBS, fixed and embedded in paraffin wax as described in section 2.3. Sections of 5μm thickness were cut and stained with haematoxylin and eosin as described in sections 2.3.3 and 2.3.4. Some sections were stained with safranin O/haematoxylin and picro-sirius red.

In some cases, cartilage was frozen as described in section 2.3.6 and embedded in OCT. Frozen sections were cut and immunolabelled for the presence of Notch 1-4 as follows.

Antibodies for Notch receptors 1, 3 and 4 were goat polyclonals against the human epitope. Antibody against Notch 2 was a rabbit polyclonal against the human epitope. Antibodies were provided by Santa Cruz. Immunolabelling for BST-1 and fibronectin-EDA was carried out as described in sections 3.2.2 and 4.2.1.1 respectively.

For immunolabelling, sections were washed twice for 5 minutes in PBS (Oxoid) prior to blocking with 2.5% normal rabbit serum (2.5% goat serum for Notch 2 slides) (Dako) in PBS for 20 minutes at room temperature. Blocking solution was wicked off using a tissue and sections were then incubated with 20μg/ml of the antibodies in PBS for 45 minutes at room temperature. Sections were washed three times for 3 minutes with PBS and incubated with a 1:100 dilution in PBS of a biotinylated rabbit anti-goat

secondary antibody (Notch2 sections biotinylated goat anti-rabbit secondary antibody) (Dako) for 45 minutes at room temperature followed by washing for three times for 3 minutes. Sections were then incubated with a 1:100 dilution in PBS of a Streptavidin-FITC conjugate (Amersham Pharmacia) for 45 minutes at room temperature followed by washing three times for 3 minutes. Sections were mounted with Vectashield (Vector) containing propidium iodide, coverslipped and viewed using fluorescence microscopy.

Control sections were incubated with the equivalent concentration of non-immune IgG of the same species the primary antibody was raised in.

7.2.3 Cell isolation and expansion

7.2.3.1 Isolation and expansion of populations of cells enriched in articular cartilage progenitor cells

Protocol was repeated as described in section 5.2.1 with the following exceptions:

Human plasma fibronectin (Sigma, Poole) was used at $10\mu gml^{-1}$ in Dulbecco's PBS with 1mM $MgCl_2$ and 1mM $CaCl_2$ (Sigma, Poole) for coating of plates.

Following isolation, 7.8ml of the 4000 cells ml^{-1} suspension of superficial zone cells (SZCs) in serum free DMEM was seeded into each of 4 coated $75cm^2$ flasks and incubated at 37°C for 20 minutes. After 20 minutes, the media was gently swirled and discarded. Expansion was carried out in media as shown in table 7.2. Fetal bovine serum (FBS) was obtained from Helena Biosciences. Human serum (HS) was obtained from Sigma (H1513). FGF-2 was obtained from R&D Systems, Oxfordshire.

7.2.3.2 Full Thickness Chondrocyte Controls

Chondrocytes were isolated from the full thickness of the articular cartilage (FTCs) of each of the donor joints. The tissue was harvested from the remaining half of the condyle and patellar groove that had not been used for superficial zone cell isolation. Cartilage was digested as described above. Following isolation, approximately 1 x 10^6 cells were seeded into each of 4 x 75cm^2 flasks and cultured in either DMEM/10% FBS and DMEM/10% HS (Donors 1 & 2), DMEM/10% FBS with and without 5ng/ml bFGF (Donor 3) or DMEM/10% FBS alone (Donors 4 & 5).

7.2.4 Chondrogenic differentiation: pellet culture

Pellets were cultured and analysed histologically as described in section 5.2.2.

Donor	Age/Sex	Cause of Death	Supplier
1	33 weeks gestation Male Neonatal	Trisomy 13	Cell Dynamics (Georgia)
2	35 days post-natal Female Neonatal	Pontus cerebellum hypoplasia	Cell Dynamics (Georgia)
3	2 years post-natal Male Paediatric	Microencephaly	Cell Dynamics (Georgia)
4	38 years old Female Adult	Cirrhosis, liver failure	ScienceCare Anatomical (Arizona)
5	45 years old Female Adult	Respiratory failure, stomach sclerosis	ScienceCare Anatomical (Arizona)

Table 7.1: Summary of human donors used in these studies.

Donor	Expansion Media
1	DMEM/10% FBS DMEM/10% HS
2	DMEM/10% FBS DMEM/10% HS
3	DMEM/10% FBS ± 5ng/ml FGF-2
4	DMEM/10% FBS
5	DMEM/10% FBS

Table 7.2: Expansion conditions for putative human superficial zone progenitor cells

7.3 RESULTS

7.3.1 Histology

7.3.1.1 Donor 1

Histological staining of articular cartilage from donor 1 revealed a deep, highly cellular, vascularised and immature cartilage tissue (figure 7.1A & B) that comprised most of the epiphysis of the femur and contained abundant sulphated glycosaminoglycans (figure 7.1C). The architecture and defined zones of articular cartilage present in the adult were absent. Large vascularised canals were present throughout the depth of the tissue, often extending to the surface. There also appeared to be a fibrous, denser staining region on the surface of the tissue that contained a greater abundance of collagen (figure 7.2C) and little or no sulphated proteoglycans (figures 7.1C & 7.2B). This region contained highly oriented collagen fibres lying parallel with the surface of the tissue as shown when sections were stained with picro-sirius red and viewed using polarised light (figure 7.1D). Higher magnification examination of this surface area revealed it to be continuous with both the vascularised canals and vascularised synovial tissue at the periphery of the joint (figure 7.2). The surface of this layer was composed of numerous flattened cells whereas deeper in this layer there were numerous chondrocyte clones indicating it to be a region of active cell proliferation (figure 7.2).

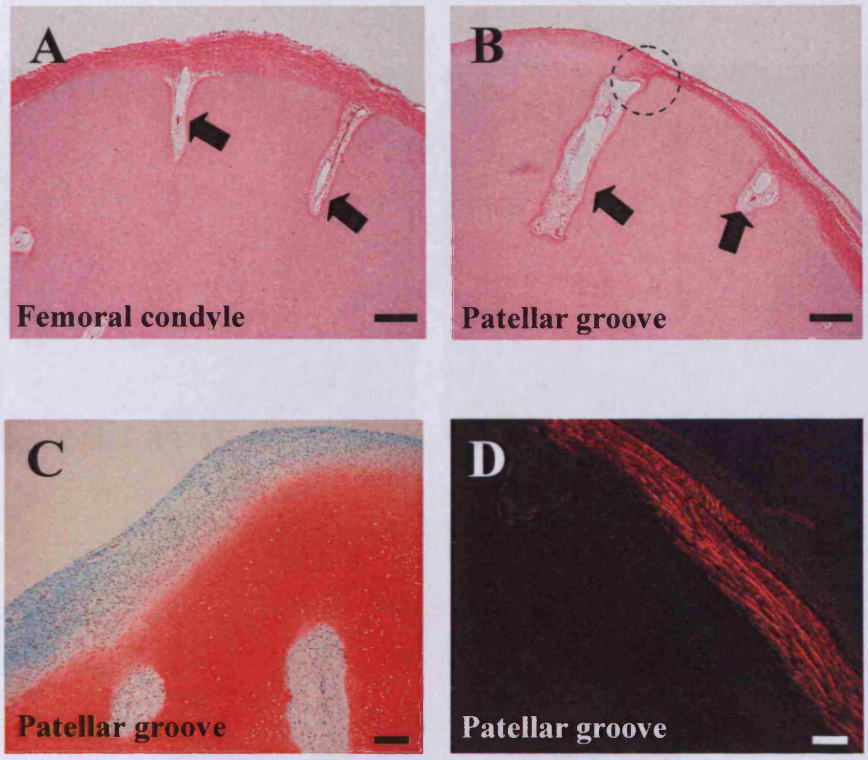


Figure 7.1: Haematoxylin and eosin (A & B) and safranin O (C) staining of donor 1 articular cartilage. A section stained with picrosiruis red and viewed using polarised light is shown in (D). Vascularised areas indicated by solid arrows. A & B bar = 1000μm. C & D bar = 400μm.

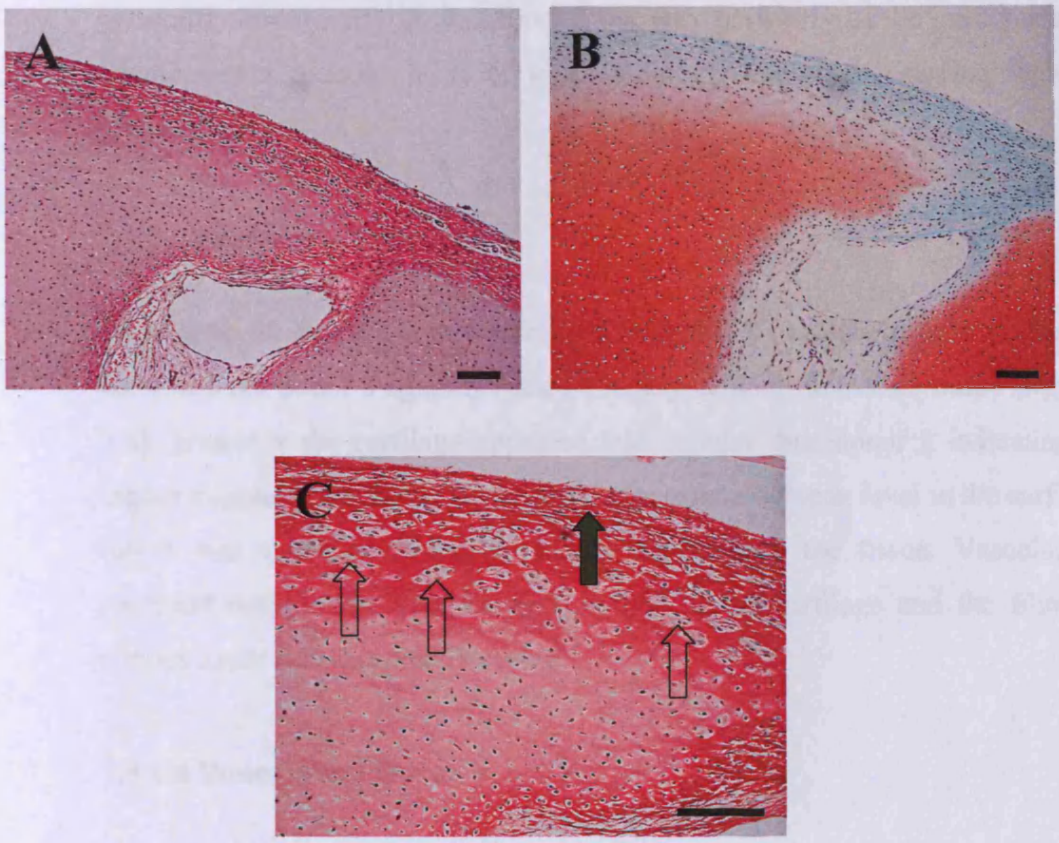


Figure 7.2: Higher magnification image of area highlighted in Figure 7.1B stained with haematoxylin and eosin (A), safranin O (B) and picro-sirius red (C). Flattened cells are indicated by solid arrows. Chondrocyte clones are indicated by open arrows. Bar in all images = 200μm.

7.3.1.2 Donor 2

Haematoxylin and eosin and safranin O staining of patellar groove cartilage derived from donor 2 again revealed a thick, highly cellular and immature tissue (figure 7.3). In contrast to donor 1, the tissue contained a much lower degree of vascularity and the fibrous surface layer evident in donor 1 was generally absent, only present toward the very periphery of the joint surface. There was a reduced level of glycosaminoglycans in the surface regions (figure 7.3B).

7.3.1.3 Donor 3

Haematoxylin and eosin and safranin O staining of patellar groove cartilage derived from donor 3 again revealed a highly cellular, immature tissue (figure 7.4). However the cartilage appeared less cellular than donor 2 indicating a higher degree of maturity. The reduced glycosaminoglycan level in the surface layers was more extensive, extending deeper into the tissue. Vascularity occurred only in the very deepest regions of the cartilage and the fibrous surface layer was completely absent.

7.3.1.4 Donor 4 and Donor 5

Haematoxylin and eosin staining revealed features typical of mature cartilage. The cellularity was relatively lower than that noted in the immature cartilage from donors 1 to 3 and the cells were present in lacunae and arranged in columns in the deeper regions. The surface zone was sparsely populated with flattened cells (arrows) and contained a high proportion of empty lacunae. The surface zone of donor 4 appeared fibrous, although in contrast to donor 1 this region was relatively acellular, possibly a result of some form of early degradative changes.

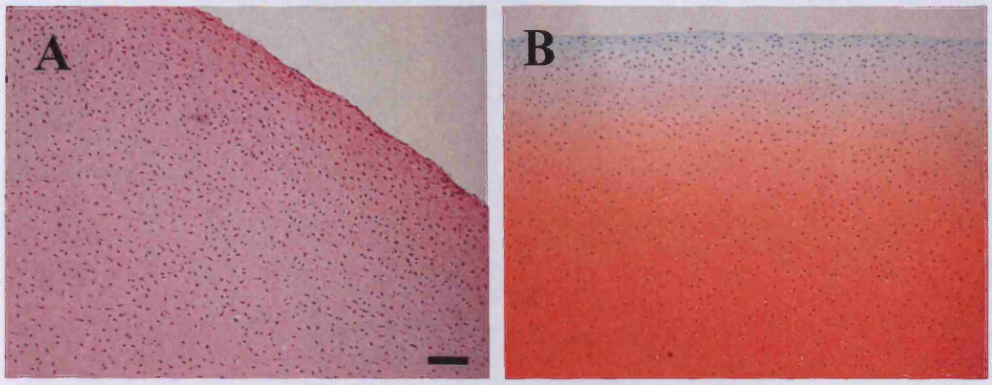


Figure 7.3: Haematoxylin and eosin (A) and safranin O (B) staining of donor 2 articular cartilage from the patellar groove. Bar = 200μm.

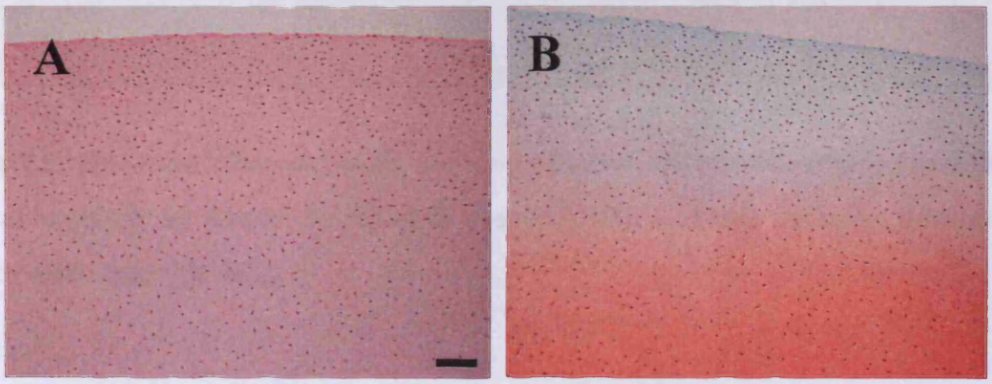


Figure 7.4: Haematoxylin and eosin (A) and safranin O (B) staining of donor 3 articular cartilage from the patellar groove. Bar = 200μm.

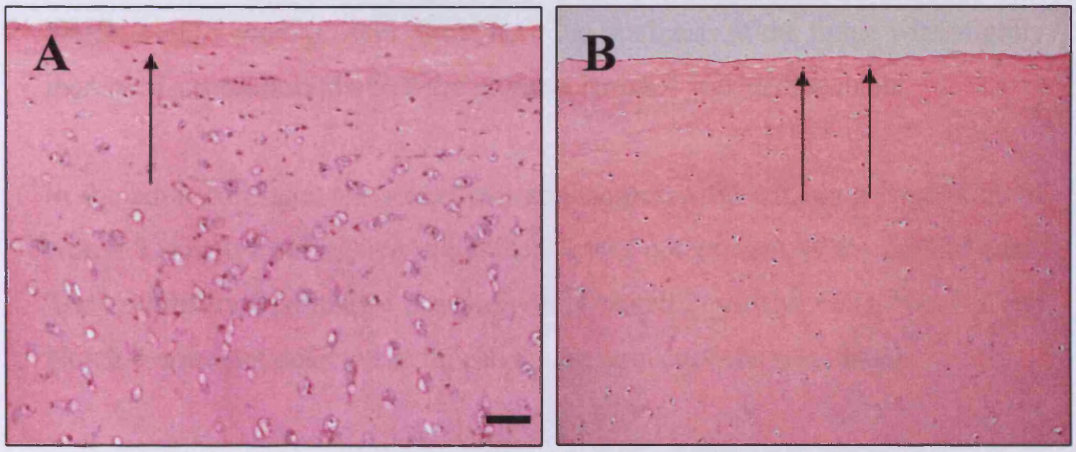


Figure 7.5: Haematoxylin and eosin staining of adult articular cartilage from the femoral condyle of donor 4 (A) and donor 5 (B). Arrows indicate flattened surface cells. Bar = 200μm.

7.3.2 Immunolabelling: Notch 1-4, fibronectin-EDA and BST-1

Figures 7.6 and 7.7 show the distribution of Notch 1-4 in normal neonatal (donor 1) and adult human (donor 4) articular cartilage. Red fluorescence is a result of propidium iodide counter staining of cell nuclei. Results are summarised in tables 7.3 and 7.4. The scores in tables 7.3 and 7.4 were obtained from an independent observer.

In the neonatal cartilage, Notch 1 was present towards the surface of the articular cartilage although it was not expressed in the uppermost cell layers. Notch 2 and 3 were present throughout the thickness of the tissue with slightly increased expression towards the surface. Notch 4 was undetectable.

In the adult cartilage, the superficial zone appeared to contain a population of Notch 3 positive cells (arrows). Notch 3 was not present in the middle zone. The middle zone contained predominantly Notch 2 positive cells. Notch 1 and Notch 4 were not detected in this study. Control sections were blank.

Figure 7.8 shows the expression of BST-1 and fibronectin-EDA in neonatal human articular cartilage. In a similar fashion to Notch 1, BST-1 was present towards the surface of the articular cartilage although it was not expressed in the uppermost cell layers. Fibronectin-EDA was abundant in the surface layers of the tissue. Expression of both proteins was absent in the deeper zones.

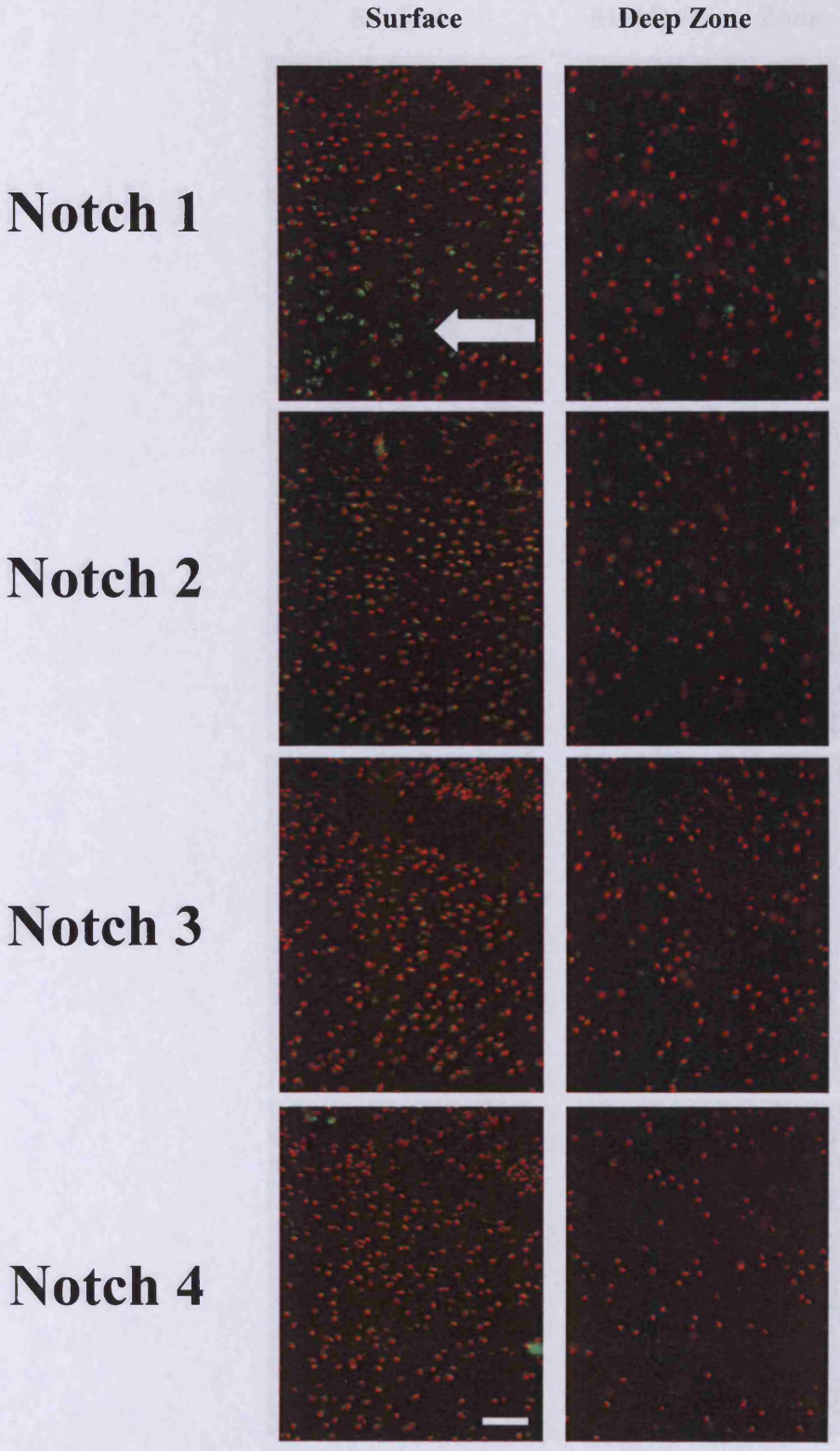


Figure 7.6: Immunostaining of Notch 1-4 in neonatal human articular cartilage (donor 1). Notch 1 expression was noted in the surface regions (white arrow) although it was not expressed in the uppermost cell layers. Bar = 100μm

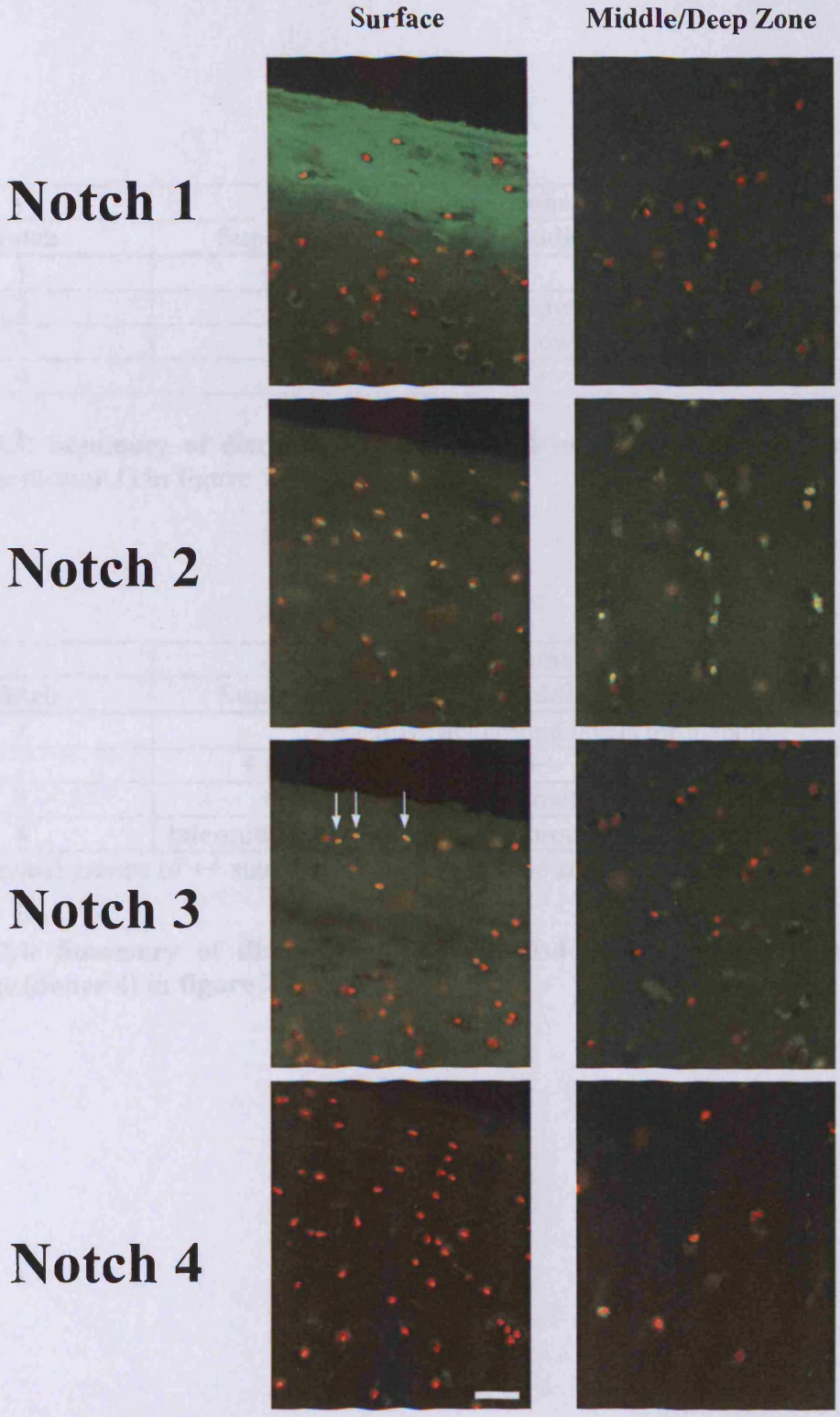


Figure 7.7: Immunostaining of Notch 1-4 in adult human articular cartilage (donor 4). Arrows illustrate Notch 3 expression in uppermost cell layers. Bar = 100μm

	Zone		
Notch	**Superfical**	**Middle**	**Deep**
1	+++	-	-
2	+	+ (low)	+ (low)
3	+	+ (low)	+ (low)
4	-	-	-

Table 7.3: Summary of distribution of Notch 1-4 in neonatal human articular cartilage (donor 1) in figure 7.6

	Zone		
Notch	**Superfical**	**Middle**	**Deep**
1	Very low/background levels throughout		
2	+ (low)	++	+
3	+++	+ (low)[#]	+ (low)
4	Intermittent v. low level stain throughout. Occasional bright cell		

[#] - occasional groups of ++ stain extending into middle zone.

Table 7.4: Summary of distribution of Notch 1-4 in adult human articular cartilage (donor 4) in figure 7.7

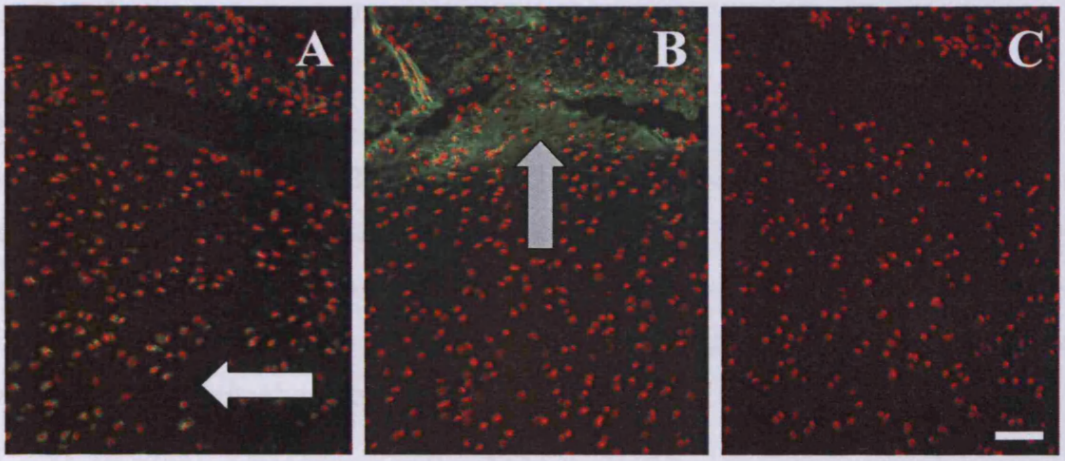


Figure 7.8: Immunostaining of BST-1 (A), fibronectin-EDA (B) at the surface of neonatal human articular cartilage (donor 1). Control of non-specific IgG is shown in (C). BST-1 expression was noted in the surface regions (white arrow) although it was not expressed in the uppermost cell layers. Fibronectin-EDA was clearly present in very surface layers of the tissue (grey arrow). Bar = 100μm

7.3.3 Growth kinetics

Donor 1

Both FTCs and SZCs derived from Donor 1 underwent between approximately 19 and 24 population doublings (pds) over approximately 100 days in culture (figure 7.9).There was little difference in growth rate between cells in human serum (HS) and foetal bovine serum (FBS), although expansion in HS promoted the growth of fibroblastic colonies (figure 7.13) which persisted throughout the expansion process.

Donor 2

Both FTCs and SZCs derived from Donor 2 underwent between approximately 14 and 16 population doublings over approximately 75 days in culture (figure 7.10). The growth rate of both cell types declined after approximately 13 population doublings. Again, as with donor 1, expansion in HS promoted the growth of fibroblastic colonies which persisted throughout the expansion process.

Donor 3

FTCs derived from Donor 3 underwent approximately 10 population doublings when cultured with or without bFGF over 30 and 50 days in culture respectively (figure 7.11). SZCs derived from donor 3 underwent 14 and 10 population doublings when cultured with and without bFGF respectively. The addition of 5ng/ml bFGF to the culture media resulted in a faster rate of growth of both SZCs and FTCs (figures 7.11 and 7.14) and the cells adopted a more spindle-shaped morphology (figure 7.15).

Donor 4

The cells underwent approximately 15 population doublings over 55 days in culture (figure 7.12). The rate of growth rapidly declined after the first passage.

Donor 5

Initial SZC growth was extremely slow, requiring 4 weeks to reach confluence. Subsequent growth was negligible. Normal chondrocytes isolated from the full thickness of the articular cartilage also expanded extremely slowly. Cells were discarded after a further 4 weeks in culture and data is not plotted. No pellet cultures initiated.

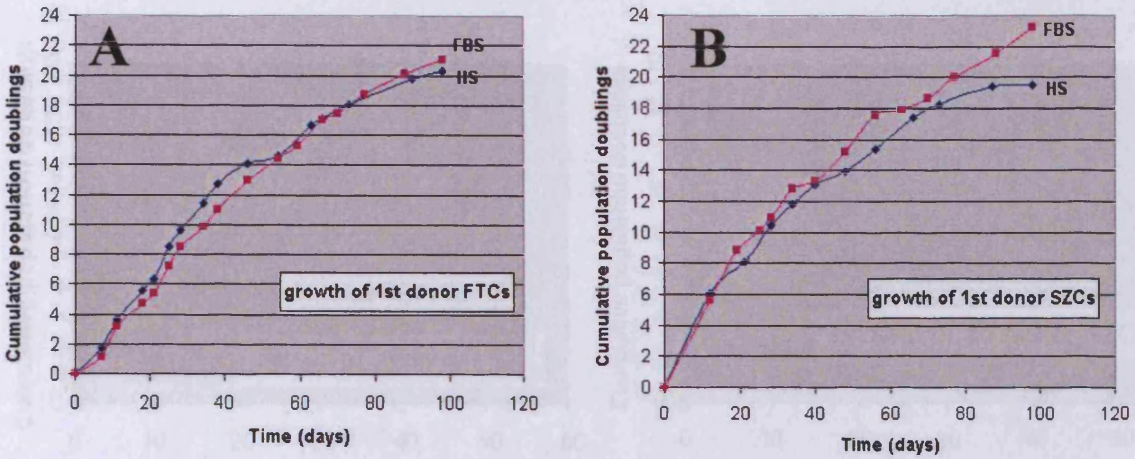


Figure 7.9: Donor 1 - Expansion of FTCs (A) and SZCs (B) in both human serum and foetal bovine serum

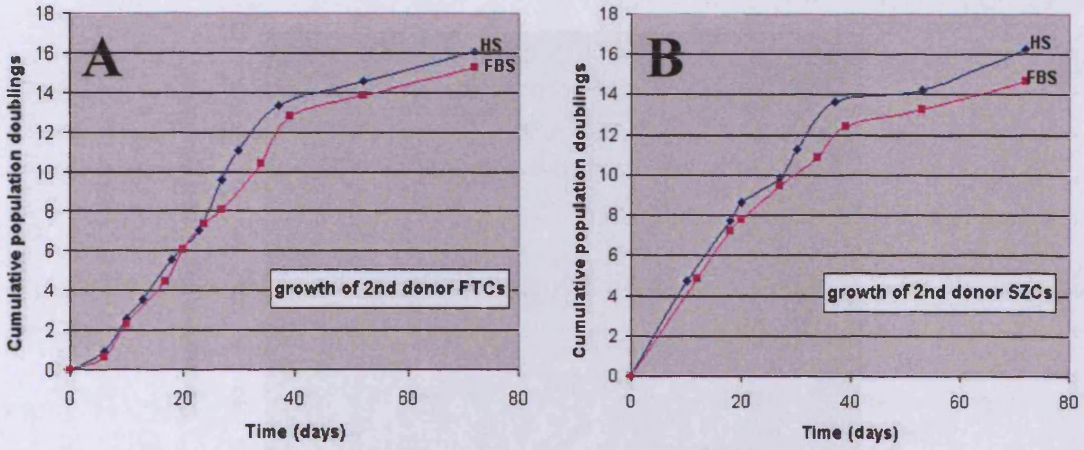


Figure 7.10: Donor 2 - Expansion of FTCs (A) and SZCs (B) in both human serum and foetal bovine serum

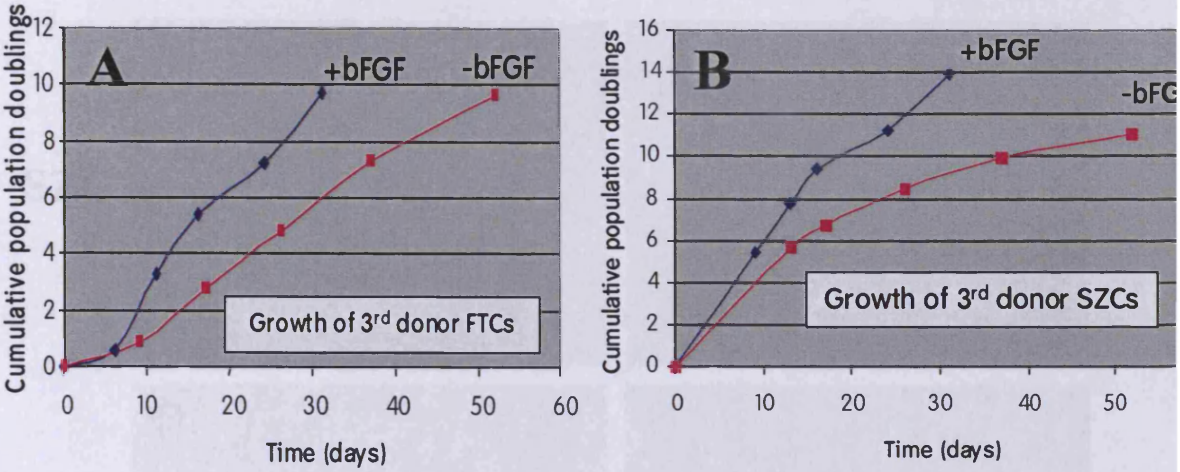


Figure 7.11: Donor 3 - Expansion of FTCs (A) and SZCs (B) in DMEM 10% FBS +/- bFGF

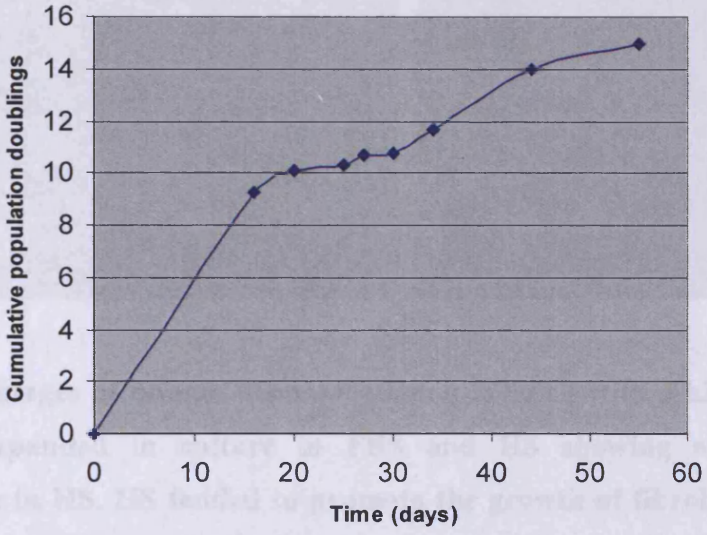


Figure 7.12: Donor 4 - Expansion of SZCs in foetal bovine serum

7.3.4 Cellular morphology

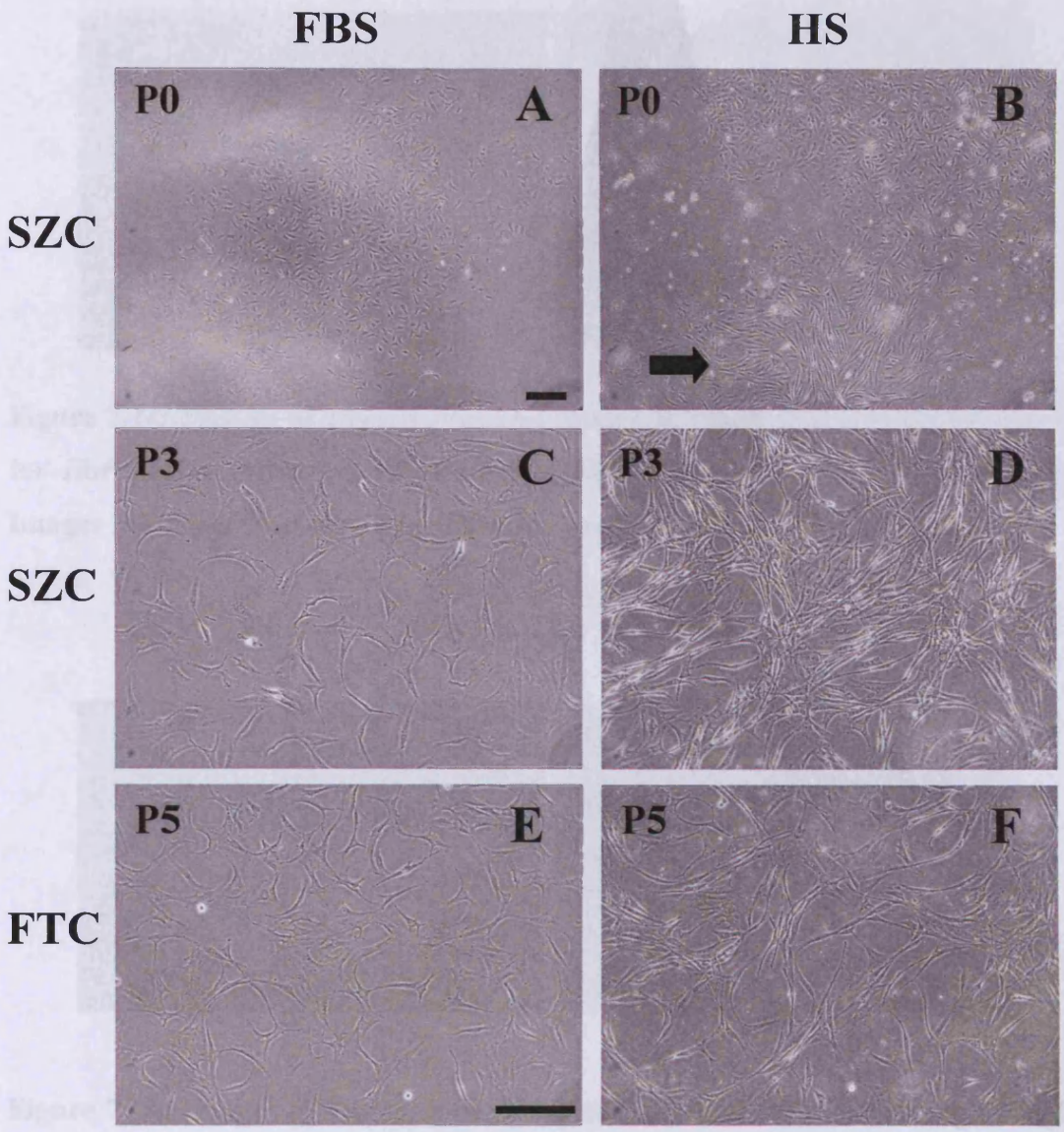


Figure 7.13: Images of human neonatal (donor 1) SZCs with a high affinity for fibronectin expanded in culture in FBS and HS showing altered growth characteristics in HS. HS tended to promote the growth of fibroblastic colonies (solid arrow) which remained throughout the expansion process as seen in (D) and (F). Bars = 100µm.

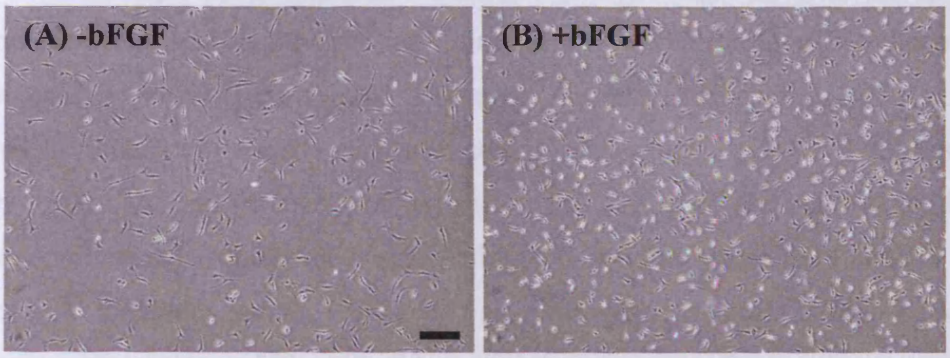


Figure 7.14: Images of human neonatal (donor 3) SZCs (P1) with a high affinity for fibronectin expanded in culture in FBS with and without 5ng/ml bFGF. Images illustrate the faster growth in the presence of bFGF. Bar = 100μm.

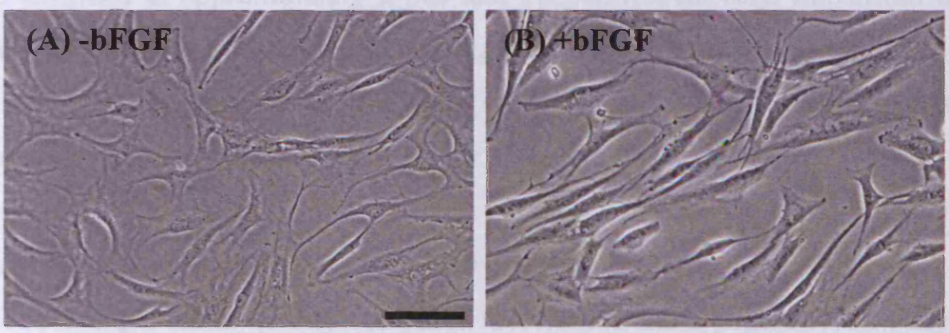


Figure 7.15: Images of human neonatal (donor 3) SZCs (P2) with a high affinity for fibronectin expanded in culture in FBS with and without 5ng/ml bFGF. Images illustrate the more spindle-shaped morphology in the presence of bFGF. Bar = 200μm.

7.3.5 Pellet Culture

Donor 1 FTCs and SZCs expanded in culture and subsequently grown in pelleted micromasses synthesised a matrix that stained strongly with Safranin O and contained abundant collagen II (figure 7.16). However, this occurred only in pellet cultures derived from cells in the early stages of expansion – up to 11 pds for SZCs and 1 pd for the FTCs. At higher levels of expansion the ability of the cells to produce a cartilagenous matrix rapidly declined. Safranin O staining was absent and the tissue was of a more fibrous nature. Collagen II staining was also absent at higher levels of expansion. Expansion in HS resulted in a slightly inferior quality cartilagenous matrix in that safranin O staining was reduced.

As with Donor 1, Donor 2 FTCs and SZCs at early stages of expansion synthesised a matrix that stained strongly with safranin O and contained abundant collagen II (figure 7.17). The quality of cartilage produced by Donor 2 FTCs and SZCs expanded in HS declined at approximately 5 pds and 10 pds respectively. Expansion in FBS resulted in a slightly poorer quality cartilagenous matrix than expansion in HS. Thus, cells derived from Donor 2 lost the ability to synthesise a cartilagenous matrix at an earlier stage than cells derived from Donor 1.

Pellet culture of expanded SZCs and FTCs derived from donor 3 gave rise to a vastly inferior cartilage matrix quality compared to donors 1 and 2 (figure 7.18). Only FTCs at P1 gave rise to a matrix that stained with safranin O. Supplementation of the expansion media with 5ng/ml bFGF resulted in a slight increase in safranin O staining at P4 and P1 for the FTCs and SZCs respectively.

Donor 4 SZCs expanded in culture to P1 and subsequently grown in pelleted micromasses synthesised a cartilage-like matrix that stained moderately with safranin O (figure 7.19). Cells were rounded and present in lacunae. At P4, safranin O staining was completely absent and the tissues were of a more fibrous nature. FTCs derived from donor 4 expanded to P1 and transferred to pellet culture synthesised a matrix that stained weakly with safranin O. Thus, as with donor 1, the SZCs retained the ability to form a cartilagenous matrix at a higher level of expansion that the FTCs.

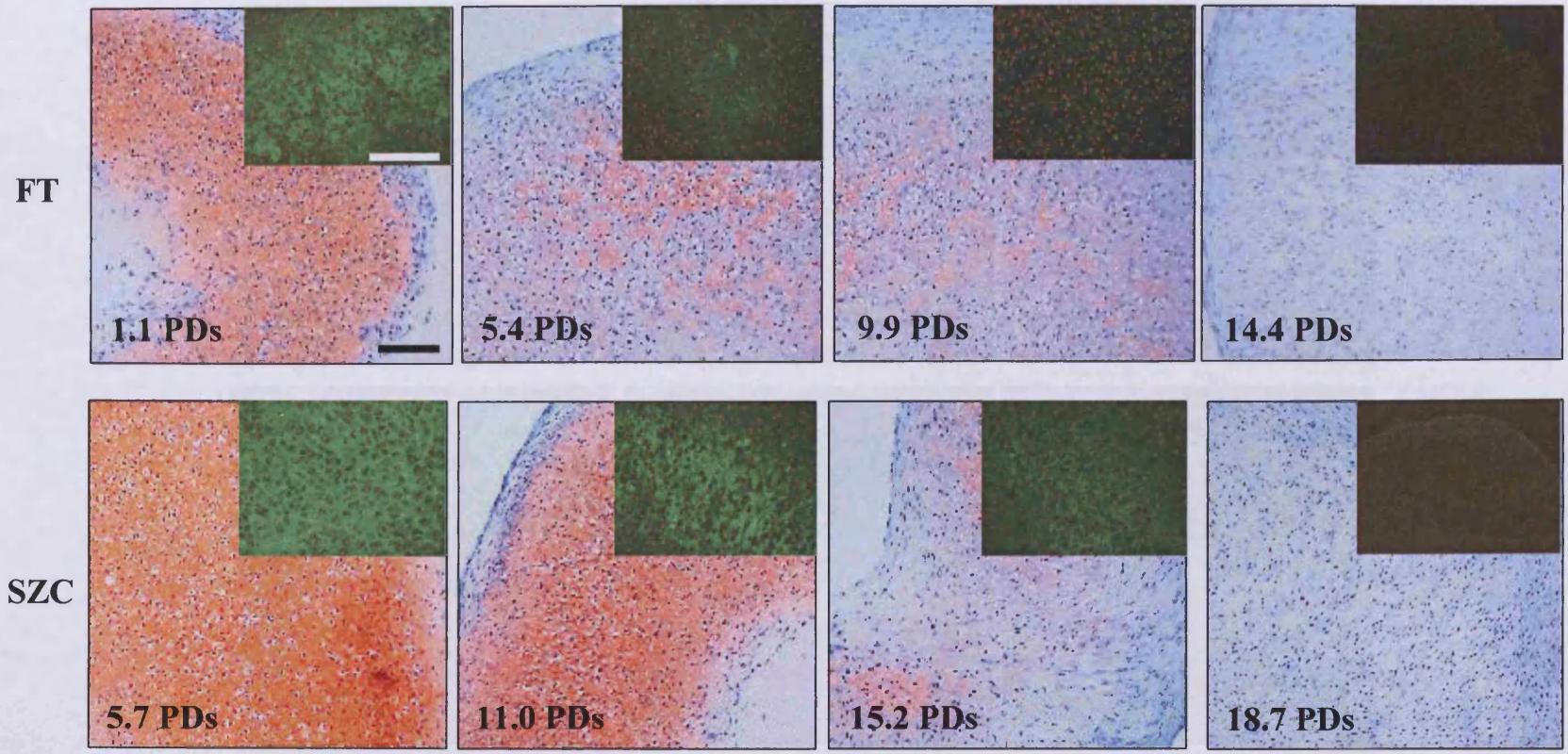


Figure 7.16: Donor 1 - Pellet culture of FBS culture expanded human SZCs and FTCs. Safranin O staining. Collagen II staining inset. Bar = 200μm.

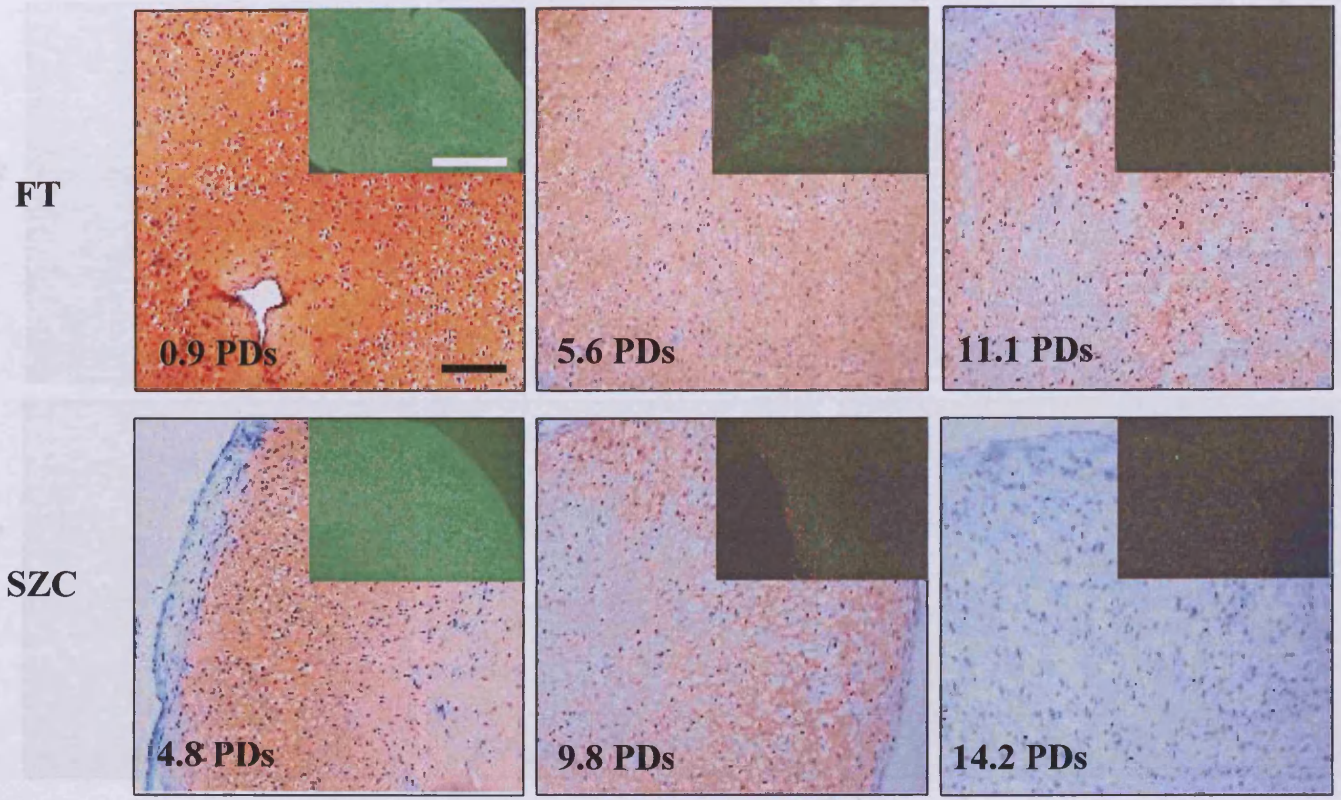


Figure 7.17: Donor 2 - Pellet culture of HS culture expanded human SZCs and FTCs. Safranin O staining. Collagen II staining inset. Bar = 200μm.

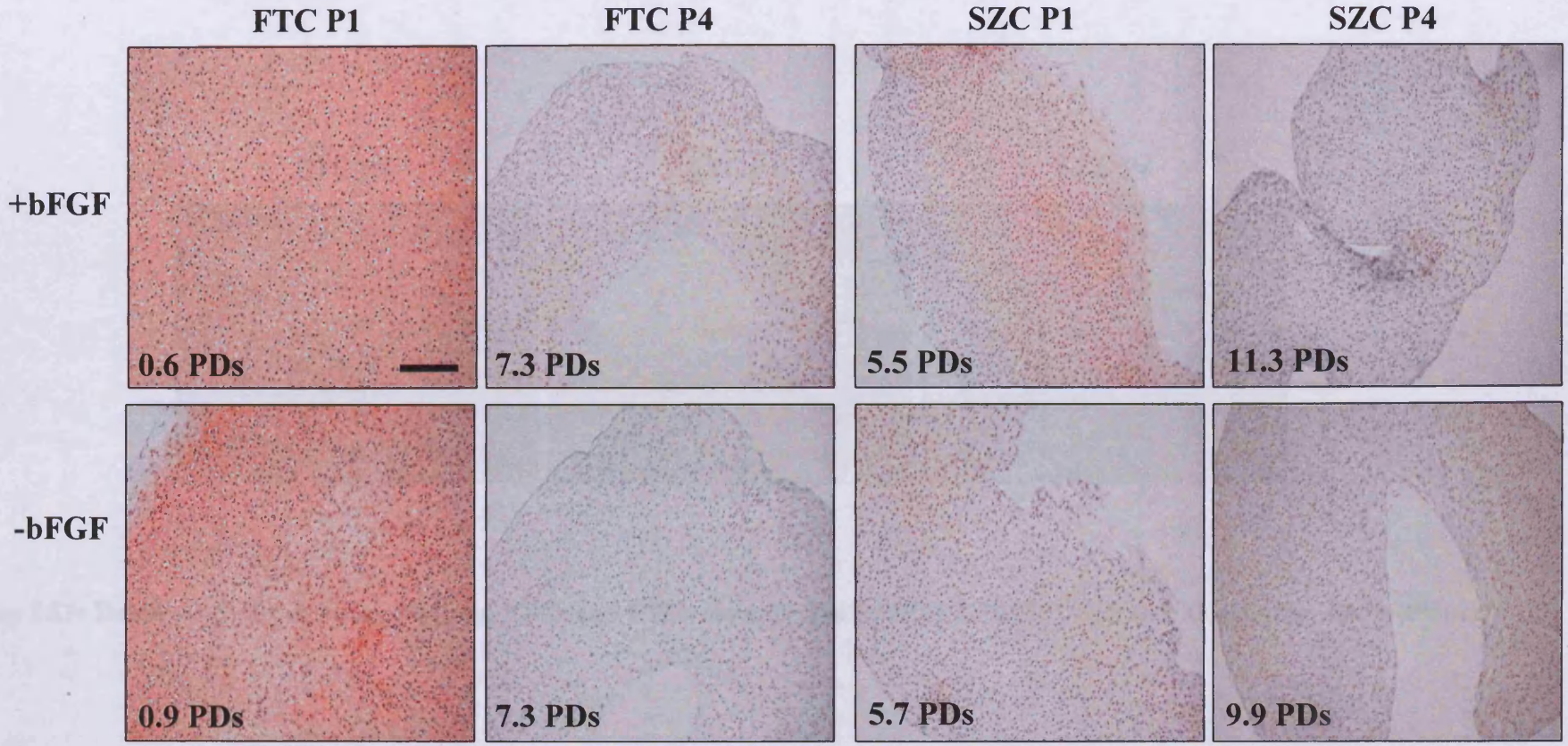


Figure 7.18: Donor 3 - Pellet culture of human SZCs and FTCs expanded in DMEM/10% FBS with and without 5ng/ml bFGF. Safranin O staining. Bar = 200μm.

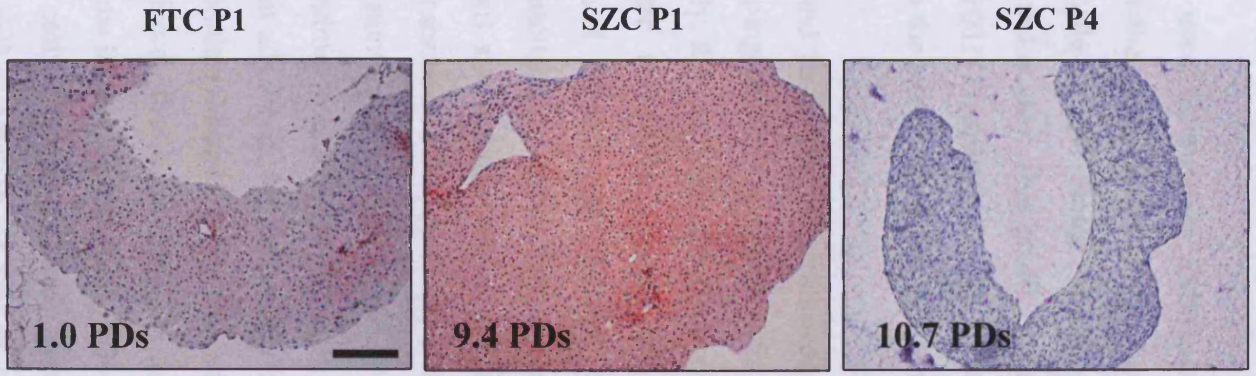


Figure 7.19: Donor 4 - Pellet culture of human SZCs and FTCs expanded in DMEM/10%FBS. Safranin O staining. Bar = 400μm.

7.4 DISCUSSION

Interesting properties exhibited by enriched populations of bovine superficial zone articular cartilage progenitor cells (SZCs) warranted attempts at isolating the cells from human articular cartilage as the value of human cells possessing similar properties in potential cell-based cartilage repair therapies would be high.

Initial studies used histological techniques and immunolabelling to gain general information on the morphology of the tissue during development and to study the expression profile of the Notch family of receptors and other proteins by the developing chondrocytes. This profile would be compared to published information (Hayes et al., 2003; Dowthwaite et al., 2004) on developing animal tissue in order to gain clues as to the existence of the cells in the human case.

The neonatal and paediatric tissue showed morphological features typical to that of normal developing articular cartilage (McDermott, 1943; Gray and Gardner, 1950). Macroscopically, the cartilage comprised most of the epiphyses of the femur and tibia. The architecture and defined zones of articular cartilage present in the adult (Buckwalter and Mankin, 1997) were absent. The cartilage was also highly vascularised. Interestingly, the surface of the most immature donor tissue (donor 1; 33 weeks gestation) showed histological features indicative of the appositional growth mechanisms described in Hayes et al., (2001) in that the surface of the tissue was composed of numerous flattened cells whereas immediately beneath these were numerous chondrocyte clones indicating it to be a region of active cell proliferation. As development advanced, the thickened fibrous layer on the surface of the tissue and vascularised canals evident in donor 1 receded together with the level of cellularity of the tissue, which further decreased into adulthood. It is important to note that the thickened fibrous layer on the surface of Donor 1 articular cartilage probably provided the bulk of the cells that were cultured. This layer was absent from the other donors so the expanded cell population of Donor 1 was taken from a different anatomical location. Some similarities existed in the expression of Notch receptor isoforms between data generated here and published data on bovine and murine tissue (Hayes et al., 2003; Dowthwaite et al., 2004). Notch 1 was expressed only in the surface regions of the developing human tissue. Notch 2 and 3 were expressed at low levels

throughout the depth of the tissue. In this study we were unable to detect any level of Notch 4 expression which is in contrast to published data for mice (Hayes et al., 2003). These data suggest Notch 1 may also play a role in determining cell fate during development of human articular cartilage as it is believed to in other species. In the adult human tissue, Notch 1 expression became absent from the surface regions and Notch 2 was confined to the middle regions. Notch 3 was expressed to a greater extent in the surface of the tissue, which is in contrast to published data (Hayes et al., 2003).

A factor that must be borne in mind when using human tissue is that it is unclear as to the effect of the pathologies that resulted in death of the donor had on the development and biology of the articular cartilage. For example, donor 1 suffered from a trisomy 13 (Patau Syndrome) which is associated with skeletal abnormalities such as polydactyly and syndactyly, although there were no obvious morphological abnormalities noted in the articular cartilage in this study. Donor 3 suffered from a microencephaly which may have resulted in disability and decreased mobility. This may have reduced the stresses and strains normally applied to the cartilage following birth. These mechanical factors play an integral role in the development of the tissue (Pitsillades, 2003) and their absence is bound to have affected the normal progression of the maturation of the tissue. In the case of the adult tissue, the cartilage will be undergoing the normal age related changes and even may be in the very initial stages of the development of osteoarthritis. It is also unknown as to the effect of the drugs the donors were receiving as part of their medical treatment prior to death, on the biology of the chondrocytes in culture.

The populations of cells enriched in superficial zone progenitor cells (SZCs) isolated from donor 1 displayed enhanced chondrogenic ability as a function of expansion in comparison to normal chondrocytes isolated from the full thickness of the cartilage of the same donor. This property was not replicated by the other donors apart from, curiously, the adult donor 4. Although this property is interesting, it is doubtful the expansion levels obtained in this study would be sufficient from a commercial perspective. The costs of establishing and maintaining an allogeneic cell bank for therapeutic use is extremely high. The establishment of a cell bank derived from more than 1 donor increases the costs dramatically due to expenses incurred through procurement of a suitable donor, screening and other regulatory and development

aspects. Therefore one would ideally develop the cell bank from 1 donor as was the case with Dermagraft™, a human fibroblast-derived dermal substitute previously marketed by Smith and Nephew for the treatment of diabetic foot ulcers. If we consider the US focal defect market to be approximately 600,000 cases per year (Medtech Insight report, 2003) and estimate a product could achieve an initial 10% penetration into that market and if we also assume we would use approximately 1 $x10^7$ cells in a tissue engineered device then we would require around 6×10^{11} cells to supply the market for one year. If we could achieve a total yield of 1×10^6 progenitor cells per donor then they would need to undergo over 18 population doublings to achieve the desired amount. Clearly there are a lot of assumptions made in this calculation but it serves to highlight the issues involved.

The inability to replicate entirely the bovine data is likely to be due to a lack of optimisation of the isolation, expansion and possibly differentiation conditions for the human cells. The optimisation process was hampered by difficulties in the procurement of tissue to the required specification. We required normal articular cartilage with no evidence of osteoarthritis or other pathologies. We were keen to obtain the whole knee to maintain the sterility of the cartilage during transit with a timeframe of less than 48 hours post mortem to cell isolation. The neonatal and paediatric tissue is particularly difficult to obtain to this specification. Obtaining tissue from amputations or surgical discard from operations is not feasible as these procedures are very rare in this age group. It was necessary to work with groups that see large numbers of potential donors, who are trained in obtaining consent, hospital liaison and working with the tissue banks and organ procurement organisations to check donor suitability, for serology, tissue harvest and shipment. The complexities of obtaining tissue to a challenging specification must be appreciated early in future research projects in order to ensure to supply of tissue does not become rate limiting.

As part of the limited optimisation process that was carried out, cells were expanded cells in both foetal bovine serum (FBS) and human serum (HS). The hypothesis being tested was that species-specific factors in the serum may be necessary for optimal expansion and differentiation. For example, bovine-specific factors present in the FBS may be necessary for the expansion and differentiation characteristics of the bovine SZC cell population. Although expansion in HS induced morphological changes in a

subpopulation of the cells in the cultures this did not lead to an increased proliferative potential or differentiative capability, rather in one case (donor 1) HS marginally decreased the matrix quality of the cartilage-like tissue produced in the pellet cultures. The factors present in HS that promoted the altered cell morphology are unknown.

This study has served to highlight species-specific differences in cell behaviour in that it is difficult to extrapolate data obtained using animal cells to the human situation. For future studies, there are a number of factors that would need to be known in order to move forward. The optimal age of donor tissue for cell isolation is unknown. This is because the fate of the cells during development and subsequent maturation of the tissue is unknown, together with the level of variability between donors. Also, the technique used to produce enriched populations of the cells used in the study was identical to that used for bovine cells. More sophisticated techniques may be required for isolation of the human cell equivalents. For example, a more detailed characterisation of possible markers would aid identification of the cells in the tissue and tracking the fate of the cells during development and ageing. It would also aid optimisation of the isolation protocol in that it would allow calculations of yields and also may enable immuno-selection techniques such as fluorescence activated cell sorting (FACS) to enrich the populations if the markers are cell surface-based. A candidate for this may be the cell surface receptor Notch 1. However, FACS analysis and colony forming studies have shown that although 75% of the bovine cells from the superficial zone express Notch 1, only 1-2% of these cells form colonies (Dowthwaite et al., 2004) indicating Notch 1 expression in itself is not a marker of progenitor chondrocytes.

The expansion conditions would also need to be optimised. Researchers looking to study chondrogenic differentiation of bone marrow derived mesenchymal stem cells (MSCs) often rigorously select the serum to be used to supplement the medium (Lennon et al., 1996). The use of suboptimal serum lots is reported to result in rapid loss of multipotentiality and slow mitotic expansion. In addition, small differences in the cell culture protocols, such as seeding density of primary and subsequent cultures or formulation of the base medium is also reported to result in reduction in chondrogenic potential (Sekiya et al., 2002; Caterson et al., 2002; Solchaga et al., 2005). Supplementation of the media with growth factors such as TGF-β and bFGF

has been suggested to promote the retention of chondrogenic potential of bovine (Martin et al., 1999) and human (Jakob et al., 2001; Barbero et al., 2004) chondrocytes and human MSCs (Mastrogiacomo et al., 2001; Solchaga et al., 2005). However, data generated in this study showed supplement of the expansion media with bFGF led to only a very limited enhancement of chondrogenesis by SZCs.

In conclusion, this study describes the first attempts to demonstrate the existence of a population of cells within the superficial zone of human adult and neonatal articular that possess the ability to undergo expansion in monolayer culture and retain the ability to undergo chondrogenic differentiation to a greater extent than normal chondrocytes. Although data in this chapter has demonstrated this is possible, further optimisation of the isolation and expansion conditions will be necessary for the human cells.

CHAPTER 8

GENERAL DISCUSSION

8. GENERAL DISCUSSION

Articular cartilage has a distinct zonal architecture with biochemical and cellular variations existing from the surface zone to the deeper calcified layers. Thus, the development of the tissue must be stringently controlled, both spatially and temporally in order for the complex structure to be established. A number of studies have highlighted the importance of the surface areas of articular cartilage in both the morphogenesis of the joint and in the regulation of subsequent growth and differentiation of the tissue (Archer et al., 1994; Ward et al., 1999; Hayes et al., 2001; Archer et al., 2003; Hayes et al., 2003). Importantly, the surface zone is believed to be responsible for the appositional growth of articular cartilage during development and this growth is believed to be driven by a population of slow cycling progenitor cells within the surface zone itself (Hayes et al., 2001; Dowthwaite et al., 2004).

This thesis has described a set of studies looking to isolate and partially characterise articular cartilage progenitor cells from the surface zone. In view of the possible role of these cells in driving development this thesis has also investigated potential applications of enriched populations of the cells in novel tissue repair or tissue engineering therapies for the treatment of defects in articular cartilage. Chapter 3 described the techniques used to identify the cells within the superficial zone of immature bovine articular cartilage and a limited characterisation in terms of some cell surface markers expressed by the cells. The differential expression of Notch 1, the cell-fate determination receptor, is believed to play a key role in the developmental process by controlling the clonality of the surface zone cells (Dowthwaite et al., 2004). Chapter 4 showed the cells are present within the surface zone in a distinct extra-cellular matrix enriched in the foetal splice-variant of fibronectin which contains the additional EDA segment. This chapter also illustrated that this specific form of fibronectin may act to modulate the behaviour of the cells and may play an important role *in vivo* in the control of proliferation and differentiation.

In terms of the potential of the cells for use in tissue engineering, a promising feature of the cells is the discovery that enriched populations of the cells can undergo extensive expansion in simple monolayer cultures and yet retain their ability to undergo chondrogenic differentiation (Chapter 5). If this could be replicated using

human cells then this would side-step well documented issues with the use of adult chondrocytes in tissue engineering such as low growth potentials and loss of chondrogenic ability following expansion (Benya and Shaffer, 1982; Evans and Georgescu, 1983; Von Der Mark et al., 1997; Binette et al., 1998). The expansion and differentiation properties of the cells may allow the generation of large allogeneic cell banks for cell transplantation therapies from only small quantities of donor cartilage tissue. Sadly, our results using human cells did not replicate entirely those obtained from bovine cells (Chapter 7). However, this is most likely due to a lack of optimisation of isolation and expansion conditions for the human cell cultures, rather than an inherent lack of ability of the cells to perform like their bovine counterparts. The optimisation process was hampered due to difficulties in the procurement of normal human articular cartilage, in particular human neonatal articular cartilage. This limitation of tissue supply places great restrictions on the range of experiments that can be carried out, thus preventing an effective optimisation of the isolation and expansion conditions.

A survey of the literature and results obtained in clinical studies indicates we are still a long way off a viable and efficacious cell-based therapy for the regeneration of articular cartilage. The current treatment closest to that of a tissue engineering approach is ACI. Although ACI is a widely used technique with promising outcomes, doubts still remain as to its superiority over simpler and cheaper approaches such as microfracture (Breinan et al., 1997; Knutson et al., 2004). However, ACI is constantly evolving with the incorporation of scaffolds and the replacement of the periosteal flap (Pavesio et al., 2003; Haddo et al., 2004; Ronga et al., 2004) which may eventually lead to improvements in efficacy, ease of technique or reductions in costs. The problems faced by ACI will be faced by all future cartilage repair therapies. Techniques such as microfracture are cheap, safe, have a long history of use and as good a level of efficacy as every other technique. In order to displace techniques such as microfracture from the operating room new therapies must obviously offer significant advantages alongside increased efficacy. This does not necessarily mean that the cell-based therapy must work to the extent that it achieves a recreation of the architecture of the native articular cartilage and restore the joint to full pain free mobility. Although this would be a long term goal, it is too optimistic to believe we can achieve this in the near future. Extension of the efficacy of current treatment

regimes using small step-wise changes would be of immediate benefit in delaying the need for total knee replacement further.

There are significant issues that need to be addressed in order to bring an effective cell based articular cartilage repair therapy to market. Cell source, promotion of lateral integration, control of differentiation and immunological aspects all require considerable attention.

The choice of cell type and source of that cell type is critical. Autologous cell sources have the benefits of the removal of the risks of disease transmission from donors and also reduce the risks of immunologic rejection. However, high costs associated with autologous cells, as seen during ACI, may drive the use of banked allogeneic cells in potential "off-the-shelf" transplantation therapies. The use of allogeneic cells immediately raises concerns of immunologic rejection. Articular cartilage is an avascular tissue and thus may be shielded from detection by circulating immune cells. Allogeneic osteochondral grafts have been in widespread use for many years with positive outcomes although concerns have been raised about immunologic rejection (Meyers et al., 1983; Czitrom et al., 1986; Garrett, 1986; Stevenson et al., 1989; Sirlin et al., 2001). Whether the osseous or chondral parts of the graft are responsible for the immune response is uncertain, although recent reports suggest there is an immune response to the cartilage component (Phipatanakul et al., 2004). In light of concerns over immunologic responses, researchers have speculated on the potential of progenitor cells such as MSCs from the bone-marrow or synovium due to their interesting immune-modulting properties. Evidence suggests bone marrow derived MSCs have immune-suppressing capabilities, for example, *in vitro*, MSCs are capable of suppressing mixed lymphocyte reactions in response to autologous or allogeneic T cells. T cell proliferation stimulated by the addition of irradiated allogeneic peripheral blood lymphocytes or dendritic cells has been shown to be greatly suppressed when the cultures contained MSCs (Barry and Murphy, 2004). Also MSCs lack HLA Class II expression and have a low expression of co-stimulatory molecules thus rendering them non-immunogenic. Class II expression has also been shown to be absent from the surface of differentiated MSCs and the differentiated cells do not elicit an alloreactive lymphocyte proliferative response (Le Blanc et al., 2003). There are also several clinical reports of the use of allogeneic donor mis-matched MSCs with little

evidence of host immune rejection or graft versus host disease (Aggarwal and Pittenger, 2005). It would be interesting to determine whether these same properties and indeed other properties of MSCs such as homing (Barry and Murphy, 2004) are also exhibited by human SZCs.

The fundamental principle behind the use of progenitor cells or MSCs is that undifferentiated cells, once delivered to the repair site, will differentiate under local signals into cells of the appropriate phenotype (Barry, 2003). These differentiated cells then contribute to the repair of the injured tissue. For example, studies have shown that scaffolds loaded with MSCs and implanted in osteochondral lesions give rise to both cartilage and bone cells. In addition, studies have shown that MSCs, when delivered by infusion to an immunocompromised mouse, can engraft to the normal myocardium and differentiate into a cardiomyocyte phenotype. Whereas such evidence indicates MSCs do indeed differentiate *in situ*, little is known about the specific local signals that drive it (Barry, 2003).

Although efforts are focussed on the use of cells on 3D scaffolds with the appropriate cues, there role of transplanted cells in repair is unknown. Solchaga et al., (2002) reported that a fibronectin coated hyaluronan-based sponge was able to organise and facilitate the reparative response following implantation within an osteochondral defect, even without pre-loading the scaffold with autologous bone marrow, suggesting an enhancement of the natural repair response by scaffold alone. Furthermore, it is unknown as to the fate of cells once implanted into the defect. The extent to which they persist or survive at the implantation site and the extent of their contribution to the repair tissue is uncertain. Although some studies have shown little contribution of the cells to repair in ACI (Breinan et al., 1997) other studies have shown that expanded chondrocytes persist at the repair tissue and contribute to the matrix formation in a goat model (Dell' Accio et al., 2003). Other studies have shown that MSCs injected intra-articularly in a goat model of OA where the whole meniscus had been previously excised led to the prevention of articular cartilage degeneration due to the removal of the meniscus through the regeneration of a 'neo-meniscus' (Murphy et al., 2003). The injected MSCs did not appear to directly contribute to the regeneration of the neo-meniscus, rather they engrafted into various joint tissues and orchestrated the repair directly carried out by other cell types within the joint space.

Although another study has shown cartilage tissue formed by MSCs derived from the synovium is unstable and rapidly resorbed when implanted subcutaneously in nude mice, more studies need to be carried out in relevant cartilage defects to confirm this (De Bari et al., 2004). The fate of the cells may also become an issue when cells are transplanted in OA joints. In these circumstances the environment is hostile, containing degradative enzymes and inflammatory cytokines, thus the cells and matrix they produce may require some protection in order to lay down an effective repair tissue.

Another aspect that requires attention is that of lateral integration. The poor integration of repair tissue with the native articular cartilage is widely recognised (Hunziker, 2001a). Poor integration must be addressed in order to achieve prolonged healing. Although various attempts have been made to resolve integration, for example using biological glues and other adhesives or brief enzymatic degradation, this still remains an issue. Without proper integration there will be constant shear stresses at the interface between the two tissues and incorrect distribution of mechanical forces over the joint surface.

In conclusion, tissue engineering is still in its infancy with many issues that need to be addressed. The widespread use of tissue engineering will depend upon the availability of validated methods for large scale culture, storage and distribution of a chondrogenic cell type.

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Dowthwaite GP, Bishop JC, Redman SN, Khan IM, Rooney P, Evans DJ, Haughton L, Bayram Z, Boyer S, Thomson B, Wolfe MS, Archer CW. (2004) **The surface of articular cartilage contains a progenitor cell population.** J Cell Sci. **117** 889-97.

Conference abstracts

Articular cartilage progenitor cells: Chondrogenic potential during expansion in monolayer culture

S Boyer, R Turner, I Honeyborne, MF Smith, BM Thomson, D Chenery, G Dowthwaite & C Archer. UK Tissue and Cell Engineering Society Annual Meeting. Cardiff. September 2003. Published in European Cells and Materials Vol. 6. Suppl. 2, 2003 (page 16)

Chondrogenic ability of articular cartilage progenitor cells during extensive sub-culture

S Boyer, R Turner, I Honeyborne, MF Smith, BM Thomson, G Dowthwaite & C Archer. Orthopaedic Research Society 49th Annual Meeting. New Orleans. February 2003

Preparation of stratified, tissue engineered implants for articular cartilage

BM Thomson, S Boyer, R Turner, D Chenery, CW Archer & M Smith. Tissue engineering 2002 3rd International S&N symposium. Atlanta. October 2002

Characterisation of an articular cartilage progenitor cell

GP Dowthwaite, JC Bishop, S Boyer, B Thomson, M Smith & CW Archer. Tissue engineering 2002 3rd International S&N symposium Atlanta. October 2002

Production of stratified, tissue-engineered articular cartilage implants

BM Thomson, S Boyer, R Turner, D Chenery, D Kidd, H Riggs, S Downes, CW Archer & M Smith. International Cartilage Repair Society. Toronto. June 2002

Tissue engineering of stratified articular cartilage

BM Thomson, S Boyer, R Turner, D Chenery, D Kidd, H Riggs, S Downes, G Dowthwaite, CW Archer & M Smith. Society for Biomaterials 28th Annual Meeting & Exposition, Tampa. April 2002

Production of stratified, tissue-engineered articular cartilage

BM Thompson, S Boyer, R Turner, D Chenery, D Kidd, H Riggs, S Downes, CW Archer, JA Watson, N Medcalf & M Smith. The International Symposium on Bio-integrated Materials and Tissue Engineering. Tokyo. March 2002

Coated biomaterials, zonal cell seeding and cartilage tissue engineering

BM Thomson, M Smith, S Boyer, R Turner, D Kidd, H Riggs, G Dowthwaite, & CW Archer. Orthopaedic Research Society. 48th Annual Meeting. Dallas. February 2002

The identification and characterisation of articular cartilage progenitor cells

CW Archer, S Redman, J Bishop, S Boyer & G Dowthwaite. Orthopaedic Research Society. 48th Annual Meeting. Dallas. February 2002

Cutting instrumentation affects post-operative tissue viability in cartilage

BM Thomson, S Boyer, S Redman, A Kwan, CWArcher & N Medcalf. 5th Annual Hilton Head Workshop & Annual ET Workshop - Engineering Tissues, South Carolina. February 2001

COATED BIOMATERIALS, ZONAL CELL-SEEDING AND CARTILAGE TISSUE ENGINEERING

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Introduction. Articular cartilage is a layered tissue, with differing cellular constituents, matrix components and matrix architecture in the superficial, mid and deep zones. Our research on cartilage wound biology has shown that a population of chondroprogenitor cells exists within the superficial zone of articular cartilage. These cells form colonies *in vitro*, express the cell signaling receptor Notch-1, produce the ECM component fibronectin-EDA, have a high affinity for fibronectin in cell adhesion assays and synthesize a cartilage-like ECM when grown in pellet cultures. Our research into tissue engineering has shown the need for a particulate cell binding material that can act as a microcarrier during the early stages of cell culture and as a 'synthetic interterritorial matrix' during construct assembly. We have found that by adsorbing different cell adhesion factors onto a particulate biomaterial and regulating the cell-seeding conditions it is possible to produce cell seeded particulate biomaterials coated with either progenitors or more mature chondrocytes. Such differentially seeded particulate biomaterials could be assembled into a tissue engineered construct that begins to re-create the zonal cell distribution of healthy cartilage.

Methods. *Synthesis and coating of the biomaterial.* A polyanionic, particulate biomaterial was synthesized by sulphating Intrasite polymer (Smith and Nephew) with sulphur trioxide-pyridine complex. The material was freeze dried, washed in PBS, autoclaved and coated with either (i) heparin-binding cell-adhesion factors, (e.g. Fibronectin or Collagen VI; 60mg/ml; 2.5 ml; 2h at 4°C), (ii) mixtures of adhesion factors absorbed from foetal calf serum (FCS; 10%; 2.5 ml; 2h at 4°C) or (iii) PBS, (control). The chemical nature of the modified polymer was confirmed by Raman, infrared spectroscopy, histological staining, (alcian blue CEC or safranin O) and elemental analysis, whilst the absorbtion of the cell adhesion factors was confirmed by immunocytochemistry.
Seeding the material with cells. Bone marrow stromal cells (BMSC) were obtained from fragmented trabecular neonatal bovine bone, grown to confluence and harvested by mild trypsinisation. Superficial zone chondrocytes (SZC) were obtained from superficial zone neonatal bovine cartilage by fine dissection and sequential digestion in 0.1% pronase and 0.04% collagenase; chondrocytes were obtained from full thickness explants of neonatal bovine articular cartilage digested with 0.1% collagenase, (Worthington type II).
Sulphated Intrasite, (SI; 4.125 mg per layer of construct) was coated with plasma fibronectin, (60ml/ml; 2.5 ml; 2h at 4°C) and seeded with 750k BMSC; or coated with serum-derived cell attachment factors, (10%; 2.5 ml; 2h at 4°C), and seeded with 750k chondrocytes. Cultures were incubated for 3h to 28 days in 20 ml of DMEM plus FCS; (10%), penicillin, streptomycin, non-essential amino acids, L-glutamine and ascorbate-2-phosphate (25mg/ml). Cell viability was assessed using MTT, (1 mg/ml; 2.5 hours), whilst matrix production was assessed using immunocytochemistry, (collagen I, Biogenesis; collagen II, NeoMarkers; collagen VI, Abcam) and histological dyes, (picrosirius red and safranin O).
Colony forming cell adhesion to cell-attachment factor coated SI. A minority of BMSC and SZC form colonies of > 16 cells when cultured on tissue culture plastic in DMEM/10%FCS for 4 and 6 days respectively. These colony forming cells were regarded as putative progenitor cells. In these experiments, colony forming cells that do not attach to the coated SI particles attach to the culture plastic and may subsequently form colonies. Cells that do bind to the SI particles are removed during the washing step, thereby reducing the number of colonies formed on the culture plastic. Sulphated Intrasite was coated with plasma fibronectin, cellular fibronectin, collagen IV, polylysine, laminin (all 60mg/ml) or PBS in 2.5ml DMEM for 2h at 4°C. The coated biomaterial was incubated with BMSC or SZC suspensions (125k cells/ml in serum free medium) for 3h at 37°C, the suspension diluted 1000 fold and aliquoted, (4 ml) to the wells of 12 well plates. FCS (0.4 ml) was then added to the wells and the cultures incubated for a further 16 - 24 hours. The wells were then washed with PBS, and the cultures incubated for a further 4 - 6 days in DMEM/10% FCS (2 ml). Colonies, (discrete clusters of > 16 cells), were visualized by phase contrast microscopy and counted blind. Results were expressed as a 'colony depletion score', defined as the number of colonies formed by cell suspensions exposed to adhesion factor pre-treated sulphated Intrasite divided by the number of colonies per well formed by cells exposed to PBS pretreated sulphated Intrasite.

Results. *Sulphation and coating of the polymer.* The sulphation of the polymer was confirmed by a distinct infrared spectroscopy peak at 1280 cm-1 and by the binding of alcian blue to the modified material in the presence of 0.9 M MgCl2 compared to <0.3 M for the unmodified material. Immunocytochemistry showed plasma fibronectin, cellular fibronectin, fibronectin-EDA and collagen VI all bound to SI. Likewise, a mixture of cell adhesion factors absorbed onto the surface of the material, (including fibronectin and vitronectin) when it was incubated with serum.
Cell growth on coated SI. Neither mature chondrocytes nor BMSC adhered to unmodified Intrasite or to sulphated Intrasite that had been pre-treated with PBS alone. Both cell types bound to the surface of the cell adhesion factor coated SI particles within 3 - 24 hours. The cells proliferated on adhesion factor coated SI, (ovine chondrocytes increased in numbers 14.4 fold over 16 days when grown on FCS-coated SI), and agglomerated the particles to form macroscopic aggregates of cells, biomaterial and extracellular matrix components. Microscopic examination of chondrocytes growing on FCS coated sulphated Intrasite showed rounded, MTT+ve cells distributed on and between the polymer particles and lying within a picrosirius red positive matrix. Immunocytochemistry showed that mature chondrocytes secreted types II and VI collagens but not type I collagen during a 27 day incubation period. In contrast, BMSC produced a matrix that contained type I but not type II collagen.
Adhesion of colony forming cells to coated SI. Microscopic examination of the diluted suspensions of cells and particles after the initial 3 hour incubation revealed a sparse distribution of particles, (some with adherent cells), together with unattached cells. Addition of FCS to these cultures for 16-24 hours allowed the cells that had not attached to the biomaterial particles to adhere to the tissue culture plastic. The subsequent washing step removed the particles and any cells that had adhered to them, leaving only those cells that had adhered to the culture plastic. Results showed that there were 46.8±3.76 colonies per well when SZC were incubated with PBS pre-treated sulphated Intrasite compared to 21.0±1.39 colonies per well when the SZC were exposed to plasma fibronectin coated sulphated Intrasite, (i.e. a colony depletion score of 0.45; $p < 0.001$). Likewise BMSC incubated with adhesion factor coated sulphated Intrasite gave colony depletion scores of plasma fibronectin, 0.49; collagen IV, 0.67; cellular fibronectin, 0.8; polylysine, 0.88 and laminin 1.26.

Discussion. These results show that colony forming cells, (i.e. putative progenitor cells), from the superficial zone of articular cartilage or bone marrow stroma adhered to plasma fibronectin coated sulphated Intrasite particles. BMSC grown upon the fibronectin coated SI to formed aggregates of cells, extracellular matrix and biomaterial. Chondrocytes attached to SI coated with serum-derived attachment factors, proliferated upon the material but retained their rounded morphology and secreted a matrix containing collagens II and VI but not collagen I, indicating that they had retained their differentiated phenotype. Assembly of these two types of cell-seeded particulates will allow the creation of a tissue engineered cartilage construct with a zonal architecture in which a layer of progenitor cells overlays a layer of more mature chondrocytic cells.

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THE IDENTIFICATION AND CHARACTERISATION OF ARTICULAR CARTILAGE PROGENITOR CELLS

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Introduction: There are two major problems which afflict current strategies in cartilage repair. One problem is tissue integration between host and reparative tissue. The second problem is the generation of a repair tissue with the structural characteristics of articular cartilage. Using the marsupial *Monodelphis domestica* as a model system, it has been shown that articular cartilage grows by apposition from the articular surface towards the subchondral bone and that this growth is driven by the proliferation of surface zone cells (1, 2), Additionally, a population of cells with an increased cell cycle time was identified within the surface zone; a property typical of many progenitor cell populations (2). The aim of our research is to identify and characterise a chondroprogenitor population from articular cartilage to enable the rapid culture of undifferentiated chondrocytes in vitro for future clinical use. Here we describe the isolation and partial characterisation of a cell population from the articular surface which exhibits differential adhesion to fibronectin, differential integrin expression and the ability to form large numbers of colonies from an initialy small seeding density; properties that are common to known progenitor cell populations of other tissues. Additionally we report on the presence of the cell surface signalling molecule Notch 1 (N1) in a subpopulation of surface zone chondrocytes and that this N1-expressing subpopulation has an enhanced ability to form large numbers of colonies from an initially low seeding density.

Materials and Methods: *Tissue culture and differential adhesion assay:* Cartilage slices were isolated from the surface (SZ), middle (MZ) and deep (DZ) zones of 7 day old bovine metacarpal-phalangeal joints by fine dissection. Slices were then incubated in pronase (0.1% in DMEM/5%FCS) for 3 hours at 37°C followed by collagenase (0.04% in DMEM/5%FCS) for 16 hours at 37°C. Chondrocytes were counted and seeded onto fibronectin (10μg ml^{-1})-coated or PBS/1% BSA-coated 35 mm dishes at 4,000 cells ml^{-1} in serum free DMEM (DMEM-) for 20 minutes. After 20 minutes, media and non-adherent cells were removed and placed in similarly treated dishes for a further 40 minutes before this media and nonadherent cells were placed in a third dish. After removal of media at 20 and 40 minutes, fresh DMEM- was added to the remaining cells which were cultured for up to 10 days. In all experiments 6 fibronectin and 6 uncoated dishes were used for each zone of cartilage. Fibronectin was used as a ligand in the experiments since it is known to be differentially expressed at the articular surface during mammalian development (3). Within three hours of plating, chondrocyte adhesion was assayed by counting the total number of cells per dish using phase contrast microscopy and expressed as a percentage of the initial seeding density. Additionally, colonies of chondrocytes consisting of 32 or more cells were counted at 0, 3, 6 and 10 days after differential adhesion (n = 6 experiments). Colony forming efficiency (CFE) was calculated by dividing the number of colonies by the initial number of adherent cells. In some experiments (n = 3) the number of cells per colony were counted to determine the average number of cells per colony. Results were analysed using the Students *t* test. *Flow cytometry*: Four hours after differential adhesion, chondrocytes were removed from dishes non-enzymatically and 2 x 10^5 cells were incubated for 3 hours with antibodies to α5 (AB1928) and β1 (MAB1951) integrin subunits and anti-N1 (SC 6014) at room temperature. Cells were centrifuged at 3,000rpm, supernatants removed and cells washed three times in PBS with centrifugation between each wash. Cells were then incubated with relevant FITC conjugated secondary antibodies for 1 hour at room temperature and washed three times in PBS as described above. Finally, labelled cells were resuspended in 500μl PBS and subjected to flow cytometry. *Notch 1 Immunolabelling and Immunomagnetic Isolation:* Frozen sections of 7 day bovine full depth articular cartilage were immunolabelled with anti-N1 antibody and localised with the appropriate secondary FITC conjugated secondary antibody. Chondrocytes were isolated by sequenti pronase/collagenase digestion from surface middle and deep zone articul cartilage and incubated with M450 tosyl-activated Dynal beads conjugated t goat anti-human N1 antibody for 4 hours at 4°C. N1 selected cells wer counted and 4,000 cells ml^{-1} subjected to differential adhesion to fibronecti for 20 minutes. Initial adhesion and CFE were assessed as described above. In all experiments, results were analysed using Students *t* test.

Results: Initial adhesion ranged between 3.5% and 14.5% of the original ce number. Significant differences in adhesion were evident between surfac zone chondrocytes seeded on fibronectin for 20 minutes (9.05% +/- 0.44) an those seeded on PBS coated dishes for 20 minutes (3.83% +/- 0.27; $p < 0.001$ and also with those seeded on fibronectin for 40 minutes (4.89% +/- 0.43; $p <$ 0.001). Middle zone chondrocytes were significantly more adherent at 2(minutes (14.53% +/- 0.86) than at 40 minutes (10.58% +/- 0.51) when seede onto fibronectin-coated dishes ($p < 0.01$). Additionally, middle zon chondrocytes were more adhesive to fibronectin at both time points compare with PBS-coated dishes ($p < 0.001$). No differences in adhesion wer observed between deep zone chondrocytes regardless of substrate or tim point ($p > 0.05$ in all cases).

At days 0 and 3, no colonies containing 32 or more cells were present in any sample. At 6 and 10 days, the CFE of surface zone chondrocytes initially cultured on fibronectin for 20 minutes was greater than that of the othe samples ($p < 0.01$ at 6 days, $p < 0.001$ at 10 days). In addition, the CFE of surface zone cells initially cultured for 20 minutes on fibronectin was greate at 10 days compared with that at 6 days ($p < 0.05$). No change in CFE was evident between 6 and 10 days for any other sample ($p > 0.05$ in all cases). The average number of cells per colony was greater in surface zone cells initially grown on fibronectin for 20 minutes at both 6 ($p < 0.05$) and 10 ($p <$ 0.01) days compared with all other samples. FACS analysis showed elevated levels of both α5 and β1 integrin subunits in surface zone cells compared with middle and deep zone cells ($p > 0.05$).

N1 immunolabelling revealed occasional N1 positive cells within uppermost 2-3 cell layers of the articular cartilage. When surface zone cells were isolated and analysed for N1 using FACS over 84% of the surface zone population were N1 positive and this result was reflected in the cell counts obtained after N1 selection. Adhesion assays performed using N1 selected chondrocytes revealed that the N1 positive cells were more adherent than either negative cells or unselecetd cells ($p > 0.05$) and that the CFE of N1 selected cells was increased 4 fold relative to negative cells and unselected cells ($p > 0.001$).

Discussion: The ability of a population of cells to form large numbers of chondrocyte colonies from a low seeding density, differences in α5β1 integrin subunit expression and differentail N1 expression when taken together with previous results demonstrating the prolonged cell cycle time at the articular surface (2), strongly suggest that a subpopulation of progenitor chondrocytes resides in the articular surface. Additionally, the prolonged adhesiveness of mid zone cells, their restricted ability to form large numbers of colonies and their relatively short cell cycle (2) strongly indicates the presence of transit amplifying cells within this zone. Furthermore, the use of N1 selection increases the CFE of surface zone cells seeded on fibronectin fourfoldrelative to unselected cells suggesting that N1 will be a useful marker in the further purification of chondroprogenitor cells. The eventual isolation and purification of such a progenitor population will prove to be vital in advancing staetegies for cartilage repair.

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**Smith and Nephew, York, England.

CHONDROGENIC ABILITY OF ARTICULAR CARTILAGE PROGENITOR CELLS DURING EXTENSIVE SUB-CULTURE

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Introduction. The use of chondrocytes in cartilage repair strategies is hampered by the limited number of cell divisions a mature chondrocyte will undergo *in vitro*. This limited growth potential in culture is further complicated by the rapid loss of phenotype, known as dedifferentiation, of chondrocytes in monolayer culture characterised by a loss of spherical morphology and concomitant decrease in collagen II and aggrecan synthesis to a fibroblastic-like morphology with upregulation of collagen I and versican [1]. Although the dedifferentiation of mature chondrocytes can be reversed by transferring cells into an environment supporting a spherical morphology eg. pellet culture, alginate or 3D scaffolds, their redifferentiation is rarely complete. The identification of a cell with extensive growth potential and which retains its chondrogenic ability after extensive growth in culture would be a major step forward in addressing these issues. Our research has identified a population of chondroprogenitor cells in the superficial zone of articular cartilage with an extended cell cycle time, a high affinity for fibronectin in cell adhesion assays and an ability to form colonies *in vitro* from a low seeding density [2]. Here we report on the isolation and extensive subculture of these cells together with an evaluation of their chondrogenic ability at various passages by transfer into pellet culture.

Methods. *Isolation of chondroprogenitor cells:* Cartilage portions were isolated from the surface zone articular cartilage of 2-3 week old bovine metatarsophalangeal joints by fine dissection and sequential digestion in pronase (0.1% w/v; 3 hours) and collagenase (0.04% w/v; overnight) in DMEM containing 5% FCS at 37°C. After isolation, 4000 superficial zone cells in serum free DMEM were seeded into wells of fibronectin coated (10μg/ml) 6-well plates and incubated at 37°C for 20 minutes. After 20 minutes, the media was removed and discarded. Fresh DMEM containing 10% FCS was added to each well. *Expansion in Culture:* When cells in the 6-well plate were approaching confluency (P0), cells were trypsinised and transferred to a 75cm^2 culture flask (P1) and then into a 175cm^2 culture flask (P2) when near-confluency was reached. Subsequent growth in 175cm^2 culture flasks was carried out by continual passage at a ratio of 1:3. At P0, P2, P5, P8, P11 and P22 aliquots of cells were removed for pellet culture. Detailed accounts of cell numbers harvested and seeded at each stage were kept throughout the process to allow the number of population doublings at each stage to be calculated. Controls for this experiment consisted of normal chondrocytes isolated from the full thickness of bovine articular cartilage placed into pellet culture immediately after isolation and also following expansion in culture to P8 (approx 13 population doublings over 34 days) to allow dedifferentiation. Quantitative real-time PCR (Taqman) for collagen I, II and aggrecan mRNA was carried out on these control monolayer cultures of normal bovine chondrocytes in order to confirm dedifferentiation had taken place. *Pellet Culture*: Aliquots of 250,000 cells were resuspended in chondrogenesis media (see below), gently centrifuged in 15ml polypropylene tubes and incubated at 37°C in a 5% CO_2 atmosphere for 14 days with media changes carried out every 2-3 days. *Chondrogenesis media*: DMEM supplemented with pen/strep; ITS premix; ascorbate 2-phosphate (100μM); dexamethasone (10^{-7}M); and TGFβ-1 (10ng/ml). *Histology and immunolabelling:* Pellets were fixed overnight in 10% neutral buffered formalin and embedded in paraffin wax. Sections of 5μm thickness were cut and stained with Safranin O/haematoxylin. For immunolabelling, sections were labelled with antibodies to collagen I and II and visualised using appropriate secondary FITC conjugated antibodies.

Results. *Cellular Morphology:* Cultures of bovine chondroprogenitor cells at P0, P2 and P16 consisted mainly of cells of a polygonal, flattened morphology. This morphology was generally maintained, although at high passage (P25), a proportion of chondroprogenitor cells began to adopt large, often binucleate, irregular morphologies. This is in contrast to cultures of normal bovine chondrocytes which consisted of predominantly spindle-shaped fibroblastic cells up to P8 (34 days). Taqman confirmed dedifferentiation of control full thickness bovine chondrocytes had taken place in that by 14 days in monolayer culture they were expressing negligible levels of collagen II and aggrecan mRNA and high levels of collagen I mRNA. *Growth:* Chondroprogenitor cells underwent approximately 58 population doublings over 134 days in culture. At P0 the cells had a doubling time of approximately 24 hours where after the cells maintained a fairly constant rate of growth with cells requiring subculture every 3-4 days. This was maintained until P28 when the rate of growth declined with subculture being required every 7-8 days. *Pellet Culture:* Chondroprogenitor cells expanded in culture up to P22 and subsequently grown in pellets synthesised a cartilage–like matrix that stained strongly with Safranin O, indicating the presence of sulphated proteoglycans (figure 1). The periphery of the pellets at all time points stained weakly with Safranin O but was positive for collagen I. P0, P2, P5, P8 and P11 (P11 = 25 population doublings) chondroprogenitor pellets were rich in collagen II whereas pellets derived from P22 (42 population doublings) chondroprogenitor cells appeared to contain low amounts of collagen II. Pellet cultures of freshly isolated normal chondrocytes appeared to be smaller in volume and histologically have less matrix and rounder cells than in chondroprogenitor pellets (figure 2). The matrix stained strongly for collagen II. In comparison, pellet culture of dedifferentiated normal chondrocytes at P8 (13 population doublings) synthesised a greater amount of matrix but this stained less strongly for safranin O and contained negligible collagen II.

Figure 1: Pellet culture of culture expanded chondroprogenitor cells. Safranin O staining. Collagen II immunostaining inset. Bar = 200μm.

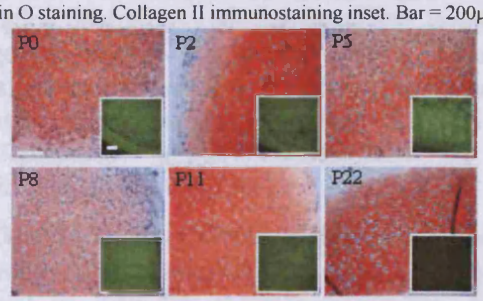
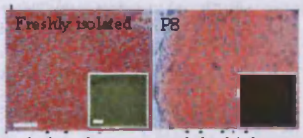


Figure 2: Pellet culture of freshly isolated and also culture expanded normal chondrocytes. Safranin O staining. Collagen II immunostaining inset. Bar = 200μm.



Discussion. This study has demonstrated the high expansion potential of articular cartilage progenitor cells in comparison to normal bovine chondrocytes. The cells retain the ability to synthesise a cartilage-like hyaline matrix rich in collagen II even after 11 passages which equated to 25 population doublings. Some collagen II was also evident at P22 (42 population doublings). In this study, normal chondrocytes isolated from bovine articular cartilage rapidly dedifferentiated in monolayer and had completely lost the ability to redifferentiate in pellet culture at P8 (13 population doublings) although it remains to be seen whether this loss of redifferentiation potential had occurred at an earlier stage of expansion. The enhanced potential of these articular cartilage progenitor cells to retain the ability to form cartilage after extensive expansion in culture is a major step forward for cartilage repair as it may enable the generation of large cell banks for use in allogeneic tissue engineering applications. Investigations are currently focusing on employing these cells in cartilage repair strategies.

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ARTICULAR CARTILAGE PROGENITOR CELLS: CHONDROGENIC POTENTIAL DURING EXPANSION IN MONOLAYER CULTURE

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INTRODUCTION: Current strategies in cartilage repair are based on the transplantation of cells from a variety of sources into the defect in question in order to generate a functional repair tissue. Difficulties arise in the use of chondrocytes due to a limited mitotic ability of mature chondrocytes *in vitro* and rapid loss of phenotype, known as dedifferentiation, in monolayer culture [1]. Previous research has identified a population of articular cartilage progenitor cells in the superficial zone of articular cartilage with an extended cell cycle time, a high affinity for fibronectin in cell adhesion assays, ability to form colonies *in vitro* from a low seeding density [2]. Here we report on the isolation and extensive subculture of these cells together with an evaluation of their chondrogenic ability at various passages by transfer into pellet culture.

METHODS: *Cell isolation:* Superficial zone cells were isolated by sequential digestion in pronase and collagenase of superficial zone cartilage from the articular cartilage of 2-3 week old bovine metatarsophalangeal joints. After isolation, 4000 superficial zone cells in serum free DMEM were seeded into wells of fibronectin coated 6-well plates and incubated at 37°C for 20 minutes. After 20 minutes, the media was removed and discarded. Fresh DMEM containing 10% FCS was added to each well. *Expansion in Culture:* Cells were subsequently transferred into 75cm^2 and 175cm^2 culture flasks. Growth was maintained by continual passaging at a ratio of 1:3. At various passages aliquots of cells were removed for pellet culture and Real-time PCR (Taqman). Controls consisted of normal chondrocytes isolated from the full thickness of bovine articular cartilage. *Pellet Culture*: Aliquots of 250,000 cells were resuspended in a serum free chondrogenic media containing TGFβ-1 and centrifuged to form a pellet. Pellets were incubated for 14 days and then paraffin embedded and sections stained with Safranin O/haematoxylin and immunolabelled with antibodies to collagen I and II. Taqman was used to quantify expression of collagen I, II, aggrecan and versican mRNA in the pellets.

RESULTS: *Growth:* Articular cartilage progenitor cells underwent approximately 61 population doublings over 162 days in culture. At P0 the cells had a doubling time of approximately 24 hours where after the cells maintained a moderately constant rate of growth with cells requiring subculture every 3-4 days. This was maintained until P28 when the rate of growth declined with subculture being required every 7-8 days. After P35 cell growth had virtually stopped. *Pellet Culture:* Articular cartilage progenitor cells expanded up to P22 and grown in pelleted micromasses synthesised a hyaline-like cartilage matrix that stained strongly with Safranin O. P1, P3, P6, P9 and P12 (25 pop doublings) pellets were rich in collagen II protein and mRNA whereas pellets derived from P22 (42 population doublings) cells contained low levels of collagen II. Pellet cultures of freshly isolated normal chondrocytes also stained strongly with Safranin O and contained abundant collagen II. Dedifferentiated normal chondrocytes at P8 contained negligible collagen II.

DISCUSSION & CONCLUSIONS: Although this study has illustrated the high expansion potential of articular cartilage progenitor cells, more importantly, the cells retain the ability to synthesise a cartilage-like hyaline matrix rich in collagen II even after 12 passages (25 pop doublings). In contrast, normal bovine articular chondrocytes rapidly dedifferentiated in monolayer and completely lost the ability to redifferentiate in pellet culture at P8 (13 pop. doublings). This important property to retain the ability to form cartilage after extensive expansion in culture may enable the generation of large cell banks for use in allogeneic tissue engineering applications. Investigations are currently focusing on developing human articular cartilage progenitor cells isolation and expansion protocols and employing these cells in cartilage repair strategies.

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The surface of articular cartilage contains a progenitor cell population

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Summary

It is becoming increasingly apparent that articular cartilage growth is achieved by apposition from the articular surface. For such a mechanism to occur, a population of stem/progenitor cells must reside within the articular cartilage to provide transit amplifying progeny for growth. Here, we report on the isolation of an articular cartilage progenitor cell from the surface zone of articular cartilage using differential adhesion to fibronectin. This population of cells exhibits high affinity for fibronectin, possesses a high colony-forming efficiency and expresses the cell fate selector gene Notch 1. Inhibition of Notch signalling abolishes colony forming ability whilst activated Notch rescues this inhibition. The progenitor population also exhibits phenotypic plasticity in its differentiation pathway in an embryonic chick tracking system, such that chondroprogenitors can engraft into a variety of connective tissue types including bone, tendon and perimysium. The identification of a chondrocyte subpopulation with progenitor-like characteristics will allow for advances in our understanding of both cartilage growth and maintenance as well as provide novel solutions to articular cartilage repair.

Key words: Cartilage, Progenitor cell, Notch

Introduction

Articular cartilage is an avascular, aneural tissue with a high matrix to cell volume ratio. The matrix comprises mainly type II collagen fibres and the high molecular weight aggregating proteoglycan aggrecan. The tissue is not, however, homogeneous with biochemical and morphological variations existing from the surface zone to the deeper calcified layer. The surface zone of the tissue is characterised by flattened, discoid cells that secrete surface zone proteoglycan (proteoglycan 4) (Schumacher et al., 1994). The mid zone of the tissue comprises rounded cells arranged in perpendicular columns and in addition to type II collagen and aggrecan, expresses cartilage intermediate layer protein (CILP) (Lorenzo et al., 1998). The deep zone and calcified zone chondrocytes express type X collagen and alkaline phosphatase (Schmid and Linsenmayer, 1985), and in the deep zone the chondrocytes are considerably larger than in the other zones.

Clearly, the differentiation and proliferation events occurring during the development of articular cartilage must, therefore, be strictly controlled both temporally and spatially in order for the distinct zonal architecture of the tissue to be established. Various studies have shown that the surface zone of articular cartilage is centrally involved in the regulation of tissue development and growth. Not only does the surface of articular cartilage play a major role in the morphogenesis of the diarthrodial joint via differential matrix synthesis (Ward et al., 1999), but the expression of many growth factors and their receptors at the articular surface (Archer et al., 1994; Hayes et al., 2001) suggest that this region represents an important signalling centre. In addition, it has been shown in vivo that the surface zone of articular cartilage is responsible for the appositional growth of articular cartilage and from these studies we hypothesised that the surface zone of articular cartilage contains a progenitor/stem cell population that allows for the appositional growth of the tissue (Hayes et al., 2001). Identification of such cells holds exciting possibilities in the field of cartilage tissue engineering because the tissue has limited inherent reparative capacity after trauma (Hunziker, 1999). Here, we describe the isolation and partial characterisation of a specific articular cartilage progenitor cell using a previously described differential adhesion assay (Jones and Watt, 1993).

Materials and Methods

Cell isolation, differential adhesion assay and tissue culture

Petri dishes (35 mm) were coated with 10 μg ml^{-1} bovine fibronectin (FN; Sigma, UK) in 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (PBS+) overnight at 4°C. Dishes were blocked with 1% bovine serum albumin (BSA) in PBS+

before chondrocytes were added. Control dishes were treated with PBS+ containing 1% BSA overnight at 4°C.

Previous studies have utilised differential adhesion to fibronectin in vitro to identify epidermal stem cells (Jones and Watt, 1993). Fibronectin is expressed in developing mammalian articular cartilage in addition to the classic fibronectin receptor integrin subunits α5 and β1 (Hynes, 1992). We therefore utilised fibronectin in an in vitro adhesion assay to identify and partially characterise articular cartilage progenitor cells. Chondrocytes were isolated from the surface, middle and deep zones of articular cartilage of 7-day-old calves by sequential pronase/collagenase digestion as previously described (Archer et al., 1990). After isolation, chondrocytes (4000 ml^{-1}) were seeded onto 35 mm plastic Petri dishes at 37°C for 20 minutes in 1:1 DMEM/F12 containing 0.1% Gentamycin (DMEM/F12–). After 20 minutes, media (and non-adherent cells) was removed and placed in a second dish for 40 minutes at 37°C before this media (and non-adherent cells) was removed and placed in a third dish. After removal of media at 20 and 40 minutes, fresh 1:1 DMEM/F12 containing 0.1% Gentamycin, 0.5 μg ml^{-1} ascorbate, 1 μg ml^{-1} glucose and 10% foetal calf serum (FCS; DMEM/F12+) was added to the remaining adherent cells which were maintained in culture for up to 10 days. In all experiments, six fibronectin-coated dishes and six untreated dishes were used for each time point and for each zone of cartilage. Controls comprised cells subjected to differential adhesion on dishes coated with 1% BSA in PBS+.

For Notch 1 selection, magnetic tosyl-activated Dynal Beads (Dynal, UK) were coated with polyclonal anti-Notch 1 antibody (5 μg ml^{-1}; Santa Cruz, CA) following the manufacturer's instructions. Freshly isolated chondrocytes from the surface, middle and deep zone were incubated with antibody-coated beads for 30 minutes at 4°C and separated from Notch-negative cells using a powerful magnet. Non-magnetic cells were aspirated and Notch-positive cells washed three times in PBS before isolated cells were resuspended in DMEM/F12 and the purified cells counted using a haemocytometer. Notch 1-positive cells were then subjected to differential adhesion on fibronectin for 20 minutes (4000 cells ml^{-1} in 35 mm dishes as described above), and initial adhesion and colony forming efficiency assayed up to 10 days as described below.

Within 3 hours of plating, initial chondrocyte adhesion was assayed by counting the total number of cells adhering to the bottom of the dish using an inverted microscope equipped with phase contrast optics and expressed as a percentage of the initial seeding density. Colonies (defined as consisting of more than 32 chondrocytes) were counted using the same microscope at 3, 6 and 10 days. Thirty-two cells were chosen as this represents a population of cells derived from more than 5 population doublings of a single cell, thereby discounting a transit amplifying cell (Jones and Watt 1993). Colony forming efficiency (CFE) was calculated by dividing the number of colonies by the initial number of adherent cells. In some experiments (n=3), the number of cells per colony was counted to determine the average number of cells per colony. Results were analysed using the Student's t-test. However, for comparative purposes, we also analysed the data in terms of colonies comprising more than 4 cells.

For γ-secretase inhibition studies, cells were isolated and subjected to differential adhesion to fibronectin as described above. Cells were maintained in media containing 50 nM N-[N-(3,5-diflurophenylacetate)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT) (Dovey et al., 2001) in 0.1% DMSO for 7 days with media changes every 48 hours. Initial adhesion and CFE were assayed as described.

For explant cultures, full-depth cartilage chips were removed and bisected. One half of each explant was cultured in the presence of 50 nM DAPT in DMEM/F12+ and 0.1% DMSO for 7 days, whereas the other half of the explant was cultured in DMEM/F12+ and 0.1% DMSO. Media was changed every 48 hours and samples fixed in 10% NBFS, wax embedded and stained with toluidine blue. Sections were examined using brightfield optics and digital images obtained. A calibrated grid was then used to count the number of cells 0-100 μm and 101-200 μm from the articular surface. Results were analysed using the Student's t-test.

In separate experiments, explants were excised and cut in half. Half of each explant was maintained in 50 nM DAPT and the other half was maintained in control media as described above for 7 days. On days 4, 5 and 6, the thymidine analogue bromodeoxyuridine (BrdU; final concentration 50 mM) was added to control and experimental media in order to identify s-phase chondrocytes. Explants were removed after 24, 48 and 72 hours' incubation in BrdU, fixed in 10% formalin and wax embedded. Dewaxed sections were then immunolabelled with monoclonal anti-BrdU (5 μg ml^{-1} in PBS) and localised using goat anti-mouse fluorescein-conjugated secondary antibody.

Immunocytochemistry

Chondrocytes were labelled with antibodies raised against α5 and β1 integrin subunits after sequential pronase/collagenase digestion and at various time points after differential adhesion. Briefly, chondrocytes (2×10^5 cells ml^{-1}) were fixed in 95% ice-cold ethanol for 10 minutes and washed in PBS. The cells were incubated with primary antibodies diluted in PBS (2 μg ml^{-1}) for 1 hour at room temperature, washed three times in PBS and incubated with appropriate FITC-conjugated secondary antibodies (2 μg ml^{-1}) diluted in 20% heat inactivated foetal calf serum in PBS. Cells were washed in PBS and mounted in Vectashield containing 1.0 mg ml^{-1} propidium iodide. Cells were then observed and photographed using a fluorescent microscope. To determine integrin, FN-extra domain A (EDA) and Notch 1 expression in vivo, full-depth articular cartilage was excised from 7-day-old bovine metacarpal-phalangeal joints and chilled by precipitate immersion in n-hexane at –80°C. Cryostat sections (10 μm) were cut on a Bright's cryostat and collected on APES (3-aminopropyltriethoxysilane)-coated slides and stored at –20°C. Sections were defrosted, post-fixed in ice-cold acetone for 5 minutes, washed in PBS and incubated with primary antibody diluted in PBS/0.01% Tween 20 (PBST) for 1 hour at room temperature. After washing in PBST, sections were incubated with relevant FITC-conjugated antibodies diluted in 20% heat-inactivated FCS in PBST for 1 hour before washing three times with PBST and mounting in Vectashield. Labelled sections were examined and photographed using either a Zeiss or an Olympus photomicroscope fitted with epifluorescent optics.

Flow cytometry

To assess integrin expression before differential adhesion, freshly isolated chondrocytes were incubated in fresh DMEM/F12– at 37°C on a roller prior to labelling for FACS analysis. After differential adhesion, chondrocytes were removed from dishes non-enzymatically (Sigma) and labelled for FACS analysis. All samples were counted and 2×10^5 cells were incubated for 3 hours with antibodies to α5 and β1 integrin subunits and Notch 1 in sextuplicate at room temperature. Cells were centrifuged at 500 ***g***, supernatants removed and cells washed three times in PBS with centrifugation between each wash. Cells were then incubated with relevant FITC-conjugated secondary antibodies for 1 hour at room temperature and washed three times in PBS as described above. Finally, labelled cells were re-suspended in 200 μl PBS and subjected to single channel FACS analysis.

Notch intracellular domain (NICD) transfection

Activated Notch constructs (Notch ICv) were obtained from Raphael Kopan (University of Washington) (Schroeter et al., 1998). Surface zone chondrocytes were subjected to differential adhesion to fibronectin and grown in the presence of 50 nM DAPT for 3 days. Excess (1 μg) plasmid DNA was transiently transfected into surface zone chondrocytes 3 days after differential adhesion using Effectene

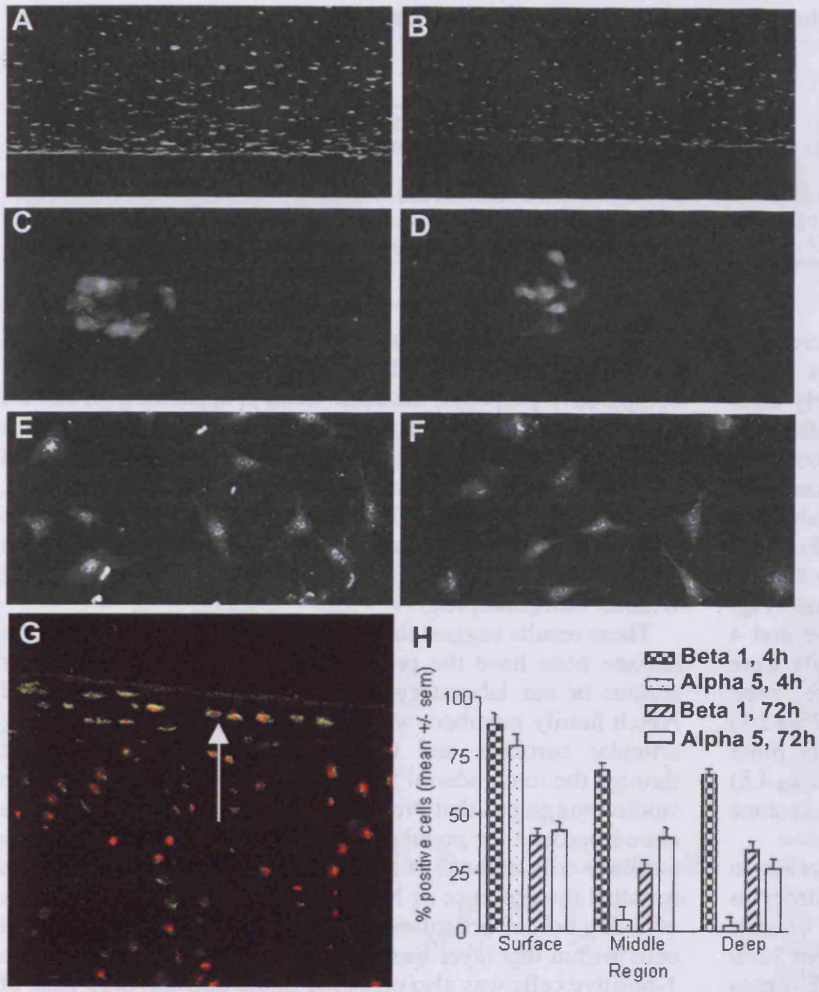

Fig. 1. Frozen sections (A,B) and isolated chondrocytes (C-F) from 7-day bovine articular cartilage immunolabelled for α5 (A,C,E) and β1 (B,D,F) integrin subunits. α5 and β1 integrin subunits are localised throughout the depth of the articular cartilage (A,B) although not every chondrocyte is labelled. Isolated chondrocytes from surface zone immediately after sequential pronase/collagenase isolation labelled with antibody to alpha α5 (C) and β1 (D) subunits. Labelling for α5 (E) and β1 (F) is also present 72 hours after differential adhesion to fibronectin. Fibronectin-EDA was localised in frozen tissue sections to the surface 2-3 cell layers (G). Integrin α5 and β1 subunit expression was assessed by flow cytometry after sequential pronase/collagenase digestion (4 hours) and 72 hours after differential adhesion assay to fibronectin (H). At 4 hours and 72 hours, there was no difference in integrin subunit expression between surface zone chondrocytes ($P>0.05$), although during this time period the overall expression of α5 and β1 subunits was significantly decreased ($P<0.01$). At 4 hours, middle zone chondrocytes had a higher expression of β5 subunits relative to β1 subunits ($P<0.01$), although there was no difference in expression after 72 hours ($P>0.05$).

reagent (Qiagen), and colonies consisting of more than 32 cells were counted 10 days after differential adhesion and colony forming efficiency calculated.

Culture and transient transfection of 293GP packaging cells

293GP cells expressing the *gag* and *pol* proteins (Burns et al., 1993) were cultured to 70-80% confluency in DMEM/F12 containing 0.5 mg ml^{-1} Gentamycin and 10% FCS. Cells were then transfected with 1 μg plasmid DNA encoding VSV-G and *lacZ* using the Quiagen Effectene kit following the manufacturer's instructions. Briefly, plasmid DNA (1 μg VSV-G and 1 μg *lacZ*) was resuspended in 148 μl condensation buffer and vortexed, 16 μl of enhancer reagent was added, mixed and incubated at room temperature for 5 minutes. Following incubation, 50 μl of Effectene reagent was added to the solution, mixed and after 10 minutes' incubation at room temperature, 1 ml of DMEM/F12+ was added. The solution was mixed and the suspension added drop-wise to 293GP cells. Transfected cells were cultured for 3 days in DMEM/F12+ and viral supernatants collected after 3 days and frozen at –80°C.

Chondroprogenitor cell isolation and infection

Surface and deep zone chondrocytes were isolated from 7-day-old bovine articular cartilage using sequential pronase/collagenase digestion and 5×10^6 cells subjected to differential adhesion to FN (10 μg ml^{-1} in PBS+) in 60 mm dishes for 20 minutes. Non-adherent cells were aspirated and chondrocytes were cultured for up to 5 days prior to infection. Chondrocytes were infected with pseudotyped retrovirus-conditioned media ($5\text{-}6\times10^6$ CFU ml^{-1}) containing 10 μg ml^{-1} polybrene for 24 hours prior to injection. Media was removed and cells washed in DMEM containing no additives, trypsinised, centrifuged and resuspended at 1×10^5 cells 10 μl^{-1}.

In ovo injections and tissue processing

After harvesting, 10 μl aliquots of cell suspension containing 1×10^5 cells (both surface and deep zone derived) were immediately injected into the proximal or distal wing bud of 3-day-old (Stage 12-14) (Hamburger and Hamilton, 1951) chick embryos which had been previously windowed. Eggs were resealed with adhesive tape and re-incubated for various times up to day 10 (Stage 36-37). Embryos were killed by cervical dislocation, a note of their developmental stage taken and embryos washed in 0.1 M PBS (pH 7.4). After washing, embryos were fixed in 2.5% paraformaldehyde in 0.1 M PBS (pH 7.4) for 1 hour at room temperature followed by 3×20 minute washes in 0.1 M PBS containing 2 mM $MgCl_2$, 0.01% deoxycholic acid and 0.02% igepal [(Octylphenoxy)polyethoxyethanol, pH 7.4]. Embryos were then reacted at 37°C overnight for *lacZ* in 0.1 M PBS containing 2 mM $MgCl_2$, 0.01% deoxycholic acid, 0.02% igepal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM spermidine trihydrochloride and 1 mg ml^{-1} X-gal previously solubilised in DMSO. Embryos were washed extensively in 0.1 M PBS, post-fixed in 10% NBFS overnight and wax embedded. Serial wax sections (8-10 μm) were taken onto 3-aminopropyl triethoxy silane-coated slides air dried overnight, dewaxed, stained with 1% eosin for 15 seconds and examined under bright field microscopy after coverslipping. In separate experiments, immunocytochemistry using antibody to bacterial gene product was performed as a control against endogenous β-galactosidase activity and also to co-localise bovine-specific type I collagen within engrafted tissues.

Results

Initially, we examined integrin and fibronectin expression in 7-day-old bovine articular cartilage using immunocytochemistry and flow cytometry (Fig. 1). Both α5 and β1 integrin subunits were expressed in the majority of chondrocytes at the surface

Table 1. Initial adhesion of chondrocytes to fibronectin

	Treatment					
Adhesion time	SFN	SPBS	MFN	MPBS	DFN	DPBS
20 minutes	9.05%±0.44*,‡	3.83%±0.27	14.53%±0.86†,§,¶	3.94%±0.19	3.59%±0.22	3.68%±0.23
40 minutes	4.89%±0.43	4.12%±0.34	10.85%±0.51††	4.2%±0.19	3.95%±0.30	4.21%±0.36

Initial adhesion to fibronectin and PBS-coated dishes. Chondrocytes were plated as described in the Materials and Methods and assayed for cell adhesion at 20 and 40 minutes. D, deep; FN, fibronectin-coated dishes; M, middle; PBS, PBS-coated dishes; S, surface. *$P<0.001$ compared with 40 minutes; †$P<0.01$ compared with 40 minutes; ‡$P<0.001$ compared with PBS control; §$P<0.01$ compared with surface FN 20; ¶$P<0.001$ compared with deep FN 20.

of the tissue with decreasing label intensity and decreasing numbers of chondrocytes labelled in the deeper zones of the cartilage (Fig. 1A,B). Using chondrocytes immediately after isolation and at various times after differential adhesion, surface, middle and deep zone chondrocytes were shown to express α5 and β1 subunits at all time points analysed regardless of substrate although differences in labelling intensity and the number of labelled cells were noted (Fig. 1C-F and data not shown). FN-EDA was localised pericellularly within the surface 2-3 cell layers of the articular cartilage (Fig. 1G). Using flow cytometry immediately after isolation and 4 hours after differential adhesion (Fig. 1H), β1 subunits were shown to be preferentially expressed by surface zone chondrocytes (88%±4.8) compared with middle (67%±2.1) and deep (62%±3.7) zone chondrocytes. Substantially more cells in the surface zone expressed α5 subunits (79%±4.8) compared with middle (5%±2.1) and deep (2.5%±1.7) zone chondrocytes.

Next, we assessed the degree of chondrocyte adhesion to fibronectin (Table 1). Surface and middle zone chondrocytes were more adherent to fibronectin than the other cohorts examined at 20 minutes. Initial adhesion ranged between 3.5% and 14.5% of the original cell number. Significant differences in adhesion were evident between surface zone chondrocytes plated on fibronectin for 20 minutes and those plated on fibronectin for 40 minutes ($P<0.001$) and cells cultured on BSA-coated dishes for 20 minutes ($P<0.001$). Middle zone chondrocytes were significantly more adhesive at 20 minutes than at 40 minutes when plated on fibronectin-coated dishes ($P<0.001$). In addition, middle zone chondrocytes were more adhesive to fibronectin at both time points compared with BSA-coated dishes ($P<0.001$). No differences in adhesion were observed between deep zone chondrocytes regardless of substrate or time point ($P>0.05$ in all cases).

If these cells with a high affinity for fibronectin are a population of chondroprogenitor cells then they should have the ability to form large numbers of colonies from an initially low seeding density, as is the case in other tissues with a clearly defined stem cell population (Jones and Watt 1993). To determine the clonality of the adhesive chondrocytes, we counted the number and size of colonies of chondrocytes subjected to differential adhesion to fibronectin (Fig. 2A). Differences in the initial adhesion of surface zone cells were reflected in CFE at 6 and 10 days that was not matched by the CFE of middle zone cells (Fig. 2B). The CFE of surface zone chondrocytes initially cultured on fibronectin for 20 minutes was greater than that of all other samples ($P<0.01$ at 6 days and $P<0.001$ at 10 days) when we applied the definition of a colony as being more than 32 cells. Indeed, using the criteria of 32 cells as indicative of a colony, no colonies were present in any other cohort besides surface zone cells initially plated on fibronectin. Using 4 cells as being indicative of a colony for comparative purposes, the same trend is apparent with surface zone cells subjected to differential adhesion to fibronectin for 20 minutes having a significantly enhanced CFE at both 6 and 10 days relative to all other cohorts (Fig. 2C,D). In addition, the average number of cells per colony was greater in surface zone cells initially grown on fibronectin for 20 minutes at both 6 (Fig. 2E) ($P<0.05$) and 10 (Fig. 2F) ($P<0.01$) days compared with all other samples.

These results suggest that a subpopulation of cells within the surface zone have the properties of a progenitor cell. Other studies in our laboratory using BALBc mice had identified Notch family members within the surface zone of developing articular cartilage and that Delta was widely distributed through the remainder of the tissue (Hayes et al., 2003). These studies suggested that Notch 1 was a suitable marker for the chondroprogenitor population and immunolabelling of bovine cartilage with a panel of antibodies to Notch family members revealed the presence of Notch 1 in the surface 2-3 cell layers of 7-day bovine articular cartilage (Fig. 3A), although not all cells within this layer were labelled. Another cohort of Notch 1-positive cells was also observed in the mid and deep zone of the tissue. Using flow cytometry, we showed that 86% of the surface zone cells isolated by pronase/collagenase digestion were Notch 1-positive compared with 10% and 34% from the middle and deep zone, respectively (Fig. 3B). Using Dynal Bead capture of Notch 1-positive surface zone chondrocytes, we showed that a Notch 1-enriched population of chondrocytes had both an increased adhesion to fibronectin ($P<0.01$) (Fig. 3C) and an increased CFE ($P<0.05$) (Fig. 3D) relative to unselected cells. These data suggest that the Delta/Notch signalling pathway may have a major influence in controlling both chondrocyte colony forming efficiency and differentiation.

To determine the role of Notch signalling in the colony forming ability of surface zone chondrocytes we cultured surface zone cells in the presence of a γ-secretase inhibitor, DAPT (Dovey et al., 2001), which is known to bind to the active site of presenillin in the γ-secretase complex and not interfere with β-catenin-mediated signalling (Kornilova et al., 2003). The γ-secretases are responsible for the cleavage of the amyloid precursor protein during the progression of Alzheimer's disease and are also responsible for the intramembranous cleavage of Notch receptors (Berezovska et al., 2000). Because of their role in Alzheimer's disease progression, much interest has focused on the development of γ-secretase inhibitors (Dovey et al., 2001), which not only prevent the accumulation of amyloid plaques but also prevent Notch family signalling (Berezovska et al., 2001). Treatment

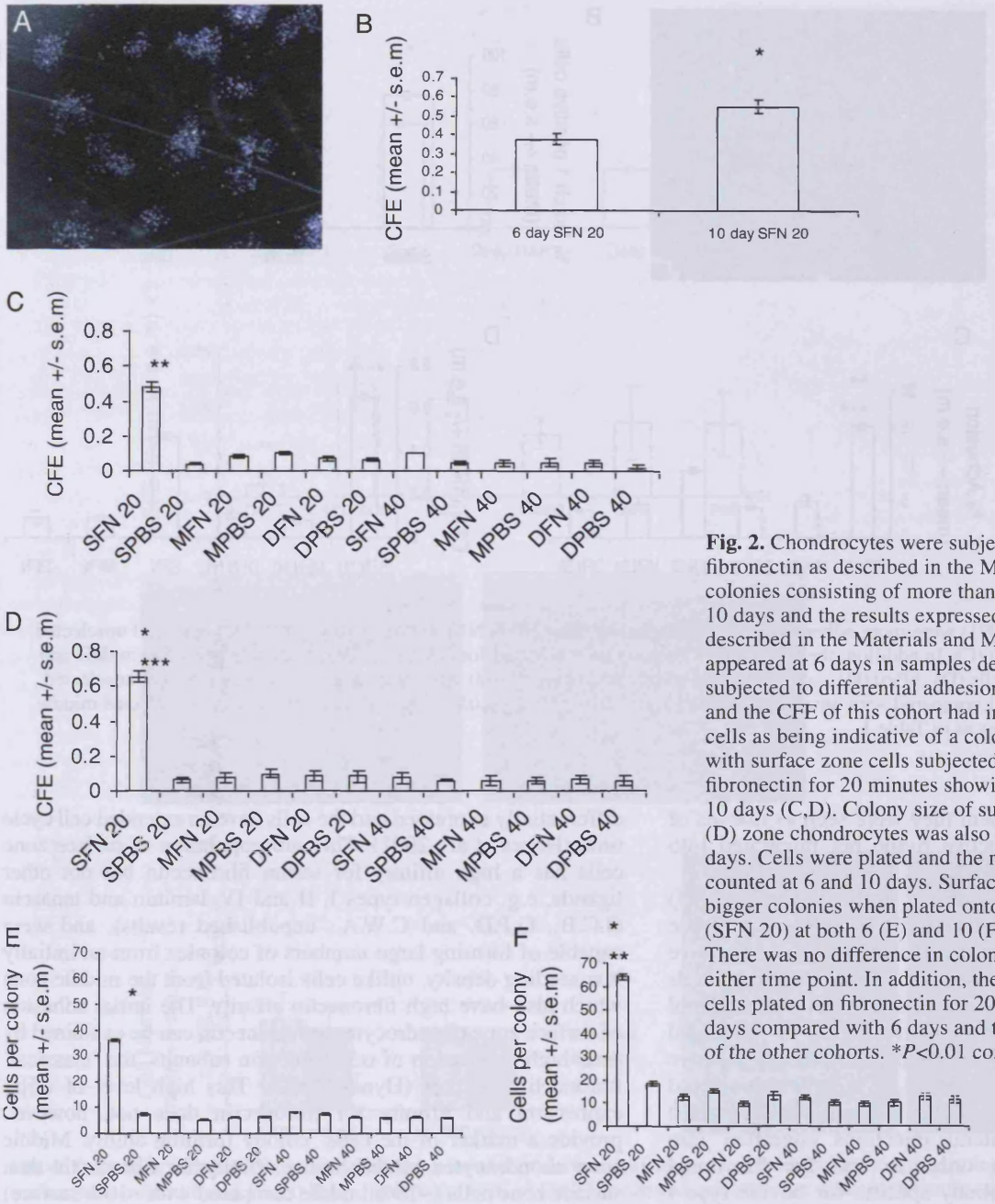

Fig. 2. Chondrocytes were subjected to differential adhesion to fibronectin as described in the Materials and Methods, and colonies consisting of more than 32 cells were counted at 6 and 10 days and the results expressed as colony forming efficiency as described in the Materials and Methods. (A,B) Colonies initially appeared at 6 days in samples derived from the surface zone subjected to differential adhesion to fibronectin for 20 minutes and the CFE of this cohort had increased by 10 days. Using 4 cells as being indicative of a colony the same trend was apparent, with surface zone cells subjected to differential adhesion to fibronectin for 20 minutes showing enhanced CFE at both 6 and 10 days (C,D). Colony size of surface (S), middle (M) and deep (D) zone chondrocytes was also assessed at 6 (E) and 10 (F) days. Cells were plated and the number of cells per colony was counted at 6 and 10 days. Surface zone chondrocytes formed bigger colonies when plated onto fibronectin for 20 minutes (SFN 20) at both 6 (E) and 10 (F) days than any other sample. There was no difference in colony size within any other cohort at either time point. In addition, the colony size of surface zone cells plated on fibronectin for 20 minutes was increased at 10 days compared with 6 days and there was no increase within any of the other cohorts. *P<0.01 compared with 6 days; **P<0.01 compared with all other cohorts at the same time point; ***P<0.001 compared with all other cohorts at the same time point. Abbreviations as in Table 1.

with DAPT did not affect the initial adhesion of chondrocytes to fibronectin (Fig. 4A), but abolished clonality at both 6 and 10 days compared with controls when the 32 cell definition was applied (Fig. 4B) such that CFE was equal to that of deep zone chondrocytes. NICD was able to rescue colony abolition when added to DAPT-treated cultures after 3 days (P<0.05) (Fig. 4C) but NICD transfection did not increase colony forming efficiency compared with controls (P>0.05) (Fig. 4C). Culture of cartilage explants in the presence of 50 nM DAPT for 7 days produced a region of hypocellular, weakly stained matrix immediately beneath the surface zone (Fig. 4D,E). The region 101-200 μm from the articular surface contained fewer cells in DAPT-treated samples compared with controls (P<0.05) (Fig. 4D-F), whereas there was no difference in cell number 0-100 μm from the articular surface (P>0.05) (Fig. 4D-F). In addition, it was shown that incubation of explants in 50 nM DAPT prevented cell proliferation as there was no evidence of BrdU incorporation in any of the treated samples examined (n=24) (Fig. 4G,H).

In order to assess the differentiation potential of the progenitor population, we infected a lineage label into the cells and injected them into the proximal limb of stage 22 chick embryos and tracked them for 1 week in ovo. Twenty-four hours after injection, β-galactosidase-positive cells were present in positions corresponding to the original injection site (Fig. 5A,B). Examination of embryos injected with labelled deep zone cells gave variable results. Labelled cells were either absent suggesting that the cells could not survive in the chick

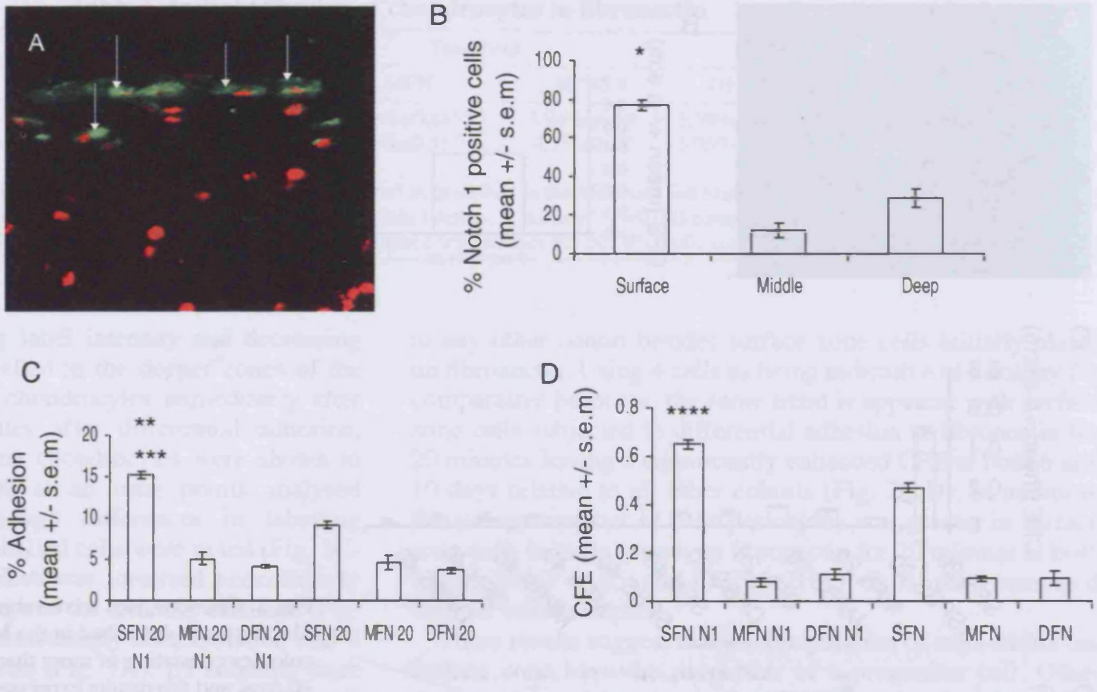

Fig. 3. Seven-day bovine articular cartilage labelled with antibody to Notch 1 (A) and counterstained with propidium iodide. Chondrocytes within the uppermost 2-3 cell layers of the surface zone (arrows) label strongly for Notch 1. Chondrocytes were labelled with anti-notch 1 antibody and subjected to single-channel FACS analysis immediately after isolation (B). 86% of surface zone cells label positively for N1 compared with 10% and 34% from middle and deep zone, respectively. (*P<0.001 compared with middle and deep.) Chondrocytes were selected immunomagnetically and subjected to differential adhesion and initial adhesion and CFE assessed. Notch 1-selected surface zone cells (SFN N1) were more adherent than N1-selected middle (MFN N1) and deep zone (DFN N1) cells and unselected cells (SFN 20, MFN 20, DFN 20) (C). In addition, the CFE of surface zone cells selected for N1 was greater than notch-positive middle and deep zone cells and unselected cells (D). *P<0.001 compared with middle and deep, **P<0.001 compared with N1 selected and unselected middle and deep cells, ***P<0.01 compared with unselected surface zone cells, ****P<0.01 compared with selected and unselected middle and deep zone cells. Abbreviations as in Table 1.

embryos, or if cells were present they were seen as masses of labelled cells in loose connective tissue not integrated into surrounding host tissue (Fig. 5K).

Examination of embryos incubated to 10 days (Stage 36) revealed β-galactosidase-positive cells in numerous tissue types, including cartilage, bone, tendon and muscle connective tissues (Fig. 5C-F). The sites of these positive cells corresponded with the sites of injection such that proximal injections gave β-galactosidase-positive cells in proximal tissues and distal injections revealed β-galactosidase-positive cells in distal structures. Furthermore, if cells were injected into the central proximal region of the limb bud, cells engrafted into the humerus. More lateral injections engrafted into tendons and perimysim. In order to test for functional engraftment, we used an antibody specific for bovine type I collagen. We found that in tendon, parallel arrays of fibrillar collagen ran along the tendon length (Fig. 5H) contrasting with dense immunofluorescence in the subperiosteal bone (Fig. 5J). In addition, both the perichondrium and articular fibrocartilage (Fig. 5G) and the perimysium (Fig. 5H) labelled with anti-bovine type I collagen antibody.

Discussion

Using differential adhesion to serum fibronectin, we have described the isolation and partial characterisation of a subpopulation of articular cartilage chondrocytes with properties akin to those of a progenitor cell and that are able to engraft into a variety of tissue types, albeit of the connective tissue lineage. These cells reside within the surface zone of articular cartilage, where the EDA isoform of fibronectin is differentially expressed and the cells have an extended cell cycle time (Hayes et al., 2001). This sub-population of surface zone cells has a high affinity for serum fibronectin but not other ligands, e.g. collagen types I, II and IV, laminin and tenascin (J.C.B., G.P.D. and C.W.A., unpublished results), and were capable of forming large numbers of colonies from an initially low seeding density, unlike cells isolated from the middle zone which also have high fibronectin affinity. The initial adhesion of surface zone chondrocytes to fibronectin can be explained by their high expression of α5β1 integrin subunits, the 'classical' fibronectin receptor (Hynes, 1992). This high level of α5β1 expression and affinity for fibronectin does not, however, provide a marker of the cells' colony forming ability. Middle zone chondrocytes exhibit higher affinity for fibronectin than surface zone cells (~15% middle compared with ~10% surface) (Table 1), but lack the ability to form colonies (Fig. 2) and may represent a transit amplifying population. In addition, the percentage of cells that possess a high colony forming efficiency within the surface zone (approximately 1-2% of the initial number adhered) is only a fraction of the number of cells expressing α5β1 subunits (approximately 75%), for this reason we could not use α5β1 integrin expression as a chondroprogenitor marker.

Previous studies have documented the expression of Notch family members during articular cartilage and growth plate development (Hayes et al., 2003; Crowe et al., 1999). Of particular interest was the specific expression of Notch 1 at the developing articular surface of mouse knee joints (Hayes et al., 2003). This specific expression in the surface zone of articular cartilage suggested that Notch 1 may provide a marker for colony forming cells in the bovine model used in the present

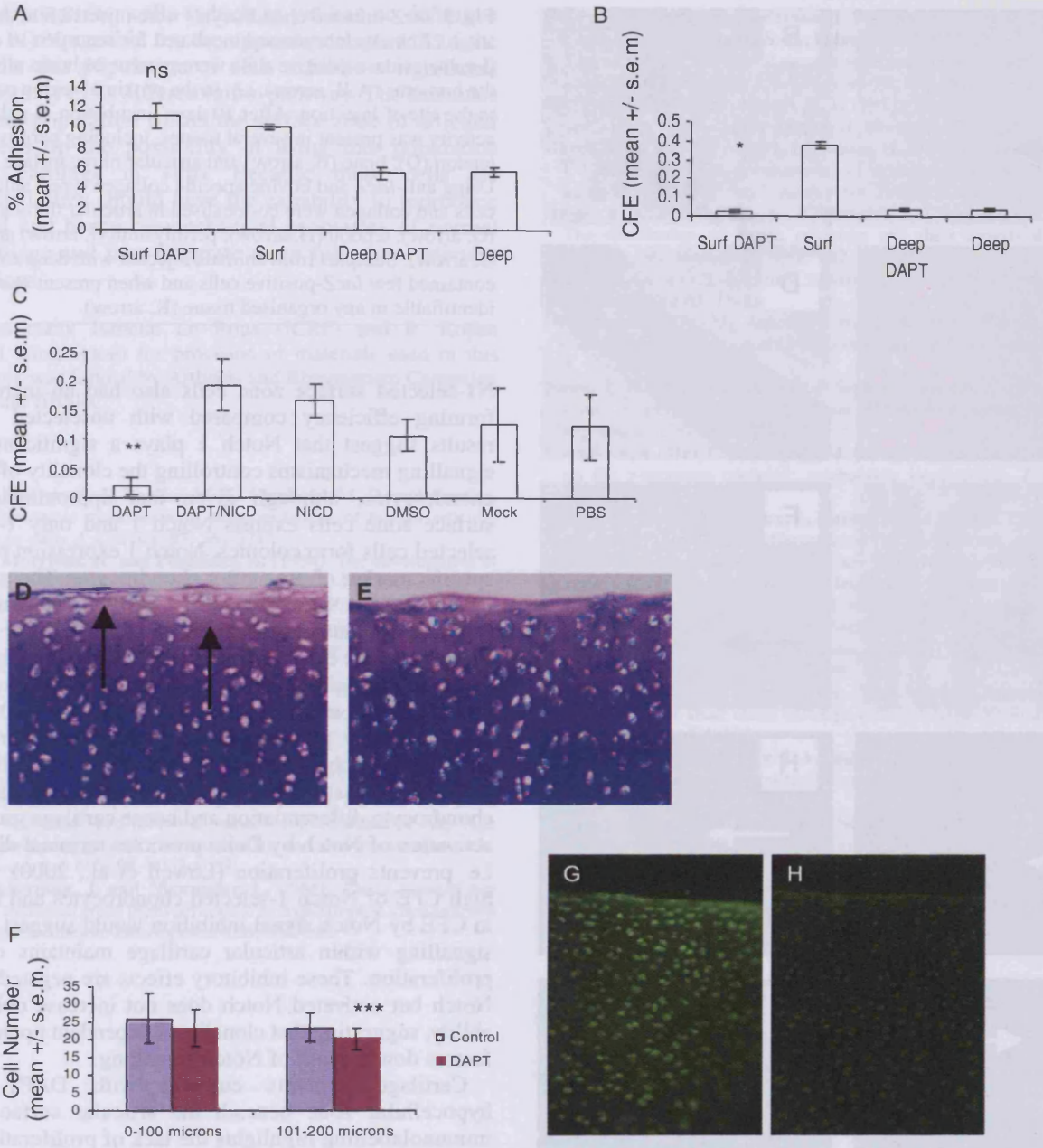

Fig. 4. Treatment with DAPT did not affect the adhesion of surface and deep zone chondrocytes to fibronectin (A) but abolished the CFE of surface zone cells at 6 and 10 (B) days. Indeed, the CFE of DAPT-incubated cells was not different from that of deep zone cells at either time point. Transfection with NICD rescued this abolition of CFE (C). NICD transfection did not increase CFE in cells not treated with DAPT ($P>0.05$). Cartilage explants were removed from 7-day bovine articular cartilage and cultured in the presence (D) or absence (E) of 50 nM DAPT for 7 days as described in Materials and Methods. Note that in the presence of DAPT, an acellular weakly stained band is present beneath the surface zone (arrows). These images represent a selection from 3 separate experiments each containing 6 explants per treatment. Note that the image in D is the other half of the explant from that shown in E. Using a graduated grid, the number of cells 0-100 and 101-200 μm from the articular surface was counted and the region 101-200 μm from the articular surface was shown to contain fewer cells in treated samples relative to controls (F). Explants were treated with DAPT for 7 days with the addition of BrdU on days 4, 5 and 6. Localisation of BrdU in controls (G) reveals cell proliferation, whereas there was no BrdU localisation in DAPT-treated samples (H). ns, $P>0.05$ compared with surf control; *$P<0.01$ compared with DAPT treated; **$P<0.05$ compared with all other cohorts, DMSO; 0.1%, dimethyl sulfoxide, Mock; no plasmid, PBS; fibronectin only.

study. Indeed, Notch 1 expression in immature bovine articular cartilage matches that in developing mouse articular cartilage, such that in both species, Notch 1 is present in the chondrocytes of the surface zone articular cartilage to a depth of 2-3 cells (see Fig. 3) (Hayes et al., 2003). Flow cytometry of freshly isolated chondrocytes revealed that Notch 1 expression was significantly increased in surface zone chondrocytes and these high levels of Notch 1 expression were maintained in surface zone cells after differential adhesion, relative to middle and deep zone chondrocytes. Using magnetic immunoselection, Notch 1-

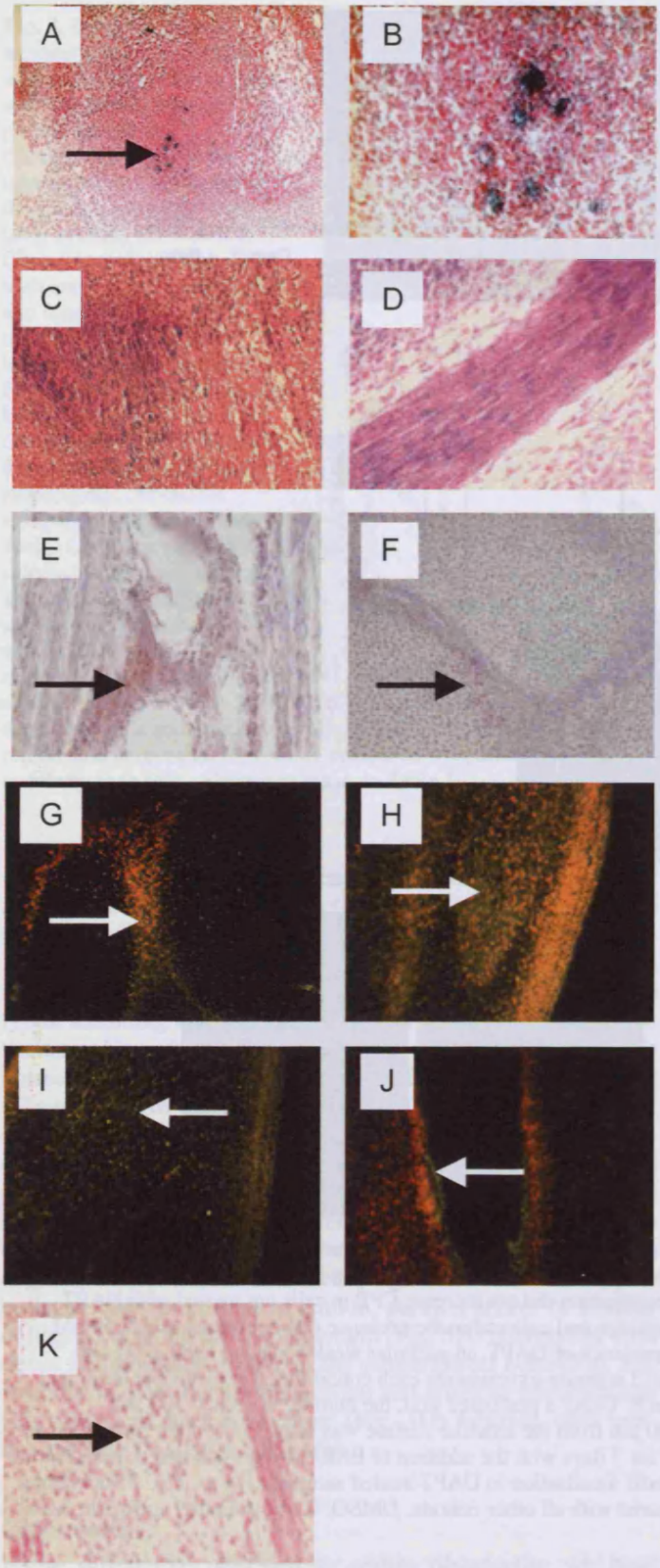

Fig. 5. *lacZ*-infected chondrocytes were injected into the wing bud of stage 22 chick embryos and incubated for stage 36 (10 days). β-galactosidase-positive cells were present 24 hours after injection in the humerus (A,B, arrow), i.e. in the proximal region corresponding to the site of injection. After 10 days' incubation, β-galactosidase activity was present in several tissues, including perimysium (C), tendon (D), bone (E, arrow) and articular fibrocartilage (F, arrow). Using anti-*lacZ* and bovine-specific collagen type I antibody, bovine cells and collagen were co-localised in articular fibrocartilage (G, arrow), tendon (H, arrow), perimysium (I, arrow) and bone (J, arrow). Samples from animals injected with deep zone cells contained few *lacZ*-positive cells and when present were not identifiable in any organised tissue (K, arrow).

positive cells were isolated from bovine articular cartilage and subjected to differential adhesion. Surface zone cells thus isolated had a higher affinity for fibronectin than N1-selected middle and deep zone cells and unselected cells (Fig. 3). These N1-selected surface zone cells also had an increased colony forming efficiency compared with unselected cells. These results suggest that Notch 1 plays a significant role in the signalling mechanisms controlling the clonality of surface zone chondrocytes, although given that approximately 75% of surface zone cells express Notch 1 and only 1-2% of these selected cells form colonies, Notch 1 expression per se is not a specific marker of progenitor chondrocytes. The precise role of Notch in the promotion of clonality or maintenance of progenitor status remains unclear, although our own studies have shown the expression of several Notch ligands (Jagged and Delta) in articular cartilage, although their expression is not specific to the articular surface (Hayes et al., 2003). Notch 1 signalling may play one of two roles in the surface zone of articular cartilage; it may function to maintain cells in a proliferative state, i.e. maintain clonality, or it may promote chondrocyte differentiation and hence cartilage growth. In skin, activation of Notch by Delta promotes terminal differentiation, i.e. prevents proliferation (Lowell et al., 2000), however the high CFE of Notch 1-selected chondrocytes and the reduction in CFE by Notch signal inhibition would suggest that Notch 1 signalling within articular cartilage maintains clonality and proliferation. These inhibitory effects are negated by activated Notch but activated Notch does not increase colony forming ability, suggesting that clonality is dependent upon rate limiting factors downstream of Notch signalling.

Cartilage explants cultured with DAPT contain a hypocellular zone beneath the articular surface and BrdU immunolabelling highlights the lack of proliferation in DAPT-treated samples. These results would indicate that Notch inhibition via DAPT inactivation of presenillin prevents chondroprogenitor proliferation, thus depleting the number of daughter cells capable of differentiating and contributing to articular cartilage growth.

At present, we cannot sate which member of the Notch family controls chondrocyte proliferation/differentiation, but the results of the immunolabelling for Notch 1 in both bovine and mouse (Hayes et al., 2003) and the enhanced clonality of Notch 1-selected chondrocytes suggests that this family member is central to cartilage growth and differentiation.

The engraftment of bovine surface zone-derived cells and their tissue-specific matrix synthesis in ovo highlights the plasticity of this cell population. This plasticity further supports our argument that these cells represent a progenitor population as plasticity is a key marker of a stem cell population (Morrison et al., 1997).

We conclude that immature articular cartilage contains a

population of progenitor cells (which as yet has no definitive marker) that is responsible for the appositional growth of the tissue and that this population of cells exhibits a significant degree of plasticity in its differentiation pathway. The existence of a progenitor population within the surface zone of articular cartilage opens up the possibility of using this population to engineer cartilage in vitro. Because these cells are undifferentiated, they should have the capability to reproduce the structural and hence biomechanical properties of normal articular cartilage and thus integrate more fully into articular cartilage lesions.

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