

ANALYSIS OF CELL SURFACE MARKERS WITHIN IMMATURE BOVINE ARTICULAR CARTILAGE

A thesis submitted to Cardiff University
for the degree of Doctor of Philosophy.

by


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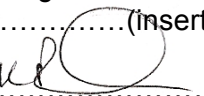
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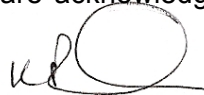
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Summary

Previous studies have shown that articular cartilage grows by apposition from the joint surface, driven by proliferation of a progenitor cell sub-population residing in the superficial zone. To date, there is no individual marker for this progenitor sub-population; however, markers located to mesenchymal stem cells have been identified in articular cartilage. Using immunofluorescence, this study demonstrated the localisation of the stem cell markers, CD44, CD49e, CD105 and CD166 to the superficial zone of bovine immature articular cartilage. CD29 and the developmental markers, Notch1, Delta1, Jagged1, Jagged2 and Msx1 were located in cells throughout the tissue. The restricted expression of the majority of these stem cell markers to predominantly the superficial zone in immature bovine complements the appositional growth model notion and greatly suggests a resident stem cell population.

To further investigate and quantify expression of these differentiation and stem cell markers, superficial zone cells were isolated, immunolabelled and analysed using flow cytometry. In addition, cells were cultured for 24 hours in monolayer for comparison and to enable epitope recovery. Stem cell marker expression was absent or reduced following cell isolation and upregulated following monolayer culture. Developmental markers displayed expression comparable to that seen in tissue following cell isolation, but expression was absent after monolayer culture. The differences observed suggest cell surface marker cleavage during cell isolation and subsequent cell adhesion and proliferation.

To assess the changes in expression, superficial zone cells were cultured for 14 days, to provide a proxy for dedifferentiated cells. Superficial zone chondrocytes were immunolabelled at day 3, 7 and 14 and analysed using flow cytometry. The majority of cell surface receptors exhibited a unimodal increase in expression indicative of a homogeneous population. The number of cells expressing CD44 increased with time in culture, from 3 to 14 days, characteristic of cells adhering to plastic. Bimodal distributions were observed with CD105 and CD117, after 14 days in culture. This expression has not previously been reported and demonstrates a distinct and discrete subset of cells equating to 1-2% of the total superficial zone population analysed, comparable to chondroprogenitor percentages previously reported.

The use of specific markers to isolate chondroprogenitors will allow for further characterisation, including a more in-depth understanding of the mechanisms of proliferation and differentiation within articular cartilage. This has the potential to lead to an improved understanding of the role of these markers and, as such, may provide us with a more beneficial cell type that could significantly contribute to the field of articular cartilage repair.

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Abbreviations

ACI	Autologous chondrocyte implantation
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
APC	Allophycocyanin
BMP	Bone morphogenetic proteins
BMSCs	Bone marrow stromal cells
BrDU	Bromodeoxyuridine
BSA	Bovine serum albumin
CD	Cluster of differentiation
CD29	β 1 integrin
CD44	Hyaluronan receptor
CD49e	α 5 integrin
CD90	Thy-1 (thymocyte differentiation antigen-1)
CD105	Endoglin
CD117	C-kit
CD166	ALCAM (activated cell adhesion molecule)
CILP	Cartilage intermediate layer protein
COMP	Cartilage oligomeric matrix protein
CS	Chondroitin sulphate
CSL	CBF1, Su (H), Lag-1
DAPI	4',6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium containing Hams F12
DSL	Delta/Serrate/LAG2
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ETOH	Ethanol
FACIT	Fibril-associated collagen interrupted triple helices
FACS	Fluorescent activated cell sorting
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FGF2	Fibroblast growth factor
FITC	Fluorescein iso-thiocyanate
FSC	Forward scatter
G1, G2 or G3	Globular domains
GAGs	Glycosaminoglycans
GDF	Growth and differentiation factor
GPI	Glycosylphosphatidylinositol
HA	Hyaluronan
HAS	Hyaluronan synthase
HGF	Hepatocyte growth factor or scatter factor
HSCs	Hematopoietic stem cells
IGD	Interglobular domain
IGF	Insulin-like growth factor
IgG	Immunoglobulin

KS	Keratin sulphate
MACI	Matrix-induced autologous chondrocyte implantation
MFI	Geometric median fluorescent intensity
MSCs	Mesenchymal stem cells
Msh	Muscle segment homoeobox-like gene
NICD	Notch intracellular domain
OA	Osteoarthritis
PBS	Phosphate buffered saline
PBS/T	PBS/Tween (polyethylenesorbitan monolaurate)
PCNA	Proliferating cell nuclear antigen
PFA	Paraformaldehyde
PG	Proteoglycans
PTHrP	Parathyroid hormone-related peptide
R-PE & PE	R-Phycoerythrin
RGD	Arginine-glycine-aspartate containing peptides
SCF	Stem cell factor
SLRPs	Small leucine-rich repeat proteoglycans
SSC	Side scatter
SZP	Surface zone protein
TGF- β	Transforming growth factor β
TS	Transverse section

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CHAPTER 1:
GENERAL INTRODUCTION

1.1 The synovial joint

There are several types of joints within the body: synarthrosis (fibrous), amphiarthrosis (cartilaginous), and diarthrosis (synovial). Synarthrosis joints are immovable and occur between cranial bones; amphiarthrosis joints permit limited movement and are present between vertebrae and at the pubic symphysis. Diarthrosis joints are freely moveable and possess a cavity that is lined with a synovial membrane. This type of joint is mainly located within the appendicular skeleton and is, otherwise, known as a synovial joint (Standring and Gray, 2008). The synovial joint consists of a variety of tissues that all contribute to the function of the joint. The ends of long bones are covered in articular cartilage that is nourished and lubricated by synovial fluid, which in turn, is produced by the synovial membrane; a fibrous capsule encapsulates the whole joint. Ligaments, tendons and muscles also surround and stabilise the joint (Figure 1.1).

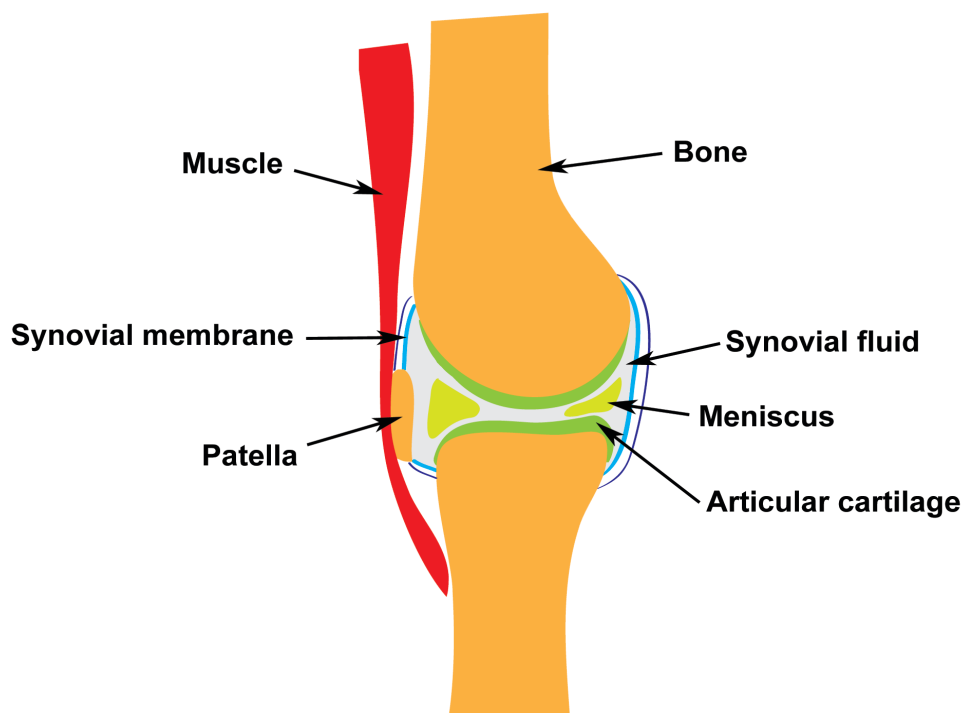


Figure 1.1: Diagrammatic presentation of a synovial joint

Articular cartilage covers the opposing surfaces of the skeletal elements, the joint space is filled with synovial fluid and, in the case of the knee, also contains fibrocartilaginous meniscus to aid stabilisation.

1.1.1 Development of the synovial joint

Synovial joints arise from cells of mesenchymal origin; the long bones that will abut the presumptive joint, are initially modelled with hyaline cartilage, that is later replaced by mineralised bone during endochondral ossification (Khan et al., 2007). Initially, the presumptive joint is a continuous structure and is first recognised histologically by a flattening of cells in the presumptive joint region, loss of type II collagen and acquisition of type I collagen (Craig et al., 1987, Hyde et al., 2007, Archer et al., 2003, Archer et al., 1994). Depending on species and joint location the presumptive joint varies, in the embryonic chick three regions are formed: two chondrogenous zones associated with the epiphyseal regions and a central region of high cell density known as the interzone (Craig et al., 1987). In mammals, the interzone consists of a thin layer of cells between the developing articular surfaces (Edwards et al., 1994, Archer et al., 2003).

As the interzone thickens, the intermediate layer is responsible for the formation of joint associated structures. The outer layer cells proliferate and differentiate into chondrocytes and go on to form the presumptive articular surfaces. These chondrocytes contribute to the initial lengthening of the elements by appositional growth at each of the epiphyses, this continues until the growth plates develop (Pacifici et al., 2006, Khan et al., 2007). Cavitation occurs through the differential synthesis and secretion of glycosaminoglycans (GAGs), in particular hyaluronan (HA). HA is synthesised throughout and prior to cavitation by cells of the chondrogenous layer in both chicken and human joints (Edwards et al., 1994, Archer et al., 1994, Craig et al., 1987, Bland and Ashhurst, 1996, Pitsillides, 1999). Cavitation is movement dependent, as paralysis of the embryo results in failure to cavitate or fusion of previously cavitated joints (Khan et al., 2007, Craig et al., 1990). It has also been shown that transforming growth factor β (TGF- β) peptide is widespread during chondrogenesis, but after differentiation there is very little present. Insulin-like growth factor (IGF) promotes chondrogenesis through stimulation of matrix secretion but again; the amount and location throughout the tissue vary with developmental stage. Fibroblast growth factor (FGF) promotes chondrocyte proliferation and inhibits terminal differentiation to hypertrophic cells (Hayes et al., 2001).

The shape of the joint appears to be regulated by bone morphogenic proteins (BMPs) and growth and differentiation factors (GDFs). The factors that stop the reticular chondrocytes from ossifying are not known. Barx-1 (homeobox family), Wnt-4 (Wnt family), GDF-5, hepatocyte growth factor (HGF; scatter factor) and parathyroid hormone-related peptide (PTHrP) may all be involved. Barx-1 and Wnt-4 are involved in initial development. GDF-5 has been seen in mouse development in all areas initially and later restricted to the interzone. HGF plays a role in early skeletal development during chondrocyte proliferation (Pacifici et al., 2000). FGF has also been shown to be involved in skeletal development (Tickle and Munsterberg, 2001). PTHrP and TGF- β are known to delay the maturation process. BMP 2-7 induce chondrocyte maturation both *in vitro* and *in vivo* (Beier et al., 2001, Broadus et al., 2007).

1.1.2 Development and maturation of articular cartilage

Articular chondrocytes develop from the chondrogenous layer of the interzone. Following cavitation the majority of growth in articular cartilage is achieved by deposition of matrix (Bland and Ashhurst, 1996), however, little is still known regarding the mechanisms or molecular basis of articular cartilage growth.

Foetal articular cartilage is isotropic in structure, as it matures it reduces in thickness and cell density and becomes more anisotropic in structure (Hayes et al., 2001, Archer et al., 2003, Hunziker et al., 2007). Early studies by Mankin (Mankin, 1962b, Mankin, 1963, Mankin, 1962a), using tritiated thymidine incorporation in immature rabbit knees identified two proliferative bands within immature articular cartilage; one below the articular surface and one above the subchondral bone, this demonstrated that articular cartilage grows by both appositional and interstitial growth. Further work, carried out by Archer et al using South American opossum (*Monodelphis domestica*) (Archer et al., 1994), established that IGF is extensively expressed in the epiphyses during early development. However, once the secondary centre of ossification has formed, the distribution of IGF is restricted to the surface of articular cartilage. Cell proliferation activity assessed by proliferating cell nuclear antigen (PCNA) was also found to have a similar distribution to IGF using the marsupial, becoming increasingly restricted to the surface layers of articular cartilage with

skeletal maturity, indicative of appositional growth. In a study by Hayes and colleagues (Hayes et al., 2001), bromodeoxyuridine (BrDU) was used to identify cycling cells in the immature South American opossum, BrDU is incorporated into replicating DNA during S-phase of the cell cycle. This study demonstrated that flattened cells of the superficial zone incorporated BrDU after 10 days, whereas, the transitional zone cells labelled after 4 days. The slow cycling time of the superficial zone cells is indicative of a chondroprogenitor or stem cell population; the shorter cycling time of the transitional zone cells is characteristic of a transit amplifying population.

The reorganisation of articular cartilage during maturation was recently investigated using the immature rabbit knee joint (Hunziker et al., 2007, Hunziker, 2009). One month after birth, the superficial zone cells were arranged perpendicular to the joint surface, but deeper layers were randomly orientated; at this stage collagen fibrils were also randomly arranged throughout the tissue. By two months, cells appeared more anisotropic and the cell density had decreased. At three months the cell arrangement was comparable to mature tissue, the depth of the tissue had decreased further and the arrangement of collagen fibrils was comparable to mature tissue. After three months, there was no significant decrease in tissue depth. During the first three months when most bone growth occurs, the bone length increase exceeds that of the decrease in cartilage depth, indicating that immature cartilage is not remodelled and, instead, must be resorbed and replaced by new tissue. Therefore, during early postnatal development articular cartilage acts as a surface growth plate for lateral and longitudinal expansion of the epiphyseal bone as well as an articulating layer; this appositional growth is accompanied by tissue resorption. Only after puberty do the superficial zone cells produce a mature anisotropic articular cartilage.

1.2 Structure of articular cartilage

There are three main types of adult cartilage: fibrocartilage, elastic cartilage and hyaline cartilage. Fibrocartilage is found between tendon and bone attachments. It consists of collagen fibres that form many thick bundles. Elastic cartilage differs from fibrocartilage and hyaline cartilage in that it comprises a matrix containing elastic fibres, it occurs in the external ear, eustachian tube and epiglottis. Hyaline cartilage is the most common; it occurs on the ventral surfaces of the ribs, the tracheal rings and at the ends of bones. It generally appears homogeneous consisting of collagen fibres embedded in a meshwork within the extracellular matrix (ECM; (Standring and Gray, 2008).

The transition from immature to mature cartilage structure occurs during adolescence, when closure of the growth plates occurs. Mature chondrocytes are usually found as single cells within lacunae surrounded by extensive ECM; the tissue lacks neuronal components and vasculature. Articular cartilage varies slightly in the mechanical properties and composition of the matrix among species, and among joints of the same individual (Hunziker, 1992, Athanasiou et al., 1991). The main functions of articular cartilage are: to protect the underlying subchondral bone, provide a low-friction gliding surface between the opposing skeletal elements and transmit load through to the underlying bone. Articular cartilage is divided into four zones that are chiefly denoted by their cellular morphology and orientation of collagen fibrils (Saamanen et al., 2010, Bhosale and Richardson, 2008).

1.3 Zones of articular cartilage

Articular cartilage consists of four layers: superficial, middle (transitional), deep (radial) and calcified zones (Figure 1.2; (Buckwalter and Mankin, 1997). The cells within these layers differ in morphology, orientation and cell density. The ECM in the specific layers differs in water, proteoglycans (PG) and collagen concentration, as well as aggregate sizes, density and metabolic activity.

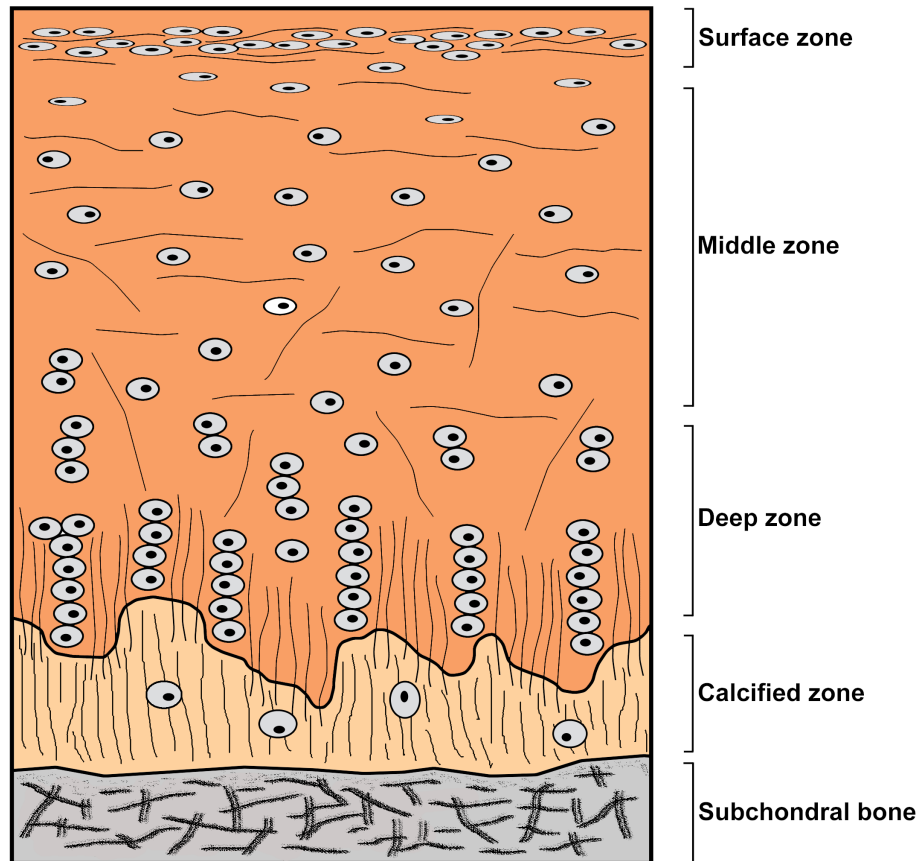


Figure 1.2: Diagram showing the zonal variation in articular cartilage

Articular cartilage is composed of four distinct zones: surface (superficial), middle, deep and calcified zones.

1.3.1 Superficial zone

The superficial, surface or tangential zone is adjacent to the joint cavity and consists of an acellular region, the lamina splendens and a deeper cellular layer (Jurvelin et al., 1996, Wu et al., 2008). The cells are flattened and ellipsoid in shape and aligned parallel to the surface. This zone is rich in collagen and the meshwork formed, gives cartilage its structure and tensile strength. This layer is relatively low in PGs compared to other regions. Quantities of fibronectin and water are highest in this zone and PGs and non-collagenous proteins bind to the meshwork.

1.3.2 Middle zone

The morphology and matrix composition of the middle or transitional zone is between that of the superficial and radial zone. The volume is usually twice that of the superficial zone. The cells in this zone are spheroidal in shape and equally spaced, they occur singly or in pairs (Hunziker, 2010). The matrix consists of larger diameter collagen fibrils that are randomly oriented, a higher PG concentration, but lower concentrations of water and collagen than the superficial layer (Buckwalter and Hunziker, 1999).

1.3.3 Deep zone

The radial or deep zone contains large spheroidal shaped cells that are aligned in columns perpendicular to the joint surface. In the upper and lower regions, they are rich in cytoplasmic organelles such as endoplasmic reticulum and Golgi apparatus. In the mid-section, they frequently contain large numbers of intermediate filaments. There is a gradual but marked, increase in cell size with depth, the cells occur singly in the upper portion, but with increasing depth usually occur in groups of three to four called chondrons (Hunziker, 1992, Hunziker, 2010). This layer contains the largest diameter collagen fibrils that are arranged perpendicular to the joint surface and penetrate the calcified cartilage beneath. The deep zone has the highest concentration of PGs and the lowest concentration of water (Buckwalter and Mankin, 1997). The alignment of these cells within the matrix is maintained by the collagen fibre organisation, termed Benninghof Arcades (Oegema and Thompson, 1995, Buckwalter and Hunziker, 1999).

1.3.4 Calcified zone

The calcified cartilage zone is adjacent to the subchondral bone. The calcified zone cells are smaller and fewer than those in the radial zone (Oegema and Thompson, 1995). The border between the calcified zone and subchondral bone is known as the tidemark, it represents a mineralisation front where the calcified zone and subchondral zone beneath, interdigitate and has a characteristically undulating appearance when viewed histologically. During postnatal growth, this front advances and the matrix

compartment becomes actively mineralised. It is generally believed that cell activity and mineralisation in this area is low or even completely absent, and that only after traumatic insults or during the early phases of osteoarthritis (OA), do chondrocytes near the tidemark become activated and induce mineralisation (Hunziker, 1992).

1.4 Extracellular matrix organisation

Articular cartilage can be further divided into compartments within each zone, reflecting differential distribution and densities of matrix constituents (Hunziker, 2010). The matrix comprises three regions: the pericellular, the territorial and the interterritorial region (Figure 1.3). The pericellular and territorial matrices bind the cell membranes and matrix macromolecules. These matrices protect the cells from damage during loading and deformation of the tissue. The principle function of the interterritorial matrix is to provide the mechanical properties of the tissue (Buckwalter and Hunziker, 1999).

1.4.1 Pericellular matrix

The pericellular matrix is attached to the cell membrane of chondrocytes and is approximately 1µm thick. A chondron is made up of a chondrocyte and its surrounding pericellular matrix, this matrix is free of fibrillar collagens, but rich in PGs (Hunziker, 1992) and non-fibrillar collagens including type VI collagen (Buckwalter and Mankin, 1997). Cytoplasmic projections extend from the chondrocytes into and through the pericellular matrix to the territorial matrix (Buckwalter and Hunziker, 1999).

1.4.2 Territorial matrix

The territorial matrix surrounds the pericellular matrix. It is characterised by a basket-like arrangement of collagen fibrils that surround individual chondrocytes and in deeper zones, surrounds chondrons (Figure 1.3; (Hunziker, 1992, Hunziker, 2010). The basket arrangement may provide mechanical protection for the chondrocytes during loading and deformation of the tissue. It is difficult to identify the boundary between the territorial and interterritorial matrices, although the fibril orientation

changes from the basket-like orientation to a more parallel arrangement (Buckwalter and Hunziker, 1999).

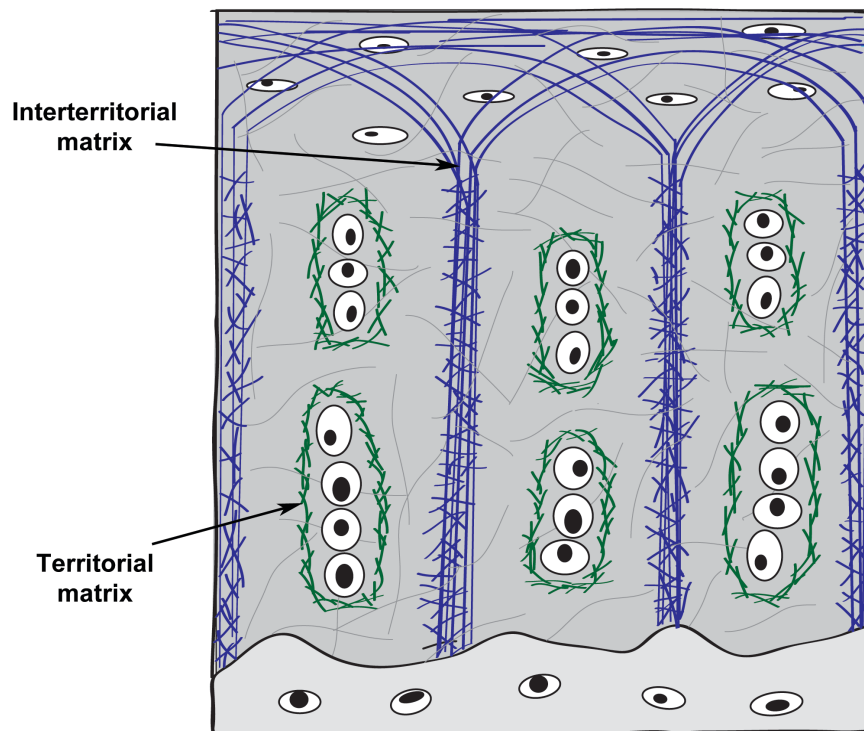


Figure 1.3: Schematic representation of adult articular cartilage

Diagram showing the organisation of the fibrillar components within the matrix of articular cartilage. The pericellular matrix (not shown in this diagram) surrounds individual chondrocytes forming chondrons. The territorial matrix contains a basket-like arrangement of collagen fibrils and organises chondrons into distinct morphological entities. The interterritorial matrix forms the bulk of the tissue (Buckwalter and Hunziker, 1999).

1.4.3 Interterritorial matrix

The interterritorial matrix makes up the majority of the volume of the extracellular space. It is characterised by cross-banded collagen fibrils or fibres running in parallel (Figure 1.3). The concentration of PGs in the interfibrillar space varies according to the zone (Hunziker, 1992). The collagen fibrils are the thickest of all three matrix regions, in the deep zone, the fibrils are large and lie perpendicular to the joint

surface. As the collagen fibrils approach the joint surface, they become more oblique until in the superficial zone, the fibrils are smaller in diameter and are orientated parallel to the joint surface (Buckwalter and Hunziker, 1999).

1.5 Composition of articular cartilage

Articular cartilage is aneural and avascular and derives its mechanical properties from its matrix. Articular cartilage consists of chondrocytes, matrix, water and matrix macromolecules. Cells make up approximately 1% of the constituents of articular cartilage in large animals, however, the percentage is higher in smaller animals (Stockwell, 1978). Mature chondrocytes depend primarily on anaerobic metabolism, as cartilage cells obtain their nutrients from synovial capillaries in the synovial membrane, which is transported in the synovial fluid to the cartilage matrix. The cartilage matrix is restrictive to the size and charge of molecules. In all layers, chondrocytes form a complex relationship with the ECM. Primary cilia may also have a role in sensing mechanical changes in the matrix (Buckwalter and Hunziker, 1999, Farnum and Wilsman, 2011).

1.5.1 The chondrocyte

Chondrocytes are the only known resident cell type within articular cartilage and are responsible for the integrity, organisation and maintenance of the ECM around them (Saamanen et al., 2010). Chondrocytes are approximately 10µm in diameter and are generally spheroidal in shape, with the exception of chondrocytes within the superficial zone that are more flattened. Mature chondrocytes are located in lacunae and their cell membranes adhere directly to the pericellular matrix, which contains PGs, non-collagenous proteins and glycoproteins (James and Uhl, 2001). Adult articular chondrocytes do not normally divide, but contribute to the maintenance and integrity of the cartilage through a combination of synthetic and catabolic activities (Cancedda et al., 2003).

1.5.2 *The cartilage collagens*

The strength of articular cartilage depends on the extensive cross-linking of collagens and the zonal arrangement. All collagen molecules are composed of three α chains characterised by a glycine-X-Y sequence, where X is commonly proline and Y hydroxyproline (Cremer et al., 1998). Several collagens are found within cartilage including types II, VI, IX, X and XI. Collagen types II and XI are classed as fibrillar collagens. They are closely associated and covalently bonded with collagen type IX and form the fibrillar meshwork characteristic of articular cartilage (Figure 1.4; (Eyre, 1995). Collagens found in articular cartilage are either: fibrillar (types II and XI) or fibril-associated (IX, XII and XIV; (Sandell, 1995).

Type II collagen is the most abundant protein in the ECM, comprising approximately 90-95% of the total and primarily provides cartilage with its tensile strength (Lin et al., 2006). It polymerises to form the major component of the large cross-banded filaments. It is composed of three identical α chains, which are synthesised as precursors to form a procollagen molecule that is released into the extracellular space, there are two alternative forms of type II procollagen, type IIA and type IIB; only the latter is produced in mature cartilaginous tissue (Sandell et al., 1991). The collagen fibrillar meshwork entraps aggregating PGs and provides a framework for the small PGs like decorin and fibromodulin to bind (Eyre et al., 1992).

Type IX collagen is an example of a fibril-associated collagen with interrupted triple helices (FACIT) and the heterotrimeric molecule consists of three different α chains (Figure 1.4B; $\alpha 1$ [IX], $\alpha 2$ [IX] and $\alpha 3$ [IX]). It accounts for at least 10% of the collagen in foetal cartilage, but only around 1% of the collagenous protein in adults (Eyre et al., 1992, Sandell, 1995, Gelse et al., 2003). Collagen type IX is sometimes also classed as a PG and is closely associated with type II and type XI collagens in articular cartilage.

Type XI collagen is thought to lie within type II fibrils (Mendler et al., 1989) and may control fibrillogenesis of type II collagen (Gelse et al., 2003). It also forms a heterotrimer $\alpha 1$ (XI), $\alpha 2$ (XI) and $\alpha 3$ (XI) (Figure 1.4C; (Eyre et al., 1992).

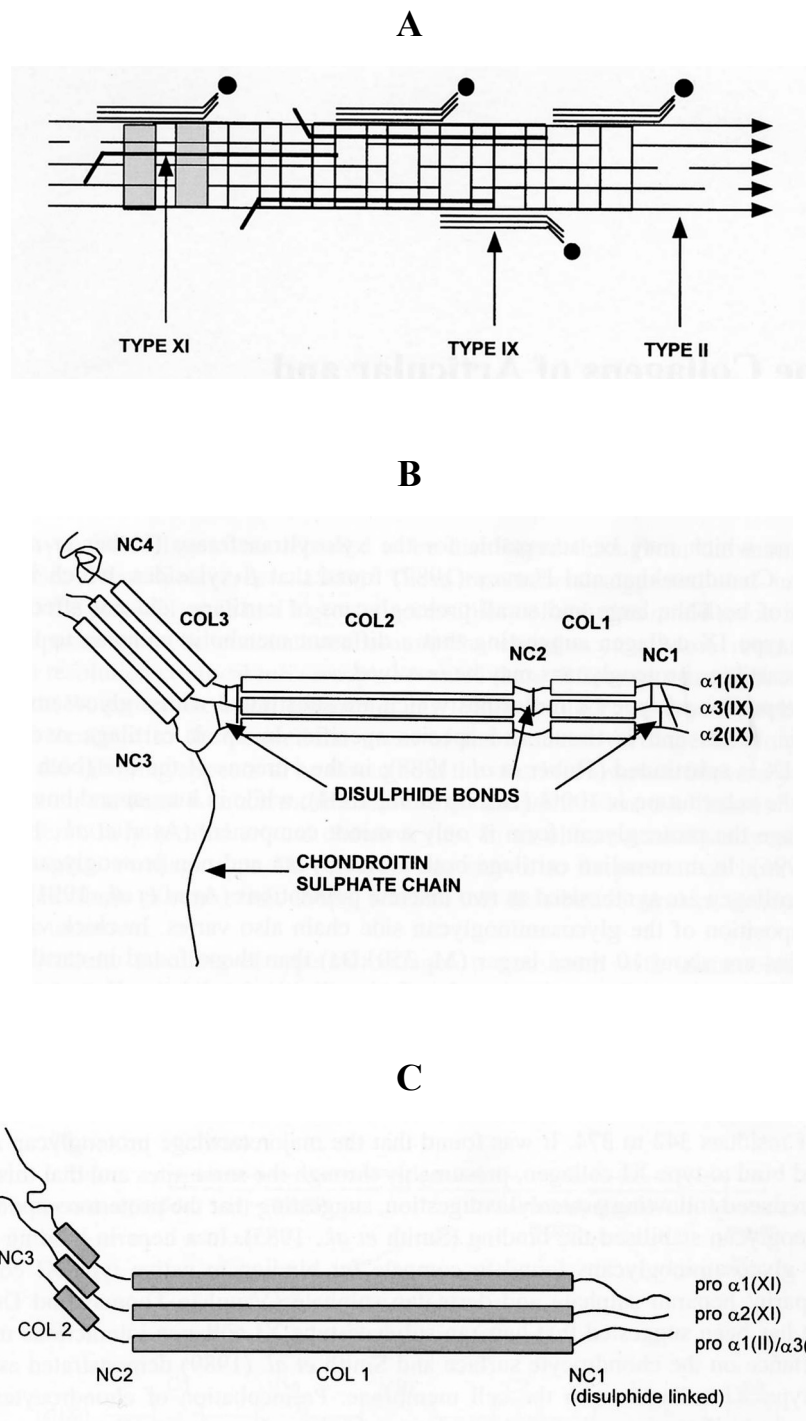


Figure 1.4: Schematic representation of the major collagen types present in articular cartilage

A). Heterotypic fibril consisting of types: II, IX and XI collagen. B). Type IX collagen. C). Type XI collagen (Duance et al., 1999).

In summary, type II, IX and XI collagens form a fibrillar meshwork that gives the tissue its form, tensile stiffness and strength. Type II forms the backbone of the heteropolymeric fibrils, with type IX located along the surface of the fibrils of type II in an anti-parallel orientation. Type XI is located within the fibrils where it is covalently linked to type II (Cremer et al., 1998, Gelse et al., 2003). Type IX binds covalently to the superficial layers of filaments projecting into the matrix that can bind to other type IX collagens. The type IX arrangement may also stabilise the extracellular framework as well as, aiding in the binding of PGs.

Other collagens are present in articular cartilage but to a lesser extent. Type VI collagen forms microfibrils concentrated within the pericellular matrix, it comprises approximately 1% of the total collagens present within articular cartilage (Poole et al., 1992). It is a globular collagen and is another short-helix molecule. It does not appear to be covalently linked to other collagen types (Cremer et al., 1998). This collagen aids in the attachment of the chondrocytes to the matrix by interacting indirectly, with matrix components such as collagen type II, HA, fibronectin and possibly with cell surface receptors (Soder et al., 2002).

Type X collagen is yet another short-helix collagen that is specific to cartilage. It is considerably shorter than types II, IX and XI, and is only found in small amounts in normal adult articular cartilage. It may have a role in the mineralisation of cartilage, as its expression is restricted to the hypertrophic zone of the growth plates of growing animals and to the deep calcified layer of mature articular cartilages (Eyre et al., 1992). During OA, however, chondrocytes nearer the articular surface, including the superficial cells retain the ability to synthesise collagen type X (Morrison et al., 1993).

Type XII and XIV collagens are similar in structure and like type IX, are FACIT collagens. Their function is poorly understood but they may act as minor interfibrillar proteins in cartilaginous tissue (Gelse et al., 2003).

1.5.3 *Cartilage proteoglycans*

ECM macromolecules consist mainly of collagen, elastin, PGs and matrix glycoproteins. PGs have a protein core to which one or more GAGs are covalently attached (Roughley, 2006, Knudson and Knudson, 2001). PGs are associated with all tissues, although connective tissues have the most abundant concentrations due to their extensive ECM. There are three classes of PGs: ECM, cell surface, and intracellular, however, intracellular PGs do not occur in cartilage (Hascall et al., 1999). The most abundant are ECM PGs, of which there are a further three types: large aggregating, large non-aggregating, and small interstitial.

GAGs are long linear polysaccharides that are composed of repeating disaccharide units. GAGs can be sulphated or non-sulphated and it is generally only the sulphated that are associated with PGs. Within articular cartilage, there are four sulphated GAGs (chondroitin sulphate, dermatan sulphate, keratan sulphate [KS] and heparan sulphate) and a single non-sulphated GAG (HA). However, the predominate GAG present in articular cartilage is chondroitin sulphate (Schnabel et al.) (Roughley and Lee, 1994, Knudson and Knudson, 2001).

There are five PGs present in the ECM of articular cartilage: aggrecan, biglycan, decorin, fibromodulin and collagen type IX. Most of the PGs exist as aggregates formed by the non-covalent association of PG with HA and link protein. The major PG in cartilage is aggrecan, which is a member of the large aggregating chondroitin sulphate PG family (Figure 1.5 and 1.6).

Aggrecan is the largest and most abundant PG comprising approximately 90% of the total found within articular cartilage (Lin et al., 2006). It is highly negatively charged, drawing in water from outside the tissue by osmosis; this addition of water causes the aggrecan matrix to swell and expand, which enables cartilage to resist compressive loads (Kiani et al., 2002). When the tissue is compressed water is displaced, which is dissipated upon removal of the compressive force by water being drawn back into the tissue. This flow of water during compressive cycling is thought to aid in the flow of nutrients from synovial fluid to the chondrocytes (Roughley and Lee, 1994).

Aggrecan consists of three globular domains (G1, G2 and G3) and three extended domains (interglobular domain [IGD], KS and CS). The majority of the aggrecan core protein lies between the G2 and G3 regions, KS and CS chains are attached between these domains. The G1 and G2 domains are connected by a short IGD, (Knudson and Knudson, 2001, Kiani et al., 2002). The G1 domain has the same structural motif as link protein, the G1 region is located at the N-terminus and interacts with HA and link protein (Knudson and Knudson, 2001, Kiani et al., 2002). This aggregation with HA is mediated through a specific protein at the N-terminus of their core proteins (the HA-binding domain). Similar domains are present in other HA-binding molecules, such as versican, link protein, and CD44 (Hardingham et al., 1992).

Loss of aggrecan is a major feature of cartilage degradation associated with disease in particular, OA (Chen et al., 2003). Chondrocytes play a vital role in the breakdown of aggrecans, by the production of specific proteinases (aggrecanases). These proteinases include aggrecanase-1 and -2, which are members of the ADAMTS metalloproteinases (a disintegrin and metalloproteinase with thrombospondin motifs; ADAMTS-4 and ADAMTS-5). These cleave the core of the protein of aggrecan at a number of sites (Handley et al., 2002).

Aggrecan plays an important role in mediating chondrocyte-chondrocyte and chondrocyte-matrix interactions through its ability to bind HA (Kiani et al., 2002). HA is a large non-sulphated GAG that is synthesised at the plasma membrane via hyaluronan synthase (HAS) into the extracellular space and is present around all chondrocytes (Roughley, 2006). HA plays an important role in the function and structural organisation of cartilage, cell adhesion, migration and differentiation mediated by cell surface receptors such as CD44 and HA-binding proteins (Lin et al., 2006). The interaction between aggrecan and HA is non-covalent and is strengthened and stabilised by link protein. Link proteins are small glycoproteins synthesised by chondrocytes that have the ability to interact with HA and a single aggrecan molecule. The aggrecan aggregate is a densely substituted PG with a bottlebrush structure (Figure 1.5; (Hardingham et al., 1992); large molecules may have more than 300 aggrecan molecules (Pitsillides, 1999). There are two populations of PG aggregates: slow and fast sedimenting types. Fast have a high CS to HA ratio and slow have a

low ratio. The superficial zone contains mostly smaller slow sedimenting aggregates. Deeper regions contain both types. The loss of larger aggregates is one of the earliest changes in OA (Buckwalter and Hunziker, 1999).

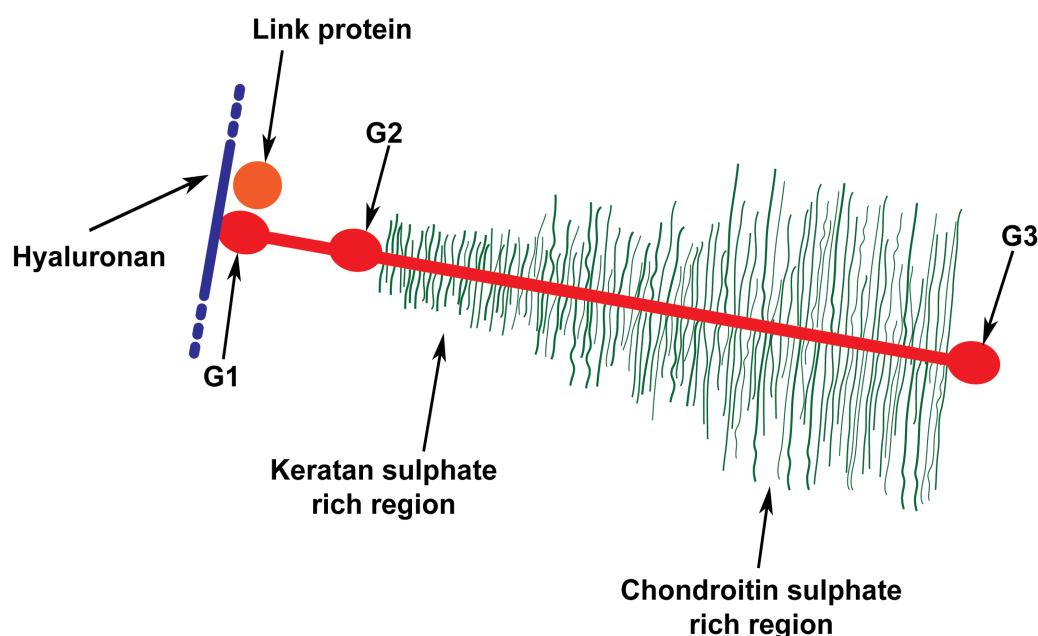


Figure 1.5 Schematic representation of aggrecan aggregate

Diagram of PG aggregate comprising aggrecan, HA and link protein.

Perlecan is a large non-aggregating PG that is commonly associated with basement membranes; it has a very large protein core (400 kDa) and two or three heparan sulphate GAG chains attached to its N-terminal. The rest of the core protein has several different structural domains that facilitate its specific interaction with cells and other specific matrix macromolecules. It interacts with ECM proteins, growth factors and receptors (Melrose et al., 2006, Arikawa-Hirasawa et al., 1999) and is enriched in the pericellular matrix in adult articular cartilage (Knudson and Knudson, 2001).

The small interstitial PGs or small leucine-rich repeat PGs (SLRPs) are ubiquitous in connective tissues. They are characterised by having relatively small core proteins that contain leucine-rich protein domain repeats that facilitate protein-protein interactions and associations. In articular cartilage they consist of decorin, biglycan,

fibromodulin and lumican, they are similar in structure but differ in GAG content. Decorin and biglycan are classified as dermatan sulphate PGs, whereas fibromodulin and lumican are KS PGs. The core proteins of the SLRPs allow interaction with fibrillar collagens, assisting in the regulation of fibril diameter during formation and possibly the interaction of fibrils in the ECM. The SLRPs also help protect the fibrils from proteolytic damage by preventing collagenases from gaining access to cleavage sites (Roughley, 2006). These leucine-rich PGs also interact with many other macromolecules, such as collagen types VI, XII and XIV, fibronectin and growth factors including TGF- β . The level of SLRP synthesis varies with age and may be affected by a variety of growth factors (Roughley, 2006).

Syndecan and glypican are cell surface PGs expressed by chondrocytes. Syndecans are transmembrane PGs that have a specific protein domain that anchors them in the plasma membrane. Glypican is a PG that is covalently linked to the cell surface by phosphoinositol in the lipid layer of the plasma membrane (Knudson and Knudson, 2001).

An example of intracellular PGs is serglycin (mast cells). Its protein core is composed of serine and glycine repeats that render it extremely resistant to proteolytic digestion. It contains many heparan or CS GAG chains attached to the core protein, this PG binds powerful proteases to its GAG chains, that are specifically released into the ECM on mast cell degranulation in inflammation and wounding (Hascall et al., 1999).

1.5.4 Non-collagenous macromolecules

There are numerous abundant proteins that are neither collagenous or PGs within articular cartilage, these macromolecules may help to organise and maintain the structure and facilitate matrix-cell signalling. The macromolecules that will be discussed in more detail are: cartilage oligomeric matrix protein (COMP), cartilage intermediate layer protein (CILP), fibronectin, tenascin and surface zone protein (SZP). This list is by no means exhaustive, but represents proteins commonly encountered. These macromolecules can be grouped into structural molecules and molecules regulating cell function.

The first structural matrix macromolecule to consider is COMP, it is a cartilage-specific pentameric protein also known as thrombospondin 5 (Lohmander et al., 1994). It is primarily found within the interterritorial matrix in mature articular cartilage, but during development it is principally located within the territorial matrix. It is thought to mediate chondrocyte attachment to the ECM by binding to collagens I, II and IX, fibronectin and aggrecan (Murray et al., 2001). The function of COMP is still unclear but it is thought to be associated with endochondral ossification and the stabilisation of the ECM (Tseng et al., 2009). During cartilage damage and disease, fragments of COMP are released into the synovial fluid and increased levels can be associated with the progression of OA. It is not yet understood whether this altered COMP distribution is a repair response or increases the damage to cartilage (Neame et al., 1999, Murray et al., 2001).

CILP is a 91.5 kDa glycoprotein located in the interterritorial matrix, and is prevalent in older tissue. It occurs within the deeper layers of cartilage and is almost absent from the superficial and calcified zones; its function in cartilage is not clear (Neame et al., 1999).

Fibronectin is a large cell matrix adhesion glycoprotein present at high concentrations in most ECM. It can bind to cells via several cell surface receptors, known as integrins. Integrin $\alpha 5 \beta 1$ is a receptor for fibronectin. The RGD peptide (arginine-glycine-aspartate) sequence is crucial for binding to this integrin and is believed to have a role in cell adhesion (Hayashi et al., 1996). Fibronectin binds to a number of matrix macromolecules including collagen and GAG chains of PGs. It is also considerably up-regulated in OA (Jones et al., 1987).

Tenascin-C is structurally related to fibronectin and is predominantly expressed in the condensing mesenchyme during development (Gluhak et al., 1996), repair and oncogenesis. However, studies show it is also produced by mature differentiated chondrocytes and incorporated into the pericellular region surrounding the chondrocytes (Qi and Scully, 2003). It is a hexameric ECM glycoprotein and its disulphide-linked sub-units consist of a series of domains that display homology to other proteins, including epidermal growth factor, fibronectin and fibrinogen (Mackie and Ramsey, 1996). Despite sharing structural features with fibronectin, tenascin-C

possesses anti-adhesion and anti-spreading properties. Tenascin-C affects attachment and spreading of a number of cell types in culture. It has been shown to interact with cell-surface receptors, including integrins and syndecans. Tenascin-C can also exhibit pro-adhesive effects on cells such as endothelial cells. It promotes the differentiation of chondrocytes from mesenchyme and perichondrium, probably via its ability to inhibit cell attachment and favouring a rounded cell shape (Mackie and Ramsey, 1996).

The final macromolecule to be discussed is SZP, which was first described by Schumacher et al (1994). SZP is secreted by superficial zone cells of articular cartilage and is homologous to lubricin (Flannery et al., 1999, Schumacher et al., 1994), which is secreted by the synovium. It is a mucin containing glycoprotein with a covalently attached PG chain. Both SZP and lubricin are boundary lubricants in synovial joints and reduce friction at the articular surface (Becerra et al., 2010).

1.5.5 Interstitial fluid

Water is the most abundant constituent of cartilage and contributes to approximately 80% of the wet weight. Some of the water can move freely within and out of the tissue, around 30% is associated with the fibrillar collagen meshwork, but the majority is associated with the large aggregating PGs that are highly negatively charged. The interstitial fluid transports gases, small proteins, metabolites and cations from the synovial fluid to the chondrocytes and vice versa (Buckwalter and Mankin, 1997).

1.6 Chondrocyte-matrix interactions

Chondrocytes exist singly, paired or in multiples surrounded by pericellular matrix and together comprise a chondron (Poole, 1997). Within the deep zone, chondrocytes are stacked into columns, whilst in other zones chondrocytes were thought to be isolated from each other. However, recently, horizontal pairs or multiples of chondrocytes have been observed within the superficial zone (Chi et al., 2004, Rolauffs et al., 2008, Schumacher et al., 2002) suggesting these cells are also in direct contact with each other. In a recent study using atomic force and two-photon

excitation microscopy interconnecting tracks between superficial zone lacunae in normal human articular cartilage were observed (Gonzalez et al., 2007). Chondrocyte attachment to the ECM appears to be mediated by matrix receptors, including integrins and annexins. Cell anchorage, migration, matrix synthesis and tissue repair are all affected by the interaction of these molecules and the ECM in combination with growth factors and cytokines (Figure 1.6; (Loeser, 1993, Aszodi et al., 2003).

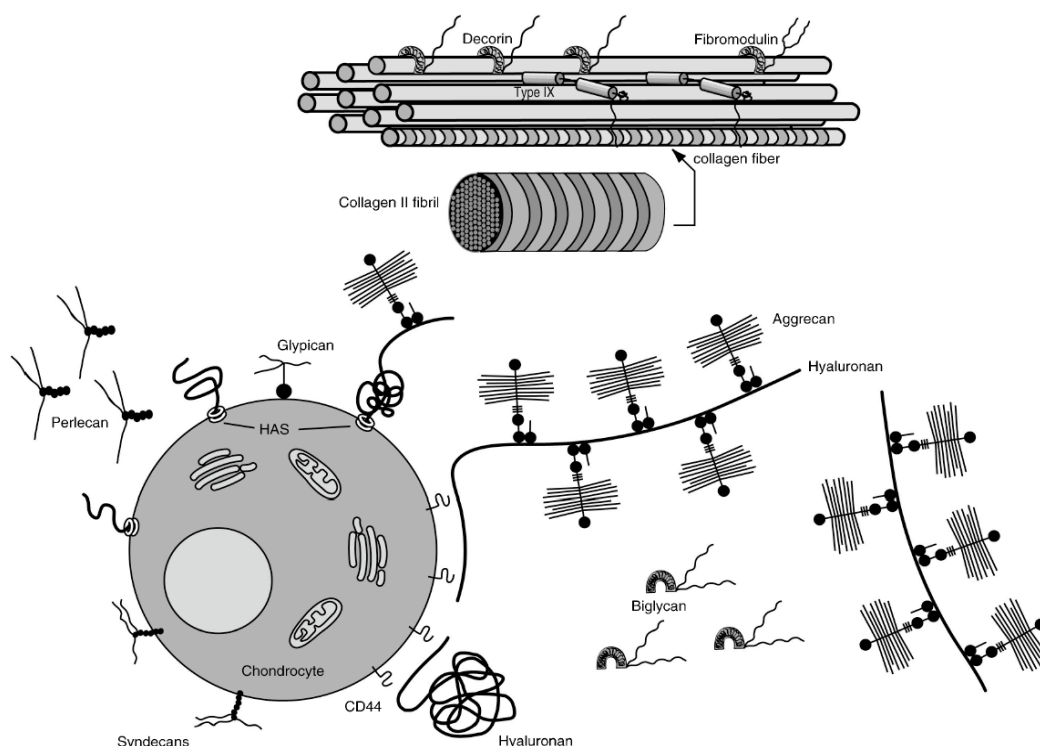


Figure 1.6: Schematic representation of proteoglycans in articular cartilage

This diagram highlights the interactions of the chondrocyte within its extracellular matrix macromolecules (Knudson and Knudson, 2001).

The interaction between chondrocytes and the ECM is partly mediated by integrins, whose cytoplasmic domains are attached to the cytoskeleton. Signalling of integrins is bidirectional; the extracellular domain of integrins can become more adhesive for the ligands, or alternatively from the ‘outside-in’, the attachment of integrins to their ligands results in a cellular response (van der Kraan et al., 2002). Chondrocytes are known to attach to a number of proteins including fibronectin, vitronectin, and

collagen types I, II, V, IX and XI and studies have suggested that integrins mediate attachments to these proteins.

Integrins are heterodimeric proteins that are composed of α and β subunits; each subunit has an extracellular and cytoplasmic domain (Reid et al., 2000). Binding of integrins with ligands within the ECM can induce cellular responses and promote cytoskeletal reorganisation (Kim et al., 2003). The most abundant integrin expressed on chondrocytes is $\alpha 5\beta 1$, the fibronectin receptor (Salter et al., 1992, van der Kraan et al., 2002). Fibronectin receptors have been shown to be mechanoreceptors and involved in the differentiation of chondrocytes (Reid et al., 2000).

Annexins are another group of transmembrane molecules that act as receptors for the ECM; annexin II, V and VI have all been observed in articular cartilage (Reid et al., 2000). Anchorin CII (the cartilage-derived annexin V) is the most abundant annexin within articular cartilage and has various functions including anti-inflammatory responses, signal transduction, and cell-matrix interaction and plays a vital role in cartilage metabolism. Anchorin CII has affinity for collagen types II and X and is expressed mainly by superficial zone chondrocytes (van der Kraan et al., 2002, Durr et al., 1993).

Another well-documented ECM receptor is CD44, which is a polymorphic glycoprotein. CD44 is a cell adhesion molecule and receptor for HA, fibronectin and collagen (Salter et al., 1992). CD44 mediated binding of HA is essential for cartilage homeostasis. When HA adherence to CD44 is restricted, it results in degradation of the cartilage ECM (Bosch et al., 2002, Knudson and Knudson, 2004).

1.7 Age-related changes in articular cartilage

The formation of superficial fibrillations is the most apparent age-related morphologic change in articular cartilage (Buckwalter and Lane, 1996, Martin and Buckwalter, 2003). These changes are rare in adolescents and young adults and it may not be possible to distinguish age-related superficial fibrillation from early stages of OA. Evidence suggests that most cases of superficial fibrillation do not progress to the deeper layers of the cartilage even after decades. Changes in the mechanical

properties of articular cartilage have been observed with aging (Barbero et al., 2003); cells accumulate intracytoplasmic filaments and may lose some of their endoplasmic reticulum, there is also a reduction in cartilage hydration and cell number. In addition, the mitotic and synthetic activities of cells decline. The water content generally decreases with age and both the PGs and collagens that form the primary components of the articular cartilage matrix macromolecular framework undergo age-related changes (Buckwalter et al., 1993, Roughley and Lee, 1994). Aggrecans become smaller and more variable in size with age. The KS content within aggrecan increases and the CS content decreases. As a result of the decrease in aggrecan size there is a reduction in the size of PG aggregates. Proteolytic degradation results in fragments within the matrix that can bind to HA where they can inhibit aggregation of fully functional molecules, HA decreases in size and increases in concentration (Thonar et al., 1986). Link proteins also become less effective with age as they undergo ongoing proteolytic alterations. Age-related changes may also occur in small PGs. Cartilage collagens also undergo changes with age: more cross-linking, increase in fibril diameter and variability, decrease in type XI in relation to type II collagen, large collagens with high amounts of cross-linking may become less flexible and make the cartilage more rigid (Barbero et al., 2003).

1.8 Articular cartilage degradation and disease

Articular cartilage commonly survives for seven or more decades of adult life without overt signs of degeneration (Roughley and Lee, 1994). The superficial zone of articular cartilage plays a vital role in the healthy joint. It provides a smooth surface with low friction. It also withstands high tensile stresses and distributes the load over the surface of the joint, thereby protecting the underlying cartilage. The superficial layer, however, is often the first region to show signs of damage during disease or following trauma. Damage to this layer exposes the deeper cartilage to damaging stresses of loading during joint movement (Aydelotte et al., 1992). Trauma to joints commonly affects younger individuals following sporting activities but can occur at any age and all components of the joint including the articular cartilage are eventually affected.

Damage to articular cartilage ultimately leads to OA. This is the commonest disease affecting articular cartilage and most often occurs in the knee, hip and shoulder joints, but any synovial joint can be affected. Symptoms include joint pain, restriction of movement, deformity and variable degrees of inflammation (Buckwalter and Mankin, 1997). The clinical observation first reported by Hunter in 1743 (Hunter, 1743) that articular cartilage once damaged would not repair still stands today. OA is the single most expensive disease in the developed world, as it progresses over a series of decades (Mollenhauer and Erdmann, 2002). OA resulting from joint injury is referred to as secondary OA. More commonly, OA develops in the absence of any known joint degeneration and is referred to as, primary or idiopathic OA and may result from several other factors such as obesity, joint instability, overuse, genetics or anatomical abnormalities (Carossino et al., 2007, Buckwalter and Martin, 2006).

Lesions in articular cartilage are often referred to as partial or full thickness-defects. Partial thickness or chondral defects are contained within the cartilaginous regions and do not extend to the calcified cartilage. Full-thickness or osteochondral defects extend down to the vascularised subchondral bone and, are thus exposed to the bone marrow pool of stem cells that fill the defect in an attempt to repair. The newly formed tissue is fibrocartilaginous and does not integrate well with the native tissue and is a poor substitute for hyaline cartilage (Redman et al., 2005, Hunziker, 1999, Cancedda et al., 2003). In OA, fraying or fibrillation of the superficial zone extends into the middle zone of the articular cartilage. There is a reduction in PGs in the fibrillated regions and clusters of chondrocytes are often evident as the tissue attempts to repair. As the fissures extend deeper they eventually reach the subchondral bone, this is often accompanied by an increase in matrix synthesis and duplication in the tidemark. The continued degradation results in further loss of the superficial zone cells, which are responsible for cell regeneration (Rosenberg, 1992). Accompanying fibrillation is an increase in water content along with a shortening of GAG chains and decrease in PG aggregation and concentration of aggrecans. All these factors disrupt the organised arrangement of the matrix and increase the permeability of the tissue. This disruption is followed by degradation of the matrix constituents including type IX and XI collagens; this increases the stresses on the remaining collagen-fibril network and chondrocytes. Type X collagen is normally associated with calcified cartilage, however, terminal differentiation of chondrocytes has been associated with

the progression of OA and can be characterised by the expression of type X collagen, resulting in increased matrix degradation and loss of type II collagen (van der Kraan et al., 2001). With the increased fibrillation there is an elevation in enzymatic degradation of the matrix, further decreasing the volume of cartilage eventually resulting in total loss of cartilage and presence of necrotic eburnated bone. Cracks with vascular invasions may also be evident with fibrocartilage pockets (Frenkel and Di Cesare, 1999). In some cases, the unsuccessful repair response leads to the formation of osteophytes. Fragments of cartilage can also detach from the articular surface and become trapped within the joint space causing obstruction and inflammation as well as further structural damage (Nelson et al., 2010).

1.9 Repair mechanisms of articular cartilage

The most suitable treatment for end-stage OA, particularly in the knee joint is prosthetic replacement. This surgical procedure is suitable for aged individuals with a sedentary lifestyle, however, is not ideal for patients under 45 years of age (Bhosale and Richardson, 2008). In younger individuals, a variety of methods have been used to repair defects and stimulate the formation of new articular cartilage, these include: penetration of the subchondral bone, lavage, mosaicplasty, soft-tissue grafts, cells transplantation and osteotomy. All of these methods are highly variable in their results and do not produce tissue comparable to articular cartilage. Many factors need to be considered when selecting a repair method for cartilage defects, such as the size of the defect and age of the patient (Bhosale and Richardson, 2008).

The original and still the most widely used method that surgeons adopt for cartilage repair involves penetration of the subchondral bone. This method includes procedures such as: abrasion arthroplasty, Pridie drilling and microfracture and mimics osteochondral defects, penetrating the bone marrow pool of stem cells. Abrasion arthroplasty uses a burr to induce bleeding from the underlying bone, producing fibrin blood clots that are then infiltrated with stem cells (Yen et al., 2008, Nelson et al., 2010). Pridie drilling and microfracture function in a similar way, they encourage the formation of fibrin blood clotting and mainly serve to give symptomatic relieve to patients. Microfracture may be an improvement on drilling as there is no over-heating

or burning of the subchondral bone (Bhosale and Richardson, 2008, Redman et al., 2005).

Joint debridement involves the arthroscopic removal of damaged cartilage and is most effective when combined with arthroscopic lavage; a process involving irrigating the joint space and removing debris. These are common methods to alleviate pain but are not known to have any biological repair response (Nelson et al., 2010). Osteotomy is a technique that accompanies debridement and lavage. It is generally conducted on joints with unilateral arthritic damage and redistributes the joint load reducing contact pressures on the cartilage surface. This procedure involves incising bone in order to change joint alignment.

Mosaicplasty is widely used to treat both chondral and osteochondral defects, during this process osteochondral plugs are harvested from low weight-bearing areas within the knee joint, harvested plugs are inserted into the defects. There is a lack of integration with the native tissue and questionable viability of the chondrocytes within the plugs (Hangody et al., 1997). This method is limited by the shortage of autologous cartilage available to harvest, so is restricted to small size defects. This method also requires damaging an equal amount of healthy tissue as it is replacing, which subsequently is subject to degradation (Chiang and Jiang, 2009). Osteochondral allografts can be used for the treatment of focal, chondral or full thickness defects and is well documented in the knee (Gomoll et al., 2011). However, this technique is not well documented in cases of advanced OA. Cartilage only allografts can be used for small chondral defects, where plugs or culture-expanded chondrocytes are inserted into the defect(s), this method has less post-operative complications associated with it as there are no immunological responses (Nelson et al., 2010).

During recent years, considerable research has been directed towards facilitating the biological repair of articular cartilage defects. Methods are diverse involving: tissue engineering, scaffolds, stem cells, or gene therapy or a combination of these (Hunziker, 2009). Cells are embedded within a matrix or scaffold, and are derived from a number of locations including expanded autologous chondrocytes, bone marrow stem cells, or allogenic stem cells. Autologous chondrocyte implantation

(ACI) was the first procedure to utilise ‘tissue engineering’ in articular cartilage. During the procedure, a small plug of cartilage is harvested and expanded in culture; the expanded cells are reintroduced into the chondral defect during a second operation under a periosteal flap. This technique was first developed by Chesterman and Smith (1968), where chondrocytes were placed in humeral defects of Dutch rabbits and adapted by Bentley and Greer (1971). This procedure was further developed by Brittberg in the clinic (Brittberg et al., 1994). ACI has also been adapted using mesenchymal stem cells to replace chondrocytes (Brittberg, 2010, Bhosale and Richardson, 2008). Alternatively, cells can be seeded into scaffolds or matrices as in matrix-induced autologous chondrocyte implantation (Broadus et al.), where chondrocytes or stem cells are contained within a collagen scaffold, a procedure that avoids the need for a periosteal flap.

Scaffolds provide a three dimensional environment in which cells can be embedded. Collagen or HA are the most common materials used for scaffolds as they are natural constituents of cartilage. However, synthetic scaffolds such as polyglycolic and polylactic acid based scaffolds are also used (Oliveira et al., 2007, Cancedda et al., 2003). These synthetic scaffolds eliminate immune responses. These procedures, however, all demand a large number of cells, requiring extended time in culture and ultimately dedifferentiation.

1.10 Resident mesenchymal progenitor cells in synovial joint tissues

Stem cells can be defined as any cell that retains a high capacity for self-renewal throughout adult life. They characteristically divide to produce a daughter cell that is a stem cell or a precursor cell; these precursor cells proliferate before differentiating and are otherwise known as transit amplifying cells (Jones and Watt, 1993, Raff, 2003).

Stem cells within synovial joints are mesodermal in origin and are often referred to as mesenchymal stem cells (MSCs). MSCs have been located in virtually all tissues and organs (Saamanen et al., 2010) and are known to home to injured or diseased tissues. However, the mechanisms that orchestrate this migration is not certain, (Chamberlain et al., 2007). Adult MSCs principally maintain tissues by serving as a cellular reserve

for tissue remodelling and renewal and are maintained at specific sites in ‘stem cell niches’. These niches have been observed in tendons, bone marrow and nodes of Ranvier at the periphery of articular cartilage (Karlsson et al., 2009, Saamanen et al., 2010, Walker et al., 2009). The regulation and survival of stem cells requires interaction with the microenvironment via cell contact, cell signalling pathways such as Notch, extracellular proteins and cell surface receptors (Walker et al., 2009, Jones and Wagers, 2008).

Unlike hematopoietic stem cells (HSCs), there is no single marker to identify MSCs. This cell type appears to be heterogeneous with most being progenitors rather than true stem cells, but all are highly clonogenic and possess the potential to differentiate into multiple mesenchymal tissues (Pittenger et al., 1999, Saamanen et al., 2010). The International Society of Cellular Therapy recommended that MSCs fulfil minimum criteria including: adhesion to plastic, expression of surface markers CD73, CD90, CD105 and tripotent differentiation capacity to a chondrogenic, osteogenic and adipogenic phenotype (Horwitz et al., 2005). As well as the cell surface markers listed above, numerous publications have listed multiple MSC markers, including CD44, CD49e, CD90, CD105 and CD166 (Diaz-Romero et al., 2005), however none are specific to this cell type (Chamberlain et al., 2007). Stem cells can be identified within a small side-population using fluorescently activated cell sorting (FACS). The weakly fluorescent cells within this side-population express high levels of an ABC transporter and actively efflux Hoechst dye from the cells (Goodell et al., 1996, Raff, 2003). This technique was first applied to isolate HSCs in the mouse but is now routinely used to select MSCs and other stem cells. Side-population cells have been identified in synovial cells and articular chondrocytes (Yamane and Reddi, 2007, Grogan et al., 2009, de la Fuente et al., 2004, Hattori et al., 2007).

1.11 Stem cells in articular cartilage

Adult articular cartilage was thought to be devoid of stem cells or progenitors but recent work has demonstrated their presence *in vivo*, and *in vitro* studies have demonstrated the presence of stem cell markers and plasticity in culture (Barbero et al., 2003, Fickert et al., 2004, Dowthwaite et al., 2004, Hayes et al., 2001, Alsalameh et al., 2004, de la Fuente et al., 2004).

In vivo, chondrocytes demonstrate little or no capacity for self-repair; this is most likely due to absence of vascularisation, limited number of progenitors and restricted mobility of the cells. However, in culture, chondrocytes are able to proliferate and gradually dedifferentiate to a fibroblast-like precursor indicated by loss of synthesis of aggrecan and type-II collagen expression. These dedifferentiated cells regain their phenotype when placed in three-dimensional culture such as agarose, micromass or pellet culture (Benya and Shaffer, 1982, Tallheden et al., 2003, Raff, 2003). Dedifferentiated monolayer chondrocytes have demonstrated plasticity by differentiating to chondrogenic, osteogenic and adipogenic phenotypes and display similar characteristics to MSCs (Tallheden et al., 2003, Barbero et al., 2003, Williams et al., 2010, Fickert et al., 2004, de la Fuente et al., 2004).

It is generally accepted that articular cartilage grows by apposition and that the progenitor cells are located at the articular surface during development and maturation (Hayes et al., 2003, Williams et al., 2009). In the skeletally immature South American opossum, BrDU highlighted slow-cycling cells within the superficial zone of articular cartilage, a characteristic expected of chondroprogenitors or a stem cell population (Hayes et al., 2001). These chondroprogenitors were isolated in our laboratory by Dowthwaite et al (2004) and were found to express the cell fate marker, Notch1 and preferentially adhered to fibronectin. When cultured, this subpopulation of cells from the superficial zone exhibited a high colony forming efficiency. This study also demonstrated the expression of integrins $\beta 1$ and $\alpha 5$ (CD29 and CD49e respectively) throughout the depth of immature bovine cartilage. However, labelling was most abundant within the superficial zone (Dowthwaite et al., 2004). Msx1, a transcription factor associated with bone formation has been located within immature bovine articular cartilage (unpublished observations) and has recently been associated with OA chondrocytes (Karlsson et al., 2010).

Recently, other stem cell markers have also been identified within articular chondrocytes including CD105 and CD166, the majority of human chondrocytes *in vitro* express CD105 with a minority expressing CD166 (Alsalameh et al., 2004, Williams et al., 2010, Fickert et al., 2004, de la Fuente et al., 2004, Grogan et al., 2009). CD44 and CD90 are also characterised as MSC markers and are located within articular cartilage (Diaz-Romero et al., 2005, Diaz-Romero et al., 2008).

CD117 is also a MSC marker and has been associated with cells within the synovial joint but as yet has not been identified within articular cartilage (Gimeno et al., 2005).

1.11.1 Differentiation markers

The Notch phenotype was originally identified in *Drosophila* by Morgan and colleagues (Morgan, 1917). It is a heterodimer transmembrane protein comprising a large extracellular domain with multiple epidermal growth factor (EGF) repeats, and a relatively small transmembrane domain (Carlson and Conboy, 2007). Notch is located in many tissues including muscle, neurons, skin, bone marrow, and cartilage (Carlson and Conboy, 2007, Chiba, 2006).

The Notch signalling cascade is initiated by cell-cell contact of the Notch receptor with one of its ligands (Gering and Patient, 2010). In mammals, four Notch homologues (Notch 1-4) interact with five transmembrane ligands (Delta-like1, Delta-like3, Delta-like4, Jagged1 and Jagged2) (Gering and Patient, 2010). The DSL (Delta/Serrate/LAG2) domain-containing proteins, Delta, Jagged1, and Jagged2, are ligands for Notch1 and 3 receptors (Shimizu et al., 2000). Cells expressing Delta or Jagged proteins in their cell membranes activate neighbouring cells. This activation results in intramolecular cleavage of the precursor protein (S1 cleavage) to form a heterodimer, composed of an extracellular and transmembrane domain on the cell membrane. When attached to one of these ligands, Notch undergoes a conformational change that enables it to be cleaved by a protease (S2 cleavage); where the extracellular domain is separated from the transmembrane subunit. The transmembrane domain is then also cleaved (S3 cleavage) allowing translocation of the NICD (Notch intracellular domain) to the nucleus. The cleaved portion enters the nucleus and binds to a dormant transcription factor of the CSL (CBF1, Su [H], Lag-1) family, converting the complex from a transcriptional repressor to a transcriptional activator and subsequent upregulation of downstream targets such as HES (Figure 1.7; (Chiba, 2006, Gering and Patient, 2010, Mumm and Kopan, 2000).

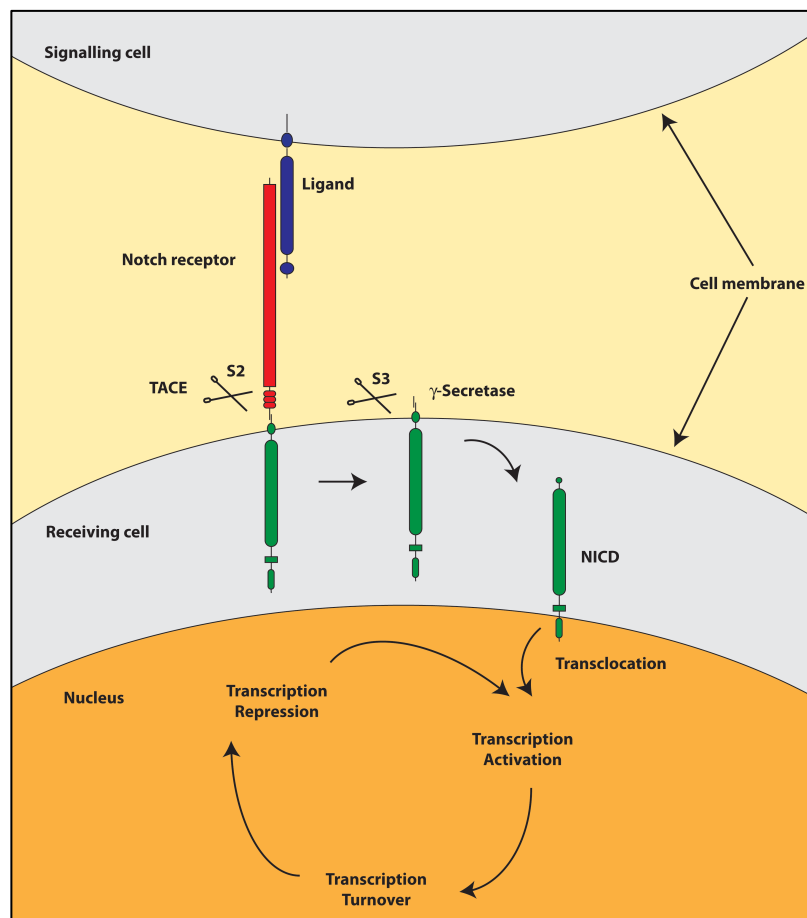


Figure 1.7: Diagram of Notch signalling pathway

Notch is activated and attaches to a ligand resulting in S2 cleavage. The transmembrane domain is further cleaved by γ -secretase during S3 cleavage to release the NICD, which translocates to the nucleus, resulting in upregulation of downstream targets (Borggreffe and Oswald, 2009).

Notch is also involved in lateral inhibition, where within a group of equivalent cells expressing both ligand and receptor, one cell differentiates and blocks neighbouring cells from adopting the same fate, by activating the notch receptor on the surrounding cells. Thus, Notch signalling controls cell fate by inhibiting or promoting differentiation (Mumm and Kopan, 2000, Gering and Patient, 2010).

Notch signalling was thought to be mono-directional although recent work by Hiratochi et al (2007) has disputed this. In this study, neuronal stem cells were

cultured with Delta1 expressing COS7 cells and the rate of neuronal development was lower than controls. Conversely, when neuronal stem cells were cultured with Notch1-expressing COS7 cells, the rate of neuronal development was significantly higher than controls. These results suggest that Delta1 expressing cells activate Notch signalling on neuronal stem cells, which help maintain an undifferentiated state. These data imply that Delta1 plays a role in bi-directional signalling (Hiratochi et al., 2007).

Notch signalling is involved in cell fate determination during differentiation, proliferation and apoptosis (Carlson and Conboy, 2007, Artavanis-Tsakonas et al., 1999). Notch expression has been observed within articular cartilage, where Notch1 was restricted to the superficial zone during development and in immature animals (Hayes et al., 2003, Dowthwaite et al., 2004, Hiraoka et al., 2006, Grogan et al., 2009). Ligands, Delta1, Jagged1 and Jagged2 have also been located within articular cartilage (Hayes et al., 2003). Notch1 has also recently been demonstrated in equine chondroprogenitors and bone marrow stromal cells (McCarthy et al., 2011).

Msx genes are a small family of homeobox-containing genes that encode transcription factors, they are related to the *Drosophila* muscle segment homeobox (msh)-like gene family. They are expressed in a variety of tissues during development and are essential in cranial-facial formation, and the development of mammary glands, teeth and limbs (Bensoussan-Trigano et al., 2011, Blin-Wakkach et al., 2001).

The presence of Msx1 and 2 within the limb bud mesenchyme are essential for normal limb development (Bensoussan-Trigano et al., 2011). In chick limb buds, high expression of Msx1 and 2 correlates with functional epithelial-mesenchymal interactions (Brown et al., 1997). Msx1 is thought to prevent differentiation and, hence, promote proliferation. It has been shown to inhibit MyoD gene expression in differentiating muscle exhibiting a negative effect on differentiation; this has been supported by forced expression of Msx1 in myogenic precursors (Blin-Wakkach et al., 2001, Hu et al., 2001). Msx genes are associated with inhibiting chondrogenic and osteogenic differentiation *in vitro* and are general inhibitors of mesenchymal and epithelial progenitor cell differentiation *in vivo* and *in vitro*. This inhibition is correlated with an upregulation in cyclin D1 activity and, therefore, preventing these

cells from exiting the cell cycle and undergoing terminal differentiation, therefore Msx does not actively promote proliferation but blocks differentiation by preventing cells from exiting the cell cycle (Hu et al., 2001). In mature tissue, Msx expression is restricted to stem cells including mammary epithelium and basal epithelium of the dermis and in pathology, it can be observed within tumours and cancer cell lines (Sarapura et al., 1997, Hu et al., 2001).

1.11.2 Stem cell markers

Integrins were first described by Hynes in 1987 and are heterodimeric glycoproteins consisting of α and β subunits (Hynes, 1987), each of which has an extracellular and cytoplasmic domain. Integrins mediate cell-cell and cell-matrix interactions and regulate many processes including matrix assembly, adhesion, migration and differentiation (Aszodi et al., 2003). Both α and β subunits have relatively small cytoplasmic tails that interact with the cytoskeletal proteins mediating changes in cell shape (Hynes, 1987, Loeser, 1993, Durr et al., 1993, Loeser et al., 1995, Loeser, 2002).

Salter et al (1992) investigated the expression of integrins within articular cartilage. The fibronectin receptors $\beta 1$ (CD29) and $\alpha 5$ (CD49e) were found to be the most abundant. Integrins $\alpha 1$ and $\alpha 3$ were also observed in this study, however their expression was weaker and much less consistent (Salter et al., 1992). CD29 mediates cell adhesion to collagen types I, II and fibronectin and these interactions are thought to play a role in cartilage formation (Enomoto et al., 1993, Loeser et al., 1995). More recently, CD29 and CD49e expression has been further demonstrated in cultured chondrocytes and intact articular cartilage, as well as MSCs (de la Fuente et al., 2004, Dowthwaite et al., 2004, Williams et al., 2010). In immature bovine cartilage, these integrins were predominantly expressed within superficial and middle zone cells, and cells from both zones demonstrated a high affinity for fibronectin. However, only superficial zone cells were able to form colonies (Dowthwaite et al., 2004). Expression was also observed in normal human chondrocytes where the majority of cells expressed CD29 and a subset expressed CD49e. However, when chondroprogenitors were expanded in culture all expressed CD49e (Williams et al.,

2010). The presence of CD29 and CD49e within the superficial zone, chondroprogenitors and within MSCs suggests that these integrins may identify a resident progenitor within articular cartilage.

CD44 is a polymorphic transmembrane protein present in many tissues (Salter et al., 1996). The amino terminus has a sequence that is homologous with link protein and aggrecan. There are several isoforms; the common forms are hematopoietic (CD44H) and epithelial (CD44E). CD44E is similar to CD44H but has an additional 135 amino acids within its extracellular domain (Salter et al., 1996). CD44H is the major isoform expressed by chondrocytes, however, smaller amounts of other isoforms do occur in articular cartilage.

Within cartilage, CD44 is the primary HA receptor, mediating both cell-cell and cell-matrix interactions. At the cell surface CD44 may regulate the pericellular matrix structure via HA (Knudson and Knudson, 2004). In addition to interacting with HA, CD44 also binds other extracellular proteins including fibronectin and collagen types I and VI (Aguiar et al., 1999, Ostergaard et al., 1997, Noonan et al., 1996). CD44 has the capacity to function as a HA receptor at the chondrocyte cell surface (Akmal et al., 2005) but can also mediate internalisation and degradation of HA (Chow et al., 1995, Aguiar et al., 1999, Nishida et al., 2000). In a study by Ostergaard et al (1997), CD44 in normal and OA cartilage, was predominantly located within the superficial zone of normal articular cartilage. Within OA cartilage, CD44 was only observed within the deep zone (Ostergaard et al., 1997, Tibesku et al., 2005).

CD90 otherwise known as thymocyte differentiation antigen 1 (Thy-1), is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein associated with lipid rafts in the outer leaflet of the plasma membrane. It is expressed in various cell types including fibroblasts, endothelial cells and stem cells (Rege and Hagood, 2006, Haeryfar et al., 2005). The processes behind CD90 signalling remain unclear, as the GPI-anchored glycoprotein lacks the transmembrane or cytoplasmic sequences generally required for signal transduction (Haeryfar et al., 2005). It is, however, expressed by HSCs, MSCs and a small sub-population of articular chondrocytes (Fickert et al., 2004, de la Fuente et al., 2004, Grogan et al., 2009, Diaz-Romero et al., 2005).

In vitro, CD90 appears to be associated with proliferating chondrocytes (Diaz-Romero et al., 2005). CD90 is also involved with regulation of the cytoskeleton and cellular adhesion (Kasahara and Sanai, 2000, Frame et al., 2002). It is suggested that CD90 expression *in vitro*, could induce re-differentiation of dedifferentiated chondrocytes (Diaz-Romero et al., 2005).

CD105 (endoglin) was first described by antibody SN6, that recognised a small population of bone marrow cells and was thought to be a marker of pluripotent stem cells (Haruta and Seon, 1986). Further studies by Gougos & Letarte, confirmed that the cell surface marker was an antigen of precursor cells (Gougos and Letarte, 1988b, Gougos and Letarte, 1988a). CD105 was later found to be expressed during angiogenesis and on endothelial cells (Duff et al., 2003).

CD105 is a 180 kDa homodimeric transmembrane glycoprotein. It has a large extracellular region, a hydrophobic transmembrane region and a short cytoplasmic tail. CD105 binds TGF- β 1, TGF- β 3, activin-A, BMP-2 and BMP-7 in the presence of ligand binding receptors types I and II. CD105 is not a true receptor; it is an accessory protein that interacts with the ligand binding receptor of multiple members of the TGF- β superfamily kinase receptor complexes (Barbara et al., 1999, Bellon et al., 1993, Arufe et al., 2009, Guerrero-Esteo et al., 2002). Among these receptors, the serine-threonine kinase receptor types I and II are necessary for all tested biological responses to TGF- β (Rius et al., 1998, Lastres et al., 1996). CD105 contains the RGD sequence on an exposed part of the extracellular domain. The RGD peptide is a key recognition structure found on ECM proteins, such as fibronectin and is recognised by cell surface integrins (Gougos and Letarte, 1990). Molecular cloning of the human endoglin cDNA demonstrated the existence of at least two different isoforms, a short and long isoform (S-endoglin and L-endoglin). L-endoglin is the predominant isoform; both differ in their cytoplasmic domains (Bellon et al., 1993, Rius et al., 1998, Lastres et al., 1996).

TGF β regulates several processes, including regulation of cellular proliferation, differentiation, migration, morphogenesis, cellular adhesion, ECM formation and can stimulate the expression of CD105 (Rius et al., 1998, Lastres et al., 1996). Another

role for CD105 on MSCs may be in mediating TGF- β signalling during chondrogenic differentiation. All TGF- β isoforms are capable of inducing MSCs towards a chondrogenic lineage (Barry et al., 1999). Indeed, a study by Parker and colleagues demonstrated that CD105 was expressed on human articular and non-articular chondrocytes at high concentrations and that it forms higher order complexes with receptor I and receptor II on the cell surface. The protein also forms a heteromeric complex with betaglycan in human chondrocytes (Parker et al., 2003). In many cells, CD105 suppresses TGF- β signalling. The marker is significantly reduced in differentiated MSCs including chondrogenic differentiation and is thought to characterise the differentiation status of MSCs derived from umbilical cord blood (Jin et al., 2009). TGF- β family members have recently been reported to control differentiation of MSCs and CD105 may play a functional role during stem cell differentiation, however, this has yet to be shown (Jin et al., 2009).

CD117 (c-kit) is a transmembrane protein of the type III subfamily of tyrosine kinases (Miettinen and Lasota, 2005). The extracellular region consists of five immunoglobulin-like domains, it has a transmembrane and juxtamembrane domain and an intracellular region consisting of two tyrosine kinase domains, connected by a kinase insert (Reilly, 2002). CD117 binds stem cell factor (SCF) which results in phosphorylation, activating several intracellular signalling molecules including transcription factors, that determine various cell responses such as: cell migration, proliferation, differentiation and adhesion (Hansen et al., 2005). CD117 has been observed in chronic inflammatory joint diseases, such as rheumatoid arthritis and OA. CD117 has been detected in articular cartilage, synovium, HSCs and MSCs (de la Fuente et al., 2004, Romanov et al., 2005, Gimeno et al., 2005).

Activated leukocyte cell adhesion molecule (ALCAM; CD166) was first discovered on thymic epithelial cells and activated leukocytes by Bowen and colleagues (Bowen et al., 1996) and is a member of the immunoglobulin superfamily of cell adhesion molecules. It has five extracellular immunoglobulin domains, a hydrophobic transmembrane domain and a short cytoplasmic tail (Swart, 2002, Bowen et al., 1996, Bruder et al., 1998, Skonier et al., 1996) and is highly conserved across multiple species.

CD166 mediates homophilic (CD166-CD166) and heterophilic (CD166-CD6) cell adhesion (Swart, 2002). Nelissen and co-workers demonstrated that CD166 homophilic cell adhesion is regulated through actin cytoskeleton dependent clustering of CD166 at the cell surface, this clustering is essential to obtain stable adhesive interactions (Nelissen et al., 2000, van Kempen et al., 2001, Arai et al., 2002). It is also suggested that CD166 may maintain stem cells or prevent differentiation (Ohneda et al., 2001). The majority of published data concern homophilic interactions of CD166, which may suggest that this is predominant over the heterophilic interaction (Swart, 2002).

CD166 expression has recently been observed in a number of other tissues including fibroblasts, neurones, synovium, cartilage and MSCs (Joo et al., 2000, Nelissen et al., 2000, Alsalameh et al., 2004, Pretzel et al., 2011). Pretzel et al (2011) were the first group to describe the localisation within human articular cartilage. CD166 was mainly located within the superficial and middle zones of OA and normal cartilage suggesting that it is a marker of chondroprogenitors (Pretzel et al., 2011).

The use of specific markers to isolate chondroprogenitors will allow for further characterisation, including a more in-depth understanding of the mechanisms of proliferation and differentiation within articular cartilage. This has the potential to lead to an improved understanding of the role of these markers and, as such, may provide us with a more beneficial cell type that could significantly contribute to the field of articular cartilage repair.

Aims of the project

Articular cartilage grows by apposition from the joint surface indicating the presence of a resident progenitor population within the superficial zone of immature tissue. Stem cells are required for tissue engineering; however, specific isolation of these cells has proved problematic. This study attempts to discover if known stem cell markers are expressed by chondroprogenitors and, will assess, if the expression is context dependent upon methods of isolation and culture.

The aim of this study is to identify the location of potential stem cell markers (CD29, CD44, CD49e, CD90, CD105, CD117 and CD166) and differentiation markers (Notch1, Delta1, Jagged1, Jagged2 and Msx1) in immature bovine articular cartilage. The study will further investigate marker expression within isolated and monolayer cultured chondrocytes to determine whether subpopulations of stem cells are present within superficial zone chondrocytes.

This will be achieved by:

- histological analysis by immunofluorescence of cell surface markers and differentiation markers;
- isolation of superficial zone chondrocytes and analysis of marker expression by flow cytometry;
- overnight culture of superficial zone chondrocytes and analysis of marker expression by flow cytometry;
- overnight culture of superficial, middle and deep zone chondrocytes and comparative analysis of marker expression by flow cytometry; and
- extended monolayer culture for 14 days, with cells analysed for marker expression by flow cytometry at days 3, 7 and 14.

CHAPTER 2:
IMMUNOHISTOCHEMICAL ANALYSIS OF STEM
CELL AND DEVELOPMENTAL MARKERS

2.1 Introduction

Articular cartilage relies on the interaction and organisation of its constituents to provide a lubricated, wear-resistant and friction-reducing surface that is resilient and slightly compressible on loading. The tissue comprises a single cell type, the chondrocyte, which accounts for 1-5% of the total volume of the mature tissue. A specialised ECM surrounds the chondrocytes. This matrix differs throughout the depth of tissue and around the cell periphery (Walsh et al., 2003).

The tissue is divided into four distinct cellular zones: superficial, middle, deep and calcified. The chondrocytes in each layer differ morphologically and in mechanical properties. The superficial zone is covered by the lamina splendens, an acellular layer of type II collagen with fibrils arranged tangentially to the direction of shear stress that acts as a gliding surface. The superficial zone consists of flattened, disc-like chondrocytes that are aligned parallel to the joint surface. The transitional zone is thicker than the superficial zone and contains chondrocytes that are spherical in shape and collagen fibres that are randomly oriented. In most joints, the chondrocytes of the deep or radial zone also consist of spherical cells but in this zone they are arranged in columns perpendicular to the joint surface. The radially arranged collagen fibres extend into the calcified zone reinforcing the attachment between cartilage and bone (Hu and Athanasiou, 2003).

Animal models are widely used to develop and evaluate tissue-engineering techniques intended for human applications. These models are capable of yielding results that are often transferable to human tissue. The use of a large animal model is likely to more closely resemble the cartilage of a human compared with a small animal model (Reinholz et al., 2004) and skeletally immature tissue allows for the opportunity to study tissue that is still developing and in which stem cell numbers may be higher. Immature tissue is also much thicker and more cellular than mature tissue (Figure 2.1) enabling more cells to be isolated from each joint (Stockwell, 1967). Large animal tissue, such as bovine, of immature and mature animals is also readily available.

There is a seven-fold reduction in cellularity during maturation from birth to adult human articular cartilage, which equates to a 60% decrease over seven decades (Stockwell, 1967, da Silva et al., 2008). The density of bovine articular cartilage is comparable with human tissue as the mature tissue is of a similar thickness (1.68mm compared to 2.26mm in human) and similar cell density (2.0 compared to 1.4 cells x 10⁴ mm³; (Stockwell, 1979). It should be noted that in this study all work will be carried out using an immature (approximately 7 days old) bovine model.

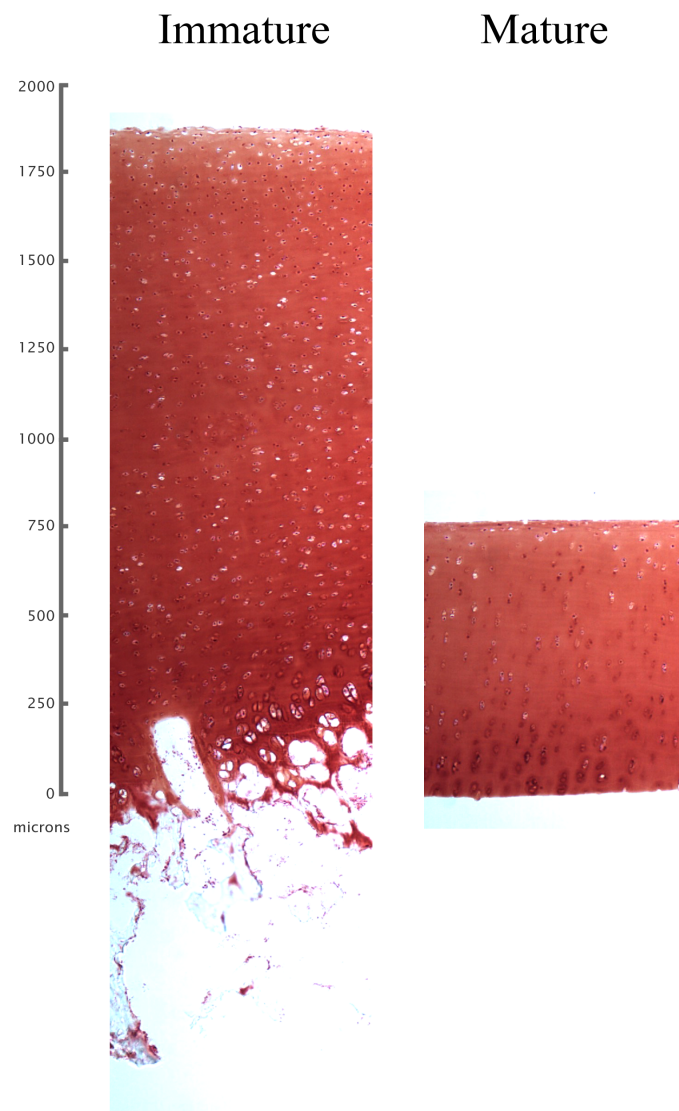


Figure 2.1: Image illustrating the difference in thickness, cellularity and organisation between immature (approximately 7 days old) and mature (18 months old) bovine articular cartilage (image kindly donated by Dr. I.M. Khan).

It has been shown that maturing articular cartilage grows by appositional growth from the articular surface therefore; a population of progenitor cells must reside within the tissue (Hayes et al., 2001). Progenitor cells have been reported within the surface zone of articular cartilage after isolation by adhesion to fibronectin *in vitro* (Dowthwaite et al., 2004). In addition, the surface zone has been centrally implicated in the regulation of tissue development and growth, factors that support the appositional growth model (Archer, 1994, Hayes et al., 2001).

Friedenstein and colleagues first identified MSCs in bone marrow in the 1970s (Friedenstein et al., 1976) cited in (Chung and Burdick, 2009) and more recently, MSCs have been shown to differentiate into several cell types including adipocytes, osteoblasts and chondrocytes (Caplan, 1991, Both et al., 2007, Kastrinaki et al., 2008).

MSCs can be characterised by the expression of particular cell-surface antigens including CD29, CD44, CD49e, CD90, CD105, CD117 and CD166 (Barry et al., 1999, Buhning et al., 2007, Majumdar et al., 2000, Romanov et al., 2005). Diaz-Romero and colleagues demonstrated a similarity in the cell surface marker expression of dedifferentiated normal human chondrocytes and MSCs. This study used a number of the above markers to discriminate between differentiated and dedifferentiated chondrocytes (Diaz-Romero et al., 2005). Other studies demonstrated that a small subpopulation of CD105/CD166 expressing cells was present within human articular cartilage that could be differentiated into chondrocytes, adipocytes and osteoblasts (Alsalameh et al., 2004). CD90 has been identified within monolayer-expanded chondrocytes (Diaz-Romero et al., 2005, Diaz-Romero et al., 2008) but has not been identified within articular cartilage *in vivo*. In addition, it has been suggested that CD90 expression is associated with proliferating cells and that the highest levels of expression are observed during the cell's proliferation phase (Diaz-Romero et al., 2005, Chen et al., 1999). CD166 is widely expressed on hematopoietic and non-haematopoietic cells such as neuronal cells, mesenchymal stem cells, endothelial cells and bone marrow stromal cells (Nelissen et al., 2000). CD166 may support not only haematopoietic development but also inhibit stem cell differentiation (Ohneda et al., 2001).

Another potential stem cell marker is Notch1, Notch and its transmembrane ligands are cell surface proteins that regulate cell differentiation during development (Artavanis-Tsakonas et al., 1999, Hayes et al., 2003). Indeed, the presence of Notch1 has been demonstrated primarily in the surface zone of immature bovine articular cartilage (Dowthwaite et al., 2004). Additionally, normal human tissue contains a small number of Notch1 positive cells that are not located primarily at the tissue surface (Hiraoka et al., 2006). The same study demonstrated an increased frequency of Notch1 expression in OA tissue (Hiraoka et al., 2006).

Msx1 is also interesting, as it is expressed by cells during embryogenesis. In mature tissue, Msx gene expression is generally associated with multipotent progenitor cells, although has also been reported within mammary and uterine tissue stem cells and within differentiated pituitary cells (Sarapura et al., 1997, Hu et al., 2001). Msx1 has been implicated in craniofacial, optic, limb and cardiac development. It has been proposed that Msx genes maintain cells in a proliferative state by blocking exit from the cell cycle, whilst not actively promoting proliferation (Hu et al., 2001).

The aim of this study is to identify the location of potential MSC markers (CD29, CD44, CD49e, CD90, CD105, CD117 and CD166) and developmental markers (Notch1, Delta1, Jagged1, Jagged2 and Msx1) in immature bovine articular cartilage.

2.2 Materials and Methods

2.2.1 Tissue isolation

Immature (approximately 7 days old) bovine limbs (removed at the carpal joint, Figure 2.2) were obtained from F. Drury & Sons abattoir, Swindon. The limbs were cleaned in detergent, skinned and incised at the level of the metacarpophalangeal joint on day of slaughter. Full-depth explants 0.5-1.0 cm² were taken using fine dissection from the lateral and medial condyles and condylar ridges from a minimum of three animals for each antibody tested (Figure 2.3).

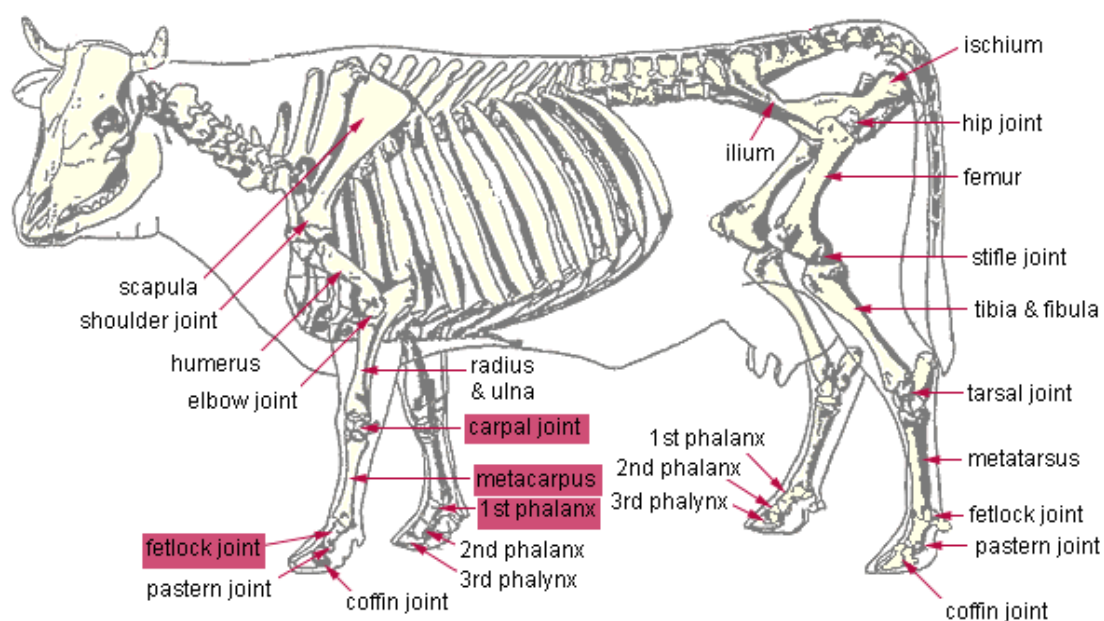


Figure 2.2: Diagram of bovine skeleton

Diagram illustrating the structure of the bovine limb, in particular, the forelimb carpal and fetlock (metacarpophalangeal) joint (adapted from Agripedia, 2009).

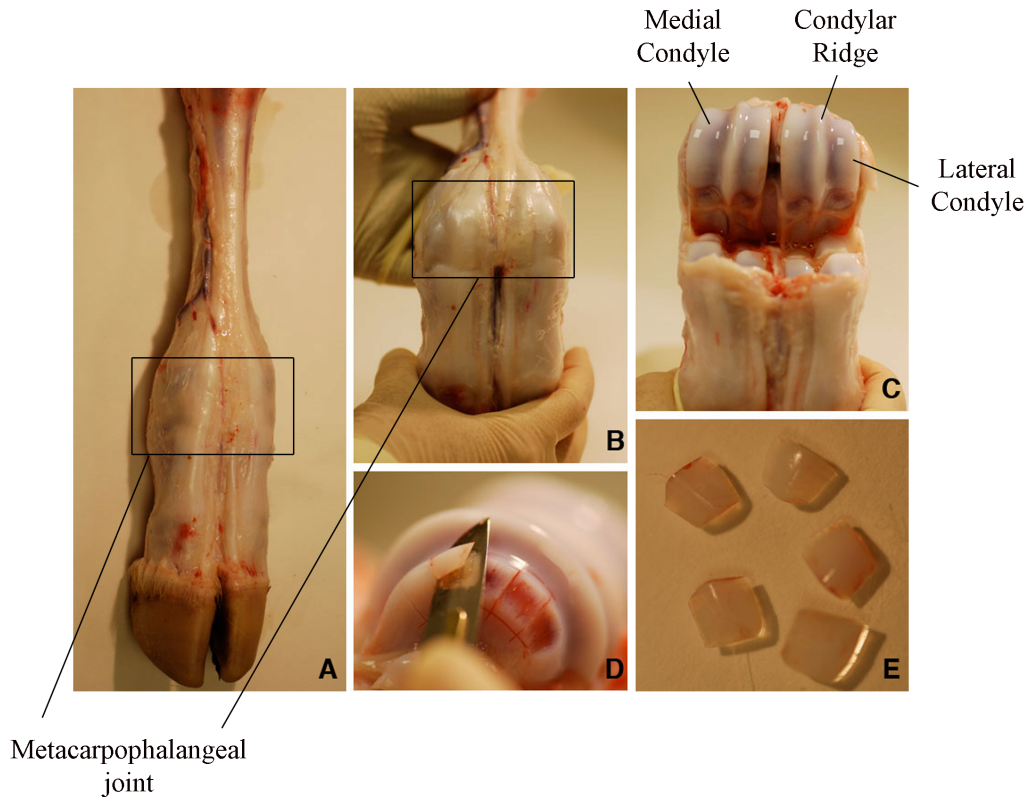


Figure 2.3: Gross morphology of the metacarpophalangeal joint demonstrating explant removal

Gross morphology of the metacarpophalangeal joint (A & B), the open joint (C), sample excision from the condyles (D) and the excised full-depth explants (E).

2.2.2 Tissue processing

Fixation should prevent antigen elution and degradation as well as preserve the position of the antigen, whether nuclear, cytoplasmic or membrane-bound, and preserve as much antigenic secondary and tertiary structure as possible for detection by antibodies (Farmilo and Stead, 2009). For this study, two predominant fixation methods were used, 2% paraformaldehyde (PFA) and 95% ethanol (ETOH).

Cryosections

Tissue was snap frozen by precipitate immersion in a pre-cooled n-hexane (Sigma-Aldrich, UK)/dry ice bath. Explants that weren't used immediately were stored at -80°C until required. Frozen, unfixed tissue was embedded in OCT embedding media (R.A. Lamb, UK). Tissue was sectioned at a thickness of 10µm using a conventional cryomicrotome (cryostat; Bright OT5000, UK) and mounted on Histobond slides (R.A. Lamb). Slides not used immediately were stored at -80°C until required.

Paraffin wax sections

Tissue was fixed in 2% PFA or 95% ETOH overnight at 4°C, and embedded in paraffin wax at 60°C paraffin wax for 1-hour x2. The embedded explants were sectioned at a thickness of 10µm using a conventional microtome (Leitz 1512, UK) and mounted on Histobond slides. Tissue was dewaxed by two washes in xylene (two minutes each) and rehydrated in descending concentrations of alcohol (2x 100%, 95% and 70%) the incubation time in each solution was 2 minutes. Finally the tissue was washed in water for 5 minutes and the sections stained with relevant antibodies.

2.2.3 Immunofluorescence optimisation

Primary and secondary antibody concentrations (Tables 2.1 and 2.2 respectively) were optimised by titration. Secondary antibodies were titrated firstly using the primary antibody of interest. If more than one primary antibody was used for a particular secondary antibody, the primary antibody that demonstrated the most extensive cellular labelling throughout the tissue was used. Jagged2 was used to optimise rabbit anti-goat/donkey anti-goat; Jagged1 was used for goat anti-rabbit; bTan20 was used for goat anti-rat; and CD166 was used for rabbit anti-mouse secondary antibody (Table 2.2). BTan20 is an antibody that recognises the intracellular domain of Notch1. Primary antibodies were titrated at 2.5, 5, 10 and 15µg ml⁻¹ using optimised secondary antibody concentrations (Table 2.1).

Background fluorescence can be a problem in articular cartilage to reduce this, primary and secondary antibodies were tested after optimisation with and without the

addition of blocking serum, and secondary antibodies were tested with and without bovine serum.

An enzymatic pre-treatment is often necessary in cartilage as large amounts of glycosaminoglycans and proteoglycans present in the tissue result in masking of antigens. Each antibody was tested on tissue fixed with 2% PFA and 95% ETOH (CD44, CD90, CD105, CD117, CD166 and Notch1 were also tested with acetone fixed tissue). Cryosections and paraffin wax sections were used and epitopes unmasked by hyaluronidase and chondroitinase digest (enzymatic pre-treatment was carried out for CD44, CD90, CD105, CD117, CD166 and Notch1).

2.2.4 Immunofluorescence of bovine articular cartilage

Immunofluorescence detection (of wax or frozen sections) of primary antibodies was used to detect the presence of stem cell and developmental markers within articular cartilage. All washes and incubations were performed at room temperature and in a humidified chamber unless otherwise stated. All antibodies and immunoglobulin-matched (IgG-matched) controls were prepared in phosphate buffered saline (PBS; Sigma-Aldrich) containing 0.1% Tween 20 (polyethylenesorbitan monolaurate; Sigma-Aldrich) and 5% normal blocking serum (for each primary antibody the serum was from the species in which the secondary antibody was raised).

Wax sections were de-waxed and rehydrated in two washes of xylene and decreasing series of alcohol (2 x 100%, 95% and 70%), the incubation time in each solution was 2 minutes. Finally, the sections were rinsed in running water for 5 minutes.

Cryosections were hydrated for 5 minutes in PBS. Tissue was fixed in fresh 2% PFA, 95% ETOH or acetone for 2 minutes at 4°C. Both paraffin wax sections and cryosections were circled with a water repellent marker (Dako pen; Dako, UK), washed 3 times in PBS/0.1% Tween 20 (PBS/T) and blocked in relevant sera for 30 minutes. Excess serum was removed by blotting the slides before incubating the sections with relevant primary antibodies for 1 hour. The tissue was washed 3 times in PBS/T and primary antibodies were detected using fluorescein iso-thiocyanate (FITC, Sigma-Aldrich) or Alexafluor 488 (Invitrogen, UK) conjugated secondary

antibodies. The secondary antibody was pre-incubated firstly with 5% bovine serum for 1 hour before applying the solution to the sections. Secondary antibodies were centrifuged at 16,000xg for 10 minutes to remove any precipitated protein (if present), before adding to sections. Finally, sections were washed 3 times in PBS/T and coverslips mounted with Vectorshield containing 4',6-Diamidino-2-phenylindole (DAPI; Vector laboratories, UK).

Antigen unmasking

Sections were digested with hyaluronidase and chondroitinase (carried out for CD44, CD90, CD105, CD117, CD166 and Notch1). Sections were fixed as previously described, washed thrice in PBS/T and then washed in Tris-acetate buffer (Sigma-Aldrich; pH6.5). Chondroitinase ABC (Sigma-Aldrich; 0.25 IU ml⁻¹) and hyaluronidase (Sigma-Aldrich; 2 IU ml⁻¹) solutions were prepared in Tris-acetate buffer, added to sections and incubated for 1 hour at 37°C. Sections were washed for 5 minutes in PBS then blocked with relevant sera. Primary and secondary antibodies were applied as described previously.

Control sections were processed as above omitting primary antibody and incubating the sections with IgG-matched controls or PBS/T. Sections were also incubated with PBS/T alone to detect autofluorescence within the tissue.

Antigen recognised	Clone Name	Source	IgG
Delta1	F-15	Santa Cruz Biotechnology, Inc. UK	Goat
Jagged1	H-66	Santa Cruz Biotechnology, Inc.	Rabbit
Jagged2	N-19	Santa Cruz Biotechnology, Inc.	Goat
Msx1	N-20	Santa Cruz Biotechnology, Inc.	Goat
Notch1	C-20	Santa Cruz Biotechnology, Inc.	Goat
Notch1	BTan20	DSHB, USA	Rat
CD29	TDM29	Millipore, UK	Mouse
CD44	IM7	Biolegend, UK	Rat
CD49e	SAM-1	Millipore	Mouse
CD90	CT-TH1	Invitrogen, UK	Mouse
CD105	SN6	Ancell, USA	Mouse
CD117	104D2	Invitrogen	Mouse
CD166	3A6	Ancell	Mouse

Table 2.1: Table of primary antibodies used

The antibodies above were tested at concentrations 2.5, 5, 10 and 15 $\mu\text{g ml}^{-1}$.

Primary Antibody	Secondary Antibody	Source	Secondary Antibody Dilution	Manufacture Recommended Concentration /Dilution
Anti-Jagged2 & IgG	Rabbit anti-goat FITC	Sigma-Aldrich	1:50, 1:100, 1:200 & 1:400	Minimum of 1:160
	Rabbit anti-goat Texas Red	Vector Laboratories	1:100 & 1:150	5-20 $\mu\text{g ml}^{-1}$ (1:300-1:75)
	Donkey anti-goat Alexafluor 488	Invitrogen	1:200 & 1:400	1-10 $\mu\text{g ml}^{-1}$ (1:2000-1:200)
Anti-Jagged1 & IgG	Goat anti-rabbit FITC	Sigma-Aldrich	1:100 & 1:200	Minimum of 1:80
	Goat anti-rabbit Texas Red	Vector Laboratories	1:100, 1:150 & 1:250	5-20 $\mu\text{g ml}^{-1}$ (1:300-1:75)
Anti-BTan20 & IgG	Goat anti-rat FITC	Sigma-Aldrich	1:100 & 1:200	1:32
Anti-CD166 & IgG	Rabbit anti-mouse FITC	Sigma-Aldrich	1:30, 1:50, 1:100, 1:200 & 1:400	1:200

Table 2.2: Table of secondary antibody concentrations used for optimisation

The primary antibodies shown above were titrated at concentrations 2.5, 5, 10 & 15 $\mu\text{g ml}^{-1}$ and each concentration was tested against the range of secondary antibody dilutions shown. The mid-range value of the secondary antibodies was determined by the manufacturers instructions or immunofluorescence carried out by co-workers.

2.3 Results

2.3.2 Immunofluorescence optimisation

Immunofluorescence optimisation is important to maximise the signal to noise ratio. Many factors have to be taken into account including tissue preparation and fixation, antibody concentration and antigen unmasking. In this study, cryosections were used to optimise all antibodies using paraformaldehyde and alcohol fixation. The optimised antibody concentrations were then tested on paraffin wax sections; CD29, CD49e and bTan20 were not tested on wax sections, as previous work in the laboratory had demonstrated valid conditions for immunofluorescence using frozen and wax sections and found them to be comparable (personal communication with Dr. Rebecca Williams). CD44, CD90, CD105, CD117, CD166, Notch1, Delta1, Jagged1, Jagged2 and Msx1 antibodies were all tested using wax and frozen sections, the cellular labelling did not differ between wax and frozen sections. However, in general more matrix autofluorescence was observed with wax sections.

All CD (cluster of differentiation) markers with the exception of CD29 were localised to the superficial zone of the tissue using paraformaldehyde and alcohol fixation. To determine whether this was an artefact of tissue preparation, the remaining CD markers were tested on wax sections and comparable results were observed with both fixations. CD44, CD90, CD105, CD117 and CD166 were also tested using acetone fixation. In addition, wax and frozen sections were treated with antigen unmasking. The cellular labelling did not alter with fixation or when pre-treated with enzymes.

Notch1, Delta1, Jagged1, Jagged2 and Msx1 were all observed throughout the tissue but not all cells were labelled. Antibody optimisation was carried out with the developmental markers, Notch1, its ligands and Msx1 but cellular labelling did not differ between fixations or tissue embedding procedures. Notch1 was also tested with acetone fixation and with antigen unmasking of both frozen and wax sections.

Following titration of the antibodies, background fluorescence was further reduced by the addition of blocking serum to the primary and secondary antibodies and pre-incubating the secondary antibody in bovine serum. Optimal antibody concentrations are shown in Tables 2.3 and 2.4.

Secondary Antibody	Optimal Dilution	Secondary Antibody	Optimal Dilution
Rabbit anti-goat Texas Red	1:150	Goat anti-rabbit Texas Red	1:150
Rabbit anti-goat FITC	1:200	Goat anti-rabbit FITC	1:200
Donkey anti-goat Alexafluor 488	1:400	Rabbit anti- mouse FITC	1:400
Goat anti-rat FITC	1:200		

Table 2.3: Table of secondary antibody optimal concentrations

The secondary antibodies were titrated using primary antibodies with the most cellular labelling: Jagged2 (goat), bTan20 (rat), Jagged1 (rabbit) and CD166 (mouse). The optimal concentrations are shown in this table.

Name (Isotype)	Optimal Dilution	Name (Isotype)	Optimal Dilution	Name (Isotype)	Optimal Dilution
Delta (goat)	10 $\mu\text{g ml}^{-1}$	BTan20 (rat)	10 $\mu\text{g ml}^{-1}$	CD90 (mouse)	10 $\mu\text{g ml}^{-1}$
Jagged1 (rabbit)	10 $\mu\text{g ml}^{-1}$	CD29 (mouse)	5 $\mu\text{g ml}^{-1}$	CD105 (mouse)	10 $\mu\text{g ml}^{-1}$
Jagged2 (goat)	10 $\mu\text{g ml}^{-1}$	CD44 (rat)	10 $\mu\text{g ml}^{-1}$	CD117 (mouse)	10 $\mu\text{g ml}^{-1}$
Msx1 (goat)	5 $\mu\text{g ml}^{-1}$	CD49e (mouse)	10 $\mu\text{g ml}^{-1}$	CD166 (mouse)	10 $\mu\text{g ml}^{-1}$
Notch1 (goat)	10 $\mu\text{g ml}^{-1}$				

Table 2.4: Table of primary antibody optimal concentrations

Following secondary antibody optimisation, the primary antibodies were titrated with concentrations 2.5, 5, 10 & 15 $\mu\text{g ml}^{-1}$. The remaining goat polyclonal antibodies were titrated with the optimum concentration of secondary antibodies.

2.3.3 Immunofluorescence of bovine articular cartilage

Results shown are representative of labelling seen with each antibody and each antibody was tested at least in triplicate.

All antibodies produced improved results when the primary antibody was incubated with 5% blocking serum and when the secondary antibody was pre-incubated with 5% bovine serum.

The results for each antibody and optimal fixatives are summarised in Table 2.5.

CD29

CD29 expression was most intense within the superficial zone of the tissue (Figure 2.4A) but was abundant throughout. It was also observed within the cytoplasm in addition to the cell membrane. The extent of the labelling did not vary with titration of the primary or secondary antibody. However, the least amount of background fluorescence was observed at $5 \mu\text{g ml}^{-1}$. The degree of labelling did not differ with either fixative (95% ETOH or 2% PFA). The antibody had already been tested on wax embedded tissue by co-workers and was not repeated in this study, as the results were comparable to frozen sections. Images shown were of cryosections fixed in paraformaldehyde (Figure 2.4).

CD44

CD44 expression was observed in a small number of cells in the most superficial 1-2 cell layers of the superficial zone (Figure 2.5A and C), no labelling was apparent in the rest of the tissue. Cells expressing the antigen were brightly labelled. There was no difference between 95% ETOH, 2% PFA or acetone fixation. Cryosections and wax embedded sections produced results that were comparable, however, wax sections produced a higher degree of autofluorescence. As the expression was limited to the superficial zone, the tissue was also digested with hyaluronidase and chondroitinase to ensure the restricted CD44 expression was not due to epitope masking. The enzymatic digest produced equivalent results. Labelling appeared superior with ethanol preserved tissue, images shown are of alcohol fixed cryosections digested with hyaluronidase and chondroitinase (Figure 2.5).

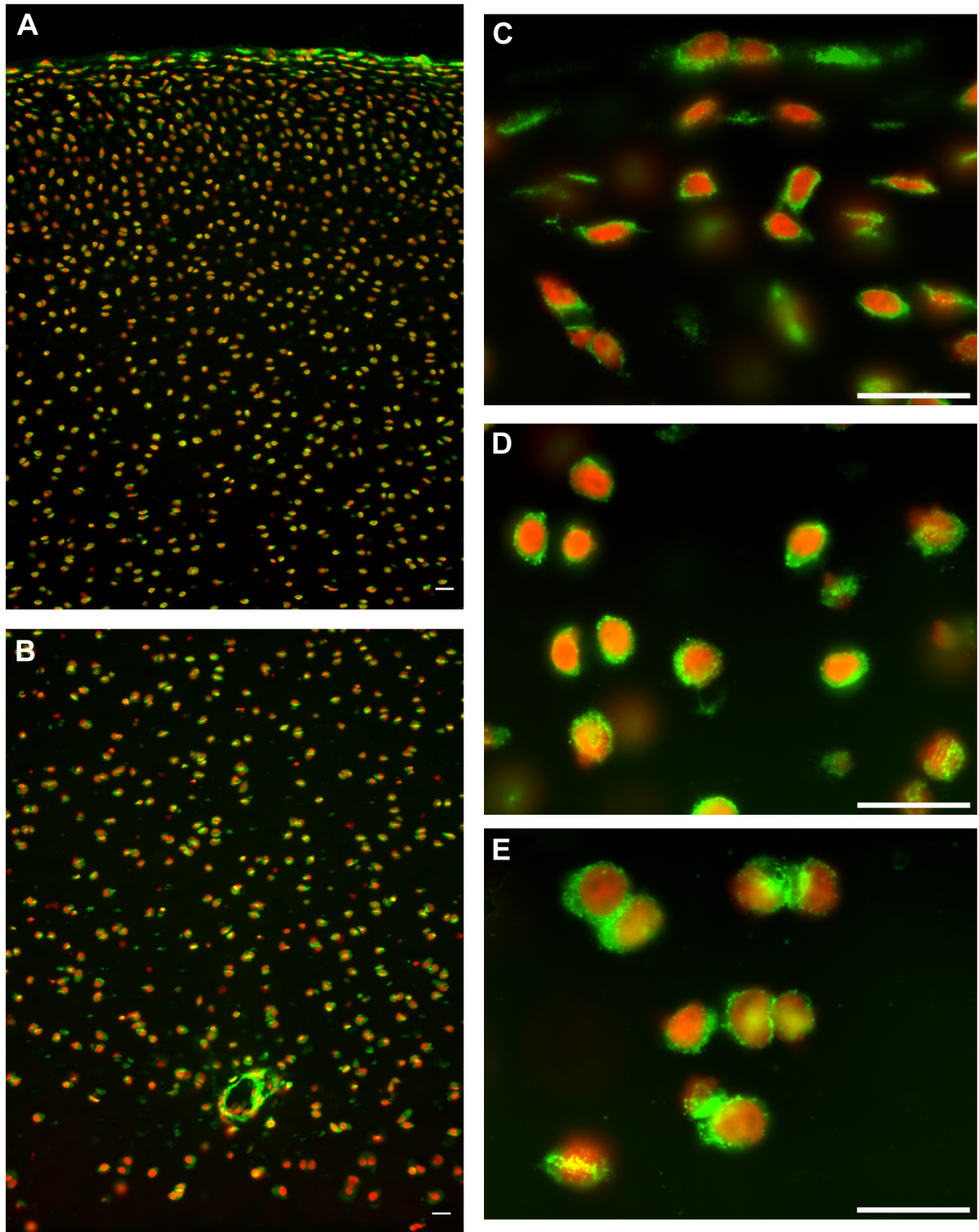


Figure 2.4: CD29 expression in full-depth immature articular cartilage

A and B: Low power images of full-depth tissue, **A:** surface and middle zones, **B:** middle and deep zones including a cartilage canal (transverse section [TS]). CD29 was distributed evenly throughout the depth of the tissue.

C, D and E: High power images (TS) of surface, middle and deep zones respectively.

Scale bars = 20µm

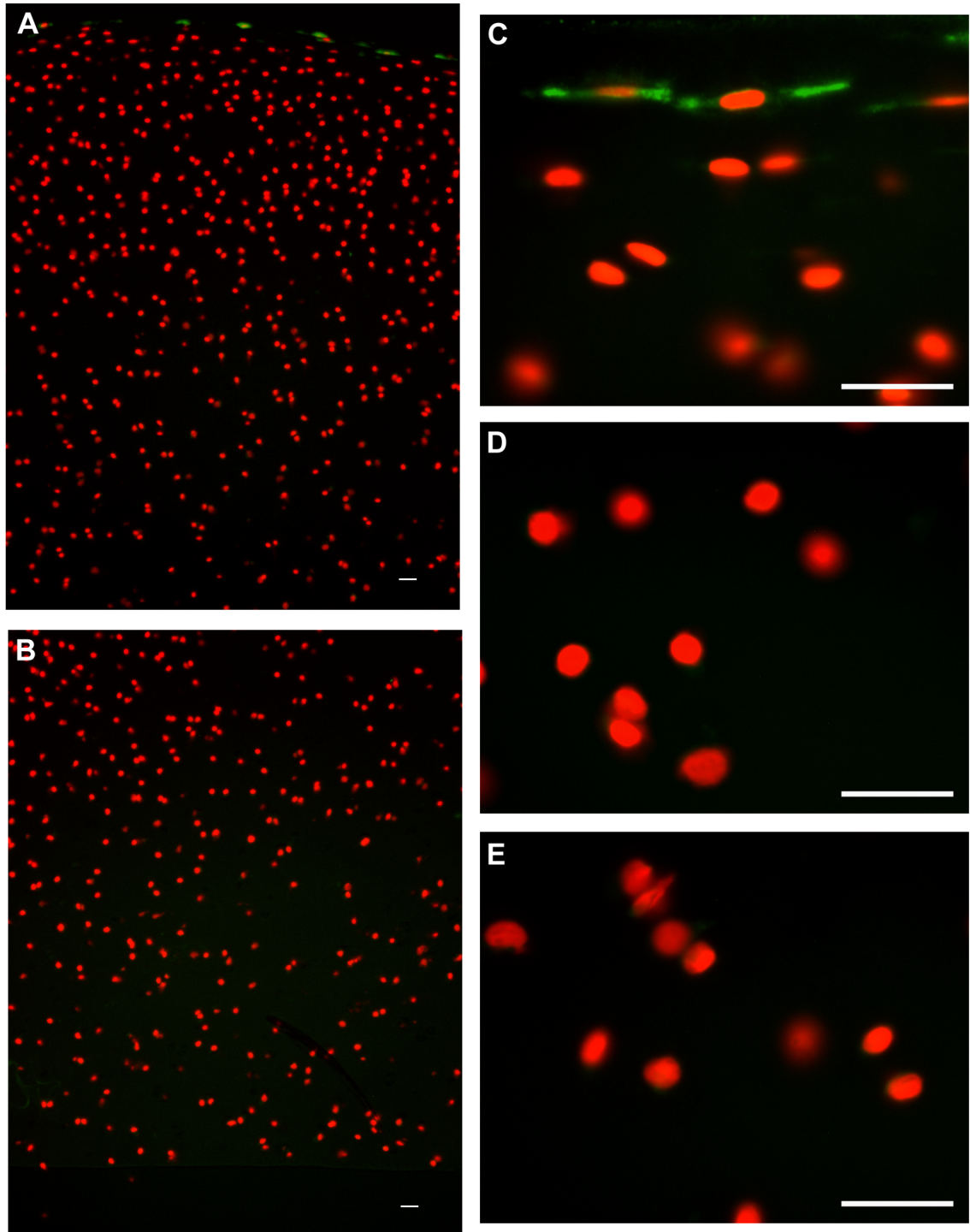


Figure 2.5: CD44 expression in full-depth immature articular cartilage

A and B: Low power images (TS) of full-depth tissue, **A:** surface and middle zones, **B:** middle and deep zones. CD44 was only evident within the most superficial layer of cells within the tissue.

C, D and E: High power images (TS) of surface, middle and deep zones respectively.

Scale bars = 20µm

CD49e

CD49e staining was carried out using cryosections only. Co-workers had previously demonstrated comparable results between wax embedded tissue and frozen sections. Sections were fixed in 95% ETOH or 2% PFA, paraformaldehyde producing superior results. Some very weak labelling was observed throughout the tissue, but intense labelling was only seen in the superficial 1-3 cell layers. Images shown are of paraformaldehyde fixed cryosections (Figure 2.6).

CD90

CD90 was not detected by immunofluorescence and only background labelling equivalent to the controls was observed. Cryosections and paraffin wax embedded sections were tested with this antibody using the fixatives: 95% ETOH, 2%PFA or acetone. The antibody was also tested after hyaluronidase and chondroitinase digest but the results did not differ. Images shown are of alcohol fixed cryosections (Figure 2.7).

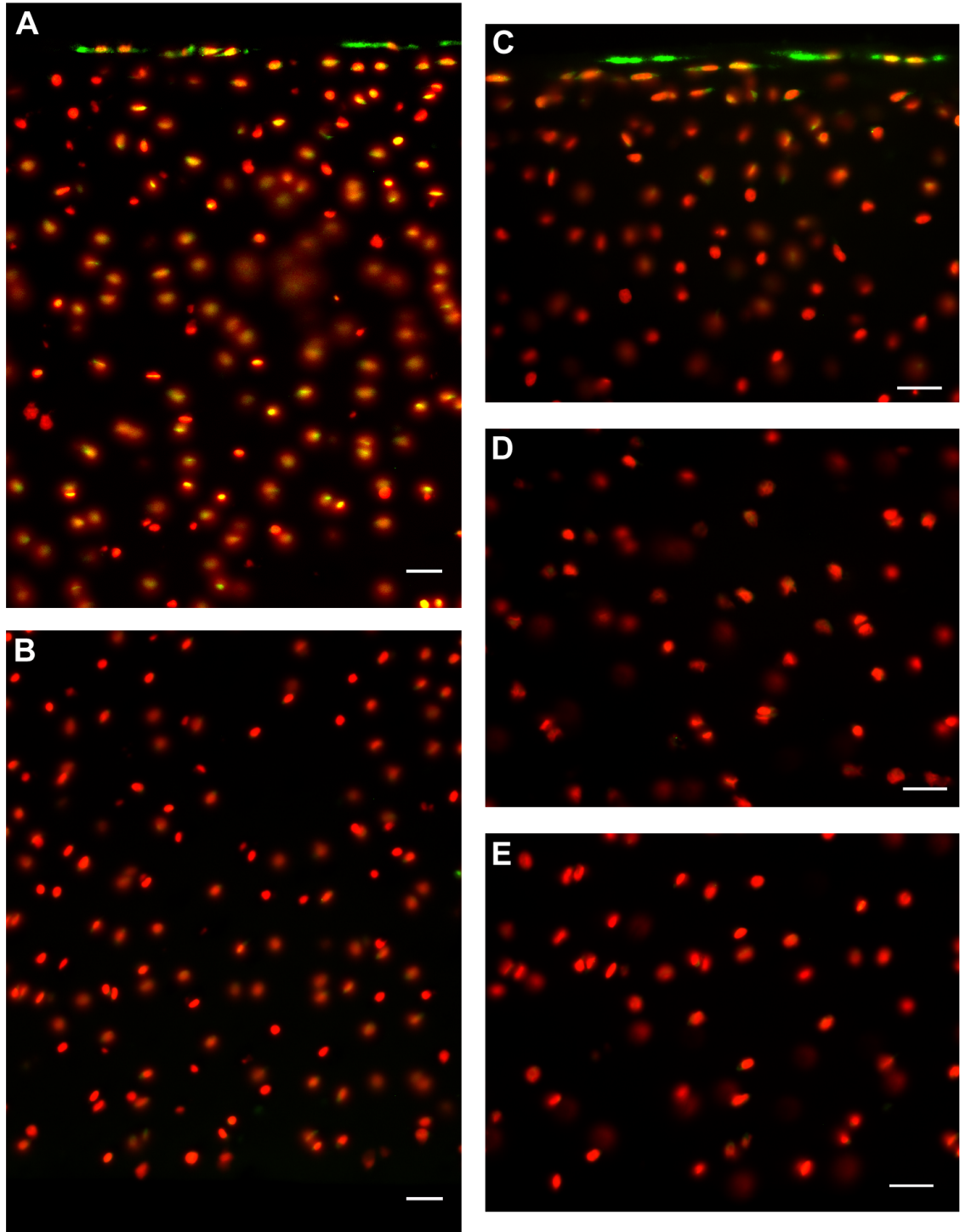


Figure 2.6: CD49e expression in full-depth immature articular cartilage

A and B: Low power images (TS) of full-depth tissue, **A:** surface and middle zones, **B:** middle and deep zones. CD49e was only observed within the upper 1-3 layers of the superficial zone.

C, D and E: High power images (TS) of surface, middle and deep zones respectively.

Scale bars = 20µm

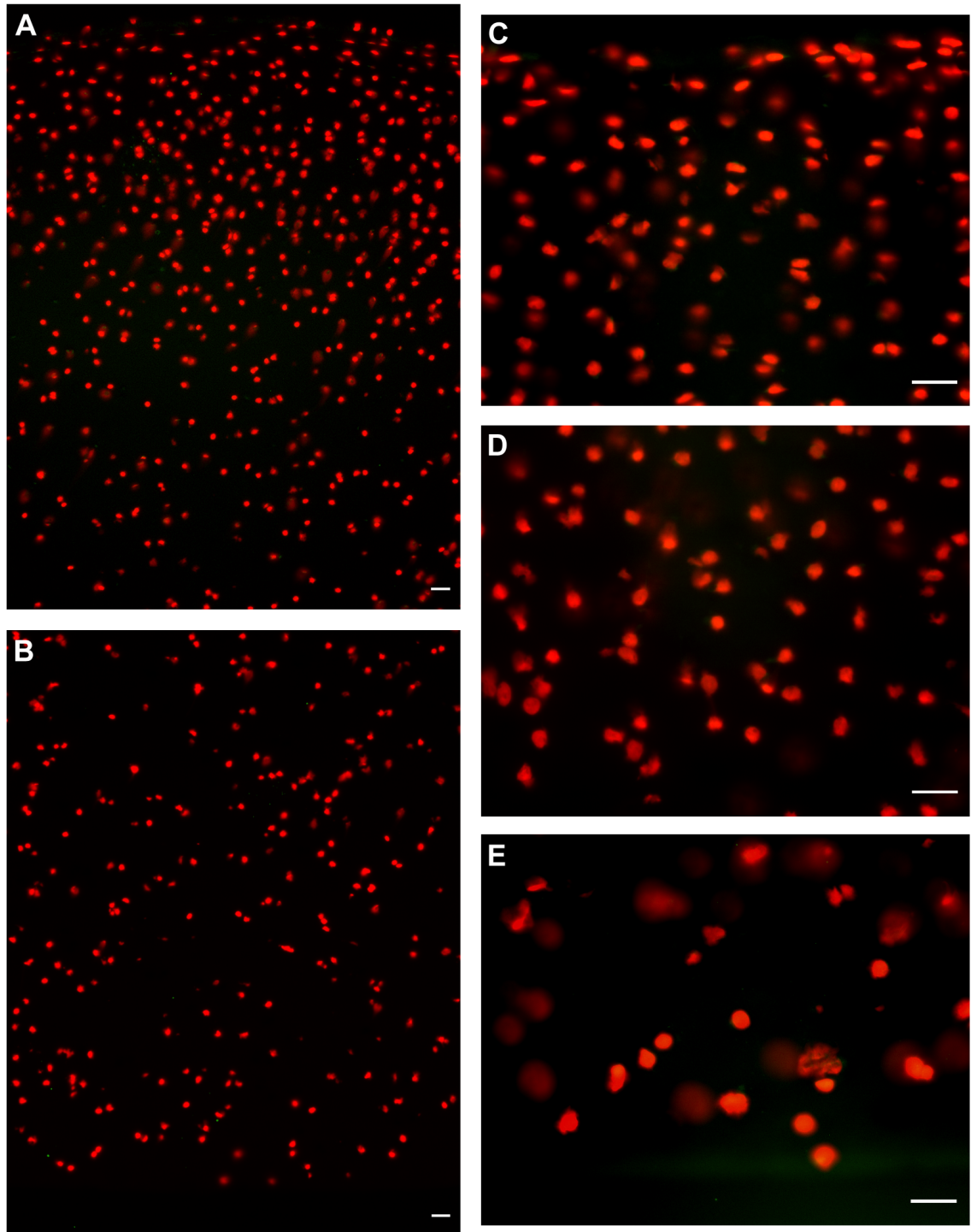


Figure 2.7: CD90 expression in full-depth immature articular cartilage

A and B: Low power images (TS) of full-depth tissue, **A:** surface and middle zones, **B:** middle and deep zones. CD90 was not detected by immunofluorescence in the tissue.

C, D and E: High power images (TS) of surface, middle and deep zones respectively.

Scale bars = 20µm

CD105

CD105 expression was observed at the articular surface within the upper 1-3 cell layers and the majority of cells in this region were labelled. This antibody was tested on cryosections and paraffin wax embedded tissue and fixed with acetone, 2% PFA or 95% ETOH. Sections were also treated with a hyaluronidase and chondroitinase digest. The antibody produced optimal results with frozen sections fixed in 2% PFA (Figure 2.8). Enzymatic digestion of the tissue had no effect on labelling.

CD117

CD117 expression was not detected by immunofluorescence and was identical to the controls. Cryosections and paraffin wax embedded sections were tested with this antibody using the fixatives: 95% ETOH, 2%PFA or acetone. The antibody was also tried after hyaluronidase and chondroitinase digest but the results did not differ. Images shown are of alcohol fixed cryosections digested with hyaluronidase and chondroitinase (Figure 2.9).

CD166

CD166 expression was confined to the superficial 1-2 cell layers of cartilage. Almost all of the cells within this superficial region expressed CD166, unlike CD44, CD49e and CD105. The antibody was tested on cryosections and paraffin wax sections and tissue fixed with 95% ETOH, 2% PFA or acetone. The antibody was also tried after unmasking with hyaluronidase and chondroitinase. Optimal results were obtained with cryosections fixed in paraformaldehyde (Figure 2.10). Enzymatic digestion of the tissue had no effect on labelling.

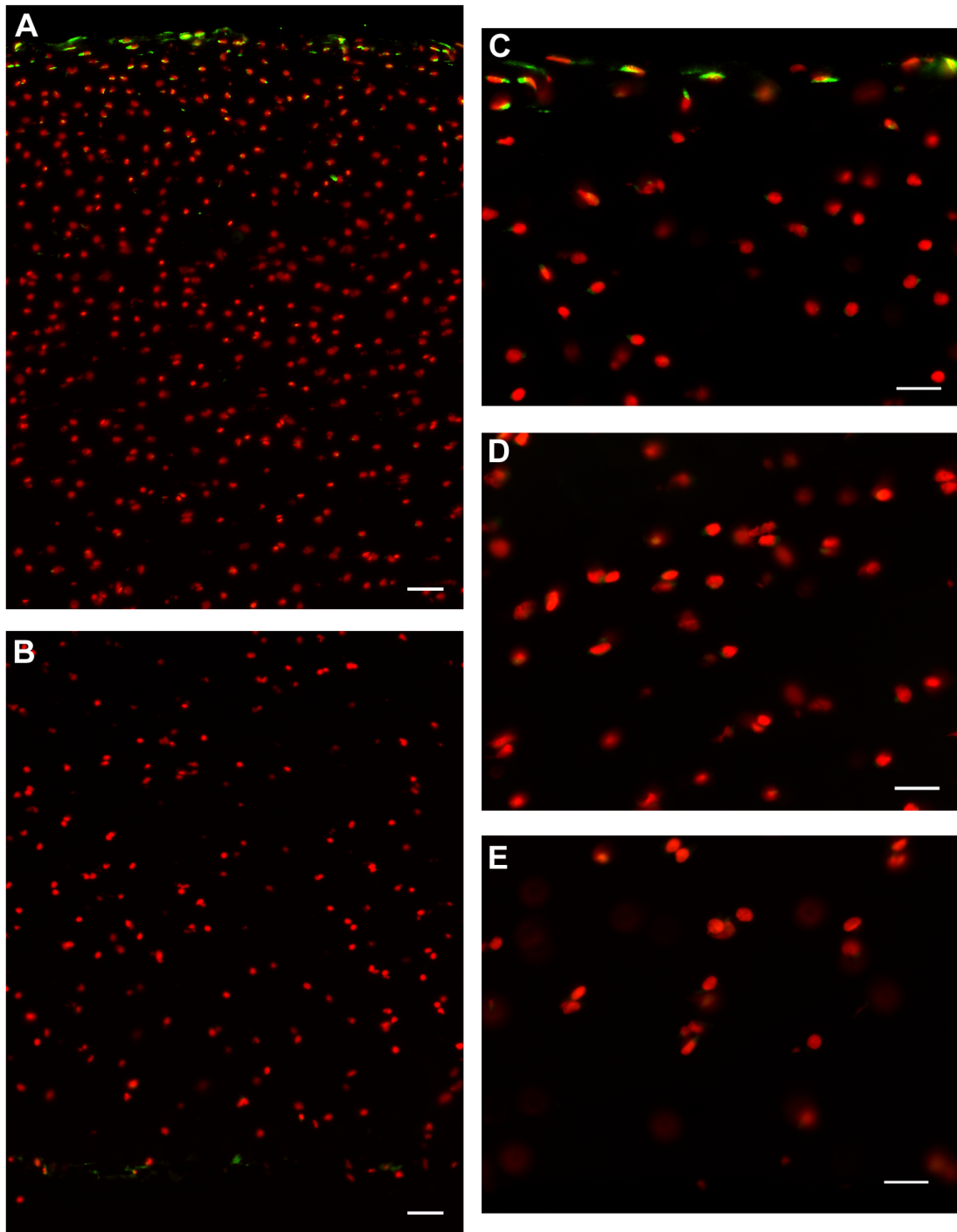


Figure 2.8: CD105 expression in full-depth immature articular cartilage

A and B: Low power images (TS) of full-depth tissue, **A:** surface and middle zones, **B:** middle and deep zones. CD105 expression was only observed within the upper 1-3 cell layers of the superficial zone.

C, D and E: High power images (TS) of surface, middle and deep zones respectively.

Scale bars = 20µm.

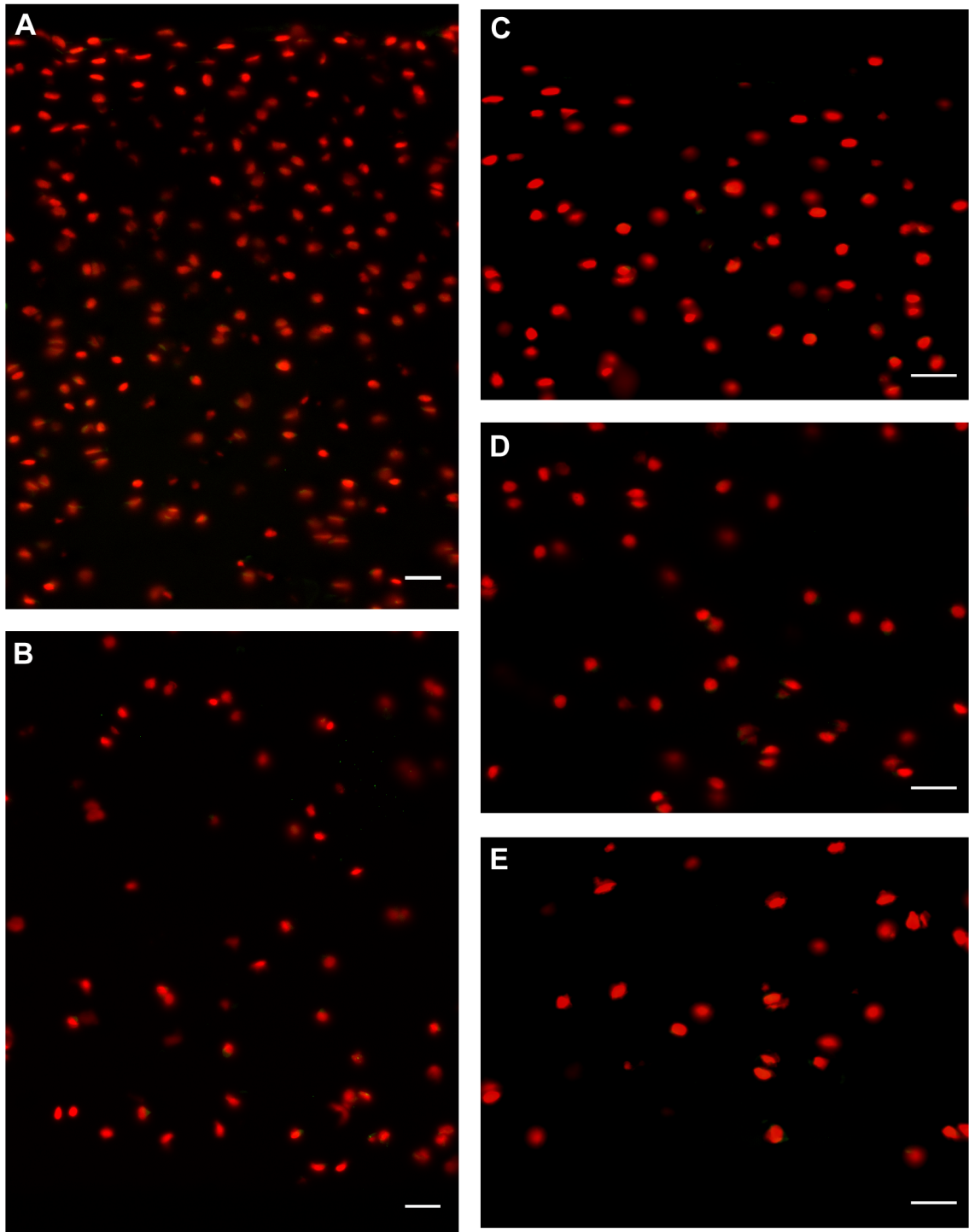


Figure 2.9: CD117 expression in full-depth immature articular cartilage

A and B: Low power images (TS) of full-depth tissue, **A:** surface and middle zones, **B:** middle and deep zones. CD117 expression was not detected by immunofluorescence in the tissue.

C, D and E: High power images (TS) of surface, middle and deep zones respectively.

Scale bars = 20 μ m

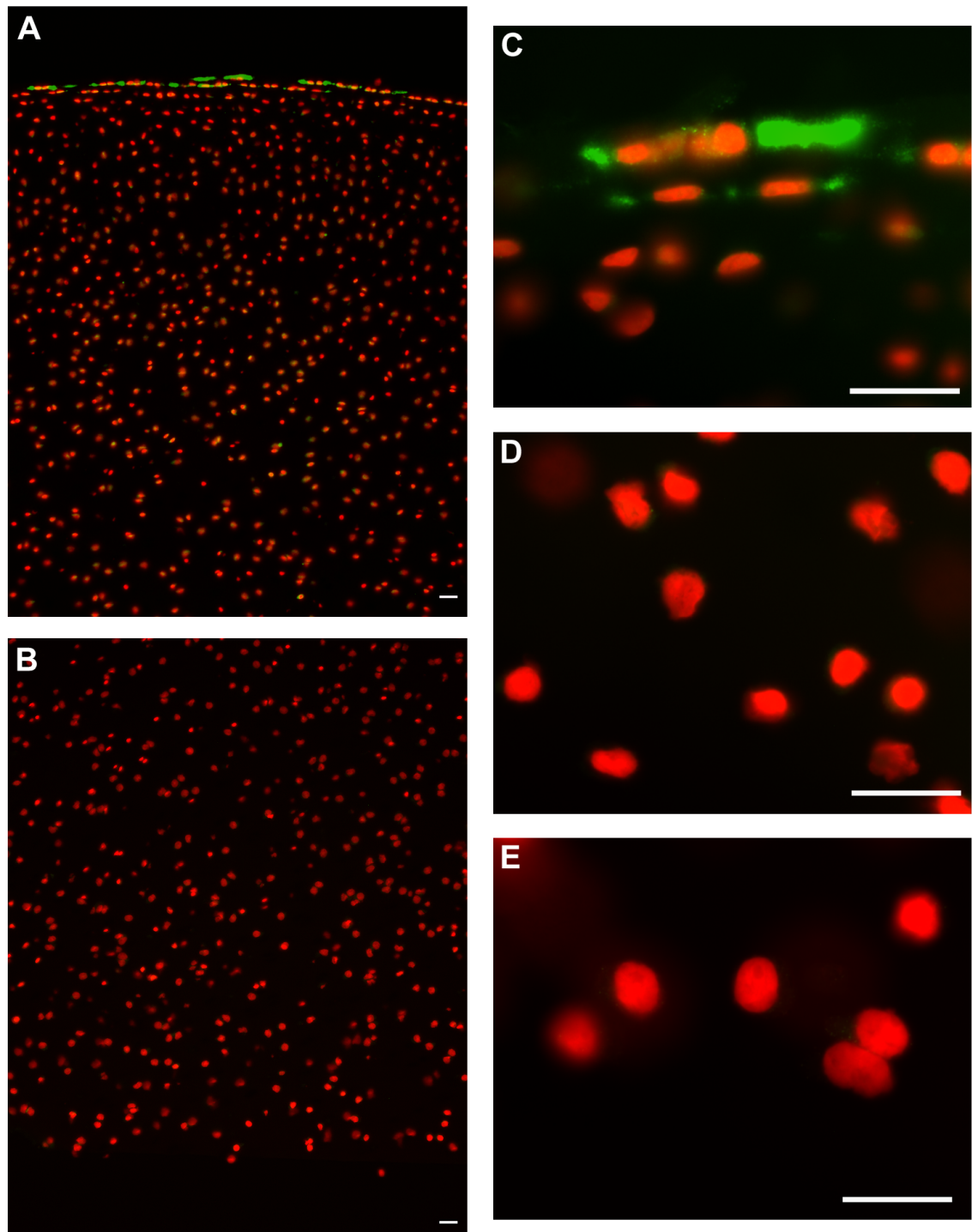


Figure 2.10: CD166 expression in full-depth immature articular cartilage

A and **B**: Low power images (TS) of full-depth tissue, **A**: surface and middle zones, **B**: middle and deep zones. CD166 labelling was only evident within the upper 1-2 cell layers of the tissue.

C, **D** and **E**: High power images (TS) of surface, middle and deep zones respectively.

Scale bars = 20µm

Notch1

C20 labelling for Notch1 was present throughout the tissue but was prevalent in the superficial and deep zones. The antibody was tested on cryosections and paraffin wax sections. The tissue was fixed in 95% ETOH, 2% PFA or acetone. The antibody was also tested after hyaluronidase and chondroitinase treatment, which had no effect on detection. Notch1 was detected on cryosections and paraffin wax sections although more background fluorescence was observed with wax sections. There was no difference between any of the fixatives and the images shown are of cryosections fixed in paraformaldehyde (Figure 2.11).

BTan20 binds to the intracellular region of Notch1, the labelling for Notch1 was comparable to C20 labelling, expression was primarily observed within the superficial and deep zones with a limited number of cells expressing the antigen within the middle zone. This antibody was only tested on cryosections that were fixed in 95% ETOH or 2% PFA. Paraformaldehyde fixed sections produced better labelling (Figure 2.12).

Two antibodies for Notch1 were used that bind to different regions of the protein, to further test the specificity of the antibodies. The distribution of labelling was comparable with both antibodies however; C20 demonstrated more intense cellular expression.

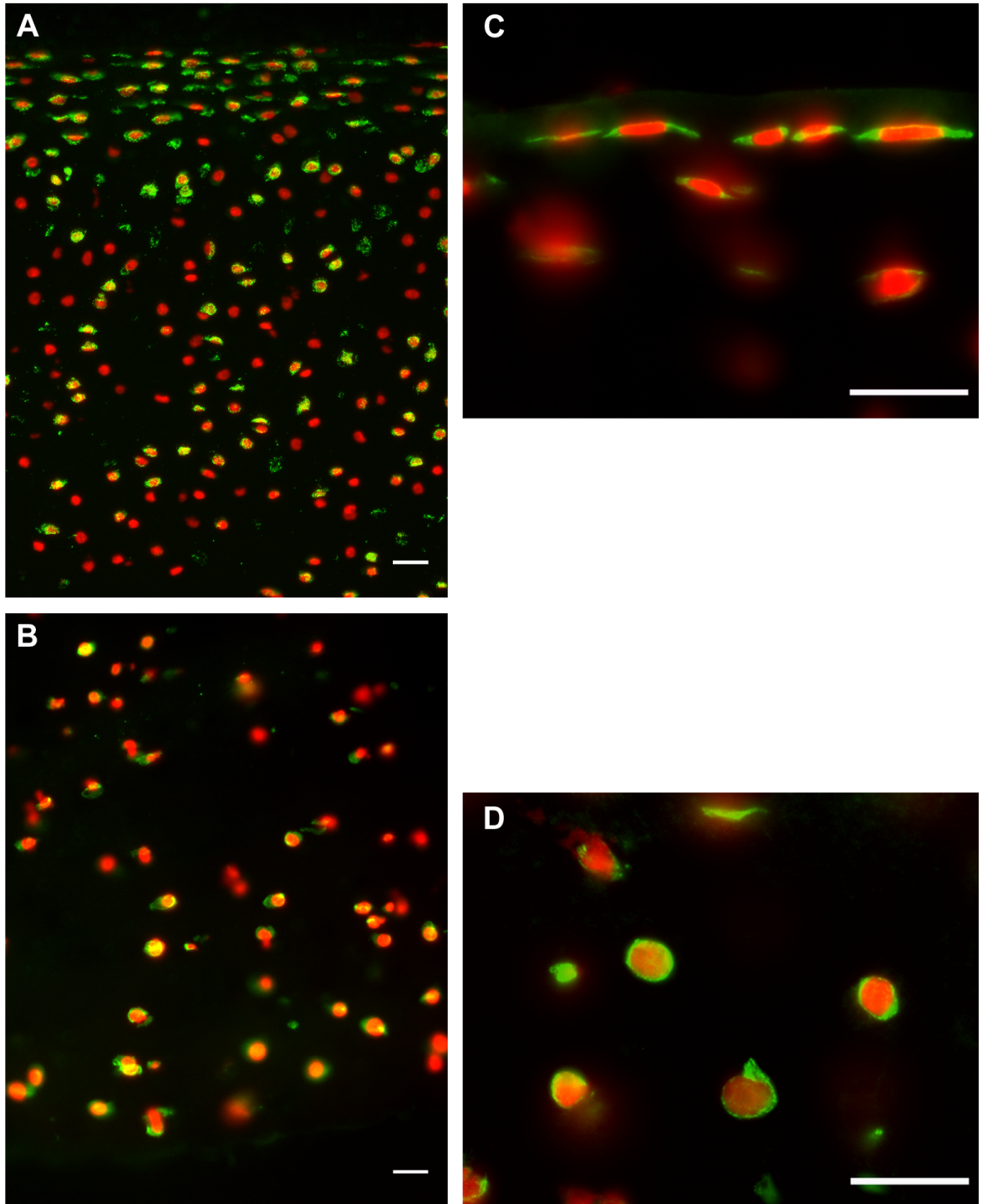


Figure 2.11: Notch1 (C20) expression in full-depth immature articular cartilage

A and **B**: Low power images (TS) of full-depth tissue, **A**: surface and middle zones, **B**: middle and deep zones. Notch1 (C20) expression was observed throughout the tissue but predominantly in the superficial and deep zones.

C, **D** and **E**: High power images (TS) of surface, middle and deep zones respectively.

Scale bars = 20µm

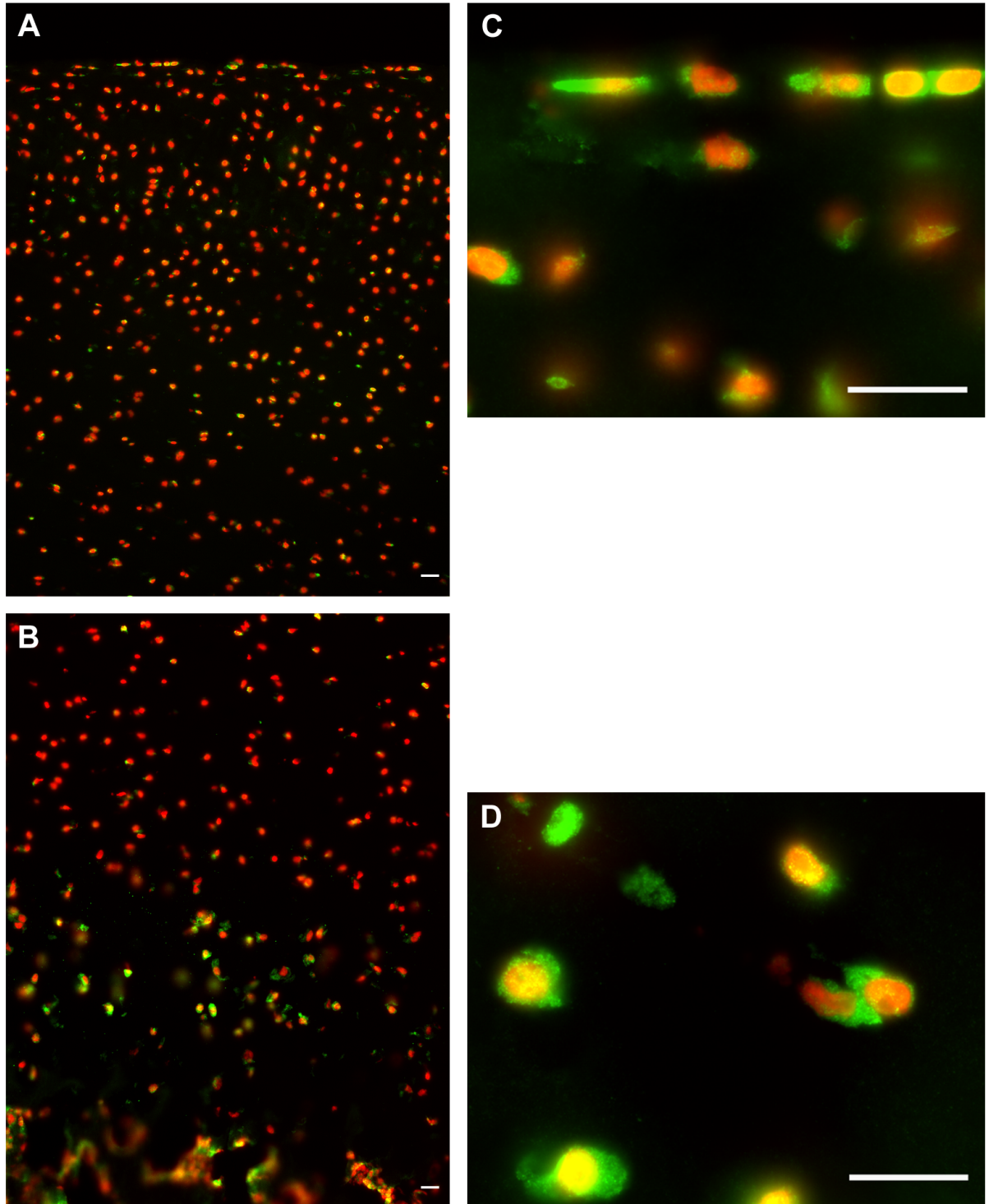


Figure 2.12: Notch1 (bTan20) expression in full-depth immature articular cartilage
A and B: Low power images (TS) of full-depth tissue, **A:** surface and middle zones, **B:** middle and deep zones. Notch1 (bTan20) expression was observed throughout the tissue but predominantly in the superficial and deep zones.
C, D and E: High power images (TS) of surface, middle and deep zones respectively.
 Scale bars = 20µm

Delta1

Delta1 labelling was observed throughout the tissue. Labelling was most extensive within the superficial and deep zones of the tissue. Delta1 was detected on frozen and wax sections, fixed in 95% ETOH or 2% PFA. Paraformaldehyde fixed frozen sections produced superior results and images shown are of this fixative (Figure 2.13).

Jagged1

Jagged1 labelling was observed primarily in the superficial and deep zones of the tissue although some weak labelling was present in the middle zone. The antibody was tested on frozen and wax sections, fixed in 95% ETOH or 2% PFA. Paraformaldehyde fixed frozen sections produced optimal results and images shown are of sections treated with this fixative (Figure 2.14).

Jagged2

Jagged2 labelling was observed throughout the tissue with intense expression observed in the deep zone. Jagged2 was detected with paraffin sections and cryosections although more background fluorescence was observed with wax sections. Labelling was seen with both fixatives (PFA and ETOH); however, the labelling was superior with paraformaldehyde fixed sections. Images shown are of cryosections fixed with paraformaldehyde (Figure 2.15).

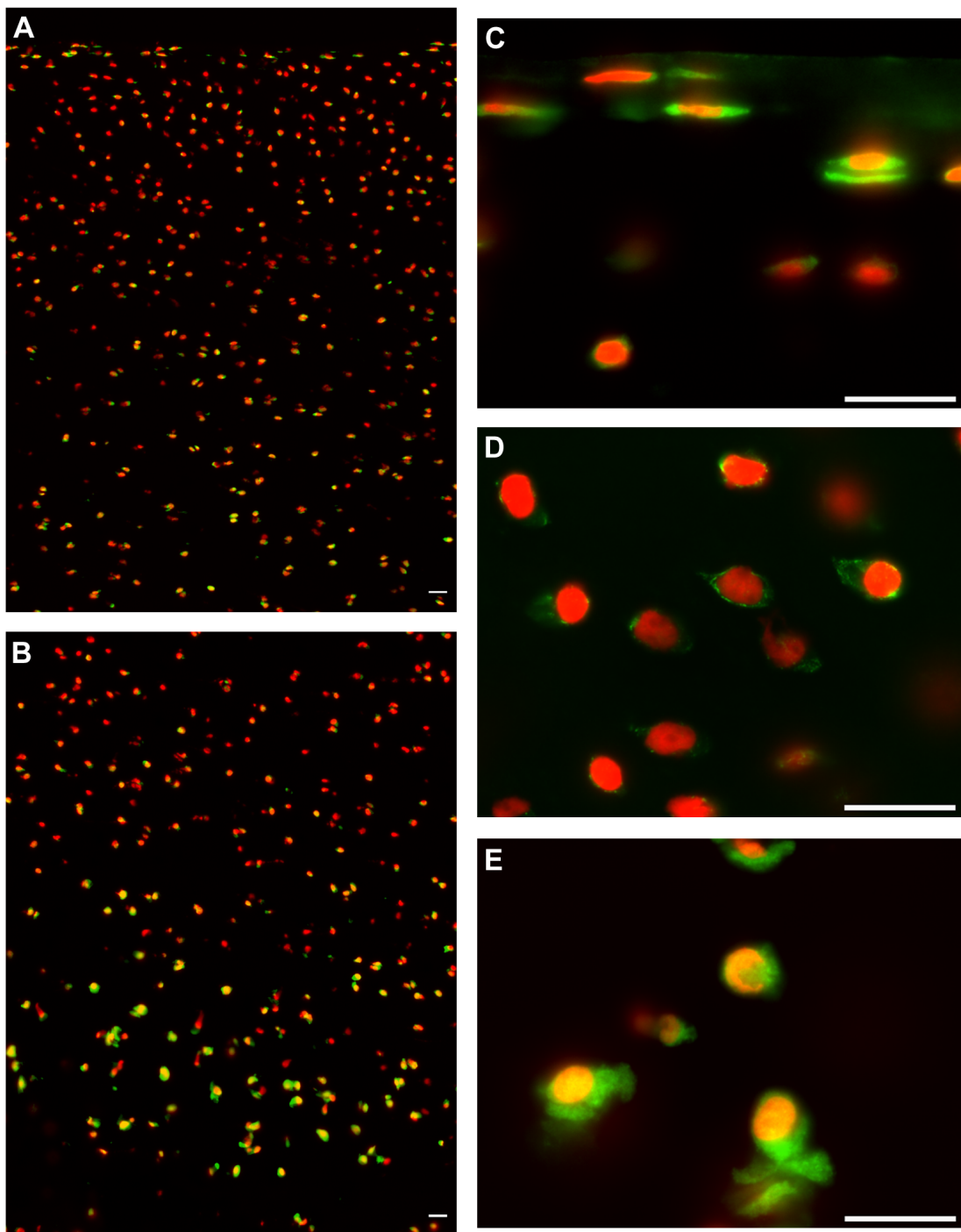


Figure 2.13: Delta1 expression in full-depth immature articular cartilage

A and **B**: Low power images (TS) of full-depth tissue, **A**: surface and middle zones, **B**: middle and deep zones. Intense Delta1 labelling was observed in the superficial and deep zones of the tissue with weaker expression in the middle zone.

C, **D** and **E**: High power images (TS) of surface, middle and deep zones respectively.

Scale bars = 20 μ m

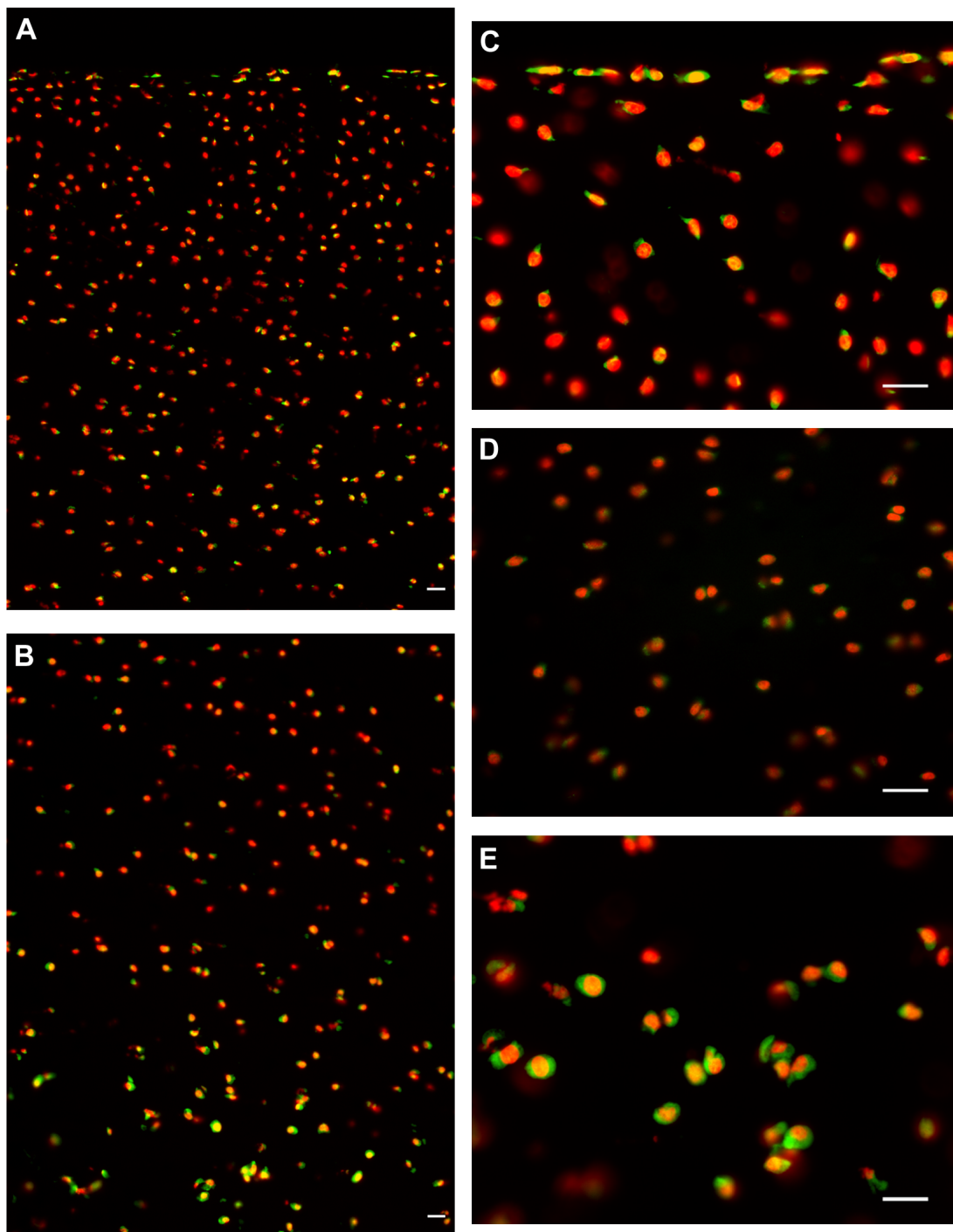


Figure 2.14: Jagged1 expression in full-depth immature articular cartilage

A and **B**: Low power images (TS) of full-depth tissue, **A**: surface and middle zones, **B**: middle and deep zones. Jagged1 expression was observed in the superficial and deep zones of the tissue with weaker expression in the middle zone.

C, **D** and **E**: High power images (TS) of surface, middle and deep zones respectively.

Scale bars = 20 μ m

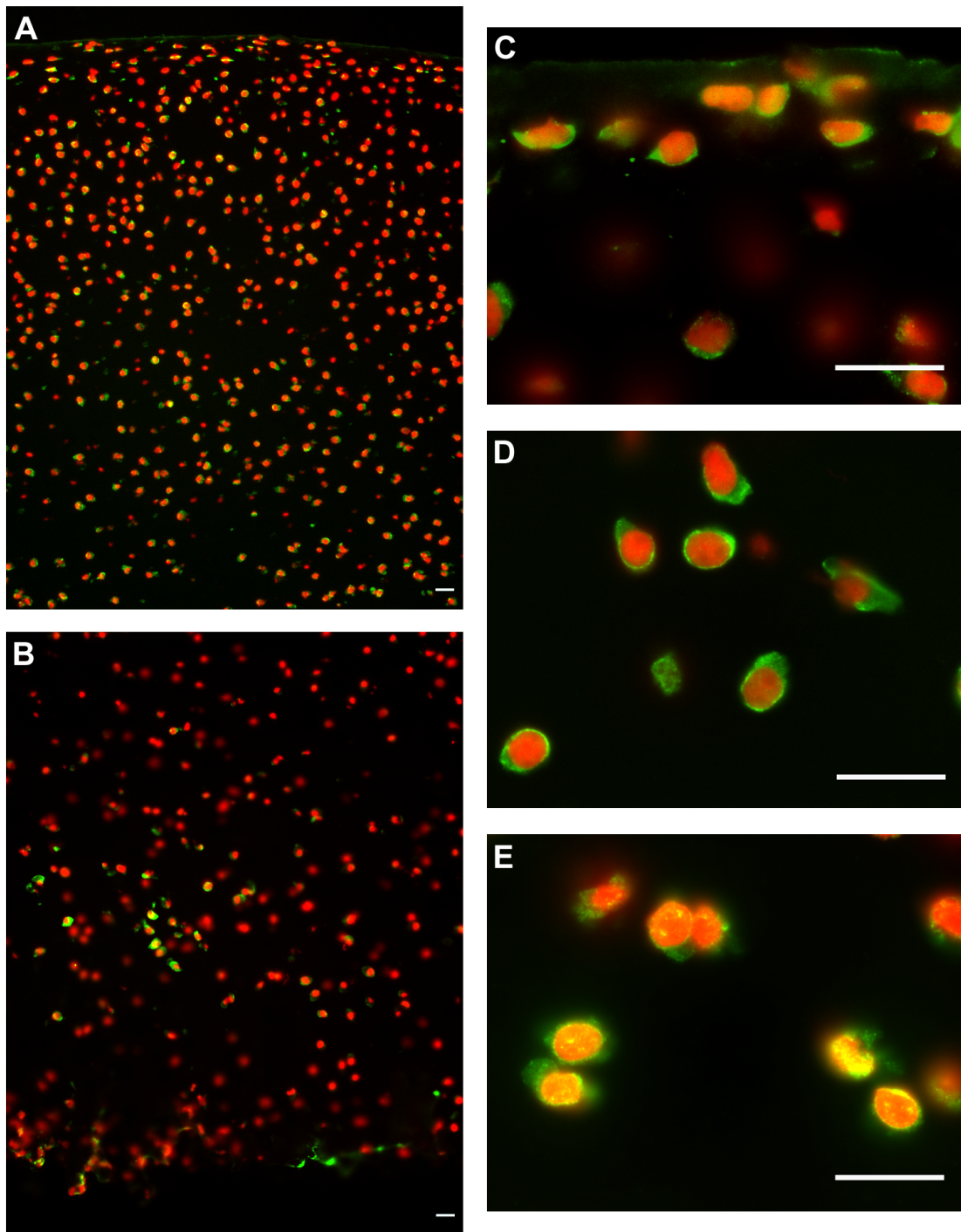


Figure 2.15: Jagged2 expression in full-depth immature articular cartilage

A and B: Low power images (TS) of full-depth tissue, **A:** surface and middle zones, **B:** middle and deep zones. Jagged2 expression was observed throughout the tissue.

C, D and E: High power images (TS) of surface, middle and deep zones respectively.

Scale bars = 20 μ m

Msx1

Msx1 labelling was observed throughout the depth of tissue, although not all cells were labelled. Msx1 was detected on cryosections and paraffin wax sections, fixed in 95% ETOH or 2% PFA. Paraformaldehyde fixed wax sections produced superior results and images shown are of sections treated with this fixative (Figure 2.16).

Controls

Controls were processed by omitting primary antibody and, instead, incubating the sections with appropriate species-specific IgG or PBS/T. Sections were also incubated with PBS/T alone to detect autofluorescence within the tissue. Controls were all negative (Figure 2.17).

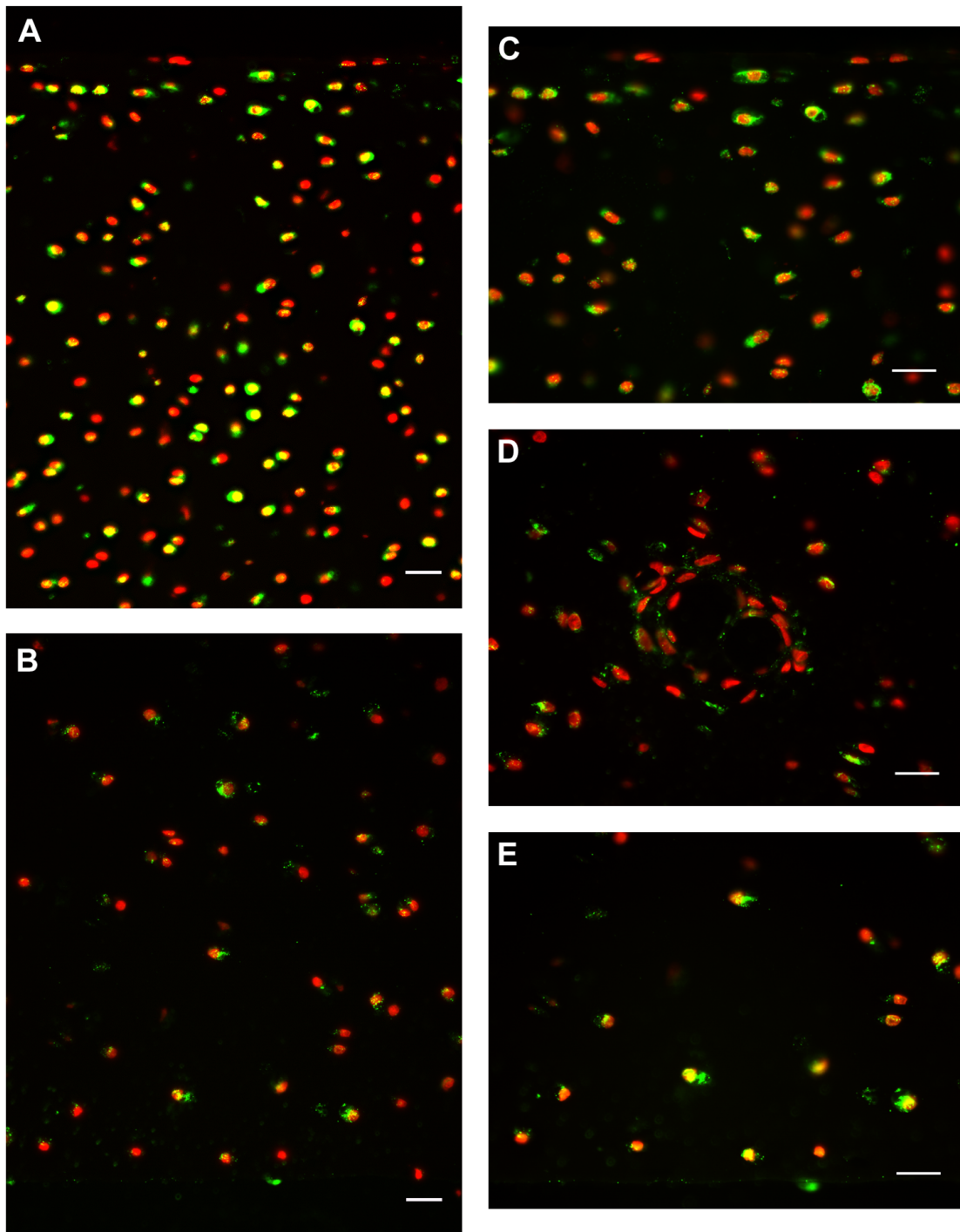


Figure 2.16: Msx1 expression in full-depth immature articular cartilage

A and **B**: Low power images (TS) of full-depth tissue, **A**: surface and middle zones, **B**: middle and deep zones. Msx1 expression was observed throughout the tissue zones but not all cells were labelled.

C, **D** and **E**: High power images (TS) of surface, middle (including cartilage canal) and deep zones respectively. Scale bars = 20µm

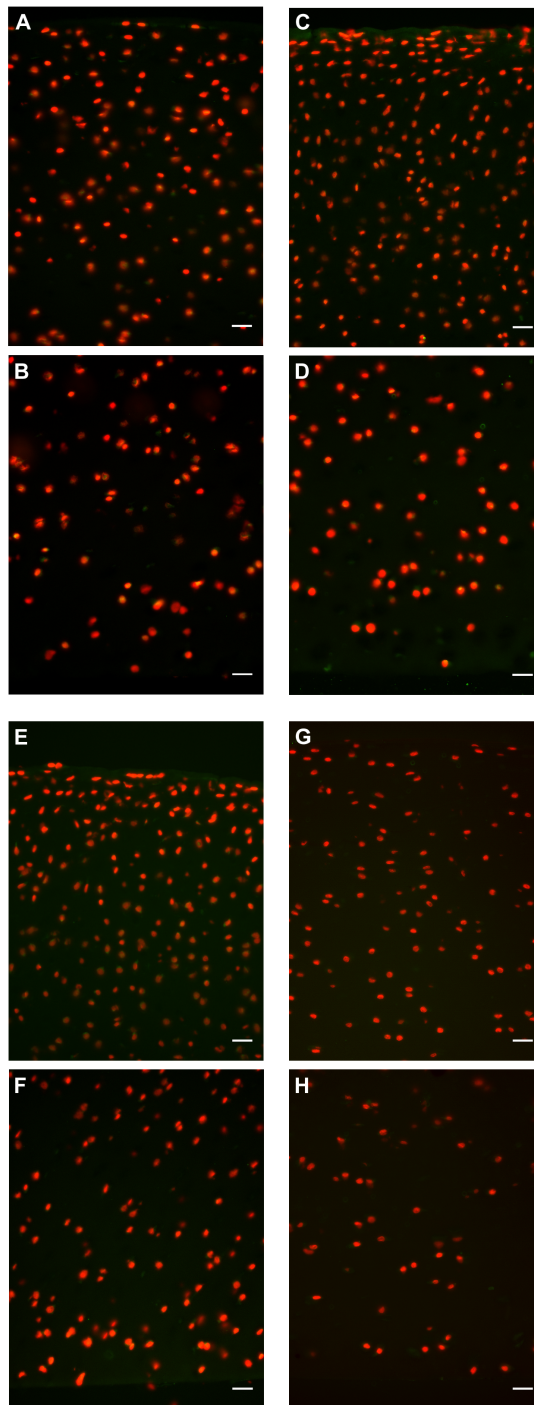


Figure 2.17: Control images in full-depth immature articular cartilage

Low power images (TS) of full-depth tissue, all controls were negative:

A and **B**: Mouse IgG control; **C** and **D**: Rat IgG control;

E and **F**: Rabbit IgG control; **G** and **H**: Goat IgG control.

A, **C**, **E** and **G**: surface and middle zones, **B**, **D**, **F** and **H**: middle and deep zones.

Scale bars = 20 μ m

Antigen recognised	Optimum Fixative	Zones in which labelling was present
CD29	95% ethanol or 2% PFA	All zones
CD44	95% ethanol	Superficial
CD49e	2% PFA	Superficial
CD90	95% ethanol or 2% PFA	No labelling
CD105	2% PFA	Superficial
CD117	95% ethanol or 2% PFA	No labelling
CD166	2% PFA	Superficial
Notch1 (C-20)	95% ethanol or 2% PFA	Mainly superficial and deep
Notch1 (bTan20)	2% PFA	Mainly superficial and deep
Delta	2% PFA	Mainly superficial and deep
Jagged1	2% PFA	Mainly superficial and deep
Jagged2	2% PFA	All zones
Msx1	2% PFA	All zones

Table 2.5: Summary of results

The table summarises the optimal conditions and results for each primary antibody used.

2.4 Discussion

In this study, all cell surface markers tested, except CD90 and CD117 were located within the superficial zone of articular cartilage. CD29 was also expressed in the middle and deep zones. The developmental markers: Notch1, Delta1, Jagged1, Jagged2 and Msx1 were located throughout the tissue but not all cells were labelled. To determine whether the restricted expression of CD44, CD49e, CD105 and CD166 and absence of CD90 and CD117 were not due to sample preparation and that optimal conditions for antibody binding were achieved, tissue was embedded in paraffin wax or snap-frozen, and a selection of fixation methods were used including: paraformaldehyde; alcohol and acetone. Acetone fixation was only used to test CD44, CD90, CD117, CD105, CD166 and Notch1. CD29 and CD49e were not tested as colleagues had already tested these and they were found to be comparable to PFA and ETOH fixation.

Wax embedding results in superior preservation of the tissue architecture compared with freezing. The processing of wax embedded tissue can lead to reduced immunoreactivity and when combined with formaldehyde fixation can result in increased tissue autofluorescence. Frozen sections generally display inferior morphological tissue preservation compared with wax sections but produce improved immunofluorescent labelling, as epitopes are better preserved (Farmilo and Stead, 2009, Hayes et al., 2008). Due to the inherent autofluorescence generally observed in articular cartilage, both frozen and wax embedded sections were used to obtain optimal results. Autofluorescence associated with adult human articular cartilage (Pretzel et al., 2011) were not as apparent using immature bovine articular cartilage and changes in fixation did not significantly affect the labelling observed in the tissue sections.

When CD44, CD90, CD105, CD117, and CD166 were pre-treated with hyaluronidase and chondroitinase no difference in expression was observed. In a similar study, Ostergaard et al (1997) also found no difference between enzymatically treated or untreated human tissue. This is in contrast to other studies where incubation with

protease K showed improved results in formaldehyde-fixed wax sections of rat and human articular cartilage (Ozbey et al., 2010, Pretzel et al., 2011).

Of the cell surface markers tested, CD29 was the most abundant, which is comparable with previous studies (Salter et al., 1992, Dowthwaite et al., 2004). In Dowthwaite et al (2004) CD49e was expressed in the majority of cells at the cell surface but the intensity and number of chondrocytes labelled decreased with tissue depth, using flow cytometry, 79% of superficial zone cells expressed CD49e compared to 5% and 2.5% of middle and deep zones chondrocytes respectively. The zonal distribution shown in the Dowthwaite study is comparable to expression observed in this Chapter, where very weak labelling was seen throughout the tissue but intense CD49e labelling was only detected within the upper 1-2 cell layers of the superficial zone.

CD166 was present within the majority of the upper 1-2 cell layers of the superficial zone. CD105 was also only located within the superficial zone but at a much lower cell intensity and fewer cells were labelled. In studies using adult rat articular cartilage, CD105 and CD166 expression were observed throughout the tissue depth, however intense CD105 labelling was observed in the superficial zone (Ozbey et al., 2010). In a second study, the distribution of CD166 in normal and OA human articular cartilage was restricted to the superficial and middle zone chondrocytes (Pretzel et al., 2011). The variations in zonal distribution of CD105 and CD166 expression observed in these studies may be due to differences in maturity of tissue or species. Differences in integrin expression between mature and foetal chondrocytes has previously been reported by Salter et al (1995) in human knees.

CD44 is the primary HA receptor, mediating both cell-cell and cell-matrix interaction (Knudson and Knudson, 2004). The reports of CD44 expression in articular cartilage vary in their zonal distribution, Bosch et al. (2002) described CD44 expression in the middle and deep zones but not in the superficial zone, whilst Ostergaard et al (1997) identified CD44 within the superficial zone and decreased expression in the middle and deep zone chondrocytes. In this study, CD44 was located in the superficial zone only. The restricted expression observed in the present study may be an artefact of tissue preparation causing receptors to be masked. However, enzymatic pre-treatment did not result in increased detection, so this is unlikely to be a factor. Variations in

expression of CD44 between this study and previous ones may be due to the immaturity of the tissue and isotropic organisation. Although CD44 is the predominant HA receptor in articular cartilage it is not the only receptor. CD44 is known to interact with other extracellular proteins such as fibronectin and this protein has been demonstrated within the superficial and middle zones of immature cartilage (Dowthwaite et al., 2004). Fibronectin adherent superficial zone cells exhibit a greater colony forming efficiency and thus enriches for chondroprogenitors.

CD90 and CD117 were not observed with any of the variations in fixation or enzyme pre-treatment. CD90 has not been reported within matrix-intact articular cartilage. Colonies (>32 cells) taken from fibronectin-adherent chondrocytes from human and equine articular cartilage and expanded in monolayer have been shown to express CD90 (Williams et al., 2010, McCarthy et al., 2011). CD90 expression has also been shown in full-depth chondrocytes cultured for 14 days in monolayer (Diaz-Romero et al., 2005). CD117 has not previously been identified within articular cartilage but has been observed in isolated chondrocytes at low levels (de la Fuente et al., 2004). It is a multipotent marker identified in mesenchymal stem cells and within synovial membranes from normal and OA patients (Hermida-Gomez et al., 2011, Gimeno et al., 2005). CD90 and CD117 are expressed in multipotent cells and may be characteristic of chondrocytes that have dedifferentiated in monolayer culture; this will be investigated further in Chapter 3.

The differentiation markers Notch1, Delta1, Jagged1 and Jagged2 were all observed throughout the tissue. Notch1 expression was most intense at the surface and deep zones. This pattern of superficial and deep zone expression was also observed with Delta1 and Jagged1 antibodies. Jagged2 expression was most intense within the deep zone.

Notch1 expression shown in this study is comparable to observations obtained by Dowthwaite and co-workers in immature bovine articular cartilage (Dowthwaite et al., 2004). Notch1 and its ligands have been studied during development in both chick and the mammalian model. In chick, the expression of Notch1, Delta1, Jagged1 and Jagged2 is widespread within the developing anlagen although as development progresses becomes restricted to the superficial fibrocartilage (Williams et al., 2009).

During mouse development, Hayes et al (2003) observed Notch1 expression at the articular surface up to birth but was later restricted to the deeper zones. Jagged2 was present throughout the developing tissue before birth and Delta1 was predominantly present within the epiphyseal and not the articular cartilage, Jagged1 was not detected. Postnatal, Jagged2 and Jagged1 were observed weakly in the deeper and middle layers respectively but then increased their distribution throughout the tissue, only absent from the first 1-2 layers of cells. Delta1 was present in the surface 2-3 cell layers and in some middle and deep zone cells (Hayes et al., 2003).

A number of studies have investigated Notch1 and its ligands within mature articular cartilage, the majority reported Notch1 expression throughout the depth of human (Hiraoka et al., 2006, Ustunel et al., 2008) or rat (Ozbey et al., 2010) articular cartilage. However, Notch1 expression has also been described at a greater density within the superficial zone or restricted to this zone in mature human articular cartilage (Grogan et al., 2009, Karlsson et al., 2007), which is similar to results observed in this study using immature bovine articular cartilage. Karlsson et al (2007) also reported that Jagged1 and Jagged2 were localised to a small number of cells within the superficial zone of human articular cartilage, but Delta1 was detected throughout the full thickness, except for the deepest layers. However, Ustunel and colleagues observed Delta1 and Jagged1 expression throughout human articular cartilage, with Delta1 and Jagged1 expression most intense within the superficial zone and deep zone respectively (Ustunel et al., 2008). The distribution of Delta1 and Jagged1 expression demonstrated by Ustunel et al (2008) are similar to results shown in this study, with Jagged1 and Delta1 expression most intense within the superficial zone of articular cartilage.

Msx1 was observed throughout the tissue but not all cells were labelled. Msx1 has not been detected in adult articular cartilage, but has been identified within the developing limb (Foerst-Potts and Sadler, 1997). Msx1 down regulation is associated with terminal differentiation of several cell types including cartilage and muscle. In muscle, Msx1-forced expression results in a highly proliferative transformed phenotype and blocks myogenic terminal differentiation through the inhibition of *MyoD*. Thus, Msx1 is thought to prevent differentiation and enhance proliferation. Msx1 is also able to decrease *Cbfa1*, essential for osteoblast differentiation, mice

lacking this factor exhibit an absence of bone formation (Blin-Wakkach et al., 2001). The presence of *Msx1* throughout immature bovine articular cartilage could be a result of proliferation occurring within the progenitor and transit amplifier populations.

It is clear from the results shown that the superficial zone is important for growth as all markers tested, with the exception of CD90 and CD117, were expressed in the superficial zone, with CD44, CD49e, CD105 and CD166 localised to this region. Indeed, Hunziker et al (2007) investigated the hypothesis of appositional growth in immature rabbits. Post-partum the articular cartilage was isotropic in chondrocytic organisation. Two months after birth, the depth of the tissue and cell density had decreased and the cells were slightly more spatially oriented. The rabbit is sexually mature at three months and exhibited an anisotropic structure with a further decrease in tissue depth, characteristic of adult articular cartilage. This study demonstrated that articular cartilage grows by a process of appositional growth from the superficial zone and tissue resorption with the articular cartilage acting as a surface growth plate. The immature cartilage is gradually resorbed and eliminated and only post puberty are the precursor cells within the superficial zone reprogrammed to produce mature-type articular cartilage (Hunziker et al., 2007, Hunziker, 2010).

Results here, and in several previous studies have described the presence of progenitor cells at the articular surface of developing or immature joints (Williams et al., 2009, Dowthwaite et al., 2004, Hayes et al., 2003). The superficial zone of immature bovine articular cartilage has already been shown to express increased levels of Notch1 (Dowthwaite et al., 2004), the Dowthwaite study also demonstrated that superficial and middle zone cells had a high affinity to fibronectin, but only the superficial zone cells formed colonies. Chondroprogenitor colonies from adult human tissue were also shown to express Notch1 whereas full-depth colonies did not (Ustunel et al., 2008). Further evidence was provided by Hayes et al (2001) where a proportion of the superficial zone exhibited an extended cell cycle time when treated with BrDu. BrDu is incorporated at S-phase of cell division, cells in the transitional zone labelled after 4-6 days, the superficial zone cells labelled after 10 days. The slow cycling time of the superficial zone cells is characteristic of a progenitor or stem cell population (Hayes et al., 2001).

This study demonstrates that the stem cell markers, CD44, CD49e, CD105 and CD166 are all localised to the articular cartilage superficial zone cells, with CD29 and the differentiation markers Notch1, Delta1, Jagged1, Jagged2 and Msx1 expressed throughout the tissue. The restricted expression of these stem cell markers predominantly to the superficial zone in immature bovine articular cartilage is akin to results observed by previous investigators (Dowthwaite et al., 2004, Hayes et al., 2003, Williams et al., 2009) and supports appositional growth. Variations in the distribution of markers observed in this study and other studies may be due to maturity of tissue or to species-specific differences. These data suggest the presence of a stem cell-like population that resides within the superficial zone of immature bovine articular cartilage. These markers can be used to isolate these stem cell-like cells from the total population. The expression of these markers within superficial zone cells will be further investigated *in vitro* in Chapter 3.

CHAPTER 3:
QUANTITATIVE STUDY OF CELL SURFACE
MARKERS IN ARTICULAR CHONDROCYTES

3.1 Introduction

In the previous Chapter, stem cell markers were predominantly expressed within the superficial zone of immature bovine articular cartilage sections. In brief, CD44, CD49e, CD105 and CD166 were restricted to the superficial zone; CD29 and the differentiation markers Notch1, its ligands and Msx1 were located throughout the depth of the cartilage; CD90 and CD117 were not detected. The results in this study are consistent with previous observations where progenitors are thought to reside within the superficial zone (Dowthwaite et al., 2004, Hayes et al., 2003, Williams et al., 2009).

Following on from this whole tissue work, we investigated the expression of the stem cell markers *in vitro* to determine the effects of cell isolation on expression. Previous work by other groups has shown that following short periods in monolayer culture, some human articular chondrocytes expressed CD29, CD44, CD49e and low levels of CD90, CD105 and CD166 (Diaz-Romero et al., 2005, Diaz-Romero et al., 2008, Bocelli-Tyndall et al., 2006). In one study, a sub-population of CD105/CD166 expressing cells after expansion and dedifferentiation were able to differentiate into chondrocytes, osteoblasts and adipocytes (Alsalameh et al., 2004) suggesting these markers could be used to isolate MSCs.

Few studies have been carried out in bovine tissue, however, Dowthwaite et al (2004) showed that in isolated immature bovine chondrocytes, superficial, middle and deep zone cells expressed CD29. The same study showed CD49e was predominantly expressed by superficial zone cells (79%) compared with middle (5%) and deep (2.5%) zone cells. It is evident from a study by Grogan et al (2009) that although 72%, 35% and 29% Notch1 labelled cells were observed within the superficial, middle and deep zones respectively, expression decreased following 24 hours in monolayer culture to 4.7% of the total population (Grogan et al., 2009). Differences in zonal expression of Notch1 were also observed in immature bovine tissue (Dowthwaite et al., 2004). Notch1 labelling was predominantly within the superficial 2-3 cell layers; following chondrocyte isolation, 86% of the isolated surface zone cells

were Notch1-positive compared with 10% and 34% from the middle and deep zone, respectively (Dowthwaite et al., 2004). Notch1, Delta1 and Jagged1 labelling was observed in human chondrocytes cultured in monolayer, however the expression of these markers was more intense in progenitor populations. Jagged2 was only observed in normal chondrocytes (Ustunel et al., 2008). From these studies, it is evident that there could be differences in marker expression between *in vivo* and *in vitro* chondrocytes and this is an important factor to consider when using these stem cell markers as a means of isolating progenitor/MSC populations.

The majority of studies investigating antigen expression of isolated or cultured chondrocytes use flow cytometry; this method enables rapid analysis and quantification of a large number of cells. A single cell suspension is injected into the machine and enters the flow cell (Figure 3.1A). Essentially, the flow cell consists of a central core containing the cell sample surrounded by an outer core of sheath fluid. The pressure of the sheath fluid against the cell sample in the narrowing flow cell creates a laminar sheath flow that carries the cells upward in single file through the centre of the flow cell where they are interrogated by one or more lasers, this is known as hydrodynamic focusing. Hydrodynamic focussing is essential to obtain information from each cell entering the flow cell. Light scattered in a forward direction, is collected by a lens known as the forward scatter channel. Forward scattered light is indicative of cell size and can distinguish between viable cells and cellular debris. Light deflected from cell organelles is detected perpendicular to the laser and is measured by the side scatter channel, which provides information regarding the granular content or cytoplasmic complexity of a cell (Figure 3.1B). Flow cytometers use a number of fluorescence channels to detect light emitted, known as photomultiplier tubes, the specificity of detection is controlled by optical filters that only permit the passage of specific band widths of light (Rahman, 2006, Carter and Ormerod, 2005).

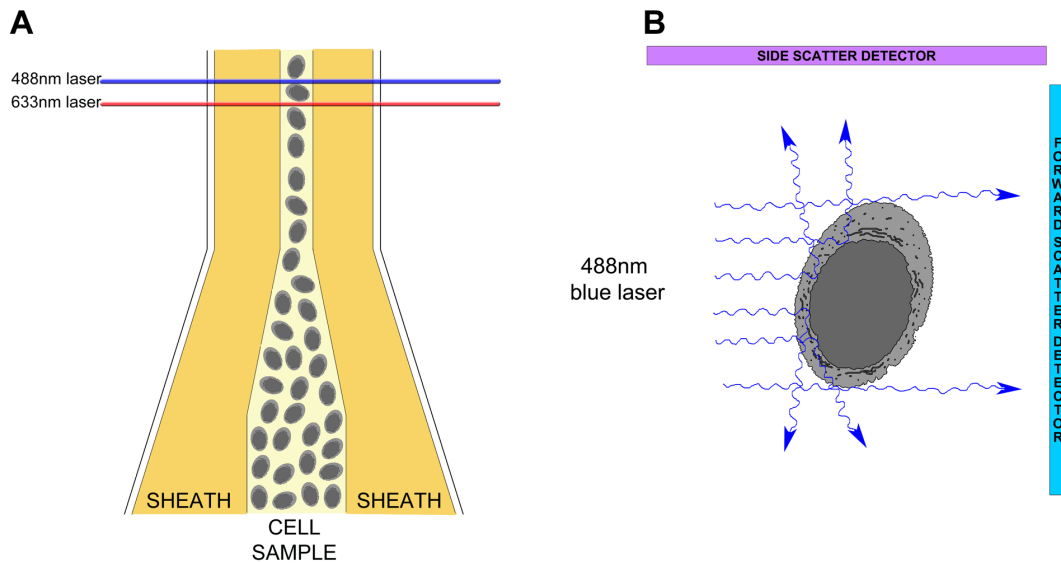


Figure 3.1: Flow cell within flow cytometer

Cells are hydrodynamically focussed within the flow cell to produce a stream of single cells for interrogation by one or more lasers (A). Light scattered in a forward direction is detected by the forward scatter channel and is indicative of particle size. The side scatter channel provides information about the granular content or cytoplasmic complexity of each cell (B)

The purpose of this Chapter is to quantify the expression of stem cell markers (CD29, CD44, CD49e, CD90, CD105, CD117 and CD166) and developmental or differentiation markers (Notch1, Delta1, Jagged1, Jagged2 and Msx1) within the superficial zone of immature bovine articular cartilage using flow cytometry; and also to determine whether sub-populations of stem cells are present within superficial zone chondrocytes. When data are combined with Chapter 2, these data will highlight any differences in expression between tissue sections and isolated cells.

3.2 Materials and Methods

3.2.1 Tissue isolation and digestion

Immature bovine limbs were cleaned and skinned as described in Chapter 2. Following the removal of the skin, the metacarpophalangeal joint was opened under sterile conditions and superficial zone explants were dissected from the joints. The explants were dissected using a #10 scalpel blade from the lateral and medial condyles as well as the condylar ridges. Superficial zone explants were removed using fine dissection, these explants were thin and translucent. In a small number of the joints, middle and deep zone explants were also excised. Middle zone explants were excised from directly below the removed superficial zone, removing approximately half of the remaining depth of tissue. The remaining cartilage was termed the deep zone and was removed from directly beneath the excised middle zone. This technique does not allow for accurate identification of the three zones but is a rapid and consistent procedure for separation of the tissue into regions. Articular cartilage from two to three joints were used from different animals for each experiment and all experiments were carried out in at least triplicate.

Explants from each region were placed into separate wells of a 6-well tissue culture plate (Corning Life Sciences; Fisher Scientific, UK) with 10% media (Dulbecco's Modified Eagle Medium containing Hams F12 and glutamine (DMEM/F12 + Glutamax; Gibco; Invitrogen, UK). The media were supplemented with 10% foetal bovine serum (FBS; Gibco), 50 $\mu\text{g ml}^{-1}$ gentamicin (Gibco), 50 $\mu\text{g ml}^{-1}$ ascorbic acid (Sigma-Aldrich) and 0.5g glucose (Sigma-Aldrich). When dissection was complete, the supplemented media (media+) were removed from the wells and the finely diced explants were transferred, using a #23 scalpel, to zone specific tubes containing 1% pronase (Roche diagnostic Ltd. UK) prepared in 10ml media+ (for each joint). Superficial and middle zone explants were enzymatically digested in 1% pronase for 15 minutes at 37°C followed by collagenase (Sigma-Aldrich; 300 IU; prepared with 10 media+ [for each joint]) for 3 hours at 37°C. Deep zone explants were treated with 1% pronase in media+ for 1 hour at 37°C followed by collagenase (900 IU) for 3

hours at 37°C. The enzymatic digestion was carried out on a rotating platform to ensure uniform digestion of the tissue. The resultant cell suspension was filtered through a 40µm cell strainer (Falcon, BD Biosciences) and the filtered cell suspension was centrifuged for 5 minutes at 400xg. The supernatant was removed and the cell pellet re-suspended in media+. The cells were counted using a haemocytometer and an average cell count calculated. Ten µl cell suspension was added to one chamber of the slide and four counts performed and an average recorded. Cell number was determined using the following calculation:

$$\text{Cells per ml} = \text{average cell count} \times \text{dilution factor} \times 10^4$$

$$\text{Total number of cells} = \text{Cells ml}^{-1} \times \text{total volume of cells (ml)}$$

Cells were re-suspended at 1.0×10^6 cells ml⁻¹ and 5×10^6 cells were transferred to each 175cm² flask (Corning Life Sciences) with the addition of a further 15ml of media+ (20ml total). The cells were cultured overnight in a humidified incubator at 37°C containing 5% CO₂. Some of the cells were immunolabelled following the cell count to determine if cell surface expression was present immediately following enzymatic digestion, an average of 6×10^6 , 5×10^6 and 18×10^6 cells were obtained from the superficial, middle and deep zones respectively.

3.2.2 Bone marrow extraction

Bone marrow contains populations of cells that are able to differentiate along mesenchymal lineages. These MSCs can be characterised by their adherence to plastic, efficacy to form colonies and the expression of a number of cell surface antigens including CD29, CD44, CD49e, CD90, CD105, CD117 and CD166. MSCs are negative for hematopoietic lineage markers CD4, CD14 and CD45 (Flores-Torales et al., 2010, Bocelli-Tyndall et al., 2006, Lee et al., 2009, Romanov et al., 2005). Notch1 has also been identified as a MSC marker (Hiraoka et al., 2006). These MSCs would provide a positive control for the stem cell and differentiation markers used in the current study.

Bone marrow was isolated from immature bovine metacarpi. The limb was washed as previously described and all soft tissue removed using a #23 scalpel before bisecting with a band saw under sterile conditions. Sawing produces a small amount of bone debris, which can contaminate the marrow. Bone debris was removed using a sterile spatula ensuring minimum contamination of the bone marrow.

The bone marrow was removed using a second sterile spatula into a sterile 100mm diameter culture dish (Corning Life Sciences). Under sterile conditions, 10ml DMEM containing 0.05mg ml^{-1} gentamicin (media+G) was added to the culture dish, the marrow was vigorously mixed using a pipette to disperse the tissue. The suspension was transferred to a 50ml tube (Corning Life Sciences), the culture dish was washed a further two times with 10ml media+G to remove as many cells as possible from the dish. The tube containing the cell suspension was gently inverted, allowing the fat to float to the top of the tube. The cell suspension beneath the fat was then strained (approximately 20ml) into a fresh sterile 50ml tube through a $40\mu\text{m}$ cell strainer. Any cells remaining in the first tube were rinsed with a further 20ml of media+G and the separation by inversion and straining repeated. The cell suspension was centrifuged at $300\times g$ for 5 minutes and the pellet re-suspended in 20ml media+G containing 10% FBS; the cell suspension was transferred to two 75cm^2 flasks (Corning Life Sciences), and a further 10ml 10% media+G added to each flask. The cells were incubated at 37°C with 5% CO_2 for four days.

After four days, the media containing all non-adherent cells was extracted and the adherent cells washed twice in media+G. After seven days in culture, the media were supplemented with 5ng ml^{-1} fibroblast growth factor (FGF2), changing the media every second day. The bone marrow stromal cells were maintained until required for flow cytometry analysis when $0.5\text{--}1.0 \times 10^6$ cells were used per antibody (dilutions shown in Table 3.1 and 3.2).

Antigen recognised	Clone Name	Conjugated fluorophore	Source	IgG
CD29	TDM29	FITC	Millipore, UK	Mouse
CD44	IM7	FITC	BD Biosciences, UK	Rat
CD49e	SAM-1	R-PE	Millipore, UK	Mouse
CD90	CT-TH1	FITC	Invitrogen, UK	Rat
CD105	SN6	APC*	Invitrogen, UK	Mouse
CD117	104D2	APC	Invitrogen, UK	Mouse
CD166	3A6	R-PE**	Ancell, USA	Mouse

Table 3.1: Conjugated primary antibodies used

The table shows the range of directly conjugated antibodies used; these antibodies were all used at $10\mu\text{g ml}^{-1}$.

*APC = Allophycocyanin

**R-PE = R-Phycoerythrin

3.2.3 Direct immunofluorescence for flow cytometry

All centrifugation steps were performed at 400xg for 3 minutes unless otherwise stated. Plated cells were washed with 10ml PBS. Accutase (10ml; Sigma-Aldrich) was added to the cells and incubated at 37°C for 5-10 minutes or until the cells became rounded and were beginning to detach from the flask. Detached cells were transferred to 50ml tubes. The flasks were washed with 10ml PBS to ensure all cells had been obtained and the liquid aspirated to the relevant 50ml tube. The cells were centrifuged and supernatant removed.

Cells were re-suspended in 1ml PBS containing 0.1% BSA (PBS/BSA) and counted using a haemocytometer as described previously. Cells were re-suspended at 1×10^6 cells ml^{-1} and 1ml of the cell suspension was transferred to 1.5ml tubes, the cells were centrifuged and the supernatant aspirated. Conjugated primary antibody or IgG-matched control was added to each tube (dilutions shown in Table 3.1), re-suspended and incubated for 45 minutes at 4°C (in the dark). Following incubation the cells were centrifuged, and washed twice in PBS/BSA with a centrifuge step between each wash. Finally, cells were re-suspended in 0.5ml PBS/BSA filtered through 30µm filters (Celltrics; Partec, UK) into 12mm FACS tubes (Falcon, BD Biosciences, UK) and viewed on a BD FACS Canto flow cytometer (BD Biosciences).

3.2.4 Indirect immunofluorescence for flow cytometry

All centrifugation steps were performed at 400xg for 3 minutes unless otherwise stated. Non-conjugated antibodies were tested using fixed and live cells with the exception of anti-Msx1, which was only tested using fixed cells. Msx1 is only present within the nucleus of the cell and antibody binding requires a compromised plasma membrane and, therefore, would not be detected in viable cells. Cells were fixed using 2% PFA; this method was found to be the superior fixation method of tissue sections and is a common and reliable fixative for cell analysis using flow cytometry. For live cells, all antibodies and controls were prepared in PBS/BSA and 5% normal blocking serum. Antibodies and controls added to fixed cells were prepared in PBS/T and 5% normal blocking serum (dilutions shown in Table 3.2).

Plated cells were washed in 10 ml PBS and detached using Accutase and centrifuged as described previously. To fix the cells, 1ml cold 2% PFA was added and the cells incubated for 2 minutes before centrifuging. Secondary antibody was prepared in advance with bovine serum ($10\mu\text{g ml}^{-1}$), incubating for 1 hour at 4°C. Following fixation, the cells were re-suspended in 1ml PBS/T and counted using a haemocytometer.

Relevant blocking serum was added to the tube containing the cells. The sample was mixed by pipetting and the mixture incubated for 15 minutes at 4°C. The cell suspension was transferred to 1.5ml tubes and centrifuged. Primary antibody or IgG-matched controls were added to each tube, re-suspended and the cells incubated for 30 minutes at 4°C. Following incubation, the cells were washed three times in PBS/T, centrifuged between each wash, supernatant removed and re-suspended in PBS/T. Secondary antibody was added to each tube (dilutions shown in Table 3.2), re-suspended and incubated for 30 minutes at 4°C in the dark. Following incubation, the cells were centrifuged and washed twice in PBS/T. Finally, the cells were re-suspended in 1ml PBS and passed through a 30µm filter to remove cell aggregates and examined on a BD FACS Canto flow cytometer.

For labelling of live cells, all reagents and wash steps were carried out in PBS/BSA.

Control samples were processed as described, omitting primary antibody and, instead, incubating the cells with appropriate IgG-matched controls, PBS/T (fixed cells) or PBS/BSA (live cells).

Antigen recognised	Clone name	IgG	Secondary antibody	Dilution	Blocking serum
Delta	F-15	Goat	Rabbit anti goat FITC	1:200	Rabbit
Jagged1	H-66	Rabbit	Goat anti-rabbit FITC	1:100	Goat
Jagged2	N-19	Goat	Rabbit anti goat FITC	1:200	Rabbit
Msx1	N-20	Goat	Rabbit anti goat FITC	1:200	Rabbit
Notch1	C-20	Goat	Rabbit anti goat FITC	1:200	Rabbit

Table 3.2: Non-conjugated primary antibodies used

The table shows the range of non-conjugated antibodies used, all were used at 5 µg ml⁻¹; the concentrations of secondary antibody are shown in the table.

3.2.5 Flow cytometry analysis

Flow cytometry was performed using a BD Canto dual laser bench top flow cytometer, equipped with 633nm and 488nm lasers and BD FACS Diva software (v 5.0.3). Single colour analysis was performed using primary antibodies conjugated to FITC, PE or APC, excitation and emission spectra for these fluorophores can be seen in Figure 3.2.

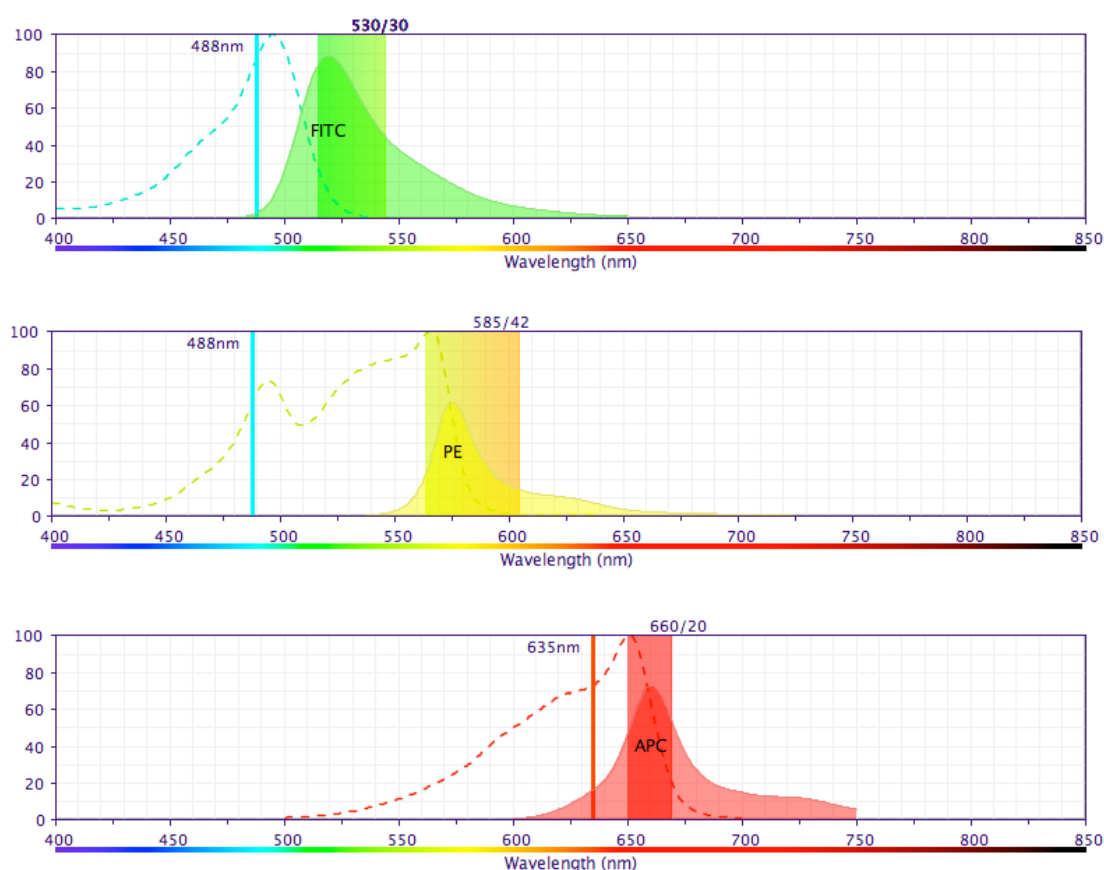


Figure 3.2: Fluorophore spectra

Spectra illustrating the excitation spectra (dotted lines) and emission spectra (solid filled histogram) of FITC (green) PE (yellow) and APC (red). The highlighted rectangular area on each graph demonstrates the band pass filter used for each fluorophore (adapted from BD Biosciences 2011).

Cells were analysed initially for forward scatter (FSC) and side scatter (SSC). FSC is a measure of relative cell size and SSC a measure of relative granularity or internal/cytoplasmic complexity. Density dot-plots were used to show the distribution of cells using the FSC and SSC parameters, voltages were adjusted so that all the cells were aligned optimally (Figure 3.3 A). An area of concentrated pixels (cells) was selected using a gate (Figure 3.3 A), which enables a sub-population to be analysed independently. This is an important step in flow cytometry analysis as it reduces the contamination from dead cells and cell doublets. Dead cells can be especially adhesive, non-specifically binding antibody, leading to false positives. An increased level of fluorescence is also observed with cell doublets. It must be noted that during the processing of each sample the whole, un-gated population was also analysed to ensure no sub-populations or labelled cells were omitted.

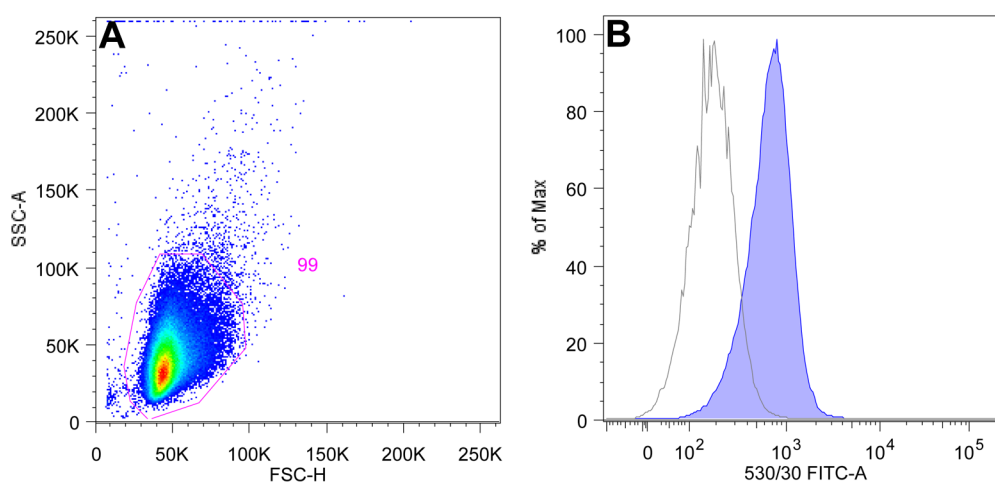


Figure 3.3: Diagram illustrating gating and voltage adjustments

The dot-plot illustrates a typical position of the cell population and gating used (A). Highest density cells are displayed as red pixels and lowest density cells are displayed as blue pixels. The cells were positioned away from the axes and cell debris. The gated population from the negative control sample was set so that the median fluorescence was close to the second logarithmic decade (grey line; B), the distribution of labelled cells (blue) was then overlaid and compared.

To illustrate antibody labelling, single parameter histograms were used to display relative fluorescence intensity on the x-axis and the cell count of the y-axis. The voltages were adjusted for individual fluorophores so that the negative controls were set to the second logarithmic (log) decade (Figure 3.3 B). If voltages are not set correctly, control samples can be set too low to analyse or conversely immunolabelled cells could be above the level of detection by the flow cytometer. Instrument settings were adjusted using either IgG-matched controls or unstained cell samples. Labelling of cells was assessed by percentage shift or fluorescent intensity of fluorescently labelled cells compared to control cells.

Results were tested for statistical significance using a paired, one-tailed t-test where the geometric mean fluorescent intensity (MFI) of control cells was compared with that of the immunolabelled cells. To compare MFI ratios between freshly isolated cells and cells cultured in monolayer overnight, statistical significance was tested using an unpaired, two-tailed Student's t-test.

3.3 Results

3.3.1 Immunofluorescence

For this study, we used Accutase to detach the chondrocytes cultured overnight. Accutase contains proteolytic and collagenolytic enzymes and is routinely used for analysis of cell surface markers by flow cytometry. Previous studies have used trypsin/EDTA (ethylenediaminetetraacetic acid) to detach chondrocytes from plastic, however, a small decrease in expression of cell surface markers was observed compared to detachment with EDTA.

All data were acquired using BD FACS Diva software but all further analysis was completed using FlowJo (version 9.2, Tree Star, Inc., Oregon, USA) from data stored in FCS 3.0 format. Gating of cells based on size and granularity (FSC and SSC) was set manually and gated cells analysed for fluorescence intensity. Where bimodal distributions of fluorescent intensity were present, the results were displayed as percentage labelled and MFI. The percentage labelled were defined by placing a delineator at the limit of the control sample fluorescence (between 0.5 and 1.0%), any cells above this level were designated as positively labelled. Where fluorescent labelling of cells was displayed as a unimodal distribution the MFI only was displayed, as the test sample distribution often overlaps that of the control.

Results shown are representative of labelling seen for each antibody, with the typical or central distributions shown. Each antibody was tested at least in triplicate from at least three animals and controls comprised unlabelled cells or IgG-matched controls. Secondary antibodies used with non-conjugated primary antibodies, were pre-incubated with bovine serum as this resulted in lower levels of autofluorescence.

3.3.2 Freshly isolated superficial zone chondrocytes

Cells were isolated using pronase and collagenase and immediately labelled with the relevant conjugated or non-conjugated primary antibody, 1.0×10^6 cells were used for each antibody.

3.3.3 Immunofluorescence with conjugated antibodies

CD29 (Integrin $\beta 1$)

All cells incubated with CD29 antibody exhibited increased MFI relative to controls (5.52 fold; Table 3.3). Gated cells, labelled with CD29 were plotted against a control sample and the distributions compared (Figure 3.4B). The geometric mean of CD29 was significantly different to the control cells ($P= 0.0296$).

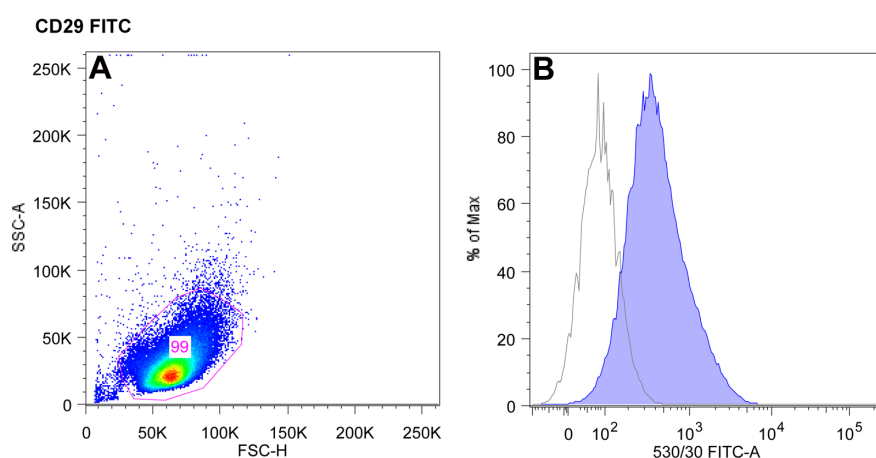


Figure 3.4: CD29 FITC expression from freshly isolated surface cells

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 99% of the total population. The gated control (grey line) and CD29 (blue) labelled cells were analysed for fluorescent intensity (B).

Geometric mean values

	Control	CD29 FITC	MFI ratio
Sample 1	120.00	785.00	6.54
Sample 2	80.90	415.00	5.13
Sample 3	83.00	406.00	4.89
		Mean	5.52

Table 3.3: CD29 FITC expression from freshly isolated surface cells

Table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted region indicates the sample represented in the histogram (Figure 3.4B).

CD44 (Hyaluronan receptor)

When cells were incubated with CD44 a small increase in MFI relative to the controls was observed (1.1 fold; Figure 3.5B and Table 3.4). This increase in fluorescence was shown to be statistically different to the control ($P = 0.0368$).

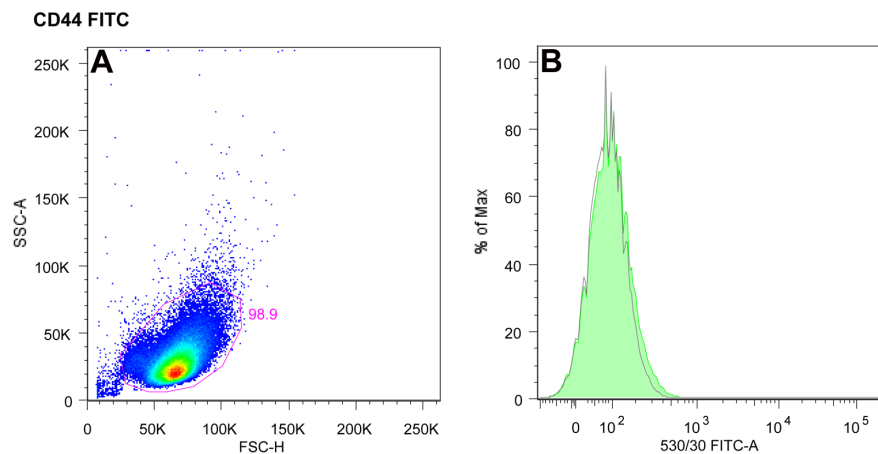


Figure 3.5: CD44 FITC expression from freshly isolated surface cells

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 98.9% of the total population. The gated control (grey line) and CD44 (green) labelled cells were analysed for fluorescent intensity (B).

Geometric mean values

	Control	CD44 FITC	MFI ratio
Sample 1	67.20	69.40	1.03
Sample 2	120.00	140.00	1.17
Sample 3	80.90	89.80	1.11
Sample 4	83.00	91.80	1.11
	Mean		1.10

Table 3.4: CD44 FITC expression from freshly isolated surface cells

Table contains the geometric fluorescence intensity for each sample acquired and the MFI ratio. The highlighted region indicates the sample represented in the histogram (Figure 3.5B).

CD49e (Integrin $\alpha 5$)

The distribution of the fluorescent intensity between CD49e and control cells was not statistically different ($P = 0.1781$; Figure 3.6 and Table 3.5).

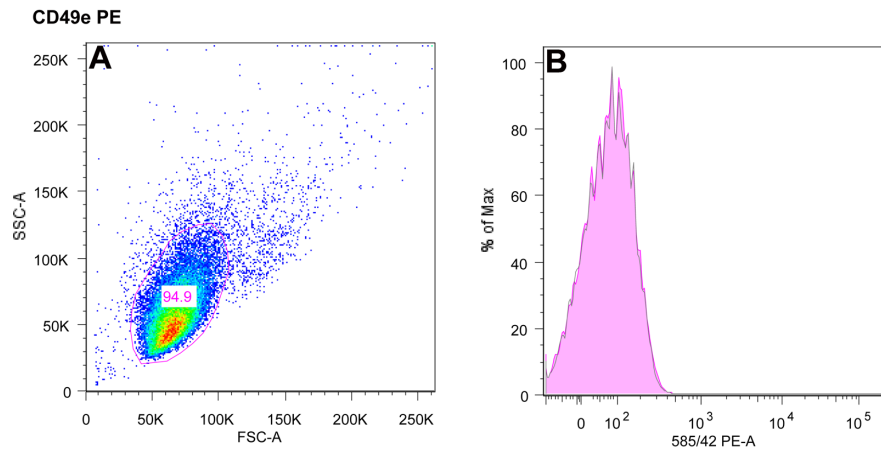


Figure 3.6: CD49e PE expression from freshly isolated surface cells

Gated cells, excluding clumped cells and debris (A) comprised 94.9% of the total population analysed. The gated control (grey line) and CD49e (pink) labelled cells were analysed for fluorescent intensity (B).

Geometric mean values

	Control	CD49e PE	MFI ratio
Sample 1	68.70	69.30	1.01
Sample 2	69.60	74.00	1.06
Sample 3	79.30	79.80	1.01
	Mean		1.03

Table 3.5: CD49e PE expression from freshly isolated surface cells

Table contains the MFI values for each sample recorded and the MFI ratio of CD49e labelled versus control cells. The highlighted region indicates the values for the histogram shown (Figure 3.6B).

CD90 (Thy-1)

Gated cells, labelled with CD90 were plotted against a control sample and the distributions compared (Figure 3.7B). Cells labelled with CD90 exhibited increased MFI compared with controls (1.23 fold; Figure 3.7B and Table 3.6). The MFI of CD90 was significantly different to the control cells ($P=0.0237$).

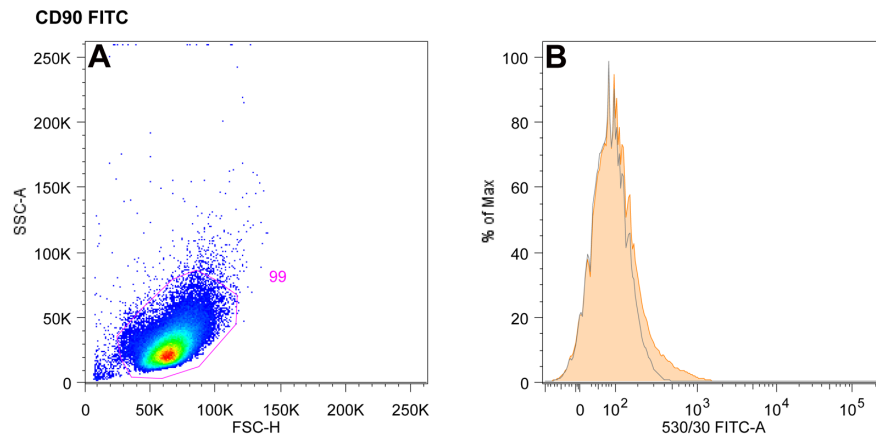


Figure 3.7: CD90 FITC expression from freshly isolated surface cells

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 99% of the total population. The gated control (grey line) and CD90 (orange) labelled cells were analysed for fluorescent intensity (B).

Geometric mean values

	Control	CD90 FITC	MFI ratio
Sample 1	120.00	152.00	1.27
Sample 2	80.90	99.20	1.23
Sample 3	83.00	99.00	1.19
		Mean	1.23

Table 3.6: CD90 FITC expression from freshly isolated surface cells

Table contains the geometric mean values for each sample tested and the MFI ratio. The highlighted region indicates the sample represented in the histogram (Figure 3.7B).

CD105 (Endoglin)

The distribution of the fluorescent intensity of both CD105 labelled and control cells were not statistically different ($P = 0.0558$; Figure 3.8B and Table 3.7).

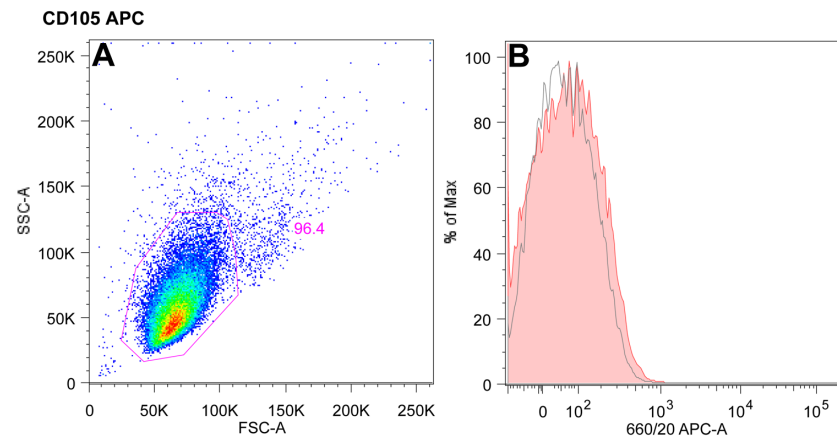


Figure 3.8: CD105 APC expression from freshly isolated surface cells

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 96.4% of the total population. The gated control (grey line) and CD105 APC (pink) labelled cells were analysed for fluorescent intensity (B).

Geometric mean values

	CD105 Control	CD105 APC	MFI ratio
Sample 1	55.60	60.70	1.09
Sample 2	73.00	98.90	1.35
Sample 3	73.30	99.20	1.35
	Mean		1.27

Table 3.7: CD105 APC expression from freshly isolated surface cells

Table contains the MFI values for each sample acquired and the MFI ratio between labelled cells and control. The highlighted region indicates the sample represented in the histogram (Figure 3.8B).

CD117 (c-kit)

The fluorescent intensity of CD117 labelled cells was equivalent to that of the control cells (Figure 3.9 and Table 3.8). The geometric means were not shown to be statistically different ($P = 0.0763$).

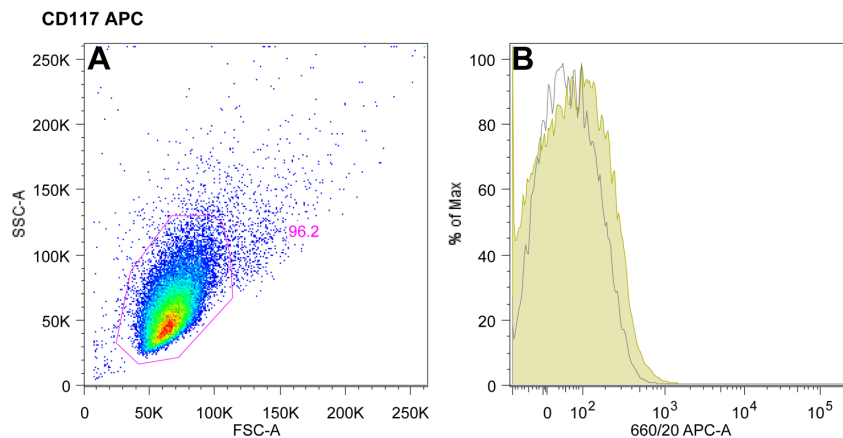


Figure 3.9: CD117 APC expression from freshly isolated surface cells

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 96.2% of the total population. The gated control (grey line) and CD117 (green) labelled cells were analysed for fluorescent intensity (B).

Geometric mean values

	CD117 Control	CD117 APC	MFI ratio
Sample 1	55.60	61.40	1.10
Sample 2	73.00	109.00	1.49
Sample 3	73.30	128.00	1.75
	Mean		1.45

Table 3.8: CD117 APC expression from freshly isolated surface cells

Table contains the geometric mean values for each sample recorded and the MFI ratio of labelled cells compared to controls. The highlighted region indicates the sample represented in the histogram (Figure 3.9B).

CD166 (ALCAM)

The fluorescent intensity of CD166 labelled cells was equivalent to that of the control (Figure 3.10B). Small increases in the geometric mean were seen with CD166 (Table 3.9) but these were shown not to be significantly different to the control ($P = 0.0633$)

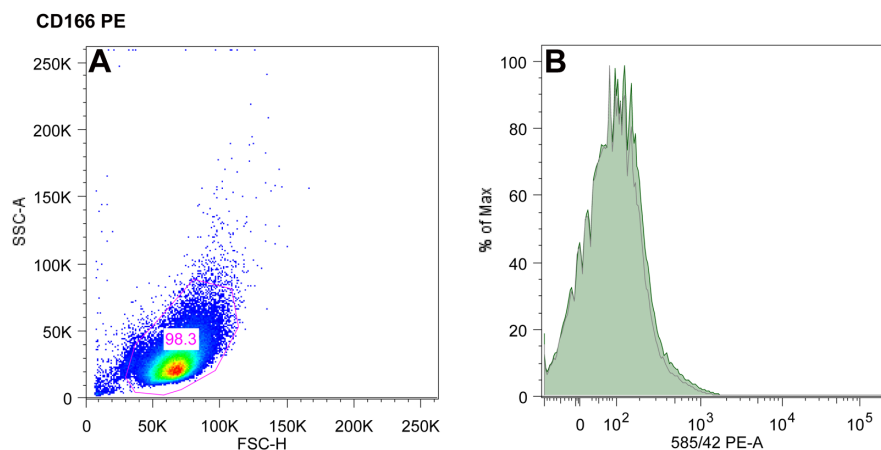


Figure 3.10: CD166 PE expression from freshly isolated surface cells

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 98.3% of the total population. The gated control (grey line) and CD166 (green) labelled cells were then analysed for fluorescent intensity (B).

Geometric mean values

	Control	CD166 PE	MFI ratio
Sample 1	84.30	91.20	1.08
Sample 2	67.10	69.00	1.03
Sample 3	88.70	99.50	1.12
	Mean		1.08

Table 3.9: CD166 PE expression from freshly isolated surface cells

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted region indicates the sample represented in the histogram (Figure 3.10B).

3.3.4 Immunofluorescence with non-conjugated antibodies

Notch1

All fixed cells incubated with Notch1 antibody exhibited increased MFI relative to controls (3.57 fold); a small increase was observed with live cells (Figure 3.11 and Table 3.10). The MFI of Notch1 labelled cells was significantly different to the control cells (live $P = 0.0110$, fixed $P = 0.0047$).

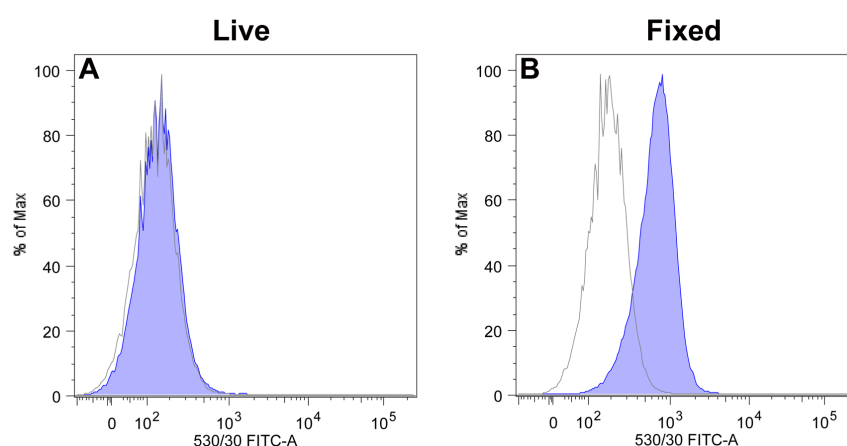


Figure 3.11: Notch1 expression from freshly isolated surface cells

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and Notch1 (blue) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Live cells				Fixed cells			
	Control	Notch1	MFI ratio		Control	Notch1	MFI ratio
Sample 1	127.00	141.00	1.11	Sample 1	191.00	720.00	3.77
Sample 2	146.00	167.00	1.14	Sample 2	168.00	544.00	3.24
Sample 3	101.00	125.00	1.24	Sample 3	178.00	658.00	3.70
		Mean	1.16			Mean	3.57

Table 3.10: Notch1 expression from freshly isolated surface cells

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted regions indicate the samples represented in the histograms above (Figure 3.11).

Delta1

There was an increase in the overall fluorescent intensity of fixed cells labelled with the Delta1 antibody. The distribution of fluorescent intensity of Delta1 and control cells appeared to be similar with live cells, however, there was a small difference in MFI (Figure 3.12 and Table 3.11). The MFI of Delta1 labelled cells was significantly different to the control cells (live $P = 0.0009$, fixed $P = 0.0060$).

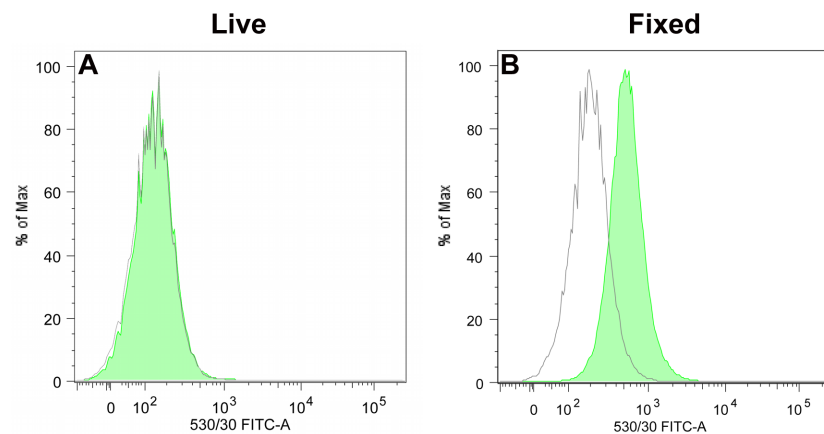


Figure 3.12: Delta1 expression from freshly isolated surface cells

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and Delta1 (green) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Live cells				Fixed cells			
	Control	Delta	MFI ratio		Control	Delta	MFI ratio
Sample 1	127.00	135.00	1.06	Sample 1	191.00	539.00	2.82
Sample 2	146.00	154.00	1.05	Sample 2	168.00	674.00	4.01
Sample 3	101.00	108.00	1.07	Sample 3	178.00	660.00	3.71
		Mean	1.06			Mean	3.51

Table 3.11: Delta1 expression from freshly isolated surface cells

The table contains the geometric mean values for each sample and the MFI ratio between labelled and control cells. The highlighted regions indicate the values used in the histograms (Figure 3.12).

Jagged1

Jagged1 labelling was not observed with live cells, however, all fixed cells labelled with Jagged1 exhibited increased MFI relative to controls (2.97 fold; Figure 3.13 and Table 3.12). The geometric mean of fixed Jagged1 cells was significantly different to the control cells ($P = 0.0138$), however, live labelled cells were not statistically different to the controls ($P = 0.1572$).

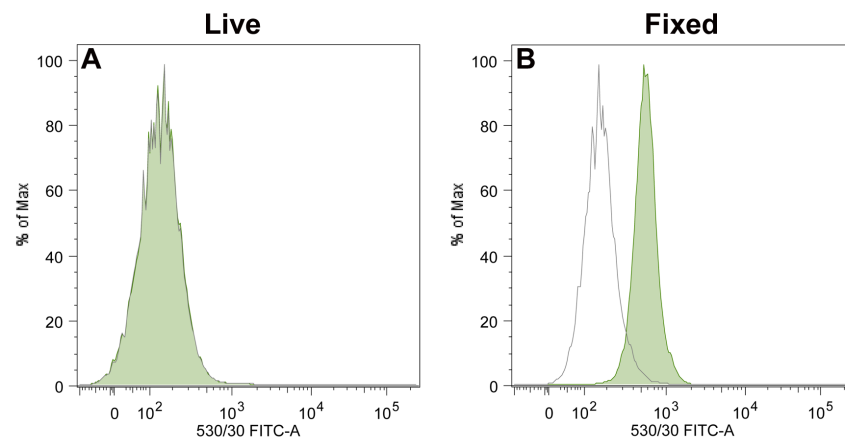


Figure 3.13: Jagged1 expression from freshly isolated surface cells

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and Jagged1 (green) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Live cells				Fixed cells			
	Control	Jagged1	MFI ratio		Control	Jagged1	MFI ratio
Sample 1	125.00	111.00	0.89	Sample 1	187.00	455.00	2.43
Sample 2	136.00	136.00	1.00	Sample 2	164.00	563.00	3.43
Sample 3	157.00	154.00	0.98	Sample 3	241.00	736.00	3.05
		Mean	0.96			Mean	2.97

Table 3.12: Jagged1 expression from freshly isolated surface cells

The table contains the MFI values for each sample carried out as well as the MFI ratio for both live and fixed cells. The highlighted regions are shown in the histograms above (Figure 3.13).

Jagged2

All fixed cells labelled with Jagged2 antibody exhibited increased MFI relative to controls (2.21 fold; Table 3.13), however, no difference was observed between live cells and their controls. Gated cells, labelled with Jagged2 were plotted against a control sample and the distributions compared (Figure 3.14A and B). The geometric mean of fixed Jagged2 cells was significantly different to the control cells ($P=0.0076$) the live cells were not statistically different to the controls ($P=0.0784$).

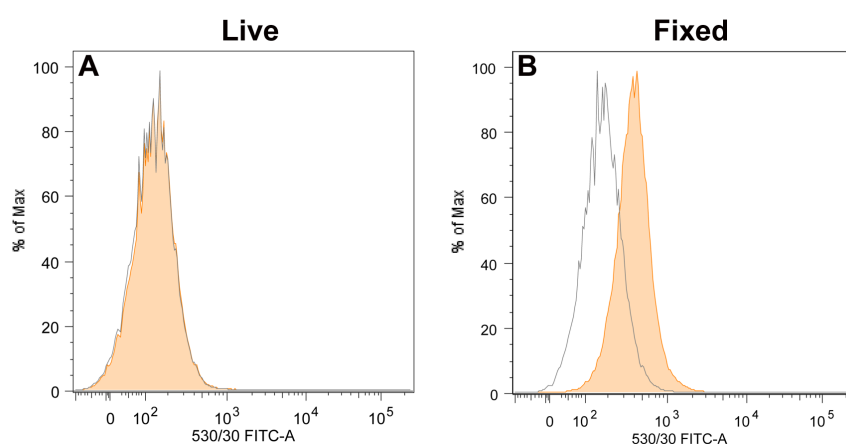


Figure 3.14: Jagged2 expression from freshly isolated surface cells

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and Jagged2 (orange) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Live cells				Fixed cells			
	Control	Jagged2	MFI ratio		Control	Jagged2	MFI ratio
Sample 1	127.00	132.00	1.04	Sample 1	191.00	356.00	1.86
Sample 2	146.00	148.00	1.01	Sample 2	168.00	390.00	2.32
Sample 3	101.00	102.00	1.01	Sample 3	178.00	435.00	2.44
		Mean	1.02			Mean	2.21

Table 3.13: Jagged2 expression from freshly isolated surface cells

The table contains the geometric mean values for each sample acquired and the MFI ratio between test and control. The highlighted regions indicate the samples used in the histograms (Figure 3.14).

Msx1

All cells incubated with Msx1 antibody exhibited increased MFI relative to controls (4.75 fold; Figure 3.15 and Table 3.14). The geometric mean of Msx1 was significantly different to the control cells ($P=0.0264$).

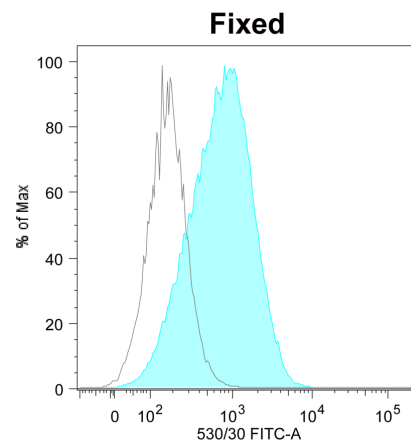


Figure 3.15: Msx1 expression from freshly isolated surface cells

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and Msx1 (blue) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Fixed cells			
	Control	Msx1	MFI ratio
Sample 1	191.00	672.00	3.52
Sample 2	168.00	704.00	4.19
Sample 3	178.00	1164.00	6.54
		Mean	4.75

Table 3.14: Msx1 expression from freshly isolated surface cells

The table contains the geometric mean values for each sample recorded and their MFI ratio. The highlighted region is shown in the histogram above (Figure 3.15).

3.3.5 Chondrocytes cultured in monolayer for 24 hours

Following enzymatic digestion, 5×10^6 cells were transferred to each 175cm^2 flask and incubated for 24 hours in 20ml media+. Cells were then labelled with the relevant conjugated or non-conjugated primary antibody, 1.0×10^6 cells were used for each antibody.

3.3.6 Immunofluorescence with conjugated antibodies

CD29 (Integrin $\beta 1$)

All cells labelled with CD29 antibody exhibited increased MFI relative to controls (12.74 fold). Gated cells, labelled with CD29 were plotted against a control sample and the distributions compared (Figure 3.16B). The geometric mean of CD29 was significantly different to the control cells ($P= 0.0056$).

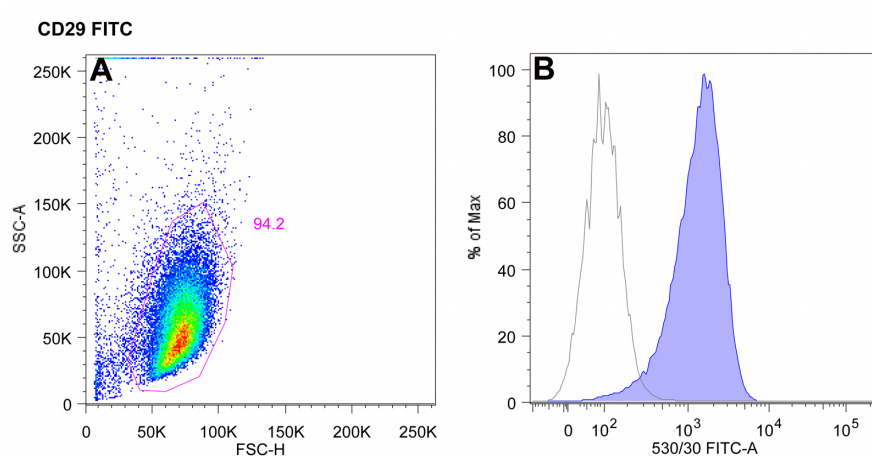


Figure 3.16: CD29 FITC expression from cells plated in monolayer overnight

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 94.2% of the total population. The gated control (grey line) and CD29 (blue) labelled cells were analysed for fluorescent intensity (B).

Geometric mean values

	Control	CD29 FITC	MFI ratio
Sample 1	95.10	1271.00	13.36
Sample 2	104.00	1485.00	14.28
Sample 3	99.30	1049.00	10.56
		Mean	12.74

Table 3.15: CD29 FITC expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted region indicates the sample represented in the histogram (Figure 3.16B).

CD44 (Hyaluronan receptor)

A sub-population was observed when cells were immunolabelled with CD44 resulting in a bimodal distribution (Figure 3.17B). There was a MFI increase of 37.25 fold between labelled and unlabelled cells (Figure 3.17B and Table 3.16). The MFI of CD44 labelled cells (+ve) was significantly different to the negative population (-ve; $P=0.0164$), the mean percentage of cells expressing CD44 was 47.00%.

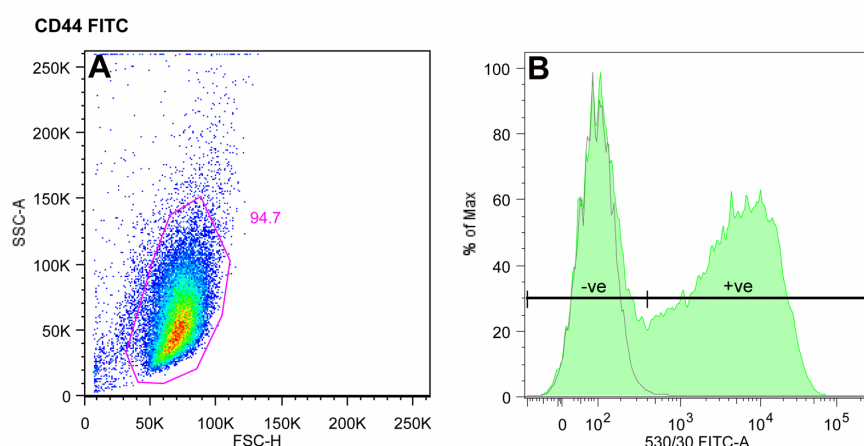


Figure 3.17: CD44 FITC expression from cells plated in monolayer overnight

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 94.7% of the total population. The gated control (grey line) and CD44 (green) labelled cells were analysed for fluorescent intensity (B). The bimodal distribution was divided into unlabelled cells (-ve) and labelled cells (+ve).

Geometric mean values

	CD44 Control	CD44 -ve	CD44 +ve	MFI ratio +ve/-ve	MFI ratio -ve/control	% +ve
Sample 1	86.00	95.80	2743.00	28.63	1.11	27.20
Sample 2	95.10	110.00	4437.00	40.34	1.16	58.30
Sample 3	104.00	125.00	5346.00	42.77	1.20	55.50
Mean				37.25	1.16	47.00

Table 3.16: CD44 FITC expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratios between CD44+ve and CD44-ve, and CD44-ve and control cells. The highlighted region indicates the sample represented in the histogram (Figure 3.17B).

CD49e (Integrin $\alpha 5$)

All cells incubated with CD49e antibody exhibited increased geometric mean relative to controls (1.71 fold; Figure 3.18B and Table 3.17). The geometric mean of CD49e was significantly different to the control cells ($P= 0.0052$).

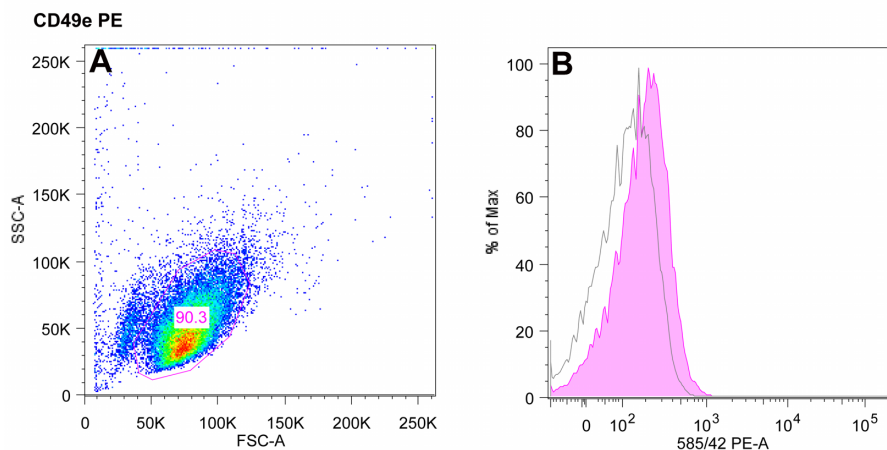


Figure 3.18: CD49e PE expression from cells plated in monolayer overnight

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 90.3% of the total population. The gated control (grey line) and CD49e (pink) labelled cells were analysed for fluorescent intensity (B).

Geometric mean values

	Control	CD49e PE	MFI ratio
Sample 1	108.00	172.00	1.59
Sample 2	54.20	105.00	1.94
Sample 3	54.20	101.00	1.86
Sample 4	54.20	79.10	1.46
	Mean		1.71

Table 3.17: CD49e PE expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted region indicates the sample represented in the histogram (Figure 3.18B).

CD90 (Thy-1)

All cells incubated with CD90 antibody exhibited a small increase in MFI relative to controls (1.32 fold; Figure 3.19 and Table 3.18). Gated cells, labelled with CD90 were plotted against a control sample and the distributions compared (Figure 3.19B). The geometric mean of CD90 was significantly different to the control cells ($P=0.0007$).

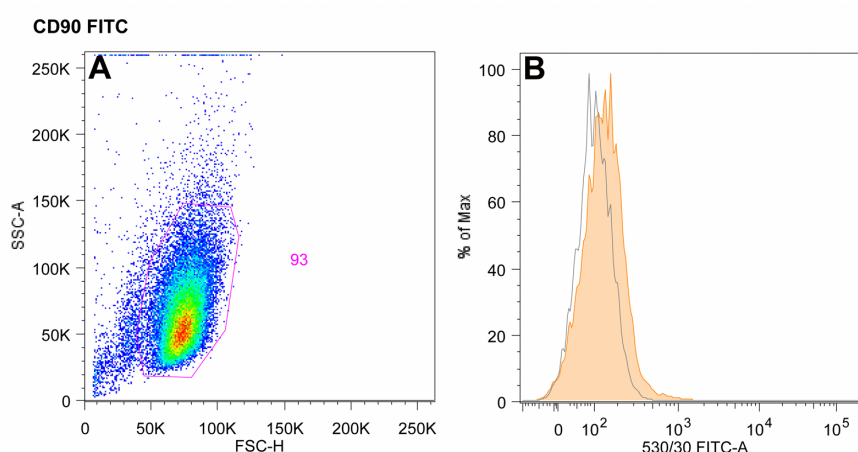


Figure 3.19: CD90 FITC expression from cells plated in monolayer overnight

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 93% of the total population. The gated control (grey line) and CD90 (orange) labelled cells were analysed for fluorescent intensity (B).

Geometric mean values

	Control	CD90 FITC	MFI ratio
Sample 1	86.00	120.00	1.40
Sample 2	95.10	118.00	1.24
Sample 3	104.00	138.00	1.33
Sample 4	99.30	131.00	1.32
		Mean	1.32

Table 3.18: CD90 FITC expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted region indicates the sample represented in the histogram (Figure 3.19B).

CD105 (Endoglin)

Cells incubated with CD105 antibody exhibited increased MFI relative to controls (1.74 fold; Figure 3.20 and Table 3.19). The geometric mean of CD105 was significantly different to the control cells ($P = 0.0048$).

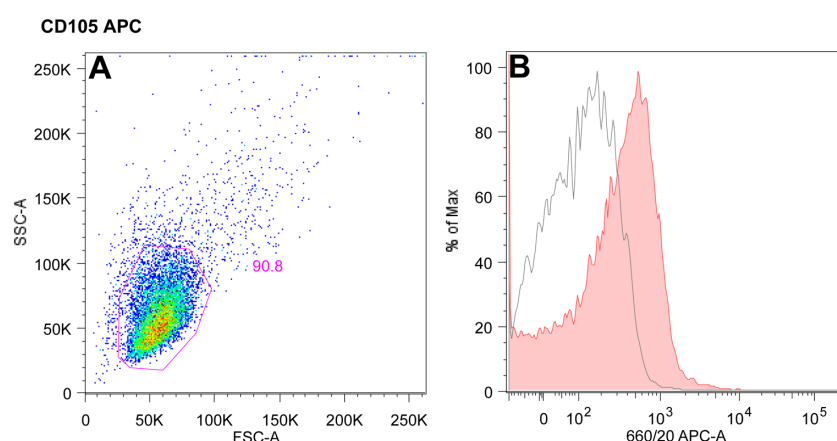


Figure 3.20: CD105 APC expression from cells plated in monolayer overnight

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 90.8% of the total population. The gated control (grey line) and CD105 (pink) labelled cells were then analysed for fluorescent intensity (B).

Geometric mean values

	Control	CD105 APC	MFI ratio
Sample 1	55.90	100.00	1.79
Sample 2	90.80	160.00	1.76
Sample 3	102.00	192.00	1.88
Sample 4	90.10	138.00	1.53
	Mean		1.74

Table 3.19: CD105 APC expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratio between labelled and control cells. The highlighted region indicates the sample represented in the histogram (Figure 3.20B).

CD117 (c-kit)

All cells incubated with CD117 antibody exhibited a small increase in MFI relative to controls (2.10 fold; Figure 3.21 and Table 3.20). Gated cells, labelled with CD117 were plotted against a control sample and the distributions compared (Figure 3.21B). The geometric mean of CD117 was significantly different to the control cells ($P=0.0035$).

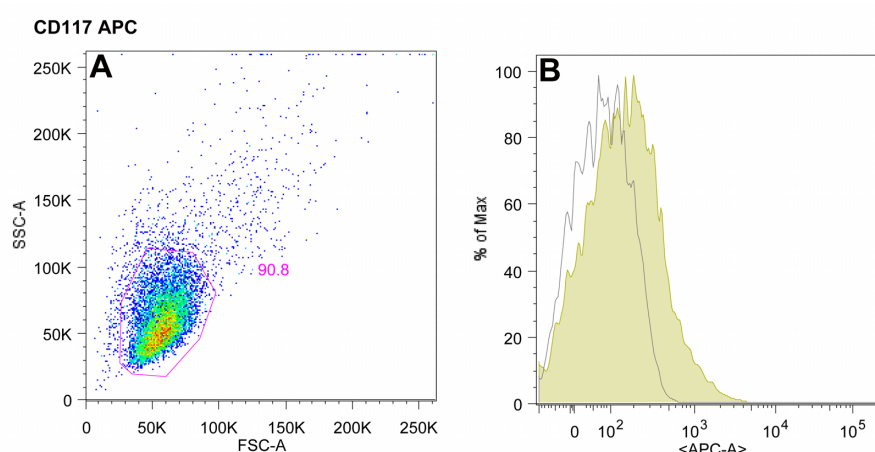


Figure 3.21: CD117 APC expression from cells plated in monolayer overnight

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 90.8% of the total population. The gated control (grey line) and CD29 (green) labelled cells were analysed for fluorescent intensity (B).

Geometric mean values

	Control	CD117 APC	MFI ratio
Sample 1	55.90	113.00	2.02
Sample 2	71.90	180.00	2.50
Sample 3	71.90	145.00	2.02
Sample 4	71.90	135.00	1.88
		Mean	2.10

Table 3.20: CD117 APC expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted region indicates the sample represented in the histogram (Figure 3.21B).

CD166 (ALCAM)

All cells incubated with CD166 antibody exhibited increased MFI relative to controls (3.83 fold; Figure 3.22B and Table 3.21). The geometric mean of CD29 was significantly different to the control cells ($P=0.0125$).

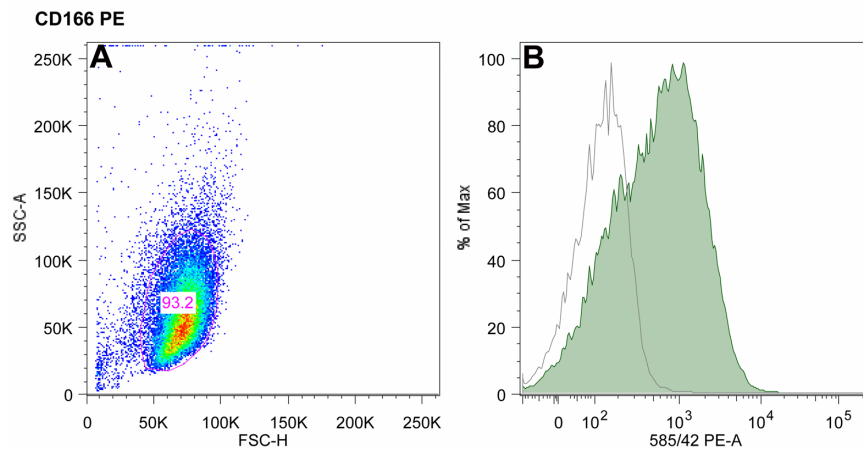


Figure 3.22: CD166 PE expression from cells plated in monolayer overnight

Cells were gated, excluding clumped cells and debris (A) this gate accounted for 93.2% of the total population. The gated control (grey line) and CD166 (green) labelled cells were analysed for fluorescent intensity (B).

Geometric mean values

	Control	CD166 PE	MFI ratio
Sample 1	126.00	368.00	2.92
Sample 2	119.00	524.00	4.40
Sample 3	134.00	557.00	4.16
	Mean		3.83

Table 3.21: CD166 PE expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted region indicates the sample represented in the histogram (Figure 3.22B).

3.3.7 Immunofluorescence with non-conjugated antibodies

Notch1

The fluorescent intensity of both fixed and live cells labelled with Notch1 was not statistically different to the controls (fixed; $P = 0.2963$ and live $P = 0.0518$; Figure 3.23 and Table 3.22).

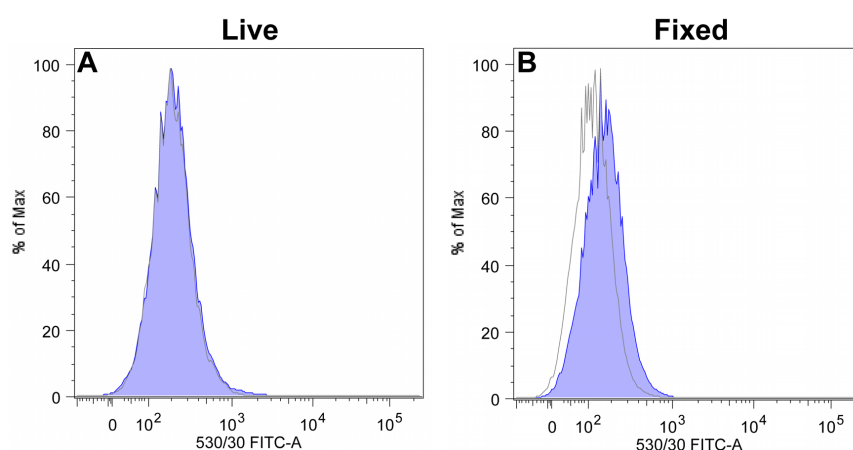


Figure 3.23: Notch1 expression from cells plated in monolayer overnight

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and Notch1 (blue) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Live cells				Fixed cells			
	Control	Notch1	MFI ratio		Control	Notch1	MFI ratio
Sample 1	117.00	116.00	0.99	Sample 1	126.00	261.00	2.07
Sample 2	110.00	119.00	1.08	Sample 2	270.00	201.00	0.74
Sample 3	198.00	205.00	1.04	Sample 3	187.00	180.00	0.96
Sample 4	133.00	138.00	1.04	Sample 4	114.00	158.00	1.39
		Mean	1.04			Mean	1.29

Table 3.22: Notch1 expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted regions indicate the samples represented in the histograms (Figure 3.23).

Delta1

Delta1 expression was not observed with live or fixed cells, (Figure 3.24 and Table 3.23). The geometric mean of Delta1 cells was not significantly different to the control cells (fixed; $P = 0.4721$ and live; $P = 0.1039$).

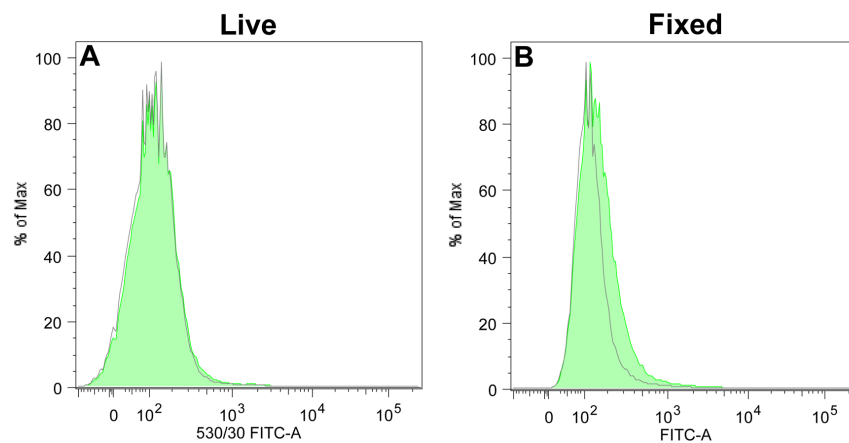


Figure 3.24: Delta1 expression from cells plated in monolayer overnight

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and Delta (green) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Live cells				Fixed cells			
	Control	Delta	MFI ratio		Control	Delta	MFI ratio
Sample 1	102.00	118.00	1.16	Sample 1	126.00	156.00	1.24
Sample 2	110.00	117.00	1.06	Sample 2	270.00	181.00	0.67
Sample 3	198.00	199.00	1.01	Sample 3	187.00	175.00	0.94
		Mean	1.08	Sample 4	114.00	196.00	1.72
						Mean	1.14

Table 3.23: Delta1 expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted regions indicate the samples represented in the histograms above (Figure 3.24).

Jagged1

Jagged1 expression was not observed with live cells, however, all fixed cells labelled with Jagged1 exhibited increased MFI relative to controls (3.73 fold; Figure 3.25 and Table 3.24). The geometric mean of fixed Jagged1 cells was significantly different to the control cells ($P = 0.0467$); the live cells were not statistically different ($P = 0.0635$).

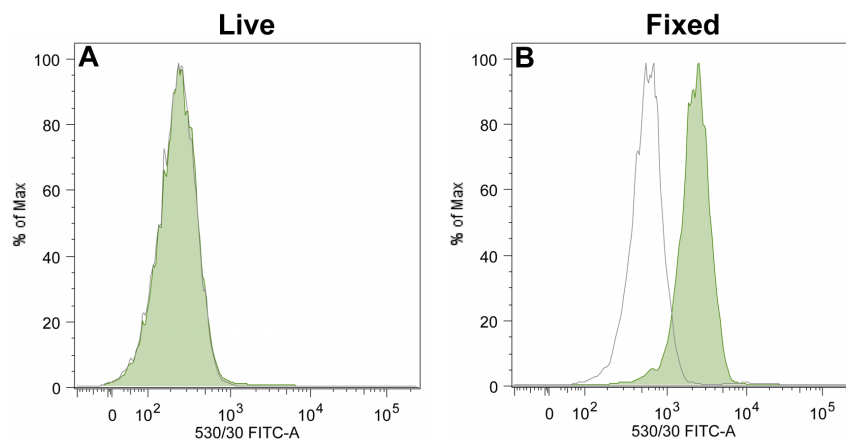


Figure 3.25: Jagged1 expression from cells plated in monolayer overnight
Cells were gated, excluding clumped cells and debris. The gated control (grey line) and Jagged1 (green) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Live cells				Fixed cells			
	Control	Jagged1	MFI ratio		Control	Jagged1	MFI ratio
Sample 1	207.00	218.00	1.05	Sample 1	2707.00	7055.00	2.61
Sample 2	227.00	239.00	1.05	Sample 2	513.00	2469.00	4.81
Sample 3	197.00	231.00	1.17	Sample 3	574.00	2160.00	3.76
		Mean	1.09			Mean	3.73

Table 3.24: Jagged1 expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted regions indicate the samples represented in the histograms above (Figure 3.25).

Jagged2

Jagged2 expression was not observed with live or fixed cells, (Figure 3.26 and Table 3.25). The geometric mean of Jagged2 cells was not significantly different to the control cells (fixed; $P = 0.4863$ and live; $P = 0.2643$).

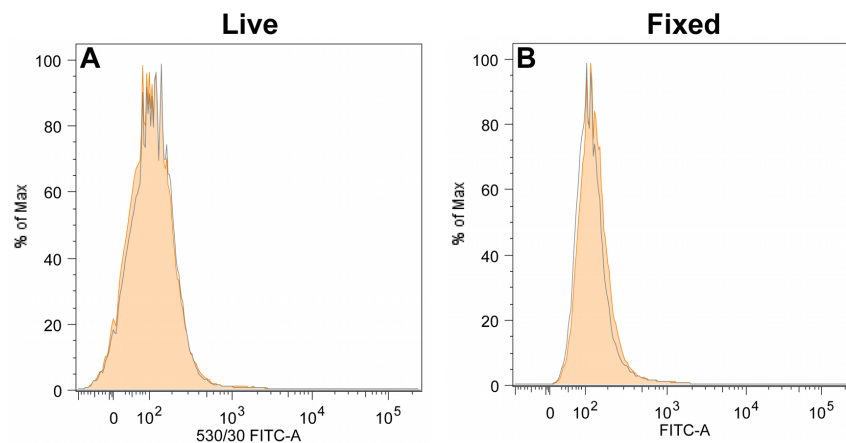


Figure 3.26: Jagged2 expression from cells plated in monolayer overnight

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and Jagged2 (orange) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Live cells				Fixed cells			
	Control	Jagged2	MFI ratio		Control	Jagged2	MFI ratio
Sample 1	102.00	106.00	1.04	Sample 1	126.00	137.00	1.09
Sample 2	110.00	106.00	0.96	Sample 2	270.00	220.00	0.81
Sample 3	133.00	141.00	1.06	Sample 3	187.00	177.00	0.95
		Mean	1.02	Sample 4	114.00	160.00	1.40
						Mean	1.06

Table 3.25: Jagged2 expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted regions indicate the samples represented in the histograms above (Figure 3.26).

Msx1

Msx1 expression was not observed. The geometric mean of Msx1 labelled cells was not statistically different to the control cells ($P = 0.4796$; Figure 3.27 and Table 3.26).

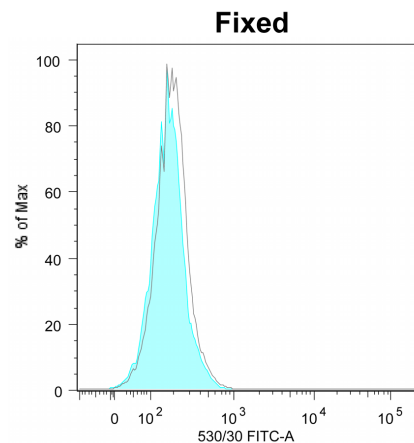


Figure 3.27: Msx1 expression from cells plated in monolayer overnight

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and Notch1 (blue) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Fixed cells			
	Control	Msx1	MFI ratio
Sample 1	270.00	175.00	0.65
Sample 2	187.00	169.00	0.90
Sample 3	114.00	217.00	1.90
	Mean		1.15

Table 3.26: Msx1 expression from cells plated in monolayer overnight

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted regions indicate the samples represented in the histograms above (Figure 3.27).

3.3.8 Expression of freshly isolated cells compared with cells cultured for 24 hours (conjugated antibodies)

When the mean MFI of cells cultured for 24 hours was compared to freshly isolated cells an increase was observed with CD29 ($P = 0.0042$), CD44 ($P = 0.0002$), CD49e ($P = 0.0037$), CD105 ($P = 0.0090$), CD117 ($P = 0.0331$) and CD166 ($P = 0.0039$) (Figure 3.28).

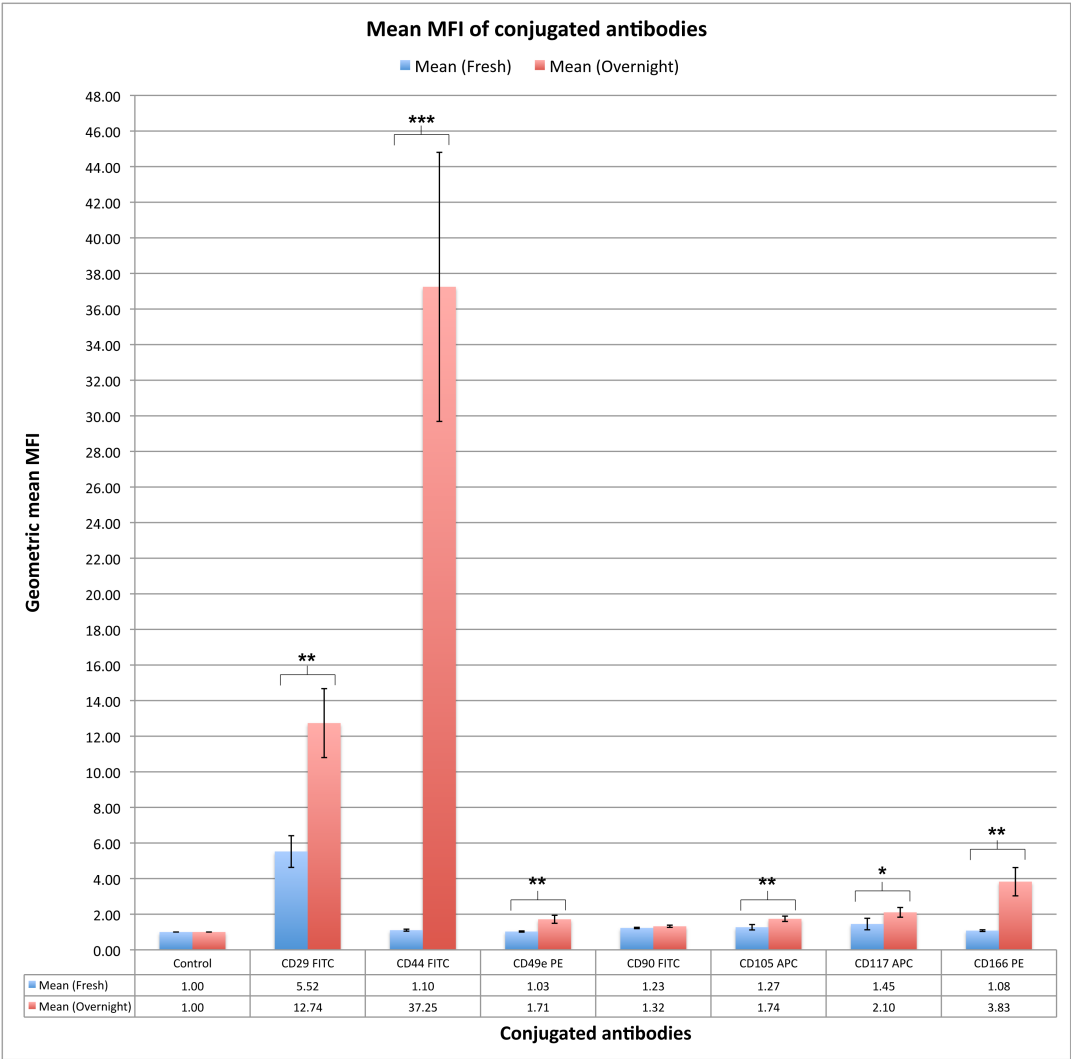


Figure 3.28: Histogram of mean MFI of conjugated antibodies

The graph illustrates the mean MFI of cells immunolabelled immediately following enzymatic digestion and cells cultured for 24 hours in monolayer. Significant differences in MFI between the time points are represented by * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

3.3.9 Expression of freshly isolated cells compared with cells cultured for 24 hours (non-conjugated antibodies)

When the mean MFI of cells cultured for 24 hours was compared to freshly isolated cells a decrease was observed with fixed cells Notch1 ($P = 0.0017$), Delta1 ($P = 0.0020$), Jagged2 ($P = 0.0028$) and Msx1 ($P = 0.0222$) (Figure 3.29). The only significant difference with live cells was seen between the Notch1 time points, with a decrease in the mean MFI when cells cultured for 24 hours were compared to freshly isolated cells ($P = 0.0243$).

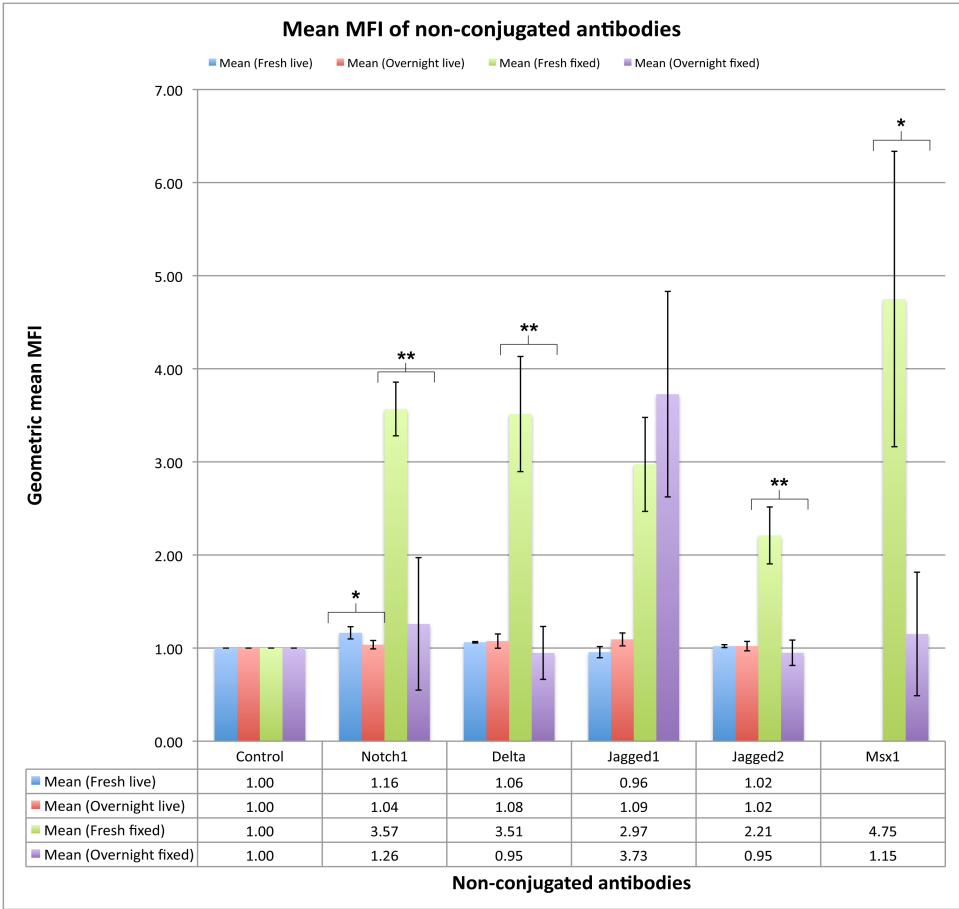


Figure 3.29: Histogram of mean MFI of non-conjugated antibodies

The graph illustrates the mean MFI of cells immunolabelled immediately following enzymatic digestion and cells immunolabelled following 24-hour culture in monolayer. Each of the time points was tested on live and fixed cells. Significant differences in MFI between the time points are represented by * ($P < 0.05$) or ** ($P < 0.01$).

3.3.10 Zonal variation in cell surface marker expression

As a number of the markers were not expressed when tested immediately following digestion, zonal variation was investigated after the cells were incubated for 24 hours in monolayer culture. Developmental markers; Notch1, Delta1, Jagged1, Jagged2 and Msx1 and the cell surface receptor CD29 were not tested for zonal variation as immunolabelling in tissue found they were expressed in all zones (Chapter 2).

The zonal variation experiments were only carried out once as a preliminary study so statistical analysis has not been carried out on any differences observed between zones.

CD44 (Hyaluronan receptor)

When cells from each zone were labelled with CD44 antibody a sub-population of cells expressed the marker, resulting in a bimodal distribution (Figure 3.30). Each zone differed in the percentage of CD44 labelled cells (surface = 25.2%, middle = 42.3% and deep = 25.4%) and the MFI ratio between labelled (+ve) and unlabelled cells (-ve; surface = 26.19 fold, middle = 30.5 fold and deep = 9.31 fold).

CD44 FITC

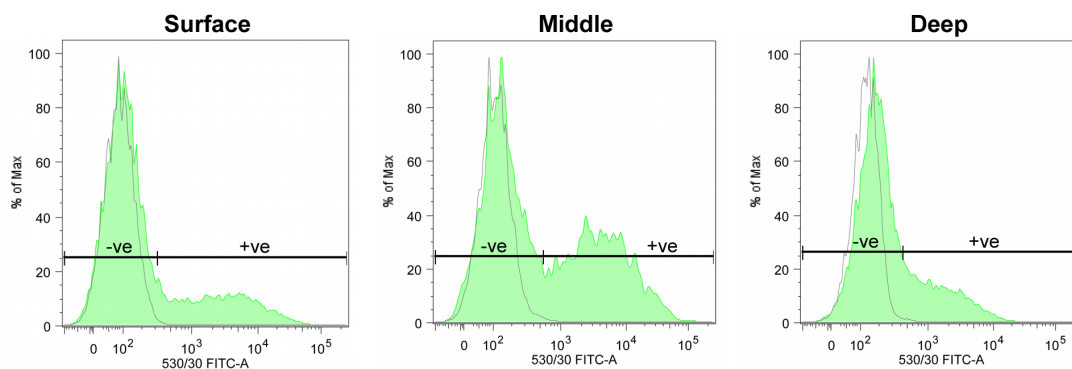


Figure 3.30: CD44 FITC expression from surface, middle and deep zone cells

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and CD44 (green) labelled cells were analysed for fluorescent intensity. The bimodal distribution was divided into unlabelled cells (-ve) and labelled cells (+ve).

CD49e (Integrin $\alpha 5$)

Cells from all zones labelled with CD49e antibody, exhibited increased geometric mean relative to controls (surface = 1.60 fold; middle = 1.61 fold and deep = 1.40 fold; Figure 3.31).

CD49e PE

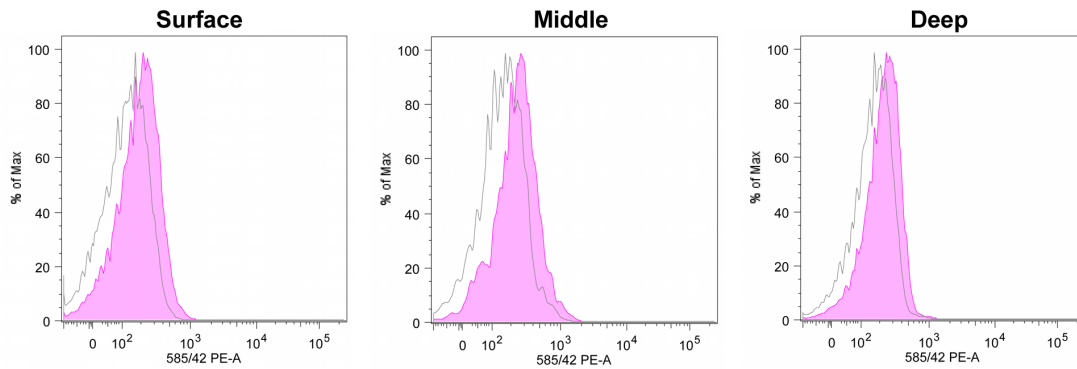


Figure 3.31: CD49e PE expression from surface, middle and deep zone cells

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and CD49e (pink) labelled cells were analysed for fluorescent intensity.

CD90 (Thy-1)

All cells labelled with CD90 antibody exhibited a small increase in MFI relative to controls (surface = 1.40 fold; middle = 1.41 fold and deep = 1.35 fold; Figure 3.32).

CD90 FITC

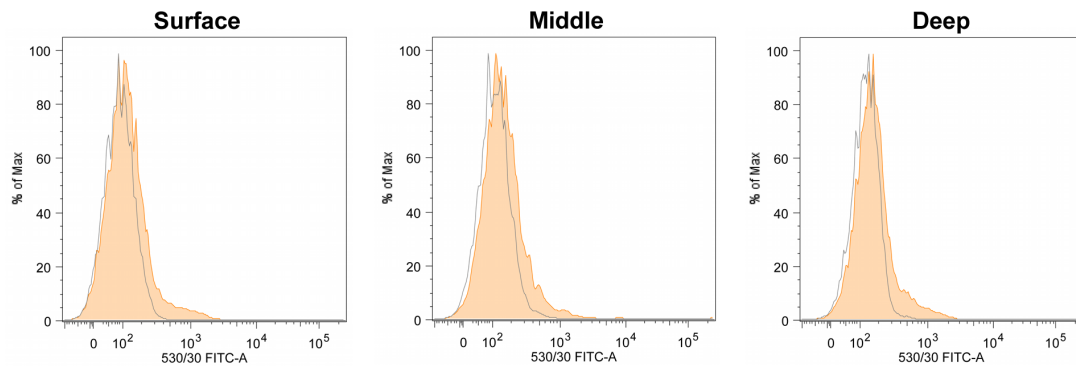


Figure 3.32: CD90 FITC expression from surface, middle and deep zone cells

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and CD90 (orange) labelled cells were analysed for fluorescent intensity.

CD105 (Endoglin)

Cells from each zone labelled with CD105 antibody exhibited increased MFI relative to controls (surface = 1.79 fold; middle = 2.11 fold and deep = 1.57 fold; Figure 3.33).

CD105 APC

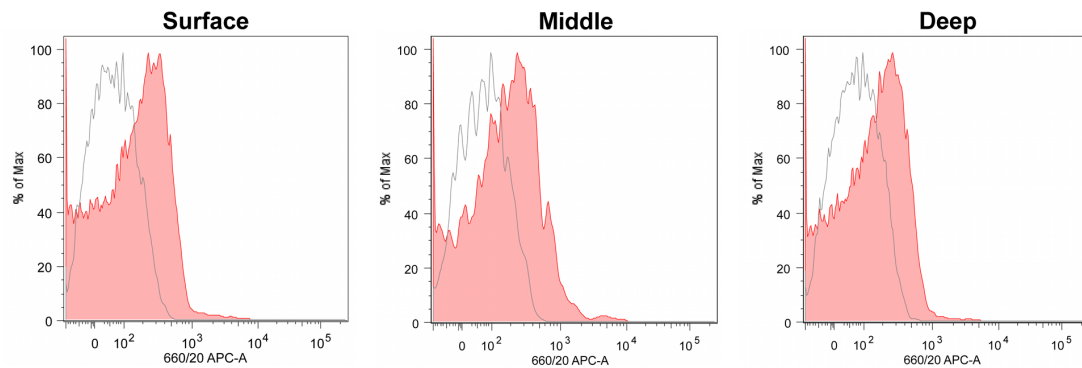


Figure 3.33: CD105 APC expression from surface, middle and deep zone cells

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and CD105 (pink) labelled cells were analysed for fluorescent intensity.

CD117 (c-kit)

Cells from all three zones labelled with CD117 antibody exhibited increased geometric mean relative to controls (surface = 2.01 fold; middle = 3.29 fold and deep = 1.57 fold; Figure 3.34).

CD117 APC

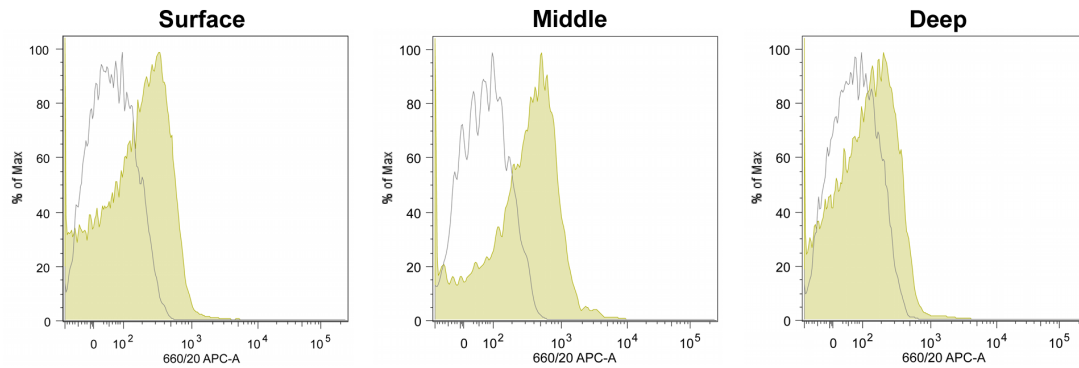


Figure 3.34: CD117 APC expression from surface, middle and deep zone cells

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and CD117 (green) labelled cells were analysed for fluorescent intensity.

CD166 (ALCAM)

When surface zone cells were incubated with CD166 antibody a subpopulation of immunolabelled cells was identified equating to 43.1% of the total population (Figure 3.35 +ve) with a 9.66 fold increase in MFI compared with the negative cells (-ve). Middle and deep zones cells exhibited an increased MFI relative to controls but the distribution was unimodal (middle = 3.99 fold and deep = 10.32 fold; Figure 3.35).

CD166 PE

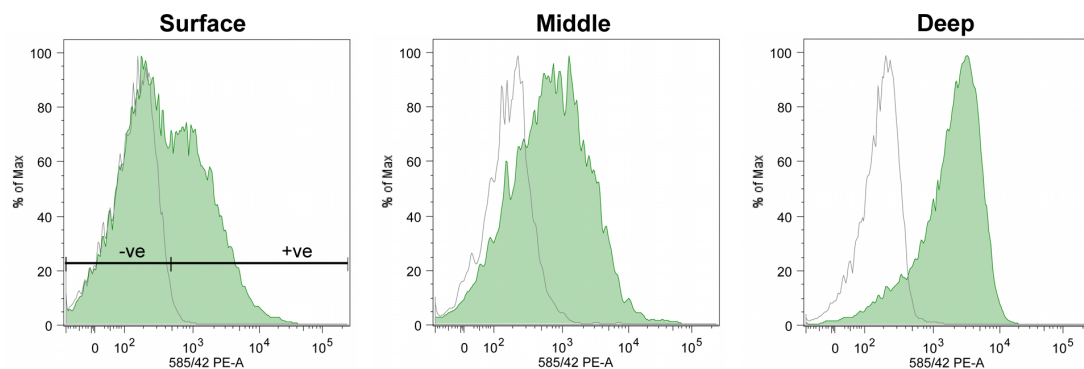


Figure 3.35: CD166 PE expression from surface, middle and deep zone cells

Cells were gated, excluding clumped cells and debris. The gated control (grey line) and CD166 (green) labelled cells were analysed for fluorescent intensity. The bimodal distribution of the surface zone cells was divided into unlabelled cells (-ve) and labelled cells (+ve).

3.3.11 Immunofluorescence with bone marrow stromal cells

A number of the markers used have been reported in bone marrow stromal cells (BMSCs). The cells were photographed after 6 days in monolayer culture when colonies are evident (Figure 3.36; A and B) and then again when the cells are confluent after 19 days in culture and prior to detachment with Accutase (Figure 3.36; C and D).

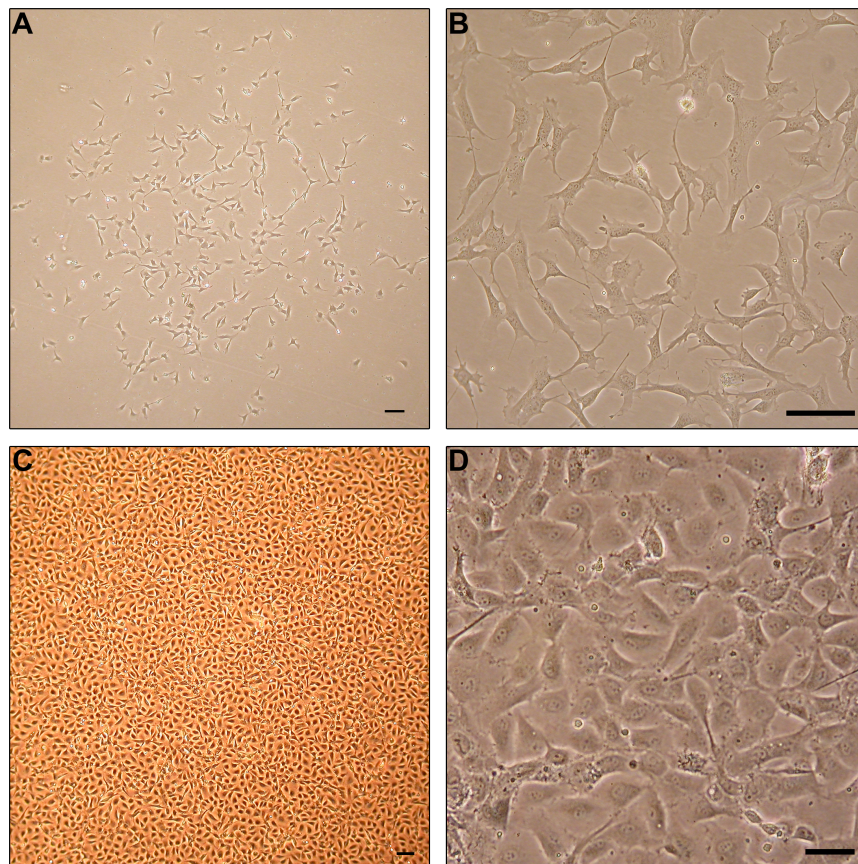


Figure 3.36: Bone marrow stromal cells in monolayer culture

Images of bone marrow stromal cells, initially forming colonies (A; low power, B; high power) then expanding to confluence (C; low power and D; high power).

Scale Bars = 100 μ m.

3.3.12 Immunofluorescence of BMSCs with conjugated antibodies

Gated cells were labelled, plotted against controls and the distributions compared (Figure 3.37). BMSCs labelled with CD29, CD44 and CD166 displayed high expression levels with increases in MFI of 25.84, 104.69 and 74.74 fold respectively. Expression was also observed with CD49e (2.06 fold). The remaining markers were only present at low levels (CD90; 1.09 fold, CD105 = 1.21 fold; and CD117 = 1.19 fold).

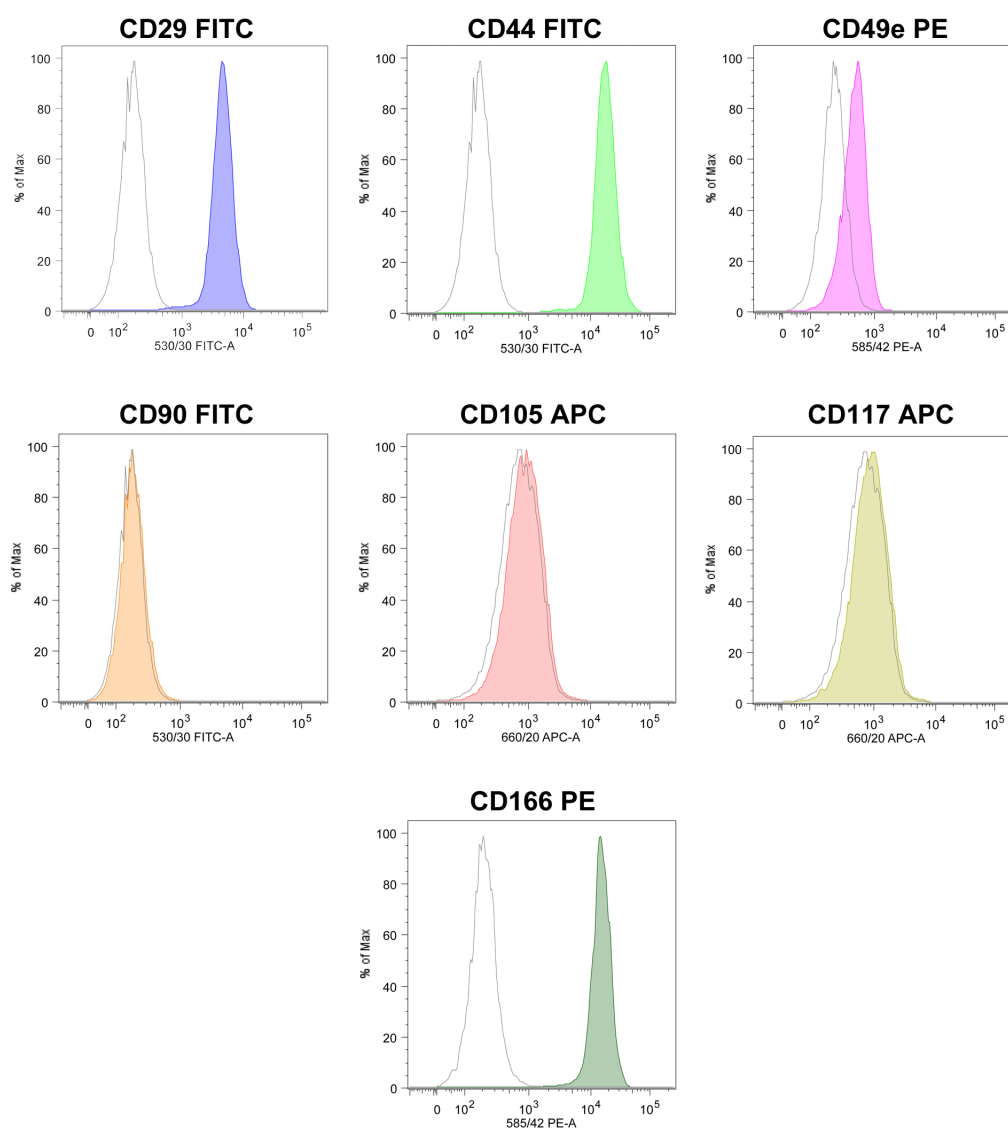


Figure 3.37: Cell surface marker expression from BMSCs plated in monolayer. Cells were gated, excluding clumped cells and debris. The gated control (grey line) and immunolabelled cells (coloured) were analysed for fluorescent intensity.

3.3.13 Immunofluorescence of BMSCs with non-conjugated antibodies

Gated cells were labelled, plotted against controls and the distributions compared (Figure 3.38). Msx1 MFI was similar to the control (1.03 fold); the remaining, transmembrane markers (Notch1, Delta1, Jagged1 and Jagged2) were all expressed with marked increases in MFI relative to controls of 8.55, 4.48, 2.76 and 3.06 fold respectively.

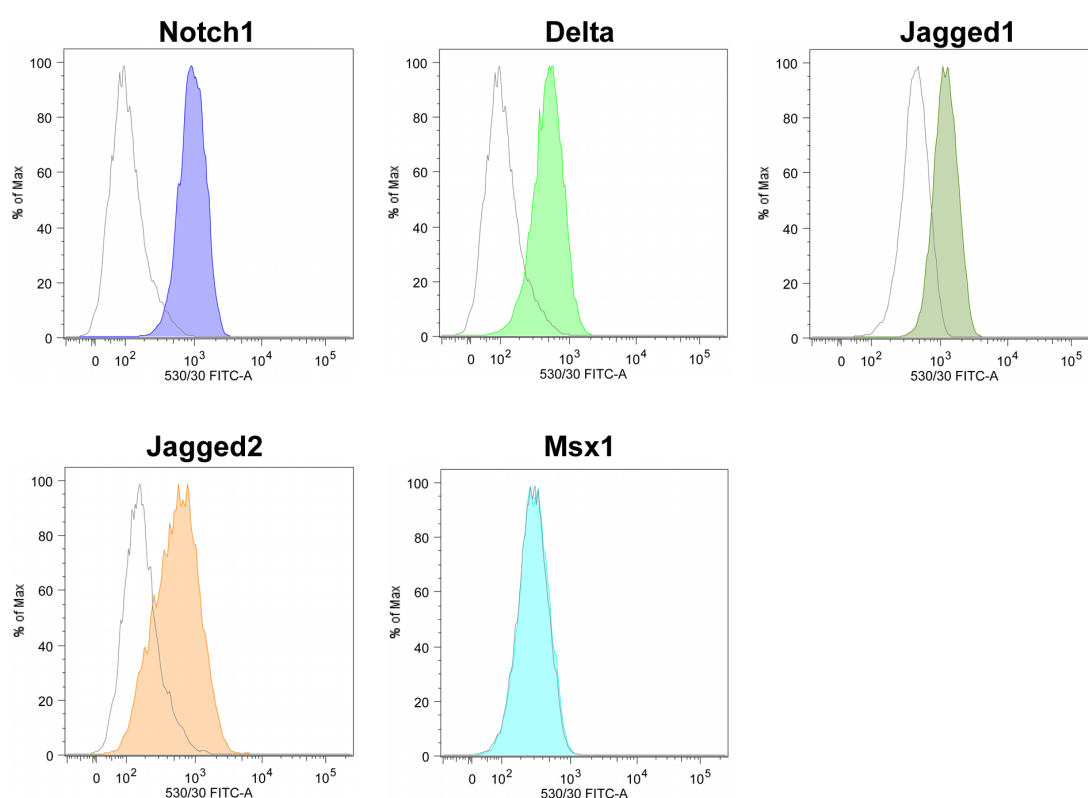


Figure 3.38: Non-conjugated antibody expression from BMSCs plated in monolayer
Cells were gated, excluding clumped cells and debris. The gated control (grey line) and immunolabelled cells (coloured) were analysed for fluorescent intensity.

3.4 Discussion

In this Chapter, experiments were carried out using chondrocytes isolated from the superficial zone. Cells isolated from the middle and deep zones were also used for comparison. Chondrocytes were immunolabelled immediately following cell isolation or following 24 hours in monolayer culture.

Freshly isolated cells were analysed using flow cytometry and the expression of cell surface markers (CD29, CD44, CD49e, CD90, CD105, CD117 and CD166) and developmental markers (Notch1, Delta1, Jagged1, Jagged2 and Msx1) studied. Cells were analysed immediately following digestion in an attempt to quantify expression levels observed *in vivo*. During enzymatic digestion, cell surface markers or transmembrane receptors may be cleaved resulting in reduced expression or absence of these markers (Diaz-Romero et al., 2005). To determine the effects of enzymatic digestion, cells were also cultured for 24 hours in monolayer to enable receptor re-expression, thus allowing us to determine the effects of pronase and collagenase digestion on marker expression.

Following enzymatic digestion, all cells expressed CD29, comparable to data obtained from tissue sections in this study. Data also correlate with labelling observed by Dowthwaite and colleagues, where CD29 was observed in the majority of bovine chondrocytes immunolabelled shortly after isolation (Dowthwaite et al., 2004). The high levels of CD29 expression demonstrate that this marker was either unaffected by the initial enzymatic digestion, or, that it was rapidly re-expressed by cells following digestion.

Small increases in the expression of CD44 and CD90 were observed in freshly isolated superficial zone chondrocytes relative to controls. The expression levels of CD44 were very low, varying from observations in tissue, where a small number of bright labelled cells were identified at the articular surface, this may suggest that the CD44 epitope is affected by the cell isolation procedure. CD90 was not detected in tissue; the variation in expression observed in isolated cells may be due to improved

epitope detection in cell suspension or increased sensitivity of flow cytometry compared to fluorescent microscopy. The expression of CD90 was comparable to labelling observed in freshly isolated human OA chondrocytes where a small number of cells expressed CD90 (Fickert et al., 2004).

CD49e, CD105, CD117 and CD166 were not detected in freshly isolated cells. The absence of expression observed with these markers in isolated cells compared to tissue could be due to loss of receptors during enzymatic digestion. In a previous study using immature bovine chondrocytes, cells were incubated in media for a short time following cell isolation prior to immunolabelling. The majority of these cells expressed CD49e, providing further evidence of the effects of pronase or collagenase on cell surface marker expression (Dowthwaite et al., 2004). The effects of pronase and collagenase on receptor antigen presence were investigated by Diaz-Romero and colleagues (2005). Freshly isolated cells were further incubated with pronase or collagenase and then immunolabelled for a selection of cell surface markers including CD44, CD49e, CD90, CD105 and CD166. CD44 and CD49e were not affected by collagenase but CD105 was almost undetectable following collagenase incubation. Pronase had a much greater effect on cell surface expression, with CD44, CD49e, CD90, CD105 and CD166 expression all reduced or exhibiting very low detectability. The results from this latter study suggest that absence or low expression levels of cell surface markers in freshly isolated cells could be the result of the enzymatic treatment rather than reflecting the normal expression levels in cells. Another study, using normal and OA human articular cartilage also commented on the variation in marker expression between cells in tissue and cells in monolayer culture. These differences in marker expression may be due to enzymatic digestion, either by cleavage of epitopes or by preferential selection of cells during cell isolation, resulting in a reduction or loss of cell expressing these markers (Grogan et al., 2009).

Expression of developmental markers investigated in this study show that cells fixed after incubation with pronase and collagenase labelled for Notch1, Delta1, Jagged1, Jagged2 and Msx1. Labelling observed with freshly isolated fixed cells was comparable to that observed in tissue, suggesting that cell isolation by enzymatic digestion preserves epitopes for these particular markers. In contrast, live freshly isolated cells showed a different labelling pattern. Statistical differences were

observed with Notch1 and Delta1 compared to controls, however, these very small consistent increases in mean fluorescence were not thought to be biologically relevant, as the increases were very minor and mirrored that of the control cells. No expression of Jagged1 or Jagged2 was observed with live cells. As Notch1 and its ligands are transmembrane receptors, the epitopes are likely to be inaccessible in live non-permeabilised cells. Msx1 was not tested on live cells, as this is a nuclear transcription factor. The latter surface markers have not previously been described in freshly isolated chondrocytes.

Following 24 hours in culture in monolayer, the expression of the majority of cell surface markers differed to that observed in freshly isolated cells. All cells continued to express CD29 but the fluorescent intensity was greater than in freshly isolated cells, demonstrating that this marker is maintained in early monolayer culture and increases its expression. There was also a significant increase in the expression of CD49e. These increases in fluorescent intensity of CD29 and CD49e have previously been reported in immature and adult bovine as well as normal human chondrocytes (Dowthwaite et al., 2004, Reid et al., 2000, Diaz-Romero et al., 2005, Diaz-Romero et al., 2008). CD29 and CD49e are integrins and, are thus, associated with cell-cell and cell-matrix interactions, therefore, increases in expression observed following 24 hours in monolayer culture may be related to cell adhesion or matrix assembly (Aszodi et al., 2003).

In contrast to freshly isolated cells, CD44 was observed in live chondrocytes cultured for 24 hours, the distribution was bimodal indicative of a sub-population of CD44 expressing cells. A previous study has reported a similar result (Diaz-Romero et al., 2005, Diaz-Romero et al., 2008). The bimodal distribution of CD44 expression observed following 24 hours in culture is most likely due to a subset of chondrocytes initiating adhesion to the plastic (Knudson and Knudson, 2004).

The expression levels of the remaining cell surface markers (CD105, CD117 and CD166) were all low, indicating weak labelling, this contrasted with freshly isolated cells that did not label for these markers. Unlike the current study, Diaz-Romero and co-workers (2005) observed unimodal distributions with all cell surface markers,

indicative of a homogeneous population with uniform expression rather than a subset of cells displaying differential expression.

The expression levels of CD90 following 24 hours in monolayer culture were very low, which were similar to results observed after enzymatic digestion. These results are comparable to studies using normal human chondrocytes (Diaz-Romero et al., 2005). CD166 expression was detected after 24 hours, again comparable to previous studies in human cells (Diaz-Romero et al., 2005, Diaz-Romero et al., 2008, Giovannini et al., 2010). The increase in expression of CD105 and CD166 compared to freshly isolated cells indicates that these markers are probably cleaved during enzymatic cell isolation.

The expression of developmental markers differed after 24 hours in monolayer to that observed in freshly isolated cells. All developmental markers tested on live cells following 24 hours in culture were identical to control cells, indicating no expression. An increase in expression of Jagged1 was observed with fixed cells. Notch1, Delta1, Jagged2 and Msx1 were all absent in fixed cells suggesting that morphological changes occur within 24 hours of monolayer culture altering levels of transmembrane receptors that are not otherwise affected by enzymatic digestion.

Other studies have demonstrated immunolabelling for Notch1 and its ligands following short periods in monolayer culture. Notch1 was observed in the majority of cells three hours after digestion (Dowthwaite et al., 2004). Notch1, Jagged1 and Delta1 were observed in monolayer cultures of chondroprogenitors, characterised by initial differential adhesion to fibronectin then expansion of clonal populations, however, these studies were in long-term cultures (Williams et al., 2010, Ustunel et al., 2008). Labelling was also observed with full-depth human chondrocytes, however, to a much lesser extent (Williams et al., 2010, Ustunel et al., 2008, Hiraoka et al., 2006). Approximately 5% of human chondrocytes at passage one expressed Notch1 (Hiraoka et al., 2006, Grogan et al., 2009). Again, these data demonstrate how culture conditions and time points in culture can affect marker expression.

As a number of the markers in this study were reduced or absent following enzymatic digestion, zonal variation was tested after cells were cultured in monolayer for 24

hours as a negative control to ascertain the effects of this short time in culture. Zonal studies were not carried out with markers that were observed throughout the tissue in the previous Chapter (such as CD29, Notch1, Delta1, Jagged1, Jagged2 and Msx1). Of the markers analysed, bimodal distributions were observed with CD44 in cells from all zones. It is interesting to note that superficial and deep zone cells appeared to express less CD44 than middle zone cells. However, in tissue sections it was the superficial zone only that labelled for the receptor. In previous studies the distribution of CD44 has been shown to vary within normal human articular cartilage, one study reported CD44 within the middle and deep zones only (Bosch et al., 2002) and another found CD44 restricted to the superficial and middle zones (Ostergaard et al., 1997). The difference between these studies may be due to the integrity of the tissue collected as 'normal cartilage'. Bosch et al (2002) harvested tissue from patients undergoing joint surgery but classed this as normal, Ostergaard and colleagues also investigated the expression of CD44 within OA cartilage and found the cell marker was restricted to the deep zone (Ostergaard et al., 1997).

Very little difference in expression was observed with CD49e, CD90 or CD105 but in all, the surface and middle zone expressed similar levels, with lower median fluorescent intensities in the deep zone cells. It is also worth mentioning that with all stem cell markers, the middle zone cells always demonstrated greater median fluorescent intensity than the surface zone cells. This pattern was also seen with CD117 with the highest MFI observed within the middle zone cells followed by surface and lowest seen with deep zone cells. The only exception was with CD166 where the highest levels of expression were observed within the deep zone. These results are interesting as similar patterns in expression were observed with CD49e, CD90, CD105 and CD117. The labelling observed with the latter markers is likely to be due to cell adhesion and possible rapid dedifferentiation of middle and deep zone cells to a less differentiated phenotype. CD166 labelling within superficial zones demonstrated a partial sub-population; this was not observed previously and may be due to a subset of cells that have not yet re-expressed the marker following enzymatic digestion. It is interesting to note that CD166 labelling was most abundant in deep zone cells. CD166 is associated with cell adhesion by clustering of CD166 at the cell surface and deep zone cells are known to adhere to plastic more rapidly than superficial zone cells (Siczkowski and Watt, 1990).

Dowthwaite and colleagues observed evidence for rapid dedifferentiation of chondrocytes in monolayer culture where the cell expression of CD29 and CD49e were observed at 4 and 72 hours in monolayer culture. After 4 hours in culture, CD49e was observed in the majority of surface zone cells but was present in very few middle or deep zone cells. After 72 hours, the superficial zone expression percentage had decreased to around 45% and the middle and deep zone cell expression had increased to around the same percentage (35-45%). CD29 was more uniformly distributed with 88%, 67% and 62% of cells expressing the cell surface marker in surface, middle or deep zone cells respectively. There was a decline in expression of CD29 after 72 hours (Dowthwaite et al., 2004). The results from the Dowthwaite study demonstrate similar changes in expression as observed in the current study.

In summary, these data demonstrate differences between freshly isolated cells and those cultured in monolayer for 24 hours. The data also highlight the differences in expression of these markers from the native tissue. The differences observed in expression from native tissue to cells cultured overnight may be explained by the increased sensitivity of flow cytometry compared to fluorescence microscopy. However, this conclusion is unlikely as some of the markers produced greater intensity labelling within the middle and deep zones, but in tissue they were not observed in these regions. The evidence from this study and others, however, seems to suggest that the differences observed, are likely due to isolation and adhesion of the cells to plastic. Zonal differences between tissue and cells were also observed, however; these are thought to be a result of culturing and not masked epitopes within the tissue.

To conclude, these data demonstrate that enzymatic digestion typically results in a reduction or loss of cell surface markers and, that, subsequent culture for 24 hours in monolayer, does not produce cell labelling equivalent to that observed in tissue. The differences in cell labelling from tissue, to cells cultured for 24 hours in monolayer, suggest a phenotypic change in the cultured cells. The next Chapter will investigate the expression of the stem cell markers on superficial zone chondrocytes during monolayer culture, to determine whether the variation in labelling continues.

CHAPTER 4:

COMPARATIVE ANALYSIS OF STEM CELL
MARKERS IN PRIMARY MONOLAYER CULTURE

4.1 Introduction

It is well known that articular chondrocytes dedifferentiate in monolayer culture. Cells re-enter the cell cycle, proliferate and become fibroblastic. There is a shift from expression of typical markers of differentiated collagens (types II, IX and XI) to markers of dedifferentiation (collagen types I and III; (Benya and Shaffer, 1982, Archer et al., 1990, Darling and Athanasiou, 2005). In a recent study, a decline in collagen type II was observed after two weeks in monolayer culture, which decreased with further culture and as, expected, chondrocytes changed from a polygonal morphology to elongated fibroblast-like phenotype in this time period (Diaz-Romero et al., 2005). Many studies have been conducted on dedifferentiation in monolayer culture, however, few studies have investigated the effects of dedifferentiation on cell surface marker expression during primary culture of articular chondrocytes particularly in immature bovine articular chondrocytes.

Mesenchymal stem cell markers are routinely used to assess progenitor characteristics in expanded chondrocyte cultures combining cell surface expression with differentiation of these cells along specific lineages, typically osteogenic, adipogenic or re-differentiation to a chondrogenic phenotype (Barbero et al., 2003, de la Fuente et al., 2004, Tallheden et al., 2003). Many of these studies are conducted using human articular chondrocytes. Here, large tissue samples are often difficult to obtain and cartilage is often from diseased or aged individuals that have relatively low cellularity. As a small number of cells are often removed from patients, these cells have to be expanded over a series of weeks if not months to enable enough cells for analysis resulting in dedifferentiation.

Alsalameh and colleagues identified a sub-population of human articular chondrocytes in primary culture that immunolabelled for both CD105 and CD166. These dual-labelled cells expressed aggrecan and type I collagen but not type II collagen. On selection and further expansion these dual-labelled cells were shown to undergo adipogenesis and osteogenesis and when placed in pellets re-expressed collagen type II (Alsalameh et al., 2004). A recent study reported less than 10% of

cells expressed CD166 but around 50% expressed CD105 after 3 weeks in monolayer and that the immunolabelling was considerably lower than that observed in the tissue. These results demonstrate the profound changes that occur in marker expression in monolayer cells. Changes could be due to enzymatic digestion initially, down regulation of protein expression in monolayer culture, or preferential loss of cells expressing these markers during the cell isolation process (Grogan et al., 2009).

In an earlier study, human chondrocytes were plated in monolayer and analysed at 14 days or allowed to dedifferentiate further and analysed at 4 weeks (Diaz-Romero et al., 2005). Cells that had been plated down in monolayer for 14 days demonstrated a marked increase in levels of expression for CD44, CD49e, CD90, CD105 and CD166 compared to levels observed at 24 hours, with the greatest increase observed with CD90 (Diaz-Romero et al., 2005). However, a more recent study by the same group reported that CD105 expression remained low throughout monolayer culture (Diaz-Romero et al., 2008). Expression of all markers following 24 hours or 14 days incubation exhibited a unimodal distribution of expression, indicative of a homogeneous population with uniform expression rather than a subset of cells displaying differential expression (Diaz-Romero et al., 2005, Diaz-Romero et al., 2008). In a further study, human chondrocytes were expanded for 2 to 18 weeks in monolayer culture, equating to 10 to 20 population doublings. The majority of the cells tested at 10 or 20 population doublings expressed CD29, suggesting the expression of this integrin is relatively constant throughout monolayer culture. The majority of cells were also positive for CD44 and CD90 with lower levels of expression observed for CD49e, CD105 and CD166. This group also demonstrated CD117 expression was very low throughout the culture period (de la Fuente et al., 2004).

Diaz-Romero and colleagues suggest that several markers including CD44, CD49e, CD90, CD105 and CD166 can be used to distinguish between differentiated and dedifferentiated chondrocytes, demonstrating a phenotypic plasticity of human articular chondrocytes. However, the expression of these markers, which are also expressed by BMSCs, may suggest a subpopulation of progenitor cells within articular cartilage that are enriched during cell isolation and subsequent *in vitro* culture (Diaz-Romero et al., 2005, Diaz-Romero et al., 2008). The aforementioned

studies demonstrate differences in cell surface marker expression between tissue, isolated cells and passaged chondrocytes. However, these studies do not investigate marker changes during early, or, primary monolayer culture.

As discussed, stem cell markers have been identified in immature chondrocytes but variations have been observed with time in culture and between species. At present, little evidence exists that focuses on the expression of these stem cell markers in immature chondrocytes. This Chapter aims to investigate changes that may occur in the cell surface markers: CD29, CD44, CD49e, CD90, CD105, CD117 and CD166 at time points during primary monolayer culture up to and including 14 days.

4.2 Materials and Methods

3.2.1 Tissue isolation and digestion

Superficial zone chondrocytes were harvested from immature bovine limbs as described in Chapter 3. Articular cartilage from two to three joints were used from different animals for each experiment and all experiments were carried out in at least triplicate. Cells were transferred to 75cm² flasks (8×10^4 cells cm²) with 10ml of media+. The cells were cultured for 3, 7 or 14 days in a humidified incubator at 37°C containing 5% CO₂. The media were changed three times a week.

3.2.3 Direct immunofluorescence for flow cytometry

Following 3, 7 or 14 days in monolayer culture, plated cells were washed with 5ml PBS. Accutase (5ml) was added to the cells and incubated at 37°C for 5-10 minutes or until the cells became rounded and were beginning to detach from the flask. Detached cells were transferred to a 50ml tube. The flasks were washed with 5ml PBS to ensure all cells had been obtained and the liquid aspirated to the 50ml tube. The cells were centrifuged at 400xg and supernatant removed.

All centrifugation steps were performed at 400xg for 3 minutes unless otherwise stated. Cells were re-suspended in 1ml PBS/BSA and counted using a haemocytometer as described previously. Cells were re-suspended at 2×10^6 cells ml⁻¹ and 1ml of the cell suspension was transferred to 1.5ml tubes. The cells were centrifuged and the supernatant aspirated. Cells were immunolabelled using CD29FITC, CD44FITC, CD49ePE, CD90FITC or CD105APC, CD117APC and CD166 PE as described in Chapter 3. Following immunolabelling, cells were viewed on a BD FACS Canto flow cytometer.

Control samples were processed as described, omitting primary antibody and, instead, incubating the cells with appropriate IgG-matched controls, or PBS/BSA.

Cells were analysed initially for forward scatter (FSC) and side scatter (SSC). Controls were set to the second log decade where possible. Labelling of cells was assessed by percentage shift or fluorescent intensity of fluorescently labelled cells compared to control cells.

Results were tested for statistical significance using a paired, one-tailed t-test where the geometric mean fluorescent intensity (MFI) of control cells was compared with that of the immunolabelled cells. MFI ratios were also compared between each time point in culture (day 3, 7 and 14); statistical significance was tested using an unpaired, two-tailed t-test.

4.3 Results

4.3.1 Flow cytometry analysis

All data were analysed using FlowJo and gated as described in Chapter 3. As with Chapter 3, bimodal distributions were displayed as percentage labelled and MFI, unimodal distributions were represented as MFI only.

Results shown are representative of labelling seen for each antibody, with the typical or central distributions shown. Each antibody was tested at least in triplicate and controls consisted of unlabelled cells or IgG-matched controls.

4.3.2 Superficial zone chondrocytes at days 3, 7 and 14 in monolayer culture

Cells were isolated using pronase and collagenase and cultured in monolayer for 3, 7 or 14 days. At each time point, the cells were detached using Accutase and labelled with the relevant conjugated primary antibody, $0.5-1.0 \times 10^6$ cells were used to test each antibody.

CD29 (Integrin $\beta 1$)

All cells incubated with CD29 antibody exhibited increased MFI relative to controls (day 3 = 17.64 fold; day 7 = 26.94 fold; and day 14 = 13.88 fold; Table 4.1; Figure 4.1). The geometric mean of CD29 was significantly different to the control cells at each time point (day 3; P= 0.0043, day7; P 0.0017 and day 14; P = 0.0171).

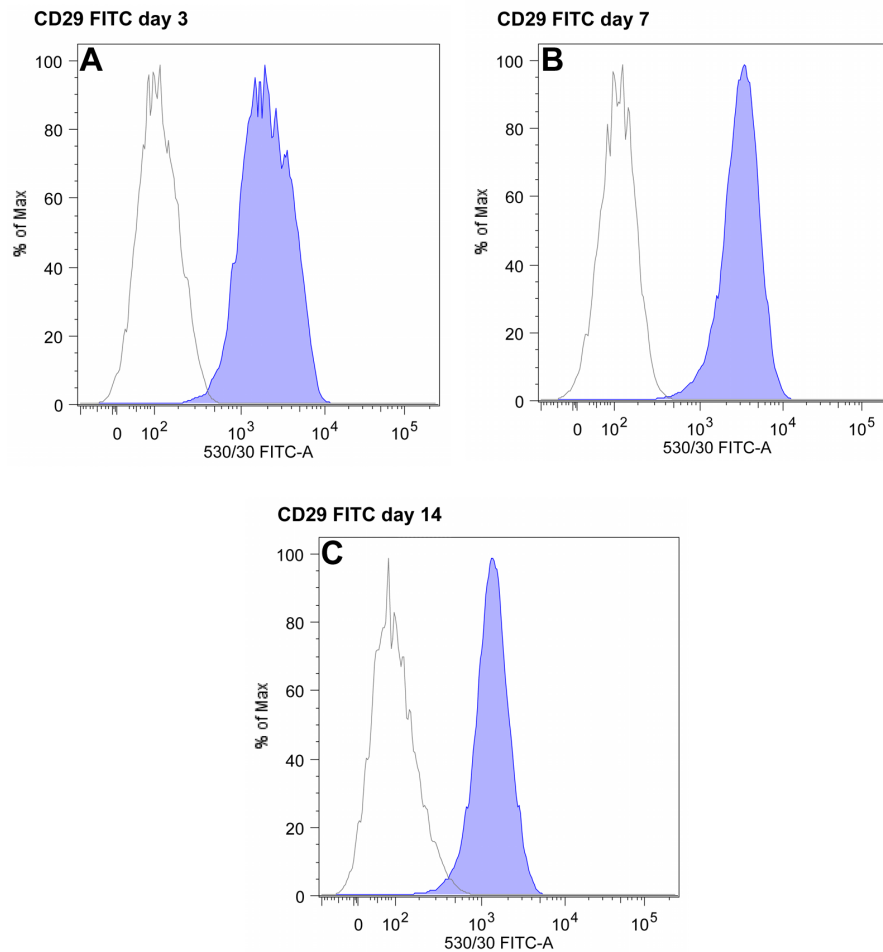


Figure 4.1: CD29 FITC expression from surface cells cultured for 3, 7 or 14 days

The gated control (grey line) and CD29 (blue) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Day 3			
	Control	CD29 FITC	MFI ratio
Sample 1	138.00	2493.00	18.07
Sample 2	113.00	1987.00	17.58
Sample 3	107.00	1848.00	17.27
	Mean		17.64

Day 7			
	Control	CD29 FITC	MFI ratio
Sample 1	119.00	3336.00	28.03
Sample 2	110.00	2735.00	24.86
Sample 3	109.00	3044.00	27.93
	Mean		26.94

Day 14			
	CD29 Control	CD29 FITC	MFI ratio
Sample 1	122.00	1802.00	14.7705
Sample 2	95.10	1309.00	13.7645
Sample 3	195.00	2554.00	13.0974
	Mean		13.88

Table 4.1: CD29 FITC expression from surface cells cultured for 3, 7 or 14 days

Table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted region indicates the sample represented in each histogram (Figure 4.1).

During monolayer culture, all cells continue to express CD29. The expression levels increase from day 3 to day 7 and decrease from 7 to 14 days in culture; these changes in geometric mean were shown to be significantly different (days 3 to 7; $P = 0.0009$, days 7 to 14; $P = 0.0003$). To test whether CD29 expression was returning to levels observed at day 3, the MFI at day 3 and day 14 were compared and found to be significantly different ($P = 0.0022$) indicating that CD29 expression on day 14 dropped below that observed at day 3.

CD44 (Hyaluronan receptor)

A subpopulation of CD44 labelled cells resulting in a bimodal distribution (Figure 4.2 A and B) was observed when cells were immunolabelled with CD44 after 3 and 7 days in culture. The geometric mean of CD44 labelled cells (+ve) was significantly different to the negative population (-ve; day 3; $P = 0.0150$, day 7; $P = 0.0089$). After 14 days in culture, all cells expressed CD44 and the geometric mean was significantly different to the control cells ($P = 0.0250$).

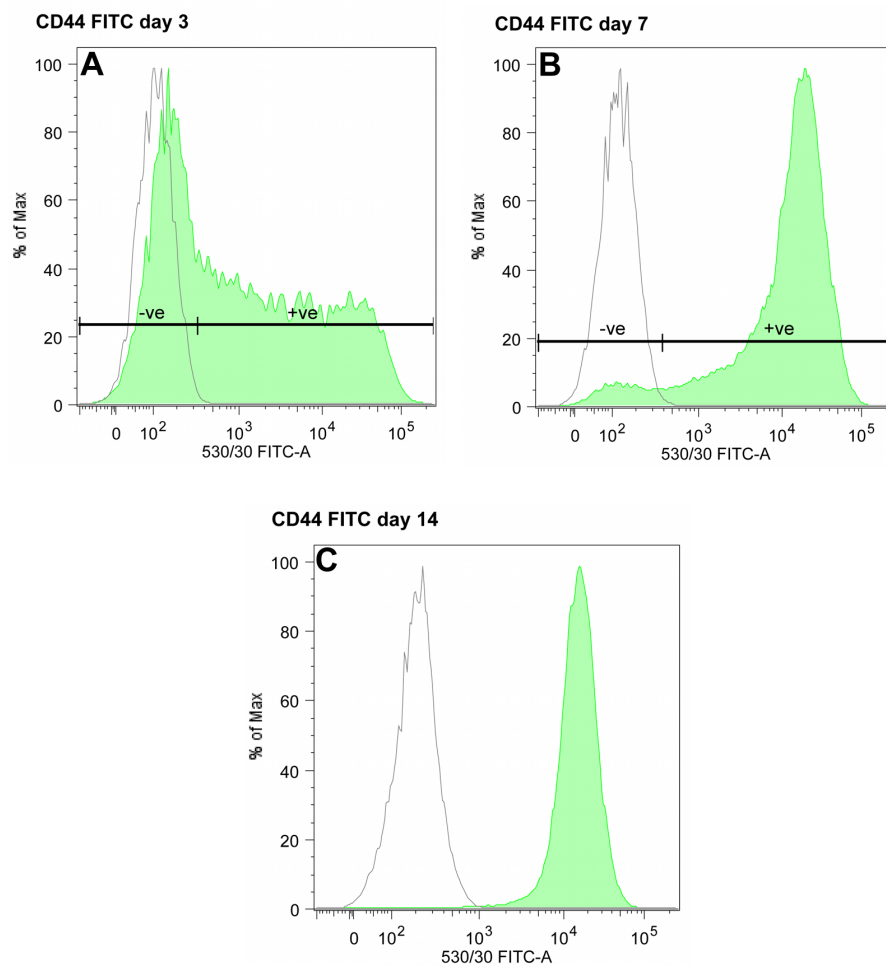


Figure 4.2: CD44 FITC expression from surface cells cultured for 3, 7 or 14 days

The gated control (grey line) and CD44 (green) labelled cells were analysed for fluorescent intensity. The bimodal distributions observed at 3 and 7 days (A and B) were divided into unlabelled cells (-ve) and labelled cells (+ve).

Geometric mean values

Day 3						
	CD44 Control	CD44 -ve	CD44 +ve	MFI ratio +ve/-ve	MFI ratio -ve/control	% +ve
Sample 1	138.00	174.00	6819.00	39.1897	1.26	48.30
Sample 2	113.00	146.00	4650.00	31.8493	1.29	62.30
Sample 3	107.00	229.00	3935.00	17.1834	2.14	59.30
			Mean	29.41	1.56	56.63

Day 7						
	CD44 Control	CD44 -ve	CD44 +ve	MFI ratio +ve/-ve	MFI ratio -ve/control	% +ve
Sample 1	119.00	137.00	12940.00	94.4526	1.15	93.50
Sample 2	110.00	131.00	17700.00	135.1145	1.19	96.70
Sample 3	109.00	129.00	11475.00	88.9535	1.18	93.40
			Mean	106.17	1.18	94.53

Day 14			
	Control	CD44 FITC	MFI ratio
Sample 1	156.00	5014.00	32.14
Sample 2	122.00	4256.00	34.89
Sample 3	95.10	7697.00	80.94
Sample 4	195.00	15203.00	77.96
		Mean	56.48

Table 4.2: CD44 FITC expression from surface cells cultured for 3, 7 or 14 days

The table contains the MFI for each cell sample acquired and the MFI ratios between CD44+ve and CD44-ve, and CD44-ve and control cells for days 3 and 7. The highlighted region indicates the sample represented in each histogram (Figure 4.2).

During monolayer culture the mean percentage of cells expressing CD44 increased from 56.63% to 94.53% from 3 to 7 days. The MFI ratio between labelled and unlabelled cells also increased (day 3 = 26.41 fold and day 7 = 106.17 fold; Table 4.2), these changes were statistically significant ($P = 0.0091$). No significant difference was observed in expression of CD44 between 7 and 14 days ($P = 0.0546$).

CD49e (Integrin $\alpha 5$)

At day 3 and day 14 a small increase in fluorescence intensity was observed between CD49e and control cells, however, this change was shown not to be statistically different (day 3; $P = 0.0987$ and day 14; $P = 0.1082$; Figure 4.3 and Table 4.3). Following 7 days in monolayer culture the increase in geometric means of immunolabelled cells was shown to be significantly different to the control cells ($P = 0.0031$).

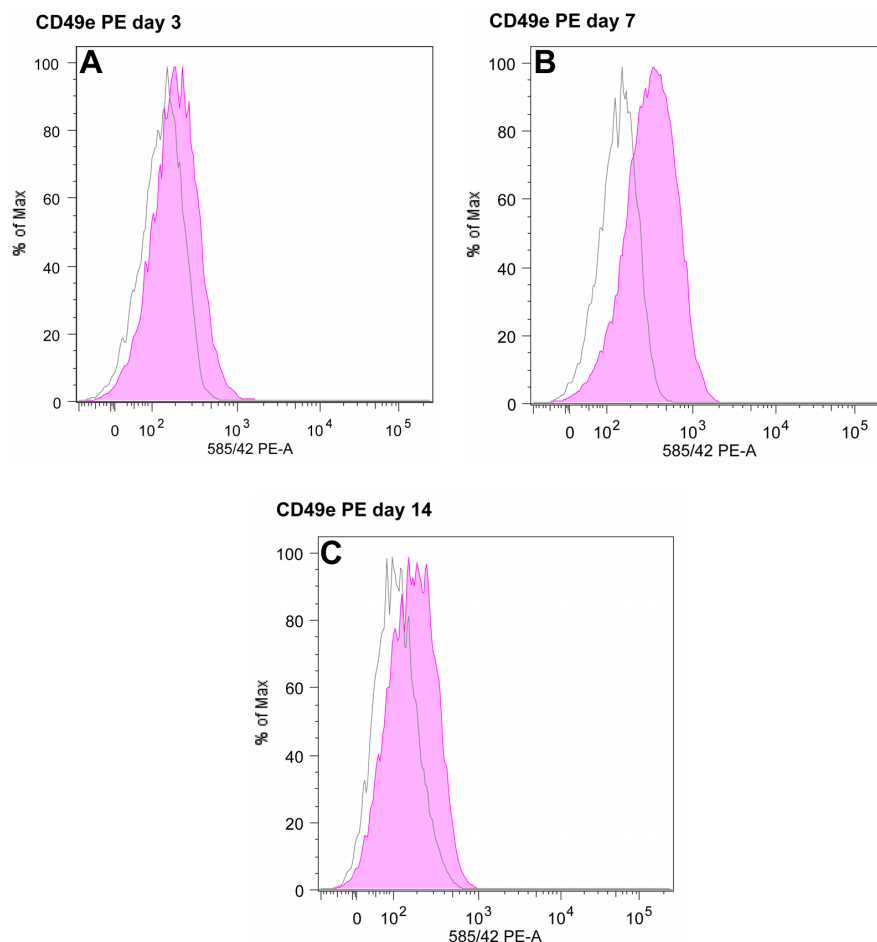


Figure 4.3: CD49e PE expression from surface cells cultured for 3, 7 or 14 days

The gated control (grey line) and CD49e (pink) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Day 3			
	Control	CD49e PE	MFI ratio
Sample 1	107.00	105.00	0.98
Sample 2	135.00	200.00	1.48
Sample 3	132.00	192.00	1.45
		Mean	1.31

Day 7			
	Control	CD49e PE	MFI ratio
Sample 1	148.00	390.00	2.64
Sample 2	147.00	346.00	2.35
Sample 3	138.00	326.00	2.36
		Mean	2.45

Day 14			
	Control	CD49e PE	MFI ratio
Sample 1	117.00	174.00	1.49
Sample 2	98.40	178.00	1.81
Sample 3	107.00	169.00	1.58
Sample 4	605.00	1142.00	1.89
		Mean	1.69

Table 4.3: CD49e PE expression from surface cells cultured for 3, 7 or 14 days

Table contains the MFI values for each sample recorded and the MFI ratio of CD49e labelled and control cells. The highlighted region indicates the sample represented in each histogram (Figure 4.3).

The expression levels of CD49e increase between day 3 and day 7 and decrease from 7 to 14 days in culture; these changes in geometric mean were significantly different (days 3 - 7; $P = 0.0036$, days 7 - 14; $P = 0.0025$). The expression of CD49e on day 14 was shown to be equivalent to that on day 3 ($P = 0.0801$).

CD90 (Thy-1)

Gated cells, labelled with CD90 were plotted against controls and the distributions compared (Figure 4.3). Cells labelled with CD90 exhibited increased MFI compared with controls at day 3 (1.35 fold) and day 7 (1.38 fold; Figure 4.4 A and B and Table 4.4). The MFI of CD90 was significantly different to the control cells (day 3; $P=0.0463$, day 7; $P=0.0137$). There was no significant difference between immunolabelled cells and control cells at day 14 ($P=0.0682$).

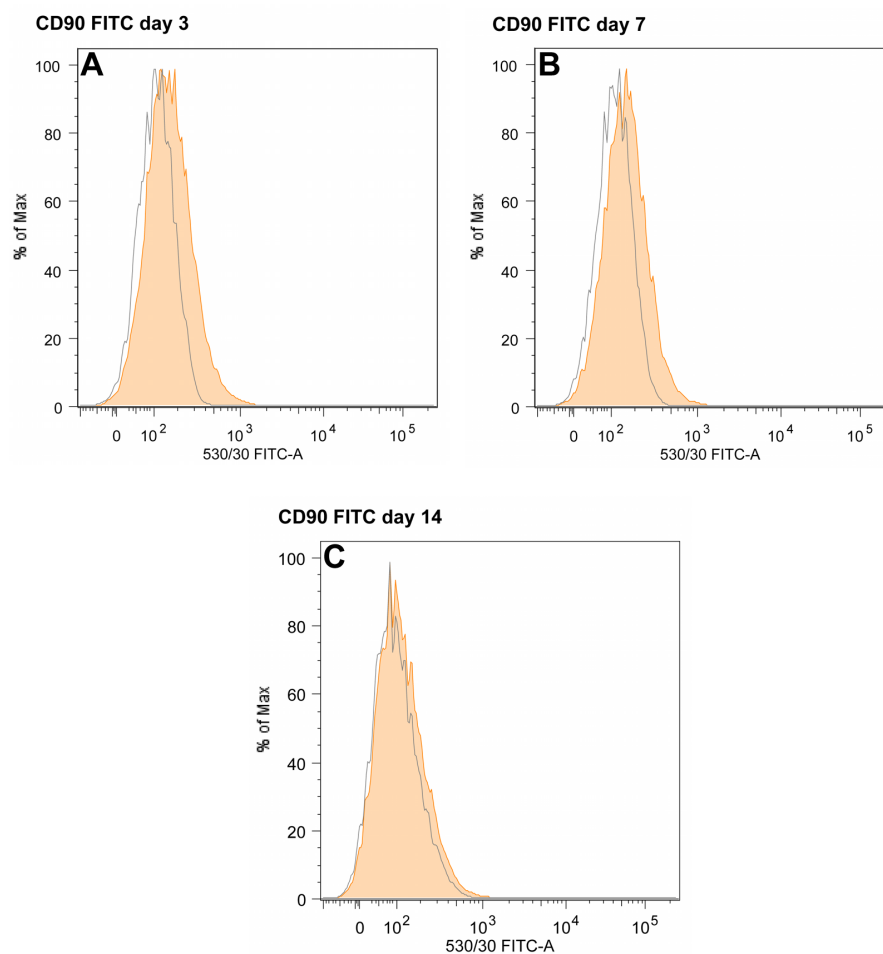


Figure 4.4: CD90 FITC expression from surface cells cultured for 3, 7 or 14 days

The gated control (grey line) and CD90 (orange) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Day 3			
	Control	CD90 FITC	MFI ratio
Sample 1	138.00	152.00	1.10
Sample 2	113.00	169.00	1.50
Sample 3	107.00	156.00	1.46
		Mean	1.35

Day 7			
	Control	CD90 FITC	MFI ratio
Sample 1	119.00	174.00	1.46
Sample 2	110.00	153.00	1.39
Sample 3	109.00	139.00	1.28
		Mean	1.38

Day 14			
	Control	CD90 FITC	MFI ratio
Sample 1	122.00	146.00	1.20
Sample 2	95.10	111.00	1.17
Sample 3	195.00	256.00	1.31
		Mean	1.23

Table 4.4: CD90 FITC expression from surface cells cultured for 3, 7 or 14 days

Table contains the geometric mean values for each sample tested and the MFI ratio. The highlighted region indicates the sample represented in each histogram (Figure 4.4).

The expression levels of CD90 were comparable at day 3 and day 7 with no significant difference observed ($P = 0.8668$). The MFI appeared to decrease between day 7 and day 14, however, this difference was shown not to be statistically significant ($P = 0.0990$).

CD105 (Endoglin)

There was no significant difference between CD105 labelled cells and control cells at day 3 ($P = 0.0602$). By day 7, cells labelled with CD105 exhibited increased MFI compared to controls (1.86 fold; Table 4.5) that was significantly different ($P = 0.0106$). At day 14 a discrete subpopulation was observed when cells were immunolabelled with CD105 (Figure 4.5 C and D). The geometric mean of CD105 labelled cells (+ve) was significantly different to the negative population (-ve; $P = 0.0353$), this discrete subpopulation accounted for on average 1.37% of the total surface population analysed.

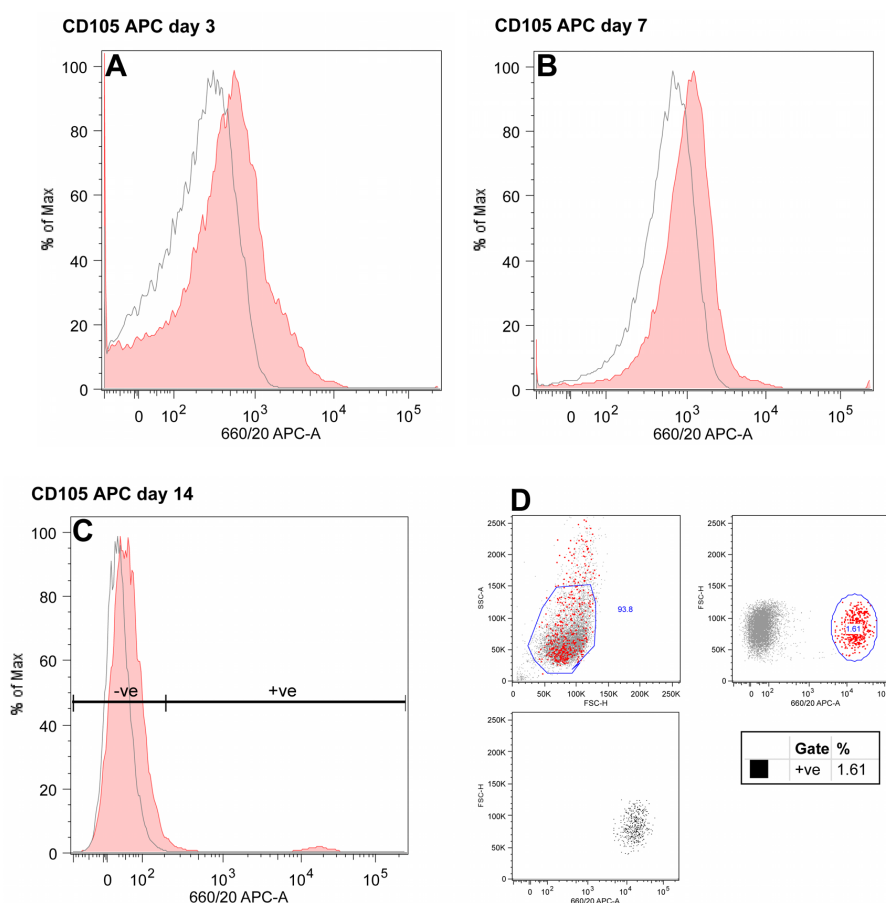


Figure 4.5: CD105 APC expression from surface cells cultured for 3, 7 or 14 days

The gated control (grey line) and CD105 APC (pink) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Day 3			
	Control	CD105 APC	MFI ratio
Sample 1	205.00	453.00	2.21
Sample 2	187.00	323.00	1.73
Sample 3	176.00	231.00	1.31
	Mean		1.75

Day 7			
	CD105 Control	CD105 APC	MFI ratio
Sample 1	527.00	928.00	1.7609
Sample 2	413.00	880.00	2.1308
Sample 3	405.00	680.00	1.6790
	Mean		1.86

Day 14						
	CD105 Control	CD105 -ve	CD105 +ve	MFI ratio +ve/-ve	MFI ratio -ve/control	% +ve
Sample 1	25.30	47.90	435.00	9.0814	1.89	2.10
Sample 2	27.10	49.30	1935.00	39.2495	1.82	1.61
Sample 3	28.80	46.50	535.00	11.5054	1.61	0.90
Sample 4	53.80	60.90	1074.00	17.6355	1.13	0.86
	Mean			19.37	1.61	1.37

Table 4.5: CD105 APC expression from surface cells cultured for 3, 7 or 14 days

Table contains the MFI values for each sample acquired and the MFI ratio between labelled cells and control. The highlighted region indicates the sample represented in each histogram (Figure 4.5).

The expression levels of CD105 were comparable at day 3 and day 7 with no significant difference observed ($P = 0.7344$). There was no significant difference in the MFI between 7 and 14 days in culture ($P = 0.0837$) but a discrete subpopulation was observed at 14 days that was not evident at 7 days.

CD117 (c-kit)

The fluorescent intensity of CD117 labelled cells was equivalent to that of the control cells at day 3 ($P = 0.0670$). At day 7 cells labelled with CD117 exhibited increased MFI compared to controls (1.79 fold; Table 4.6) that was significantly different ($P = 0.0058$). At day 14, a discrete subpopulation was observed when cells were immunolabelled with CD117 (Figure 4.6 C and D). The geometric mean of CD117 labelled cells (+ve) was significantly different to the negative population (-ve; $P = 0.0246$), this discrete subpopulation accounted for 1.31% of the total cell population analysed.

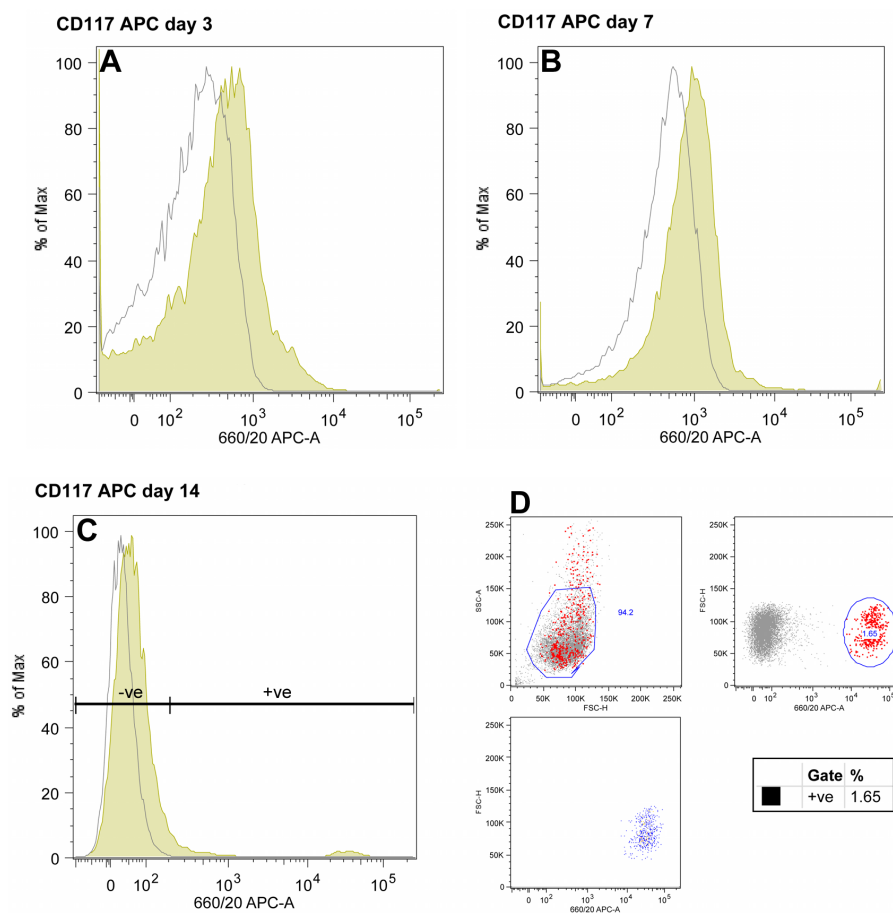


Figure 4.6: CD117 APC expression from surface cells cultured for 3, 7 or 14 days

The gated control (grey line) and CD117 (green) labelled cells were analysed for fluorescent intensity.

Geometric mean values

Day 3			
	Control	CD117 APC	MFI ratio
Sample 1	205.00	350.00	1.71
Sample 2	187.00	612.00	3.27
Sample 3	176.00	308.00	1.75
		Mean	2.24

Day 7			
	CD117 Control	CD117 APC	MFI ratio
Sample 1	527.00	948.00	1.7989
Sample 2	413.00	770.00	1.8644
Sample 3	405.00	692.00	1.7086
		Mean	1.79

Day 14						
	CD117 Control	CD117 -ve	CD117 +ve	MFI ratio +ve/-ve	MFI ratio -ve/control	% +ve
Sample 1	25.30	49.20	384.00	7.8049	1.94	1.78
Sample 2	27.10	52.80	1322.00	25.0379	1.95	1.65
Sample 3	28.80	57.10	429.00	7.5131	1.98	0.87
Sample 4	53.80	66.60	1060.00	15.9159	1.24	0.93
			Mean	14.07	1.78	1.31

Table 4.6: CD117 APC expression from surface cells cultured for 3, 7 or 14 days

Table contains the geometric mean values for each sample recorded and the MFI ratio of labelled cells compared to controls. The highlighted region indicates the sample represented in each histogram (Figure 4.6).

The expression levels of CD117 were comparable at day 3 and day 7 with no significant difference observed ($P = 0.4305$). There was no significant difference in the MFI between 7 and 14 days in culture ($P = 0.0542$) but a discrete subpopulation was observed at 14 days that was not evident at 7 days.

CD166 (ALCAM)

At day 3 and day 7, cells labelled with CD166 exhibited increased MFI compared to controls (day 3 = 5.12 fold; day 7 = 17.48 fold; Table 4.7; Figure 4.7) that were significantly different (day 3; $P = 0.0444$, day 7; $P = 0.0326$). At day 14 increases were observed in the geometric mean of CD166 compared to the control (Figure 4.7 and Table 4.7) but these were shown not to be significantly different to the control cells ($P = 0.0540$).

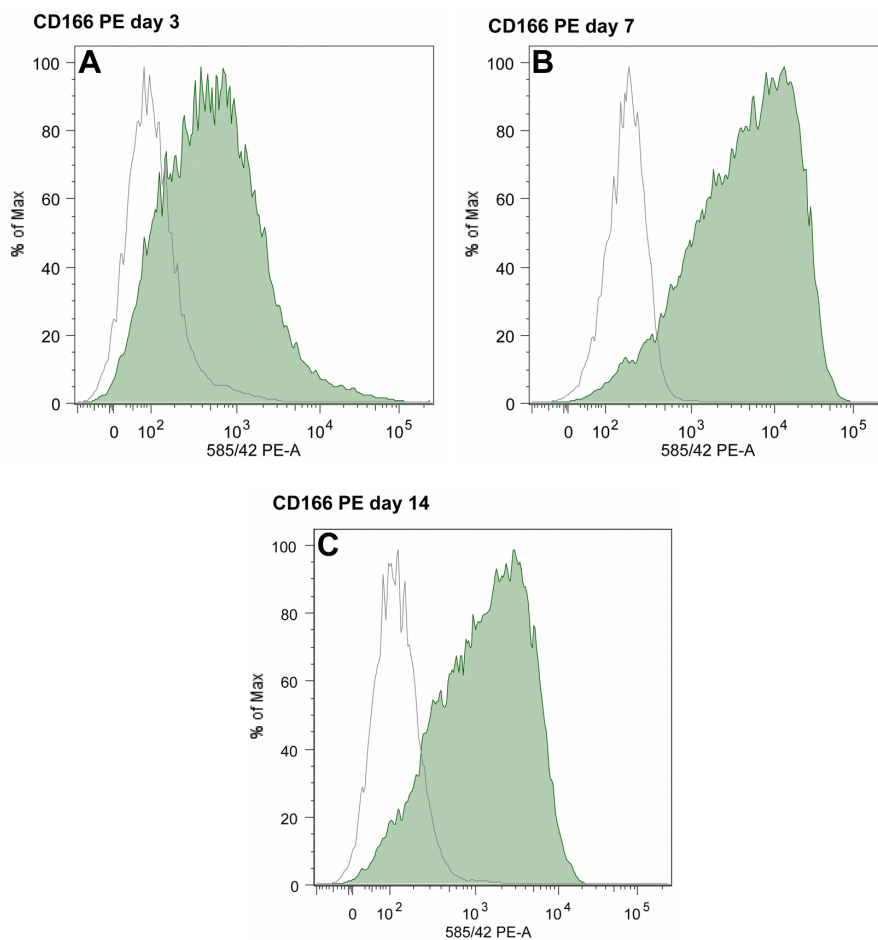


Figure 4.7: CD166 PE expression from surface cells cultured for 3, 7 or 14 days

The gated control (grey line) and CD166 (green) labelled cells were then analysed for fluorescent intensity.

Geometric mean values

Day 3			
	Control	CD166 PE	MFI ratio
Sample 1	107.00	525.00	4.91
Sample 2	222.00	1407.00	6.34
Sample 3	187.00	771.00	4.12
		Mean	5.12

Day 7			
	Control	CD166 PE	MFI ratio
Sample 1	181.00	4191.00	23.15
Sample 2	173.00	4015.00	23.21
Sample 3	282.00	1717.00	6.09
		Mean	17.48

Day 14			
	Control	CD166 PE	MFI ratio
Sample 1	113.00	591.00	5.23
Sample 2	118.00	2036.00	17.25
Sample 3	121.00	1216.00	10.05
		Mean	10.84

Table 4.7: CD166 PE expression from surface cells cultured for 3, 7 or 14 days

The table contains the MFI for each cell sample acquired and the MFI ratio between test and control. The highlighted region indicates the sample represented in each histogram (Figure 4.7).

The expression levels of CD166 were comparable at all three time points with no significant difference observed between day 3 and day 7 ($P = 0.0974$) or day 7 and day 14 ($P = 0.3768$).

4.3.3 Comparison of expression of directly conjugated antibodies from freshly isolated surface cells to day 14 in monolayer culture

When the mean MFI of cells cultured for 24 hours was compared to freshly isolated cells an increase was observed with CD29 ($P = 0.0042$), CD44 ($P = 0.0002$), CD49e ($P = 0.0037$), CD105 ($P = 0.0090$), CD117 ($P = 0.0331$) and CD166 ($P = 0.0039$) (Figures 4.8 and 4.9).

CD29 was the only antibody to demonstrate an increase between 24-hour culture and day 3 ($P = 0.0127$). The other cell surface markers did not show any significant differences during this time.

When the mean MFI of cells cultured for 3 days was compared to cells cultured for 7 days, an increase in expression was observed with CD29 ($P = 0.0009$), CD44 ($P = 0.0091$) and CD49e ($P = 0.0036$).

Differences in mean MFI between cells cultured for 7 and 14 days was only observed with CD29 ($P = 0.0003$) and CD49e ($P = 0.0025$).

It is interesting to note that expression of CD29 and CD49e demonstrated a similar pattern from days 3 to 7; both increased from day 3 to day 7 and declined in expression by day 14, with expression of CD49e at day 14 comparable to expression levels at day 3. CD105 and CD117 also demonstrated similar expression levels during the culture period, both markers identifying a discrete sub-population of approximately 1-2% of the total superficial zone cells analysed.

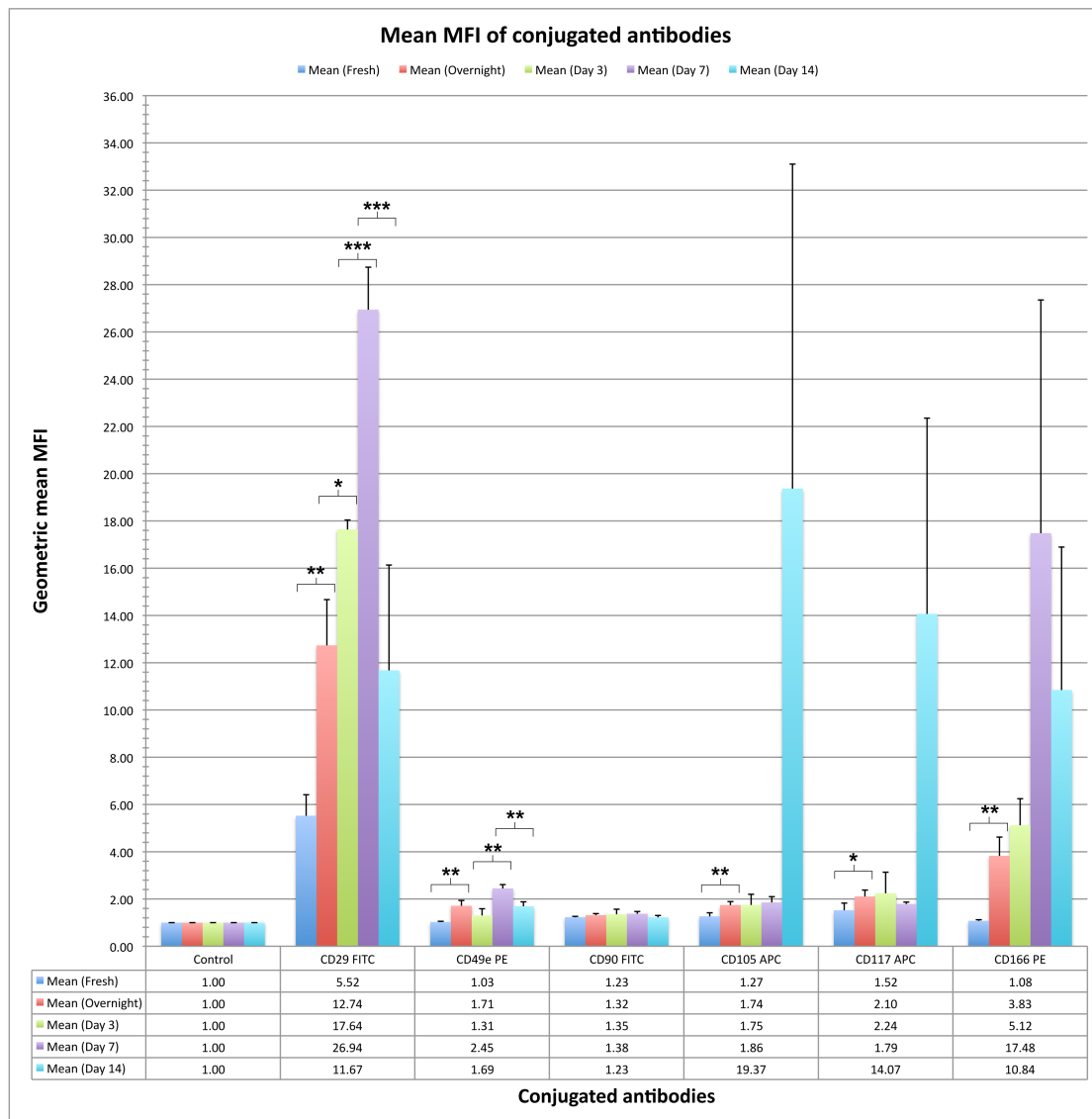


Figure 4.8: Mean MFI of conjugated antibodies

The graph illustrates the mean MFI of cells immunolabelled at each time point tested. Significant differences in MFI between the time points are represented by * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

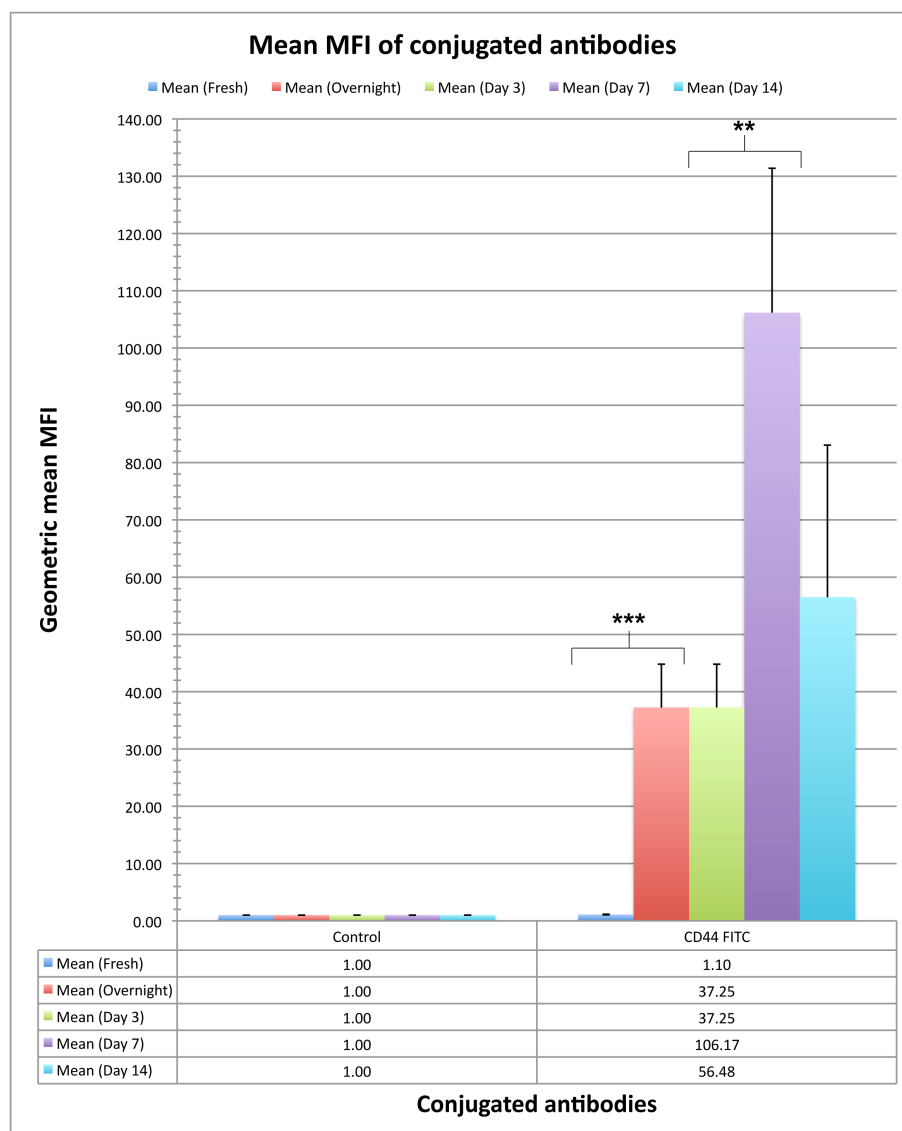


Figure 4.9: Mean MFI of CD44

The graph illustrates the mean MFI of cells immunolabelled for CD44 at each time point tested. Significant differences in MFI between the time points are represented by * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

4.4 Discussion

Results from the previous Chapter demonstrated differences between cell surface marker expression in freshly isolated cells and cells cultured for 24 hours. Differences in cell surface marker expression were also evident between cultured cells and native tissue. As cell labelling of the surface markers (CD29, CD44, CD49e, CD90, CD105, CD117 and CD166) differed in a 24-hour culture period, the aim of this Chapter was to investigate the cell surface profile of immature chondrocytes up to and including 14 days in monolayer to investigate dedifferentiation.

As with the previous Chapter, cells were analysed using flow cytometry following 3, 7 or 14 days in monolayer culture. Cells were cultured at high density to minimise dedifferentiation (Watt, 1988). Previous studies describe reductions in expression of chondrogenic markers, such as type II collagen and an adoption of a more fibroblastic phenotype, typified by the expression of type I collagen, after 14 days, so this time point was chosen to represent dedifferentiated chondrocytes (Diaz-Romero et al., 2005, Schnabel et al., 2002).

Expression of CD29 increased from freshly isolated cells to cells cultured for 24 hours, and this trend continued to day 7. At day 14, the expression had decreased to expression levels below those observed at day 3. The expression levels of CD49e demonstrated a similar pattern to CD29, increasing from day 3 to day 7 and then, declining from day 7 to day 14; the expression levels at day 14 were shown to be equivalent to those at day 3. The expression levels of CD49e were much lower than CD29 and remained low throughout culture.

CD29 ($\beta 1$) and CD49e ($\alpha 5$) are components of the $\alpha 5\beta 1$ complex that binds fibronectin. Although the levels of expression between these markers were quite different, the patterns of labelling displayed similarities, suggesting possible fibronectin binding. Superficial zone cells that adhere to fibronectin, have been shown to demonstrate greater colony forming efficiency, indicative of stem cells. Fibronectin adherence has also been demonstrated in middle zone cells; however,

these cells do not form colonies (Dowthwaite et al., 2004). Fibronectin has since been used to enrich for chondroprogenitors within superficial zone cells and full-depth cartilage across species (McCarthy et al., 2011), (Williams et al., 2010) Williams et al. 2010). In a recent study by co-workers, the majority of cultured human full-depth chondrocytes expressed CD29, but CD49e was only expressed by a discrete subset (less than 1%). In addition, when chondroprogenitors were immunolabelled all expressed CD49e, suggesting that CD49e is upregulated within progenitor populations and could be a marker of such cells (Williams et al., 2010).

CD44 expression continued to increase following overnight incubation. By day 3, an average of 57% of cells expressed CD44, which increased to around 95% by day 7. By day 14, all cells expressed the receptor. This increase in expression is most likely due to adhesion of the cultured cells to plastic. CD44 is associated with cell adhesion so, although, interesting to investigate during monolayer culture it is not thought to represent a stem cell marker within articular cartilage. Previous studies have also reported CD44 upregulation during extended monolayer culture in human chondrocytes (Williams et al., 2010, Diaz-Romero et al., 2008).

CD90 expression remained low throughout culture and, although, no statistical differences in CD90 expression were calculated during culture in the present study; the trend was similar to that observed with CD29 and CD49e, with an increase from day 3 to day 7 and decrease in expression from day 7 to day 14. These results are comparable to those observed by Fickert and co-workers, investigating CD90 expression in expanded human OA chondrocytes (Fickert et al., 2004). However, these data are dramatically different to observations recorded by Diaz-Romero and colleagues, who reported a 450-fold increase in expression of CD90, over a 14-day period in monolayer (Diaz-Romero et al., 2005). In our laboratory, increased expression was observed following monolayer expansion of human chondroprogenitors; increased expression was also observed in culture expanded full-depth human chondrocytes, but to a lesser extent than chondroprogenitors (Williams et al., 2010). These differences in results could be species-specific or due to tissue maturity.

CD105 and CD117 expression in chondrocytes displayed similar trends towards increased expression throughout monolayer culture. Unimodal distributions in expression were observed at day 3 and day 7, indicating in each case, a homogeneous population. However, after immunolabelling cells at day 14, a distinct subpopulation of cells was observed. This population was more immunofluorescent than at any previous time point and accounted for approximately 1-2% percentage of the total superficial zone population analysed. This distinct subset of cells was further analysed by back-gating to determine the FSC and SSC of each subset. The population of cells were distributed centrally within the main population of cells analysed, indicating they had equivalent size and cytoplasmic components as the rest of the population and were not dead or apoptotic. This comparable expression pattern of CD105 and CD117 has not previously been reported in any tissue. It is interesting to note, that CD117 has not been demonstrated in native articular cartilage, this may be because it is only present at low levels, undetectable by microscopy or, because it is upregulated during monolayer culture. Further studies are needed to determine if these sub-populations of CD105 and CD117 are due to dedifferentiation and whether these sub-populations are present with ongoing culture. Previous studies have reported high expression of CD105 in cultured human chondrocytes (Williams et al., 2010, Alsalameh et al., 2004). Co-workers reported that 1-2% of fibronectin-adherent cells from the superficial zone form colonies and that these colonies display stem/progenitor cell characteristics, such as multipotency and extended lifespan (Dowthwaite et al., 2004). This equates to the percentage of cells expressing CD105 and CD117, following 14 days in culture and may represent a progenitor sub-population.

Chondrocytes demonstrated a steady increase in CD166 expression from day 3 to day 7 but by day 14 had decreased, which is similar to the pattern observed with CD29, CD49e and CD90. CD105 and CD166 have been discussed previously as suitable candidates for progenitor markers (Alsalameh et al., 2004). The latter markers have previously been described in both OA and normal human articular chondrocytes, but CD166, unlike CD105 was restricted to the surface and middle zones of the tissue. In the study by Alsalameh and colleagues, the majority of chondrocytes expressed CD105, but only 0.5% expressed CD166 (Alsalameh et al., 2004). CD166 expressing cells demonstrated increased osteogenic potential compared to non-expressing cells.

However, both cell types demonstrated adipogenic potential (Pretzel et al., 2011). Co-workers also recently observed differences in adipogenic potential between chondroprogenitors and full-depth chondrocytes (Williams et al., 2010).

CD166 labelling observed in the aforementioned studies, as well as, labelling seen in this study (Chapter 2), suggest CD166 expression is indicative of a chondroprogenitor population. However, in monolayer culture, no subpopulation was observed. CD166 is associated with cell adhesion, growth and migration (Pretzel et al., 2011, Arai et al., 2002, Nelissen et al., 2000, van Kempen et al., 2001). Hence, CD166 may be up-regulated in monolayer culture conditions, resulting in the labelling of all cultured cells and thus, masking the labelling of chondroprogenitors.

In conclusion, these results demonstrate that cell surface expression of immature chondrocytes change during monolayer culture. The differences observed in this study vary from previous publications, which may be due to tissue maturity or species-specific expression variability. The majority of cell surface receptors exhibited a unimodal increase in expression indicative of a homogeneous population of cells. The number of cells expressing CD44 increased with time in culture, from 3 to 14 days, characteristic of cells adhering to plastic, with all cells adhering by day 14.

Bimodal distributions were also observed with CD105 and CD117, after 14 days in culture. This expression has not previously been reported and demonstrates a distinct and discrete subset of cells equating to 1-2% of the total surface zone population analysed, equivalent to the percentage of chondroprogenitor cells theorised to be present in the surface zone of immature bovine articular cartilage.

Unlike previous work, this study identified a general trend of increased expression to day 7, and then a decrease in expression to day 14. The increase in expression of these cell surface markers during culture is probably associated with cell adhesion, proliferation and migration of the cells during culture. In addition, CD90 and CD166 have been implicated in the regulation of the cytoskeleton (Frame et al., 2002, Kasahara and Sanai, 2000, Nelissen et al., 2000). Further characterisation of progenitors, should lead to a better understanding of the mechanisms of proliferation and differentiation within articular cartilage, improving our understanding of the role

of these markers and may significantly contribute to the field of articular cartilage repair.

CHAPTER 5:
GENERAL DISCUSSION

5.1 General Discussion

Adult articular cartilage has a poor intrinsic response to injury, caused by a lack of blood vessels, nerve supply and low cellularity. Tissue damage progresses from the articular surface to the subchondral bone resulting in loss of zonal arrangement, and painful, limited joint movement. The majority of current repair strategies, rely on infiltration of stem cells from the bone marrow pool, resulting in an inferior, fibrocartilaginous repair tissue. If resident stem/progenitor cells could be identified and isolated from within articular cartilage, these cells could be expanded *in vitro* and would ultimately result in an improved hyaline-like repair when re-implanted into the joint.

Mature cartilage possesses an anisotropic structure critical for its functional integrity. Immature cartilage, differentiated from the mesenchymal lineage, is isotropic and functions as a scaffold for bone formation and synovial joint components (Hunziker, 2010). The re-organisation of immature cartilage to produce the mature tissue, involves resorption and replacement of all but the most superficial cells, where tissue specific stem cells reside (Hunziker et al., 2007). Self-renewing, slowly proliferating stem cells are responsible for both lateral and appositional growth of articular cartilage. These stem cells supply the rapidly dividing transit amplifying cells beneath (Hunziker et al., 2007). It is only at sexual maturity that superficial zone cells are reprogrammed to produce mature articular cartilage. The processes that govern the maturation of cartilage are not known but many studies have provided evidence for an appositional growth model including Hayes et al (2001), where BrDU incorporation identified slow-cycling cells within the superficial zone of immature cartilage, suggesting the presence of a stem cell population (Hayes et al., 2001). In a more recent study, superficial zone cells expressed Notch1 and, on, adhesion to fibronectin, showed greater colony-forming efficiency than non-adherent cells from the same region. Middle zone cells were more fibronectin adherent but did not form colonies (Dowthwaite et al., 2004).

Mesenchymal stem cells have been identified in virtually all tissues and organs (Saamanen et al., 2010). Unlike hematopoietic stem cells, there is no single marker to identify MSCs. This cell type appears to be heterogeneous with most being progenitors rather than true stem cells, but all are highly clonogenic and possess the potential to differentiate into multiple mesenchymal tissues (Pittenger et al., 1999, Saamanen et al., 2010). CD44, CD49e, CD105 and CD166 are all expressed by MSCs and are thought to represent suitable markers of this cell type, however, they are not restricted to stem cells. In the current study, these cell surface markers were all localised to the superficial zone of immature articular cartilage, which is a novel observation. The identification of CD44, CD49e, CD105 and CD166 cell labelling within the superficial zone provides further evidence that chondroprogenitors reside in the superficial zone of immature articular cartilage and that cartilage develops by appositional growth.

CD29, CD90 and CD117 are also expressed by MSCs, however, the expression varied from that observed with CD44, CD49e, CD105 and CD166. CD29 was observed throughout the tissue and CD90 and CD117 were not detected in tissue. CD44, CD49e, CD105 and CD166 were all localised to the superficial zone. These observations suggest that CD29 expression is not restricted to chondroprogenitors.

The developmental markers Notch1, its ligands and Msx1 were located throughout the tissue but not all cells expressed these molecules. As these markers are not confined to the superficial zone of the tissue, Notch1, Delta1, Jagged1, Jagged2 and Msx1 are not thought to be restricted to chondroprogenitors. Notch1 and its ligands have previously been described as being present during development and maturation of articular tissue in mouse and chick limbs (Hayes et al., 2003, Williams et al., 2010). Notch1 has also previously been observed within the superficial zone of immature bovine articular cartilage, further contributing to the evidence for appositional growth. Notch1 is expressed by stem cells but is not restricted to them (Dowthwaite et al., 2004). This study was the first to investigate the expression of Notch1 and its ligands within immature bovine articular cartilage. Although Notch1 is highly expressed within the superficial zone, it is also expressed within the deeper zones, including the rapidly dividing transit amplifying population. Notch1 is thought to be a marker of chondroprogenitors as Notch1 selected cells exhibit an increased

colony-forming efficiency compared with cells not expressing the protein but its expression is not restricted to these cells (Dowthwaite et al., 2004). Notch1 has also been implicated in determining cell fate during differentiation, proliferation and apoptosis (Carlson and Conboy, 2007, Artavanis-Tsakonas et al., 1999).

The restricted expression of the stem cell markers; CD44, CD49e, CD105 and CD166 within the superficial zone greatly suggests that these markers may represent a cohort of progenitors/stem cells within articular cartilage. These markers have been identified in MSCs (Majumdar et al., 2000, Segawa et al., 2009, Sudo et al., 2007) and within culture-expanded chondrocytes (Diaz-Romero et al., 2005, Diaz-Romero et al., 2008, Williams et al., 2010, Alsalameh et al., 2004, de la Fuente et al., 2004). The extent of expression of these markers reported in previous publications varies greatly; these disparities are most likely due to species differences, maturity or structural integrity of the tissue as well as cell isolation procedures and subsequent monolayer culture.

Marker detection within chondrocytes is highly context dependent and also subject to differences brought about by variations in fixations and immunodetection protocols. A major obstacle in identifying receptors expressed by chondrocytes within the tissue is epitope masking caused by tissue preparation and fixation (such as dehydration and cross-linking) or extracellular matrix components. A number of fixatives and antigen unmasking methods were investigated in this study to reduce epitope masking although little difference was observed between them. To further investigate and quantify expression of cell surface and differentiation markers within articular cartilage, chondrocytes were isolated by enzymatic digestion and analysed by flow cytometry.

Freshly isolated cells exhibited reduced or absent expression of cell surface markers, with the exception of CD29. These data suggest that cell surface receptors are cleaved during incubation with pronase and collagenase, or, that they have low expression levels and, indicates that CD29 expression is either not affected by this digestion of the extracellular matrix or that it is rapidly re-expressed at the cell surface. Notch1, its ligands and Msx1 expression were all comparable to expression observed in tissue, implying that these transmembrane or nuclear (Msx1) markers are

not affected by cell isolation. These data suggest that the developmental markers would be suitable candidates to isolate progenitor cells, as their expression after isolation is maintained and comparable to that *in vivo*. However, isolation using these markers would only enrich for stem cells as these receptors are not restricted to stem cells.

As enzymatic digestion had a notable effect on cell surface marker expression, chondrocytes were cultured for 24 hours in monolayer to enable receptor re-expression. The expression levels were markedly different to those observed in freshly isolated cells, and from the native tissue. To determine if the expression levels differed throughout the tissue, middle and deep zone cells were isolated in addition to superficial zone cells and cultured for 24 hours. There was considerable disparity between expression of these cells and that observed within the tissue. These differences in expression could be due to improved sensitivity achieved by flow cytometry. However, this is unlikely as the isolated middle zone cells, or in the case of CD166, deep zone cells, exhibited a greater degree of expression than superficial zone cells, this would have been observed in tissue. This difference in expression may be due to contamination during explant removal from the tissue but is more likely due to cell adhesion and proliferation of the cells in culture. Indeed, this was investigated by Siczkowski and Watt (1990) where middle and deep zone cells adhered more rapidly than superficial zone cells to plastic. Resulting in rapid dedifferentiation of these cells.

A technique becoming increasingly applied within stem cell research is the Hoechst assay first developed by Goodell (1996) for murine HSCs. Stem cells within a heterogeneous population are identified by differential efflux of Hoechst 33342 by ABC transporters producing a 'side population' by flow cytometry as the stem cells actively pump out the dye. This has previously been shown by Hattori et al (2007) in immature bovine cartilage where surface, middle and deep zone cells were isolated and cultured in monolayer. A side population was observed with superficial zone cells but was absent from middle and deep zone chondrocytes. Within the superficial zone, the side population relative to the total number of cells was around 0.1% and was absent in controls (Hattori et al., 2007). This side population provides further

evidence of a stem cell population within the superficial zone of immature articular cartilage.

Notch1, Delta1, Jagged2 and Msx1 were not expressed after 24-hours in culture, this depletion once again suggests a change in expression from tissue to cultured cells. As all markers were affected by cell isolation and subsequent monolayer culture, the effects of continued monolayer culture were investigated. It is appropriate to consider repair strategies at this point, as tissue-engineering procedures using autologous MSCs or chondrocytes, involve expansion of these cells in culture. Any variation in expression *in vitro* may affect their suitability as chondroprogenitor markers. So determining changes in expression during culture will provide evidence for their suitability as markers of stem cells.

On further expansion in monolayer, increases in expression of stem cells markers continued, suggesting adaption and possible dedifferentiation of chondrocytes in monolayer. Comparable expression was observed between CD29 and CD49e and between CD105 and CD117. CD105 and CD117 were the only receptors to exhibit bimodal labelling at day 14, indicating a discrete sub-population of cells that were considerably brighter than the remaining cell population. CD44 expression was also initially distributed bimodally, with the percentage of cells expressing the receptor increasing at each time point until at day 14, all cells expressed CD44. This protein has a key role in cell adhesion and the distribution profile almost certainly highlights the increasing cell-cell and cell-plastic adhesion that occurs during continued monolayer culture.

The remaining cell surface markers (CD29, CD49e, CD90 and CD166) all presented with unimodal distributions, characteristic of homogeneous populations. Within adult articular cartilage, stem cell markers have been shown to increase in expression with continued monolayer culture (Bocelli-Tyndall et al., 2006, Diaz-Romero et al., 2005, Diaz-Romero et al., 2008, Tallheden et al., 2003). Differences in expression of these cell surface antigens is most likely due to dedifferentiation of cultured chondrocytes to a more naive cell type, exhibiting a similar surface antigen profile to MSCs (Diaz-Romero et al., 2008). Dedifferentiation of chondrocytes within monolayer culture has been reported previously, the phenotype is lost and cultured cells switch synthesis

from type II to type I collagen (Benya et al., 1978, Benya and Shaffer, 1982, Archer et al., 1990, Darling and Athanasiou, 2005). *In vitro* culture dedifferentiated chondrocytes demonstrate phenotypic plasticity, displaying multilineage potential, capable of chondrogenic, osteogenic and adipogenic differentiation (Barbero et al., 2003, Tallheden et al., 2003).

Evidence from this study and previous studies on cultured adult human chondrocytes (de la Fuente et al., 2004, Williams et al., 2010, Diaz-Romero et al., 2008), suggest stem cell markers are generally upregulated in monolayer culture, probably due, in part, to increased proliferation and cell adhesion and also dedifferentiation as previously discussed. Data from this study suggest that chondroprogenitor sub-populations, as observed within the surface zone of the tissue, could potentially be masked by upregulation of the total cell population during monolayer culture. The expression levels of these markers would need to be considerably higher on chondroprogenitors, than the expression levels of dedifferentiated cultured cells, to enable identification of progenitor cells within the total population. This marked difference in expression levels was observed with both CD105 and CD117, following 14 days in monolayer culture. There was a bright, discrete, subset of cells accounting for 1-2% of the total superficial zone cell population analysed, which was comparable to chondroprogenitor percentages previously reported (Dowthwaite et al., 2004).

In conclusion, from the experiments conducted, CD49e, CD105, CD117 and CD166 are all potentially useful cell surface markers to investigate in order to specifically isolate tissue specific stem cells. CD49e, CD105 and CD166 were all restricted to the superficial zone of immature cartilage in patterns consistent with the known localisation of stem cells at the surface of immature articular cartilage. The CD49e cell labelling pattern appeared to parallel that of CD29, suggesting an ability to bind fibronectin, which has previously been shown to significantly enrich for a stem/progenitor cell population in immature articular cartilage (Dowthwaite et al., 2004). Co-workers also observed a subset of CD49e expressing cells in full-depth human chondrocytes after extended time in monolayer (Williams et al., 2010). CD105 and CD117 also require further investigation as they were expressed by a subset of superficial zone cells following monolayer culture. CD166, in addition to its expression at the articular surface has also been reported primarily in the surface

and middle zones of adult articular cartilage and in low numbers of cultured chondrocytes (Pretzel et al., 2011, Alsalameh et al., 2004).

In terms of future work, it would be interesting to investigate CD49e, CD105, CD117 and CD166 by multicolour flow cytometry, to establish any co-expression. It would be particularly interesting to determine whether the sub-population of CD105 and CD117 expressing cells observed at day 14 were the same population of cells. Also, it would be informative to establish the differentiation potential of cells labelled for the above stem cell markers by cell sorting and establish whether stem cell marker expressing cells exhibit a greater colony-forming efficiency than non-expressing cells and their osteogenic and adipogenic potential as well as their redifferentiation to a chondrogenic phenotype. This would provide us with the most suitable markers to isolate cartilage stem cells, potentially producing a functional repair tissue *in vivo*, and thus, improving tissue engineering procedures for cartilage defects.

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PUBLICATIONS

ECM VII: Cartilage & Joint Repair

IDENTIFYING PROGENITOR CELLS WITHIN ARTICULAR CARTILAGE

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INTRODUCTION: Previous studies have shown that articular cartilage grows by apposition from the articular surface driven by proliferation of a progenitor cell sub-population that resides in the surface zone. This study concentrates on identifying markers for this progenitor cell population. Cell surface receptors CD105 and CD166 are known markers of progenitor/stem cells in endothelial cells, bone marrow constituents as well as other tissues and have recently been reported within normal and osteoarthritic human articular cartilage^{1,2}. Notch 1, Delta and Jagged 1 and 2 are known to be expressed by stem/progenitor cells during various stages of limb development³. Localisation of these markers to progenitor cells in articular cartilage would enable selective isolation facilitating further characterisation of the chondroprogenitor.

METHODS: Full depth cartilage explants were taken from 7-day-old bovine metacarpal-phalangeal joints. The tissue was then snap frozen and cryosectioned. Immunofluorescence was carried out using antibodies for the cell surface markers CD105, CD166 (Ancell, USA), Notch 1, Delta, Jagged 1 and Jagged 2 (Santa Cruz, USA). Surface zone explants were also enzymatically digested and the resulting cell suspension, filtered and counted. Cells were plated in monolayer at 30,000 cells/cm² and cultured for 4 days. Cells were lifted using accutase (Sigma,UK), then immunolabelled using directly conjugated CD105RPE and CD166FITC (Ancell). Cells were analysed using flow cytometry (BD FACSCanto).

RESULTS: Positive immunolabelling was observed for all markers. Jagged 2 occurred throughout the thickness of the cartilage, Delta was also present throughout the tissue at low levels but highly expressed at the articular surface. CD105, CD166 and Jagged 1 were localised to the superficial zone cells. Notch 1 labelling was found within the superficial layer and also in the deep zone. Labelling of CD105, CD166, Jagged 1 and Notch 1 were

predominately present as clusters within the superficial layer (Figure 1).

Using flow cytometry surface zone cells were labelled for all the previous mentioned markers. CD105 labelled 10% of the surface population and CD166 labelled 45% of the surface population.

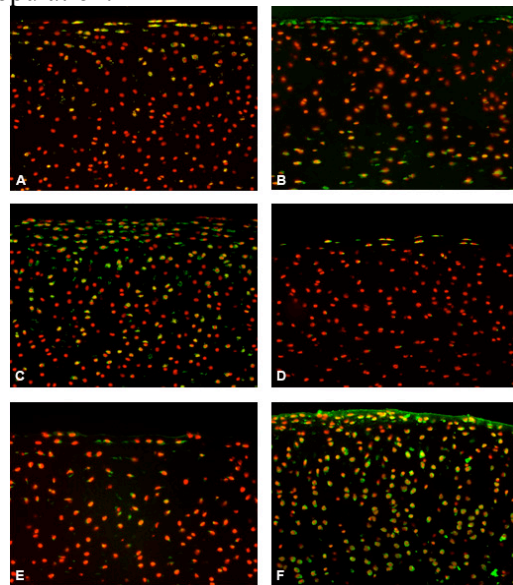


Fig.1: Immunofluorescence showing superficial zone labelling of CD105 (A), CD166 (B) Notch 1(C), Delta (D) and Jagged 1 (E) and uniform labeling throughout the tissue for Jagged 2 (F)

DISCUSSION & CONCLUSIONS: The above results demonstrate that CD105, CD166, Jagged 1, Delta and Notch 1 are promising markers for chondroprogenitor cells. At present, studies are concentrating on sorting labelled cells using flow cytometry (BD FACS Aria) to select the progenitor cells that can be expanded for use in monolayer and pellet systems.

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Identification and Clonal Characterisation of a Progenitor Cell Sub-Population in Normal Human Articular Cartilage

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Abstract

Background: Articular cartilage displays a poor repair capacity. The aim of cell-based therapies for cartilage defects is to repair damaged joint surfaces with a functional replacement tissue. Currently, chondrocytes removed from a healthy region of the cartilage are used but they are unable to retain their phenotype in expanded culture. The resulting repair tissue is fibrocartilaginous rather than hyaline, potentially compromising long-term repair. Mesenchymal stem cells, particularly bone marrow stromal cells (BMSC), are of interest for cartilage repair due to their inherent replicative potential. However, chondrocyte differentiated BMSCs display an endochondral phenotype, that is, can terminally differentiate and form a calcified matrix, leading to failure in long-term defect repair. Here, we investigate the isolation and characterisation of a human cartilage progenitor population that is resident within permanent adult articular cartilage.

Methods and Findings: Human articular cartilage samples were digested and clonal populations isolated using a differential adhesion assay to fibronectin. Clonal cell lines were expanded in growth media to high population doublings and karyotype analysis performed. We present data to show that this cell population demonstrates a restricted differential potential during chondrogenic induction in a 3D pellet culture system. Furthermore, evidence of high telomerase activity and maintenance of telomere length, characteristic of a mesenchymal stem cell population, were observed in this clonal cell population. Lastly, as proof of principle, we carried out a pilot repair study in a goat *in vivo* model demonstrating the ability of goat cartilage progenitors to form a cartilage-like repair tissue in a chondral defect.

Conclusions: In conclusion, we propose that we have identified and characterised a novel cartilage progenitor population resident in human articular cartilage which will greatly benefit future cell-based cartilage repair therapies due to its ability to maintain chondrogenicity upon extensive expansion unlike full-depth chondrocytes that lose this ability at only seven population doublings.

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Introduction

Articular cartilage displays a poor repair capacity. Consequently, the aim of cartilage cell therapy procedures is to repair damaged joint surfaces with a functional replacement tissue. As an avascular tissue, cartilage comprises a single cell type – the chondrocyte, which is organised into three distinct layers – the surface, mid and deep zones [1]. Chondrocytes required for cell-based therapies are isolated and expanded *in vitro* to generate sufficient numbers of cells for surgical procedures. However, extensive expansion results in the progressive dedifferentiation of the chondrocytes. In culture, human chondrocytes show an inability to retain a chondrogenic potential past 7 population doublings, even after cultivation in a chondrogenically permissive environment [2,3,4]. In order to combat this

problem of dedifferentiation, research has focussed on the use of growth factors and 3D culture systems as a way of maintaining the chondrogenic potential of these cells [5,6,7,8]. Although these modifications, to some extent, have proved successful they would be unsuitable as a method of expanding cells for use in cell-based repair therapies and, as such, monolayer culture is a limiting factor for chondrocyte efficacy. Additionally, when chondrocytes are used in cell-based tissue engineering, the resulting repair tissue is unpredictable and often fibrocartilaginous. It is argued that this fibrocartilage is biochemically and biomechanically inferior to native cartilage thus compromising long-term repair of the cartilage defect [9,10,11].

The loss of the chondrogenic phenotype during monolayer culture means that the size of defect that can be treated is limited

since only a defined amount of cartilage can be harvested from the joint periphery. One way to overcome this cell source limitation would be to use an alternative cell type that maintains its inherent proliferative capacity, such as a mesenchymal stem cell (MSC) population [12]. Recently, studies have demonstrated that cells obtained from a range of adult tissues eg. adipose, epidermal, dental pulp and bone marrow exhibit mesenchymal/progenitor type properties; they can differentiate into multiple lineages and express putative stem cell markers and as such, could be used for cell-based repair therapies [13,14,15,16,17,18,19,20]. In particular, studies have highlighted that MSCs obtained from bone marrow could be used in cartilage repair procedures as bone marrow stromal cells (BMSC) can be directed towards the chondrogenic lineage [21]. However, articular cartilage is a permanent cartilage and the phenotype generated by BMSCs is endochondral, which will terminally differentiate, an unfavourable outcome if one wants to repair permanent articular cartilage. At present, the type of cartilage generated by stem cells from other tissue types is poorly characterised [22,23].

Instead of utilising MSCs from different tissue sources for cartilage repair strategies, it would be logical to use cells from the same tissue as these cells may possess the developmental repertoire of the native tissue, impacting on the morphogenesis of the repair tissue in a beneficial way. Previously, research has hypothesised that foetal and immature articular cartilage develops appositionally – that is from the surface to the deep zone [24]. In support of these data, the identification of a chondroprogenitor population in the surface of bovine articular cartilage has been described and this has opened up the possibility that human cartilage could also contain a cartilage progenitor population [25,26]. Identification and characterisation of this cartilage progenitor population in human tissue has begun in earnest. Many studies relying on the use of several putative stem cell markers to determine a method by which these cells can be isolated from a full-depth articular chondrocyte population [27,28,29]. As of yet, however, no suitable single marker has been established and it is becoming increasingly evident that a high percentage of mature chondrocytes also express these markers thus confounding previous studies, especially in the absence of clonal analysis [30,31]. As cell surface marker expression can alter in monolayer culture, then it is important that other classical stem cell characteristics are relied on to determine whether a true cartilage progenitor population has been isolated [31,32].

This study describes the isolation of a distinct cartilage progenitor population resident in normal human cartilage of varying ages. The methodologies employed result in the generation of large numbers of cells from a single cell. Upon characterisation of this cell population derived from a single cell, we demonstrate its progenitor-like properties. As such, we propose that the human cartilage progenitor population characterised in this study is a suitable candidate for advancing cell-based tissue repair therapies for cartilage defects.

Materials and Methods

Ethics Statement

Cartilage tissue samples were obtained from patients who underwent knee surgery. South East Wales NHS Research Ethics Committee specifically approved this study and institutional safety and ethical guidelines were followed. Written informed consent was obtained from each patient, and extensive precautions were taken to preserve the privacy of the participants donating tissue. All caprine *in vivo* studies were approved by the Office for Food Safety and Animal Health Graubunden, permitting the project to

take place under the title PROCART. Experiments were carried out under the strict guidelines of the AO Research Institute, Switzerland.

Cell isolation & fibronectin adhesion assay

Full-depth normal human articular cartilage samples from femoral chondyles (n = 9; mean age 30.0 yrs, range 10–57) were obtained. Chondrocytes were isolated by sequential pronase (70 U ml⁻¹, 1 hour at 37°C) and collagenase (300 U ml⁻¹, 3 hours at 37°C) digest. Isolated cells were plated down as a full-depth chondrocyte population (total cell mass from surface, mid and deep zones) or subjected to a fibronectin adhesion assay as described [33]. Briefly, six well plates were coated with 10 µg ml⁻¹ fibronectin (FN; Sigma, UK) in 0.1 M phosphate buffered saline (PBS, pH7.4) containing 1 mM MgCl and 1 mM CaCl₂ (PBS+) overnight at 4°C. Control dishes were treated with PBS+ containing 0.1% BSA overnight at 4°C. Isolated full-depth chondrocytes (4000 cells ml⁻¹) were seeded onto the coated plates for 20 mins at 37°C in Dulbeccos Modified Eagle Medium (DMEM). In all experiments, a minimum of 3 fibronectin and 3 BSA coated dishes were used. After 20 mins, media and non-adherent cells were removed. Fresh DMEM containing Penicillin 10000 µg ml⁻¹/Streptomycin 10000 U ml⁻¹, 0.1 mM ascorbic acid, 0.5 mg ml⁻¹ L-glucose, 100 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine and 10% fetal bovine serum (FBS) (DMEM+) were added to the remaining adherent cells. Within 18 hours of plating, the number of cells adhered was counted.

Twelve days after plating, colonies (defined as a cluster of more than 32 cells, as this represents a population of cells derived from more than 5 population doublings of a single cell, thereby discounting a transit amplifying cell cohort) were counted and colony forming efficiency calculated [33]. Colonies were selected and isolated using sterile cloning rings (Sigma, UK). Colonies were expanded in DMEM+ plus 1 ng ml⁻¹ Transforming Growth Factor – β2 (TGF-β2; PeproTech, UK) and 5 ng ml⁻¹ Fibroblast Growth Factor-2 (FGF-2; PeproTech, UK). At each passage, the number of cells obtained and re-plated was recorded. From these data, population doublings from 4 specimens, with at least 3 clones from each specimen, were calculated using the equation: $n = [\log(\text{final cell count}) - \log(\text{number of cells initially plated})]/0.301$ [34].α

Flow cytometry

One million full-depth chondrocytes (passage 5) were washed in PBS and incubated for 1 hour at 4°C with conjugated antibodies to CD105-FITC (Ansell), CD166-RPE (Ansell), CD44-FITC (Pharminogen), CD29-FITC (Chemicon) or CD49e-PE (Chemicon) at a concentration of 10 µg ml⁻¹. Cells were centrifuged at 2000×g, supernatants removed and cells washed three times in PBS. Finally, labelled cells were re-suspended in 1 ml PBS and subjected to single channel Fluorescently Activated Cell Sorting (FACS) analysis. The appropriate IgG controls were run in parallel. A minimum of three clones were analysed for each antibody.

Immunocytochemistry

Monolayer cultures of full-depth chondrocytes and cartilage progenitors were fixed and subjected to immunofluorescence for antibodies to anti-human Notch 1 (Developmental Studies Hybridoma Bank, USA), anti-human CD90 (BD Pharminogen, USA), anti-human STRO-1 (R&D Systems, USA), anti-rabbit Jagged 1 (Santa Cruz Biotech, USA) and anti-goat Delta 1 (Santa Cruz Biotech). Monolayer cultures subjected to chondrogenic induction were analysed for anti-mouse Sox9 (Abcam, UK),

aggrecan-IGD; 6B4+ and 2B6 (both kind gifts of Professor Bruce Caterson, Cardiff University), collagen type I (10 $\mu\text{g ml}^{-1}$; Abcam) and collagen type II (DSHB). Full protocols can be found in supporting information (Text S1). Pellets from a minimum of 3 different clonal cell lines were labelled for each antibody.

Cell differentiation assays

Expanded clonal cell lines and full-depth chondrocytes were trypsinised and aliquots of 0.5×10^6 cells per 1 ml of media were centrifuged at $2000 \times g$ for 5 mins in 1.5 ml Eppendorf tubes to form spherical pellets. For chondrogenic differentiation, pellets were suspended in DMEM supplemented with ITS (10 $\mu\text{g ml}^{-1}$ insulin, 5.5 $\mu\text{g ml}^{-1}$ transferrin, 5 ng ml^{-1} selenium; GIBCO, UK), 100 mg ml^{-1} Gentamicin, 50 $\mu\text{g ml}^{-1}$ L-ascorbic acid, 1 mg ml^{-1} D-glucose, 2 mM L-glutamine, 10 mM HEPES, 10^{-7} M dexamethasone, 2% FBS and 10 ng ml^{-1} TGF- β 2. For osteogenic differentiation pellets were suspended in DMEM, 10% FBS, 10 mM β -glycerophosphate, 10 nM dexamethasone and 0.1 mM L-ascorbic-acid-2-phosphate. Pellets were cultured for 21 days with a medium change every other day.

Monolayer cultures of cartilage progenitor cell lines and full-depth chondrocytes were also established and cultured in chondrogenic differentiation media or adipogenic differentiation media or adipogenic maintenance media as previously described [35]. Cell monolayers cultured in adipogenic medium were fixed in 10% NBFS for 10 mins. For lipid detection, a stock solution (0.5% Oil Red O in 100% isopropanol) was diluted at a ratio of 4:6 with dH_2O and the cells incubated for 1 hour.

Preparation of pellets for analysis

At 21 days, cell pellets were fixed in 4% para-formaldehyde, embedded in paraffin and cross-sectioned at 8 μm or following fixation in 10% neutral buffered formalin, the pellets were fully dehydrated and processed into Technovit 9100 New[®] (TAAB Laboratories, UK) using the chemical catalytic method fully described by Yang *et al.*, [36] with fully destabilised resin at all steps [37]. The pellets embedded in Technovit 9100 New[®] resin sections were cut using glass knives to a thickness of 1 μm .

Pellet analysis

Wax embedded pellet sections were dewaxed in xylene and hydrated in a decreasing graded alcohol series. Technovit 9100[®]New embedded pellets were de-acrylated in 2-methoxyethyl acetate (Sigma, Dorset, UK) for 3–4 hours followed by rehydration through xylene, and a series of graded ethanol. Resin autofluorescence was quenched by immersing tissue sections in 1% sodium borohydride and equilibrated in PBS as previously described [37]. Pellets sections were then subjected to standard histological protocols for safranin O and toluidine blue staining for proteoglycan detection. Alizarin red and von Kossa staining were used to determine the extent of mineralisation and picro-sirius red staining was performed to demonstrate collagen fibril synthesis within the pellet matrix. Immunofluorescent analysis for cartilage (Collagen type I & II, 2B6, aggrecan) and osteogenic (Collagen type X, alkaline phosphatase) components was performed, as described above and more fully in supporting information (Text S1).

PCR analysis

At 21 days, cell pellets cultured in osteogenic differentiation media were placed into buffer RLT (Qiagen, UK). Cell monolayers from adipogenic cultures were lysed from the tissue plastic using buffer RLT (Qiagen, UK). RNA was extracted from

pellet and monolayer cells using a kit (RNAEasy, Qiagen, UK) with a DNaseI incubation step. RNA was quantified using a Nanodrop 2000c spectrophotometer and 100 ng of each sample was used for reverse transcription. Polymerase chain reactions were performed using the following primer combinations for lipoprotein lipase forward 5'CTGAAGACACAGCTGAGGAC3', reverse 5'CTGGTGAATGTGTGTAAGAC3', osteonectin forward 5'TCCACAGTACCGGATTCTCTCT3', reverse 5'TCT-ATGTTAGCACCTTGTCTCCAG3', 18S rRNA forward 5'G-ATGGGCGGCGGAAAATAG3', reverse 5'GCGTGGATTCTGCATAATGGT3'. PCR reactions were amplified using the following conditions 95°C 2 min-1 cycle, 95°C 30 sec, 53°C 30 sec, 72°C 30 sec-35 cycles, 72°C 5 min-1 cycle.

Cytogenetic analysis

Cytogenetic investigations were undertaken on 1 clonal cell line from 1 specimen (10 year-old female; PD = 31.3) and 2 clonal cell lines from a different specimen (54 year-old female; PD = 23.6 & 28.5). Cultures were selected for harvest when cell growth was almost confluent. Chromosome preparations were obtained using a modification of conventional techniques explained in detail in supporting information (Text S2) [38]. The karyotype was determined by microscopic examination after modification of the Giemsa staining and banding analysis [39,40].

Telomere length analysis

Two full-depth chondrocyte populations (1 at PD10 and 1 at PD12) and 3 clonal cell lines (1 at PD 29, the second at PD26 and PD39 and the third at PD29 and PD38), were washed in sterile PBS, pelleted at 1.0×10^6 cells and frozen in liquid nitrogen for telomere length analysis. DNA extractions and single length telomere analysis (STELA) reactions at the 17p and XpYp telomeres were carried out as described previously and in full in supporting information (Text S3) [41]. Corresponding telomerase activity using real time quantitative repeat amplification procedure (RTQ-TRAP) was carried out for the corresponding full-depth chondrocyte and clonal cell lines, described in full in supporting information (Text S4).

In ovo injections and tissue processing

Cell engraftments: Clonal cells were expanded, harvested, and aliquots of between 1×10^4 – 1×10^5 cells re-suspended in media and immediately injected into the limb bud of 3-day-old (st23) [42] chick embryos which had previously been windowed. Eggs were resealed with adhesive tape and re-incubated up to day 10 (st36–37). Embryos were killed by cervical dislocation, a note of their developmental stage taken and the hind-limbs removed. The limbs were washed in 0.1 M PBS (pH 7.4) and fixed in 10% NBFS for 1 hour at room temperature before being processed to wax. Sections were cut (8 μm) and subjected to immunohistochemistry, protocol previously described, for anti-human collagen-1 (Abcam, UK) at a dilution of 1:1000 to detect engrafted cells. The antibody did not cross-react to chick type I collagens or to other chick collagens. To determine specificity of the antibody, positive controls were carried out on human cartilage tissue sections and negative controls were run on non-injected chick limbs.

In situ hybridisation for human-specific Alu genomic repeats: *In situ* hybridisation for human Alu genomic repeats was performed as described previously [4,43].

Caprine in vivo repair study

In order to obtain proof-of principle, a pilot study was carried out using 6 young, mature female goats. In the first surgical

procedure a circular 6 mm defect was created in the lateral femoral condyle whilst preserving the calcified layer. The protocol followed for cartilage digestion and chondroprogenitor isolation was identical to that described here for human cartilage progenitors. Colony forming efficiency and population doublings for the goat chondroprogenitors were calculated. Cells were pelleted into a 3D culture system and cultured in chondrogenic induction media, as described previously in this manuscript. Immunohistological analysis was performed on goat chondroprogenitor pellets for Collagen type II ($10 \mu\text{g}/\text{ml}^{-1}$; DSHB) and aggrecan-IGD;6B4 (kind gift from Professor Bruce Caterson, Cardiff University) as described previously. RNA was isolated from the chondrogenic induced pellet cultures, transcribed to cDNA and polymerase chain reaction (PCR) performed to determine Collagen type II and Sox 9 mRNA expression.

Three weeks after the initial isolation, in a second surgical procedure, 2×10^5 cells were loaded onto a 5 mm diameter type I/III collagen membrane (Chondro-Gide®, Geistlich AG). Three goats received a membrane with goat chondroprogenitors (mean population doubling, 21.4) whilst another three goats received a membrane seeded with full-depth goat chondrocytes (mean population doubling, 3.5). After debridement of the margins of the lesion from the previous surgery the membrane was inserted into the defect and sutured with 8 individual suture points. The operated limb was immobilised for 2 weeks using a sling. The animals were sacrificed at 20 months after the second surgery. The operated knees were dissected, formalin fixed, decalcified and prepared for routine histology and immunocytochemistry as described above. Repair tissue grading was performed independently, in a blinded manner, by 3 researchers, based on the International Cartilage Repair Society (ICRS) scoring system.

Results

FACS analysis

Fluorescently activated cell sorting (FACS) analysis was utilised to label the full-depth chondrocytes for a series of putative cartilage stem cell markers. Analysis of full-depth chondrocytes for CD105 (endoglin), CD166 (ALCAM), CD44 and CD29 ($\beta 1$ integrin) all showed expression in over 95% of the viable cell population (Fig. 1A–1D). However, expression of CD49e, $\alpha 5$ -integrin, was observed in a distinct population, 0.7%, within the viable full-depth chondrocyte population (Fig. 1E). On analysis of the clonal population, isolated using the fibronectin adhesion assay, almost 100% of the cells labelled for CD49e expression (data not shown).

Fibronectin adhesion assay

Fifty percent of the samples showed a CFE (colony forming efficiency) of less than 0.1 with the remaining 40% demonstrating a CFE of 0.2 (Fig. 2A). Discrete colonies that form (comprising more than 32 cells) were selected and cultured in monolayer (Fig. 2C) and clonal cell proliferative capacity calculated. The cells were able to proliferate to over 60 population doublings, taking over 200 days (Fig. 2B). Clones obtained from the same sample proliferated at similar rates. Differences in growth kinetics were observed between the clones from different samples. Morphologically, the clonal cell lines showed a fibroblastic phenotype in early population doubling monolayer cultures that were retained in long-term cultures. There were no obvious differences in the size of the cells between early and late population doublings (Fig. 2D & E).

Monolayer cultures

Monolayer cultures of cartilage progenitors at population doublings greater than 30 demonstrated positive labelling for stem

cell markers CD90 (Fig. 3A) and STRO-1 (Fig. 3B). Notch 1 receptor and its corresponding ligands, Delta 1 and Jagged 1, which are involved in signalling pathways of stem cell differentiation, were also expressed in the cartilage progenitor monolayers (Fig. 3C–3E). Although expression of these putative stem cell markers were observed in full-depth chondrocytes, it was to a lesser extent (Fig. 3K, 3L). Analysis of chondrogenic phenotype markers, in monolayer clonal cell lines post-30 population doublings and subjected to chondrogenic induction by TGF β 2, resulted in the observed expression of collagen type I, collagen type II, chondroitin-4-sulphate (2B6), aggrecan-IGD (6B4+) and the chondrogenic transcription factor, Sox9 (Fig. 3F–3J). The induction of cartilage gene expression and immuno-detectable matrix synthesis in monolayer clonal cell lines after 30 population doublings is significant and noteworthy. Corresponding immunoglobulin controls for the antibodies were all negative (Fig. 3M–3O).

Differentiation assays

At 30 population doublings or greater, clonal cells were pelleted into 3D Eppendorf cultures and maintained for 21 days in media containing factors to promote either chondrogenic or osteogenic differentiation. Four clonal cell lines and a full-depth chondrocyte sample cultured as pellets in chondrogenic differentiation media, both showed a smooth surface morphology (Fig. 4A, 4C). Toluidine blue and safranin O staining demonstrated glycosaminoglycan synthesis 21 days after chondrogenic culture (Fig. 4B, 4C, 4F, 4G). The presence of collagen fibrils within the cartilage progenitor pellet matrix was indicated by picro-sirius red staining (Fig. 4D) and on further analysis, detection of collagen type I (Fig. 4I) and collagen type II (Fig. 4J), by immunohistochemical methods, was revealed and indicated a differentiation process similar to that found during early development [44]. Positive labelling for aggrecan core protein, 6B4, was observed intensely in the outer regions of the pellet with weaker labelling demonstrated in central regions (Fig. 4K). Labelling for 2B6 was present throughout the pellet matrix (Fig. 4L). Significantly, neither alkaline phosphatase (Fig. 4M) nor collagen type X (Fig. 4O) were observed in pellets cultured in chondrogenic media.

The cartilage progenitor pellets cultured in osteogenic differentiation media showed a rough surface topography and were slightly smaller in size compared to the full-depth chondrocyte pellets (Fig. 5A, 5D). When stained for mineralisation, cartilage progenitor pellets showed extensive regions of calcium deposition as indicated by both von Kossa (Fig. 5E) and alizarin red stain (Fig. 5F). The full-depth chondrocyte pellets displayed only small regions of mineralisation (Fig. 5B). PCR analysis for osteonectin, a marker for early bone development, demonstrated mRNA expression in both the full-depth and cartilage progenitor pellets (Fig. 5M). Osteogenic differentiation was not evident as alkaline phosphatase activity (Fig. 5G) was seen at a very low level and collagen type X (Fig. 5I) expression was entirely absent in the cartilage progenitor pellets.

Clonal cells and full-depth chondrocytes cultured as monolayers in adipogenic media stained positively for lipid deposition (Fig. 5J, 5K) whereas those cultured in control media did not (Fig. 5L). PCR analysis for lipo-protein lipase showed mRNA expression in the full-depth and cartilage progenitor population (Fig. 5M).

Cytogenetic Analysis

Examination of the GTG-banded preparations from 3 flasks from a clonal cell line from one specimen (54 year old, PD = 31.3) revealed a normal female karyotype, 46, XX (Fig. 5A). However, 2 of 12 cells examined from one of the culture flasks showed a

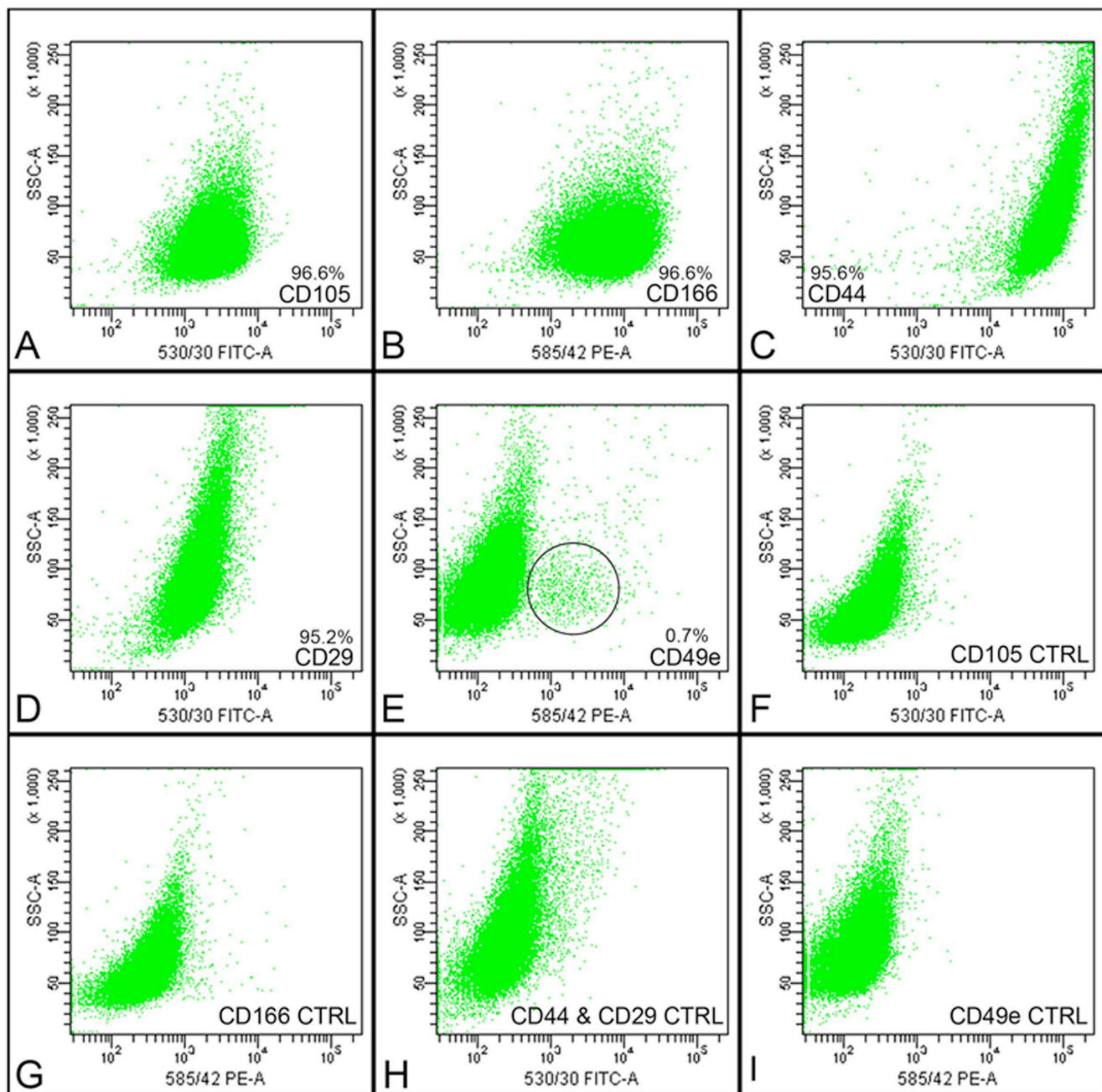


Figure 1. Flow cytometric analysis of full-depth chondrocytes. Full-depth chondrocytes were labelled for the putative stem cell surface markers CD105 (A), CD166 (B), CD44 (C), CD29 (D) and CD49e (E). Note the subpopulation of cells labelled for CD49e. Corresponding immunoglobulin control samples were analysed for each marker at each experimental run (F–I).
doi:10.1371/journal.pone.0013246.g001

terminal deletion of the long arm of chromosome 20, from q11.2 to qter (Fig. 6B). This anomaly was not observed in the other 2 culture flasks. The clonal cell line from the second specimen (10 year old, PD = 23.5) showed a normal female 46, XX karyotype in 70 cells examined. However, 10 of the 70 cells examined had an apparently balanced translocation involving the short arm of chromosome 7 and the long arm of chromosome 16. The breakpoints appeared to be at p13 and q22 respectively. The karyotype from the second clonal cell line (PD = 28.5) from the second specimen showed a normal female karyotype, 46, XX in 50 cells examined from 2 flasks.

Telomere length and telomerase activity analysis

Two clonal cell lines were analysed, one of which is depicted for greater clarity although data from both were very similar. The clonal cell line and the full-depth chondrocyte population undergo telomere erosion with progressive cell divisions (Fig. 6C). It is interesting to note that the distributions within the clonal cell lines display less heterogeneity that is normally observed in bulk populations of cells or tissues where a typical SD of around 3 kb would be expected. In the clonal cell line, it is apparent that subsets of cells with a larger distribution are present and the proportion of cells within this subset is increasing with time in

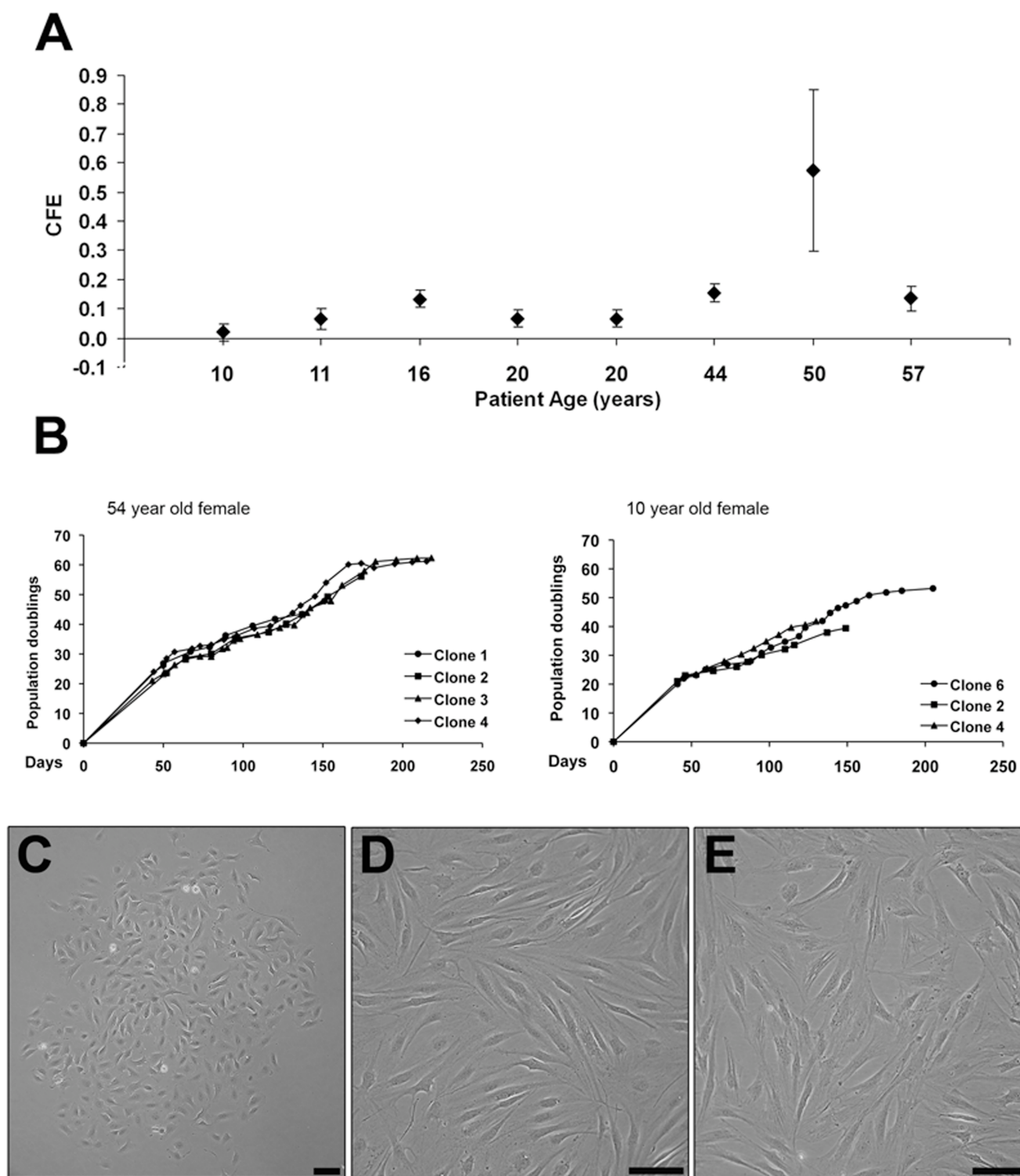


Figure 2. Population data from clonal cell lines. Colony forming efficiency calculated from 8 samples that were subjected to the fibronectin adhesion assay (as described in Materials & Methods) (A). Population doublings data from 2 representative samples demonstrating proliferative rate of the clonal cell lines during a period of over 200 days (B). Phase contrast microscopic appearance of a representative fibronectin adhered colony (C) and clonal monolayers at low (<30) and high (>30) population doublings (D & E respectively). Scale bar = 100 μ m.
doi:10.1371/journal.pone.0013246.g002

culture (Fig. 6C, arrowed). Full-depth chondrocytes displayed almost baseline levels of telomerase activity in both low (PD12) and high (PD49) population doubling cultures (Fig. 6D). The clonal cell line at a high population doubling (PD39) exhibited the

greatest telomerase activity, even higher than that of the low population doubling (PD26) clonal cell line, possibly due to the presence of a subset of cells showing a high telomere length. When converted to HL60 cell equivalent units, the clonal cell line showed

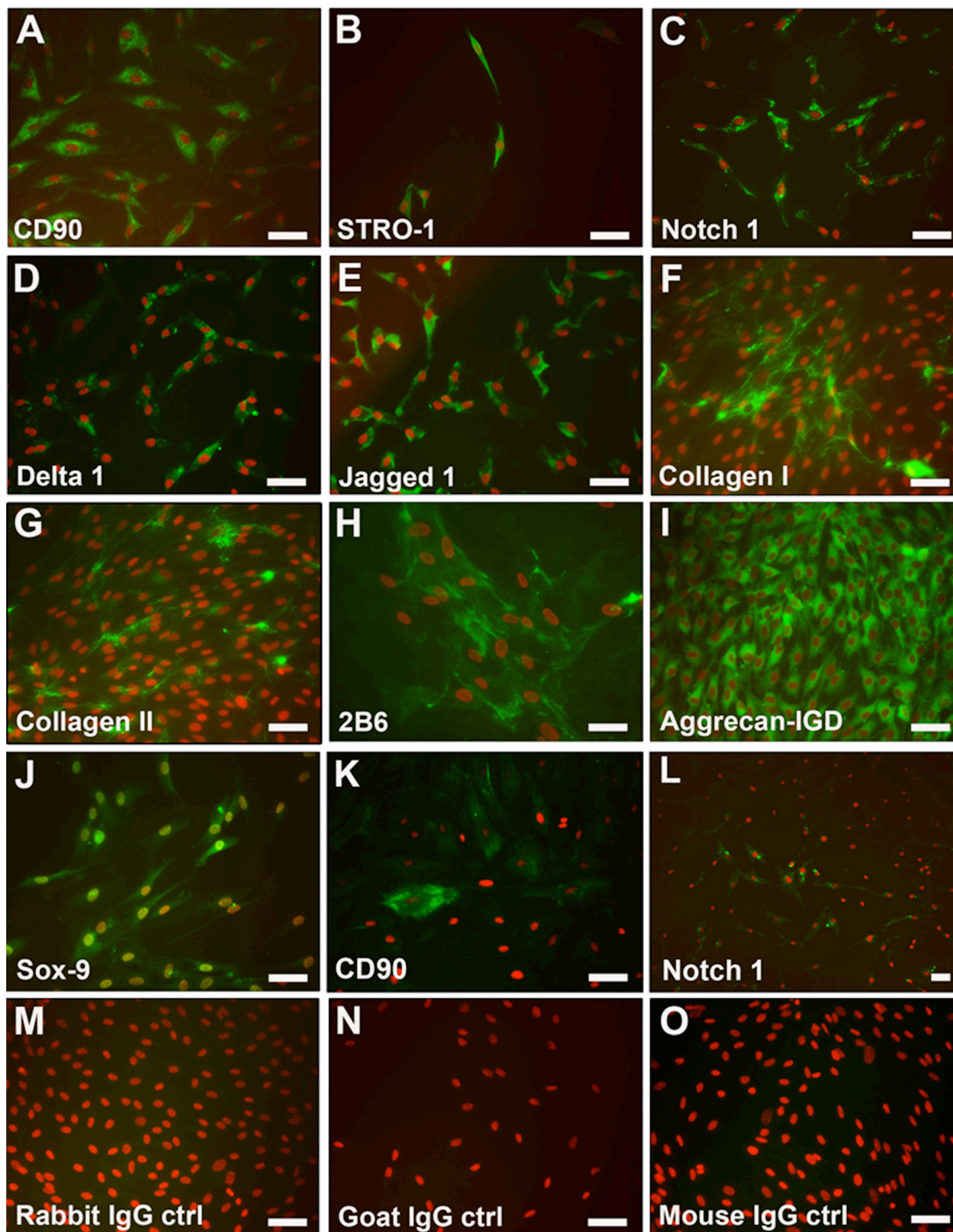


Figure 3. Phenotype of cartilage progenitor and full-depth chondrocyte cell lines. Monolayer cultures of expanded clonal cell lines at over 30 population doublings express the putative stem cell markers, CD90 (A) and STRO-1 (B) when localised using immunofluorescent labelling (green). Members of the Notch signalling family, Notch 1 (C), Delta 1 (D) and Jagged 1 (E) were expressed in clonal monolayer cultures. Markers of the chondrogenic phenotype, collagen type II (G), 2B6 (H), aggrecan-IGD (I), Sox9 (J), and also, collagen type I (F) were present in clonal monolayer cultures at over 30 population doublings. Full-depth chondrocytes show fewer cells with positive expression of CD90 (K) and Notch 1 (L) within the monolayer cultures. Corresponding immunoglobulin controls, rabbit (M), goat (N) and mouse (O), were all negative. Scale bars = 50 μ m. doi:10.1371/journal.pone.0013246.g003

3.1-fold greater telomerase activity than full-depth chondrocytes at low population doubling and at the high population doubling this figure increased to a 10.3-fold difference.

Engraftment of cartilage progenitor cell lines

In order to test for *in vivo* plasticity, cartilage progenitor cells were injected into st23 chick hind limbs. A high number of cells

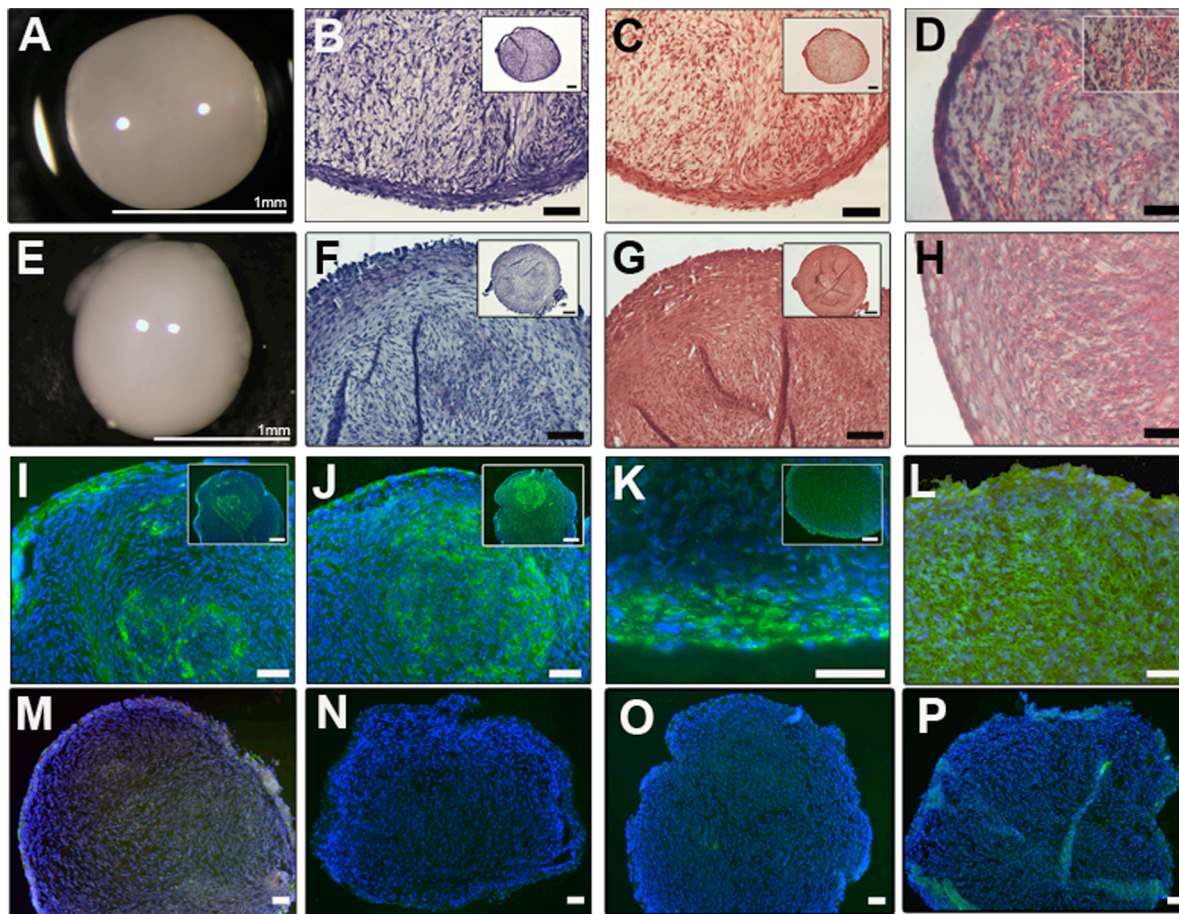


Figure 4. Chondrogenic differentiation of cartilage progenitor and full-depth chondrocyte populations. Gross morphology of a representative 3D cartilage progenitor (A) and full depth chondrocyte (B) pellet cultured in chondrogenic media for 21 days. Pellets display a shiny smooth surface. Toluidine blue (B, F) and safranin O (C, G) stained pellets demonstrate the presence of glycosaminoglycans within the pellet matrix. Picro-sirius red staining highlights synthesis of collagen fibres in the pellet matrix (D, H). Immunohistochemistry of the donal pellets demonstrates both collagen type I (I) and collagen type II (J) within the chondrogenic pellet matrix. Aggrecan labelling was present on the outer edge of the chondrogenic pellet (K) and 2B6 expression was present throughout the pellet matrix (L). Pellets cultured in chondrogenic media show no alkaline phosphatase (M) or collagen type X expression (N). Representative examples of negative controls for mouse monoclonal (O) and rabbit polyclonal (P) antibody protocols. Scale bars = 50 μ m. doi:10.1371/journal.pone.0013246.g004

labelling positively for human collagen type I were observed in the perichondrium and alongside the prehypertrophic region of the developing anlagen (Fig. 7A, 7B). Controls determined that the human collagen type I antibody was specific for human collagen type I and did not recognise chick collagen type I or other chick collagens (Fig. 7D, 7E). *In situ* hybridisation for human Alu repeats, on a representative section from a chick limb 7 days after human clonal cell transplantation showed human Alu-positive nuclei stained black in the peri-articular tissues (Fig. 7C). Control sections were negative (Fig. 7F).

Caprine *in vivo* repair study

In order to determine whether articular cartilage progenitors are suitable candidates for cartilage repair, we carried out a proof of principle pilot study on goats. Chondroprogenitors obtained from the goat study demonstrated an average CFE of 0.2. Positive collagen type II and aggrecan labelling was observed by both immunohistological and PCR analysis in the 3D chondrogenic pellet cultures.

The ICRS scoring system designed by Brittberg and Peterson [45] was used to evaluate cartilage repair in the goat tissue sections. Histological repair scores for full-depth chondrocytes

ranged from 5 (abnormal) to 10 (nearly normal) whilst repair scores for chondroprogenitors ranged from 7 (abnormal) to 10 (nearly normal). Overall, full-depth chondrocytes had a mean score of 7.8 and chondroprogenitors had a mean score of 8.2 thus suggesting no overall differences between the two experimental groups. Examples of excellent lateral integration in both full-depth chondrocyte (Fig. 7G, 7I) and chondroprogenitor (Fig. 7H, 7J) treatment groups were observed. In some of the samples, there was no obvious demarcating border and it was difficult to tell where the defect actually was. In all of the samples, both full-depth chondrocyte (Fig. 7K) and chondroprogenitor seeded membranes (Fig. 7L) showed evidence of collagen type II positive repair tissue.

Discussion

Previous research has relied on cell surface markers, originally designated to characterise bone marrow stromal cells, as a means of isolating possible stem cell populations from articular cartilage [27,29,46]. In particular, research suggested that CD105 and CD166 as possible candidates of cartilage stem cell markers but our study and others have demonstrated that a sub-population of cartilage stem cells cannot be isolated from these markers alone as

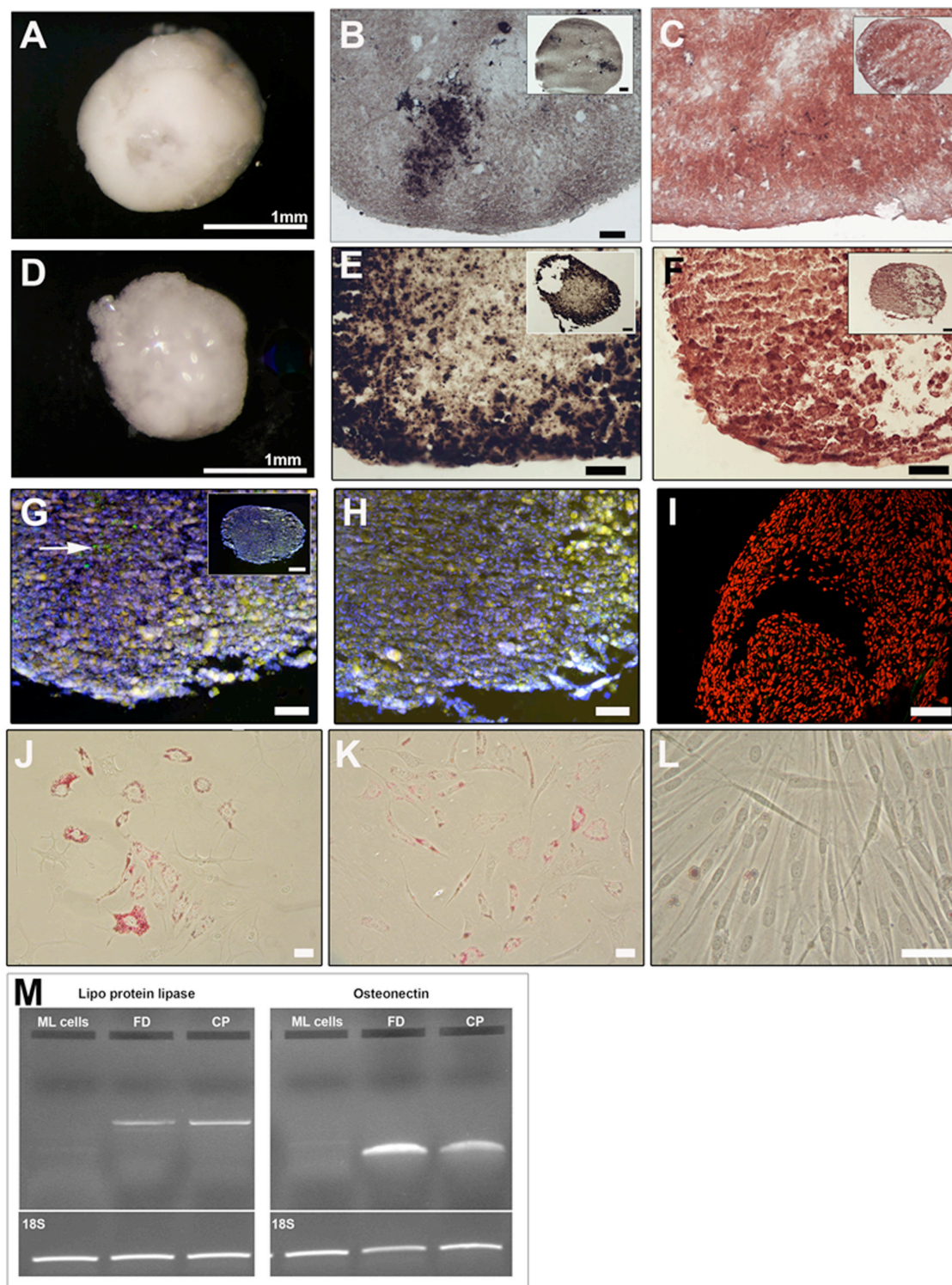


Figure 5. Lineage differentiation of clonal and full depth cell populations. Pellets cultured in osteogenic differentiation media show a rough surface topography in the full depth chondrocytes (A) and in cartilage progenitor cell pellets (D). Regions of mineralisation are indicated in the pellets cultured in osteogenic media by von Kossa (B, E) and alizarin red staining (C, F). Alkaline phosphatase (G;arrowed) was present in a small region of the osteogenic cartilage progenitor cultured pellets and collagen type X was absent (I). Representative example of a negative control for mouse monoclonal antibody protocol (H). Monolayer clonal cells cultured in adipogenic differentiation media show positive staining with Oil Red O in both cartilage progenitor cells (J) and full depth chondrocytes (K). Control cultures did not stain with Oil Red O (L). Scale bars = 50 µm. mRNA expression for lipo-protein lipase (bp 505) and osteonectin (bp 107) in non-treated monolayer (ML) chondrocytes, treated full-depth chondrocytes (FD) and treated cartilage progenitors (CP) (M).

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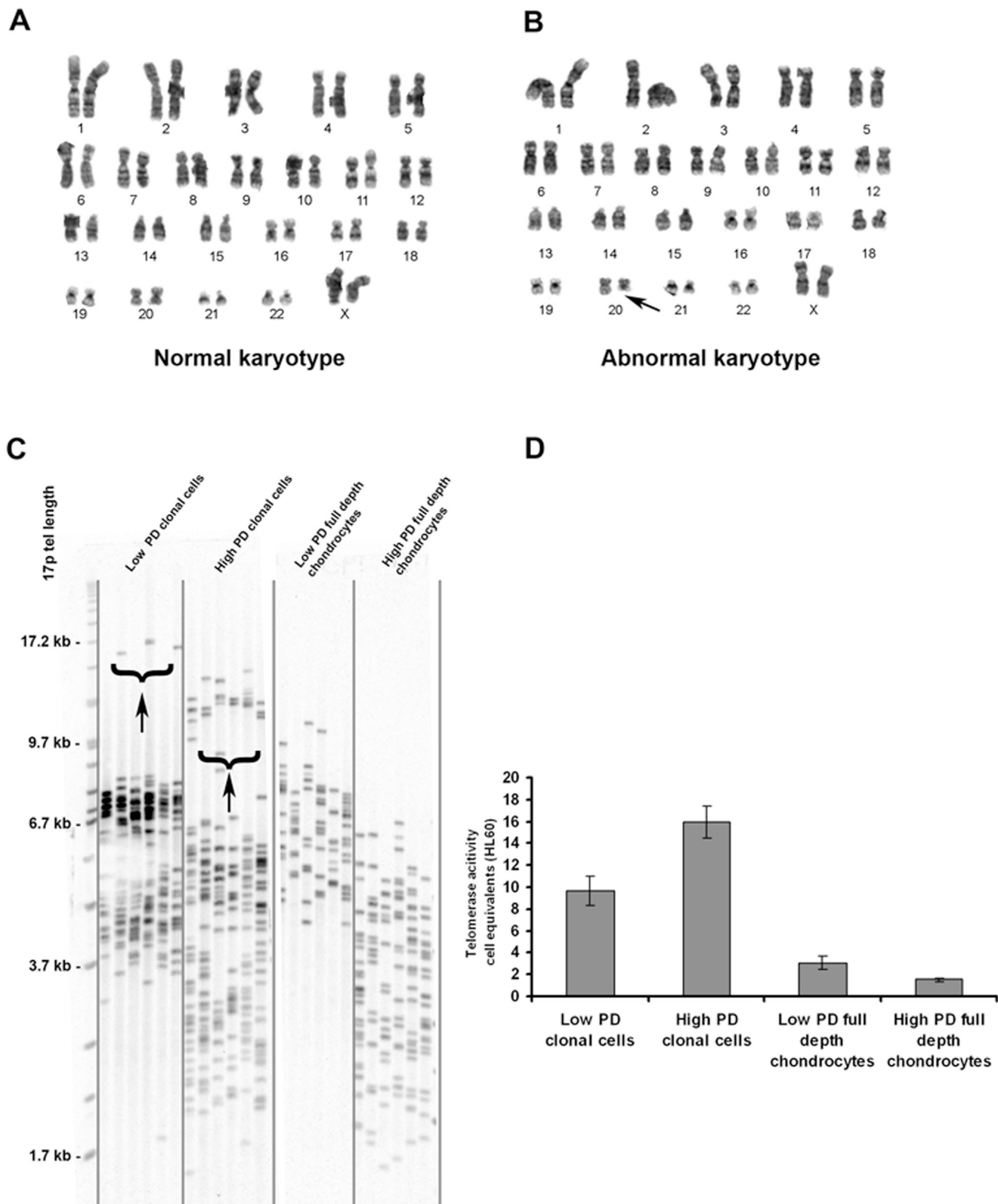


Figure 6. Cytogenetic Analysis. Normal female, 46, XX karyotype was observed in a clonal cell line at 31.3 population doublings (A). In one flask from the same cell line, 2 out of 12 cells displayed an abnormal karyotype; a deletion on the long arm of chromosome 20 at q11.2 (B; arrowed). **Telomere length analysis and real-time quantitative telomerase repeat amplification procedure (RTQ-TRAP) of telomerase activity in clonal cell lines and full-depth chondrocyte populations.** STELA reveals both clonal cell lines and full-depth chondrocytes undergo telomere erosion (C). It is interesting to note a subpopulation of cells in the clonal cell line show a larger distribution which is increasing with time in culture (arrowed). RTQ-TRAP analyses of telomerase activity in HL60 cell equivalents showed low population doubling (<30) clonal cells displaying a 3.1-fold

greater activity than low population full depth chondrocytes (D). High population doubling clonal cells show a 10.6-fold greater activity than full depth chondrocytes at a high population doubling (>30). PD = population doubling.
doi:10.1371/journal.pone.0013246.g006

they are widely expressed by mature chondrocytes in the native tissue [29,30,47]. Isolating a possible stem cell population reliant on stem cell marker expression, however, could prove problematic for cell-based repair therapies. In a clinical situation, isolation of the correct cell population in suitable numbers would be dependent on an extremely consistent method of cartilage digestion and culture conditions, as these parameters play a significant role in cell surface marker expression. Indeed, work from our own laboratory and others have demonstrated that these putative markers vary during culture [48]. Additionally, clinically accredited FACs machines have yet to come on-stream.

In light of these studies, we decided to isolate what we now characterise as a cartilage progenitor population using the fibronectin adhesion assay, selecting for discrete colonies (of over 32 cells to eliminate transit amplifiers) which could then be expanded to high numbers [33]. Currently, generation of high numbers of chondrogenically competent full-depth chondrocytes for cell-based repair strategies is not achievable due to the loss of Sox-9 expression and concomitant loss of the cartilage phenotype [26,49]. Expansion of our cartilage progenitor clonal populations to a high population doubling suggests that this method of isolation is indeed selecting for cartilage progenitor populations.

It is interesting to observe that a sub-population (0.7%) of cells within the total chondrocyte cell mass express CD49e ($\alpha 5$ integrin) and indeed the clonal cell lines obtained by adhesion to fibronectin express this marker (99%) when expanded. The expression of this marker may explain the high affinity of these progenitor cells for fibronectin. Whether or not CD49e can be utilised as a specific marker for this cartilage progenitor cell population remains to be determined. However, the colony forming efficiency of these cells indicates that they are present at a ratio of 1:100, which is in the order of 0.7%.

The cytogenetic analyses demonstrated that cartilage progenitors that had undergone 31.3 population doublings are largely normal but with some anomalies that are likely due to the high PD number and culture conditions. This was particularly apparent in the deletion of the short arm of chromosome 20 in one flask yet not apparent in the other two flasks containing the same clonal cell line. It can be considered cautionary and underlies the necessity of carrying out a karyotype analysis when generating cells for clinical application.

It is well documented that when full depth chondrocytes in monolayer culture lose their rounded cell configuration, there is a loss of phenotype associated with the number of division cycles the cell undergoes and the eventual loss of Sox9 expression [5,50,51,52,53]. Monolayer cartilage progenitor populations that have been isolated in this study, although demonstrating a flattened morphology in culture, show expression of putative stem cell markers, yet when induced to undergo chondrogenic differentiation by addition of TGF β 2, markers of the chondrogenic phenotype are expressed and translated into immunodetectable proteins. Using the bovine equivalent progenitor cells, we have demonstrated that the extended chondrogenic potential of these cells is related to the maintenance of Sox9 expression during extended monolayer culture and we would suggest that this is likely with the human equivalent cells [26].

Another effect of long-term chondrocyte culture is cell senescence due to a shortening of telomeres, the DNA sequences necessary for chromosome replication [54,55,56]. As true stem cells maintain their telomere length and show unlimited replicative

capacity, this provided a way of characterising the isolated cartilage progenitor population and also strengthened the case for their suitability for cell-based repair therapies [57]. Full-depth chondrocytes and cartilage progenitors investigated in our study displayed eroded telomeres with time in culture consistent with growth kinetic data. However, if we study the results in more detail, a sub-population of cells within the cartilage progenitor population is observed that maintain their telomere length. Indeed, this sub-population of cells maintains their telomere length and increases in number with time in culture. However, we do not find this sub-population expanding beyond 60 PDs and may senesce in a telomere independent fashion.

The cartilage progenitor population express a number of chondrogenic markers and matrix components when induced to undergo chondrogenesis *in vitro*, including collagen type I and collagen type II. In culture, collagen type I is often considered an indicator of fibrocartilage. However, the expression of collagen type II in the chondrogenic cell pellets suggests that the cartilage progenitors are following a developmental process in their matrix synthesis, synthesising firstly collagen type I and secondly collagen type II as the cells mature [44]. Evidence of co-localisation of the two collagens at the articular surfaces was described in a study by Archer *et al* [58] and it is interesting to note that collagen type I is observed in the pellets in the outer layer of cells, which appear flattened – similar to the flattened interzone cells and perichondrium in the developing limb, as described in the same study. Osteogenically induced cultures show signs of mineralisation when cultured as a 3D pellet. However, there is no evidence of bone formation *per se*; neither collagen type X protein expression nor alkaline phosphatase activity were evident in the chondrogenic or osteogenically induced 3D cultured pellets. These data highlight that cartilage progenitors neither undergo chondrogenic terminal differentiation nor true osteogenesis. Notably, many published studies assay osteogenesis of stem cells in monolayer culture through analysis of expression of alkaline phosphatase and the appearance of calcification by von Kossa staining that *de facto*, is not an assay for bone formation. Indeed, if the cells are demonstrating a restricted differentiation potential *in vitro*, then the ability of these cells to form stable ectopic cartilage that doesn't undergo terminal differentiation *in vivo* would be a very desirable factor for using these cells in clinical applications for cartilage repair strategies. In contrast, current research into BMSCs for cartilage regeneration procedures demonstrates terminal differentiation and extracellular matrix (ECM) calcification *in vitro* upon chondrogenic induction, ultimately resulting in failure of transplantation [16,22,59,60].

Multi-lineage potential is often used to characterise a mesenchymal stem cell population. The results from our study demonstrate that the cartilage progenitor cells have the capacity to differentiate into the adipogenic lineage, whilst retaining a restricted differentiation potential during osteogenic differentiation, as discussed above. However, the full-depth chondrocytes are also positive for differentiation into these lineages, albeit to a lesser extent. This can be explained as such; during the isolation procedure, progenitor cells are part of the full-depth chondrocyte culture and are likely to have become enriched during the cell expansion process. This small progenitor population within the full-depth chondrocytes would, therefore, undergo differentiation when cultured in the appropriate media, as we have observed.

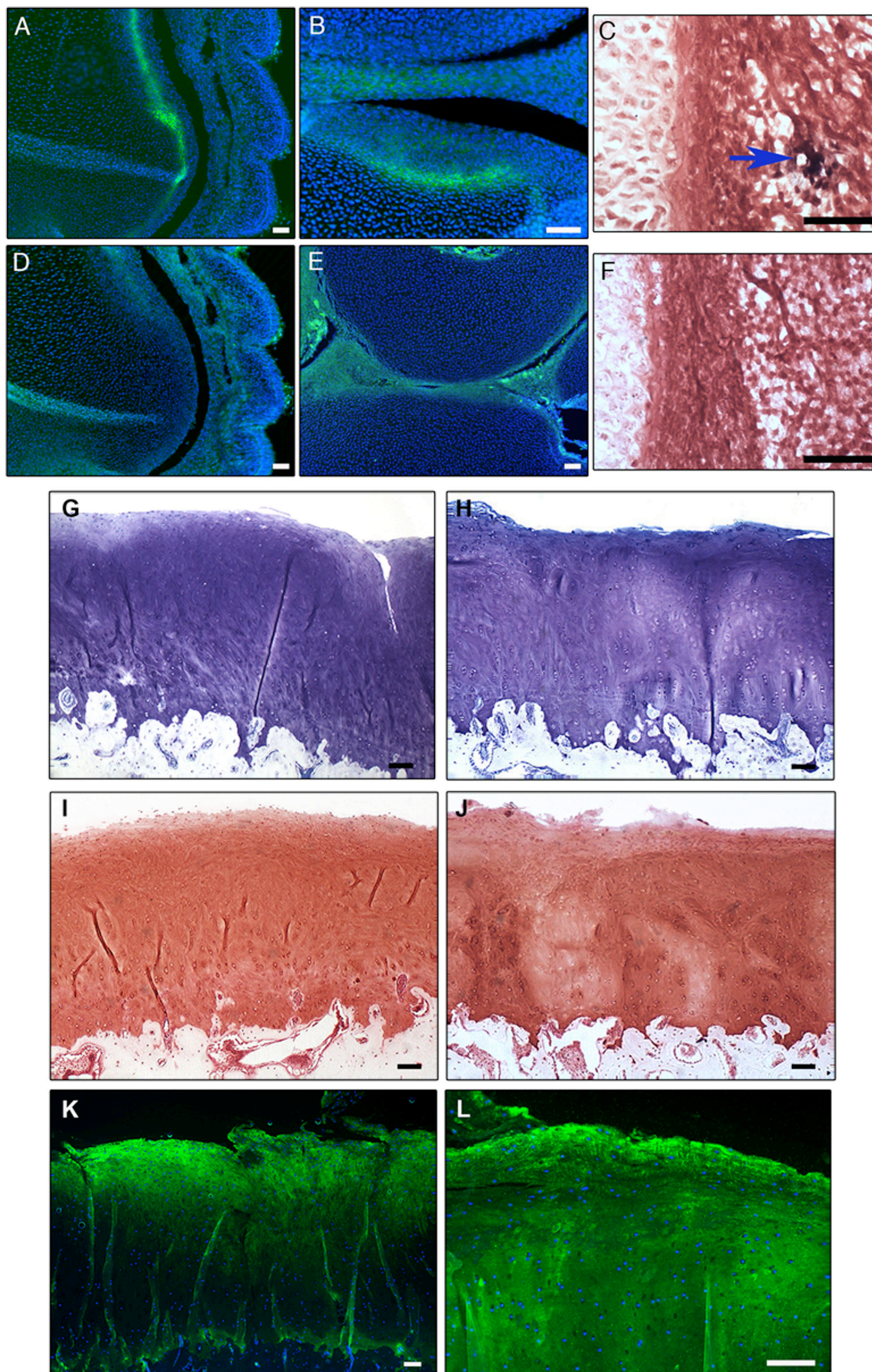


Figure 7. Engraftment of human cartilage progenitor cells into developing chick hind limbs. Cells expressing human collagen type I are present in the growth plate of the developing chick limb at st36 (A). The high power image demonstrates human collagen type I expression in the surface region of the developing cartilage anlagen (B). IgG negative control (D). Expression of chick collagen type I is not evident in st36 chick limb (E). ISH for human Alu repeats on a representative section from a st36 chick limb (C). The Alu-positive nuclei are stained black (arrowed). Negative control for ISH for the Alu repeats (F). Scale bars = 50 μ m. ***In vivo* implantation of goat chondroprogenitors into cartilage defects.** Histological stained sections of repair tissue in the caprine *in vivo* repair model. Toluidine blue stained sections of repair tissue containing the membrane seeded with full-depth chondrocytes (G) and chondroprogenitors (H) shows examples of an integrated repair tissue. Safranin O staining demonstrated proteoglycan synthesis in the repair tissue in defects treated with full-depth chondrocyte seeded membranes (I) and chondroprogenitor seeded membranes (J). Repair tissue in defects treated with chondrocyte seeded membranes (K) or chondroprogenitor seeded membranes (L), labelled positive for collagen type II. Scale bars = 100 μ m.
doi:10.1371/journal.pone.0013246.g007

Characteristically, mesenchymal stem cells can migrate and engraft into tissues, including regions of injury, and undergo site-specific differentiation [61,62,63]. It is encouraging to see that data from our engraftment studies demonstrated that the cartilage progenitor population can migrate to its specific milieu within the developing limb. The isolated cartilage progenitor populations largely engrafted within the perichondrium – an already known niche of stem cells and the cells also migrated to the surface of the articular cartilage – where data by Dowthwaite *et al* [25] has demonstrated the presence of a progenitor population. However, engrafted cells were observed in other connective tissues including dermis but were not seen outside tissues of the connective tissue lineage, lending further support to progenitor status.

To determine the potential of chondroprogenitors in *in vivo* cartilage repair, we have used the goat as our model. The goat chondroprogenitors were isolated and characterised in the same manner as the human chondroprogenitors. The caprine *in vivo* repair study revealed examples of excellent integration in cartilage defects treated with Chondro-Gide® membrane seeded with goat chondroprogenitors. When subjected to the ICRS scoring system to evaluate cartilage repair, however, there was no difference in repair between defects that were treated with chondroprogenitors and those treated with the current gold standard of expanded full-depth chondrocytes. Although, no differences in repair tissue were observed at the 20-month end point of the study, the main advantage of these cells over full-depth chondrocytes is the ability to generate very large cell numbers that retain their phenotype, from a single cell. As a result, it would be feasible to treat much larger defects than is currently possible.

Conclusion

The main findings from these data lead to the conclusion that human articular cartilage contains a cartilage progenitor population that retains a stem cell-like phenotype until appropriate induction of chondrogenic differentiation is required. As such, we propose that

this cell population is an ideal candidate to use in the development of enhanced protocols for future cell-based tissue engineering procedures that require cells with an extensive replication capacity and restricted differentiation potential. As a single cell can generate millions of cells that retain their chondrogenic phenotype *in vivo*, this isolation procedure would ensure sufficient numbers of cells, that maintain their ability to form cartilage, are readily available for surgical procedures. These characteristics are favourable if the transplanted cells are to physically contribute to the repair and maintenance of the cartilage defect long-term.

Supporting Information

Text S1 Immunohistochemistry.

Found at: doi:10.1371/journal.pone.0013246.s001 (0.04 MB DOC)

Text S2 Cytogenetic Analysis.

Found at: doi:10.1371/journal.pone.0013246.s002 (0.04 MB DOC)

Text S3 Telomere length analysis.

Found at: doi:10.1371/journal.pone.0013246.s003 (0.04 MB DOC)

Text S4 RTQ-TRAP.

Found at: doi:10.1371/journal.pone.0013246.s004 (0.04 MB DOC)

Author Contributions

Conceived and designed the experiments: RW IMK HEM CWA. Performed the experiments: RW IMK KR LN HEM TA SKS REJ DMB HL SR GPD. Analyzed the data: RW IMK KR HEM SKS DMB HL SR HMS CWA. Contributed reagents/materials/analysis tools: RW DMB SR JD JF TB CWA. Wrote the paper: RW IMK HEM SKS DMB SR CWA.

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